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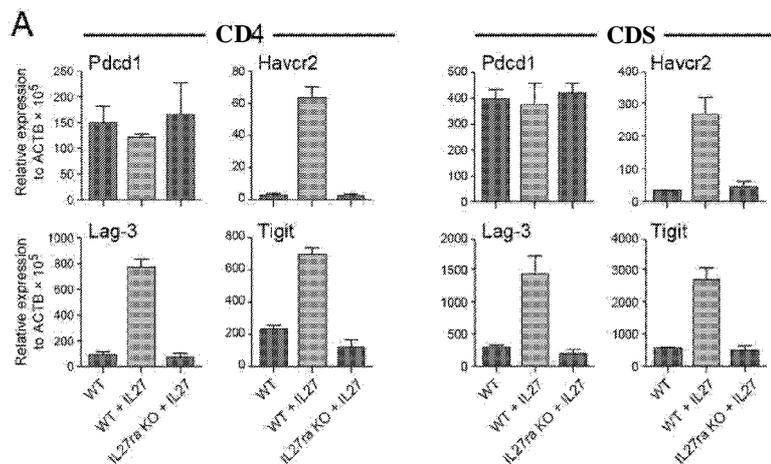
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[Continued on nextpage]

(54) Title: MODULATION OF NOVEL IMMUNE CHECKPOINT TARGETS

FIG. 1A



(57) Abstract: Dysfunctional or exhausted T cells arise in chronic diseases including chronic viral infections and cancer, and express high levels of co-inhibitory receptors. Therapeutic blockade of these receptors has clinical efficacy in the treatment of cancer. While co-inhibitory receptors are co-expressed, the triggers that induce them and the transcriptional regulators that drive their co-expression have not been identified. The immunoregulatory cytokine IL-27 induces a gene module in T cells that includes several known co-inhibitory receptors (Tim-3, Lag-3, and TIGIT). The present invention provides a novel immunoregulatory network as well as novel cell surface molecules that have an inhibitory function in the tumor microenvironment. The present invention further provides the novel discovery that the transcription factors Prdml and c-Maf cooperatively regulate the expression of the co-inhibitory receptor module. This critical molecular circuit underlies the co-expression of co-inhibitory receptors in dysfunctional T cells and identifies novel regulators of T cell dysfunction.

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MODULATION OF NOVEL IMMUNE CHECKPOINT TARGETS**RELATED APPLICATIONS AND INCORPORATION BY REFERENCE**

[0001] Reference is made to U.S. provisional application serial No. 62/239,548 filed on October 9, 2015.

[0002] The foregoing applications, and all documents cited therein or during their prosecution ("apln cited documents") and all documents cited or referenced in the apln cited documents, and all documents cited or referenced herein ("herein cited documents"), and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

FIELD OF THE INVENTION

[0003] The present disclosure relates to the modulation of T cell dysfunction.

FEDERAL FUNDING LEGEND

[0004] This invention was made with government support under grant numbers NS076410, AI0562999, NS045937, AI039671, AI045757, AI073748, CA187975 and awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0005] The following discussion is merely provided to aid the reader in understanding the disclosure and is not admitted to describe or constitute prior art thereto.

[0006] T cell dysfunction or exhaustion is a state of T cell differentiation that arises in chronic disease settings such as chronic viral infections and cancer. Dysfunctional T cells exhibit diverse deficits in effector functions, including impaired proliferative capacity, cytotoxicity, and production of pro-inflammatory cytokines (Pardoll, D. M. (2012) *Nature reviews. Cancer* **12**, 252-264; Wherry and Kurachi, (2015) *Nature reviews Immunology* **15**, 486-499). Consequently, dysfunctional T cells are poor mediators of both viral and tumor clearance. Dysfunctional T cells express high levels of co-inhibitory receptors, such as

Programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and blockade of these receptors is associated with recovery of effector T cell responses in multiple experimental models of chronic viral infection and Consequently, exhausted T cells are poor mediators of viral and/or tumor clearance. Exhausted T cells have been noted to express high levels of co-inhibitory receptors, such as PD-1 and CTLA-4, and blockade of these receptors has been associated with the recovery of effector T cell responses in experimental models of chronic viral infection and cancer (Leach, D. R., et al, (1996) *Science* **271**, 1734-1736; Barber, D. L. et al., (2006) *Nature* **439**, 682-687; Mahoney et al., (2015) *Nature reviews Drug discovery* **14**, 561-584; Wherry and Kurachi, 2015). Indeed, therapeutic blockade of CTLA-4 and PD-1 has been successfully translated to the clinic for the treatment several human cancers (Hodi, F. S. et al, (2010) *The New England journal of medicine* **363**, 711-723; Robert, C. et al, (2011) *The New England journal of medicine* **364**, 2517-2526, Hamid, O. et al, (2013) *The New England journal of medicine* **369**, 134-144; Topalian et al, (2012) *The New England journal of medicine* **366**, 2443-2454).

[0007] CTLA-4 and PD-1 are not the only co-inhibitory receptors that are expressed by dysfunctional T cells. In fact, as described herein, dysfunctional T cells express multiple co-inhibitory receptors including TIM-3, LAG-3, and TIGIT, indicating shared regulatory mechanisms driving their expression. Importantly, as dysfunctional T cells accumulate expression of co-inhibitory receptors they develop a "deep" state of dysfunction and begin to produce IL-10, which further contributes to local immune suppression (Wherry, E. J. (2011) *Nature immunology* **12**, 492-499). Thus, the co-expression of co-inhibitory receptors on dysfunctional T cells has important functional consequences. Indeed, combination therapies that simultaneously target multiple co-inhibitory pathways, such as CTLA-4 together with PD-1, or PD-1 together with TIM-3, LAG-3, or TIGIT, are more potent at restoring anti-tumor immunity than blockade of single co-inhibitory targets in both humans and in experimental mouse tumor models (Wolchok, J. D. et al. (2013) *The New England journal of medicine* **369**, 122-133; Woo, S. R. et al. (2012) *Cancer research* **72**, 917-927; Johnston, R. J. et al. (2014) *Cancer cell* **26**, 923-937; Fourcade, J. et al. (2014) *Cancer research* **74**, 1045-1055). Together these observations raise the important issue of understanding how co-inhibitory receptors are induced and co-regulated in exhausted or dysfunctional T cells.

[0008] Dysfunctional T cells express multiple co-inhibitory receptors in addition to CTLA-4 and PD-1, including T-cell immunoglobulin and mucin-domain containing-3 (Tim-3), Lymphocyte-activation gene 3 (Lag-3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT); (Anderson et al, (2016) *Immunity* **44**, 989-1004; Wherry and Kurachi,

2015). The extent of co-inhibitory receptor co-expression is directly correlated to the severity of dysfunctional phenotype (Wherry and Kurachi, 2015). Thus, combination therapies that simultaneously target multiple co-inhibitory pathways, such as PD-1 together with CTLA-4 are more efficacious at restoring anti-tumor immunity than blockade of single co-inhibitory targets in both mouse tumor models and patients (Fourcade et al, 2014; Johnston et al, 2014; Sakuishi et al, (2010) *The Journal of experimental medicine* 207, 2187-2194; Wolchok et al, 2013; Woo et al, 2012). Unfortunately, even with combination therapy, a substantial number of patients fail to respond to immune checkpoint blockade, highlighting the importance of identifying additional co-inhibitory receptors that could be targeted for cancer immunotherapy.

[0009] The co-expression and co-regulation of co-inhibitory receptors in dysfunctional T cells, suggests that there might be a common trigger that induces them and common regulatory mechanisms that control their expression in dysfunctional T cells. If such common triggers and regulators exist, they may facilitate the development of more efficacious therapies that will simultaneously antagonize multiple co-inhibitory receptors. However, such common mechanisms have not been identified to date.

[0010] One compelling candidate for a common trigger is IL-27, a heterodimeric cytokine and a member of the IL-12 family of cytokines that is produced by antigen presenting cells. Although IL-27 was initially shown to promote pro-inflammatory Type 1 immune responses, emerging evidence suggests that this cytokine plays an important role in the resolution of tissue inflammation (Yoshida and Hunter, (2015) *Annual review of immunology* 33, 417-443). IL-27 administration *in vivo* suppresses the pathogenicity of primed effector T cells and inhibits the development of autoimmunity (Fitzgerald et al, (2007a) *Journal of immunology* 179, 3268-3275). Consistent with a suppressive function for IL-27, IL-27ra (WSX-1) deficient mice exhibit increased inflammation during *Toxoplasma gondii* infection and exacerbated disease in a model of central nervous system autoimmunity (Awasthi et al, (2007) *Nature immunology* 8, 1380-1389; Hirahara et al, (2012) *Immunity* 36, 1017-1030; Villarino et al, (2003) *Immunity* 19, 645-655). Indeed, Applicants (Awasthi et al, 2007) and others (Fitzgerald et al, 2007a; Stumhofer et al, (2007) *Nature immunology* 8, 1363-1371) have shown that exposure of naive T cells to IL-27 induces IL-10-secreting Type 1 regulatory (Tr1) cells that are immune suppressive. Moreover, Applicants have recently shown that IL-27 induces Tim-3 (Zhu et al, (2015) *Nature communications* 6, 6072), which has been shown to cooperate with PD-1 in promoting a dysfunctional phenotype in T cells (Sakuishi et al, 2010). Taken together, these observations raise the

possibility that IL-27 may be one of the triggers that induces multiple co-inhibitory receptors, which in turn promote T cell dysfunction in effector T cells.

[0011] Citation or identification of any document in this application is not an admission that such document is available as prior art to the present invention.

SUMMARY OF THE INVENTION

[0012] Here, Applicants used a systems biology approach, to find that IL-27 signaling drives the expression of a gene module that includes not only Tim-3, but also Lag-3, TIGIT, and IL-10, all molecules that are associated with T cell dysfunction. The IL-27-induced transcriptional module significantly overlaps with the gene signatures that define dysfunctional T cells in chronic viral infection and cancer, as well as with gene signatures associated with other suppressed or tolerant T cell states. Applicants further identify a number of novel molecules within the IL-27-induced gene module that mediate T cell dysfunction and can be modulated to improve anti-tumor T cell responses *in vivo*. Finally, using network-based approaches Applicants identify Prdml and c-Maf as key transcriptional regulators that cooperatively drive the inhibitory gene module. Our study defines a new role for IL-27 signaling in immune regulation and uncovers the downstream regulatory network that drives the expression of an inhibitory gene module that sets the stage for the development of dysfunctional phenotype in effector T cells.

[0013] Accordingly, the methods and compositions described herein are based, in part, on the discovery of target gene(s) that are involved in T cell dysfunction, including but not limited to, T cell exhaustion and T cell non-responsiveness. Accordingly, provided herein are methods and compositions for modulating T cell dysfunction by modulating the expression, activity and/or function of at least one target gene or gene product, for example, the target genes listed herein in Table 1, Table 10, Table 11, Table 12, Table 13 or the pairs of target genes listed herein in Table 2, or any combination thereof.

[0014] In one aspect, provided herein is a method of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13 or any combination thereof.

[0015] In one embodiment of this aspect and all other aspects provided herein, the T-cell dysfunction is T-cell exhaustion.

[0016] In another embodiment of this aspect and all other aspects provided herein, the modulation of T-cell exhaustion comprises a decrease in the exhausted T-cell phenotype, such that functional T-cell activity is increased.

[0017] In another embodiment of this aspect and all other aspects provided herein, the modulation of T-cell exhaustion comprises an increase in the exhausted T-cell phenotype, such that functional T-cell activity is decreased.

[0018] In another embodiment of this aspect and all other aspects provided herein, the selected target gene or gene product or a combination thereof is/are identified as participating in the inhibition of functional T-cell activity.

[0019] In another embodiment of this aspect and all other aspects provided herein, the modulating agent inhibits the expression, activity and/or function of the selected target gene or gene product or combination thereof.

[0020] In another embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the promotion of functional T-cell activity.

[0021] In another embodiment of this aspect and all other aspects provided herein, the modulating agent promotes or activates the expression, activity and/or function of the selected target gene or gene product or combination thereof.

[0022] In another embodiment of this aspect and all other aspects provided herein, the method further comprises contacting the dysfunctional T-cell with modulating agents that modulate the expression, activity and/or function of at least two target genes or gene products selected from the target genes listed in Table 1, Table 2, or any combination thereof.

[0023] In another embodiment of this aspect and all other aspects provided herein, the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody or antigen-binding fragment thereof agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

[0024] In another embodiment of this aspect and all other aspects provided herein, the methods can further comprise contacting the dysfunctional T-cell with an agent or treatment selected from the group consisting of a PD-1 inhibitor, CTLA4 inhibitor, chemotherapy, radiation therapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an activator or agonist for OX-40, 4-1BB, GITR, CD226, KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and/or SLAMF7.

[0025] Another aspect provided herein relates to a method of treating a condition involving or characterized by the presence of T cells exhibiting an exhausted or dysfunctional

phenotype, the method comprising administering an amount of a modulating agent effective to modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, or any combination thereof.

[0026] In one embodiment of this aspect and all other aspects provided herein, the condition is cancer or a persistent infection.

[0027] In another embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

[0028] In another embodiment of this aspect and all other aspects provided herein, the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

[0029] In another embodiment of this aspect and all other aspects provided herein, a selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

[0030] In another embodiment of this aspect and all other aspects provided herein, the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

[0031] In another embodiment of this aspect and all other aspects provided herein, the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody or antigen-binding fragment agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

[0032] Provided herein in another aspect is a pharmaceutical composition for modulating T cell dysfunction, the composition comprising a first modulating agent and a second modulating agent that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13 or any combination thereof.

[0033] Another aspect provided herein relates to a pharmaceutical composition for modulating T cell dysfunction, the composition comprising a first modulating agent that inhibits the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13 or any combination thereof and a second modulating agent that promotes the expression, activity and/or function of one or more target genes or gene products thereof

selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13 or any combination thereof.

[0034] Also provided herein, in another aspect, is a pharmaceutical composition for modulating T cell dysfunction, the composition comprising a modulating agent that modulates the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, , Table 10, Table 11, Table 12, Table 13 or any combination thereof and an agent selected from the group consisting of a PD-1 inhibitor, a CTLA4 inhibitor, chemotherapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-IBB, GITR, CD226, KLRC2, KLRE1, KLRK1, IL12RB 1, IL1R, and SLAMF7.

[0035] Also provided herein, in another aspect, are pharmaceutical compositions for modulating T cell dysfunction, the composition comprising at least one modulating agent that modulates the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13 or any combination thereof. In another aspect, the pharmaceutical compositions comprise at least two modulating agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13 or any combination thereof.

[0036] Also provided herein, in another aspect, are pharmaceutical compositions for modulating T cell dysfunction, the composition comprising at least one modulating agent that modulates the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, Table 9 or any combination thereof. In another aspect, the pharmaceutical compositions comprise at least two modulating agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, Table 9 or any combination thereof.

[0037] In one embodiment of this aspect and all other aspects provided herein, the T cell dysfunction comprises T cell exhaustion.

[0038] In another embodiment of this aspect and all other aspects provided herein, the T cell exhaustion occurs in an individual with cancer or a persistent infection.

[0039] Another aspect provided herein relates to a pharmaceutical composition for modulating T cell dysfunction, the composition comprising an inhibitor of the expression

and/or activity of PDPN, an inhibitor of the expression and/or activity of PROCR, or a combination thereof.

[0040] Also provided herein in another aspect is a pharmaceutical composition for modulating T cell dysfunction comprising: (a) an inhibitor of the expression and/or activity of PDPN and an inhibitor of the expression and/or activity of PROCR; and (b) an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of TIGIT, LAG3, LILRB4, and KLRC1; and/or an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R, and SLAMF7.

[0041] Provided herein in another aspect is a pharmaceutical composition for modulating an IL-27-regulated co-inhibitory module comprising: (a) an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM, and KLRC1; and (b) an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

[0042] In one embodiment of this aspect and all other aspects provided herein, the composition further comprises an inhibitor of the expression and/or activity of TIM-3.

[0043] In another embodiment of this aspect and all other aspects provided herein, the composition further comprises an inhibitor of the expression and/or activity of PD-1.

[0044] In another embodiment of this aspect and all other aspects provided herein, the composition further comprises an inhibitor of the expression and/or activity of CTLA4.

[0045] In another embodiment of this aspect and all other aspects provided herein, the composition further comprises an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of PD-1. In another embodiment of this aspect and all other aspects provided herein, the composition further comprises an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of CTLA4. In another embodiment of this aspect and all other aspects provided herein, the composition further comprises an inhibitor of the expression and/or activity of CTLA4 and an inhibitor of the expression and/or activity of PD-1. In another embodiment of this aspect and all other aspects provided herein, the composition further comprises an inhibitor of the expression and/or activity of CTLA4, and an inhibitor of the expression and/or activity of PD-1 and an inhibitor of the expression and/or activity of TIM-3.

[0046] In another embodiment of this aspect and all other aspects provided herein, the inhibitors and activators are selected from an antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog.

[0047] In another embodiment of this aspect and all other aspects provided herein, the antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog is selected from: an anti-CTLA4 antibody, an anti-PD-1 antibody, or aPDL-1 antagonist. In certain embodiments, the antibody or antigen binding fragment thereof is selected from the group consisting of: nivolumab, pembrolizumab, lambrolizumab, ipilimumab, and atezolizumab.

[0048] Another aspect provided herein relates to a method of modulating an IL-27-regulated co-inhibitory module in a subject in need thereof, the method comprising administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of PDPN, an inhibitor of the expression and/or activity of PROCR, or a combination thereof.

[0049] An additional aspect provided herein relates to a method of modulating an IL-27-regulated co-inhibitory module in a subject in need thereof, the method comprising: (a) administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of PDPN, and an inhibitor of the expression and/or activity of PROCR; and (b) administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of an inhibitor of the expression and/or activity of TIGIT, LAG3, LILRB4, and KLRC1; and/or an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

[0050] Also provided herein in another aspect is a method of modulating an IL-27-regulated co-inhibitory module in a subject in need thereof, the method comprising: (a) administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM and KLRC1; and (b) administering a pharmaceutical composition comprising an activator the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

[0051] In one embodiment of this aspect and all other aspects provided herein, the method further comprises administering an inhibitor of the expression and/or activity of TIM-3.

[0052] In another embodiment of this aspect and all other aspects provided herein, the method further comprises administering an inhibitor of the expression and/or activity of PD-1.

[0053] In another embodiment of this aspect and all other aspects provided herein, the method further comprises administering an inhibitor of the expression and/or activity of CTLA-4.

[0054] In another embodiment of this aspect and all other aspects provided herein, the method further comprises administering an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of PD-1.

[0055] In another embodiment of this aspect and all other aspects provided herein, the inhibitors and activators are selected from an antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog.

[0056] In another embodiment of this aspect and all other aspects provided herein, the antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog is selected from the group consisting of: an anti-CTLA4 antibody, an anti-PD-1 antibody, or aPDL-1 antagonist. In certain embodiments, the antibody or antigen binding fragment thereof is selected from the group consisting of: nivolumab, pembrolizumab, lambrolizumab, ipilimumab, and atezolizumab.

[0057] In another embodiment of this aspect and all other aspects provided herein, the subject in need thereof has a disease or disorder characterized by T-cell exhaustion.

[0058] In another embodiment of this aspect and all other aspects provided herein, the subject in need thereof is diagnosed as having a cancer or tumor.

[0059] In another embodiment of this aspect and all other aspects provided herein, the subject in need thereof is diagnosed as having a chronic or persistent infection.

[0060] Also provided herein in another aspect is a method of modulating T cell dysfunction, the method comprising contacting a dysfunctional T cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the group consisting of: the subset of genes

listed in Table 5, the subset of genes listed in Table 6, the subset of genes listed in Table 7, the subset of genes listed in Table 8, and the subset of genes listed in Table 9.

[0061] In one embodiment of this aspect and all other aspects provided herein, the T cell dysfunction is T cell exhaustion.

[0062] In another embodiment of this aspect and all other aspects provided herein, the modulation of T cell exhaustion comprises a decrease in the exhausted T cell phenotype, such that T cell activation is increased.

[0063] In another embodiment of this aspect and all other aspects provided herein, the modulation of T cell exhaustion comprises an increase in the exhausted T cell phenotype, such that T cell activation is decreased.

[0064] In another embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

[0065] In another embodiment of this aspect and all other aspects provided herein, the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

[0066] In another embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

[0067] In another embodiment of this aspect and all other aspects provided herein, the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

[0068] In another embodiment of this aspect and all other aspects provided herein, the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

[0069] Also provided herein in another aspect is a method of treating a condition involving or characterized by the presence of T cells exhibiting an exhausted phenotype, the method comprising administering an amount of a modulating agent effective to modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the group consisting of: the subset of genes listed in Table 5, the subset of genes listed in Table 6, the subset of genes listed in Table 7, the subset of genes listed in Table 8, and the subset of genes listed in Table 9.

[0070] In one embodiment of this aspect and all other aspects provided herein, the condition is cancer or a persistent infection.

[0071] In another embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

[0072] In another embodiment of this aspect and all other aspects provided herein, the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

[0073] In another embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

[0074] In another embodiment of this aspect and all other aspects provided herein, the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

[0075] In another embodiment of this aspect and all other aspects provided herein, the agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

[0076] Another aspect provided herein relates to a method of determining the presence of T cells exhibiting an exhausted phenotype, the method comprising detecting, in a sample comprising T cells, a level of expression, activity and/or function of one or more genes or expression products thereof selected from the target genes listed in Table 1, Table 2 or any combination thereof, and comparing the detected level to a reference, wherein a difference in the detected level relative to the reference indicates the presence of T cells exhibiting an exhausted phenotype.

[0077] In one embodiment of this aspect and all other aspects provided herein, the sample is from an individual with cancer or a persistent infection.

[0078] In some aspects, provided herein are methods of treating a disease or disorder characterized by aberrant or unwanted T-cell functional activity in a subject in need thereof, the method comprising administering a therapeutically effective amount of a modulating agent effective to modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, or any combination thereof.

[0079] In one embodiment of this aspect and all other aspects provided herein, the disease or disorder is an autoimmune disease or graft vs. host disease.

[0080] In one embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation and the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

[0081] In another embodiment of this aspect and all other aspects provided herein, the selected target gene(s) is/are identified as participating in the promotion of T cell activation and the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

[0082] In one embodiment of this aspect and all other aspects provided herein, the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

[0083] In one embodiment of this aspect and all other aspects provided herein, the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

[0084] In some aspects, provided herein are methods of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5. In one embodiment of this aspect and all other aspects provided herein, two or more target genes or gene products thereof selected from the target genes listed in Table 5 are modulated.

[0085] In some aspects, provided herein are methods of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 6. In one embodiment of this aspect and all other aspects provided herein, two or more target genes or gene products thereof selected from the target genes listed in Table 6 are modulated.

[0086] In some aspects, provided herein are methods of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 7. In one embodiment of this

aspect and all other aspects provided herein, two or more target genes or gene products thereof selected from the target genes listed in Table 7 are modulated.

[0087] In some aspects, provided herein are methods of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 8. In one embodiment of this aspect and all other aspects provided herein, two or more target genes or gene products thereof selected from the target genes listed in Table 8 are modulated.

[0088] In some aspects, provided herein are methods of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 9. In one embodiment of this aspect and all other aspects provided herein, two or more target genes or gene products thereof selected from the target genes listed in Table 9 are modulated.

[0089] In one embodiment of this aspect and all other aspects provided herein, the T-cell dysfunction is T-cell exhaustion.

[0090] In one embodiment of this aspect and all other aspects provided herein, the modulation of T-cell exhaustion comprises a decrease in the exhausted T-cell phenotype, such that functional T-cell activity is increased.

[0091] In another embodiment of this aspect and all other aspects provided herein, the modulation of T cell exhaustion comprises an increase in the exhausted T cell phenotype, such that T cell activation is decreased.

[0092] In one embodiment of this aspect and all other aspects provided herein, the selected target gene or gene product or a combination thereof is/are identified as participating in the inhibition of functional T-cell activity.

[0093] In one embodiment of this aspect and all other aspects provided herein, the modulating agent inhibits the expression, activity and/or function of the selected target gene or gene product or combination thereof.

[0094] In one embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the promotion of functional T-cell activity.

[0095] In one embodiment of this aspect and all other aspects provided herein, the modulating agent promotes or activates the expression, activity and/or function of the selected target gene or gene product or combination thereof.

[0096] In one embodiment of this aspect and all other aspects provided herein, the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

[0097] In one embodiment of this aspect and all other aspects provided herein, the method further comprises contacting the dysfunctional T-cell with an agent or treatment selected from the group consisting of a PD-1 inhibitor, a CTLA4 inhibitor, chemotherapy, radiation therapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and/or SLAMF7.

[0098] Also provided herein in another aspect is method of treating a condition involving or characterized by the presence of T cells exhibiting a dysfunctional or exhausted phenotype, the method comprising administering an amount of a modulating agent effective to modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9.

[0099] In one embodiment of this aspect and all other aspects provided herein, the condition is cancer or a persistent infection.

[00100] In one embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

[00101] In one embodiment of this aspect and all other aspects provided herein, the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

[00102] In one embodiment of this aspect and all other aspects provided herein, the selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

[00103] In one embodiment of this aspect and all other aspects provided herein, the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

[00104] In one embodiment of this aspect and all other aspects provided herein, the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

[00105] In some aspects, provided herein are pharmaceutical compositions for modulating T cell dysfunction, the composition comprising a first modulating agent and a second modulating agent that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9.

[00106] In some aspects, provided herein are pharmaceutical compositions for modulating T cell dysfunction, the composition comprising a first modulating agent that inhibits the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9 and a second modulating agent that promotes the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9.

[00107] In another aspect, the present invention provides for an isolated immune cell modified to comprise an altered expression or activity of at least one gene listed in Table 1 or Table 2. The immune cell may be a T cell, preferably a CD8⁺ T cell. In preferred embodiments, the immune cell is a CD8⁺ T cell. The immune cell may display tumor specificity. The immune cell may have been isolated from a tumor of a subject, preferably the immune cell is a tumor infiltrating lymphocyte. The immune cell may comprise a tumor-specific T cell receptor or a tumor-specific chimeric antigen receptor (CAR). Not being bound by a theory, modulation of expression or activity results in a more activated or less dysfunctional T cell. Not being bound by a theory, dysfunctional autologous T cells may be used for generating a CAR T cell. Alternatively, non-dysfunctional T cells may be used to generate CAR T cells that are modified to prevent them from becoming dysfunctional. The isolated immune cell may be modified to comprise downregulated or abolished expression or activity of at least one gene listed in Table 1 or Table 2. An endogenous gene may be modified, whereby the cell comprises downregulated or abolished expression or activity of at least one gene listed in Table 1 or Table 2. The endogenous gene may be modified using a nuclease. The nuclease may comprise (i) a DNA-binding portion configured to specifically bind to the endogenous sequence of at least one gene listed in Table 1 or Table 2 and (ii) a DNA cleavage portion. The DNA-binding portion may comprise a zinc finger protein or DNA-binding domain thereof, a transcription activator-like effector (TALE) protein or DNA-binding domain thereof, or an RNA-guided protein or DNA-binding domain thereof. The DNA-binding portion may comprise (i) a Cas protein modified to eliminate its nuclease activity, or (ii) DNA-binding domain of a Cas protein. The DNA cleavage portion may

comprise FokI or variant thereof or DNA cleavage domain of FokI or variant thereof. The nuclease may be an RNA-guided nuclease, such as a Cas protein. The cell may comprise a protein comprising a DNA-binding portion configured to specifically bind to at least one gene listed in Table 1 or Table 2. The protein may be a heterologous repressor protein capable of repressing the transcription of at least one gene listed in Table 1 or Table 2. The heterologous repressor protein may comprise at least a DNA-binding portion configured to specifically bind to at least one gene listed in Table 1 or Table 2, preferably to the endogenous promoter of the gene. The heterologous repressor protein may comprise (i) a DNA-binding portion configured to specifically bind to at least one gene listed in Table 1 or Table 2, preferably to the endogenous promoter of the gene, and (ii) a transcription repression portion. The DNA-binding portion may comprise a zinc finger protein or DNA-binding domain thereof, TALE protein or DNA-binding domain thereof, or RNA-guided nuclease protein or DNA-binding domain thereof. The DNA-binding portion may comprise (i) a Cas protein modified to eliminate its nuclease activity, or (ii) DNA-binding domain of a Cas protein.

[00108] In another aspect, the present invention provides for an isolated immune cell modified to comprise an agent capable of inducibly altering expression or activity of at least one gene listed in Table 1 or Table 2. The agent may comprise: a nuclease capable of modifying at least one gene listed in Table 1 or Table 2, such as to downregulate or abolish expression of the gene, such as the nuclease as defined in any embodiment herein; or a heterologous repressor protein capable of repressing the transcription of the gene, such as the heterologous repressor protein as defined in any embodiment herein.

[00109] In another aspect, the present invention provides for an isolated immune cell modified to comprise an altered expression or activity of PDPN. The immune cell may be a T cell, preferably a CD8⁺ T cell. In preferred embodiments, the immune cell is a CD8⁺ T cell. The immune cell may display tumor specificity. The immune cell may have been isolated from a tumor of a subject, preferably the immune cell is a tumor infiltrating lymphocyte. The immune cell may comprise a tumor-specific T cell receptor or a tumor-specific chimeric antigen receptor (CAR). Not being bound by a theory, modulation of expression or activity results in a more activated or less dysfunctional T cell. Not being bound by a theory, dysfunctional autologous T cells may be used for generating a CAR T cell. Alternatively, non-dysfunctional T cells may be used to generate CAR T cells that are modified to prevent them from becoming dysfunctional. The isolated immune cell may be modified to comprise downregulated or abolished expression or activity of PDPN. The endogenous PDPN gene

may be modified, whereby the cell comprises downregulated or abolished expression or activity of PDPN. The endogenous PDPN gene may be modified using a nuclease. The nuclease may comprise (i) a DNA-binding portion configured to specifically bind to the endogenous PDPN gene and (ii) a DNA cleavage portion. The DNA-binding portion may comprise a zinc finger protein or DNA-binding domain thereof, a transcription activator-like effector (TALE) protein or DNA-binding domain thereof, or an RNA-guided protein or DNA-binding domain thereof. The DNA-binding portion may comprise (i) a Cas protein modified to eliminate its nuclease activity, or (ii) DNA-binding domain of a Cas protein. The DNA cleavage portion may comprise FokI or variant thereof or DNA cleavage domain of FokI or variant thereof. The nuclease may be an RNA-guided nuclease, such as a Cas protein. The cell may comprise a protein comprising a DNA-binding portion configured to specifically bind to the endogenous PDPN gene. The protein may be a heterologous repressor protein capable of repressing the transcription of the endogenous PDPN gene. The heterologous repressor protein may comprise at least a DNA-binding portion configured to specifically bind to the endogenous PDPN gene, preferably to the endogenous PDPN gene promoter. The heterologous repressor protein may comprise (i) a DNA-binding portion configured to specifically bind to the endogenous PDPN gene, preferably to the endogenous PDPN gene promoter, and (ii) a transcription repression portion. The DNA-binding portion may comprise a zinc finger protein or DNA-binding domain thereof, TALE protein or DNA-binding domain thereof, or RNA-guided nuclease protein or DNA-binding domain thereof. The DNA-binding portion may comprise (i) a Cas protein modified to eliminate its nuclease activity, or (ii) DNA-binding domain of a Cas protein.

[00110] In another aspect, the present invention provides for an isolated immune cell modified to comprise an agent capable of inducibly altering expression or activity of PDPN. The agent may comprise: a nuclease capable of modifying the endogenous PDPN gene, such as to downregulate or abolish expression of PDPN, such as the nuclease as defined in any embodiment herein; or a heterologous repressor protein capable of repressing the transcription of the endogenous PDPN gene, such as the heterologous repressor protein as defined in any any embodiment herein.

[00111] In another aspect, the present invention provides for an isolated immune cell modified to comprise an altered expression or activity of PRDM1 and/or c-MAF. The immune cell may be a T cell, preferably a CD8⁺ T cell. In preferred embodiments, the immune cell is a CD8⁺ T cell. The immune cell may display tumor specificity. The immune cell may have been isolated from a tumor of a subject, preferably the immune cell is a tumor

infiltrating lymphocyte. The immune cell may comprise a tumor-specific chimeric antigen receptor (CAR). Not being bound by a theory, modulation of expression or activity results in a more activated or less dysfunctional T cell. Not being bound by a theory, dysfunctional autologous T cells may be used for generating a CAR T cell. Alternatively, non-dysfunctional T cells may be used to generate CAR T cells that are modified to prevent them from becoming dysfunctional. The isolated immune cell may be modified to comprise downregulated or abolished expression or activity of PRDMI and/or c-MAF. The endogenous PRDMI and c-MAF gene may be modified, whereby the cell comprises downregulated or abolished expression or activity of PRDMI and/or c-MAF. Preferably, the cell comprises downregulated or abolished expression or activity of PRDMI and c-MAF.

[00112] Alternatively, the endogenous PRDMI and c-MAF genes may be modified, whereby the cell comprises upregulated expression or activity of PRDMI and/or c-MAF. Alternatively, expression or activity may be modified by introducing a transgene. Not being bound by a theory, providing an immune cell with abolished expression or activity of both PRDMI and c-MAF results in decreasing a dysfunctional phenotype of the immune cell or renders the immune cell more resistant to becoming dysfunctional, whereas a dysfunctional phenotype is not affected when only one of PRDMI or c-MAF has abolished expression or activity. Not being bound by a theory, providing an immune cell with increased expression or activity of either one of or both of PRDMI and/or c-MAF results in increasing a dysfunctional phenotype of the immune cell.

[00113] The endogenous PRDMI and c-MAF genes may be modified using a nuclease. The nuclease may comprise (i) a DNA-binding portion configured to specifically bind to the endogenous PRDMI and/or c-MAF gene and (ii) a DNA cleavage portion. The DNA-binding portion may comprise a zinc finger protein or DNA-binding domain thereof, a transcription activator-like effector (TALE) protein or DNA-binding domain thereof, or an RNA-guided protein or DNA-binding domain thereof. The DNA-binding portion may comprise (i) a Cas protein modified to eliminate its nuclease activity, or (ii) DNA-binding domain of a Cas protein. The DNA cleavage portion may comprise FokI or variant thereof or DNA cleavage domain of FokI or variant thereof. The nuclease may be an RNA-guided nuclease, such as a Cas protein. More than one guide RNA may be used to target PRDMI and/or c-MAF. In certain embodiments, multiple guides target each gene. The cell may comprise a protein comprising a DNA-binding portion configured to specifically bind to the endogenous PRDMI and/or c-MAF gene. The protein may be a heterologous repressor protein capable of repressing the transcription of the endogenous PRDMI and/or c-MAF gene. The

heterologous repressor protein may comprise at least a DNA-binding portion configured to specifically bind to the endogenous PRDM1 and/or c-MAF gene, preferably to the endogenous PRDM1 and/or c-MAF gene promoter. The heterologous repressor protein may comprise (i) a DNA-binding portion configured to specifically bind to the endogenous PRDM1 and/or c-MAF gene, preferably to the endogenous PRDM1 and/or c-MAF gene promoter, and (ii) a transcription repression portion. The DNA-binding portion may comprise a zinc finger protein or DNA-binding domain thereof, TALE protein or DNA-binding domain thereof, or RNA-guided nuclease protein or DNA-binding domain thereof. The DNA-binding portion may comprise (i) a Cas protein modified to eliminate its nuclease activity, or (ii) DNA-binding domain of a Cas protein.

[00114] In another aspect, the present invention provides for an isolated immune cell modified to comprise an agent capable of inducibly altering expression or activity of PRDM1 and/or c-MAF. The agent may comprise: a nuclease capable of modifying the endogenous PRDM1 and/or c-MAF gene, such as to downregulate or abolish expression of PRDM1 and c-MAF, such as the nuclease as defined in any embodiment herein; or a heterologous repressor protein capable of repressing the transcription of the endogenous PRDM1 and c-MAF gene, such as the heterologous repressor protein as defined in any any embodiment herein. The agent may comprise more than one nuclease. In certain embodiments, the agent comprises more than one TALE or zinc finger protein, whereby one TALE or Zinc finger targets PRDM1 and one targets c-MAF. In other embodiments, the agent comprises more than two nucleases, capable of targeting multiple genes. In certain embodiments, a CRISPR-Cas system is used and multiple guide RNAs are used to target the CRISPR enzyme to multiple gene targets.

[00115] In another aspect, the present invention provides for an isolated immune cell modified to comprise an altered expression or activity of PROCR. The immune cell may be a T cell, preferably a CD8⁺ T cell. In preferred embodiments, the immune cell is a CD8⁺ T cell. The immune cell may display tumor specificity. The immune cell may have been isolated from a tumor of a subject, preferably the immune cell is a tumor infiltrating lymphocyte. The immune cell may comprise a tumor-specific chimeric antigen receptor (CAR). Not being bound by a theory, modulation of expression or activity results in a more activated or less dysfunctional T cell. Not being bound by a theory, dysfunctional autologous T cells may be used for generating a CAR T cell. Alternatively, non-dysfunctional T cells may be used to generate CAR T cells that are modified to prevent them from becoming dysfunctional. The isolated immune cell may be modified to comprise downregulated or

abolished expression or activity of PROCR. The endogenous PROCR gene may be modified, whereby the cell comprises downregulated or abolished expression or activity of PROCR. The endogenous PROCR gene may be modified using a nuclease. The nuclease may comprise (i) a DNA-binding portion configured to specifically bind to the endogenous PROCR gene and (ii) a DNA cleavage portion. The DNA-binding portion may comprise a zinc finger protein or DNA-binding domain thereof, a transcription activator-like effector (TALE) protein or DNA-binding domain thereof, or an RNA-guided protein or DNA-binding domain thereof. The DNA-binding portion may comprise (i) a Cas protein modified to eliminate its nuclease activity, or (ii) DNA-binding domain of a Cas protein. The DNA cleavage portion may comprise FokI or variant thereof or DNA cleavage domain of FokI or variant thereof. The nuclease may be an RNA-guided nuclease, such as a Cas protein. The cell may comprise a protein comprising a DNA-binding portion configured to specifically bind to the endogenous PROCR gene. The protein may be a heterologous repressor protein capable of repressing the transcription of the endogenous PROCR gene. The heterologous repressor protein may comprise at least a DNA-binding portion configured to specifically bind to the endogenous PROCR gene, preferably to the endogenous PROCR gene promoter. The heterologous repressor protein may comprise (i) a DNA-binding portion configured to specifically bind to the endogenous PROCR gene, preferably to the endogenous PROCR gene promoter, and (ii) a transcription repression portion. The DNA-binding portion may comprise a zinc finger protein or DNA-binding domain thereof, TALE protein or DNA-binding domain thereof, or RNA-guided nuclease protein or DNA-binding domain thereof. The DNA-binding portion may comprise (i) a Cas protein modified to eliminate its nuclease activity, or (ii) DNA-binding domain of a Cas protein.

[00116] In another aspect, the present invention provides for an isolated immune cell modified to comprise an agent capable of inducibly altering expression or activity of PROCR. The agent may comprise: a nuclease capable of modifying the endogenous PROCR gene, such as to downregulate or abolish expression of PROCR, such as the nuclease as defined in any embodiment herein; or a heterologous repressor protein capable of repressing the transcription of the endogenous PROCR gene, such as the heterologous repressor protein as defined in any any embodiment herein.

[00117] The isolated immune cell according to any embodiment described herein, may be further modified to comprise: an altered expression or activity of PDPN; an altered expression or activity of PRDM1 and/or c-MAF; an altered expression or activity of PROCR; an altered expression or activity of any one or more of PD1, CTLA4, TIGIT, TIM3, LAG3,

or PDL1; an altered expression or activity of any one or more of TIGIT, LAG3, LILRB4, or KLRC1; an altered expression or activity of any one or more of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, or SLAMF7; an altered expression or activity of any one or more of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM or KLRC1; an altered expression or activity of any one or more of BTLA, TIGIT, HAVCR2 (TIM-3), LAG3, PDPN, IL10RA, IL1R2, PROCR, LILRB4, KLRC1, KLRC2, KLRE1, TNFSF9 (4-1BB), KLRK1, IL12RB1, IL1R1, or SLAMF7; an agent capable of inducibly altering expression or activity of PDPN; an agent capable of inducibly altering expression or activity of PRDM1 and c-MAF; an agent capable of inducibly altering expression or activity of PROCR; an agent capable of inducibly altering expression or activity of any one or more of PD1, CTLA4, TIGIT, TIM3, LAG3, or PDL1; an agent capable of inducibly altering expression or activity of any one or more of TIGIT, LAG3, LILRB4, or KLRC1; an agent capable of inducibly altering expression or activity of any one or more of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, or SLAMF7; an agent capable of inducibly altering expression or activity of any one or more of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM or KLRC1; or an agent capable of inducibly altering expression or activity of any one or more of BTLA, TIGIT, HAVCR2 (TIM-3), LAG3, PDPN, IL10RA, IL1R2, PROCR, LILRB4, KLRC1, KLRC2, KLRE1, TNFSF9 (4-1BB), KLRK1, IL12RB1, IL1R1, or SLAMF7. The agent may comprise more than one nuclease. In certain embodiments, the agent comprises more than one TALE or zinc finger protein, whereby one TALE or Zinc finger targets one gene and one targets another gene. In other embodiments, the agent comprises more than two nucleases, capable of targeting multiple genes. In certain embodiments, a CRISPR-Cas system is used and multiple guide RNAs are used to target the CRISPR enzyme to multiple gene targets.

[00118] In another aspect, the present invention provides for a cell population of immune cells as defined in any embodiment herein.

[00119] In another aspect, the present invention provides for a method for generating the modified immune cell of any embodiment described herein, the method comprising (i) providing an isolated immune cell, and (ii) modifying said isolated immune cell such as to comprise an altered expression or activity of PDPN, PROCR, or PRDM1 and/or c-MAF, preferably PRDM1 and c-MAF.

[00120] In another aspect, the present invention provides for a method for generating the modified immune cell of any embodiment described herein, the method comprising (i) providing an isolated immune cell, and (ii) modifying said isolated immune cell such as to

comprise an agent capable of inducibly altering expression or activity of PDPN, PROCR, or PRDM1 and c-MAF.

[00121] In certain embodiments, the step of providing the isolated immune cell comprises providing the immune cell isolated from a subject, or isolating the immune cell from a subject. The immune cell isolated from the subject preferably expresses PDPN, PROCR, and/or PRDM1 and c-MAF. The immune cell isolated from the subject may be dysfunctional or may be not dysfunctional. Not being bound by a theory, a dysfunctional cell may be modulated to have an activation phenotype and a nondysfunctional cell may be modulated to have an enhanced activation phenotype. The immune cell isolated from the subject may express a signature of dysfunction as defined herein. The method may further comprise the step of expanding the isolated immune cell prior to and/or subsequent to the modification.

[00122] In another aspect, the present invention provides for a pharmaceutical composition comprising the isolated immune cell or the cell population according to any embodiment described herein. The isolated immune cell or the cell population may be for use in therapy. The isolated immune cell or the cell population may be for use in immunotherapy or adoptive immunotherapy, preferably immunotherapy or adoptive immunotherapy of a proliferative disease, such as a tumor or cancer, or a chronic infection, such as a chronic viral infection. The isolated immune cell or cell population may be for use according in a subject, wherein the subject has been determined to comprise immune cells which: express PDPN, PROCR and/or PRDM1 and/or c-MAF, preferably PRDM1 and c-MAF; are dysfunctional, or are not dysfunctional; or express a signature of dysfunction as defined herein.

[00123] In another aspect, the present invention provides for a method of treating a subject in need thereof, preferably a subject in need of immunotherapy or adoptive immunotherapy, more preferably immunotherapy or adoptive immunotherapy of a proliferative disease, such as a tumor or cancer, or a chronic or persistent infection, such as a chronic viral infection, comprising administering to said subject the isolated immune cell or the cell population of any embodiment described herein. The method may further comprise administering to said subject one or more other active pharmaceutical ingredient, preferably wherein said one or more other active pharmaceutical ingredient is useful in immunotherapy or adoptive immunotherapy, or wherein said one or more other active pharmaceutical ingredient is useful in the treatment of a proliferative disease, such as a tumor or cancer, or a chronic infection, such as a chronic viral infection. The one or more other active pharmaceutical ingredient may be: an agonist of a cell molecule, such as a cell surface molecule, which when activated is capable of upregulating immune response, such as one or more of an agonist of 4-1BB, an

agonist of OX40, an agonist of GITR, an agonist of STING, an agonist of TLR, or an agonist of BTLA; and/or an inhibitor of a cell molecule, such as a cell surface molecule, which when not inhibited is capable of downregulating immune response, such as a checkpoint inhibitor, or such as one or more of an antagonist of PDI, an antagonist of CTLA4, an antagonist of BTLA, an antagonist of TIGIT, an antagonist of TIM3, an antagonist of LAG3, an antagonist of VISTA, an antagonist of LILRB4, an antagonist of CD160, an antagonist of CD274, or an antagonist of IDO. The subject may comprise immune cells which: express PDPN, PROCR, PRDMI and/or c-MAF; are dysfunctional, or are not dysfunctional; or express a signature of dysfunction as defined herein. Non-limiting examples on immunotherapeutics that may be used in the claimed methods or in conjunction with the claimed compositions include IMP321, BMS-986016, LAG525, TSR022, MTIG7192A, TRX518, INCAGN01876, GWN323, MEDI1873, MEDI9447, PF-05082566 (utomilumab), BMS-663513 (urelumab), MOXR0916, MEDI6469, MEDI6383, PF04518600, KHK4083, and combinations of two or more thereof.

[00124] In another aspect, the present invention provides for a method of treating a subject in need thereof, preferably a subject in need of immunotherapy or adoptive immunotherapy, more preferably immunotherapy or adoptive immunotherapy of a proliferative disease, such as a tumor or cancer, or a chronic infection, such as a chronic viral infection, comprising: providing an isolated immune cell from the subject, or isolating an immune cell from a subject; modifying said isolated immune cell such as to comprise an altered expression or activity of PDPN, PROCR, and/or PRDMI and/or c-MAF, or modifying said isolated immune cell such as to comprise an agent capable of inducibly altering expression or activity of PDPN, PROCR, and/or PRDMI and c-MAF; and reintroducing the modified isolated immune cell to the subject. The immune cell isolated from the subject: may express PDPN, PROCR, and/or PRDMI and c-MAF; may be dysfunctional or is not dysfunctional; or may express a signature of dysfunction as defined herein. The method may further comprise the step of expanding the isolated immune cell prior to and/or subsequent to the modification, and before reintroduction to the subject. The subject may additionally be treated with known immunotherapies, including but not limited to, IMP321, BMS-986016, LAG525, TSR022, MTIG7192A, TRX518, INCAGN01876, GWN323, MEDI1873, MEDI9447, PF-05082566 (utomilumab), BMS-663513 (urelumab), MOXR0916, MEDI6469, MEDI6383, PF04518600, KHK4083, and combinations of two or more thereof.

[00125] In another aspect, the present invention provides for a method of detecting dysfunctional immune cells comprising detection of a gene expression signature comprising

one or more markers selected from the group consisting of Abcal, Adam8, Adam9, Alcam, Ccl5, Ccl9, Ccl9, Ccl9, Ccr2, Ccr5, Cd68, Cd93, Cxcl10, Cysltr2, Ddrl, Entpd1, Entpd1, Epcam, Gabarapl1, Gcntl, Gpr65, Haver2, Ifitml, Ifitm3, 1110, IllOra, I112rb1, I113ral, Illr1, Illr2, 1121, I12ra, I12rb, 1133, I16st, Inhba, Isg20, Klrc2, Klrc2, Klrc2, Klrc2, Klrc2, Klrc2, Klrdl, Klrkl, Lag3, Lamp2, Lpar3, Ly75, Ly75, Nampt, Olfml, Pdpn, Pglyrpl, Procr, Pstpipl, Ptpn3, Sdcl, Sdc4, Selp, Sema7a, Slamf7, Sppl, Tgfb3, Tigit, Tnfrsf8, Tnfsf9, Vldlr, Bst2, Btla, Cell, Ccr4, Cd226, Cd401g, Cd83, Cd8a, Csf2, Cxcl3, Cxcr4, Ifitm3, Isg20, Lap3, Lif, Serpincl, Timp2, Tnfsf11, Acvrl1, Ada, Are, Bmp2, Bmprla, ccl22, Ccr6, Ccr8, Cdl60, Cd200r4, Cd24a, Cd70, Cd74, Cmtm7, Csf1, Ctla2a, Ctla2b, Ctsd, Ctsl, Dkl, Enpep, Enpp1, Eps8, F2r, Fgf2, Flt31, H2-AM, Hspbl, Ifhgrl, I112rb2, 1118, I118r1, I118rap, 112, 1124, I127ra, 114, I14ra, I17r, Itga4, Itga7, Itga9, Klrc1, Klrel, Lpar2, Lta, Ly6a, Ly6e, Nlgn2, Nrpl, Flt31, H2-Ab2, Hspb2, Ifngr2, I112rb3, 1119, I118r2, I118rap, 1146, 1168, I127ra, 115, Smpdl, Tgdb3, Tirap, Tnfrsf13c, Tnfrsf23, Tnfsf10, Tnfsf4, Trem12, Trpcl, Trpm4, Tspan32, and Xcll; or selected from the group consisting of ABCA1, ADAM8, ADAM9, ALCAM, CCL5, CCL15, CCL23, CCL15-CCL14, CCR2, CCR2, CD68, CD93, CXCL10, CYSLTR2, DDR1, ENTPD1, EPCAM, GABARAPL1, GCNT1, GPR65, HAVCR2, IFITM1, IFITM1, IL10, IL10RA, IL12RB1, IL13RA1, IL1R1, IL1R2, IL21, IL2RA, IL2RB, IL33, IL6ST, INHBA, ISG20, KLRC4-KLRK1, KLRC4, KLRC1, KLRC3, KLRC2, KLRD1, KLRK1, LAG3, LAMP2, LPAR3, LY75-CD302, LY75, NAMPT, OLFM1, PDPN, PGLYRP1, PROCR, PSTPIP1, PTPN3, SDC1, SDC4, SELP, SEMA7A, SLAMF7, SPP1, TGFB3, TIGIT, TNFRSF8, TNFSF9, VLDLR, BST2, BTLA, CCL1, CCR4, CD226, CD40LG, CD83, CD8A, CSF2, CXCL13, CXCR4, IFITM1, ISG20, LAP3, LIF, SERPINC1, TIMP2, TNFSF11, ACVRL1, ADA, BMPR1A, CCR5, CD160, CD24, CMTM7, CSF1, CTSD, CTSL1, CYSLTR2, ENPP1, EPS8, F2R, FLT3LG, HSPB1, IFNGR1, IL18, IL18R1, IL18RAP, IL24, IL24, IL27RA, IL27RA, IL4R, IL7R, ITGA4, ITGA7, LY6E, NLGN2, NRPI, OSM, PDE4B, PEAR1, PLXNC1, PRNP, PRNP, PRNP, PTRPJ, SIPRI, SDC1, SELL, SEMA4D, SERPINE2, SERPINE2, SMPD1, TIRAP, TNFSF10, TRPC1, TRPM4, and XCL1.

[00126] In another aspect, the present invention provides for a method of detecting dysfunctional immune cells comprising detection of a gene expression signature comprising one or more markers selected from the group consisting of ABCA1, ADAM8, ADAM9, ALCAM, CCL5, CCL9, CCR2, CCR5, CD68, CD93, CTLA2A, CXCL10, CYSLTR2, ENTPD1, EPCAM, GABARAPL1, GCNT1, GPR65, HAVCR2, IFITM1, IFITM3, IL10IL10RA, IL12RB1, IL13RA1, IL1R1, IL1R2, IL21, IL2RA, IL2RB, IL33, IL6ST,

INHBA, ISG20, KLRC2, KLRD1, KLRE1, KLRK1, LAG3, LAMP2, LILRB4, LPAR3, LY75, NAMPT, OLFM1, PDPN, PGLYRP1, PROCR, PSTPIP1, PTPN3, SDC1, SDC4, SELP, SEMA7A, SLAMF7, SPP1, TGFB3, TIGIT, TNFRSF8, TNFSF9, and VLDLR.

[00127] In another aspect, the present invention provides for a method of detecting dysfunctional immune cells comprising detection of a gene expression signature comprising one or more markers selected from the group consisting of IL33, KLRC2, KLRD1, KLRE1, OLFM1, PDPN, PTPN3, SDC1, TNFSF9, VLDLR, PROCR, GABARAPL1, SPP1, ADAM8, LPAR3, CCL9, CXCL10, CCR2, IL10RA, IL2RB, CD68, KLRK1, IL12RB2, IL6ST, IL7R, INHBA, ISG20, LAMP2, LY75, NAMPT, S1PR1, IL21, IL13RA1, TIGIT, CCR5, ALCAM, HAVCR2, LAG3, IL1R2, CYSLTR2, ENTPD1, GCNT1, IFITM3, IL2RA, PGLYRP1, CD93, ADAM9, LILRB4, IL-10, CTLA2A, and GPR65.

[00128] Any of the signatures described herein may comprise at least two markers, or at least three markers, or at least four markers, or at least five markers, or six or more markers, such as wherein the signature consists of two markers, three markers, four markers, or five markers. Any of the signatures described herein may comprise two or more markers, and wherein: one of said two or more markers is PDPN; one of said two or more markers is PROCR; or two of said two or more markers are PDPN and PROCR.

[00129] In another aspect, the present invention provides for a method of isolating a dysfunctional immune cell comprising binding of an affinity ligand to a signature gene as defined in any embodiment herein, wherein the signature gene is expressed on the surface of the immune cell.

[00130] In another aspect, the present invention provides for a kit of parts comprising means for detection of the signature of dysfunction as defined in any embodiment herein.

[00131] Accordingly, it is an object of the invention not to encompass within the invention any previously known product, process of making the product, or method of using the product such that Applicants reserve the right and hereby disclose a disclaimer of any previously known product, process, or method. It is further noted that the invention does not intend to encompass within the scope of the invention any product, process, or making of the product or method of using the product, which does not meet the written description and enablement requirements of the USPTO (35 U.S.C. §112, first paragraph) or the EPO (Article 83 of the EPC), such that Applicants reserve the right and hereby disclose a disclaimer of any previously described product, process of making the product, or method of using the product. It may be advantageous in the practice of the invention to be in compliance with Art. 53(c) EPC and Rule 28(b) and (c) EPC. All rights to explicitly disclaim any embodiments that are

the subject of any granted patent(s) of applicant in the lineage of this application or in any other lineage or in any prior filed application of any third party is explicitly reserved. Nothing herein is to be construed as a promise.

[00132] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

[00133] These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[00134] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[00135] The following detailed description, given by way of example, but not intended to limit the invention solely to the specific embodiments described, may best be understood in conjunction with the accompanying drawings.

[00136] **Figure 1A-M.** illustrates that IL-27 induces multiple co-inhibitory receptors on CD4⁺ and CD8⁺ T cells. CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes (TILs) harvested from WT mice bearing B16F10 melanoma tumors. **A)** Naive T cells from either wild type (WT) or IL-27ra deficient mice (IL27ra KO) were stimulated with anti-CD3/CD28 in the presence or absence of IL-27 as indicated. Expression of the indicated co-inhibitory molecules was examined by real-time PCR at 96hr (CD4) and 72hr (CD8), n >3, error bars indicate s.e.m. **B)** Surface expression of co-inhibitory receptors on T cells stimulated as in (A) was determined by flow cytometry. Representative data are shown. **C)** Co-expression analysis of co-inhibitory and co-stimulatory receptor mRNA expression as determined by single cell RNAseq (316 and 516 for CD4⁺ and CD8⁺ respectively). For visualization purposes negative correlation values were set to zero. **D)** Protein expression by CyTOF for 23,656 CD4⁺ and 36,486 CD8⁺ TILs. Co-expression was analyzed using Spearman correlation. For visualization purposes negative correlation values were set to zero. **E)** TILs were harvested from WT and IL27ra KO mice bearing B16F10 melanoma and analyzed

using CyTOF. CyTOF data were analyzed using vi-SNE. Polygons indicating clusters 1, 2 (in CD8⁺ T cells), 3 and 4 (in CD4⁺ T cells) are shown. **F)** The within groups sum of squared error (SSE) plot. The location of the elbow or a bend in the resulting plot suggests a suitable number of clusters for the k-means algorithm, which in this case is somewhere between 7 and 11 clusters. **G)** Gap statistics for estimating the optimal number of clusters using k-means from 1 up to 12 clusters using bootstrapping and first SE max method. This method suggested 9 clusters as optimal. **H)** Applying k-means clustering with (k=9) on our CyTOF data resulted in clear distinction between clusters 1, 2, 3 and 4. Visualization of cluster distribution using two-dimensional non-linear embedding of the protein expression profiles by t-SNE. **I)** CyTOF expression analysis of co-inhibitory and co-stimulatory receptors in TILs harvested from B16F10 melanoma tumor-bearing WT and IL17Ra KO mice from Figure 1A and 1J using t-SNE. **J)** vi-SNE plot highlighting the distribution of CD8⁺ TILs from WT (red) and IL27ra KO (blue) mice in clusters 1 and 2 and CD4⁺ TILs from WT (red) and IL27ra KO (blue) mice in clusters 3 and 4. Pie charts show the distribution of WT or IL27ra KO CD8⁺ and CD4⁺ TILs in each cluster. Bar graphs show the mean of signal intensity for PD-1, Tim-3, Lag-3, and TIGIT from WT and IL27ra KO TILs. Error bars are the standard error and p-values for significance are calculated using standard t-test (**p < 0.01). **K)** Expression of PD-1, Tim-3, Lag-3, TIGIT, and IL-10 on CD8⁺ TILs obtained from WT and IL27ra KO mice bearing B16F10 melanoma was determined by flow cytometry. Thyl.1-IL-10 reporter mice crossed with WT and IL27ra KO mice were used for IL-10 expression analysis. **L,M)** Impact of IL-27 signaling on co-inhibitory receptor expression in TILs. Pie charts show the distribution of CD8⁺ and CD4⁺ TILs from WT and IL27ra KO mice bearing B16F10 melanoma between clusters 1 and 2 for CD8⁺ and between clusters 3 and 4 for CD4⁺ TILs as determined by k-means clustering of CyTOF protein expression data. Data are from independent WT and IL27ra KO TILs samples from that shown in Figure 1J.

[00137] Figure 2A-2B. IL-27 inducing inhibitory molecules. FIG. 2A. Naive T cells from either wild type or IL-27ra deficient were stimulated in the presence or absence of IL-27 as indicated. Expression of known co-inhibitory molecules was examined by real-time PCR at 96hr. N ≥ 3, error bars indicate SD **FIG. 2B.** Surface expression of co-inhibitory receptors on T cells stimulated as in was examined by flow cytometry. Representative data are shown.

[00138] Figure 3A-3B. IL-27 inducing inhibitory molecules. FIG. 3A. Naive T cells from either wild type or IL-27ra deficient were stimulated in the presence or absence of IL-27 as indicated. Expression of known co-inhibitory molecules was examined by real-time PCR at

72hr. $N \geq 3$, error bars indicate SD. **FIG. 3B.** Surface expression of co-inhibitory receptors on T cells stimulated as in was examined by flow cytometry. Representative data are shown.

[00139] **Figure 4.** TILs were harvested from WT and IL27ra deficient mice bearing B16F10 melanoma and analyzed using CyTOF. Right panel shows TILs from WT (blue) and IL27ra KO (red). All data were analyzed using vi-SNE. Right top). Graphical representation of the distribution of CD8⁺ TILs in cluster 1 and cluster 2 in WT and IL27ra KO CD8⁺ TILs.

[00140] **Figure 5.** IL-27 inducing inhibitory molecules and PD-1 expression in TILs. Surface molecule expression on TILs from WT and IL27ra^{-/-}. Surface molecules expression on CD8 TILs obtained from WT and WSX- $\Gamma^{-/-}$ mice bearing B16F10 melanoma was analyzed by FACS.

[00141] **Figure 6A-0.** The IL-27-driven gene signature overlaps with multiple signatures of T cell dysfunction and tolerance and includes cytokines and cell-surface molecules. Temporal analysis of gene expression during the differentiation of **A)** CD4⁺ and **B)** CD8⁺ T cells from WT and IL27ra KO mice upon IL-27 stimulation over different time points. Data were obtained using a custom nanostring code-set containing probes (**Table 16**) for regulatory genes on T cells. Data shown are representative of 3 different experiments. Naive CD4⁺ and CD8⁺ T cells from either WT or IL-27ra KO mice were stimulated with anti-CD3/CD28 in the presence or absence of IL-27 and harvested at 96hr (CD4) and 72hr (CD8) for global gene expression analysis. **C)** Naive CD4⁺ and CD8⁺ T cells from either wild type or IL-27ra KO mice were stimulated with anti-CD3/CD28 in the presence or absence of IL-27 and harvested at 96hr (CD4) and 72hr (CD8) for global gene expression analysis. Expression level of 118 genes encoding cell surface receptors and cytokines are shown as a heatmap. **D)** Naive CD4⁺ and CD8⁺ T cells from either WT or IL-27ra KO mice were stimulated with anti-CD3/CD28 in the presence or absence of IL-27 and harvested at 96hr (CD4) and 72hr (CD8) for global gene expression analysis. 118 genes encoding cell surface receptors and cytokines are shown as in Figure 6C. **E)** Pearson correlation between the samples described in (D) for all 1,392 genes that were differentially expressed between WT CD4⁺ T cells stimulated in the presence or absence of IL-27 (Fold change >2 and FDR < 0.2). **F)** Corresponding gene expression heatmap for all 1,392 genes in (E). **G)** Graphical representation of the overlap of IL-27-signature up-regulated genes with genes expressed in several different dysfunctional or tolerant T cell states. The width of the gray bars reflects the extent of overlap across groups. **H)** IL-27 driven surface molecules overlapped with regulatory signatures. Five different T cells from regulatory state: CD8 TILs from cancer environment, virus-antigen specific CD8 T cells from chronic virus infection, anergic CD4 T

cells, over stimulated CD4 T cells by anti-CD3 antibody, tolerated CD4 T cells. All the molecules shown were differentially expressed by IL-27 stimulation and appeared on Venn figures overlapped with each regulatory T cell state. Highlighted molecules were further biologically validated. **I)** Pearson correlation between WT CD4⁺ and CD8⁺ T cells for the 1,392 genes that were differentially expressed between WT CD4⁺ T cells stimulated in the presence or absence of IL-27 (Fold change >2 and FDR < 0.2). **J)** IL-27 signature genes were compared to T cell signatures obtained from five states of T cell impairment/tolerance/dysfunction. Number (left panel) and frequency (right panel) of overlapping genes between the IL-27 signature and each signature is depicted. P values were determined by hypergeometric test: Anergy - 3.2e-05, Nasal anti-CD3 - 4.7e-21, Cancer - 1.2e-33, Specific tolerance - 4e-14 and Viral exhaustion - 1.7e-26. **K)** Graphical representation of IL-27-driven soluble and cell surface molecules that overlap between dysfunctional CD8⁺ T cell signatures from cancer and chronic viral infection. All of the molecules depicted were induced by IL-27 stimulation. The shaded background reflects the ranking based on the extent of overlap with the T cell states depicted in G. **L)** Pdpn and Procr protein and mRNA expression was determined in T cells from WT and IL27Ra KO stimulated with anti-CD3/CD28 in the presence or absence of IL-27. CD4⁺ cells were analyzed at 96hr (CD4) and CD8⁺ cells at 72hr (CD8). Representative flow cytometry and qPCR data are shown. **M)** Pdpn and Procr expression on CD8⁺ TILs. Representative flow cytometry data showing Pdpn and Procr expressions with PD-1 and Tim-3 on CD8⁺ TILs obtained from WT and IL27ra KO mice bearing B16F10 melanoma. **N)** TILs from WT mice bearing B16F10 melanoma were stimulated with PMA and Ionomycin. Cytokine production in Procr⁺ or Procr⁻ CD8⁺ TILs is shown. Thy1.1-IL-10 reporter mice were used for IL-10 expression analysis. Statistical significance was determined by paired-t-test (*p < 0.05; **p < 0.01). **O)** panels I-VI, tSNE plots of the 516 CD8⁺ single-cell TILs (dots) harvested from WT mice bearing B16F10 melanoma tumor. Cells are colored in each panel by the relative average expression of the genes in the overlap of the IL-27 gene signature with the signatures for each of the indicated states of T cell non-responsiveness. The contour plot marks the region of highly scored cells by taking into account only those cells that have a signature score above the mean.

[00142] Figure 7A-E. Role of Procr in T cell dysfunction and anti-tumor immunity. **A)** Lack of Procr signaling (EPCRdd) suppresses tumor growth (B16 melanoma). WT (n=8) and Procr^{""} (n=8) mice were implanted with B16F10 melanoma and the change of tumor size were plotted. Left panel, mean tumor size \pm s.e.m. **p < 0.01; ***p < 0.001, t-test. Right

panel, linear regression, $p < 0.001$. Data are from two experiments and are representative of a total of 4 independent experiments. **B)** Top panels, representative flow cytometry data showing cytokine production of CD8⁺ TILs from WT and Procr^{d/d} mice bearing B16F10 melanoma. Bottom panels, summary data. * $p < 0.05$, t-test. **C)** Left panels, representative flow cytometry data showing Tim-3 and PD-1 expression on CD8⁺ TILs from WT and Procr^{d/d} mice bearing B16F10 melanoma. Right panels, summary data. ** $p < 0.01$; *** $p < 0.001$, t-test. **D-E)** T cell intrinsic effects of Procr. 5×10^5 CD8⁺ T cells from wild type or Procr^{dd} mice were transferred along with 1×10^6 wild type CD4⁺ T cells to Rag1^{-/-} mice. On day 2, 5×10^5 B16F10 cells were implanted. **D)**, mean tumor size \pm s.e.m, * $p < 0.05$, t-test. **E)**, linear regression, * $p < 0.05$.

[00143] **Figure 8.** Exemplary data indicating that PROCR is on exhausted CD8 T cells.

[00144] **Figure 9.** Reduced accumulation of exhausted T cells in PR^{d/d} mice.

[00145] **Figure 10A-C.** IL-7R expression on PD-1⁺Tim3⁺ CD8⁺ TILs from wild type and Pdpn cKO mice. TILs were obtained from WT and Pdpn cKO mice bearing B16F10 melanoma and stained for the expression of IL-7R. **A)** Representative flow cytometry data. **B)** Summary data, error bars are the standard error and p-values for significance are calculated using standard t-test (* $p < 0.05$). **C)** Pdpn deficient CD8 T cells maintain IL-7R on PD-1⁺Tim3⁺ cells. IL-7R expression on PD-1⁺Tim3⁺ CD8 TILs is increased in CD4CrePdpnfl/fl mice compared to Pdpnfl/fl mice. TILs were obtained from Pdpnfl/fl and CD4CrePdpnfl/fl mice bearing B16F10 melanoma and stained for the expression of IL-7R and IL-2Ra. Representative data is shown as flow-cytometric schemes and the data from multiple experiments are combined and shown as plots. The t-test provided the statistical p values (* $p < 0.05$). The bars represent the SD.

[00146] **Figure 11A-C.** Role of Pdpn in T cell dysfunction and anti-tumor immunity. **A)** Pdpn fl/fl (WT, n=5) and CD4crePdpnfl/fl (Pdpn cKO, n=5) mice were implanted with B16F10 melanoma. Left panel, mean tumor size \pm s.e.m. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, t-test. Right panel, linear regression $p < 0.001$. Data shown are representative of 3 independent experiments. **B)** Top panels, representative flow cytometry data showing cytokine production of CD8⁺ TILs from WT and Pdpn cKO bearing B16F10 melanoma. Bottom panels, summary data. * $p < 0.05$; *** $p < 0.001$, t-test. **C)** Pdpn deficient CD8 T cells lose PD-1⁺Tim3^{high} sub-population. Lack of Pdpn lost Tim-3^{high} population of CD8 TILs. Left panels, representative flow cytometry data showing Tim-3 and PD-1 expression on CD8⁺ TILs from WT and Pdpn cKO bearing B16F10 melanoma. Right panels, summary data. * $p < 0.05$, t-test.

[00147] Figure 12A-D. Prdml regulate multiple co-inhibitory molecules on T cells in cancer. **A)** Network model based on gene expression data of naive CD8⁺ T cells from Prdml^{f/f} (WT) or CD4^{cre}Prdml^{f/f} (Prdml cKO) mice stimulated in the presence of IL-27 and ChIPseq data for Prdml. Green arrows designate genes up-regulated by Prdml and red arrows designate genes down-regulated by Prdml. Gray arrows designate potential Prdml binding sites on each gene promoter. **B)** Prdml expression in naive CD8⁺ T cell stimulated in the presence of IL-27 and in PD-1⁺Tim-3⁺ CD8⁺ (DP) compared to PD-1⁻Tim-3⁻ CD8⁺ (DN) TILs as determined by global gene expression profiling. *p<0.05 **C)** Representative flow cytometry data showing PD-1, Tim-3, Tigit, Lag3, Procr, and Pdpn expression on CD8⁺ TILs from WT and Prdml cKO mice bearing B16F10 melanoma. *p<0.05, ***p<0.001. **D)** WT (n=5) and Prdml cKO (n=5) mice were implanted with B16F10 melanoma. Mean tumor size \pm s.e.m is shown. Data are representative of 3 independent experiments.

[00148] Figure 13A-D. c-Maf regulates multiple co-inhibitory molecules on T cells in cancer. **A)** Left panel, gene expression in CD8⁺ TILs from WT and Prdml cKO mice bearing B16F10 melanoma was analyzed by n-counter code-set of 397 genes. Differentially expressed genes are shown as a heatmap. Red designates up-regulated genes and blue designates down-regulated genes. Right panel, expression of c-Maf in CD8⁺ TILs from WT and Prdml cKO mice as determined by qPCR. *p < 0.05, t-test. **B)** Expression shown as representative contour plots for PD-1, Tim-3, Tigit, Lag3, Procr, and Pdpn expression on CD8⁺ TILs from Prdml KO and CD4^{cre}c-Maf^{f/f} (c-Maf cKO) as determined by flow cytometry and summarized below *p < 0.05, t-test. **C)** Frequency of co-inhibitory receptor expression of prdml cKO (gray bar) and c-Maf cKO (open bar) CD8⁺ TILs relative to WT (filled bar). **D)** Left panel, c-Maf^{f/f} (WT, n=5) and c-Maf cKO (n=5) mice were implanted with B16F10 melanoma. Mean tumor size \pm s.e.m is shown. Data are representative of 3 independent experiments. Right panel, expression of Prdml in CD8⁺ TILs from WT and c-Maf cKO mice as determined by qPCR.

[00149] Figure 14A-G. Prdml and c-Maf together regulate a co-inhibitory gene module that determines anti-tumor immunity. **A)** Network model based on coupling gene expression data of naive CD8⁺ T cells from Prdml cKO or c-Maf cKO mice stimulated in the presence of IL-27 and CHIP data for Prdml and c-Maf. Green arrows indicate up-regulated genes and red arrows indicate down-regulated genes. Gray arrows indicate potential binding on each promoter region by either Prdml or c-Maf. **B)** Top panels, representative flow cytometry data shown as contour plots for PD-1, Tim-3, Tigit, Lag3, Procr, and Pdpn expression on CD8⁺ TILs from WT and CD4^{cre}Prdml^{f/f}c-Maf^{f/f} (cDKO) bearing B16F10 melanoma. Bottom

panels, summary of expression data by flowcytometry. **p < 0.01; ***p < 0.001, t-test. C) Top panels, representative flow cytometry data showing cytokine production from CD8⁺ TILs WT and Prdml^{fl/fl}c-Maf^{fl/fl} cDKO bearing B16F10 melanoma. Bottom panels, summary data *p < 0.05, t-test. **p<0.01 D) Top panel, WT (n=14) and CD4^{cre}Prdml^{fl/fl}c-Maf^{fl/fl} cDKO (n=8) mice were implanted with B16F10 melanoma. Mean tumor size \pm s.e.m is shown. *p < 0.05, **p < 0.01, t-test. Bottom panel, Linear regression ***p < 0.001. Data shown are pooled from 3 independent experiments. E) 940 differentially expressed genes between CD8⁺ TILs from wild type control (WT) and CD4^{cre}Prdml^{fl/fl}c-Maf^{fl/fl} (cDKO) bearing B16F10 melanoma. (adj. P. value<0.05, likelihood ratio test and FDR correction) (top panel) and their corresponding expression pattern in PD-1⁺Tim-3⁺ CD8⁺ (DP), PD-1⁺Tim-3⁻ CD8⁺ (SP) and PD-1⁻Tim-3⁻ CD8⁺ (DN) TILs (bottom panel). F) Co-inhibitory receptor expression in CD4⁺ TILs from Prdml/c-Maf cDKO mice. Top panels, representative flow cytometry data for TILs from WT and Prdml/c-Maf cDKO stained for PD-1, Tim-3, TIGIT, Pdpn, and Procr expression. Bottom panels show summary data. *p < 0.05, t-test. G) A tSNE plot of the 516 CD8⁺ single-cell tumor-infiltrating lymphocytes (TILs) harvested from WT mice bearing B16F10 melanoma tumors, colored by the relative signature score for co-inhibitory module and the cDKO signature (shown in (E)). The contour plot marks the region of highly scored cells by taking into account only those cells that have a signature score above the mean.

[00150] Figure 15A-C. Comparison of gene expression between Prdml/c-Maf cDKO TILs and CD8⁺ TILs populations from wild type mice. A) Barcode enrichment plot displaying two gene sets in a ranked gene list. The ranked gene list was defined as fold change in gene expression between Prdml/c-Maf cDKO and WT CD8⁺ TILs. The three gene sets consist of differentially expressed genes between: PD-1⁺Tim-3⁺ CD8⁺ (DP) and PD-1⁻Tim-3⁻ CD8⁺ (DN) TILs, PD-1⁺Tim-3⁺ CD8⁺ (DP) TILs and Memory CD8⁺, and PD-1⁺Tim-3⁻ CD8⁺ (SP) and PD-1⁻Tim-3⁻ CD8⁺ (DN) TILs. B) This analysis was followed by four statistical tests (one-sample Kolmogorov-Smirnov test, mean-rank gene set test (wilcoxGST), hypergeometric and competitive gene set test accounting for inter-gene correlation) for enrichment of these signatures in the DKO expression profile. C) WT versus DKO volcano plot, in green are all the genes that were up-regulated in the PD-1⁻Tim-3⁻ CD8⁺ (DN) TILs and in red are all the genes that were up-regulated in the PD-1⁺Tim-3⁺ CD8⁺ (DP) TILs.

[00151] Figure 16. NKG2A is co-expressed with PD-1+Tim3+ CD8 T cells.

[00152] Figure 17. Lilrb4 is co-expressed with PD-1+Tim3+ CD8 T cells and blocking antibody slightly suppress tumor growth (B16 melanoma).

[00153] **Figure 18.** Cysltr2 (LT2) deficiency enhances tumor growth. WT and LT2 KO mice were injected with B16F10 melanoma cells on day 0 and the change in tumor size was plotted (WT: N=5, LT2 KO: N=5). Linear regression following ANOVA was performed between the groups.

[00154] **Figure 19.** Cysltr2 (LT2) deficiency reduces IL-2 production by CD8 TILs. Cytokine production from CD8 TILs was analyzed by intracellular cytokine staining using FACS. Representative data are shown as flow-cytometric schemes and the data from multiple experiments are combined and shown as plots.

[00155] **Figure 20.** Comparison of expression levels between exhausted CD8 cells and memory cells for the target genes. Those genes that were up-regulated in the memory cells can be associated with survival/stimulatory/inhibitory-of-inhibitory effects.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[00156] Unless otherwise defined, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. By means of further guidance, term definitions are included to better appreciate the teaching of the invention. When specific terms are defined in connection with a particular aspect of the invention or a particular embodiment of the invention, such connotation is meant to apply throughout this specification, i.e., also in the context of other aspects or embodiments of the invention, unless otherwise defined.

[00157] As used herein, the term "unresponsiveness" includes refractivity to activating receptor-mediated stimulation. Such refractivity is generally antigen-specific and persists after exposure to the antigen has ceased. Unresponsive immune cells can have a reduction of at least 10%, at least 20%, at least at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or even 100% in cytotoxic activity, cytokine production, proliferation, trafficking, phagocytotic activity, or any combination thereof, relative to a corresponding control immune cell of the same type.

[00158] As described herein, the terms "modulating" or "to modulate" generally means either reducing or inhibiting the activity or expression of, or alternatively increasing the activity or expression of, a given entity or effect. As non-limiting examples, one can modulate the activity or expression of a target or antigen, such as at least one of the target genes listed in Table 1 (e.g., PROCR and/or PDPN), as measured using a suitable *in vitro*, cellular or *in vivo* assay, such as those described herein in the Examples. As another non-

limiting example, one can modulate a T cell phenotype, including e.g., exhaustion or responsiveness to stimulation. As another non-limiting example, one can modulate a disease phenotype, e.g. an autoimmune or other immune disease phenotype. In particular, "modulating" or "to modulate" can mean either reducing or inhibiting the activity or expression of, or alternatively increasing a (relevant or intended) biological activity or expression of, a target or antigen, or a phenotype, as measured using a suitable *in vitro*, cellular or *in vivo* assay (which will usually depend on the target or antigen involved), by at least 5%, at least 10%, at least 25%, at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to activity of the target or antigen in the same assay under the same conditions but without the presence of the inhibitor/antagonist agents or activator/agonist agents described herein.

[00159] As will be clear to the skilled person, "modulating" can also involve effecting a change (which can either be an increase or a decrease) in affinity, avidity, specificity and/or selectivity of a target or antigen for one or more of its ligands, binding partners, partners for association into a homomultimeric or heteromultimeric form, or substrates; and/or effecting a change (which can either be an increase or a decrease) in the sensitivity of the target or antigen for one or more conditions in the medium or surroundings in which the target or antigen is present (such as pH, ion strength, the presence of co-factors, etc.), compared to the same conditions but without the presence of a modulating agent. Again, this can be determined in any suitable manner and/or using any suitable assay known per se, depending on the target or antigen involved. In particular, an action as an inhibitor/antagonist or activator/agonist can be such that an intended biological or physiological activity is increased or decreased, respectively, by at least 5%, at least 10%, at least 25%, at least 50%, at least 60%, at least 70%, at least 80%, or 90% or more, compared to the biological or physiological activity in the same assay under the same conditions but without the presence of the inhibitor/antagonist agent or activator/agonist agent. Modulating can, for example, also involve allosteric modulation of the target or antigen; and/or reducing or inhibiting the binding of the target or antigen to one of its substrates or ligands and/or competing with a natural ligand, substrate for binding to the target or antigen. Modulating can also involve activating the target or antigen or the mechanism or pathway in which it is involved. Modulating can for example also involve effecting a change in respect of the folding or conformation of the target or antigen, or in respect of the ability of the target or antigen to fold, to change its conformation (for example, upon binding of a ligand), to associate with other (sub)units, or to disassociate. Such a change will have a functional effect.

[00160] The terms "decrease", "reduced", "reduction", or "inhibit" are all used herein to mean a decrease or lessening of a property, level, or other parameter by a statistically significant amount. In some embodiments, "reduce," "reduction" or "decrease" or "inhibit" typically means a decrease by at least 10% as compared to a reference level (*e.g.*, the absence of a given treatment) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% , or more. As used herein, "reduction" or "inhibition" does not encompass a complete inhibition or reduction as compared to a reference level. "Complete inhibition" is a 100% inhibition as compared to a reference level. A decrease can be preferably down to a level accepted as within the range of normal for an individual without a given disorder.

[00161] The terms "increased" /"increase" or "enhance" or "activate" are all used herein to generally mean an increase of a property, level, or other parameter by a statistically significant amount; for the avoidance of any doubt, the terms "increased", "increase" or "enhance" or "activate" means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 1-fold, at least about a 1.5-fold, at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, at least about a 20-fold increase, at least about a 50-fold increase, at least about a 100-fold increase, at least about a 1000-fold increase or more as compared to a reference level.

[00162] A "pharmaceutical composition" refers to a composition that usually contains an excipient, such as a pharmaceutically acceptable carrier that is conventional in the art and that is suitable for administration to cells or to a subject. In addition, compositions for topical (*e.g.*, oral mucosa, respiratory mucosa) and/or oral administration can be in the form of solutions, suspensions, tablets, pills, capsules, sustained-release formulations, oral rinses, or powders, as known in the art and described herein. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, University of the Sciences in Philadelphia (2005) Remington: The Science and Practice of Pharmacy with Facts and Comparisons, 21 st Ed.

[00163] The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00164] As used herein, the term "pharmaceutically acceptable carrier" can include any material or substance that, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. The term "pharmaceutically acceptable carriers" excludes tissue culture media.

[00165] As used herein, the term "comprising" means that other elements can also be present in addition to the defined elements presented. The use of "comprising" indicates inclusion rather than limitation.

[00166] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00167] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00168] Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. As used herein, the singular forms "a", "an", and "the" include both singular and plural referents unless the context clearly dictates otherwise.

[00169] The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints.

[00170] The terms "about" or "approximately" as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, are meant to encompass variations of and from the specified value, such as variations of +/-10% or less, preferably +1-5% or less, more preferably +1-1% or less, and still more preferably +/-0.1% or less of and from the specified value, insofar such variations are appropriate to

perform in the disclosed invention. It is to be understood that the value to which the modifier "about" or "approximately" refers is itself also specifically, and preferably, disclosed.

[00171] Whereas the terms "one or more" or "at least one", such as one or more members or at least one member of a group of members, is clear *per se*, by means of further exemplification, the term encompasses *inter alia* a reference to any one of said members, or to any two or more of said members, such as, e.g., any >3, >4, >5, ≥6 or ≥7 etc. of said members, and up to all said members. In another example, "one or more" or "at least one" may refer to 1, 2, 3, 4, 5, 6, 7 or more.

[00172] The term "optional" or "optionally" means that the subsequent described event, circumstance or substituent may or may not occur, and that the description includes instances where the event or circumstance occurs and instances where it does not.

[00173] It should be understood that this invention is not limited to the particular methodologies, protocols, and reagents, etc., described herein and as such can vary therefrom. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

IL-27 and IL-27 Signaling Pathways

[00174] IL-27 is a heterodimeric cytokine of the IL-6 and IL-12 family composed of the IL-27p28 and EBI3 subunits. IL-27p28 and EBI3 are produced primarily by antigen-presenting cells after stimulation by microbial products or inflammatory mediators. The IL-27 receptor is composed of WSX-1 (also known as T cell cytokine receptor), a type I cytokine receptor, and glycoprotein 130 (gp130), a receptor subunit utilized by several other IL-6 and IL-12 family members. Although gp130 expression is ubiquitous, WSX-1 expression is largely restricted to leukocytes, including T cells, natural killer (NK) cells, human monocytes, and human mast cells. IL-27 binds specifically to WSX-1, and EBI3 is required for signal transduction (E.D. Tait Wojno and C.A. Hunter, Trends Immunol. 2012 Feb; 33(2):91-7).

[00175] Accordingly, the term "IL-27," as used herein, refers to the heterodimer composed of: the mature form of the precursor IL-27p28 polypeptide having the amino acid sequence

of:

MGQTAGDLGWRLSLLLLPLLLVQAGVWGFPRPPGRPQLSLQELRREFTVSLHLARK
 LLSEVRGQAHRFAESHLPGVNLVLLPLGELPDVSLTFQAWRRLSDPERLCFISTTLQ
 PFHALLGGLGTQGRWTNMERMQLWAMRLDLRDLQRHLRFQVLAAGFNLPEEEEE

EEEEEEERKGLLPGALGSALQGPAQVSWPQLLSTYRLLHSLELVLSRAVRELLLLSKA
 GHSVWPLGFPTLSPQP (SEQ ID NO: 1), as described by, *e.g.*, NP_663634.2, together
 with any naturally occurring allelic, splice variants, and processed forms (*e.g.*, the mature
 form IL-27p28(29-243)) thereof, and the mature form of the precursor EBI3 or IL-27B
 polypeptide having the amino acid sequence of:

MTPQLLLALVLWASCPPCSGRKGPPAALTLPRVQCRASRYPIAVDCSWTLPPAPNST
 SPVSFIATYRLGMAARGHSWPCLQQTPTSTSTITDVQLFSMAPYVLNVTAVHPWGS
 SSSFVPFITEHIIKPDPEGVRLSPLAERQLQVQWEPPGSWPFPEIFSLKYWIRYKRQGA
 ARFHRVGPIEATSFILRAVRPRARYYVQVAAQDLTDYGELSDWSLPATATMSLGK

(SEQ ID NO: 2), as described by, *e.g.*, NP_005 746.2, together with any naturally occurring
 allelic, splice variants, and processed forms (*e.g.*, the mature form IL-27B(21-229)) thereof.
 Typically, IL-27 refers to human IL-27. Specific residues of IL-27 can be referred to as, for
 example, "IL-27(62)."

[00176] IL-27 was initially described as a proinflammatory cytokine that promoted T
 helper (Th)1 responses. Subsequent studies in multiple models of infectious and autoimmune
 disease demonstrated an anti-inflammatory role for IL-27 in Th1, Th2 and Th17 responses,
 and recent work has shown that IL-27 can induce T cells to produce the anti-inflammatory
 cytokine IL-10. The consequences of IL-27 signaling appear to depend, in part, on the
 immunological context, the temporal regulation of IL-27 production, and tissue- and cell-
 specific expression of components of the IL-27 receptor (E.D. Tait Wojno and C.A. Hunter,
Trends Immunol. 2012 Feb;33(2):91-7).

[00177] IL-27 has been shown to promote the generation of Tr-1 cells that produce IL-10
 by inducing expression of the activator protein-1 family transcription factor c-Maf. c-Maf
 directly transactivates the 1110 promoter to upregulate IL-10, and binds to the promoter of the
 common γ chain cytokine IL-21 to elicit IL-21 production that maintains IL-10 producers.
 Moreover, IL-27 signaling upregulates expression of the aryl hydrocarbon receptor (AhR),
 which partners with c-Maf to optimize interactions with the 1110 and 1121 promoters, further
 supporting Tr-1 development. IL-27-mediated IL-10 production also depends on STAT1 and
 STAT3 signaling, and the inducible co-stimulator (ICOS). IL-27 signaling is also believed to
 elicit Tfh responses by inducing c-Maf and IL-21 that promote Tfh activity. However, IL-27
 alone does not cause CD4⁺ T cells to differentiate into functional Tfh, and IL-27 signaling is
 not required for the generation of antibody responses in models of infection, allergy and
 autoimmunity. IL-27 also has direct effects on B cells. IL-27 has also been shown to regulate
 regulatory T cell (Treg) populations and acts as an antagonist of inducible Treg

differentiation (E.D. Tait Wojno and C.A. Hunter, Trends Immunol. 2012 Feb;33(2):91-7). Recently, it was also demonstrated that IL-27 priming of naive CD4 and CD8 T cells upregulates expression of PD-L1 in a STAT1-dependent manner and such IL-27 primed cells can limit *in trans* the effect of pathogenic IL-17-producing Th17 cells in vitro and in vivo (HiraharaK. et al, Immunity. 2012 Jun 29;36(6): 1017-30).

[00178] As demonstrated herein, IL-27 plays a critical role in the development of T cell exhaustion, and drives an IL-27 inhibitory gene module in which the expression and activity of a variety of co-inhibitory and co-stimulatory molecules are induced.

T cell dysfunction

[00179] As used herein, the term "T cell dysfunction" refers to a state in which a T cell or population of T cells fail to respond with effector function when stimulated with antigen and/or stimulatory cytokines sufficient to elicit an effector response in non-dysfunctional T cells. The term encompasses T cell tolerance, a normal state required to avoid self-reactivity, as well as T cell ignorance, T cell exhaustion, and T cell anergy.

[00180] As used herein, in regard to T cell tolerance, thymocytes that express a T cell receptor with affinity for self antigen/MHC complexes are actively deleted (referred to herein as central tolerance, involving negative selection). As used herein, in regard to peripheral tolerance, self-reactive T cells that escape negative selection are inactivated in the periphery by deletion, suppression by regulatory T cells and/or induction of an imprinted cell-intrinsic program resulting in a state of functional unresponsiveness. Self-tolerant T cells have been exposed to self antigen.

[00181] As used herein, in regard to T cell ignorance, self-reactive peripheral T cells are "unaware of self-antigen, e.g., due to physical sequestration of the antigen from immune surveillance, or because the level of self-antigen and/or its presentation is too low to elicit a response.

[00182] As used herein, T cell anergy, originally referred to the absence of delayed skin test hypersensitivity responses to recall antigens in cancer patients, now commonly also refers to the dysfunctional state of T cells stimulated in vitro in the absence of co-stimulatory signals. Anergic T cells induced in vitro fail to produce IL-2 or to proliferate in response to later antigen stimulation under optimal conditions. An *in vivo* state referred to as T cell anergy or adaptive tolerance involves unresponsiveness as a result of suboptimal stimulation.

[00183] T cell exhaustion is a state of functional hyporesponsiveness to stimuli that tends to occur with chronic exposure to antigen, e.g., in chronic infection or in cancer. Exhausted T cells fail to induce effector function following stimulation with CD28 and TCR/CD3 cross-

linking, and express one or more of eomesodermin (Eomes), and the transcription factor(s) Blimp-1, T-bet, BATF, and NFAT. Exhausted T cells also generally express PD-1 and TIM-3. In one embodiment, T cell exhaustion can be assessed by an *in vitro* assay comprising contacting a T cell with a CD28 stimulus and measuring the degree of response. An exhausted T cell will fail to respond to stimulation with CD28. Other methods for measuring T cell exhaustion include proliferation assays or cytotoxic assays and/or are known in the art (see *e.g.*, Yi *et al.* (2010) *Immunol* 129(4):474-481).

[00184] T cell dysfunction and the similarities and differences between the various types of dysfunction are discussed by Schietinger and Greenberg, Trends in Immunol. 35: 51-60, 2014, "Tolerance and exhaustion: defining mechanisms of T cell dysfunction," the contents of which are incorporated herein by reference.

[00185] As used herein, the terms "functional exhaustion" or "unresponsiveness" refer to a state of a cell where the cell does not perform its usual function or activity in response to normal input signals, and includes refractivity of immune cells to stimulation, such as stimulation via an activating receptor or a cytokine. Such a usual function or activity includes, but is not limited to, proliferation or cell division, entrance into the cell cycle, cytokine production, cytotoxicity, trafficking, phagocytotic activity, or any combination thereof. Normal input signals can include, but are not limited to, stimulation via a receptor (*e.g.*, T cell receptor, B cell receptor, co-stimulatory receptor). Unresponsive immune cells can have a reduction of at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or even 100% in one or more effector functions, such as cytotoxic activity, cytokine production, proliferation, trafficking, phagocytotic activity, or any combination thereof, relative to a corresponding control immune cell of the same type. In some particular embodiments of the aspects described herein, a cell that is functionally exhausted is a CD4 or helper T lymphocyte that expresses the CD4 cell surface marker. Such CD4 cells normally proliferate, and/or produce cytokines, such as IL-2, TNF α , IFN γ , IL-4, IL-5, IL-17, or a combination thereof, in response to T cell receptor and/or co-stimulatory receptor stimulation. Thus, a functionally exhausted or unresponsive CD4 T cell is one which has a reduction in proliferation, and/or cytokine production, such as IL-2, TNF α , IFN γ , in response to normal input signals. The cytokines produced by CD4 T cells act, in part, to activate and/or otherwise modulate, *i.e.*, "provide help," to other immune cells such as B cells and CD8 $^{+}$ cells. In some particular embodiments of the aspects described herein, a cell that is functionally exhausted is a CD8 or cytotoxic T

lymphocyte that expresses the CD8 cell surface marker. Such CD8 cells normally proliferate, engage in cytotoxic or cytolytic activity, and/or produce cytokines, such as IL-2 and IFN γ , or a combination thereof, in response to T cell receptor and/or co-stimulatory receptor stimulation. Thus, a functionally exhausted or unresponsive CD8 T cell is one which has a reduction in proliferation, cytotoxic activity, and/or cytokine production, such as IL-2, TNF α , IFN γ , in response to normal input signals.

[00186] As used herein, the term "reduces T cell tolerance" means that a given treatment or set of conditions leads to reduced T cell tolerance as evidenced by an increase in one or more T cell effector functions, *e.g.*, greater T cell proliferation, cytokine production, responsiveness, and/or ability or receptiveness with regards to activation. Methods of measuring T cell activity are known in the art. By way of non-limiting example, T cell tolerance can be induced by contacting T cells with recall antigen, anti-CD3 in the absence of costimulation, and/or ionomycin. Levels of, *e.g.* LDH-A, RAB10, and/or ZAP70 (both intracellular or secreted) can be monitored, for example, to determine the extent of T cell tolerogenesis (with levels of IL-2, interferon- γ and TNF correlating with increased T cell tolerance). The response of cells pre-treated with, *e.g.* ionomycin, to an antigen can also be measured in order to determine the extent of T cell tolerance in a cell or population of cells, *e.g.* by monitoring the level of secreted and/or intracellular IL-2 and/or TNF- α (see, *e.g.* Macian et al. Cell 2002 109:719-731; which is incorporated by reference herein in its entirety). Other characteristics of T cells having undergone adaptive tolerance is that they have increased levels of Fyn and ZAP-70/Syk, Cbl-b, GRAIL, Ikaros, CREM (cAMP response element modulator), B lymphocyte-induced maturation protein-1 (Blimp-1), PD1, CD5, and SHP2; increased phosphorylation of ZAP-70/Syk, LAT, PLC γ 1/2, ERK, PKC- Θ /IKBA; increased activation of intracellular calcium levels; decreased histone acetylation or hypoacetylation and/or increased CpG methylation at the IL-2 locus. Thus, in some embodiments, modulation of one or more of any of these parameters can be assayed to determine whether one or more modulating agents modulates an immune response in vivo or modulates immune tolerance.

[00187] Modulation of T cell tolerance can also be measured by determining the proliferation of T cells in the presence of a relevant antigen assayed, *e.g.* by a ^3H -thymidine incorporation assay, flow cytometry based assay, such as CFSE or other fluorochrome-based proliferation assay, or cell number. Markers of T cell activation after exposure to the relevant antigen can also be assayed, *e.g.* flow cytometry analysis of cell surface markers indicative of

T cell activation (e.g. CD69, CD30, CD25, and HLA-DR). Reduced T cell activation in response to antigen-challenge is indicative of tolerance induction. Conversely, increased T cell activation in response to antigen-challenge is indicative of reduced tolerance.

[00188] Modulation of T cell tolerance can also be measured, in some embodiments, by determining the degree to which the modulating agent inhibits or increase the activity of its target. For example, the SEB model can be used to measure T cell tolerance and modulation thereof. In normal mice, neonatal injection of staphylococcal enterotoxin B (SEB) induces tolerance in T cells that express reactive T cell receptor (TCR) V beta regions. If, in the presence of an IL-27 or NFIL-3 modulating, T cells expressing reactive TCR V beta regions (e.g., Vbeta8) display a statistically significant reduction or increase in T cell activity than T cells not contacted with the modulating agent, the modulating agent is one that modulates T cell tolerance.

[00189] Other *in vivo* models of peripheral tolerance that can be used in some aspects and embodiments to measure modulation in T cell tolerance using the modulating agents described herein include, for example, models for peripheral tolerance in which homogeneous populations of T cells from TCR transgenic and double transgenic mice are transferred into hosts that constitutively express the antigen recognized by the transferred T cells, e.g., the H-Y antigen TCR transgenic; pigeon cytochrome C antigen TCR transgenic; or hemagglutinin (HA) TCR transgenic. In such models, T cells expressing the TCR specific for the antigen constitutively or inducibly expressed by the recipient mice typically undergo an immediate expansion and proliferative phase, followed by a period of unresponsiveness, which is reversed when the antigen is removed and/or antigen expression is inhibited. Accordingly, if, in the presence of one or more modulating agents, for example, in such models if the T cells proliferate or expand, show cytokine activity, etc. significantly more than T cells in the absence of the inhibitory agent, than that agent is one that reduces T cell tolerance. Such measurements of proliferation can occur *in vivo* using T cells labeled with BrDU, CFSE or another intravital dye that allows tracking of proliferation prior to transferring to a recipient animal expressing the antigen, or cytokine reporter T cells, or using *ex vivo* methods to analyze cellular proliferation and/or cytokine production, such as thymidine proliferation assays, ELISA, cytokine bead assays, and the like.

[00190] Modulation of T cell tolerance can also be assessed by examination of tumor infiltrating lymphocytes or T lymphocytes within lymph nodes that drain from an established tumor. Such T cells exhibit features of "exhaustion" through expression of cell surface molecules, such as TIM-3, for example, and decreased secretion of cytokines such as

interferon- γ . Accordingly, if, in the presence of an inhibitory agent, increased quantities of T cells with, for example, 1) antigen specificity for tumor associated antigens are observed (e.g. as determined by major histocompatibility complex class I or class II tetramers which contain tumor associated peptides) and/or 2) that are capable of secreting high levels of interferon- γ and cytolytic effector molecules such as granzyme-B, relative to that observed in the absence of the inhibitory agent, this would be evidence that T cell tolerance had been reduced.

Target Genes/Gene Products that modulate T cell function/dysfunction

[00191] Provided herein are target genes, gene products, and combinations thereof that are useful in modulating T cell dysfunction, particularly T cell exhaustion. Any of the target genes/gene products can be targeted alone or in any combination thereof. Also provided herein are novel gene signatures for detecting and isolating T cells having a particular phenotype, particularly dysfunctional T cells.

<i>Table 1: Genes that modulate T cell function/dysfunction</i>		
Bst2	NM_004335.3	SEQ ID NO: 3.
Btla	NM_001085357.1	SEQ ID NO: 4.
Ccl9	NM_011338.2(Mus Musculus)	SEQ ID NO: 5.
Ccr4	NM_005508.4	SEQ ID NO: 6.
Cd40lg	NM_011616.2(Mus Musculus)	SEQ ID NO: 7.
Cxcr4	NM_001008540.1	SEQ ID NO: 8.
Gpr65	NM_003608.3	SEQ ID NO: 9.
Il33	NM_001199640.1	SEQ ID NO: 10.
Klrc2	NM_002260.3	SEQ ID NO: 11.
Klrd1	NM_001114396.1	SEQ ID NO: 12.
Klre1	NM_153590.3(Mus Musculus)	SEQ ID NO: 13.
Lif	NM_001257135.1	SEQ ID NO: 14.
Lpar3	NM_012152.2	SEQ ID NO: 15.
Olfm1	NM_001282611.1	SEQ ID NO: 16.
Pdpr	NM_001006624.1	SEQ ID NO: 17.
Ptpn3	NM_001145368.1	SEQ ID NO: 18.
Sdc1	NM_001006946.1	SEQ ID NO: 19.
Timp2	NM_003255.4	SEQ ID NO: 20.
Tnfrsf9 (4-1BB)	NM_001561.5	SEQ ID NO: 21.
Vldlr	NM_001018056.1	SEQ ID NO: 22.
Entpd1	NM_001098175.1	SEQ ID NO: 23.
Il13ra1	NM_001560.2	SEQ ID NO: 24.
Il6st	NM_001190981.1	SEQ ID NO: 25.
Inhba	NM_002192.2	SEQ ID NO: 26.
Lamp2	NM_001122606.1	SEQ ID NO: 27.

Lap3	NM_015907.2	SEQ ID NO: 28.
Ly75	NM_002349.3	SEQ ID NO: 29.
Nampt	NM_005746.2	SEQ ID NO: 30.
Ccl5	NM_001278736.1	SEQ ID NO: 31.
Cd83	NM_001040280.1	SEQ ID NO: 32.
Klrkl	NM_007360.3	SEQ ID NO: 33.
Sema7a	NM_001146029.1	SEQ ID NO: 34.
Serpincl	NM_000488.3	SEQ ID NO: 35.
Ccr2	NM_001123041.2	SEQ ID NO: 36.
Ifitm1	NM_003641.3	SEQ ID NO: 37.
Il12rb1	NM_001290023.1	SEQ ID NO: 38.
Il1rl1	NM_000877.3	SEQ ID NO: 39.
Sdc4	NM_002999.3	SEQ ID NO: 40.
Slamf7	NM_001282588.1	SEQ ID NO: 41.
Tgfb3	NM_003239.3	SEQ ID NO: 42.
Adam9	NM_003816.2	SEQ ID NO: 43.
Cd93	NM_012072.3	SEQ ID NO: 44.
Tigit	NM_173799.3	SEQ ID NO: 45.
Ccr5	NM_000579.3	SEQ ID NO: 46.
Adam8	NM_001109.4	SEQ ID NO: 47.
Cd68	NM_001040059.1	SEQ ID NO: 48.
Isg20	NM_001303233.1	SEQ ID NO: 49.
Itih1	NM_000572.2	SEQ ID NO: 50.
Il10ra	NM_001558.3	SEQ ID NO: 51.
Itih2	NM_001207006.2	SEQ ID NO: 52.
Il2rb	NM_000878.3	SEQ ID NO: 53.
Abca1	NM_005502.3	SEQ ID NO: 54.
Alcam	NM_001243280.1	SEQ ID NO: 55.
Cysltr2	NM_001308465.1	SEQ ID NO: 56.
Gcnt1	NM_001097633.1	SEQ ID NO: 57.
Havcr2(Tim-3)	NM_032782.4	SEQ ID NO: 58.
Gabarapl1	NM_031412.2	SEQ ID NO: 59.
Il2ra	NM_000417.2	SEQ ID NO: 60.
Sppl1	NM_000582.2	SEQ ID NO: 61.
Cxcl10	NM_001565.3	SEQ ID NO: 62.
Ifitm3	NM_021034.2	SEQ ID NO: 63.
Il1r2	NM_001261419.1	SEQ ID NO: 64.
Lag3	NM_002286.5	SEQ ID NO: 65.
Pglyrp1	NM_005091.2	SEQ ID NO: 66.
Il1rb4	NM_001278426.3	SEQ ID NO: 67.
Kircl1	NM_001304448.1	SEQ ID NO: 68.
Procr	NM_006404.4	SEQ ID NO: 69.

Table 2 cont'd

[00192] In one embodiment, at least two target genes are modulated using a combination of inhibitors and/or activators as described herein. In one embodiment, the at least two target genes are selected from the gene pairs listed in Table 2. In one embodiment, one or more target genes to be modulated are positive regulators of T cell function as listed in Table 3. In another embodiment, the one or more target genes to be modulated are negative regulators of T cell function as listed in Table 4.

Table 3: Positive Regulators of T cell function

Klrc2	Klre1	Tnfsf9 (4-1BB)	Klrk1
Il12rb1	Il1r1	Slamf7	

Table 4: Negative Regulators of T cell function

Btla	Tigit	Havcr2(Tim-3)	Lag3
Pdpc	Il10ra	Il1r2	Procr
Lilrb4	Klrc1		

[00193] In some embodiments, two or more target genes are modulated using two or more modulating agents as described herein. In some embodiments, at least three target genes are modulated; in other embodiments at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 or more target genes are modulated in the methods and/or compositions provided herein.

[00194] In some embodiments, at least one pair of target genes as listed in Table 2 is modulated in combination with at least one additional target gene as listed in Tables 1, 3, or 4.

[00195] In some embodiments, two or more target genes selected from Table 4 are modulated using two or more modulating agents as described herein.

[00196] As described herein, T cells isolated from a cancer environment express an IL-27 inhibitory gene module in which the expression and activity of a subset of co-inhibitory and co-stimulatory molecules are induced, as described in FIG. 6H and listed in Table 5.

[00197] Accordingly, in some embodiments, one or more target genes selected from Table 5 are modulated using one or more modulating agents as described herein, for the treatment of certain disorders, such as cancer. In some embodiments, two or more target genes selected from Table 5 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as cancer.

LAG3	PDPN	PROCR	SDC1
CTLA2A	KLRE1	GPR65	KLRD1
IL33	OLFM1	KLRC2	PTPN3
TNFSF9	VLDLR	CCR5	ADAM9
CYSLTR2	CCL9	LPAR3	CD93
ENTPD1	IFITM3	ADAM8	GABARAPL1
SPP1	IL1R2	PGLYRP1	IL2RA
GCNT1	ALCAM	TIGIT	HAVCR2

[00198] As described herein, T cells isolated under conditions of a chronic viral infection express an IL-27 inhibitory gene module in which the expression and activity of a subset of co-inhibitory and co-stimulatory molecules are induced, as described in FIG. 6H and listed in Table 6.

[00199] Accordingly, in some embodiments, one or more target genes selected from Table 6 are modulated using one or more modulating agents as described herein, for the treatment of certain disorders, such as chronic infections. In some embodiments, two or more target genes selected from Table 6 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as chronic infections.

Table 6: Chronic Infection Associated IL-27 driven molecules

LAG3	ADAM9	CD93	CYSLTR2
IL1R2	PGLYRP1	IL2RA	ENTPD1
IFITM3	GCNT1	ALCAM	TIGIT
HAVCR2	IL13RA1	IL10	IL21
CCR2	IL10RB	IL10RA	CXCL10
CD68	KLKR1	LILRB4	IL12RB2
IL6ST	IL7R	LNHBA	NAMPT
S1PR1	LSG20	LAMP2	LY75

[00200] As described herein, T cells isolated under anergic conditions express an IL-27 inhibitory gene module in which the expression and activity of a subset of co-inhibitory and co-stimulatory molecules are induced, as described in FIG. 6H and listed in Table 7.

[00201] Accordingly, in some embodiments, one or more target genes selected from Table 7 are modulated using one or more modulating agents as described herein, for the treatment of certain disorders, such as conditions involving anergy. In some embodiments, two or more target genes selected from Table 7 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as conditions involving anergy.

Table 7: Anergy Associated IL-27 driven molecules

LAG3	IL1R2	PGLYRP1	IL2RA
CXCL10	CD68	KLKR1	CCL5
GABARAPL1	SPP1	TNFRSF8	ABCA1
SEMA7A	CCR5		

[00202] As described herein, T cells isolated under conditions of nasal tolerance express an IL-27 inhibitory gene module in which the expression and activity of a subset of co-inhibitory and co-stimulatory molecules are induced, as described in FIG. 6H and listed in Table 8.

[00203] Accordingly, in some embodiments, one or more target genes selected from Table 8 are modulated using one or more modulating agents as described herein, for the treatment of certain disorders, such as conditions in which tolerance is to be induced (e.g., autoimmunity). In some embodiments, two or more target genes selected from Table 8 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as conditions in which tolerance is to be induced (e.g., autoimmunity).

Table 8: Nasal Tolerance Associated IL-27 driven molecules

LAG3	ADAM8	GABARAPL1	CYSLTR2
IL1R2	PGLYRP1	IL2RA	ENTPD1
IFITM3	GCNT1	ALCAM	TIGIT
HAVCR2	SPP1	IL10	IL21
CCR2	IL10RB	IL10RA	CXCL10
TNFRSF8	ABCA1	IL12RB1	IL1R1
SDC4	IFITM1	SLAMF7	TGFB3

[00204] As described herein, T cells isolated under conditions of skin tolerance express an IL-27 inhibitory gene module in which the expression and activity of a subset of co-inhibitory and co-stimulatory molecules are induced, as described in FIG. 6H and listed in Table 9.

[00205] Accordingly, in some embodiments, one or more target genes selected from Table 9 are modulated using one or more modulating agents as described herein, for the treatment of certain disorders, such as conditions in which tolerance is to be induced (e.g., autoimmunity). In some embodiments, two or more target genes selected from Table 9 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as conditions in which tolerance is to be induced (e.g., autoimmunity).

Table 9: Skin Tolerance Associated IL-27 driven molecules

LAG3	ALCAM	TIGIT	HAVCR2
IL10	IL21	IL13RA1	CCR5
CXCL10	CCL5	CTSB	KLRC1
LPAR3	CCL9		

[00206] In some embodiments, one or more target genes selected from Tables 8 and 9 are modulated using one or more modulating agents as described herein, for the treatment of certain disorders, such as conditions in which tolerance is to be induced (e.g., autoimmunity).

In some embodiments, two or more target genes selected from Tables 8 and 9 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as conditions in which tolerance is to be induced (e.g., autoimmunity).

[00207] As described further herein, 1,392 genes were identified that were differentially expressed between WT CD4⁺ T cells stimulated in the presence or absence of IL-27. In certain embodiments differential expression of these genes may be used as a gene signature to identify or detect T cells with a dysfunctional phenotype. In other embodiments, differentially expressed genes may be modulated or targeted with an agent capable of modulating expression or activity of a gene. In certain preferred embodiments, genes that encode cell surface receptors or cytokines are targeted for modulation. Not being bound by a theory, cell surface receptors or cytokines facilitate targeting by a therapeutic agent. Not being bound by a theory, cell surface receptors or cytokines facilitate detection or isolation of cells without destroying the cell, such as by cell sorting, particularly FACS or magnetic sorting. Cell surface receptors or cytokines found to be differentially expressed between WT CD4⁺ T cells stimulated in the presence or absence of IL-27 are described in Table 10, FIG. 6C and 6D. Table 10 lists the mouse and human gene names. The present invention may use the corresponding genes in any mammal, preferably human. Accordingly, in some embodiments, one or more target genes selected from Table 10 are modulated using one or more modulating agents as described herein for the treatment of certain disorders, such as cancer. In some embodiments, two or more target genes selected from Table 10 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as cancer.

Table 10			
<i>Table 10a: Mouse genes encoding cell surface receptors and cytokines differentially expressed between WT CD4⁺ T cells stimulated in the presence or absence of IL-27</i>			
Up-regulated			Down-Regulated
Abca1	Ifitm3	Lamp2	Bst2
Adam8	Il10	Lpar3	Btla
Adam9	Il10ra	Ly75	Ccl1
Alcam	Il12rb1	Ly75	Ccr4
Ccl5	Il13ra1	Nampt	Cd226
Ccl9	Il1r1	Olfm1	Cd40lg
Ccl9	Il1r2	Pdpm	Cd83
Ccl9	Il21	Pglyrp1	Cd8a
Ccr2	Il2ra	Procr	Csf2
Ccr5	Il2rb	Pstpip1	Cxcl13
Cd68	Il33	Ptpn3	Cxcr4

Cd93	Il6st	Sdc1	Ifitm3
Cxcl10	Inhba	Sdc4	Isg20
Cysltr2	Isg20	Selp	Lap3
Ddr1	Klrc2	Sema7a	Lif
Entpd1	Klrc2	Slamf7	Serpinc1
Entpd1	Klrc2	Spp1	Timp2
Epcam	Klrc2	Tgfb3	Tnfsf11
Gabarapl1	Klrc2	Tigit	
Gent1	Klrc2	Tnfrsf8	
Gpr65	Klrd1	Tnfsf9	
Havcr2	Klrk1	Vldlr	
Ifitm1	Lag3		

Table 10b: Human genes encoding cell surface receptors and cytokines differentially expressed between WT CD4+ T cells stimulated in the presence or absence of IL-27

<u>Up-regulated</u>		<u>Down-regulated</u>	
ABCA1	IFITM1	LAMP2	BST2
ADAM8	IL10	LPAR3	BTLA
ADAM9	IL10RA	LY75-CD302	CCL1
ALCAM	IL12RB1	LY75	CCR4
CCL5	IL13RA1	NAMPT	CD226
CCL15	IL1R1	OLFM1	CD40LG
CCL23	IL1R2	PDPN	CD83
CCL15-CCL14	IL21	PGLYRP1	CD8A
CCR2	IL2RA	PROCR	CSF2
CCR2	IL2RB	PSTPIP1	CXCL13
CD68	IL33	PTPN3	CXCR4
CD93	IL6ST	SDC1	IFITM1
CXCL10	INHBA	SDC4	ISG20
CYSLTR2	ISG20	SELP	LAP3
DDR1	KLRC4-KLRK1	SEMA7A	LIF
ENTPDI	KLRC4	SLAMF7	SERPINC1
EPCAM	KLRC1	SPP1	TIMP2
GABARAPL1	KLRC3	TGFB3	TNFSF11
GCNT1	KLRC2	TIGIT	
GPR65	KLRD1	TNFRSF8	
HAVCR2	KLRK1	TNFSF9	
IFITM1	LAG3	VLDLR	

*The up- and down-regulated genes were determined over a 96h time-course. Therefore the same gene can be both up-regulated and down-regulated at different time points along the differentiation.

[00208] As described herein, IL-27-signatures of up-regulated and down-regulated genes with overlapping expression in several different dysfunctional or tolerant T cell states were identified (Table 11, Fig. 6G and 6H). Not being bound by a theory, T cells become exhausted after having cancer or chronic infection or become tolerant after prolonged exposure to antigens. Thus, in certain embodiments the identified genes may be used as a gene signature to identify or detect T cells with a dysfunctional phenotype. In other embodiments, the overlapping genes may be modulated or targeted with an agent capable of

modulating expression or activity of a gene for the treatment of certain disorders, such as cancer. Accordingly, in some embodiments, one or more target genes selected from Table 11 are modulated using one or more modulating agents as described herein. In some embodiments, two or more target genes selected from Table 11 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as cancer. In some embodiments, genes that are up-regulated in Table 11 are modulated by down-regulation of expression or activity. In some embodiments, genes that are down-regulated in Table 11 are modulated by up-regulation of expression or activity.

Table 11							
<i>Table 11a: IL-27-signature of up-regulated mouse genes expressed in several different dysfunctional or tolerant T cell states.</i>							
1700012 B09Rik	Cdh17	Ets1	Havcr2	Klrc2	Nfia	Rab31	Sqrdl
AA46719 7	Cdk6	Etv6	Hhat	Klrc2	Nfil3	Ramp3	Srgap3
Abca1	Cdkn2d	F2rl1	Hhex	Klrd1	Nkg7	Rbp1	Stat1
Abcb9	Cds2	Fam129b	Hif1a	Klre1	Oas2	Rfk	Stat3
Acadl	Cebpd	Fam20a	Hlx	Klrk1	Ociad2	Rgs1	Stom
Adam19	Cela1	Fbxw7	Hopx	Ksr1	Oit3	Rhoc	Styk1
Adam8	Cercam	Ffar2	Hpse	Lag3	Olfm1	Rhoq	Syt11
Adam9	Chac1	Fgl2	Id2	Lama5	Ormdl3	Ripk3	Tbx21
Agpat3	Chit1	Fhit	Ier3	Lamp2	Osr2	Rnf125	Tcp11l2
Ahnak	Chm	Filip1	Ih1	Lat2	Ovol2	Rnh1	Tgfb3
Ahr	Chst11	Flot1	Iitm1	Lgals3	Padi2	Rorc	Tigit
Ahr	Chst2	Fndc3a	Iitm3	Lgals3bp	Parp14	Runx2	Timp1
Ak1	Clip3	Frmd4b	Igf2bp2	Lilrb4	Pdpn	S100a4	Tmcc3
Akr1b8	Clybl	Gabarapl1	Il10	Litaf	Pfkip	S100a6	Tnfrsf8
Akr1b8	Cnih2	Galc	Il10ra	Lpar3	Pglyrp1	Sccpdh	Tnfsf9
Akt2	Copz2	Gatm	Il12rb1	Lpxn	Phactr2	Sdc1	Tor2a
Alcam	Creb3l2	Gbe1	Il13ra1	Lrrk1	Pik3ap1	Sdc4	Tpbp
Aldoc	Ctla2a	Gbp3	Il1r1	Ltbp3	Piwil2	Sdcbp2	Tpd52
Anxa2	Cxcl10	Gbp3	Il1r2	Ly75	Pkp2	Sec24d	Trib3
Anxa3	Cysltr1	Gbp6	Il21	Ly75	Plac8	Selenbp1	Tspan4
Aplp1	Cysltr2	Gcnt1	Il2ra	Maf	Plekhf1	Selm	Tspan5
Aqp9	Dapk2	Gem	Il2rb	Map3k5	Plekho2	Selp	Ttc39b
Arfgap3	Dclk1	Gemin8	Il33	Med12l	Plekho2	Sema7a	Ttc39c
Arhgap1 8	Ddr1	Gfra1	Il6st	Mettl7a1	Plod2	Serpinb1a	Tubb6
Arl5a	Dhx58	Gimap7	Impa2	Mmp15	Ppme1	Serpinb6b	Tulp4
Armcx3	Dock9	Gja1	Inhba	Ms4a6d	Ppp1r3b	Serpinb9	Ubac2
Asb2	Dst	Glg1	Irf1	Ms4a6d	Pqlc3	Serpinf1	Upp1
Atf6	E330009J 07Rik	Glrx	Irf4	Mt1	Prdm1	Sigirr	Usp18

Atp6v0d2	Eaf2	Gmfg	Irf8	Mtl	Prexl	Skap2	Uspi8
Auh	Ecml	Gmppa	Irf9	Mtl	Prfl	Slamf7	Vldlr
Bcl2l15	Egln3	Gnb5	Isgl5	Mtl	Procr	Slc2a3	Wdr54
Bnip3	Elmo2	Gnpda2	Isg20	Mtl	Prss2	Slc2a3	Wdr81
C3	Emilin2	Golga7	Jun	Mtl	Prss2	Slc39a14	Zbpl
Ccl5	Empl	Gpm6b	Junb	Mt2	Prss2	Slc41a2	Zeb2
Ccl9	Enpp2	Gpr65	Kctd11	Mxd1	Psmb9	Slc4all	Zfp36
Ccl9	Entpd1	Gpt2	Klf10	Mxil	Pstpipl	Slc7a3	
Ccl9	Entpd1	Gsn	Klhl24	Nampt	Ptpn1	Sord	
Ccr2	Epcam	Gsn	Klrc2	Ndrgl	Ptpn3	Sox5	
Ccr5	Ern1	Gsn	Klrc2	Neb	Pygl	Spats2	
Cd68	Eroll	Gzmb	Klrc2	Nedd4	Rabllfip5	Sppl	
Cd93	Errfil	Gzmc	Klrc2	Nek6	Rab27a	Sqrdl	

Table 10b: IL-27-signature of down-regulated mouse genes expressed in several different dysfunctional or tolerant T cell states.

Aatf	Cd40lg	Dph5	Gucylb3	Lrigl	Phb	Rrs1	Tafld
Adil	Cd83	Dus4l	Hells	Marcksil	Phlda1	Rtp4	Timm9
Agpat5	Cd8a	Egr3	Hist2h3cl	Mettil	Pkp4	Sema4b	Timp2
Akr1c18	Cdk5r1	Eomes	Id3	Mmachc	Pmepal	Sema4c	Tm4sf5
Akr1c18	Chd9	Fam26f	Idi2	Mpegl	Prkcdbp	Serpinb6b	Tmem97
Akr1c18	Cnksr3	Fhit	Ifih1	Mtap	Prmt1	Serpinb9	Tnfaip8
Akr1c18	Cnn3	Ftsj3	Ifitm3	Myb	Prmt3	Serpincl	Tnfsfll
Atp2a3	Cpd	Galnt6	Ipcefl	Ndufa4	Pter	Sh3bp5	Toplmt
Bst2	Crtam	Gchl	Irf6	Ndufaf4	Ptger4	Shmtl	Tratl
Btla	Csell	Gemin4	Irgml	Nhp2	Pus7l	Slamf6	Tripl3
Cacna1a	Csf2	Gfil	Isg20	Noc4l	Rcll	Slamf9	Trpml
Cadm1	Cxcl3	Gnaq	Kbtbd8	Nolcl	Rcsd1	Slcl9al	Tsr2
Camkk2	Cxcr4	Gnl3	Klf10	Nopl6	Rfc4	Snhg7	Ttc27
Capn3	D930015E06Rik	Gpatch4	Ktil2	Nop2	Rnmtll	Snhg7	Umps
Ccdc86	Dapll	Gpd1l	Lad1	Nop56	Rppl4	Snhg7	Utp20
Cell	Ddit4	Gramd1b	Lap3	Nr4a3	Rpp40	St6gall	Wdr77
Ccr4	Ddx18	Grwd1	Lgals3bp	Pde7a	Rragd	St8sia4	Zbtbl0
Cd226	Dennd5a	Gucyla3	Lif	Pde8a	Rrpl5	Stc2	Zfp608

Table 11c: IL-27-signature of up-regulated human genes expressed in several different dysfunctional or tolerant T cell states.

ABCA1	CD93	ETS1	HAVCR2	KLRC2	NEDD4	PYGL	SPATS2
ABCB9	CDH17	ETV6	HHAT	KLRC3	NEK6	RAB11FIP5	SPP1
ACADL	CDK6	F2RL1	HHEX	KLRC4	NFIA	RAB27A	SQRDL
ADAM19	CDKN2D	FAM129B	HIF1A	KLRC4-KLRK1	NFIL3	RAB31	SRGAP3
ADAM8	CDS2	FAM20A	HLX	KLRD1	NKG7	RAMP3	STAT1
ADAM9	CEBPD	FBXW7	HOPX	KLRK1	OAS2	RBP1	STAT3
AGPAT3	CELA1	FFAR2	HPSE	KSR1	OCIAD2	RFK	STOM
AHNAK	CERCAM	FGL2	ID2	LAG3	OIT3	RGS1	STYK1
AHR	CHAC1	FHIT	IER3	LAMA5	OLFM1	RHOQ	SYT11

AK1	CHIT1	FILIP1	IFIH1	LAM P2	ORM DL3	RIPK3	TBX21
AKR1B10	CHM	FLOT1	IFITM 1	LAT2	OSR2	RNF125	TCP11L2
AKR1B15	CHST11	FNDC3A	IFITM 1	LGALS3	OVOL2	RNH1	TGFB3
AKT2	CHST2	FRM D4B	IGF2BP2	LGALS3BP	PADI2	RORC	TIG IT
ALCAM	CLIP3	GABARAP LI	IL10	LITAF	PARP14	RUNX2	TIM P1
ALDOC	CLYBL	GALC	IL10RA	LPAR3	PDPN	S100A4	TMCC3
ANXA2	CNIH2	GATM	IL12RB1	LPXN	PFKP	S100A6	TNFRSF8
ANXA3	COPZ2	GBE1	IL13RA1	LRRK1	PG LYRP1	SCCPDH	TNFSF9
APLP1	CREB3L2	GBP4	IL1R1	LTBP3	PHACTR2	SDC1	TOR2A
AQP9	CXCL10	GBP6	IL1R2	LY75	PIK3AP1	SDC4	TPBG
ARFGAP3	CYSLTR1	GBP7	IL21	LY75-CD302	PIWI L2	SDCBP2	TPD52
ARHGAP 18	CYSLTR2	GCNT1	IL2RA	MAF	PKP2	SEC24D	TRIB3
ARL5A	DAPK2	GEM	IL2RB	MAP3K5	PLAC8	SELENBP1	TSPAN4
ARMCX3	DLCK1	GEMI N8	IL33	M ED12L	PLEKH F1	SELP	TSPAN5
ASB2	DDR1	GFRA1	IL6ST	M ETTL7A	PLEKH02	SEMA7A	TTC39B
ATF6	DHX58	GIMAP7	IMPA2	M MP15	PLOD2	SERPINB1	TTC39C
ATP6V0D 2	DOCK9	GJA1	INHBA	MS4A6A	PPME1	SERPINB6	TUBB6
AUH	DST	GLG1	IRF1	MS4A6E	PPP1R3B	SERPINB9	TULP4
BCL2L15	EAF2	GLRX	IRF4	MT1B	PQLC3	SERPINF1	UBAC2
BNIP3	ECM 1	GM FG	IRF8	MT1E	PRDM 1	SIG IRR	UPP1
C1orf97	EG LN3	GM PPA	IRF9	MT1F	PREX1	SKAP2	USP18
C15orf48	ELM02	GNB5	ISG 15	MT1G	PRF1	SLAMF7	USP41
C3	EM ILIN2	GNPDA2	ISG20	MT1M	PROCR	SLC2A14	VLDLR
CCL15	EM P1	GOLGA7	JUN	MT1X	PRSS1	SLC2A3	WDR54
CCL15-CCL14	ENPP2	GPM6B	JUNB	MT2A	PRSS2	SLC39A14	WDR81
CCL23	ENTPD1	GPR65	KCTD11	MXD1	PRSS3	SLC41A2	ZBP1
CCL5	EPCAM	GPT2	KIAA1147	MXI 1	PSMB9	SLC4A11	ZEB2
CCR2	ERN 1	GSN	KLF10	NAMPT	PSTPI P1	SLC7A3	ZFP36
CCR2	ER01A	GZMB	KLH L24	NDRG1	PTPN1	SORD	
CD68	ERRFI 1	GZMB	KLRC1	NEB	PTPN3	SOX5	

Table lid: IL-27-signature of down-regulated human genes expressed in several different dysfunctional or tolerant T cell states.

AATF	CD40LG	EG R3	HIST2H3C	LRIG 1	PDE8A	RRS1	TIM P2
ADI 1	CD83	EOMES	ID3	MARCKSL 1	PHB	RTP4	TM4SF5
AGPAT5	CD8A	FAM26F	IDI2	M ETTLI	PHLDA1	SEMA4B	TMEM97
AKR1C1	CDK5R1	FHIT	IFIH1	M MACHC	PKP4	SEMA4C	TNFAIP8
AKR1C2	CHD9	FTSJ3	IFITM 1	M PEG 1	PM EPA1	SERPINB6	TNFSF11
AKR1C3	CNN3	GALNT6	IPCEF1	M RM3	PRKCDBP	SERPINB9	TOP1MT
AKR1C4	CPD	GCH 1	IPCEF1	MTAP	PRMT1	SERPIN C1	TRAT1
ATP2A3	CRTAM	GEMI N4	IRF6	MYB	PRMT3	SH3BP5	TRIP13
BST2	CSE1L	GFI1	IRGM	NDUFA4	PTER	SHMT1	TRPM 1
BTLA	CSF2	GNAQ	ISG20	NDUFAF4	PTGER4	SLAMF6	TSR2
CACNA1A	CXCL13	GNL3	KBTBD8	NHP2	PUS7L	SLAMF9	TTC27
CADMI	CXCR4	GPATCH4	KIAA0922	NOC4L	RCL1	SLC19A1	UM PS

CAM KK2	DAPL1	GPD1L	KLF10	N0LC1	RCSD1	SNORA17 B	UTP20
CAPN3	DDIT4	GRAM D1 B	KTI 12	NOP16	RFC4	ST6GAL1	WDR77
CCDC86	DDX18	GRWD1	LAD1	NOP2	RPP14	ST8SIA4	ZBTB10
CCL1	DEN ND5A	GUCY1A3	LAP3	NOP56	RPP40	STC2	ZNF608
CCR4	DPH5	GUCY1B3	LGALS3BP	NR4A3	RRAGD	TAF1D	
CD226	DUS4L	HELLS	LIF	PDE7A	RRP15	TIM M9	

[00209] As described herein, genes were identified that were up-regulated in response to IL-27 signaling and overlap with dysfunctional CD8⁺ T cell signatures from cancer and chronic viral infection (Table 12, Fig. 6K). Not being bound by a theory, these genes may be negative regulators of T cell function or be regulators of the T cell dysfunctional program and are targets for modulation. Down-regulation of the genes that are up-regulated in response to IL-27 signaling may result in an enhanced immune response and reactivation of exhausted T cells. Thus, in certain embodiments the identified genes may be used as a gene signature to identify or detect T cells with a dysfunctional phenotype. In other embodiments, the overlapping genes may be modulated or targeted with an agent capable of modulating expression or activity of a gene for the treatment of certain disorders, such as cancer. Accordingly, in some embodiments, one or more target genes selected from Table 12 are modulated using one or more modulating agents as described herein. In some embodiments, two or more target genes selected from Table 12 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as cancer. In preferred embodiments, genes selected from Table 12 are modulated by downregulation of expression or activity.

Table 12: Genes up-regulated under IL-27 signaling that overlap between dysfunctional CD8⁺ T cell signatures from cancer and chronic viral infection.

Il33	Adam8	Isg20	Cysltr2
Klrc2	Lpar3	Lamp2	Entpd1
Klrd1	Ccl9	Ly75	Gcnt1
Klre1	Cxcl10	Nampt	Ifitm3
Olfm1	Ccr2	S1pr1	Il2ra
Pdpn	Il10ra	Il21	Pglyrp1
Ptpn3	Il2rb	Il13ra1	Cd93
Sdc1	Cd68	Tigit	Adam9
Tnfsf9	Klrk1	Ccr5	Lilrb4
Vldlr	Il12rb2	Alcam	IL-10
Procr	Il6st	Havcr2	Ctla2a
Gabarapl1	Il7r	Lag3	Gpr65

Spp1	Inhba	Ilr2	
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[00210] As described herein, genes were identified that are enriched in a population of dysfunctional CD8⁺ T cells that had high scores for the disclosed signature associated with IL-27 signaling (i.e. the gene expression signature shown in Table 11). Not being bound by a theory, these genes may be negative regulators of CD8⁺T cell function or be regulators of the T cell dysfunctional program and are targets for modulation. Down-regulation of the genes that are up-regulated in CD8⁺ T cells bearing an IL-27 signaling signature may result in an enhanced immune response and reactivation of exhausted T cells. Thus, described herein are genes that were identified as up-regulated or down-regulated in CD8⁺ TILs which exhibited expression signatures similar to those associated with IL-27 signaling (Table 13). Not being bound by a theory, up-regulation of the genes that are down-regulated in CD8⁺ T cells bearing an IL-27 signaling signature may result in an enhanced immune response and reactivation of exhausted T cells. Thus, in certain embodiments the enriched genes may be used as a gene signature to identify or detect CD8⁺ T cells with a dysfunctional phenotype. In other embodiments, the enriched genes may be modulated or targeted with an agent capable of modulating expression or activity of a gene for the treatment of certain disorders, such as cancer. Accordingly, in some embodiments, one or more target genes selected from Table 13 are modulated using one or more modulating agents as described herein. In some embodiments, two or more target genes selected from Table 13 are modulated using two or more modulating agents as described herein, for the treatment of certain disorders, such as cancer. In preferred embodiments, up-regulated genes selected from Table 13a are modulated by down-regulation of expression or activity. In preferred embodiments, down-regulated genes selected from Table 13b are modulated by up-regulation of expression or activity.

Table 13							
<i>Table 13a: Up-regulated mouse genes that were in enriched in CD8+ TILs with high score for the IL-27 signature</i>							
5-Mar	Ccdc127	Evi2b	H2-Q2	Lrrc58	Pdxdc1	Sh2d2a	Tsc22d4
1600014C 10Rik	Ccdc82	Fam102a	H2-Q4	Lrrn4cl	Phc3	Sh3glb1	Ttc39b
1700017B 05Rik	Ccdc88c	Fam149b	H2-Q4	Luc7l2	Phf1	Sipa1l1	Tyr
2810474O 19Rik	Ccl5	Fam189b	H2-Q4	Luc7l2	Phf20l1	Skil	Uba7
4932438A 13Rik	Ccni	Fam65b	H2-Q4	Macf1	Pigv	Sla2	Ube2h

A230046K							
03Rik	Ccr5	Fam65b	H2-Q4	Maoa	Pik3cg	Slc35e2	Ubr4
Aak1	Cd244	Fcho1	H2-Q4	Map3k1	Pik3r1	Slc35e2	Ulk3
Abcb1a	Cd300a	Fgl2	H2-T10	Mbd4	Pitpnc1	Slc7a14	Unkl
Abcg1	Cd300a	Fli1	H2-T10	Mcmcdc2	Plcg1	Slc9a9	Usp48
Abr	Cd38	Fmn1	H2-T10	Mfap1a	Pnpla7	Slfn5	Usp9x
Abt1	Cdc14b	Foxn3	H2-T10	Mfsd11	Pot1b	Slfn8	Utp23
Acad9	Cdkn1b	Fryl	H2-T10	Mgea5	Ppm1k	Slfn8	Utrn
Acsbg1	Celf2	Fut8	H2-T10	Mier1	Ppp1r12a	Soat2	Vasp
Adam19	Chrna1	Fyco1	Hdac4	Miip	Ppp1r12b	Son	Vps13a
Adar	Cic	Gabpb2	Herc1	Milr1	Ppp1r16b	Sorl1	Vps37b
Adcy7	Cnppd1	Gak	Hip1	Mplkip	Ppp1r18	Spata13	Vps54
Ahnak	Colec12	Galnt2	Hipk1	Mpv17	Ppp3cc	Spata13	Wasf2
Akap13	Cpne8	Gbp7	Hmha1	Mpv17l	Prex1	Spn	Wbp2
Akna	Crebbp	Gbp7	Hnrnpd	Mpv17l	Prrc2b	Srrm2	Wdr34
Alox8	Csnk1g1	Gbp9	Hnrnpl	Mtfmt	Prrc2c	Stim1	Wdr92
Ankrd11	Ctcf1	Ggnbp2	Ifnar1	Mtmr1	Ptpn22	Stk10	Whsc111
Ankrd12	Ctsa	Ghdc	Ifngr1	Myh9	Purb	Stxbp2	Wipf1
Ankrd13a	Ctsd	Gimap3	Igf2r	Myo1f	Pxmp4	Suv420h1	Wnk1
Ankrd44	Ctsd	Gimap3	Ikbip	Mysm1	Rab33b	Syne1	Wtap
Ankrd44	Cxcr2	Gimap4	Ii16	Nabp1	Rapgef6	Synj2bp	Xaf1
Aplf	Cxcr6	Gimap6	Intu	Nbeal2	Rapgef6	Sytl2	Xiap
Arhgef1	Cybrd1	Gimap8	Irak1	Nbr1	Rassf2	Tab2	Xiap
Arid1a	Cyld	Gjc3	Irak2	Ncoa3	Rbm41	Tacc1	Xpo7
Arid4a	Cytip	Gje1	Irf2bpl	Ncor1	Rbm5	Tbc1d14	Yipf4
Arid4b	Dcaf10	Glrx2	Irga4	Neu3	Rdh1	Tbc1d24	Ypel5
Arid5a	Dclre1c	Gm11127	Irgal	Neur3	Rgs1	Tecpr1	Zbtb44
Arl4c	Ddx58	Gm11127	Irgav	Nktr	Rgs3	Tet2	Zc3h12a
Arsb	Decr2	Gm11127	Kansl1	Nlrc5	Ripply3	Tigit	Zc3hav1
Ash1l	Dennd1c	Gm11127	Kdm5a	Nlrp1a	Rmi2	Tmem127	Zcchc11
Asxl2	Dennd4a	Gm11127	Kif21b	Nmrk1	Rnf139	Tmem63a	Zcchc6
Atf7	Dgat1	Gm11127	Klf13	Notch1	Rnf166	Tmem69	Zfp113
Atp2b1	Dgka	Gm7102	Klf6	Npc2	Rnf167	Tmem88b	Zfp202
Atp2b4	Dnajc14	Gmeb1	Klrc2	Nsd1	Rnf168	Tmf1	Zfp277
Atxn1	Dock10	Gng2	Klrc2	Nsl1	Rock1	Tnfrsf10b	Zfp316
Azi2	Dock2	Gpsm3	Klrc2	Nup210	Rprd2	Tnfrsf10b	Zfp3612
B4galnt2	Dtx3l	Grap2	Klrc2	Oas3	Rsbnl1	Tnfrsf10b	Zfp488
Baiap3	Dusp11	Grina	Klrc2	Olfr1033	Runx2	Tnfrsf10b	Zfp605
Bcl11b	Dusp5	Grk4	Klrc2	Omd	Runx3	Tnfsf10	Zfp781
Birc6	E030030I 06Rik	Grk6	Lbh	Osbp13	S1pr4	Tnrc6a	Zfp9
Bnip3l	Eif2ak2	Gsk3b	Ldb1	P2ry10	Samhd1	Tnrc6b	Zmym5
Brip1	Elf1	Gtdc1	Leng8	Padi2	Sap18	Tor4a	Zmynd8
Btbd16	Entpd1	Gzmk	Lime1	Pak2	Sec62	Tprkb	Zscan26
Camk4	Entpd1	H2-Q2	Lime1	Pan3	Selplg	Trappc9	Zyg11b
Car5b	Ep300	H2-Q2	Lipi	Pced1b	Serinc3	Trim12c	
Casc4	Ep400	H2-Q2	Lnpep	Pcnt	Serpina3i	Trim65	
Cbfa2t2	Epsti1	H2-Q2	Loxl2	Pdcd4	Serpina3i	Trp53i11	
Cblb	Etsl	H2-Q2	Lpp	Pde3b	Sfil	Trp53inpl	

Table 13b: Up-regulated mouse cell surface and cytokine genes that were enriched in CD8+ TILs with high score for the IL-27 signature

Cast	Cd200rl	Csfl	Flot2	Il12rb2	Klrcl	Ncor2	Smpd1
Ccl3	Cd200rl	Ctla4	Gpil	Il18rap	Klrcl	Nrpl	Spn
Ccl3	Cd200r4	Ctsb	Gprl60	Irak2	Klrcl	Pdcdl	Tnfrsf9
Ccl3	Cd200r4	Cx3crl	Hcst	Itga4	Klrcl	Pearl	Trpv2
Ccl4	Cd244	Cxcr6	Icos	Itgal	Klrcl	Selplg	
Ccr12	Cd38	Erp44	Ifng	Itgav	Klrcl	Sema4d	
Cd164	Cd3g	Fasl	Ifngrl	Itgb2	Lgalsl	Serpine2	
Cast	Cd200rl	Csfl	Flot2	Il12rb2	Klrcl	Ncor2	

Table 13c: Up-regulated human genes that were enriched in CD8+ TILs with high score for the IL-27 signature

5-Mar	CCDC127	FAM149B1	HLA-C	LRRN4CL	PDXDC1	SH3GLB1	UBA7
AAK1	CCDC82	FAM189B	HLA-C	LUC7L2	PHC3	SIPA1L1	UBE2H
ABC1	CCDC88C	FAM65B	HLA-E	MACF1	PHF1	SKIL	UBR4
ABCG1	CCL5	FCH1	HLA-E	MAOA	PHF20L1	SLA2	ULK3
ABR	CCN1	FGF2	HLA-E	MAP3K1	PIGV	SLC35E2	UNKL
ABT1	CCR2	FLU	HLA-E	MBD4	PIK3CG	SLC35E2B	USP48
ACAD9	CD244	FMNL1	HLA-F	MCMDC2	PIK3R1	SLC7A14	USP9X
ACSBG1	CD300A	FOXN3	HLA-F	MFAP1	PITPNC1	SLC9A9	UTP23
ADAM19	CD300C	FRYL	HLA-F	MFSD11	PLCG1	SLFN11	UTRN
ADAR	CD38	FUT8	HLA-F	MGEA5	PNPLA7	SLFN13	VASP
ADCY7	CDC14B	FYCO1	HLA-G	MIR1	POT1	SLFN5	VPS13A
AHNAK	CDK6	GABPB2	HLA-G	MIRP	PPM1K	SOAT2	VPS37B
AKAP13	CDKN1B	GAK	HLA-G	MILR1	PPP1R12A	SON	VPS54
AKNA	CELF2	GALNT2	HLA-G	MPLKIP	PPP1R12B	SORL1	WASF2
ALOX15B	CHRNA1	GBP4	HNRNP	MPLKIP	PPP1R16B	SPATA13	WBP2
ANKRD11	CIC	GBP6	HNRNP	MPV17	PPP1R18	SPN	WDR34
ANKRD12	CNPPD1	GBP7	IFNAR1	MPV17L	PPP3CC	SRRM2	WDR92
ANKRD13A	C0LEC12	GNBP2	IFNGR1	MTFMT	PREX1	STIM1	WHSC1L1
ANKRD44	CPNE8	GHDC	IGF2R	MTMR1	PRRC2B	STK10	WIPF1
APLF	CREBBP	GIMAP1-GIMAP5	IKBIP	MYH9	PRRC2C	STXBP2	WNK1
ARHGAP45	CSNK1G1	GIMAP4	IL16	MYO1F	PTPN22	SYNE1	WTAP
ARHGEF1	CTCF	GIMAP5	INTU	MYSM1	PURB	SYNJ2BP	XAF1
ARID1A	CTSA	GIMAP6	IRAK1	NABP1	PXMP4	SYTL2	XIAP
ARID4A	CTSD	GIMAP8	IRAK2	NBEAL2	RAB33B	TAB2	XP07
ARID4B	CXCR2	GJC3	IRF2BPL	NBR1	RAPGEF6	TACC1	YIPF4
ARID5A	CXCR6	GJE1	ITGA4	NCOA3	RASSF2	TBC1D14	YPEL5
ARL4C	CYBRD1	GLRX2	ITGAL	NCOR1	RBM41	TECPR1	ZBTB44
ARSB	CYLD	GMEB1	ITGAV	NEU3	RBM5	TET2	ZC3H12A
ASH1L	CYTIP	GNG2	KANSL1	NEURL3	RDH16	TIGIT	ZC3HAV1
ASXL2	DCAF10	GPSM3	KDM5A	NKTR	RGS1	TMEM127	ZCCHC11

ATF7	DCLRE1C	GRAP2	KIAA1033	NLRC5	RGS3	TMEM63A	ZCCHC6
ATP2B1	DDX58	GRINA	KIAA1109	NLRP1	RIPPLY3	TMEM69	ZFP36L2
ATP2B4	DECR2	GRK4	KIAA1551	NMRK1	RMI2	TMEM88B	ZMYM5
ATXN1	DENND1C	GRK6	KIF21B	NOTCH1	RNF139	TMF1	ZMYND8
AZI2	DENND4A	GSK3B	KLF13	NPC2	RNF166	TNFRSF10A	ZNF202
B4GALNT2	DGAT1	GTDC1	KLF6	NSD1	RNF167	TNFRSF10B	ZNF25
BAIAP3	DGKA	GZMK	KLRC1	NSL1	RNF168	TNFRSF10C	ZNF277
BCL11B	DOCK10	HDAC4	KLRC2	NTN3	ROCK1	TNFRSF10D	ZNF3
BIRC6	DOCK2	HERC1	KLRC3	NUP210	RPRD2	TNFSF10	ZNF316
BIRC8	DTX3L	HIP1	KLRC4	OAS3	RSBN1L	TNRC6A	ZNF488
BNIP3L	DUSP11	HIPK1	KLRC4-KLRK1	OMD	RUNX2	TNRC6B	ZNF605
BRIP1	DUSP5	HLA-A	KMT5B	OR5M3	RUNX3	TOR4A	ZNF781
BTBD16	EIF2AK2	HLA-A	LBH	OSBPL3	S1PR4	TP53I11	ZSCAN26
C15orf39	ELF1	HLA-A	LDB1	P2RY10	SAMHD1	TP53I1NP1	ZYG11B
C19orf12	ENTPD1	HLA-A	LENG8	PADI2	SAP18	TPRKB	
C7orf55-LUC7L2	EP300	HLA-B	LIME1	PAK2	SEC62	TRAPPC9	
CA5B	EP400	HLA-B	LIP1	PAN3	SELPLG	TRIM5	
CAMK4	EPST11	HLA-B	LNPEP	PCED1B	SERINC3	TRIM65	
CASC4	ETS1	HLA-B	LOXL2	PCNT	SERPINA3	TSC22D4	
CBFA2T2	EVI2B	HLA-C	LPP	PDCD4	SFI1	TTC39B	
CBLB	FAM102A	HLA-C	LRRC58	PDE3B	SH2D2A	TYR	

Table 13d: Up-regulated human cell surface and cytokine genes that were enriched in CD8+ TILs with high score for the IL-27 signature

CAST	CD200R1	CSF1	FLOT2	IL12RB2	KLRC1	NRP1	SPN
CCL18	CD200R1	CTLA4	GPI	IL18RAP	KLRC2	PDCD1	TNFRSF9
CCL3	CD200R1L	CTSB	GPR160	IRAK2	KLRC3	PEAR1	TRPV2
CCL3L3	CD200R1L	CX3CR1	HCST	ITGA4	KLRC4	SELPLG	
CCL4	CD244	CXCR6	ICOS	ITGAL	KLRC4-KLRK1	SEMA4D	
CCRL2	CD38	ERP44	IFNG	ITGAV	LGALS1	SERPINE2	
CD164	CD3G	FASLG	IFNGR1	ITGB2	NCOR2	SMPD1	

Table 13e: Down-regulated mouse genes that were enriched in CD8+ TILs with high score for the IL-27 signature

1810022K09Rik	Cdk4	Eif5a	lars	Ndufa4	Polr2h	Rpn1	Tcp1
2810004N23Rik	Cebpz	Eif5a	ldh3a	Ndufab1	Polr2j	Rpn2	Tex30
Aatf	Chchd1	Eif6	Il2ra	Ndufaf2	Ppa1	Rps19bp1	Tfdp1
Abce1	Chchd2	Eif6	Imp4	Ndufb4	Ppan	Rps27l	Tfric
Abcf2	Chchd4	Emc2	Impdh2	Ndufb6	Ppan	Rrp1	Thoc5
Adpgk	Cinp	Emc6	lpo4	Ndufc2	Ppat	Rrp15	Thumpd3

Adrm1	Cirh1a	Eno3	lpo5	Ndufc2	Ppib	Rrp9	Thyn1
Aen	Cisd1	Enoph1	Jtb	Ndufs3	Ppid	Rrsl	TimmlO
Aga	Cks1b	Erh	Kars	Ndufs8	Ppie	Rslld1	Timml3
Ahcy	Clnsla	Exosc1	Kpna2	Ndufv1	Ppif	Rsl24d1	Timml7a
Aifm1	Cluh	Exosc5	Ktil2	Ndufv2	Ppp5c	Ruvbl1	Timm23
Akr7a5	Cops3	Exosc7	Lad1	Nfkb1a	Prdx1	Ruvbl2	Timm23
Aldh18a1	Cops6	Fam136a	Lap3	Nfkb1b	Prdx4	Samm50	Timm50
Aldh9a1	Cox6b1	Fam162a	Ldha	Nhp2	Preld1	Sarnp	Timm8a1
Alg8	Cox7c	Fam96a	Letm1	Nhp2l1	Prmt1	Sdf2l1	Tkt
Anapc15	Crtam	Fbl	Llph	Nme1	Prmt5	Sdhaf1	Tmal6
Anapc5	Csell	Fdps	Lsm2	Nme2	Prmt7	Sec13	Tma7
Anp32e	Ctps	Fdxl1	Lsm7	Nobl	Prps1	Sec61b	Tmed2
Apex1	Ctsz	Fdxl1	Lta	Noc4l	Psat1	Serbpl	Tmem14c
Api5	Cycl	Fkbpla	Lyar	Nolcl	Psm2	Set	Tmem14c
Aprt	Cyca	Fkbp2	M6pr	Nop1O	Psm2	Set	Tmem97
Arfl	Dad1	Fkbp4	Magoh	Nop16	Psm3	Sf3b5	Tnfrsf9
Atad3a	Dapll	Ftsj3	Manf	Nop2	Psm5	Sfxn1	Tomm22
Atad3a	Dars	G3bpl	Mat2a	Nop56	Psm6	Shmt1	Tomm40
Atad3a	Dbi	Gadd45b	Mcm2	Nop58	Psm5	Shmt2	Tomm5
Atp5a1	Dctpp1	Gars	Mcm3	Nsun2	Psm11	Siva1	Tpil
Atp5b	Ddbl	Gart	Mcm5	Ntmt1	Psm3	Skpla	Trp53
Atp5e	Ddx1	Gcsh	Mcm7	Nude	Psm6	Skpla	Tsrl
Atp5e	Ddx18	Gfer	Med11	Nudtl9	Psm7	Slc19a1	Tubalb
Atp5g1	Ddx21	Gins2	Mettl1	Nudt21	Psmg1	Slc1a5	Tubg1
Atp5g2	Ddx27	Gnl3	Mif	Nudt5	Psmg2	Slc25a39	Tufm
Atp5g3	Ddx39	Gpatch4	Mphosph6	Nup54	Ptbpl	Smyd2	Txn1
Atp5j	Dkcl	Gps1	Mrpl12	Nup62	Ptges3	Smyd5	Txn2
Atp5j2	Dnajb11	Gpx1	Mrpl20	Nutf2-ps1	Ptpn6	Snrpal	Txn14a
Atp5k	Dnajc19	Gramdlb	Mrpl23	Ost4	Pus1	Snrpd1	U2af1
Atpif1	Dohh	Grwdl	Mrpl23	Ostc	Pus11	Snrpd3	U2af1
Banfl	Dpagt1	Gtf2f2	Mrpl28	P4hb	Pwp2	Snrpe	Uchl3
Bcap29	Dpy30	Gtf2h1	Mrpl3	Pa2g4	Pwp2	Snrpf	Uchl5
Bccip	Drg2	Gtpbp4	Mrpl30	Pa2g4	Pycl	Snrpg	Uck2
Bola2	Dtymk	Gypc	Mrpl30	Paics	Rabggtb	Spcs3	Uhrfl
Bola2	Duspl4	Hars	Mrpl30	Parpl	Rad51	Spr	Umps
Bopl	Dut	Haus7	Mrpl38	Pbdcl	Rael	Srm	Ung
Brix1	Ebnalbp2	Hax1	Mrpl42	Pcbpl	Ran	Srsf1O	Uqcr1O
Bsg	Eef1d	Hint1	Mrpl52	Pdcd2l	Ranbpl	Srsf3	Uqcrb
Bud31	Eef1e	Hivep3	Mrpl8b	Pdia6	Rars	Srsf6	Uqcrcl
Bzw2	Eftud2	Hmbs	Mrps26	Pebpl	Rbbp7	Srsf7	Uqcrq
Clqbp	Eif2b1	Hn11	Mrps28	Pes1	Rbfa	Ssb	Usmg5
Cacybp	Eif2b3	Hnrnpa1	Mrps36	Pfdn2	Rbm38	Ssr2	UsplO
Cad	Eif2s1	Hnrnpa1	Mrps5	Pgkl	Reel	Sssca1	Vars
Calr	Eif2s2	Hnrnpc	Mrps6	Phb	Rcc2	Stat5a	Vcp
Canx	Eif2s3x	Hnrnpc	Mrto4	Phb2	Rcll	Stip1	Wdr12
Ccdc86	Eif2s3x	Hnrnpc	Ms4a4c	Phf5a	Rel	Stmn1	Wdr18
Cell	Eif3a	Hnrnpc	Ms4a4c	Phgdh	Rexo2	Stom12	Wdr4
Ccnel	Eif3c	Hnrnpc	Mtap	Pigu	Rfc3	Strap	Wdr43
Ccr7	Eif3c	Hnrnrm	Mtap	Plrg1	Rfc4	Stt3a	Wdr46

Cct2	Eif3d	Hsp90abl	Mtch2	Pmfl	Rgcc	Suc1gl	Wdr61
Cct3	Eif3e	Hsp90bl	Mthfd 1	Pmpcb	Rnmtll	Syncrip	Wdr74
Cct4	Eif3g	Hspa4	Mthfd2	Pnol	Rnpsl	Syngr2	Wdr75
Cct5	Eif3i	Hspa5	Mybbpla	Pola2	Rpf2	Tafld	Wdr77
Cct7	Eif3l	Hspa9	Naa20	Pold2	Rpl22ll	Taf6	Xcll
Cct8	Eif3m	Hspbpl	Naa25	Poldip2	Rpl26	Tagln2	Xcll
Cd83	Eif4a l	Hspdl	Nasp	Polrle	Rpl30	Tbcb	Ywhae
Cdca7	Eif4a3	Hspel	Ncl	Polr2c	Rpl35	Tbrg4	Ywhaq
Cdk2	Eif4e	Hsphl	Ndufa l 2	Polr2f	Rpl36al	Tceb2	Zfp593

Table 13f: Down-regulated mouse cell surface and cytokine genes that were in enriched in CD8+ TILs with high score for the IL-27 signature

Clqbp	Hnrnpu	Itgb7	Wnt4
Ccnd2	Hsp90aa l	Slprl	Xcll
Ccr7	Hspa9	Sell	Xcll
Cd69	Il7r	Tnfsfl4	

Table 13g: Down-regulated human genes that were in enriched in CD8+ TILs with high score for the IL-27 signature

AATF	CDK2	EIF5A	IDH3A	NDU FB6	PPI B	RSL24D1	TIM M 13
ABCE1	CDK4	EIF5AL1	IL2RA	NDUFC2	PPI D	RUVBL1	TIM M 17A
ABCF2	CEBPZ	EIF6	IM P4	NDU FC2-KCTD14	PPI E	RUVBL2	TIM M23
ADPG K	CHCH D1	EMC2	IM PDH2	NDUFS3	PPI F	SAM M50	TIM M23B
ADRM 1	CHCH D2	EMC6	IP04	NDUFS8	PPP5C	SARN P	TIM M50
AEN	CHCH D4	EN03	IP05	NDU FV1	PRDX1	SDF2L1	TIM M8A
AGA	ciNP	EN0PH 1	JTB	NDU FV2	PRDX4	SDHAF1	TKT
AHCY	CISDI	ERH	KARS	NFKBIA	PRELIDI	SEC13	TMA16
AIFM 1	CKS1B	EXOSC1	KPNA2	NFKBI B	PRMT1	SEC61B	TMA7
AKR7A2	CLNS1A	EXOSC5	KTI 12	NHP2	PRMT5	SERBP1	TM ED2
ALDH 18A 1	CLU H	EXOSC7	LAD1	NM E1	PRMT7	SET	TM EM 14B
ALDH9A1	C0PS3	FAM 136A	LAP3	NM E2	PRPS1	SETSI P	TM EM 14C
ALG8	C0PS6	FAM 162A	LDHA	NOB1	PSAT1	SF3B5	TM EM97
ANAPC15	C0X6B1	FAM96A	LETM 1	NOC4L	PSMA2	SFXN 1	TNFRSF9
ANAPC5	C0X7C	FBL	LLPH	NOLC1	PSMA3	SHMT1	TOM M22
ANP32E	CRTAM	FDPS	LSM2	NOP10	PSM B5	SHMT2	TOM M40
APEX1	CSE1L	FDX2	LSM7	NOP16	PSM B6	SIVA1	TOM M5
API5	CTPS1	FKBP1A	LTA	NOP2	PSMC5	SKP1	TP53
APRT	CTSZ	FKBP2	LYAR	NOP56	PSM D11	SLC19A1	TPI 1
ARF1	CYC1	FKBP4	M6PR	NOP58	PSM D3	SLC1A5	TSR1
ATAD3A	CYCS	FTSJ3	MAGOH	NSU N2	PSM D6	SLC25A39	TUBA1B
ATAD3B	DAD1	G3BP1	MAN F	NTMT1	PSM D7	SMYD2	TUBG1
ATAD3C	DAPL1	GADD45B	MAT2A	NUDC	PSMG1	SMYD5	TUFM
ATP5A1	DARS	GARS	MCM2	NUDT19	PSMG2	SNRPA1	TXN
ATP5B	DBI	GART	MCM3	NUDT21	PTBP1	SNRPD1	TXN2
ATP5E	DCTPP1	GCSH	MCM5	NUDT5	PTG ES3	SNRPD3	TXN L4A
ATP5EP2	DDB1	GFER	MCM7	NUP54	PTPN6	SNRPE	U2AF1
ATP5G 1	DDX1	GINS2	M ED11	NUP62	PUS1	SNRPF	U2AF1L5
ATP5G2	DDX18	GNL3	M E TTL1	NUTF2	PUSL1	SNRPG	UCH L3

ATP5G3	DDX21	GPATCH4	M IF	OST4	PWP2	SNU 13	UCH L5
ATP5 1	DDX27	GPS1	M PHOSPH 6	OSTC	PYCRL	SPCS3	UCK2
ATP5J	DDX39A	GPX1	M RM3	P4H B	RABGGTB	SPR	UHRF1
ATP5J2	DKC1	GRAM D1 B	M RPL12	PA2G4	RAD51	SRM	UM PS
ATPIF1	DNAJ B11	GRWD1	M RPL20	PAICS	RAE1	SRSF10	UNG
BAN F1	DNAJC19	GTF2F2	M RPL23	PARP1	RAN	SRSF3	UQCR10
BCAP29	DOH H	GTF2H 1	M RPL28	PBDC1	RAN BP1	SRSF6	UQCRB
BCCIP	DPAGT1	GTPBP4	M RPL3	PCBP1	RARS	SRSF7	UQCRC1
B0LA2	DPY30	GYPC	M RPL30	PDCD2L	RBBP7	SSB	UQCRQ
B0LA2B	DRG2	HARS	M RPL38	PDIA6	RBFA	SSR2	USMG5
B0P1	DTYM K	HAUS7	M RPL42	PEBP1	RBM38	SSSCA1	USP10
BRIX1	DUSP14	HAX1	M RPL52	PES1	RCC1	STAT5A	UTP4
BSG	DUT	HINT1	M RPS18B	PFDN2	RCC2	STI P1	VAR5
BUD31	EBNA1BP 2	HIVEP3	M RPS26	PGK1	RCL1	STM N 1	VCP
BZW2	EEF1D	HM BS	M RPS28	PH B	REL	STOM L2	WDR12
Clorf131	EEF1E1-BLOC1S5	HN1L	M RPS36	PHB2	REX02	STRAP	WDR18
C1QBP	EFTU D2	HNRNPA1	M RPS5	PH F5A	RFC3	STT3A	WDR4
C8orf59	EIF2B1	HNRNPA1 L2	M RPS6	PHGDH	RFC4	SUCLG1	WDR43
CACYBP	EIF2B3	HNRN PC	M RT04	PIGU	RGCC	SYNCRIP	WDR46
CAD	EIF2S1	HNRN PCL 1	MS4A4A	PLRG1	RNPS1	SYNGR2	WDR61
CALR	EIF2S2	HNRN PCL 2	MS4A4E	PMF1	RPF2	TAF1D	WDR74
CANX	EIF2S3	HNRN PCL 3	MTAP	PMPCB	RPL22L1	TAF6	WDR75
CCDC86	EIF3A	HNRN PCL 4	MTCH2	PNOI	RPL26	TAGLN2	WDR77
CCL1	EIF3C	HNRNPM	MTH FD1	POLA2	RPL30	TBCB	XCL1
CCN E1	EIF3CL	HSP90AB1	MTH FD2	POLD2	RPL35	TBRG4	XCL2
CCR7	EIF3D	HSP90B1	MYBBP1A	POLDI P2	RPL36A	TCEB2	YWHA E
CCT2	EIF3E	HSPA4	NAA20	POLR1E	RPN 1	TCP1	YWHAQ
CCT3	EIF3G	HSPA5	NAA25	POLR2C	RPN2	TEX30	ZNF593
CCT4	EIF3I	HSPA9	NASP	POLR2F	RPS19BP1	TFDP1	
CCT5	EIF3L	HSPBP1	NCL	POLR2H	RPS27L	TFRC	
CCT7	EIF3M	HSPD1	NDUFA12	POLR2J	RRP1	THOC5	
CCT8	EIF4A1	HSPE1	NDUFA4	PPA1	RRP15	THUMPD3	
CD83	EIF4A3	HSPH 1	NDUFAB1	PPAN	RRP9	THYN 1	
CDCA7	EIF4E	IARS	NDUFAF2	PPAN-P2RY11	RRS1	TIM M 10	
Table 13h: Down-regulated human cell surface and cytokine genes that were in enriched in CD8+ TILs with high score for the IL-27 signature							
C1QBP		HNRNPU		ITG B7		WNT4	
CCN D2		HSP90AA1		S1PR1		XCL1	
CCR7		HSPA9		SELL		XCL2	

CD69	IL7R	TNFSF14	
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[00211] As described herein, Prdml and c-Maf together regulate a co-inhibitory gene module that determines anti-tumor immunity. Applicants describe that anti-tumor immunity can be modulated upon modulating both genes (e.g., see Figs. 12-14). Accordingly, in some embodiments, anti-tumor immunity is modulated using two or more modulating agents as described herein for the treatment of certain disorders, such as cancer. In preferred embodiments, Prdml and c-Maf are modulated by downregulation of expression or activity. In other embodiments, Prdml and c-Maf are modulated by upregulation of expression or activity.

[00212] Because Prdml and c-Maf each regulate numerous co-inhibitory receptors, it may be advantageous to modulate express of only one of Prdml or c-Maf at a time. Thus, in some embodiments, Prdml or c-Maf are modulated by downregulation of expression or activity. In other embodiments, Prdml or c-Maf are modulated by upregulation of expression or activity. In preferred embodiments, Prdml and c-Maf are modulated by downregulation of expression or activity. In preferred embodiments, Prdml and c-Maf are modulated by upregulation of expression or activity.

[00213] In one embodiment, at least one target gene selected from the list in Table 1, Table 10, Table 11, or Table 12 or the combination of Prdml and/or c-Maf is modulated in combination with a treatment selected from the group consisting of: an immune checkpoint inhibitor, a CTLA-4 inhibitor, a PD-1 inhibitor, chemotherapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-IBB and/or GITR. In some embodiments, the combination of modulation of at least one target gene selected from the list in Table 1, Table 10, Table 11, or Table 12 or the combination of Prdml and/or c-Maf in combination with a treatment selected from the group consisting of: an immune checkpoint inhibitor, a CTLA-4 inhibitor, a PD-1 inhibitor, chemotherapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-IBB and/or GITR produces a synergistic effect (e.g., the effect of the agents used in combination is greater than the sum of the effect of each agent alone).

[00214] In one embodiment, the methods, compositions and uses described herein comprise modulation of PDPN expression, activity and/or function, PROCR expression, activity, and/or function, or modulation of the combination of Prdml and c-Maf expression, activity and/or function, and at least one additional target gene/gene product or combination selected from the group consisting of those listed in Table 1, Table 10, Table 11, or Table 12

or the combination of Prdml and c-Maf. In another embodiment, the methods, compositions and uses described herein comprise modulation of PDPN expression, activity and/or function, PROCR expression, activity, and/or function, or modulation of the combination of Prdml and c-Maf expression, activity and/or function, and at least one additional target gene/gene product selected from the group consisting of TIGIT, LAG3, LILRB4, and KLRC1. In another embodiment, the methods, compositions and uses described herein comprise inhibition of PDPN expression, activity and/or function, PROCR expression, activity, and/or function, or modulation of the combination of Prdml and c-Maf expression, activity and/or function, and inhibition of at least one additional target gene/gene product selected from the group consisting of TIGIT, LAG3, LILRB4, and KLRC1. In another embodiment, the methods, compositions, and uses described herein comprise inhibition of PDPN, PROCR, at least one additional target gene/gene product selected from the group consisting of TIGIT, LAG3, LILRB4, and KLRC1, and activation of expression, activity, and/or function of at least one of the target genes/gene products selected from the group consisting of: CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7. In another embodiment, the methods, compositions, and uses described herein comprise inhibition of the combination of Prdml and c-Maf, at least one additional target gene/gene product selected from the group consisting of TIGIT, LAG3, LILRB4, and KLRC1, and activation of expression, activity, and/or function of at least one of the target genes/gene products selected from the group consisting of: CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7. In one embodiment, a combination therapy comprising (i) a treatment selected from the group consisting of: an immune checkpoint inhibitor, a CTLA-4 inhibitor, a PD-1 inhibitor, chemotherapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-1BB and/or GITR, (ii) modulation of PDPN, PROCR or the combination of Prdml and c-Maf (iii) optionally modulating at least one additional target gene/gene product selected from the group consisting of TIGIT, LAG3, LILRB4, and KLRC1 and (iv) optionally inducing activation of expression, activity, and/or function of at least one of the target genes/gene products selected from the group consisting of: CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7 is used in the methods and compositions described herein.

[00215] In one embodiment, at least one target gene selected from the list in Table 1, Table 10, Table 11, or Table 12 or the combination of Prdml and/or c-Maf is modulated in an immune cell. In certain embodiments, the immune cell is a CD8⁺ T cell. In other

embodiments, the immune cell is modulated *ex vivo* and is used in an adoptive cell transfer therapy. In certain embodiments, autologous T cells are used in a personalized therapy. In other embodiments, a cell is provided with at least one gene modulated selected from the list in Table 1, Table 10, Table 11, or Table 12 or the combination of Prdml and/or c-Maf. In preferred embodiments, the cell is a CD8⁺ T cell. The CD8⁺ T cell may be a chimeric antigen receptor (CAR) T cell, described further herein.

[00216] In one embodiment, at least one target gene selected from the list in Table 1, Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, Table 11, Table 12, or Table 13 is used as part of a gene signature or biomarker signature to detect and/or isolate an immune cell, preferably a T cell with a specific immune state. In some embodiments, the biomarker or gene signature may comprise, consist essentially of, or consist of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 or more genes disclosed in Table 1, Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, Table 11, Table 12, or Table 13. For example, disclosed herein, a gene signature for dysfunctional T cell associated with chronic infection can comprise any combination of the genes disclosed in Table 6.

[00217] In some embodiments, the gene signature may comprise, consist essentially of, or consist of all types of genes, for instance genes that encode transcription factors, cell signaling molecule, cell surface receptors, or cytokines. In some embodiments, the gene signature may comprise, consist essentially of, or consist of genes that encode transcription factors, cell surface receptors, and cytokines. In some embodiments, the gene signature may comprise, consist essentially of, or consist of genes that encode cell surface receptors and cytokines. Not being bound by a theory, cell surface receptors or cytokines facilitate detection or isolation of cells without destroying the cell, such as by cell sorting, particularly FACS or magnetic sorting. In preferred embodiments, dysfunctional T cells are detected.

[00218] Detection may be part of a diagnostic assay or may be used as a method of determining whether a patient is suitable for administering an immunotherapy or another type of therapy. For example, detection of the disclosed gene or biomarker signatures may be performed in or to determine whether a patient is responding to a given treatment or, if the patient is not responding, if this may be due to T cell dysfunction. Such detection is informative regarding the types of therapy the patient is best suited to receive. For example, whether the patient should receive immunotherapy. Non-limiting examples on immunotherapeutics (exemplary embodiments also shown in Table 14) that may be used in the claimed methods or in conjunction with the claimed compositions include IMP321, BMS-

986016, LAG525, TSR022, MTIG7192A, TRX518, INCAGN01876, GWN323, MEDI1873, MEDI9447, PF-05082566 (utomilumab), BMS-663513 (urelumab), MOXR0916, MEDI6469, MEDI6383, PF045 18600, KHK4083, and combinations of two or more thereof. In preferred embodiments the immunotherapy may comprise administering at least one check point inhibitor.

Table 14	
Target	Active agents investigated in clinical trials
Lag-3	IMP321, BMS-986016, LAG525
Tim-3	TSR022
Tigit	MTIG7192A
Gitr (CD357)	TRX518, INCAGN01876, GWN323, MEDI1873
CD73	MEDI9447,
4-1BB (CD137, TNFRSF9)	PF-05082566 (utomilumab), BMS-663513 (urelumab)
OX40 (CD134)	MOXR0916, MEDI6469, MEDI6383, PF04518600, KHK4083

[00219] In some embodiments, a patient that is not responding to ACT may benefit from use of the detection methods to determine whether the adoptive cells are dysfunctional, and if so, what course of treatment could correct the dysfunction.

[00220] In some embodiments, the disclosed gene signature can be detected using methods disclosed herein or methods known in the art. For example, the disclosed gene signatures immunofluorescence, mass cytometry (CyTOF), FACS, drop-seq, RNA-seq, single cell qPCR, MERFISH (multiplex (in situ) RNA FISH), microarray and/or by in situ hybridization. Other methods including absorbance assays and colorimetric assays are known in the art and may be used herein. In some aspects, measuring expression of signature genes comprises measuring protein expression levels. Protein expression levels may be measured, for example, by performing a Western blot, an ELISA or binding to an antibody array. In another aspect, measuring expression of said genes comprises measuring RNA expression levels. RNA expression levels may be measured by performing RT-PCR, Northern blot, an array hybridization, or RNA sequencing methods.

Signature Genes

[00221] As used herein a signature may encompass any gene or genes, or protein or proteins, whose expression profile or whose occurrence is associated with a specific cell type, subtype, or cell state of a specific cell type or subtype within a population of cells. Increased or decreased expression or activity or prevalence may be compared between different cells in order to characterize or identify for instance specific cell (sub)populations. A gene signature

as used herein, may thus refer to any set of up- and down-regulated genes between different cells or cell (sub)populations derived from a gene-expression profile. For example, a gene signature may comprise a list of genes differentially expressed in a distinction of interest. It is to be understood that also when referring to proteins (e.g. differentially expressed proteins), such may fall within the definition of "gene" signature.

[00222] The signatures as defined herein (being it a gene signature, protein signature or other genetic signature) can be used to indicate the presence of a cell type, a subtype of the cell type, the state of the microenvironment of a population of cells, a particular cell type population or subpopulation, and/or the overall status of the entire cell (sub)population. Furthermore, the signature may be indicative of cells within a population of cells in vivo. The signature may also be used to suggest for instance particular therapies, or to follow up treatment, or to suggest ways to modulate immune systems. The signatures of the present invention may be discovered by analysis of expression profiles of single-cells within a population of cells from isolated samples (e.g. blood samples), thus allowing the discovery of novel cell subtypes or cell states that were previously invisible or unrecognized. The presence of subtypes or cell states may be determined by subtype specific or cell state specific signatures. The presence of these specific cell (sub)types or cell states may be determined by applying the signature genes to bulk sequencing data in a sample. Not being bound by a theory, a combination of cell subtypes having a particular signature may indicate an outcome. Not being bound by a theory, the signatures can be used to deconvolute the network of cells present in a particular pathological condition. Not being bound by a theory the presence of specific cells and cell subtypes are indicative of a particular response to treatment, such as including increased or decreased susceptibility to treatment. The signature may indicate the presence of one particular cell type. In one embodiment, the novel signatures are used to detect multiple cell states or hierarchies that occur in subpopulations of immune cells that are linked to particular pathological condition (e.g. cancer), or linked to a particular outcome or progression of the disease, or linked to a particular response to treatment of the disease.

[00223] The signature according to certain embodiments of the present invention may comprise or consist of one or more genes and/or proteins, such as for instance 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 or more. In certain embodiments, the signature may comprise or consist of two or more genes and/or proteins, such as for instance 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48,

59, or 50 or more. In certain embodiments, the signature may comprise or consist of three or more genes and/or proteins, such as for instance 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, or 50 or more. In certain embodiments, the signature may comprise or consist of four or more genes and/or proteins, such as for instance 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, or 50 or more. In certain embodiments, the signature may comprise or consist of five or more genes and/or proteins, such as for instance 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, or 50 or more. In certain embodiments, the signature may comprise or consist of six or more genes and/or proteins, such as for instance 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, or 50 or more. In certain embodiments, the signature may comprise or consist of seven or more genes and/or proteins, such as for instance 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, or 50 or more. In certain embodiments, the signature may comprise or consist of eight or more genes and/or proteins, such as for instance 8, 9, 10 or more. In certain embodiments, the signature may comprise or consist of nine or more genes and/or proteins, such as for instance 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, or 50 or more. In certain embodiments, the signature may comprise or consist of ten or more genes and/or proteins, such as for instance 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, or 50 or more. For example, a signature for use in the disclosed detection methods can include a combination of genes either Table 1, Table 2, Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, Table 11, Table 12, or Table 13. It is to be understood that a signature according to the invention may for instance also include a combination of genes or proteins.

[00224] It is to be understood that "differentially expressed" genes/proteins include genes/proteins which are up- or down-regulated as well as genes/proteins which are turned on or off. When referring to up- or down-regulation, in certain embodiments, such up- or down-regulation is preferably at least two-fold, such as two-fold, three-fold, four-fold, five-fold, or more, such as for instance at least ten-fold, at least 20-fold, at least 30-fold, at least 40-fold, at

least 50-fold, or more. Alternatively, or in addition, differential expression may be determined based on common statistical tests, as is known in the art.

[00225] As discussed herein, differentially expressed genes/proteins may be differentially expressed on a single cell level, or may be differentially expressed on a cell population level. Preferably, the differentially expressed genes/proteins as discussed herein, such as constituting the gene signatures as discussed herein, when as to the cell population level, refer to genes that are differentially expressed in all or substantially all cells of the population (such as at least 80%, preferably at least 90%, such as at least 95% of the individual cells). This allows one to define a particular subpopulation of cells. As referred to herein, a "subpopulation" of cells preferably refers to a particular subset of cells of a particular cell type which can be distinguished or are uniquely identifiable and set apart from other cells of this cell type. The cell subpopulation may be phenotypically characterized, and is preferably characterized by the signature as discussed herein. A cell (sub)population as referred to herein may constitute of a (sub)population of cells of a particular cell type characterized by a specific cell state.

[00226] When referring to induction, or alternatively suppression of a particular signature, preferable is meant induction or alternatively suppression (or upregulation or downregulation) of at least one gene/protein of the signature, such as for instance at least to, at least three, at least four, at least five, at least six, or all genes/proteins of the signature.

[00227] Signatures may be functionally validated as being uniquely associated with a particular immune phenotype. Induction or suppression of a particular signature may consequentially be associated with or causally drive a particular immune phenotype.

[00228] Various aspects and embodiments of the invention may involve analyzing gene signatures, protein signature, and/or other genetic signature based on single cell analyses (e.g. single cell RNA sequencing) or alternatively based on cell population analyses, as is defined herein elsewhere.

[00229] In further aspects, the invention relates to gene signatures, protein signature, and/or other genetic signature of particular immune cell subpopulations, as defined herein. The invention hereto also further relates to particular immune cell subpopulations, which may be identified based on the methods according to the invention as discussed herein; as well as methods to obtain such cell (sub)populations and screening methods to identify agents capable of inducing or suppressing particular immune cell (sub)populations.

[00230] The invention further relates to various uses of the gene signatures, protein signature, and/or other genetic signature as defined herein, as well as various uses of the

immune cells or immune cell (sub)populations as defined herein. Particular advantageous uses include methods for identifying agents capable of inducing or suppressing particular immune cell (sub)populations based on the gene signatures, protein signature, and/or other genetic as defined herein. The invention further relates to agents capable of inducing or suppressing particular immune cell (sub)populations based on the gene signatures, protein signature, and/or other genetic signature as defined herein, as well as their use for modulating, such as inducing or repressing, a particular gene signature, protein signature, and/or other genetic signature. In related aspects, modulating, such as inducing or repressing, a particular gene signature, protein signature, and/or other genetic signature may modify overall immune cell composition, such as activated or dysfunctional immune cell composition, or distribution, or functionality.

[00231] As used herein the term "signature gene" means any gene or genes whose expression profile is associated with a specific cell type, subtype, or cell state of a specific cell type or subtype within a population of cells. The signature gene can be used to indicate the presence of a cell type, a subtype of the cell type, the state of the microenvironment of a population of cells, and/or the overall status of the entire cell population. Furthermore, the signature genes may be indicative of cells within a population of cells *in vivo*. Not being bound by a theory, the signature genes can be used to deconvolute the cells present in a tumor based on comparing them to data from bulk analysis of a tumor sample. The signature gene may indicate the presence of one particular cell type. In one embodiment, the signature genes may indicate that dysfunctional or activated tumor infiltrating T-cells are present. The presence of cell types within a tumor may indicate that the tumor will be resistant to a treatment. In one embodiment the signature genes of the present invention are applied to bulk sequencing data from a tumor sample to transform the data into information relating to disease outcome and personalized treatments. In one embodiment, the novel signature genes are used to detect multiple cell states that occur in a subpopulation of tumor cells that are linked to resistance to targeted therapies and progressive tumor growth. In preferred embodiments, immune cell states of tumor infiltrating lymphocytes are detected.

[00232] In one embodiment, the signature genes are detected by immunofluorescence, mass cytometry (CyTOF), FACS, drop-seq, RNA-seq, single cell qPCR, MERFISH (multiplex (in situ) RNA FISH), microarray and/or by in situ hybridization. Other methods including absorbance assays and colorimetric assays are known in the art and may be used herein. In some aspects, measuring expression of signature genes comprises measuring protein expression levels. Protein expression levels may be measured, for example, by

performing a Western blot, an ELISA or binding to an antibody array. In another aspect, measuring expression of said genes comprises measuring RNA expression levels. RNA expression levels may be measured by performing RT-PCR, Northern blot, an array hybridization, or RNA sequencing methods.

Modulating Agents

[00233] Provided herein are methods and compositions comprising one or more modulating agents that modulate the expression, activity and/or function of one or more target genes in Table 1, Table 10, Table 11, Table 12, or Table 13 or that modulate the expression, activity and/or function of the combination of Prdml and c-Maf and/or Prdml and c-Maf, individually, or pairs of target genes as shown in Table 2, or combinations thereof as described herein in any of Tables 3-9. In one embodiment, one or a combination of modulating agents is used to modulate T cell exhaustion. In some embodiments, the combination of modulating agents has a synergistic effect compared to the effect of each agent alone.

[00234] In some embodiments, the modulating agent is an activator of the expression, activity and/or function of one or more target genes. In some embodiments, where the desired effect is to increase non-responsiveness of a T-cell (*e.g.*, in autoimmune disease and/or transplants), an agent that induces an increase in the expression, activity and/or function of a negative regulator of T cell function from the list of target genes, such as in Table 4, will induce an increase in T cell non-responsiveness or exhaustion. Where the desired effect is to decrease T-cell exhaustion, an activating agent that increases the expression, activity and/or function of a positive regulator of T cell function from the list of target genes, such as in Table 3, can be used.

[00235] In some embodiments, the modulating agent is an inhibitor of the expression, activity, and/or function of one or more target genes listed in Table 1, Table 10, Table 11, or Table 12 or the combination of Prdml and c-Maf and/or Prdml and c-Maf, individually, or the pairs of target genes as shown in Table 2, or other combinations thereof as described herein. Where the desired effect of the inhibiting agent is to reduce T-cell exhaustion, an agent that inhibits the expression, activity and/or function of a negative regulator of T-cell function (see *e.g.*, Table 4) will induce a reduction in T-cell exhaustion. Where the desired effect of the inhibiting agent is to increase T-cell non-responsiveness (*e.g.*, autoimmune disease and/or transplant), an agent that inhibits the expression, activity and/or function of a positive regulator of T-cell function (*e.g.*, those listed in Table 4 and/or Tables 8-9), will induce T-cell non-responsiveness.

[00236] In some embodiments, one or more modulating agents are used in combination with the methods and compositions described herein. In some embodiments, two or more modulating agents are used in combination with the methods and compositions described herein. One of skill in the art will appreciate that, depending on the identities of the selected target genes or proteins, one can employ both inhibiting agents and activating agents in the same method and/or composition provided that the agents are employed with a common goal (*i.e.*, to produce a similar biological effect such as reduction of T-cell exhaustion) such that the agents work together additively, or preferably synergistically, towards the desired overall biological effect. In some embodiments, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 or more agents are formulated or administered in combination.

[00237] *Inhibitors:* As used herein, the terms "inhibitor," "antagonist," and "silencing agent," refer to a molecule or agent that significantly blocks, inhibits, reduces, or interferes with one or more target genes listed in Table 1, Table 10, Table 11, or Table 12 or the combination of Prdml and c-Maf or Prdml and c-Maf, individually, their biological activity *in vitro*, *in situ*, and/or *in vivo*, including activity of downstream pathways mediated by gene signaling. In some embodiments, the inhibitor or antagonist will modulate markers of T-cell exhaustion, such as, for example, lack of/reduction in proliferation, lack of/reduction in cytokine production, lack of/reduction in cytotoxic activity, lack of/reduction in trafficking or migration, transcription factor induction, IL-10 induction, and/or elicitation of a cellular response to IL-27. Exemplary inhibitors contemplated for use in the various aspects and embodiments described herein include, but are not limited to, antibodies or antigen-binding fragments thereof that specifically bind to one or more target genes listed in Table 1, Table 10, Table 11, or Table 12, or gene products thereof, or one or more subunits of the target gene(s)/product(s); anti-sense molecules directed to a nucleic acid encoding the target protein or subunits thereof; short interfering RNA ("siRNA") molecules directed to a nucleic acid encoding the target protein or subunits thereof; RNA or DNA aptamers that bind to the target gene or gene product or a subunit thereof; gene product structural analog; soluble variant proteins or fusion polypeptides thereof; DNA targeting agents, such as CRISPR systems, Zinc finger binding proteins, TALEs or TALENS; and small molecule agents that target or bind to the target gene or subunit(s) thereof. In some embodiments of the compositions, methods, and uses described herein, the inhibitor inhibits some or all of IL-27 mediated signal transduction. Exemplary assays to measure inhibition or reduction of downstream IL-27 signaling pathway activities are known to those of ordinary skill in the art and/or are provided herein.

[00238] As used herein, an inhibitor or antagonist has the ability to reduce the activity and/or expression of the target gene in a cell (e.g., T cells, such as CD8+ or CD4+ T cells) by at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95 %, at least 98%, at least 99%, or more, relative to the activity or expression level in the absence of the antagonist.

[00239] In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist is a monoclonal antibody.

[00240] In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist is an antibody fragment or antigen-binding fragment. The terms "antibody fragment," "antigen binding fragment," and "antibody derivative" as used herein, refer to a protein fragment that comprises only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen.

[00241] In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist is a chimeric antibody derivative of an antagonist antibody or antigen-binding fragment thereof.

[00242] The inhibitor or antagonist antibodies and antigen-binding fragments thereof described herein can also be, in some embodiments, a humanized antibody derivative.

[00243] In some embodiments, the inhibitor or antagonist antibodies and antigen-binding fragments thereof described herein, *i.e.*, antibodies that are useful for decreasing T cell exhaustion, include derivatives that are modified, *i.e.*, by the covalent attachment of any type of molecule to the antibody, provided that the covalent attachment does not prevent the antibody from binding to the target antigen, *e.g.*, one or more target gene products from Table 1, Table 10, Table 11, or Table 12.

[00244] In some embodiments of the compositions, methods, and uses described herein, fully human antibodies are used, which are particularly desirable for the therapeutic treatment of human patients.

[00245] In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist is a small molecule inhibitor or antagonist, including, but is not limited to, small peptides or peptide-like molecules, soluble peptides, and synthetic non-peptidyl organic or inorganic compounds. A small molecule inhibitor or antagonist can have a molecular weight of any of about 100 to about 20,000 daltons (Da), about 500 to about 15,000 Da, about 1000 to about 10,000 Da. In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist comprises a small molecule

that binds the target gene product selected from the genes listed in Table 1, Table 2, Table 10, Table 11, or Table 12 or the combination of Prdml and c-Maf or Prdml and c-Maf, individually.

[00246] In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist is an RNA or DNA aptamer that binds or physically interacts with a target gene/ gene product, and blocks interactions between the gene product and a binding partner.

[00247] In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist comprises at least one structural analog of a target gene/ gene product as listed in Table 1, Table 10, Table 11, or Table 12 or the combination of Prdml and c-Maf, or Prdml and c-Maf, individually. The term "structural analogs" as used herein, refers to compounds that have a similar three dimensional structure as the target gene or portion thereof, under physiological conditions *in vitro* or *in vivo*, wherein the binding of the analog in the signaling pathway reduces a desired biological activity. Suitable structural analogs can be designed and synthesized through molecular modeling of protein binding. The structural analogs and receptor structural analogs can be monomers, dimers, or higher order multimers in any desired combination of the same or different structures to obtain improved affinities and biological effects.

[00248] In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist comprises at least one soluble peptide, or portion of the target gene product, or fusion polypeptide thereof. In some such embodiments, the soluble peptide is fused to an immunoglobulin constant domain, such as an Fc domain, or to another polypeptide that modifies its *in vivo* half-life, *e.g.*, albumin.

[00249] In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist comprises at least one antisense molecule capable of blocking or decreasing the expression of a desired target gene by targeting nucleic acids encoding the gene or subunit thereof. Methods are known to those of ordinary skill in the art for the preparation of antisense oligonucleotide molecules that will specifically bind one or more target gene(s) without cross-reacting with other polynucleotides. Exemplary sites of targeting include, but are not limited to, the initiation codon, the 5' regulatory regions, including promoters or enhancers, the coding sequence, including any conserved consensus regions, and the 3' untranslated region. In some embodiment of these aspects and all such aspects described herein, the antisense oligonucleotides are about 10 to about 100 nucleotides in length, about 15 to about 50 nucleotides in length, about 18 to about 25 nucleotides in length,

or more. In certain embodiments, the oligonucleotides further comprise chemical modifications to increase nuclease resistance and the like, such as, for example, phosphorothioate linkages and 2'-O-sugar modifications known to those of ordinary skill in the art.

[00250] In some embodiments of the compositions, methods, and uses described herein, an inhibitor or antagonist comprises at least one siRNA molecule capable of blocking or decreasing the expression of a target gene product or a subunit thereof. Generally, one would prepare siRNA molecules that will specifically target one or more mRNAs without cross-reacting with other polynucleotides. siRNA molecules for use in the compositions, methods, and uses described herein can be generated by methods known in the art, such as by typical solid phase oligonucleotide synthesis, and often will incorporate chemical modifications to increase half-life and/or efficacy of the siRNA agent, and/or to allow for a more robust delivery formulation. Alternatively, siRNA molecules are delivered using a vector encoding an expression cassette for intracellular transcription of siRNA.

[00251] Inhibitors or antagonists for use in the compositions, methods, and uses described herein can be identified or characterized using methods known in the art, such as protein-protein binding assays, biochemical screening assays, immunoassays, and cell-based assays, which are well known in the art.

[00252] *Activators:* Also provided herein, in other aspects, are compositions comprising activators or agonists for use in the methods and compositions described herein.

[00253] As used herein, the terms "activator," "agonist," or "activating agent," refer to a molecule or agent that mimics or up-regulates (*e.g.*, increases, potentiates or supplements) the expression and/or biological activity of a target gene/gene product *in vitro*, *in situ*, and/or *in vivo*, including downstream pathways mediated by gene signaling. In some embodiments, an activator or agonist as described herein can modulate markers of T-cell exhaustion, such as, for example, transcription factor induction (*e.g.*, NFIL3 or T-bet induction), IL-10 induction, histone acetylation at the *TIMS* locus, TIM-3 mRNA or protein upregulation, and/or elicitation of a cellular response to IL-27. An "activator" of a given polypeptide can include the polypeptide itself, in that supplying the polypeptide itself will increase the level of the function provided by the polypeptide. An activator or agonist can be a protein or derivative thereof having at least one bioactivity of the wild-type target gene/gene product. An activator or agonist can also be a compound that up-regulates expression of the desired target gene product or its subunits. An activator or agonist can also be a compound which increases the interaction of the target gene with its receptor, for example. Exemplary activators or agonists

contemplated for use in the various aspects and embodiments described herein include, but are not limited to, antibodies or antigen-binding fragments thereof that specifically bind to a target gene/gene product or subunits thereof; RNA or DNA aptamers that bind to the target gene/ gene product; structural analogs or soluble mimics or fusion polypeptides thereof; DNA targeting agents, such as CRISPR systems, Zinc finger binding proteins, and TALES; and small molecule agents that target or bind to a target gene product binding partner and act as functional mimics.

[00254] As used herein, an agonist has the ability to increase or enhance the activity and/or expression of a target gene/ gene product in a cell (e.g., T cells, such as CD8+ or CD4+ T cells) by at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95 %, at least 98%, at least 99%, at least 100%, at least 1.5-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 25-fold, at least 50-fold, at least 100-fold, at least 1000-fold, or more relative to the activity or expression level in the absence of the activator or agonist.

[00255] In some embodiments of the compositions, methods, and uses described herein, the activator or agonist increases or enhances signal transduction mediated by the target gene/gene product. In some embodiments of the compositions and methods described herein, the activator or agonist increases or enhances transcription factor induction or activation.

[00256] In some embodiments of the compositions, methods, and uses described herein, the binding sites of the activators or agonists, such as an antibody or antigen-binding fragment thereof, are directed against an interaction site between the target gene product and one or more of its binding partners. By binding to an interaction site, an activator or agonist described herein can mimic or recapitulate the binding of the target gene product to its partner and increase the activity or expression of the target gene product, and downstream signaling consequences.

[00257] In some embodiments of the compositions, methods, and uses described herein, an activator or agonist is a monoclonal antibody. In some embodiments of the compositions, methods, and uses described herein, an activator or agonist is an antibody fragment or antigen-binding fragment.

[00258] In some embodiments of the compositions, methods, and uses described herein, an activator or agonist is a chimeric antibody derivative of the agonist antibodies and antigen-binding fragments thereof.

[00259] In some embodiments of the compositions, methods, and uses described herein, an activator or agonist is a humanized antibody derivative.

[00260] In some embodiments, the activator or agonist antibodies and antigen-binding fragments thereof described herein, *i.e.*, antibodies that are useful for increasing T cell exhaustion, include derivatives that are modified, *i.e.*, by the covalent attachment of any type of molecule to the antibody, provided that covalent attachment does not prevent the antibody from binding to the target antigen.

[00261] The activator or agonist antibodies and antigen-binding fragments thereof described herein can be generated by any suitable method known in the art.

[00262] In some embodiments, the activator or agonist antibodies and antigen-binding fragments thereof described herein are fully human antibodies or antigen-binding fragments thereof, which are particularly desirable for the therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art, and as described in more detail elsewhere herein.

[00263] In some embodiments of the compositions, methods, and uses described herein, an activator or agonist is a small molecule activator or agonist, including, but not limited to, small peptides or peptide-like molecules, soluble peptides, and synthetic non-peptidyl organic or inorganic compounds. A small molecule activator or agonist can have a molecular weight of any of about 100 to about 20,000 daltons (Da), about 500 to about 15,000 Da, or about 1000 to about 10,000 Da.

[00264] In some embodiments of the compositions, methods, and uses described herein, an activator or agonist is an RNA or DNA aptamer that binds or physically interacts with a target gene product and one or more of its binding partners, and enhances or promotes protein-protein interactions.

[00265] In some embodiments of the compositions, methods, and uses described herein, an activator or agonist comprises at least one structural analog of a target gene or gene product as listed in Table 1, Table 10, Table 11, or Table 12 or the combination of Prdml and c-Maf, or Prdml and c-Maf, individually. The term "structural analog," as used herein, refers to compounds that have a similar three dimensional structure as all or a portion of the desired target gene product under physiological conditions *in vitro* or *in vivo*, wherein the binding at least partially mimics or increases a biological activity mediated by the target gene product. Suitable structural analogs can be designed and synthesized through molecular modeling of binding of a target gene product and its binding partner(s). The structural analogs can be monomers, dimers, or higher order multimers in any desired combination of the same or different structures to obtain improved affinities and biological effects.

[00266] Activators or agonists for use in the compositions, methods, and uses described herein can be identified or characterized using methods known in the art, such as protein-protein binding assays, biochemical screening assays, immunoassays, and cell-based assays, which are well known in the art.

[00267] With respect to general information on CRISPR-Cas Systems, components thereof, and delivery of such components, including methods, materials, delivery vehicles, vectors, particles, AAV, and making and using thereof, including as to amounts and formulations, all useful in the practice of the instant invention, reference is made to: US Patents Nos. 8,999,641, 8,993,233, 8,945,839, 8,932,814, 8,906,616, 8,895,308, 8,889,418, 8,889,356, 8,871,445, 8,865,406, 8,795,965, 8,771,945 and 8,697,359; US Patent Publications US 2014-0310830 (US APP. Ser. No. 14/105,031), US 2014-0287938 A1 (U.S. App. Ser. No. 14/213,991), US 2014-0273234 A1 (U.S. App. Ser. No. 14/293,674), US2014-0273232 A1 (U.S. App. Ser. No. 14/290,575), US 2014-0273231 (U.S. App. Ser. No. 14/259,420), US 2014-0256046 A1 (U.S. App. Ser. No. 14/226,274), US 2014-0248702 A1 (U.S. App. Ser. No. 14/258,458), US 2014-0242700 A1 (U.S. App. Ser. No. 14/222,930), US 2014-0242699 A1 (U.S. App. Ser. No. 14/183,512), US 2014-0242664 A1 (U.S. App. Ser. No. 14/104,990), US 2014-0234972 A1 (U.S. App. Ser. No. 14/183,471), US 2014-0227787 A1 (U.S. App. Ser. No. 14/256,912), US 2014-0189896 A1 (U.S. App. Ser. No. 14/105,035), US 2014-0186958 (U.S. App. Ser. No. 14/105,017), US 2014-0186919 A1 (U.S. App. Ser. No. 14/104,977), US 2014-0186843 A1 (U.S. App. Ser. No. 14/104,900), US 2014-0179770 A1 (U.S. App. Ser. No. 14/104,837) and US 2014-0179006 A1 (U.S. App. Ser. No. 14/183,486), US 2014-0170753 (US App Ser No 14/183,429); European Patents EP 2 784 162 B1 and EP 2 771 468 B1; European Patent Applications EP 2 771 468 (EP13818570.7), EP 2 764 103 (EP13824232.6), and EP 2 784 162 (EP14170383.5); and PCT Patent Publications PCT Patent Publications WO 2014/093661 (PCT/US2013/074743), WO 2014/093694 (PCT/US2013/074790), WO 2014/093595 (PCT/US2013/074611), WO 2014/093718 (PCT/US2013/074825), WO 2014/093709 (PCT/US2013/074812), WO 2014/093622 (PCT/US2013/074667), WO 2014/093635 (PCT/US2013/074691), WO 2014/093655 (PCT/US2013/074736), WO 2014/093712 (PCT/US2013/074819), WO2014/093701 (PCT/US2013/074800), WO2014/018423 (PCT/US2013/051418), WO 2014/204723 (PCT/US2014/041790), WO 2014/204724 (PCT/US2014/041800), WO 2014/204725 (PCT/US2014/041803), WO 2014/204726 (PCT/US2014/041804), WO 2014/204727 (PCT/US2014/041806), WO 2014/204728 (PCT/US2014/041808), WO 2014/204729 (PCT/US2014/041809). Reference is also made to US provisional patent

applications 61/758,468; 61/802,174; 61/806,375; 61/814,263; 61/819,803 and 61/828,130, filed on January 30, 2013; March 15, 2013; March 28, 2013; April 20, 2013; May 6, 2013 and May 28, 2013 respectively. Reference is also made to US provisional patent application 61/836,123, filed on June 17, 2013. Reference is additionally made to US provisional patent applications 61/835,931, 61/835,936, 61/836,127, 61/836, 101, 61/836,080 and 61/835,973, each filed June 17, 2013. Further reference is made to US provisional patent applications 61/862,468 and 61/862,355 filed on August 5, 2013; 61/871,301 filed on August 28, 2013; 61/960,777 filed on September 25, 2013 and 61/961,980 filed on October 28, 2013. Reference is yet further made to: PCT Patent applications Nos: PCT/US2014/041803, PCT/US20 14/04 1800, PCT/US2014/041809, PCT/US2014/041804 and PCT/US20 14/04 1806, each filed June 10, 2014 6/10/14; PCT/US20 14/04 1808 filed June 11, 2014; and PCT/US2014/62558 filed October 28, 2014, and US Provisional Patent Applications Serial Nos.: 61/915,150, 61/915,301, 61/915,267 and 61/915,260, each filed December 12, 2013; 61/757,972 and 61/768,959, filed on January 29, 2013 and February 25, 2013; 61/835,936, 61/836,127, 61/836,101, 61/836,080, 61/835,973, and 61/835,931, filed June 17, 2013; 62/010,888 and 62/010,879, both filed June 11, 2014; 62/010,329 and 62/010,441, each filed June 10, 2014; 61/939,228 and 61/939,242, each filed February 12, 2014; 61/980,012, filed April 15,2014; 62/038,358, filed August 17, 2014; 62/054,490, 62/055,484, 62/055,460 and 62/055,487, each filed September 25, 2014; and 62/069,243, filed October 27, 2014. Reference is also made to US provisional patent applications Nos. 62/055,484, 62/055,460, and 62/055,487, filed September 25, 2014; US provisional patent application 61/980,012, filed April 15, 2014; and US provisional patent application 61/939,242 filed February 12, 2014. Reference is made to PCT application designating, inter alia, the United States, application No. PCT/US14/41806, filed June 10, 2014. Reference is made to US provisional patent application 61/930,214 filed on January 22, 2014. Reference is made to US provisional patent applications 61/915,251; 61/915,260 and 61/915,267, each filed on December 12, 2013. Reference is made to US provisional patent application USSN 61/980,012 filed April 15, 2014. Reference is made to PCT application designating, inter alia, the United States, application No. PCT/US 14/4 1806, filed June 10, 2014. Reference is made to US provisional patent application 61/930,214 filed on January 22, 2014. Reference is made to US provisional patent applications 61/915,251; 61/915,260 and 61/915,267, each filed on December 12, 2013.

[00268] Mention is also made of US application 62/091,455, filed, 12-Dec-14, PROTECTED GUIDE RNAS (PGRNAS); US application 62/096,708, 24-Dec-14,

PROTECTED GUIDE RNAS (PGRNAS); US application 62/091,462, 12-Dec-14, DEAD GUIDES FOR CRISPR TRANSCRIPTION FACTORS; US application 62/096,324, 23-Dec-14, DEAD GUIDES FOR CRISPR TRANSCRIPTION FACTORS; US application 62/091,456, 12-Dec-14, ESCORTED AND FUNCTIONALIZED GUIDES FOR CRISPR-CAS SYSTEMS; US application 62/091,461, 12-Dec-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR GENOME EDITING AS TO HEMATOPOETIC STEM CELLS (HSCs); US application 62/094,903, 19-Dec-14, UNBIASED IDENTIFICATION OF DOUBLE-STRAND BREAKS AND GENOMIC REARRANGEMENT BY GENOME-WISE INSERT CAPTURE SEQUENCING; US application 62/096,761, 24-Dec-14, ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED ENZYME AND GUIDE SCAFFOLDS FOR SEQUENCE MANIPULATION; US application 62/098,059, 30-Dec-14, RNA-TARGETING SYSTEM; US application 62/096,656, 24-Dec-14, CRISPR HAVING OR ASSOCIATED WITH DESTABILIZATION DOMAINS; US application 62/096,697, 24-Dec-14, CRISPR HAVING OR ASSOCIATED WITH AAV; US application 62/098,158, 30-Dec-14, ENGINEERED CRISPR COMPLEX INSERTIONAL TARGETING SYSTEMS; US application 62/151,052, 22-Apr-15, CELLULAR TARGETING FOR EXTRACELLULAR EXOSOMAL REPORTING; US application 62/054,490, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING PARTICLE DELIVERY COMPONENTS; US application 62/055,484, 25-Sep-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,537, 4-Dec-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/054,651, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US application 62/067,886, 23-Oct-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US application 62/054,675, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN NEURONAL CELLS/TISSUES; US application 62/054,528, 24-Sep-14, DELIVERY, USE

AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN IMMUNE DISEASES OR DISORDERS; US application 62/055,454, 25-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING CELL PENETRATION PEPTIDES (CPP); US application 62/055,460, 25-Sep-14, MULTIFUNCTIONAL-CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; US application 62/087,475, 4-Dec-14, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/055,487, 25-Sep-14, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,546, 4-Dec-14, MULTIFUNCTIONAL CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; and US application 62/098,285, 30-Dec-14, CRISPR MEDIATED IN VIVO MODELING AND GENETIC SCREENING OF TUMOR GROWTH AND METASTASIS.

[00269] Each of these patents, patent publications, and applications, and all documents cited therein or during their prosecution ("appln cited documents") and all documents cited or referenced in the appln cited documents, together with any instructions, descriptions, product specifications, and product sheets for any products mentioned therein or in any document therein and incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. All documents (e.g., these patents, patent publications and applications and the appln cited documents) are incorporated herein by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

[00270] Also with respect to general information on CRISPR-Cas Systems, mention is made of the following (also hereby incorporated herein by reference):

- > Multiplex genome engineering using CRISPR/Cas systems. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., & Zhang, F. Science Feb 15;339(6121):819-23 (2013);
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each of which is incorporated herein by reference, may be considered in the practice of the instant invention, and discussed briefly below:

- > Cong et al. engineered type II CRISPR-Cas systems for use in eukaryotic cells based on both *Streptococcus thermophilus* Cas9 and also *Streptococcus pyogenes* Cas9 and demonstrated that Cas9 nucleases can be directed by short RNAs to induce precise cleavage of DNA in human and mouse cells. Their study further showed that Cas9 as converted into a nicking enzyme can be used to facilitate homology-directed repair in eukaryotic cells with minimal mutagenic activity. Additionally, their study demonstrated that multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several at endogenous genomic loci sites within the mammalian genome, demonstrating easy programmability and wide applicability of the RNA-guided nuclease technology. This ability to use RNA to program sequence specific DNA cleavage in cells defined a new class of genome engineering tools. These studies further showed that other CRISPR loci are likely to be transplantable into mammalian cells and can also mediate mammalian genome cleavage. Importantly, it can be envisaged that several aspects of the CRISPR-Cas system can be further improved to increase its efficiency and versatility.
- > Jiang et al. used the clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated Cas9 endonuclease complexed with dual-RNAs to introduce precise mutations in the genomes of *Streptococcus pneumoniae* and *Escherichia coli*. The approach relied on dual-RNA:Cas9-directed cleavage at the targeted genomic site to kill unmutated cells and circumvents the need for selectable markers or counter-selection systems. The study reported reprogramming dual-RNA:Cas9 specificity by changing the sequence of short CRISPR RNA (crRNA) to make single- and multinucleotide changes carried on editing templates. The study showed that simultaneous use of two crRNAs enabled multiplex mutagenesis. Furthermore, when the approach was used in combination with recombineering, in *S. pneumoniae*, nearly 100% of cells that were recovered using the described approach contained the desired mutation, and in *E. coli*, 65% that were recovered contained the mutation.
- > Wang et al. (2013) used the CRISPR/Cas system for the one-step generation of mice carrying mutations in multiple genes which were traditionally generated in multiple steps by sequential recombination in embryonic stem cells and/or time-consuming

intercrossing of mice with a single mutation. The CRISPR/Cas system will greatly accelerate the in vivo study of functionally redundant genes and of epistatic gene interactions.

- > Konermann et al. (2013) addressed the need in the art for versatile and robust technologies that enable optical and chemical modulation of DNA-binding domains based CRISPR Cas9 enzyme and also Transcriptional Activator Like Effectors
- > Ran et al. (2013-A) described an approach that combined a Cas9 nickase mutant with paired guide RNAs to introduce targeted double-strand breaks. This addresses the issue of the Cas9 nuclease from the microbial CRISPR-Cas system being targeted to specific genomic loci by a guide sequence, which can tolerate certain mismatches to the DNA target and thereby promote undesired off-target mutagenesis. Because individual nicks in the genome are repaired with high fidelity, simultaneous nicking via appropriately offset guide RNAs is required for double-stranded breaks and extends the number of specifically recognized bases for target cleavage. The authors demonstrated that using paired nicking can reduce off-target activity by 50- to 1,500-fold in cell lines and to facilitate gene knockout in mouse zygotes without sacrificing on-target cleavage efficiency. This versatile strategy enables a wide variety of genome editing applications that require high specificity.
- > Hsu et al. (2013) characterized SpCas9 targeting specificity in human cells to inform the selection of target sites and avoid off-target effects. The study evaluated >700 guide RNA variants and SpCas9-induced indel mutation levels at >100 predicted genomic off-target loci in 293T and 293FT cells. The authors that SpCas9 tolerates mismatches between guide RNA and target DNA at different positions in a sequence-dependent manner, sensitive to the number, position and distribution of mismatches. The authors further showed that SpCas9-mediated cleavage is unaffected by DNA methylation and that the dosage of SpCas9 and sgRNA can be titrated to minimize off-target modification. Additionally, to facilitate mammalian genome engineering applications, the authors reported providing a web-based software tool to guide the selection and validation of target sequences as well as off-target analyses.
- > Ran et al. (2013-B) described a set of tools for Cas9-mediated genome editing via non-homologous end joining (NHEJ) or homology-directed repair (HDR) in mammalian cells, as well as generation of modified cell lines for downstream functional studies. To minimize off-target cleavage, the authors further described a double-nicking strategy using the Cas9 nickase mutant with paired guide RNAs. The

protocol provided by the authors experimentally derived guidelines for the selection of target sites, evaluation of cleavage efficiency and analysis of off-target activity. The studies showed that beginning with target design, gene modifications can be achieved within as little as 1-2 weeks, and modified clonal cell lines can be derived within 2-3 weeks.

- > Shalem et al. described a new way to interrogate gene function on a genome-wide scale. Their studies showed that delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library targeted 18,080 genes with 64,751 unique guide sequences enabled both negative and positive selection screening in human cells. First, the authors showed use of the GeCKO library to identify genes essential for cell viability in cancer and pluripotent stem cells. Next, in a melanoma model, the authors screened for genes whose loss is involved in resistance to vemurafenib, a therapeutic that inhibits mutant protein kinase BRAF. Their studies showed that the highest-ranking candidates included previously validated genes NF1 and MED12 as well as novel hits NF2, CUL3, TADA2B, and TADA1. The authors observed a high level of consistency between independent guide RNAs targeting the same gene and a high rate of hit confirmation, and thus demonstrated the promise of genome-scale screening with Cas9.
- > Nishimasu et al. reported the crystal structure of *Streptococcus pyogenes* Cas9 in complex with sgRNA and its target DNA at 2.5 Å resolution. The structure revealed a bilobed architecture composed of target recognition and nuclease lobes, accommodating the sgRNA:DNA heteroduplex in a positively charged groove at their interface. Whereas the recognition lobe is essential for binding sgRNA and DNA, the nuclease lobe contains the UNH and RuvC nuclease domains, which are properly positioned for cleavage of the complementary and non-complementary strands of the target DNA, respectively. The nuclease lobe also contains a carboxyl-terminal domain responsible for the interaction with the protospacer adjacent motif (PAM). This high-resolution structure and accompanying functional analyses have revealed the molecular mechanism of RNA-guided DNA targeting by Cas9, thus paving the way for the rational design of new, versatile genome-editing technologies.
- > Wu et al. mapped genome-wide binding sites of a catalytically inactive Cas9 (dCas9) from *Streptococcus pyogenes* loaded with single guide RNAs (sgRNAs) in mouse embryonic stem cells (mESCs). The authors showed that each of the four sgRNAs tested targets dCas9 to between tens and thousands of genomic sites, frequently

characterized by a 5-nucleotide seed region in the sgRNA and an NGG protospacer adjacent motif (PAM). Chromatin inaccessibility decreases dCas9 binding to other sites with matching seed sequences; thus 70% of off-target sites are associated with genes. The authors showed that targeted sequencing of 295 dCas9 binding sites in mESCs transfected with catalytically active Cas9 identified only one site mutated above background levels. The authors proposed a two-state model for Cas9 binding and cleavage, in which a seed match triggers binding but extensive pairing with target DNA is required for cleavage.

- > Piatt et al. established a Cre-dependent Cas9 knockin mouse. The authors demonstrated in vivo as well as ex vivo genome editing using adeno-associated virus (AAV)-, lentivirus-, or particle-mediated delivery of guide RNA in neurons, immune cells, and endothelial cells.
- > Hsu et al. (2014) is a review article that discusses generally CRISPR-Cas9 history from yogurt to genome editing, including genetic screening of cells.
- > Wang et al. (2014) relates to a pooled, loss-of-function genetic screening approach suitable for both positive and negative selection that uses a genome-scale lentiviral single guide RNA (sgRNA) library.
- > Doench et al. created a pool of sgRNAs, tiling across all possible target sites of a panel of six endogenous mouse and three endogenous human genes and quantitatively assessed their ability to produce null alleles of their target gene by antibody staining and flow cytometry. The authors showed that optimization of the PAM improved activity and also provided an on-line tool for designing sgRNAs.
- > Swiech et al. demonstrate that AAV-mediated SpCas9 genome editing can enable reverse genetic studies of gene function in the brain.
- > Konermann et al. (2015) discusses the ability to attach multiple effector domains, e.g., transcriptional activator, functional and epigenomic regulators at appropriate positions on the guide such as stem or tetraloop with and without linkers.
- > Zetsche et al. demonstrates that the Cas9 enzyme can be split into two and hence the assembly of Cas9 for activation can be controlled.
- > Chen et al. relates to multiplex screening by demonstrating that a genome-wide in vivo CRISPR-Cas9 screen in mice reveals genes regulating lung metastasis.
- > Ran et al. (2015) relates to SaCas9 and its ability to edit genomes and demonstrates that one cannot extrapolate from biochemical assays.

- > Shalem et al. (2015) described ways in which catalytically inactive Cas9 (dCas9) fusions are used to synthetically repress (CRISPRi) or activate (CRISPRa) expression, showing advances using Cas9 for genome-scale screens, including arrayed and pooled screens, knockout approaches that inactivate genomic loci and strategies that modulate transcriptional activity.
- > Xu et al. (2015) assessed the DNA sequence features that contribute to single guide RNA (sgRNA) efficiency in CRISPR-based screens. The authors explored efficiency of CRISPR/Cas9 knockout and nucleotide preference at the cleavage site. The authors also found that the sequence preference for CRISPRi/a is substantially different from that for CRISPR/Cas9 knockout.
- > Pamas et al. (2015) introduced genome-wide pooled CRISPR-Cas9 libraries into dendritic cells (DCs) to identify genes that control the induction of tumor necrosis factor (Tnf) by bacterial lipopolysaccharide (LPS). Known regulators of Tlr4 signaling and previously unknown candidates were identified and classified into three functional modules with distinct effects on the canonical responses to LPS.
- > Ramanan et al (2015) demonstrated cleavage of viral episomal DNA (cccDNA) in infected cells. The HBV genome exists in the nuclei of infected hepatocytes as a 3.2kb double-stranded episomal DNA species called covalently closed circular DNA (cccDNA), which is a key component in the HBV life cycle whose replication is not inhibited by current therapies. The authors showed that sgRNAs specifically targeting highly conserved regions of HBV robustly suppresses viral replication and depleted cccDNA.
- > Nishimasu et al. (2015) reported the crystal structures of SaCas9 in complex with a single guide RNA (sgRNA) and its double-stranded DNA targets, containing the 5'-TTGAAT-3' PAM and the 5'-TTGGGT-3' PAM. A structural comparison of SaCas9 with SpCas9 highlighted both structural conservation and divergence, explaining their distinct PAM specificities and orthologous sgRNA recognition.
- > Zetsche et al. (2015) reported the characterization of Cpf1, a putative class 2 CRISPR effector. It was demonstrated that Cpf1 mediates robust DNA interference with features distinct from Cas9. Identifying this mechanism of interference broadens our understanding of CRISPR-Cas systems and advances their genome editing applications.
- > Shmakov et al. (2015) reported the characterization of three distinct Class 2 CRISPR-Cas systems. The effectors of two of the identified systems, C2c1 and C2c3, contain

RuvC like endonuclease domains distantly related to CpfI. The third system, C2c2, contains an effector with two predicted HEPN RNase domains.

[00271] Also, "Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing", Shengdar Q. Tsai, Nicolas Wyvekens, Cyd Khayter, Jennifer A. Foden, Vishal Thapar, Deepak Reyon, Mathew J. Goodwin, Martin J. Aryee, J. Keith Joung Nature Biotechnology 32(6): 569-77 (2014), relates to dimeric RNA-guided FokI Nucleases that recognize extended sequences and can edit endogenous genes with high efficiencies in human cells.

[00272] In addition, mention is made of PCT application PCT/US 14/70057, Attorney Reference 47627.99.2060 and BI-2013/107 entitled "DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING PARTICLE DELIVERY COMPONENTS (claiming priority from one or more or all of US provisional patent applications: 62/054,490, filed September 24, 2014; 62/010,441, filed June 10, 2014; and 61/915,118, 61/915,215 and 61/915,148, each filed on December 12, 2013) ("the Particle Delivery PCT"), incorporated herein by reference, with respect to a method of preparing an sgRNA-and-Cas9 protein containing particle comprising admixing a mixture comprising an sgRNA and Cas9 protein (and optionally HDR template) with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol; and particles from such a process. For example, wherein Cas9 protein and sgRNA were mixed together at a suitable, e.g., 3:1 to 1:3 or 2:1 to 1:2 or 1:1 molar ratio, at a suitable temperature, e.g., 15-30C, e.g., 20-25C, e.g., room temperature, for a suitable time, e.g., 15-45, such as 30 minutes, advantageously in sterile, nuclease free buffer, e.g., IX PBS. Separately, particle components such as or comprising: a surfactant, e.g., cationic lipid, e.g., 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); phospholipid, e.g., dimyristoylphosphatidylcholine (DMPC); biodegradable polymer, such as an ethylene-glycol polymer or PEG, and a lipoprotein, such as a low-density lipoprotein, e.g., cholesterol were dissolved in an alcohol, advantageously a C1-6 alkyl alcohol, such as methanol, ethanol, isopropanol, e.g., 100% ethanol. The two solutions were mixed together to form particles containing the Cas9-sgRNA complexes. Accordingly, sgRNA may be pre-complexed with the Cas9 protein, before formulating the entire complex in a particle. Formulations may be made with a different molar ratio of different components known to promote delivery of nucleic acids into cells (e.g. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-ditetradecanoyl-sn-glycero-3-phosphocholine (DMPC), polyethylene glycol (PEG), and

cholesterol) For example DOTAP : DMPC : PEG : Cholesterol Molar Ratios may be DOTAP 100, DMPC 0, PEG 0, Cholesterol 0; or DOTAP 90, DMPC 0, PEG 10, Cholesterol 0; or DOTAP 90, DMPC 0, PEG 5, Cholesterol 5. DOTAP 100, DMPC 0, PEG 0, Cholesterol 0. That application accordingly comprehends admixing sgRNA, Cas9 protein and components that form a particle; as well as particles from such admixing. Aspects of the instant invention can involve particles; for example, particles using a process analogous to that of the Particle Delivery PCT, e.g., by admixing a mixture comprising sgRNA and/or Cas9 as in the instant invention and components that form a particle, e.g., as in the Particle Delivery PCT, to form a particle and particles from such admixing (or, of course, other particles involving sgRNA and/or Cas9 as in the instant invention).

[00273] In general, the CRISPR-Cas or CRISPR system is as used in the foregoing documents, such as WO 2014/093622 (PCT/US2013/074667) and refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a tracr (transactivating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), or "RNA(s)" as that term is herein used (e.g., RNA(s) to guide Cas, such as Cas9, e.g. CRISPR RNA and transactivating (tracr) RNA or a single guide RNA (sgRNA) (chimeric RNA)) or other sequences and transcripts from a CRISPR locus. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell. In some embodiments, direct repeats may be identified in silico by searching for repetitive motifs that fulfill any or all of the following criteria: 1. found in a 2Kb window of genomic sequence flanking the type II CRISPR locus; 2. span from 20 to 50 bp; and 3. interspaced by 20 to 50 bp. In some embodiments, 2 of these criteria may be used, for instance 1 and 2, 2 and 3, or 1 and 3. In some embodiments, all 3 criteria may be used.

[00274] In embodiments of the invention the terms guide sequence and guide RNA, i.e. RNA capable of guiding Cas to a target genomic locus, are used interchangeably as in foregoing cited documents such as WO 2014/093622 (PCT/US2013/074667). In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g. the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). In some embodiments, a guide sequence is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guide sequence is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. Preferably the guide sequence is 10-30 nucleotides long. The ability of a guide sequence to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide sequence to be tested and a control guide sequence different from the test guide sequence, and comparing binding or rate of cleavage at the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art.

[00275] In a classic CRISPR-Cas systems, the degree of complementarity between a guide sequence and its corresponding target sequence can be about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or 100%; a guide or RNA or sgRNA can be about

or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length; or guide or RNA or sgRNA can be less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length; and advantageously tracr RNA is 30 or 50 nucleotides in length. However, an aspect of the invention is to reduce off-target interactions, e.g., reduce the guide interacting with a target sequence having low complementarity. Indeed, in the examples, it is shown that the invention involves mutations that result in the CRISPR-Cas system being able to distinguish between target and off-target sequences that have greater than 80% to about 95% complementarity, e.g., 83%-84% or 88-89% or 94-95% complementarity (for instance, distinguishing between a target having 18 nucleotides from an off-target of 18 nucleotides having 1, 2 or 3 mismatches). Accordingly, in the context of the present invention the degree of complementarity between a guide sequence and its corresponding target sequence is greater than 94.5% or 95% or 95.5% or 96% or 96.5% or 97% or 97.5% or 98% or 98.5% or 99% or 99.5% or 99.9%, or 100%. Off target is less than 100% or 99.9% or 99.5% or 99% or 99% or 98.5% or 98% or 97.5% or 97% or 96.5% or 96% or 95.5% or 95% or 94.5% or 94% or 93% or 92% or 91% or 90% or 89% or 88% or 87% or 86% or 85% or 84% or 83% or 82% or 81% or 80% complementarity between the sequence and the guide, with it advantageous that off target is 100% or 99.9% or 99.5% or 99% or 99% or 98.5% or 98% or 97.5% or 97% or 96.5% or 96% or 95.5% or 95% or 94.5% complementarity between the sequence and the guide.

[00276] In particularly preferred embodiments according to the invention, the guide RNA (capable of guiding Cas to a target locus) may comprise (1) a guide sequence capable of hybridizing to a genomic target locus in the eukaryotic cell; (2) a tracr sequence; and (3) a tracr mate sequence. All (1) to (3) may reside in a single RNA, i.e. an sgRNA (arranged in a 5' to 3' orientation), or the tracr RNA may be a different RNA than the RNA containing the guide and tracr sequence. The tracr hybridizes to the tracr mate sequence and directs the CRISPR/Cas complex to the target sequence.

[00277] The methods according to the invention as described herein comprehend inducing one or more mutations in a eukaryotic cell (in vitro, i.e. in an isolated eukaryotic cell) as herein discussed comprising delivering to cell a vector as herein discussed. The mutation(s) can include the introduction, deletion, or substitution of one or more nucleotides at each target sequence of cell(s) via the guide(s) RNA(s) or sgRNA(s). The mutations can include the introduction, deletion, or substitution of 1-75 nucleotides at each target sequence of said cell(s) via the guide(s) RNA(s) or sgRNA(s). The mutations can include the introduction,

deletion, or substitution of 1, 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, or 75 nucleotides at each target sequence of said cell(s) via the guide(s) RNA(s) or sgRNA(s). The mutations can include the introduction, deletion, or substitution of 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, or 75 nucleotides at each target sequence of said cell(s) via the guide(s) RNA(s) or sgRNA(s). The mutations include the introduction, deletion, or substitution of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, or 75 nucleotides at each target sequence of said cell(s) via the guide(s) RNA(s) or sgRNA(s). The mutations can include the introduction, deletion, or substitution of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, or 75 nucleotides at each target sequence of said cell(s) via the guide(s) RNA(s) or sgRNA(s). The mutations can include the introduction, deletion, or substitution of 40, 45, 50, 75, 100, 200, 300, 400 or 500 nucleotides at each target sequence of said cell(s) via the guide(s) RNA(s) or sgRNA(s).

[00278] For minimization of toxicity and off-target effect, it will be important to control the concentration of Cas mRNA and guide RNA delivered. Optimal concentrations of Cas mRNA and guide RNA can be determined by testing different concentrations in a cellular or non-human eukaryote animal model and using deep sequencing to analyze the extent of modification at potential off-target genomic loci. Alternatively, to minimize the level of toxicity and off-target effect, Cas nickase mRNA (for example *S. pyogenes* Cas9 with the D10A mutation) can be delivered with a pair of guide RNAs targeting a site of interest. Guide sequences and strategies to minimize toxicity and off-target effects can be as in WO 2014/093622 (PCT/US2013/074667); or, via mutation as herein.

[00279] Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (comprising a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. Without wishing to be bound by theory, the tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence.

[00280] The nucleic acid molecule encoding a Cas is advantageously codon optimized Cas. An example of a codon optimized sequence, is in this instance a sequence optimized for

expression in a eukaryote, e.g., humans (i.e. being optimized for expression in humans), or for another eukaryote, animal or mammal as herein discussed; see, e.g., SaCas9 human codon optimized sequence in WO 2014/093622 (PCT/US20 13/074667). Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species other than human, or for codon optimization for specific organs is known. In some embodiments, an enzyme coding sequence encoding a Cas is codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a mammal, including but not limited to human, or non-human eukaryote or animal or mammal as herein discussed, e.g., mouse, rat, rabbit, dog, livestock, or non-human mammal or primate. In some embodiments, processes for modifying the germ line genetic identity of human beings and/or processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal, and also animals resulting from such processes, may be excluded. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at www.kazusa.or.jp/codon/ and these tables can be adapted in a number of ways. See Nakamura, Y., et al. "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" *Nucl. Acids Res.* 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a Cas correspond to the most frequently used codon for a particular amino acid.

[00281] In certain embodiments, the methods as described herein may comprise providing a Cas transgenic cell in which one or more nucleic acids encoding one or more guide RNAs are provided or introduced operably connected in the cell with a regulatory element comprising a promoter of one or more gene of interest. As used herein, the term "Cas transgenic cell" refers to a cell, such as a eukaryotic cell, in which a Cas gene has been genomically integrated. The nature, type, or origin of the cell are not particularly limiting according to the present invention. Also the way how the Cas transgene is introduced in the cell is may vary and can be any method as is known in the art. In certain embodiments, the Cas transgenic cell is obtained by introducing the Cas transgene in an isolated cell. In certain other embodiments, the Cas transgenic cell is obtained by isolating cells from a Cas transgenic organism. By means of example, and without limitation, the Cas transgenic cell as referred to herein may be derived from a Cas transgenic eukaryote, such as a Cas knock-in eukaryote. Reference is made to WO 2014/093622 (PCT/US 13/74667), incorporated herein by reference. Methods of US Patent Publication Nos. 20120017290 and 20110265198 assigned to Sangamo Biosciences, Inc. directed to targeting the Rosa locus may be modified to utilize the CRISPR Cas system of the present invention. Methods of US Patent Publication No. 20130236946 assigned to Collectis directed to targeting the Rosa locus may also be modified to utilize the CRISPR Cas system of the present invention. By means of further example reference is made to Piatt et. al. (Cell; 159(2):440-455 (2014)), describing a Cas9 knock-in mouse, which is incorporated herein by reference. The Cas transgene can further comprise a Lox-Stop-polyA-Lox(LSL) cassette thereby rendering Cas expression inducible by Cre recombinase. Alternatively, the Cas transgenic cell may be obtained by introducing the Cas transgene in an isolated cell. Delivery systems for transgenes are well known in the art. By means of example, the Cas transgene may be delivered in for instance eukaryotic cell by means of vector (e.g., AAV, adenovirus, lentivirus) and/or particle and/or nanoparticle delivery, as also described herein elsewhere.

[00282] It will be understood by the skilled person that the cell, such as the Cas transgenic cell, as referred to herein may comprise further genomic alterations besides having an integrated Cas gene or the mutations arising from the sequence specific action of Cas when complexed with RNA capable of guiding Cas to a target locus, such as for instance one or more oncogenic mutations, as for instance and without limitation described in Piatt et al. (2014), Chen et al, (2014) or Kumar et al.. (2009).

[00283] In some embodiments, the Cas sequence is fused to one or more nuclear localization sequences (NLSs), such as about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or

more NLSs. In some embodiments, the Cas comprises about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the amino-terminus, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the carboxy-terminus, or a combination of these (e.g. zero or at least one or more NLS at the amino-terminus and zero or at one or more NLS at the carboxy terminus). When more than one NLS is present, each may be selected independently of the others, such that a single NLS may be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies. In a preferred embodiment of the invention, the Cas comprises at most 6 NLSs. In some embodiments, an NLS is considered near the N- or C-terminus when the nearest amino acid of the NLS is within about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, or more amino acids along the polypeptide chain from the N- or C-terminus. Non-limiting examples of NLSs include an NLS sequence derived from: the NLS of the SV40 virus large T-antigen, having the amino acid sequence PKKKRKV (SEQ ID NO: X); the NLS from nucleoplasm[^] (e.g. the nucleoplasm[^] bipartite NLS with the sequence KRPAATKKAGQAKKKK) (SEQ ID NO: X); the c-myc NLS having the amino acid sequence PAAKRVKLD (SEQ ID NO: X) or RQRRNELKRSP (SEQ ID NO: X); the hRNPA1 M9 NLS having the sequence NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY (SEQ ID NO: X); the sequence RMRIZFKNKGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV (SEQ ID NO: X) of the IBB domain from importin-alpha; the sequences VSRKRPRP (SEQ ID NO: X) and PPKKARED (SEQ ID NO: X) of the myoma T protein; the sequence POPKKKPL (SEQ ID NO: X) of human p53; the sequence SALIKKKKKMAP (SEQ ID NO: X) of mouse c-abl IV; the sequences DRLRR (SEQ ID NO: X) and PKQKKRK (SEQ ID NO: X) of the influenza virus NS1; the sequence RKLKKKIKKL (SEQ ID NO: X) of the Hepatitis virus delta antigen; the sequence REKKKFLKRR (SEQ ID NO: X) of the mouse Mx1 protein; the sequence KRKGDEV DGVDEVAKKKS K (SEQ ID NO: X) of the human poly(ADP-ribose) polymerase; and the sequence RKCLQAGMNLEARKTKK (SEQ ID NO: X) of the steroid hormone receptors (human) glucocorticoid. In general, the one or more NLSs are of sufficient strength to drive accumulation of the Cas in a detectable amount in the nucleus of a eukaryotic cell. In general, strength of nuclear localization activity may derive from the number of NLSs in the Cas, the particular NLS(s) used, or a combination of these factors. Detection of accumulation in the nucleus may be performed by any suitable technique. For example, a detectable marker may be fused to the Cas, such that location within a cell may be visualized, such as in combination with a means for detecting the location of the nucleus (e.g. a stain specific for the nucleus such as DAPI). Cell nuclei may also be isolated from cells, the

contents of which may then be analyzed by any suitable process for detecting protein, such as immunohistochemistry, Western blot, or enzyme activity assay. Accumulation in the nucleus may also be determined indirectly, such as by an assay for the effect of CRISPR complex formation (e.g. assay for DNA cleavage or mutation at the target sequence, or assay for altered gene expression activity affected by CRISPR complex formation and/or Cas enzyme activity), as compared to a control not exposed to the Cas or complex, or exposed to a Cas lacking the one or more NLSs.

[00284] In certain embodiments, the DNA-targeting agent may comprise a transcription activator-like effector (TALE) protein or DNA-binding domain thereof. Hence, certain embodiments may make use of isolated, non-naturally occurring, recombinant or engineered DNA binding proteins that comprise TALE monomers or TALE monomers or half monomers as a part of their organizational structure that enable the targeting of nucleic acid sequences with improved efficiency and expanded specificity.

[00285] Naturally occurring TALEs or "wild type TALEs" are nucleic acid binding proteins secreted by numerous species of proteobacteria. TALE polypeptides contain a nucleic acid binding domain composed of tandem repeats of highly conserved monomer polypeptides that are predominantly 33, 34 or 35 amino acids in length and that differ from each other mainly in amino acid positions 12 and 13. In advantageous embodiments the nucleic acid is DNA. As used herein, the term "polypeptide monomers", "TALE monomers" or "monomers" will be used to refer to the highly conserved repetitive polypeptide sequences within the TALE nucleic acid binding domain and the term "repeat variable di-residues" or "RVD" will be used to refer to the highly variable amino acids at positions 12 and 13 of the polypeptide monomers. As provided throughout the disclosure, the amino acid residues of the RVD are depicted using the IUPAC single letter code for amino acids. A general representation of a TALE monomer which is comprised within the DNA binding domain is XI-II-(X12X13)-X14-33 or 34 or 35, where the subscript indicates the amino acid position and X represents any amino acid. XI2X13 indicate the RVDs. In some polypeptide monomers, the variable amino acid at position 13 is missing or absent and in such monomers, the RVD consists of a single amino acid. In such cases the RVD may be alternatively represented as X*, where X represents X12 and (*) indicates that X13 is absent. The DNA binding domain comprises several repeats of TALE monomers and this may be represented as (XI-II-(X12X13)-X14-33 or 34 or 35)_z, where in an advantageous embodiment, z is at least 5 to 40. In a further advantageous embodiment, z is at least 10 to 26.

[00286] The TALE monomers have a nucleotide binding affinity that is determined by the identity of the amino acids in its RVD. For example, polypeptide monomers with an RVD of NI preferentially bind to adenine (A), monomers with an RVD of NG preferentially bind to thymine (T), monomers with an RVD of HD preferentially bind to cytosine (C) and monomers with an RVD of NN preferentially bind to both adenine (A) and guanine (G). In yet another embodiment of the invention, monomers with an RVD of IG preferentially bind to T. Thus, the number and order of the polypeptide monomer repeats in the nucleic acid binding domain of a TALE determines its nucleic acid target specificity. In still further embodiments of the invention, monomers with an RVD of NS recognize all four base pairs and may bind to A, T, G or C. The structure and function of TALEs is further described in, for example, Moscou et al., *Science* 326:1501 (2009); Boch et al., *Science* 326:1509-1512 (2009); and Zhang et al., *Nature Biotechnology* 29:149-153 (2011), each of which is incorporated by reference in its entirety.

[00287] The polypeptides used in methods of certain embodiments of the invention are isolated, non-naturally occurring, recombinant or engineered nucleic acid-binding proteins that have nucleic acid or DNA binding regions containing polypeptide monomer repeats that are designed to target specific nucleic acid sequences.

[00288] As described herein, polypeptide monomers having an RVD of HN or NH preferentially bind to guanine and thereby allow the generation of TALE polypeptides with high binding specificity for guanine containing target nucleic acid sequences. In a preferred embodiment of the invention, polypeptide monomers having RVDs RN, NN, NK, SN, NH, KN, HN, NQ, HH, RG, KH, RH and SS preferentially bind to guanine. In a much more advantageous embodiment of the invention, polypeptide monomers having RVDs RN, NK, NQ, HH, KH, RH, SS and SN preferentially bind to guanine and thereby allow the generation of TALE polypeptides with high binding specificity for guanine containing target nucleic acid sequences. In an even more advantageous embodiment of the invention, polypeptide monomers having RVDs HH, KH, NH, NK, NQ, RH, RN and SS preferentially bind to guanine and thereby allow the generation of TALE polypeptides with high binding specificity for guanine containing target nucleic acid sequences. In a further advantageous embodiment, the RVDs that have high binding specificity for guanine are RN, NH RH and KH. Furthermore, polypeptide monomers having an RVD of NV preferentially bind to adenine and guanine. In more preferred embodiments of the invention, monomers having RVDs of H*, HA, KA, N*, NA, NC, NS, RA, and S* bind to adenine, guanine, cytosine and thymine with comparable affinity.

[00289] The predetermined N-terminal to C-terminal order of the one or more polypeptide monomers of the nucleic acid or DNA binding domain determines the corresponding predetermined target nucleic acid sequence to which the polypeptides of the invention will bind. As used herein the monomers and at least one or more half monomers are "specifically ordered to target" the genomic locus or gene of interest. In plant genomes, the natural TALE-binding sites always begin with a thymine (T), which may be specified by a cryptic signal within the non-repetitive N-terminus of the TALE polypeptide; in some cases this region may be referred to as repeat 0. In animal genomes, TALE binding sites do not necessarily have to begin with a thymine (T) and polypeptides of the invention may target DNA sequences that begin with T, A, G or C. The tandem repeat of TALE monomers always ends with a half-length repeat or a stretch of sequence that may share identity with only the first 20 amino acids of a repetitive full length TALE monomer and this half repeat may be referred to as a half-monomer. Therefore, it follows that the length of the nucleic acid or DNA being targeted is equal to the number of full monomers plus two.

[00290] As described in Zhang et al, Nature Biotechnology 29: 149-153 (2011), TALE polypeptide binding efficiency may be increased by including amino acid sequences from the "capping regions" that are directly N-terminal or C-terminal of the DNA binding region of naturally occurring TALEs into the engineered TALEs at positions N-terminal or C-terminal of the engineered TALE DNA binding region. Thus, in certain embodiments, the TALE polypeptides described herein further comprise an N-terminal capping region and/or a C-terminal capping region.

[00291] An exemplary amino acid sequence of a N-terminal capping region is:

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M D P I R S R T P S P A R E L L S G P Q P D G V Q P T A D R G V S
P

P A G G P L D G L P A R R T M S R T R L P S P P A P S P A F S A D
S

F S D L L R Q F D P S L F N T S L F D S L P P F G A H H T E A A T
G

E W D E V Q S G L R A A D A P P P T M R V A V T A A R P P R A K P
A

P R R R A A Q P S D A S P A A Q V D L R T L G Y S Q Q Q Q E K I K
P

K V R S T V A Q H H E A L V G H G F T H A H I V A L S Q H P A A L
G

T V A V K Y Q D M I A A L P E A T H E A I V G V G K Q W S G A R A
L

E A L L T V A G E L R G P P L Q L D T G Q L L K I A K R G G V T A
V
    
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E A V H A W R N A L T G A P L N (SEQ ID NO: 147)

[00292] An exemplary amino acid sequence of a C-terminal capping region is:

R P A L E S I V A Q L S R P D P A L A A L T N D H L V A L A C L G
 G R P A L D A V K K G L P H A P A L I K R T N R R I P E R T S H R
 V A D H A Q V V R V L G F F Q C H S H P A Q A F D D A M T Q F G M
 S R H G L L Q L F R R V G V T E L E A R S G T L P P A S Q R W D R
 I L Q A S G M K R A K P S P T S T Q T P D Q A S L H A F A D S L E
 R D L D A P S P M H E G D Q T R A S (SEQ ID NO: 148)

[00293] As used herein the predetermined "N-terminus" to "C terminus" orientation of the N-terminal capping region, the DNA binding domain comprising the repeat TALE monomers and the C-terminal capping region provide structural basis for the organization of different domains in the d-TALEs or polypeptides of the invention.

[00294] The entire N-terminal and/or C-terminal capping regions are not necessary to enhance the binding activity of the DNA binding region. Therefore, in certain embodiments, fragments of the N-terminal and/or C-terminal capping regions are included in the TALE polypeptides described herein.

[00295] In certain embodiments, the TALE polypeptides described herein contain a N-terminal capping region fragment that included at least 10, 20, 30, 40, 50, 54, 60, 70, 80, 87, 90, 94, 100, 102, 110, 117, 120, 130, 140, 147, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260 or 270 amino acids of an N-terminal capping region. In certain embodiments, the N-terminal capping region fragment amino acids are of the C-terminus (the DNA-binding region proximal end) of an N-terminal capping region. As described in Zhang et al, *Nature Biotechnology* 29:149-153 (2011), N-terminal capping region fragments that include the C-terminal 240 amino acids enhance binding activity equal to the full length capping region, while fragments that include the C-terminal 147 amino acids retain greater than 80% of the efficacy of the full length capping region, and fragments that include the C-terminal 117 amino acids retain greater than 50% of the activity of the full-length capping region.

[00296] In some embodiments, the TALE polypeptides described herein contain a C-terminal capping region fragment that included at least 6, 10, 20, 30, 37, 40, 50, 60, 68, 70, 80, 90, 100, 110, 120, 127, 130, 140, 150, 155, 160, 170, 180 amino acids of a C-terminal capping region. In certain embodiments, the C-terminal capping region fragment amino acids are of the N-terminus (the DNA-binding region proximal end) of a C-terminal capping region. As described in Zhang et al, *Nature Biotechnology* 29:149-153 (2011), C-terminal

capping region fragments that include the C-terminal 68 amino acids enhance binding activity equal to the full length capping region, while fragments that include the C-terminal 20 amino acids retain greater than 50% of the efficacy of the full length capping region.

[00297] In certain embodiments, the capping regions of the TALE polypeptides described herein do not need to have identical sequences to the capping region sequences provided herein. Thus, in some embodiments, the capping region of the TALE polypeptides described herein have sequences that are at least 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical or share identity to the capping region amino acid sequences provided herein. Sequence identity is related to sequence homology. Homology comparisons may be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs may calculate percent (%) homology between two or more sequences and may also calculate the sequence identity shared by two or more amino acid or nucleic acid sequences. In some preferred embodiments, the capping region of the TALE polypeptides described herein have sequences that are at least 95% identical or share identity to the capping region amino acid sequences provided herein.

[00298] Sequence homologies may be generated by any of a number of computer programs known in the art, which include but are not limited to BLAST or FASTA. Suitable computer program for carrying out alignments like the GCG Wisconsin Bestfit package may also be used. Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[00299] In certain embodiments, the DNA-targeting agent may comprise a zinc finger protein or DNA-binding domain thereof. Artificial zinc-finger (ZF) technology allows to provide programmable DNA-binding domains, and involves arrays of ZF modules to target new DNA-binding sites in the genome. Each finger module in a ZF array targets three DNA bases. A customized array of individual zinc finger domains is assembled into a ZF protein (ZFP). ZFPs can comprise a functional domain. The first synthetic zinc finger nucleases (ZFNs) were developed by fusing a ZF protein to the catalytic domain of the Type IIS restriction enzyme FokI. (Kim, Y. G. et al, 1994, Chimeric restriction endonuclease, Proc. Natl. Acad. Sci. U.S.A. 91, 883-887; Kim, Y. G. et al, 1996, Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc. Natl. Acad. Sci. U.S.A. 93, 1156-1160). Increased cleavage specificity can be attained with decreased off target activity by use of paired ZFN heterodimers, each targeting different nucleotide sequences separated by a short

spacer. (Doyon, Y. et al, 2011, Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. Nat. Methods 8, 74-79).

[00300] In certain embodiments, the protein comprising the DNA-targeting agent may further comprise one or more suitable effector portions or domains. The terms "effector domain" or "regulatory and functional domain" refer to a polypeptide sequence that has an activity other than binding to the nucleic acid sequence recognized by the nucleic acid binding domain. By combining a nucleic acid binding domain with one or more effector domains, the polypeptides of the invention may be used to target the one or more functions or activities mediated by the effector domain to a particular target DNA sequence to which the nucleic acid binding domain specifically binds.

[00301] In some embodiments, the activity mediated by the effector domain is a biological activity. For example, in some embodiments the effector domain may be a transcriptional inhibitor (i.e., a repressor domain), such as an mSin interaction domain (SID). SID4X domain or a Kruppel-associated box (KRAB) or fragments of the KRAB domain. In some embodiments the effector domain may be an enhancer of transcription (i.e. an activation domain), such as the VP16, VP64 or p65 activation domain. In some embodiments, the nucleic acid binding portion may be linked, for example, with an effector domain that includes but is not limited to a transposase, integrase, recombinase, resolvase, invertase, protease, DNA methyltransferase, DNA demethylase, histone acetylase, histone deacetylase, nuclease, transcriptional repressor, transcriptional activator, transcription factor recruiting, protein nuclear-localization signal or cellular uptake signal. In some embodiments, the effector domain may be a protein domain which exhibits activities which include but are not limited to transposase activity, integrase activity, recombinase activity, resolvase activity, invertase activity, protease activity, DNA methyltransferase activity, DNA demethylase activity, histone acetylase activity, histone deacetylase activity, nuclease activity, nuclear-localization signaling activity, transcriptional repressor activity, transcriptional activator activity, transcription factor recruiting activity, or cellular uptake signaling activity. Other preferred embodiments of the invention may include any combination the activities described herein.

Adoptive Cell Transfer (ACT)

[00302] The immune cells of the present invention may be used for adoptive cell transfer. Adoptive cell therapy (ACT) can refer to the transfer of cells, most commonly immune-derived cells, back into the same patient or into a new recipient host with the goal of transferring the immunologic functionality and characteristics into the new host. If possible,

use of autologous cells helps the recipient by minimizing GVHD issues. The adoptive transfer of autologous tumor infiltrating lymphocytes (TIL) (Besser et al., (2010) Clin. Cancer Res 16 (9) 2646-55; Dudley et al., (2002) Science 298 (5594): 850-4; and Dudley et al., (2005) Journal of Clinical Oncology 23 (10): 2346-57.) or genetically re-directed peripheral blood mononuclear cells (Johnson et al., (2009) Blood 114 (3): 535-46; and Morgan et al, (2006) Science 314(5796) 126-9) has been used to successfully treat patients with advanced solid tumors, including melanoma and colorectal carcinoma, as well as patients with CD19-expressing hematologic malignancies (Kalos et al, (2011) Science Translational Medicine 3 (95): 95ra73).

[00303] Aspects of the invention involve the adoptive transfer of immune system cells, such as T cells, specific for selected antigens, such as tumor associated antigens (see Maus et al, 2014, Adoptive Immunotherapy for Cancer or Viruses, Annual Review of Immunology, Vol. 32: 189-225; Rosenberg and Restifo, 2015, Adoptive cell transfer as personalized immunotherapy for human cancer, Science Vol. 348 no. 6230 pp. 62-68; Restifo et al, 2015, Adoptive immunotherapy for cancer: harnessing the T cell response. Nat. Rev. Immunol. 12(4): 269-281; and Jenson and Riddell, 2014, Design and implementation of adoptive therapy with chimeric antigen receptor-modified T cells. Immunol Rev. 257(1): 127-144). Various strategies may for example be employed to genetically modify T cells by altering the specificity of the T cell receptor (TCR) for example by introducing new TCR α and β chains with selected peptide specificity (see U.S. Patent No. 8,697,854; PCT Patent Publications: WO2003020763, WO2004033685, WO2004044004, WO20051 14215, WO2006000830, WO2008038002, WO2008039818, WO2004074322, WO20051 13595, WO2006125962, WO2013166321, WO2013039889, WO2014018863, WO2014083173; U.S. Patent No. 8,088,379).

[00304] As an alternative to, or addition to, TCR modifications, chimeric antigen receptors (CARs) may be used in order to generate immunoresponsive cells, such as T cells, specific for selected targets, such as malignant cells, with a wide variety of receptor chimera constructs having been described (see U.S. Patent Nos. 5,843,728; 5,851,828; 5,912,170; 6,004,811; 6,284,240; 6,392,013; 6,410,014; 6,753,162; 8,211,422; and, PCT Publication W092 15322).

[00305] In general, CARs are comprised of an extracellular domain, a transmembrane domain, and an intracellular domain, wherein the extracellular domain comprises an antigen-binding domain that is specific for a predetermined target. While the antigen-binding domain of a CAR is often an antibody or antibody fragment (e.g., a single chain variable fragment,

scFv), the binding domain is not particularly limited so long as it results in specific recognition of a target. For example, in some embodiments, the antigen-binding domain may comprise a receptor, such that the CAR is capable of binding to the ligand of the receptor. Alternatively, the antigen-binding domain may comprise a ligand, such that the CAR is capable of binding the endogenous receptor of that ligand.

[00306] The antigen-binding domain of a CAR is generally separated from the transmembrane domain by a hinge or spacer. The spacer is also not particularly limited, and it is designed to provide the CAR with flexibility. For example, a spacer domain may comprise a portion of a human Fc domain, including a portion of the CH3 domain, or the hinge region of any immunoglobulin, such as IgA, IgD, IgE, IgG, or IgM, or variants thereof. Furthermore, the hinge region may be modified so as to prevent off-target binding by FcRs or other potential interfering objects. For example, the hinge may comprise an IgG4 Fc domain with or without a S228P, L235E, and/or N297Q mutation (according to Kabat numbering) in order to decrease binding to FcRs. Additional spacers/hinges include, but are not limited to, CD4, CD8, and CD28 hinge regions.

[00307] The transmembrane domain of a CAR may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions of particular use in this disclosure may be derived from CD8, CD28, CD3, CD45, CD4, CD5, CDS, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD 134, CD137, CD 154, TCR. Alternatively the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR. A glycine-serine doublet provides a particularly suitable linker.

[00308] Alternative CAR constructs may be characterized as belonging to successive generations. First-generation CARs typically consist of a single-chain variable fragment of an antibody specific for an antigen, for example comprising a VL linked to a VH of a specific antibody, linked by a flexible linker, for example by a CD8a hinge domain and a CD8a transmembrane domain, to the transmembrane and intracellular signaling domains of either CD3 ζ or FcR γ (scFv-CD3C or scFv-FcR γ ; see U.S. Patent No. 7,741,465; U.S. Patent No. 5,912,172; U.S. Patent No. 5,906,936). Second-generation CARs incorporate the intracellular domains of one or more costimulatory molecules, such as CD28, OX40 (CD134), or 4-1BB

(CD137) within the endodomain (for example scFv-CD28/OX40/4-1BB-CD3C; see U.S. Patent Nos. 8,911,993; 8,916,381; 8,975,071; 9,101,584; 9,102,760; 9,102,761). Third-generation CARs include a combination of costimulatory endodomains, such a CD3 ζ -chain, CD97, GDI la-CD18, CD2, ICOS, CD27, CD154, CDS, OX40, 4-1BB, CD2, CD7, LIGHT, LFA-1, NKG2C, B7-H3, CD30, CD40, PD-1, or CD28 signaling domains (for example scFv-CD28-4-1BB-CD3C or scFv-CD28-OX40-CD3C; see U.S. Patent No. 8,906,682; U.S. Patent No. 8,399,645; U.S. Pat. No. 5,686,281; PCT Publication No. WO2014134165; PCT Publication No. WO2012079000). Alternatively, costimulation may be orchestrated by expressing CARs in antigen-specific T cells, chosen so as to be activated and expanded following engagement of their native α TCR, for example by antigen on professional antigen-presenting cells, with attendant costimulation. In addition, additional engineered receptors may be provided on the immunoresponsive cells, for example to improve targeting of a T-cell attack and/or minimize side effects.

[00309] Alternatively, T-cells expressing CARs may be further modified to reduce or eliminate expression of endogenous TCRs in order to reduce off-target effects. Reduction or elimination of endogenous TCRs can reduce off-target effects and increase the effectiveness of the T cells (U.S. 9,181,527). T cells stably lacking expression of a functional TCR may be produced using a variety of approaches. T cells internalize, sort, and degrade the entire T cell receptor as a complex, with a half-life of about 10 hours in resting T cells and 3 hours in stimulated T cells (von Essen, M. et al. 2004. *J. Immunol.* 173:384-393). Proper functioning of the TCR complex requires the proper stoichiometric ratio of the proteins that compose the TCR complex. TCR function also requires two functioning TCR zeta proteins with ITAM motifs. The activation of the TCR upon engagement of its MHC-peptide ligand requires the engagement of several TCRs on the same T cell, which all must signal properly. Thus, if a TCR complex is destabilized with proteins that do not associate properly or cannot signal optimally, the T cell will not become activated sufficiently to begin a cellular response.

[00310] Accordingly, in some embodiments, TCR expression may be eliminated using RNA interference (e.g., shRNA, siRNA, miRNA, etc.), CRISPR, or other methods that target the nucleic acids encoding specific TCRs (e.g., TCR- α and TCR- β) and/or CD3 chains in primary T cells. By blocking expression of one or more of these proteins, the T cell will no longer produce one or more of the key components of the TCR complex, thereby destabilizing the TCR complex and preventing cell surface expression of a functional TCR.

[00311] In some instances, CAR may also comprise a switch mechanism for controlling expression and/or activation of the CAR. For example, a CAR may comprise an

extracellular, transmembrane, and intracellular domain, in which the extracellular domain comprises a target-specific binding element that comprises a label, binding domain, or tag that is specific for a molecule other than the target antigen that is expressed on or by a target cell. In such embodiments, the specificity of the CAR is provided by a second construct that comprises a target antigen binding domain (e.g., an scFv or a bispecific antibody that is specific for both the target antigen and the label or tag on the CAR) and a domain that is recognized by or binds to the label, binding domain, or tag on the CAR. See, e.g., WO 2013/044225, WO 2016/000304, WO 2015/057834, WO 2015/057852, WO 2016/070061, US 9,233,125, US 2016/0129109. In this way, a T-cell that expresses the CAR can be administered to a subject, but the CAR cannot bind its target antigen until the second composition comprising an antigen-specific binding domain is administered.

[00312] Alternative switch mechanisms include CARs that require multimerization in order to activate their signaling function (see, e.g., US 2015/0368342, US 2016/0175359, US 2015/0368360) and/or an exogenous signal, such as a small molecule drug (US 2016/0166613, Yung et al, Science, 2015), in order to elicit a T-cell response. Some CARs may also comprise a "suicide switch" to induce cell death of the CAR T-cells following treatment (Buddee et al., PLoS One, 2013) or to downregulate expression of the CAR following binding to the target antigen (WO 2016/01 1210).

[00313] Alternative techniques may be used to transform target immunoresponsive cells, such as protoplast fusion, lipofection, transfection or electroporation. A wide variety of vectors may be used, such as retroviral vectors, lentiviral vectors, adenoviral vectors, adeno-associated viral vectors, plasmids or transposons, such as a Sleeping Beauty transposon (see U.S. Patent Nos. 6,489,458; 7,148,203; 7,160,682; 7,985,739; 8,227,432), may be used to introduce CARs, for example using 2nd generation antigen-specific CARs signaling through CD3 ζ and either CD28 or CD137. Viral vectors may for example include vectors based on HIV, SV40, EBV, HSV or BPV.

[00314] Cells that are targeted for transformation may for example include T cells, Natural Killer (NK) cells, cytotoxic T lymphocytes (CTL), regulatory T cells, human embryonic stem cells, tumor-infiltrating lymphocytes (TIL) or a pluripotent stem cell from which lymphoid cells may be differentiated. T cells expressing a desired CAR may for example be selected through co-culture with γ -irradiated activating and propagating cells (AaPC), which co-express the cancer antigen and co-stimulatory molecules. The engineered CAR T-cells may be expanded, for example by co-culture on AaPC in presence of soluble factors, such as IL-2 and IL-21. This expansion may for example be carried out so as to provide memory CAR⁺ T

cells (which may for example be assayed by non-enzymatic digital array and/or multi-panel flow cytometry). In this way, CAR T cells may be provided that have specific cytotoxic activity against antigen-bearing tumors (optionally in conjunction with production of desired chemokines such as interferon- γ). CAR T cells of this kind may for example be used in animal models, for example to treat tumor xenografts.

[00315] Approaches such as the foregoing may be adapted to provide methods of treating and/or increasing survival of a subject having a disease, such as a neoplasia, for example by administering an effective amount of an immunoresponsive cell comprising an antigen recognizing receptor that binds a selected antigen, wherein the binding activates the immunoreponsive cell, thereby treating or preventing the disease (such as a neoplasia, a pathogen infection, an autoimmune disorder, or an allogeneic transplant reaction).

[00316] Additionally, the disclosed biomarker signature (e.g., the genes displayed in Tables 5-13 or a selection of genes therefrom) may be used to identify CAR T cells or other cells used in ACT that are dysfunctional or exhausted. Using the disclosed biomarkers as a diagnostic platform allows clinicians to identify whether a patient's response to the ACT is due to cell dysfunction, and if it is, the levels of up-regulation and down-regulation across the biomarker signature will allow problems to be addressed. For example, if a patient receiving ACT is non-responsive, the cells administered as part of the ACT may be assayed by an assay disclosed herein to determine the relative level of expression of a disclosed biomarker signature (e.g., Tables 5-13 or a selection of genes therefrom). If a particular inhibitory receptor or molecule is up-regulated in the ACT cells, the patient may be treated with an inhibitor of that receptor or molecule. If a particular stimulatory receptor or molecule is down-regulated in the ACT cells, the patient may be treated with an agonist of that receptor or molecule.

[00317] In one embodiment, the treatment can be administered into patients undergoing an immunosuppressive treatment. The cells or population of cells, may be made resistant to at least one immunosuppressive agent due to the inactivation of a gene encoding a receptor for such immunosuppressive agent. Not being bound by a theory, the immunosuppressive treatment should help the selection and expansion of the immunoresponsive or T cells according to the invention within the patient.

[00318] The administration of the cells or population of cells according to the present invention may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The cells or population of cells may be administered to a patient subcutaneously, intradermally, intratumorally,

intranodally, intramedullary, intramuscularly, intrathecally, by intravenous or intralymphatic injection, or intraperitoneally. In some embodiments, the disclosed CARs may be delivered or administered into a cavity formed by the resection of tumor tissue (i.e. intracavity delivery) or directly into a tumor prior to resection (i.e. intratumoral delivery). In one embodiment, the cell compositions of the present invention are preferably administered by intravenous injection.

[00319] The administration of the cells or population of cells can consist of the administration of 10^4 - 10^9 cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight including all integer values of cell numbers within those ranges. Dosing in CAR T cell therapies may for example involve administration of from 10^6 to 10^9 cells/kg, with or without a course of lymphodepletion, for example with cyclophosphamide. The cells or population of cells can be administered in one or more doses. In another embodiment, the effective amount of cells are administered as a single dose. In another embodiment, the effective amount of cells are administered as more than one dose over a period time. Timing of administration is within the judgment of managing physician and depends on the clinical condition of the patient. The cells or population of cells may be obtained from any source, such as a blood bank or a donor. While individual needs vary, determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions are within the skill of one in the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administered will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired.

[00320] In another embodiment, the effective amount of cells or composition comprising those cells are administered parenterally. The administration can be an intravenous administration. The administration can be directly done by injection within a tumor.

[00321] To guard against possible adverse reactions, engineered immunoresponsive cells may be equipped with a transgenic safety switch, in the form of a transgene that renders the cells vulnerable to exposure to a specific signal. For example, the herpes simplex viral thymidine kinase (TK) gene may be used in this way, for example by introduction into allogeneic T lymphocytes used as donor lymphocyte infusions following stem cell transplantation (Greco, et al, Improving the safety of cell therapy with the TK-suicide gene. *Front. Pharmacol.* 2015; 6: 95). In such cells, administration of a nucleoside prodrug such as ganciclovir or acyclovir causes cell death. Alternative safety switch constructs include inducible caspase 9, for example triggered by administration of a small-molecule dimerizer

that brings together two nonfunctional icasp9 molecules to form the active enzyme. A wide variety of alternative approaches to implementing cellular proliferation controls have been described (see U.S. Patent Publication No. 20130071414; PCT Patent Publication WO2011146862; PCT Patent Publication WO2014011987; PCT Patent Publication WO2013040371; Zhou et al. BLOOD, 2014, 123/25:3895 - 3905; Di Stasi et al., The New England Journal of Medicine 2011; 365:1673-1683; Sadelain M, The New England Journal of Medicine 2011; 365:1735-173; Ramos et al, Stem Cells 28(6): 1107-15 (2010)).

[00322] In a further refinement of adoptive therapies, genome editing may be used to tailor immunoresponsive cells to alternative implementations, for example providing edited CAR T cells (see Poirot et al, 2015, Multiplex genome edited T-cell manufacturing platform for "off-the-shelf adoptive T-cell immunotherapies, Cancer Res 75 (18): 3853). Cells may be edited using any CRISPR system and method of use thereof as described herein. CRISPR systems may be delivered to an immune cell by any method described herein. In preferred embodiments, cells are edited *ex vivo* and transferred to a subject in need thereof. Immunoresponsive cells, CAR T cells or any cells used for adoptive cell transfer may be edited. Editing may be performed to eliminate potential alloreactive T-cell receptors (TCR), disrupt the target of a chemotherapeutic agent, block an immune checkpoint, activate a T cell, and/or increase the differentiation and/or proliferation of functionally exhausted or dysfunctional CD8+ T-cells (see PCT Patent Publications: WO2013176915, WO2014059173, WO2014172606, WO2014184744, and WO2014191128). Editing may result in inactivation of a gene.

[00323] By inactivating a gene it is intended that the gene of interest is not expressed in a functional protein form. In a particular embodiment, the CRISPR system specifically catalyzes cleavage in one targeted gene thereby inactivating said targeted gene. The nucleic acid strand breaks caused are commonly repaired through the distinct mechanisms of homologous recombination or non-homologous end joining (NHEJ). However, NHEJ is an imperfect repair process that often results in changes to the DNA sequence at the site of the cleavage. Repair via non-homologous end joining (NHEJ) often results in small insertions or deletions (Indel) and can be used for the creation of specific gene knockouts. Cells in which a cleavage induced mutagenesis event has occurred can be identified and/or selected by well-known methods in the art.

[00324] T cell receptors (TCR) are cell surface receptors that participate in the activation of T cells in response to the presentation of antigen. The TCR is generally made from two chains, α and β , which assemble to form a heterodimer and associates with the CD3-

transducing subunits to form the T cell receptor complex present on the cell surface. Each α and β chain of the TCR consists of an immunoglobulin-like N-terminal variable (V) and constant (C) region, a hydrophobic transmembrane domain, and a short cytoplasmic region. As for immunoglobulin molecules, the variable region of the α and β chains are generated by V(D)J recombination, creating a large diversity of antigen specificities within the population of T cells. However, in contrast to immunoglobulins that recognize intact antigen, T cells are activated by processed peptide fragments in association with an MHC molecule, introducing an extra dimension to antigen recognition by T cells, known as MHC restriction. Recognition of MHC disparities between the donor and recipient through the T cell receptor leads to T cell proliferation and the potential development of graft versus host disease (GVHD). The inactivation of TCR α or TCR β can result in the elimination of the TCR from the surface of T cells preventing recognition of alloantigen and thus GVHD. However, TCR disruption generally results in the elimination of the CD3 signaling component and alters the means of further T cell expansion.

[00325] Allogeneic cells are rapidly rejected by the host immune system. It has been demonstrated that, allogeneic leukocytes present in non-irradiated blood products will persist for no more than 5 to 6 days (Boni, Muranski et al. 2008 Blood 1;112(12):4746-54). Thus, to prevent rejection of allogeneic cells, the host's immune system usually has to be suppressed to some extent. However, in the case of adoptive cell transfer the use of immunosuppressive drugs also have a detrimental effect on the introduced therapeutic T cells. Therefore, to effectively use an adoptive immunotherapy approach in these conditions, the introduced cells would need to be resistant to the immunosuppressive treatment. Thus, in a particular embodiment, the present invention further comprises a step of modifying T cells to make them resistant to an immunosuppressive agent, preferably by inactivating at least one gene encoding a target for an immunosuppressive agent. An immunosuppressive agent is an agent that suppresses immune function by one of several mechanisms of action. An immunosuppressive agent can be, but is not limited to a calcineurin inhibitor, a target of rapamycin, an interleukin-2 receptor α -chain blocker, an inhibitor of inosine monophosphate dehydrogenase, an inhibitor of dihydrofolic acid reductase, a corticosteroid or an immunosuppressive antimetabolite. The present invention allows conferring immunosuppressive resistance to T cells for immunotherapy by inactivating the target of the immunosuppressive agent in T cells. As non-limiting examples, targets for an immunosuppressive agent can be a receptor for an immunosuppressive agent such as: CD52,

glucocorticoid receptor (GR), a FKBP family gene member and a cyclophilin family gene member.

[00326] Immune checkpoints are inhibitory pathways that slow down or stop immune reactions and prevent excessive tissue damage from uncontrolled activity of immune cells. In certain embodiments, the immune checkpoint targeted is the programmed death-1 (PD-1 or CD279) gene (*PDCDI*). In other embodiments, the immune checkpoint targeted is cytotoxic T-lymphocyte-associated antigen (CTLA-4). In additional embodiments, the immune checkpoint targeted is another member of the CD28 and CTLA4 Ig superfamily such as BTLA, LAG3, ICOS, PDL1 or KIR. In further additional embodiments, the immune checkpoint targeted is a member of the TNFR superfamily such as CD40, OX40, CD137, GITR, CD27 or TIM-3.

[00327] Additional immune checkpoints include Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) (Watson HA, et al, SHP-1: the next checkpoint target for cancer immunotherapy? *Biochem Soc Trans.* 2016 Apr 15;44(2):356-62). SHP-1 is a widely expressed inhibitory protein tyrosine phosphatase (PTP). In T-cells, it is a negative regulator of antigen-dependent activation and proliferation. It is a cytosolic protein, and therefore not amenable to antibody-mediated therapies, but its role in activation and proliferation makes it an attractive target for genetic manipulation in adoptive transfer strategies, such as chimeric antigen receptor (CAR) T cells. Immune checkpoints may also include T cell immunoreceptor with Ig and ITIM domains (TIGIT/Vstm3/WUCAM/VSIG9) and VISTA (Le Mercier I, et al., (2015) Beyond CTLA-4 and PD-1, the generation Z of negative checkpoint regulators. *Front. Immunol.* 6:418).

[00328] WO2014172606 relates to the use of MT1 and/or MT1 inhibitors to increase proliferation and/or activity of exhausted CD8+ T-cells and to decrease CD8+ T-cell exhaustion (e.g., decrease functionally exhausted or unresponsive CD8+ immune cells). In certain embodiments, metallothioneins are targeted by gene editing in adoptively transferred T cells.

[00329] In certain embodiments, targets of gene editing may be at least one targeted locus involved in the expression of an immune checkpoint protein. Such targets may include, but are not limited to CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, ICOS (CD278), PDL1, KIR, LAG3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244 (2B4), TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBR2, TGFBR1, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1,

FOXP3, PRDM1, BATF, VISTA, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, MT1, MT2, CD40, OX40, CD137, GITR, CD27, SHP-1 or TIM-3. In preferred embodiments, the gene locus involved in the expression of PD-1 or CTLA-4 genes is targeted. In other preferred embodiments, combinations of genes are targeted, such as but not limited to PD-1 and TIGIT. In preferred embodiments, the novel genes or gene combinations described herein are targeted or modulated.

[00330] In other embodiments, at least two genes are edited. Pairs of genes may include, but are not limited to PD1 and TCRA, PD1 and TCR- β , CTLA-4 and TCRA, CTLA-4 and TCR β , LAG3 and TCRA, LAG3 and TCR β , Tim3 and TCRA, Tim3 and TCR β , BTLA and TCRA, BTLA and TCR β , BY55 and TCRA, BY55 and TCR β , TIGIT and TCRA, TIGIT and TCR β , B7H5 and TCRA, B7H5 and TCR β , LAIR1 and TCRA, LAIR1 and TCR β , SIGLEC10 and TCRA, SIGLEC10 and TCR β , 2B4 and TCRA, 2B4 and TCR β .

[00331] Whether prior to or after genetic modification of the T cells, the T cells can be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and 7,572,631. T cells can be expanded *in vitro* or *in vivo*.

[00332] Immune cells may be obtained using any method known in the art. In one embodiment T cells that have infiltrated a tumor are isolated. T cells may be removed during surgery. T cells may be isolated after removal of tumor tissue by biopsy. T cells may be isolated by any means known in the art. In one embodiment the method may comprise obtaining a bulk population of T cells from a tumor sample by any suitable method known in the art. For example, a bulk population of T cells can be obtained from a tumor sample by dissociating the tumor sample into a cell suspension from which specific cell populations can be selected. Suitable methods of obtaining a bulk population of T cells may include, but are not limited to, any one or more of mechanically dissociating (e.g., mincing) the tumor, enzymatically dissociating (e.g., digesting) the tumor, and aspiration (e.g., as with a needle).

[00333] The bulk population of T cells obtained from a tumor sample may comprise any suitable type of T cell. Preferably, the bulk population of T cells obtained from a tumor sample comprises tumor infiltrating lymphocytes (TILs).

[00334] The tumor sample may be obtained from any mammal. Unless stated otherwise, as used herein, the term "mammal" refers to any mammal including, but not limited to, mammals of the order Logomorpha, such as rabbits; the order Carnivora, including Felines (cats) and Canines (dogs); the order Artiodactyla, including Bovines (cows) and Swines

(pigs); or of the order Perssodactyla, including Equines (horses). The mammals may be non-human primates, e.g., of the order Primates, Ceboidea, or Simiiformes (monkeys) or of the order Anthropoidea (humans and apes). In some embodiments, the mammal may be a mammal of the order Rodentia, such as mice and hamsters. Preferably, the mammal is a non-human primate or a human. An especially preferred mammal is the human.

[00335] T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, spleen tissue, and tumors. In certain embodiments of the present invention, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll separation. In one preferred embodiment, cells from the circulating blood of an individual are obtained by apheresis or leukapheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one embodiment, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In one embodiment of the invention, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Initial activation steps in the absence of calcium lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca-free, Mg-free PBS. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

[00336] In another embodiment, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient. A specific subpopulation of T cells, such as CD28+, CD4+, CD45RO+, and CD45RA+ T cells, can be further isolated by positive or negative selection techniques. For example, in one preferred embodiment, T cells are isolated by incubation with anti-CD3/anti-CD28 (i.e., 3x28)-conjugated beads, such as DYNABEADS® M-450 CD3/CD28 T, or XCYTE DYNABEADS™ for a time period sufficient for positive selection of the desired T cells. In one embodiment, the time period is about 30 minutes. In a further embodiment, the time period ranges from 30 minutes to 36 hours or longer and all

integer values there between. In a further embodiment, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In yet another preferred embodiment, the time period is 10 to 24 hours. In one preferred embodiment, the incubation time period is 24 hours. For isolation of T cells from patients with leukemia, use of longer incubation times, such as 24 hours, can increase cell yield. Longer incubation times may be used to isolate T cells in any situation where there are few T cells as compared to other cell types, such in isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immunocompromised individuals. Further, use of longer incubation times can increase the efficiency of capture of CD8+ T cells.

[00337] Enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. A preferred method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CDlib, CD 16, HLA-DR, and CD8.

[00338] Further, monocyte populations (i.e., CD14+ cells) may be depleted from blood preparations by a variety of methodologies, including anti-CD14 coated beads or columns, or utilization of the phagocytotic activity of these cells to facilitate removal. Accordingly, in one embodiment, the invention uses paramagnetic particles of a size sufficient to be engulfed by phagocytotic monocytes. In certain embodiments, the paramagnetic particles are commercially available beads, for example, those produced by Life Technologies under the trade name Dynabeads™. In one embodiment, other non-specific cells are removed by coating the paramagnetic particles with "irrelevant" proteins (e.g., serum proteins or antibodies). Irrelevant proteins and antibodies include those proteins and antibodies or fragments thereof that do not specifically target the T cells to be isolated. In certain embodiments the irrelevant beads include beads coated with sheep anti-mouse antibodies, goat anti-mouse antibodies, and human serum albumin.

[00339] In brief, such depletion of monocytes is performed by preincubating T cells isolated from whole blood, apheresed peripheral blood, or tumors with one or more varieties of irrelevant or non-antibody coupled paramagnetic particles at any amount that allows for removal of monocytes (approximately a 20:1 bead:cell ratio) for about 30 minutes to 2 hours at 22 to 37 degrees C, followed by magnetic removal of cells which have attached to or engulfed the paramagnetic particles. Such separation can be performed using standard methods available in the art. For example, any magnetic separation methodology may be used

including a variety of which are commercially available, (e.g., DYNAL® Magnetic Particle Concentrator (DYNAL MPC®)). Assurance of requisite depletion can be monitored by a variety of methodologies known to those of ordinary skill in the art, including flow cytometric analysis of CD14 positive cells, before and after depletion.

[00340] For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (e.g., particles such as beads) can be varied. In certain embodiments, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and beads. For example, in one embodiment, a concentration of 2 billion cells/ml is used. In one embodiment, a concentration of 1 billion cells/ml is used. In a further embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells, or from samples where there are many tumor cells present (i.e., leukemic blood, tumor tissue, etc). Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

[00341] In a related embodiment, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (e.g., particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4+ T cells express higher levels of CD28 and are more efficiently captured than CD8+ T cells in dilute concentrations. In one embodiment, the concentration of cells used is 5×10^6 /ml. In other embodiments, the concentration used can be from about 1×10^5 /ml to 1×10^9 /ml, and any integer value in between.

[00342] T cells can also be frozen. Wishing not to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After a washing step to remove plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, one method involves

using PBS containing 20% DMSO and 8% human serum albumin, or other suitable cell freezing media, the cells then are frozen to -80° C at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled freezing immediately at -20° C. or in liquid nitrogen.

[00343] T cells for use in the present invention may also be antigen-specific T cells. For example, tumor-specific T cells can be used. In certain embodiments, antigen-specific T cells can be isolated from a patient of interest, such as a patient afflicted with a cancer or an infectious disease. In one embodiment neopeptides are determined for a subject and T cells specific to these antigens are isolated. Antigen-specific cells for use in expansion may also be generated in vitro using any number of methods known in the art, for example, as described in U.S. Patent Publication No. US 20040224402 entitled, Generation And Isolation of Antigen-Specific T Cells, or in U.S. Pat. Nos. 6,040,177. Antigen-specific cells for use in the present invention may also be generated using any number of methods known in the art, for example, as described in Current Protocols in Immunology, or Current Protocols in Cell Biology, both published by John Wiley & Sons, Inc., Boston, Mass.

[00344] In a related embodiment, it may be desirable to sort or otherwise positively select (e.g. via magnetic selection) the antigen specific cells prior to or following one or two rounds of expansion. Sorting or positively selecting antigen-specific cells can be carried out using peptide-MHC tetramers (Altaian, et al, *Science*. 1996 Oct. 4; 274(5284):94-6). In another embodiment the adaptable tetramer technology approach is used (Andersen et al, 2012 Nat Protoc. 7:891-902). Tetramers are limited by the need to utilize predicted binding peptides based on prior hypotheses, and the restriction to specific HLAs. Peptide-MHC tetramers can be generated using techniques known in the art and can be made with any MHC molecule of interest and any antigen of interest as described herein. Specific epitopes to be used in this context can be identified using numerous assays known in the art. For example, the ability of a polypeptide to bind to MHC class I may be evaluated indirectly by monitoring the ability to promote incorporation of ¹²⁵I labeled P2-microglobulin (β 2m) into MHC class I/p2m/peptide heterotrimeric complexes (see Parker et al, *J. Immunol.* 152:163, 1994).

[00345] In one embodiment cells are directly labeled with an epitope-specific reagent for isolation by flow cytometry followed by characterization of phenotype and TCRs. In one T cells are isolated by contacting the T cell specific antibodies. Sorting of antigen-specific T cells, or generally any cells of the present invention, can be carried out using any of a variety of commercially available cell sorters, including, but not limited to, MoFlo sorter

(DakoCytomation, Fort Collins, Colo.), FACSAria™, FACSArray™, FACSVantage™, BD™ LSR II, and FACSCalibur™ (BD Biosciences, San Jose, Calif.).

[00346] In a preferred embodiment, the method comprises selecting cells that also express CD3. The method may comprise specifically selecting the cells in any suitable manner. Preferably, the selecting is carried out using flow cytometry. The flow cytometry may be carried out using any suitable method known in the art. The flow cytometry may employ any suitable antibodies and stains. Preferably, the antibody is chosen such that it specifically recognizes and binds to the particular biomarker being selected. For example, the specific selection of CD3, CD8, TIM-3, LAG-3, 4-1BB, or PD-1 may be carried out using anti-CD3, anti-CD8, anti-TIM-3, anti-LAG-3, anti-4-1BB, or anti-PD-1 antibodies, respectively. The antibody or antibodies may be conjugated to a bead (e.g., a magnetic bead) or to a fluorochrome. Preferably, the flow cytometry is fluorescence-activated cell sorting (FACS). TCRs expressed on T cells can be selected based on reactivity to autologous tumors. Additionally, T cells that are reactive to tumors can be selected for based on markers using the methods described in patent publication Nos. WO2014133567 and WO2014133568, herein incorporated by reference in their entirety. Additionally, activated T cells can be selected for based on surface expression of CD107a.

[00347] In one embodiment of the invention, the method further comprises expanding the numbers of T cells in the enriched cell population. Such methods are described in U.S. Patent No. 8,637,307 and is herein incorporated by reference in its entirety. The numbers of T cells may be increased at least about 3-fold (or 4-, 5-, 6-, 7-, 8-, or 9-fold), more preferably at least about 10-fold (or 20-, 30-, 40-, 50-, 60-, 70-, 80-, or 90-fold), more preferably at least about 100-fold, more preferably at least about 1,000 fold, or most preferably at least about 100,000-fold. The numbers of T cells may be expanded using any suitable method known in the art. Exemplary methods of expanding the numbers of cells are described in patent publication No. WO 2003057171, U.S. Patent No. 8,034,334, and U.S. Patent Application Publication No. 2012/0244133, each of which is incorporated herein by reference.

[00348] In one embodiment, *ex vivo* T cell expansion can be performed by isolation of T cells and subsequent stimulation or activation followed by further expansion. In one embodiment of the invention, the T cells may be stimulated or activated by a single agent. In another embodiment, T cells are stimulated or activated with two agents, one that induces a primary signal and a second that is a co-stimulatory signal. Ligands useful for stimulating a single signal or stimulating a primary signal and an accessory molecule that stimulates a second signal may be used in soluble form. Ligands may be attached to the surface of a cell,

to an Engineered Multivalent Signaling Platform (EMSP), or immobilized on a surface. In a preferred embodiment both primary and secondary agents are co-immobilized on a surface, for example a bead or a cell. In one embodiment, the molecule providing the primary activation signal may be a CD3 ligand, and the co-stimulatory molecule may be a CD28 ligand or 4-1BB ligand.

Treatment of Chronic Immune Conditions

[00349] A "cancer" or "tumor" as used herein refers to an uncontrolled growth of cells which interferes with the normal functioning of the bodily organs and systems. A subject that has a cancer or a tumor is a subject having objectively measurable cancer cells present in the subject's body. Included in this definition are benign and malignant cancers, as well as dormant tumors or micrometastases. Cancers which migrate from their original location and seed vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs. Hemopoietic cancers, such as leukemia, are able to out-compete the normal hemopoietic compartments in a subject, thereby leading to hemopoietic failure (in the form of anemia, thrombocytopenia and neutropenia) ultimately causing death.

[00350] By "metastasis" is meant the spread of cancer from its primary site to other places in the body. Cancer cells can break away from a primary tumor, penetrate into lymphatic and blood vessels, circulate through the bloodstream, and grow in a distant focus (metastasize) in normal tissues elsewhere in the body. Metastasis can be local or distant. Metastasis is a sequential process, contingent on tumor cells breaking off from the primary tumor, traveling through the bloodstream, and stopping at a distant site. At the new site, the cells establish a blood supply and can grow to form a life-threatening mass. Both stimulatory and inhibitory molecular pathways within the tumor cell regulate this behavior, and interactions between the tumor cell and host cells in the distant site are also significant.

[00351] Metastases are most often detected through the sole or combined use of magnetic resonance imaging (MRI) scans, computed tomography (CT) scans, blood and platelet counts, liver function studies, chest X-rays and bone scans in addition to the monitoring of specific symptoms.

[00352] Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include, but are not limited to, basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and CNS cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric

cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); lymphoma including Hodgkin's and non-Hodgkin's lymphoma; melanoma; myeloma; neuroblastoma; oral cavity cancer (*e.g.*, lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; as well as other carcinomas and sarcomas; as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

[00353] In some embodiments of these methods and all such methods described herein, the methods further comprise administering a tumor or cancer antigen to a subject being administered the one or more agents described herein.

[00354] A number of tumor antigens have been identified that are associated with specific cancers. As used herein, the terms "tumor antigen" and "cancer antigen" are used interchangeably to refer to antigens which are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (*i.e.*, not expressed) in normal cells, those that are expressed only at certain stages of differentiation and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (*e.g.*, activated ras oncogene), suppressor genes (*e.g.*, mutant p53), and fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried

on RNA and DNA tumor viruses. Many tumor antigens have been defined in terms of multiple solid tumors: MAGE 1, 2, & 3, defined by immunity; MART-1/Melan-A, gp100, carcinoembryonic antigen (CEA), HER-2, mucins (*i.e.*, MUC-1), prostate-specific antigen (PSA), and prostatic acid phosphatase (PAP). In addition, viral proteins such as hepatitis B (HBV), Epstein-Barr (EBV), and human papilloma (HPV) have been shown to be important in the development of hepatocellular carcinoma, lymphoma, and cervical cancer, respectively. However, due to the immunosuppression of patients diagnosed with cancer (including T cell exhaustion), the immune systems of these patients often fail to respond to the tumor antigens.

[00355] Additionally, neoantigens have been described that are subject specific. Neoantigens specific for a subject result from abundant intra-tumor and inter-tumor heterogeneity. In one instance, Ott et al., (*Hematol. Oncol. Clin. N. Am.* 28 (2014) 559-569) discusses the advantages of neoantigens in the context of melanoma. Ott et al, discusses the "NeoVax" approach and shows how tumor neoantigens provide optimal immunogenicity and tumor specificity compared to native antigens such as overexpressed or selectively expressed antigens commonly used in cancer vaccines (see, e.g., Figure 2 on page 565). Van Rooij et al. (*Journal of Clinical Oncology* 31(32):e439-e442) shows the critical role of neoantigens in antitumor immune responses. Gubin et al. (2014) (*Nature* 515:577-581), identified tumor-specific mutant antigens (*i.e.* neoantigens) by sequencing and found that peptide vaccines incorporating these mutant epitopes induced tumor rejection comparably to checkpoint inhibitor therapies (e.g. targeting CTLA-4 or PD-1). Rajasagi et al. (2014), (*Blood* 124(3):453-62) used whole-exome sequencing to identify neoantigenic peptides in patients with chronic lymphocytic leukemia. Significantly, CLL patients showing long-term remission had long-lived cytotoxic T cell responses against neoantigenic mutations. Rizvi et al. (2014) (*Science Express* 10.1126/science.aaal348) discloses that in non-small cell lung cancer, whole exome sequencing revealed that a higher neoantigen burden correlated with progression-free survival and efficacy of anti-PD-1 therapy. Neoantigen-specific T cell responses also paralleled tumor regression.

[00356] In some embodiments of these methods and all such methods described herein, the methods further comprise administering one or more anti-cancer therapies or agents to a subject in addition to the one or more agents described herein.

[00357] The term "anti-cancer therapy" refers to a therapy useful in treating cancer. Examples of anti-cancer therapeutic agents include, but are not limited to, *e.g.*, surgery, chemotherapeutic agents, growth inhibitory agents, cytotoxic agents, agents used in radiation

therapy, anti-angiogenesis agents, apoptotic agents, anti-tubulin agents, and other agents to treat cancer, such as anti-HER-2 antibodies (e.g., HERCEPTIN®), anti-CD20 antibodies, an epidermal growth factor receptor (EGFR) antagonist (e.g., a tyrosine kinase inhibitor), HER1/EGFR inhibitor (e.g., erlotinib (TARCEVA®)), platelet derived growth factor inhibitors (e.g., GLEEVEC™ (Imatinib Mesylate)), a COX-2 inhibitor (e.g., celecoxib), interferons, cytokines, antagonists (e.g., neutralizing antibodies) that bind to one or more of the following targets ErbB2, ErbB3, ErbB4, PDGFR-beta, BlyS, APRIL, BCMA or VEGF receptor(s), TRAIL/Apo2, and other bioactive and organic chemical agents, etc. Combinations thereof are also specifically contemplated for the methods described herein.

[00358] The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At²¹¹, I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹², P³² and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including active fragments and/or variants thereof.

[00359] In some embodiments of these methods and all such methods described herein, the methods further comprise administering a chemotherapeutic agent to the subject being administered the one or more agents or combination thereof described herein.

[00360] Non-limiting examples of chemotherapeutic agents can include alkylating agents such as thiotepa and CYTOXAN® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramidate, triethylenethiophosphoramidate and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlormaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammall and

calicheamicin omegall (see, *e.g.*, Agnew, Chem. Intl. Ed. Engl, 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozotocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziqune; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziqune; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE® Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumburg, Ill.), and TAXOTERE® doxetaxel (Rhone-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine;

NAVELBINE, vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (TYKERB.); inhibitors of PKC-alpha, Raf, H-Ras, EGFR (e.g., erlotinib (TARCEVA®)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above. In addition, the methods of treatment can further include the use of radiation or radiation therapy.

[00361] In certain embodiments, the one or more additional agents are synergistic in that they increase immunogenicity after treatment. In one embodiment the additional agent allows for lower toxicity and/or lower discomfort due to lower doses of the additional therapeutic agents or any components of the therapy described herein. In another embodiment the additional agent results in longer lifespan due to increased effectiveness of the therapy described herein. Chemotherapeutic treatments that enhance the immunological response in a patient have been reviewed (Zitvogel et al, Immunological aspects of cancer chemotherapy. *Nat Rev Immunol.* 2008 Jan;8(1):59-73). Additionally, chemotherapeutic agents can be administered safely with immunotherapy without inhibiting vaccine specific T-cell responses (Perez et al., A new era in anticancer peptide vaccines. *Cancer* May 2010). In one embodiment the additional agent is administered to increase the efficacy of the therapy described herein. In one embodiment the additional agent is a chemotherapy treatment. In one embodiment low doses of chemotherapy potentiate delayed-type hypersensitivity (DTH) responses. In one embodiment the chemotherapy agent targets regulatory T-cells. In one embodiment cyclophosphamide is the therapeutic agent. In one embodiment cyclophosphamide is administered prior to treatment with a target gene or gene product modulator. In one embodiment cyclophosphamide is administered as a single dose before treatment (Walter et al, Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nature Medicine*; 18:8 2012). In another embodiment, cyclophosphamide is administered according to a metronomic program, where a daily dose is administered for one month (Ghiringhelli et al, Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol Immunother* 2007 56:641-648). In another embodiment taxanes are administered before treatment to enhance

T-cell and NK-cell functions (Zitvogel et al., 2008). In another embodiment a low dose of a chemotherapeutic agent is administered with the therapy described herein. In one embodiment the chemotherapeutic agent is estramustine. In one embodiment the cancer is hormone resistant prostate cancer. A >50% decrease in serum prostate specific antigen (PSA) was seen in 8.7% of advanced hormone refractory prostate cancer patients by personalized vaccination alone, whereas such a decrease was seen in 54% of patients when the personalized vaccination was combined with a low dose of estramustine (Itoh et al, Personalized peptide vaccines: A new therapeutic modality for cancer. *Cancer Sci* 2006; 97: 970-976). In another embodiment glucocorticoids are not administered with or before the therapy described herein (Zitvogel et al, 2008). In another embodiment glucocorticoids are administered after the therapy described herein. In another embodiment Gemcitabine is administered before, simultaneously, or after the therapy described herein to enhance the frequency of tumor specific CTL precursors (Zitvogel et al, 2008). In another embodiment 5-fluorouracil is administered with the therapy described herein as synergistic immune effects were seen with a peptide based vaccine (Zitvogel et al, 2008). In another embodiment an inhibitor of Braf, such as Vemurafenib, is used as an additional agent. Braf inhibition has been shown to be associated with an increase in melanoma antigen expression and T-cell infiltrate and a decrease in immunosuppressive cytokines in tumors of treated patients (Frederick et al, BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. *Clin Cancer Res*. 2013; 19:1225-1231). In another embodiment, an inhibitor of tyrosine kinases is used as an additional agent. In one embodiment the tyrosine kinase inhibitor is used before treatment with the therapy described herein. In one embodiment the tyrosine kinase inhibitor is used simultaneously with the therapy described herein. In another embodiment the tyrosine kinase inhibitor is used to create a more immune permissive environment. In another embodiment the tyrosine kinase inhibitor is sunitinib or imatinib mesylate. It has previously been shown that favorable outcomes could be achieved with sequential administration of continuous daily dosing of sunitinib and recombinant vaccine (Farsaci et al, Consequence of dose scheduling of sunitinib on host immune response elements and vaccine combination therapy. *Int J Cancer*; 130: 1948-1959). Sunitinib has also been shown to reverse type-1 immune suppression using a daily dose of 50 mg/day (Finke et al., Sunitinib Reverses Type-1 Immune Suppression and Decreases T-Regulatory Cells in Renal Cell Carcinoma Patients. *Clin Cancer Res* 2008; 14(20)). In another embodiment additional targeted therapies are administered in combination with the therapy described herein. Doses of targeted therapies

has been described previously (Alvarez, Present and future evolution of advanced breast cancer therapy. *Breast Cancer Research* 2010, 12(Suppl 2):S1). In another embodiment temozolomide is administered with the therapy described herein. In one embodiment temozolomide is administered at 200 mg/day for 5 days every fourth week of the therapy described herein. Results of a similar strategy have been shown to have low toxicity (Kyte et al., Telomerase Peptide Vaccination Combined with Temozolomide: A Clinical Trial in Stage IV Melanoma Patients. *Clin Cancer Res*; 17(13) 2011). In another embodiment the target gene or gene product modulator therapy is administered with an additional therapeutic agent that results in lymphopenia. In one embodiment the additional agent is temozolomide. An immune response can still be induced under these conditions (Sampson et al, Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma. *Neuro-Oncology* 13(3):324-333, 2011).

[00362] In one embodiment the method may comprise administering the target gene or gene product modulator therapy within a standard of care for a particular cancer. In another embodiment the target gene or gene product modulator therapy is administered within a standard of care where addition of the therapy is synergistic with the steps in the standard of care.

[00363] In another aspect, the combination therapy described herein provides selecting the appropriate point to administer the target gene or gene product modulator therapy in relation to and within the standard of care for the cancer being treated for a patient in need thereof. The therapy can be effectively administered even within the standard of care that includes surgery, radiation, or chemotherapy. The standards of care for the most common cancers can be found on the website of National Cancer Institute (www.cancer.gov/cancertopics). The standard of care is the current treatment that is accepted by medical experts as a proper treatment for a certain type of disease and that is widely used by healthcare professionals. Standard of care is also called best practice, standard medical care, and standard therapy. Standards of Care for cancer generally include surgery, lymph node removal, radiation, chemotherapy, targeted therapies, antibodies targeting the tumor, and immunotherapy. Immunotherapy can include checkpoint blockers (CBP), chimeric antigen receptors (CARs), and adoptive T-cell therapy. The therapy described herein can be incorporated within the standard of care. The therapy described herein may also be administered where the standard of care has changed due to advances in medicine.

[00364] Incorporation of the target gene or gene product modulator therapy described herein may depend on a treatment step in the standard of care that can lead to activation of the immune system. Treatment steps that can activate and function synergistically with the therapy have been described herein. The therapy can be advantageously administered simultaneously or after a treatment that activates the immune system.

[00365] Incorporation of the therapy described herein may depend on a treatment step in the standard of care that causes the immune system to be suppressed. Such treatment steps may include irradiation, high doses of alkylating agents and/or methotrexate, steroids such as glucosteroids, surgery, such as to remove the lymph nodes, imatinib mesylate, high doses of TNF, and taxanes (Zitvogel et al., 2008). The target gene or gene product modulator therapy may be administered before such steps or may be administered after. Advantageously, the treatment is administered as part of adoptive T-cell therapy.

[00366] In one embodiment the therapy may be administered after bone marrow transplants and peripheral blood stem cell transplantation. Bone marrow transplantation and peripheral blood stem cell transplantation are procedures that restore stem cells that were destroyed by high doses of chemotherapy and/or radiation therapy. After being treated with high-dose anticancer drugs and/or radiation, the patient receives harvested stem cells, which travel to the bone marrow and begin to produce new blood cells. A "mini-transplant" uses lower, less toxic doses of chemotherapy and/or radiation to prepare the patient for transplant. A "tandem transplant" involves two sequential courses of high-dose chemotherapy and stem cell transplant. In autologous transplants, patients receive their own stem cells. In syngeneic transplants, patients receive stem cells from their identical twin. In allogeneic transplants, patients receive stem cells from their brother, sister, or parent. A person who is not related to the patient (an unrelated donor) also may be used. In some types of leukemia, the graft-versus-tumor (GVT) effect that occurs after allogeneic BMT and PBSCT is crucial to the effectiveness of the treatment. GVT occurs when white blood cells from the donor (the graft) identify the cancer cells that remain in the patient's body after the chemotherapy and/or radiation therapy (the tumor) as foreign and attack them. Immunotherapy with the therapy described herein can take advantage of this by increasing immunity after a transplant.

[00367] In one embodiment the therapy is administered to a patient in need thereof with a cancer that requires surgery. In one embodiment the combination therapy described herein is administered to a patient in need thereof in a cancer where the standard of care is primarily surgery followed by treatment to remove possible micro-metastases, such as breast cancer. Breast cancer is commonly treated by various combinations of surgery, radiation therapy,

chemotherapy, and hormone therapy based on the stage and grade of the cancer. Adjuvant therapy for breast cancer is any treatment given after primary therapy to increase the chance of long-term survival. Neoadjuvant therapy is treatment given before primary therapy. Adjuvant therapy for breast cancer is any treatment given after primary therapy to increase the chance of long-term disease-free survival. Primary therapy is the main treatment used to reduce or eliminate the cancer. Primary therapy for breast cancer usually includes surgery, a mastectomy (removal of the breast) or a lumpectomy (surgery to remove the tumor and a small amount of normal tissue around it; a type of breast-conserving surgery). During either type of surgery, one or more nearby lymph nodes are also removed to see if cancer cells have spread to the lymphatic system. When a woman has breast-conserving surgery, primary therapy almost always includes radiation therapy. Even in early-stage breast cancer, cells may break away from the primary tumor and spread to other parts of the body (metastasize). Therefore, doctors give adjuvant therapy to kill any cancer cells that may have spread, even if they cannot be detected by imaging or laboratory tests.

[00368] In one embodiment the target gene or gene product modulator therapy is administered consistent with the standard of care for Ductal carcinoma *in situ* (DCIS). The standard of care for this breast cancer type is:

1. Breast-conserving surgery and radiation therapy with or without tamoxifen.
2. Total mastectomy with or without tamoxifen.
3. Breast-conserving surgery without radiation therapy.

[00369] The therapy may be administered before breast conserving surgery or total mastectomy to shrink the tumor before surgery. In another embodiment the therapy can be administered as an adjuvant therapy to remove any remaining cancer cells.

[00370] In another embodiment patients diagnosed with stage I, II, IIIA, and Operable IIIC breast cancer are treated with the therapy as described herein. The standard of care for this breast cancer type is:

1. Local-regional treatment:
 - Breast-conserving therapy (lumpectomy, breast radiation, and surgical staging of the axilla).
 - Modified radical mastectomy (removal of the entire breast with level I–II axillary dissection) with or without breast reconstruction.
 - Sentinel node biopsy.
2. Adjuvant radiation therapy postmastectomy in axillary node-positive tumors:

- For one to three nodes: unclear role for regional radiation (infra/supraclavicular nodes, internal mammary nodes, axillary nodes, and chest wall).
- For more than four nodes or extranodal involvement: regional radiation is advised.

3. Adjuvant systemic therapy

[00371] In one embodiment the therapy is administered as a neoadjuvant therapy to shrink the tumor. In another embodiment the therapy is administered as an adjuvant systemic therapy.

[00372] In another embodiment patients diagnosed with inoperable stage IIIB or IIIC or inflammatory breast cancer are treated with the therapy as described herein. The standard of care for this breast cancer type is:

1. Multimodality therapy delivered with curative intent is the standard of care for patients with clinical stage IIIB disease.

2. Initial surgery is generally limited to biopsy to permit the determination of histology, estrogen-receptor (ER) and progesterone-receptor (PR) levels, and human epidermal growth factor receptor 2 (HER2/neu) overexpression. Initial treatment with anthracycline-based chemotherapy and/or taxane-based therapy is standard. For patients who respond to neoadjuvant chemotherapy, local therapy may consist of total mastectomy with axillary lymph node dissection followed by postoperative radiation therapy to the chest wall and regional lymphatics. Breast-conserving therapy can be considered in patients with a good partial or complete response to neoadjuvant chemotherapy. Subsequent systemic therapy may consist of further chemotherapy. Hormone therapy should be administered to patients whose tumors are ER-positive or unknown. All patients should be considered candidates for clinical trials to evaluate the most appropriate fashion in which to administer the various components of multimodality regimens.

[00373] In one embodiment the therapy is administered as part of the various components of multimodality regimens. In another embodiment the therapy is administered before, simultaneously with, or after the multimodality regimens. In another embodiment the therapy is administered based on synergism between the modalities. In another embodiment the therapy is administered after treatment with anthracycline-based chemotherapy and/or taxane-based therapy (Zitvogel et al, 2008). The therapy may also be administered after radiation.

[00374] In another embodiment the therapy described herein is used in the treatment in a cancer where the standard of care is primarily not surgery and is primarily based on systemic treatments, such as Chronic Lymphocytic Leukemia (CLL).

[00375] In another embodiment patients diagnosed with stage I, II, III, and IV Chronic Lymphocytic Leukemia are treated with the therapy as described herein. The standard of care for this cancer type is:

1. Observation in asymptomatic or minimally affected patients
2. Rituximab
3. Ofatumomab
4. Oral alkylating agents with or without corticosteroids
5. Fludarabine, 2-chlorodeoxyadenosine, or pentostatin
6. Bendamustine
7. Lenalidomide
8. Combination chemotherapy.

combination chemotherapy regimens include the following:

- o Fludarabine plus cyclophosphamide plus rituximab.
 - o Fludarabine plus rituximab as seen in the CLB-9712 and CLB-901 1 trials.
 - o Fludarabine plus cyclophosphamide versus fludarabine plus cyclophosphamide plus rituximab.
 - o Pentostatin plus cyclophosphamide plus rituximab as seen in the MAYO-MC0183 trial, for example.
 - o Ofatumumab plus fludarabine plus cyclophosphamide.
 - o CVP: cyclophosphamide plus vincristine plus prednisone.
 - o CHOP: cyclophosphamide plus doxorubicin plus vincristine plus prednisone.
 - o Fludarabine plus cyclophosphamide versus fludarabine as seen in the E2997 trial [NCT00003764] and the LRF-CLL4 trial, for example.
 - o Fludarabine plus chlorambucil as seen in the CLB-901 1 trial, for example.
9. Involved-field radiation therapy.
 10. Alemtuzumab
 11. Bone marrow and peripheral stem cell transplantations are under clinical evaluation.
 12. Ibrutinib

[00376] In one embodiment the therapy is administered before, simultaneously with or after treatment with Rituximab or Ofatumomab. As these are monoclonal antibodies that target B-cells, treatment with the combination therapy may be synergistic. In another embodiment the therapy is administered after treatment with oral alkylating agents with or without corticosteroids, and Fludarabine, 2-chlorodeoxyadenosine, or pentostatin, as these treatments may negatively affect the immune system if administered before. In one

embodiment bendamustine is administered with the therapy in low doses based on the results for prostate cancer described herein. In one embodiment the therapy is administered after treatment with bendamustine.

[00377] As used herein, the terms "chemotherapy" or "chemotherapeutic agent" refer to any chemical agent with therapeutic usefulness in the treatment of diseases characterized by abnormal cell growth. Such diseases include tumors, neoplasms and cancer as well as diseases characterized by hyperplastic growth. Chemotherapeutic agents as used herein encompass both chemical and biological agents. These agents function to inhibit a cellular activity upon which the cancer cell depends for continued survival. Categories of chemotherapeutic agents include alkylating/alkaloid agents, antimetabolites, hormones or hormone analogs, and miscellaneous antineoplastic drugs. Most if not all of these agents are directly toxic to cancer cells and do not require immune stimulation. In one embodiment, a chemotherapeutic agent is an agent of use in treating neoplasms such as solid tumors. In one embodiment, a chemotherapeutic agent is a radioactive molecule. One of skill in the art can readily identify a chemotherapeutic agent of use (*e.g.* see Slapak and Kufe, Principles of Cancer Therapy, Chapter 86 in Harrison's Principles of Internal Medicine, 14th edition; Perry *et al.*, Chemotherapy, Ch. 17 in Abeloff, Clinical Oncology 2^{sup}.nd ed., 2000 Churchill Livingstone, Inc; Baltzer L, Berkery R (eds): Oncology Pocket Guide to Chemotherapy, 2nd ed. St. Louis, Mosby-Year Book, 1995; Fischer D S, Knobf M F, Durivage H J (eds): The Cancer Chemotherapy Handbook, 4th ed. St. Louis, Mosby-Year Book, 1993).

[00378] By "radiation therapy" is meant the use of directed gamma rays or beta rays to induce sufficient damage to a cell so as to limit its ability to function normally or to destroy the cell altogether. It will be appreciated that there will be many ways known in the art to determine the dosage and duration of treatment. Typical treatments are given as a one-time administration and typical dosages range from 10 to 200 units (Grays) per day.

[00379] By "reduce" or "inhibit" in terms of the cancer treatment methods described herein is meant the ability to cause an overall decrease preferably of 20% or greater, 30% or greater, 40% or greater, 45% or greater, more preferably of 50% or greater, of 55% or greater, of 60% or greater, of 65% or greater, of 70% or greater, and most preferably of 75% or greater, 80% or greater, 85% or greater, 90% or greater, or 95% or greater, for a given parameter or symptom. Reduce or inhibit can refer to, for example, the symptoms of the disorder being treated, the presence or size of metastases or micrometastases, the size of the primary tumor, or the presence or the size of a dormant tumor.

[00380] In other embodiments of the methods of treating chronic immune conditions by decreasing T cell exhaustion described herein, the subject being administered the one or more agents has or has been diagnosed as having a persistent infection with a bacterium, virus, fungus, or parasite.

[00381] "Persistent infections" refer to those infections that, in contrast to acute infections, are not effectively cleared by the induction of a host immune response. During such persistent infections, the infectious agent and the immune response reach equilibrium such that the infected subject remains infectious over a long period of time without necessarily expressing symptoms. Persistent infections often involve stages of both silent and productive infection without rapidly killing or even producing excessive damage of the host cells. Persistent infections include for example, latent, chronic and slow infections. Persistent infection occurs with viruses including, but not limited to, human T-Cell leukemia viruses, Epstein-Barr virus, cytomegalovirus, herpes viruses, varicella-zoster virus, measles, papovaviruses, prions, hepatitis viruses, adenoviruses, parvoviruses and papillomaviruses.

[00382] In a "chronic infection," the infectious agent can be detected in the subject at all times. However, the signs and symptoms of the disease can be present or absent for an extended period of time. Non-limiting examples of chronic infection include hepatitis B (caused by hepatitis B virus (HBV)) and hepatitis C (caused by hepatitis C virus (HCV)) adenovirus, cytomegalovirus, Epstein-Barr virus, herpes simplex virus 1, herpes simplex virus 2, human herpesvirus 6, varicella-zoster virus, hepatitis D virus, papilloma virus, parvovirus B19, polyomavirus BK, polyomavirus JC, measles virus, rubella virus, human immunodeficiency virus (HIV), human T cell leukemia virus I, and human T cell leukemia virus II. Parasitic persistent infections can arise as a result of infection by, for example, Leishmania, Toxoplasma, Trypanosoma, Plasmodium, Schistosoma, and Encephalitozoon.

[00383] In a "latent infection," the infectious agent (such as a virus) is seemingly inactive and dormant such that the subject does not always exhibit signs or symptoms. In a latent viral infection, the virus remains in equilibrium with the host for long periods of time before symptoms again appear; however, the actual viruses cannot typically be detected until reactivation of the disease occurs. Non-limiting examples of latent infections include infections caused by herpes simplex virus (HSV)-1 (fever blisters), HSV-2 (genital herpes), and varicella zoster virus VZV (chickenpox-shingles).

[00384] In a "slow infection," the infectious agents gradually increase in number over a very long period of time during which no significant signs or symptoms are observed. Non-

limiting examples of slow infections include AIDS (caused by HIV-1 and HIV-2), lentiviruses that cause tumors in animals, and prions.

[00385] In addition, persistent infections that can be treated using the methods described herein include those infections that often arise as late complications of acute infections. For example, subacute sclerosing panencephalitis (SSPE) can occur following an acute measles infection or regressive encephalitis can occur as a result of a rubella infection.

[00386] The mechanisms by which persistent infections are maintained can involve modulation of virus and cellular gene expression and modification of the host immune response. Reactivation of a latent infection can be triggered by various stimuli, including changes in cell physiology, superinfection by another virus, and physical stress or trauma. Host immunosuppression is often associated with reactivation of a number of persistent virus infections.

[00387] Additional examples of infectious viruses include: *Retroviridae*; *Picornaviridae* (for example, polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (such as strains that cause gastroenteritis); *Togaviridae* (for example, equine encephalitis viruses, rubella viruses); *Flaviridae* (for example, dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (for example, coronaviruses); *Rhabdoviridae* (for example, vesicular stomatitis viruses, rabies viruses); *Filoviridae* (for example, ebola viruses); *Paramyxoviridae* (for example, parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (for example, influenza viruses); *Bungaviridae* (for example, Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); *Arena viridae* (hemorrhagic fever viruses); *Reoviridae* (e.g., reoviruses, orbiviruses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and HSV-2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses); *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (such as African swine fever virus); and unclassified viruses (for example, the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (*i.e.*, Hepatitis C); Norwalk and related viruses, and astroviruses). The compositions, methods, and uses described herein are contemplated for use in treating infections with these viral agents.

[00388] Examples of fungal infections include but are not limited to: aspergillosis; thrush (caused by *Candida albicans*); cryptococcosis (caused by *Cryptococcus*); and histoplasmosis. Thus, examples of infectious fungi include, but are not limited to, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis*, *Candida albicans*. The compositions, methods, and uses described herein are contemplated for use in treating infections with these fungal agents.

[00389] Examples of infectious bacteria include: *Helicobacter pylori*, *Borelia burgdorferi*, *Legionella pneumophila*, *Mycobacteria* sps (such as *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenuis*, *Leptospira*, and *Actinomyces israelii*. The compositions, methods, and uses described herein are contemplated for use in treating infections with these bacterial agents. Other infectious organisms (such as protists) include: *Plasmodium falciparum* and *Toxoplasma gondii*. The compositions, methods, and uses described herein are contemplated for use in treating infections with these agents.

[00390] In some embodiments, the methods described herein comprise administering an effective amount of the one or more modulators (*i.e.*, inhibitor or activator) described herein to a subject or immune cell, preferably a T cell, in order to alleviate a symptom of persistent infection. As used herein, "alleviating a symptom of a persistent infection" is ameliorating any condition or symptom associated with the persistent infection. Alternatively, alleviating a symptom of a persistent infection can involve reducing the infectious microbial (such as viral, bacterial, fungal or parasitic) load in the subject relative to such load in an untreated control. As compared with an equivalent untreated control, such reduction or degree of prevention is at least 5%, 10%, 20%, 40%, 50%, 60%, 80%, 90%, 95%, or more as measured by any standard technique. Desirably, the persistent infection is cleared, or pathogen replication has been suppressed, as detected by any standard method known in the art, in which case the persistent infection is considered to have been treated. A patient who is being treated for a

persistent infection is one who a medical practitioner has diagnosed as having such a condition. Diagnosis can be by any suitable means. Diagnosis and monitoring can involve, for example, detecting the level of microbial load in a biological sample (for example, a tissue biopsy, blood test, or urine test), detecting the level of a surrogate marker of the microbial infection in a biological sample, detecting symptoms associated with persistent infections, or detecting immune cells involved in the immune response typical of persistent infections (for example, detection of antigen specific T cells that are anergic and/or functionally impaired).

Autoimmune Disease

[00391] As used herein, an "autoimmune disease" refers to a class of diseases in which a subject's own antibodies react with host tissue or in which immune effector T cells are autoreactive to endogenous self-peptides and cause destruction of tissue. Thus an immune response is mounted against a subject's own antigens, referred to as self-antigens. A "self-antigen" as used herein refers to an antigen of a normal host tissue. Normal host tissue does not include cancer cells.

[00392] Modulation of T cell dysfunction as described herein can promote tolerance or dampen an inappropriate, unwanted, or undesirable immune response, thereby permitting treatment of autoimmune disease and/or conditions associated with transplants (*e.g.*, graft vs. host disease).

[00393] Accordingly, in some embodiments of these methods and all such methods described herein, the autoimmune diseases to be treated or prevented using the methods described herein, include, but are not limited to: rheumatoid arthritis, Crohn's disease or colitis, multiple sclerosis, systemic lupus erythematosus (SLE), autoimmune encephalomyelitis, myasthenia gravis (MG), Hashimoto's thyroiditis, Goodpasture's syndrome, pemphigus (*e.g.*, pemphigus vulgaris), Grave's disease, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, scleroderma with anti-collagen antibodies, mixed connective tissue disease, polymyositis, pernicious anemia, idiopathic Addison's disease, autoimmune-associated infertility, glomerulonephritis (*e.g.*, crescentic glomerulonephritis, proliferative glomerulonephritis), bullous pemphigoid, Sjogren's syndrome, insulin resistance, and autoimmune diabetes mellitus (type 1 diabetes mellitus; insulin-dependent diabetes mellitus), gastritis, autoimmune hepatitis, hemolytic anemia, autoimmune hemophilia, autoimmune lymphoproliferative syndrome (ALPS), autoimmune uveoretinitis, glomerulonephritis, Guillain-Barre syndrome, and psoriasis. Autoimmune disease has been recognized also to encompass atherosclerosis and Alzheimer's disease.

[00394] In some embodiments of the methods of promoting T cell tolerance, the subject being administered the one or more agents as described herein has or has been diagnosed with host versus graft disease (HVGD). In a further such embodiment, the subject being treated with the methods described herein is an organ or tissue transplant recipient. In other embodiments of the methods of promoting T cell tolerance by increasing T cell exhaustion described herein, the methods are used for increasing transplantation tolerance in a subject. In some such embodiments, the subject is a recipient of an allogenic transplant. The transplant can be any organ or tissue transplant, including but not limited to heart, kidney, liver, skin, pancreas, bone marrow, skin or cartilage. "Transplantation tolerance," as used herein, refers to a lack of rejection of the donor organ by the recipient's immune system.

Dosage, Administration and Efficacy

[00395] The terms "subject" and "individual" as used in regard to any of the methods described herein are used interchangeably herein, and refer to an animal, for example a human, recipient of the bispecific or multispecific polypeptide agents described herein. For treatment of disease states which are specific for a specific animal such as a human subject, the term "subject" refers to that specific animal. The terms "non-human animals" and "non-human mammals" are used interchangeably herein, and include mammals such as rats, mice, rabbits, sheep, cats, dogs, cows, pigs, and non-human primates. The term "subject" also encompasses any vertebrate including but not limited to mammals, reptiles, amphibians and fish. However, advantageously, the subject is a mammal such as a human, or other mammals such as a domesticated mammal, *e.g.* dog, cat, horse, and the like. Production mammal, *e.g.* cow, sheep, pig, and the like are also encompassed in the term subject.

[00396] As used herein, in regard to any of the compositions, methods, and uses comprising one or more modulating agents (*i.e.*, inhibitors or activators) or combinations thereof described herein, or adoptive cell transfer, the terms "treat," "treatment," "treating," or "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with, a disease or disorder. The term "treating" includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with a chronic immune condition, such as, but not limited to, a chronic infection or a cancer. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of at least slowing of progress or worsening of symptoms that would be expected in absence

of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. The term "treatment" of a disease also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[00397] The term "effective amount" as used herein refers to the amount of one or more modulating agents (*i.e.*, inhibitor or activator), or combinations thereof described herein, needed to alleviate at least one or more symptom of the disease or disorder being treated, and relates to a sufficient amount of pharmacological composition to provide the desired effect, *i.e.*, reverse the functional exhaustion of antigen-specific T cells in a subject having a chronic immune condition, such as cancer or hepatitis C. The term "therapeutically effective amount" therefore refers to an amount of the one or more modulating agents (*i.e.*, one or more inhibitor(s) and/or activator(s)), or combinations thereof described herein, using the methods as disclosed herein, that is sufficient to provide a particular effect when administered to a typical subject. An effective amount as used herein would also include an amount sufficient to delay the development of a symptom of the disease, alter the course of a symptom of the disease (for example but not limited to, slow the progression of a symptom of the disease), or reverse a symptom of the disease. Thus, it is not possible to specify the exact "effective amount". However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation. Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. Compositions, methods, and uses that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the one or more modulators (*i.e.*, inhibitor and/or activator)), or combinations thereof described herein, which achieves a half-maximal inhibition of measured function or activity) as determined in cell culture, or in an appropriate animal model. Levels in plasma can be measured, for example, by high performance liquid chromatography. The

effects of any particular dosage can be monitored by a suitable bioassay. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment. For example, increased production of one or more cytokines, such as IL-2 or TNF α or IFN γ , decreased production of cytokines such as IL-10, increased expression of granzyme B or CD 107a, increased ability to proliferate, or increased cytotoxicity are effector functions that can be used to determine whether a treatment is efficacious in a subject.

Modes of Administration

[00398] The one or more modulating agents (*i.e.*, inhibitors and/or activators), or combinations thereof described herein, described herein can be administered to a subject in need thereof or a cell *ex vivo* by any appropriate route which results in an effective treatment in the subject or a modified cell. As used herein, the terms "administering," and "introducing" are used interchangeably and refer to the placement of one or more modulating agents (*i.e.*, inhibitor and/or activator), or a combination thereof, into a subject or cell by a method or route which results in at least partial localization of such agents at a desired site, such as a site of inflammation, or such as the cell surface or internally in the cell, such that a desired effect(s) is produced.

[00399] In some embodiments, the one or more modulators (*i.e.*, inhibitor and/or activator) or combination thereof is administered to a subject having a chronic immune condition by any mode of administration that delivers the agent systemically or to a desired surface or target, and can include, but is not limited to, injection, infusion, instillation, and inhalation administration. To the extent that polypeptide agents can be protected from inactivation in the gut, oral administration forms are also contemplated. "Injection" includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrastemal injection and infusion. In preferred embodiments, the one or more modulating agents (*i.e.*, inhibitors and/or activators) for use in the methods described herein are administered by intravenous infusion or injection.

[00400] The phrases "parenteral administration" and "administered parenterally" as used herein, refer to modes of administration other than enteral and topical administration, usually by injection. The phrases "systemic administration," "administered systemically", "peripheral administration" and "administered peripherally" as used herein refer to the administration of the one or more modulating agents (*i.e.*, inhibitor or activator), or combination thereof, other

than directly into a target site, tissue, or organ, such as a tumor site, such that it enters the subject's circulatory system and, thus, is subject to metabolism and other like processes.

[00401] For the clinical use of the methods described herein, administration of the one or more modulating agents (*i.e.*, inhibitors or activators), or combinations thereof described herein, can include formulation into pharmaceutical compositions or pharmaceutical formulations for parenteral administration, *e.g.*, intravenous; mucosal, *e.g.*, intranasal; ocular, or other mode of administration. In some embodiments, the one or more modulating agents (*i.e.*, inhibitors and/or activators), or combinations thereof described herein, can be administered along with any pharmaceutically acceptable carrier compound, material, or composition which results in an effective treatment in the subject. Thus, a pharmaceutical formulation for use in the methods described herein can contain one or more modulating agents (*i.e.*, inhibitor and/or activator), or combination thereof, as described herein in combination with one or more pharmaceutically acceptable ingredients.

[00402] The phrase "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent, media, encapsulating material, manufacturing aid (*e.g.*, lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in maintaining the stability, solubility, or activity of, one or more modulating agents (*i.e.*, inhibitor and/or activator), or combination thereof. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) excipients, such as cocoa butter and suppository waxes; (8) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (9) glycols, such as propylene glycol; (10) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol (PEG); (11) esters, such as ethyl oleate and ethyl laurate; (12) agar; (13) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (14) alginic acid; (15) pyrogen-free water;

(16) isotonic saline; (17) Ringer's solution; (19) pH buffered solutions; (20) polyesters, polycarbonates and/or polyanhydrides; (21) bulking agents, such as polypeptides and amino acids (22) serum components, such as serum albumin, HDL and LDL; (23) C2-C12 alcohols, such as ethanol; and (24) other non-toxic compatible substances employed in pharmaceutical formulations. Release agents, coating agents, preservatives, and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable carrier" or the like are used interchangeably herein.

[00403] The one or more modulating agents (*i.e.*, inhibitors and/or activators) or combinations thereof described herein can be specially formulated for administration of the compound to a subject in solid, liquid or gel form, including those adapted for the following: (1) parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; (2) topical application, for example, as a cream, ointment, or a controlled-release patch or spray applied to the skin; (3) intravaginally or intrarectally, for example, as a pessary, cream or foam; (4) ocularly; (5) transdermally; (6) transmucosally; or (79) nasally. Additionally, a bispecific or multispecific polypeptide agent can be implanted into a patient or injected using a drug delivery system. See, for example, Urquhart, *et al.*, *Ann. Rev. Pharmacol. Toxicol.* 24: 199-236 (1984); Lewis, ed. "Controlled Release of Pesticides and Pharmaceuticals" (Plenum Press, New York, 1981); U.S. Pat. No. 3,773,919; and U.S. Pat. No. 3,270,960.

[00404] Further embodiments of the formulations and modes of administration of the compositions comprising the one or more modulating agents (*i.e.*, inhibitors and/or activators), or combinations thereof described herein, that can be used in the methods described herein are described below.

[00405] Parenteral Dosage Forms. Parenteral dosage forms of the one or more modulating agents (*i.e.*, inhibitors or activators), or combinations thereof, can also be administered to a subject with a chronic immune condition by various routes, including, but not limited to, subcutaneous, intravenous (including bolus injection), intramuscular, and intraarterial. Since administration of parenteral dosage forms typically bypasses the patient's natural defenses against contaminants, parenteral dosage forms are preferably sterile or capable of being sterilized prior to administration to a patient. Examples of parenteral dosage forms include, but are not limited to, solutions ready for injection, dry products ready to be dissolved or suspended in a pharmaceutically acceptable vehicle for injection, suspensions ready for injection, controlled-release parenteral dosage forms, and emulsions.

[00406] Suitable vehicles that can be used to provide parenteral dosage forms of the disclosure are well known to those skilled in the art. Examples include, without limitation: sterile water; water for injection USP; saline solution; glucose solution; aqueous vehicles such as but not limited to, sodium chloride injection, Ringer's injection, dextrose injection, dextrose and sodium chloride injection, and lactated Ringer's injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and propylene glycol; and non-aqueous vehicles such as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

[00407] Aerosol formulations. The one or more modulating agents (*i.e.*, inhibitor or activator) described herein or combinations thereof can be packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutane with conventional adjuvants. An IL-27 or NFIL-3 modulator (*i.e.*, inhibitor or activator), or combinations thereof described herein, can also be administered in a non-pressurized form such as in a nebulizer or atomizer. The one or more modulating agents (*i.e.*, inhibitor and/or activator), or combinations thereof described herein, can also be administered directly to the airways in the form of a dry powder, for example, by use of an inhaler.

[00408] Suitable powder compositions include, by way of illustration, powdered preparations of the one or more modulating agents (*i.e.*, inhibitor and/or activator), or combinations thereof described herein, thoroughly intermixed with lactose, or other inert powders acceptable for intrabronchial administration. The powder compositions can be administered via an aerosol dispenser or encased in a breakable capsule which can be inserted by the subject into a device that punctures the capsule and blows the powder out in a steady stream suitable for inhalation. The compositions can include propellants, surfactants, and co-solvents and can be filled into conventional aerosol containers that are closed by a suitable metering valve.

[00409] Aerosols for the delivery to the respiratory tract are known in the art. See for example, Adjei, A. and Garren, J. *Pharm. Res.*, 1: 565-569 (1990); Zanen, P. and Lamm, J.-W. J. *Int. J. Pharm.*, 114: 111-115 (1995); Gonda, I. "Aerosols for delivery of therapeutic and diagnostic agents to the respiratory tract," in *Critical Reviews in Therapeutic Drug Carrier Systems*, 6:273-313 (1990); Anderson *et al.*, *Am. Rev. Respir. Dis.*, 140: 1317-1324 (1989)) and have potential for the systemic delivery of peptides and proteins as well (Patton and Platz, *Advanced Drug Delivery Reviews*, 8:179-196 (1992)); Timsina *et. al*, *Int. J. Pharm.*, 101: 1-13 (1995); and Tansey, I. P., *Spray Technol. Market*, 4:26-29 (1994); French, D. L.,

Edwards, D. A. and Niven, R. W., *Aerosol Sci.*, 27: 769-783 (1996); Visser, I, *Powder Technology* 58: 1-10 (1989)); Rudt, S. and R. H. Muller, *J. Controlled Release*, 22: 263-272 (1992); Tabata, Y, and Y. Ikada, *Biomed. Mater. Res.*, 22: 837-858 (1988); Wall, D. A., *Drug Delivery*, 2: 10 1-20 1995); Patton, J. and Platz, R, *Adv. Drug Del. Rev.*, 8: 179-196 (1992); Bryon, P., *Adv. Drug. Del. Rev.*, 5: 107-132 (1990); Patton, J. S., *et al.*, *Controlled Release*, 28: 15 79-85 (1994); Damms, B. and Bains, W., *Nature Biotechnology* (1996); Niven, R. W., *et al.*, *Pharm. Res.*, 12(9); 1343-1349 (1995); and Kobayashi, S., *et al.*, *Pharm. Res.*, 13(1): 80-83 (1996), contents of all of which are herein incorporated by reference in their entirety.

[00410] The formulations of the one or more modulating agents (*i.e.*, inhibitors and/or activators), or combinations thereof described herein, further encompass anhydrous pharmaceutical compositions and dosage forms comprising the disclosed compounds as active ingredients, since water can facilitate the degradation of some compounds. For example, the addition of water (*e.g.*, 5%) is widely accepted in the pharmaceutical arts as a means of simulating long-term storage in order to determine characteristics such as shelf life or the stability of formulations over time. See, *e.g.*, Jens T. Carstensen, *Drug Stability: Principles & Practice*, 379-80 (2nd ed., Marcel Dekker, NY, N.Y.: 1995). Anhydrous pharmaceutical compositions and dosage forms of the disclosure can be prepared using anhydrous or low moisture containing ingredients and low moisture or low humidity conditions. Pharmaceutical compositions and dosage forms that comprise lactose and at least one active ingredient that comprises a primary or secondary amine are preferably anhydrous if substantial contact with moisture and/or humidity during manufacturing, packaging, and/or storage is expected. Anhydrous compositions are preferably packaged using materials known to prevent exposure to water such that they can be included in suitable formulary kits. Examples of suitable packaging include, but are not limited to, hermetically sealed foils, plastics, unit dose containers (*e.g.*, vials) with or without desiccants, blister packs, and strip packs.

[00411] Controlled and Delayed Release Dosage Forms. In some embodiments of the aspects described herein, the one or more modulating agents (*i.e.*, inhibitor and/or activator), or combinations thereof described herein, can be administered to a subject by controlled- or delayed-release means. Ideally, the use of an optimally designed controlled-release preparation in medical treatment is characterized by a minimum of drug substance being employed to cure or control the condition in a minimum amount of time. Advantages of controlled-release formulations include: 1) extended activity of the drug; 2) reduced dosage frequency; 3) increased patient compliance; 4) usage of less total drug; 5) reduction in local

or systemic side effects; 6) minimization of drug accumulation; 7) reduction in blood level fluctuations; 8) improvement in efficacy of treatment; 9) reduction of potentiation or loss of drug activity; and 10) improvement in speed of control of diseases or conditions. (Kim, Cherng-ju, *Controlled Release Dosage Form Design, 2* (Technomic Publishing, Lancaster, Pa.: 2000)). Controlled-release formulations can be used to control a compound of formula (I)'s onset of action, duration of action, plasma levels within the therapeutic window, and peak blood levels. In particular, controlled- or extended-release dosage forms or formulations can be used to ensure that the maximum effectiveness of a compound of formula (I) is achieved while minimizing potential adverse effects and safety concerns, which can occur both from under-dosing a drug (*i.e.*, going below the minimum therapeutic levels) as well as exceeding the toxicity level for the drug.

[00412] A variety of known controlled- or extended-release dosage forms, formulations, and devices can be adapted for use with the one or more modulating agents (*i.e.*, inhibitors or activators), or combinations thereof described herein. Examples include, but are not limited to, those described in U.S. Pat. Nos.: 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5,674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; 5,733,566; and 6,365,185 B1, each of which is incorporated herein by reference in their entireties. These dosage forms can be used to provide slow or controlled-release of one or more active ingredients using, for example, hydroxypropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems (such as OROS® (Alza Corporation, Mountain View, Calif. USA)), multilayer coatings, microparticles, liposomes, or microspheres or a combination thereof to provide the desired release profile in varying proportions. Additionally, ion exchange materials can be used to prepare immobilized, adsorbed salt forms of the disclosed compounds and thus effect controlled delivery of the drug. Examples of specific anion exchangers include, but are not limited to, DUOLITE® A568 and DUOLITE® AP143 (Rohm&Haas, Spring House, Pa. USA).

[00413] In some embodiments of the methods described herein, the one or more modulating agents (*i.e.*, inhibitors and/or activators), or combinations thereof described herein, for use in the methods described herein is administered to a subject by sustained release or in pulses. Pulse therapy is not a form of discontinuous administration of the same amount of a composition over time, but comprises administration of the same dose of the composition at a reduced frequency or administration of reduced doses. Sustained release or pulse administrations are particularly preferred when the disorder occurs continuously in the subject, for example where the subject has continuous or chronic symptoms of a viral

infection. Each pulse dose can be reduced and the total amount of the one or more modulating agents (*i.e.*, inhibitor or activator), or combinations thereof described herein, administered over the course of treatment to the subject or patient is minimized.

[00414] The interval between pulses, when necessary, can be determined by one of ordinary skill in the art. Often, the interval between pulses can be calculated by administering another dose of the composition when the composition or the active component of the composition is no longer detectable in the subject prior to delivery of the next pulse. Intervals can also be calculated from the *in vivo* half-life of the composition. Intervals can be calculated as greater than the *in vivo* half-life, or 2, 3, 4, 5 and even 10 times greater the composition half-life. Various methods and apparatus for pulsing compositions by infusion or other forms of delivery to the patient are disclosed in U.S. Pat. Nos. 4,747,825; 4,723,958; 4,948,592; 4,965,251 and 5,403,590.

[00415] In one embodiment, RNA interfering agents used in the methods described herein are taken up actively by cells *in vivo* following intravenous injection, e.g., hydrodynamic injection, without the use of a vector, illustrating efficient *in vivo* delivery of the RNA interfering agents, e.g., the siRNAs used in the methods of the invention. Exemplary delivery methods for RNA interfering agents may also be used to deliver any of CRISPR/Cas system, Zinc finger, or TALE.

[00416] Other strategies for delivery of the RNA interfering agents, e.g., the siRNAs or shRNAs, used in the methods of the invention, can also be employed, such as, for example, delivery by a vector, e.g., a plasmid or viral vector, e.g., a lentiviral vector. Such vectors can be used as described, for example, in Xiao-Feng Qin et al. Proc. Natl. Acad. Sci. U.S.A., 100: 183- 188. Other delivery methods include delivery of the RNA interfering agents, e.g., the siRNAs or shRNAs of the invention, using a basic peptide by conjugating or mixing the RNA interfering agent with a basic peptide, e.g., a fragment of a TAT peptide, mixing with cationic lipids or formulating into particles.

[00417] As noted, the dsRNA, such as siRNA or shRNA can be delivered using an inducible vector, such as a tetracycline inducible vector. Methods described, for example, in Wang et al. Proc. Natl. Acad. Sci. 100: 5103-5106, using pTet-On vectors (BD Biosciences Clontech, Palo Alto, CA) can be used. In some embodiments, a vector can be a plasmid vector, a viral vector, or any other suitable vehicle adapted for the insertion and foreign sequence and for the introduction into eukaryotic cells. The vector can be an expression vector capable of directing the transcription of the DNA sequence of the agonist or antagonist nucleic acid molecules into RNA. Viral expression vectors can be selected from a group

comprising, for example, reteroviruses, lentiviruses, Epstein Barr virus-, bovine papilloma virus, adenovirus- and adeno-associated-based vectors or hybrid virus of any of the above. In one embodiment, the vector is episomal. The use of a suitable episomal vector provides a means of maintaining the antagonist nucleic acid molecule in the subject in high copy number extra chromosomal DNA thereby eliminating potential effects of chromosomal integration.

[00418] Methods of delivering RNAi agents, e.g., an siRNA, or vectors containing an RNAi agent, to the target cells (e.g., basal cells or cells of the lung and/or respiratory system or other desired target cells) are well known to persons of ordinary skill in the art. In some embodiments, a RNAi agent can be administered to a subject via aerosol means, for example using a nebulizer and the like. In alternative embodiments, administration of a RNAi agent (e.g. can include, for example (i) injection of a composition containing the RNA interfering agent, e.g., an siRNA, or (ii) directly contacting the cell, e.g., a cell of the respiratory system, with a composition comprising an RNAi agent, e.g., an siRNA. In another embodiment, RNAi agents, e.g., an siRNA can be injected directly into any blood vessel, such as vein, artery, venule or arteriole, via, e.g., hydrodynamic injection or catheterization. In some embodiments an RNAi inhibitor can delivered to specific organs, for example the liver, bone marrow or systemic administration. Administration can be by a single injection or by two or more injections.

[00419] In some embodiments, a RNAi agent is delivered in a pharmaceutically acceptable carrier. One or more RNAi agents can be used simultaneously, e.g. one or more gene silencing RNAi agent inhibitors of target gene(s) can be together. The RNA interfering agents, can be delivered singly, or in combination with other RNA interfering agents, e.g., siRNAs, such as, for example siRNAs directed to other cellular genes. A gene silencing-RNAi agent inhibitor of target gene(s) can also be administered in combination with other pharmaceutical agents which are used to treat or prevent diseases or disorders.

[00420] In one embodiment, specific cells are targeted with RNA interference, limiting potential side effects of RNA interference caused by non-specific targeting of RNA interference. The method can use, for example, a complex or a fusion molecule comprising a cell targeting moiety and an RNA interference binding moiety that is used to deliver RNAi effectively into cells. For example, an antibody -protamine fusion protein when mixed with an siRNA, binds siRNA and selectively delivers the siRNA into cells expressing an antigen recognized by the antibody, resulting in silencing of gene expression only in those cells that express the antigen which is identified by the antibody. In some embodiments, the antibody can be any antibody which identifies an antigen expressed on cells expressing the target gene

or gene product. In some embodiments, the antibody is an antibody which binds to the target gene product antigen, but where the antibody can or does not inhibit the target gene product function. In some embodiments, the siRNA can be conjugated to an antagonist of the target gene product, for example where the antagonist is a polypeptide, and where the conjugation with the RNAi does not interrupt the function of the antagonist.

[00421] In some embodiments, a siRNA or RNAi binding moiety is a protein or a nucleic acid binding domain or fragment of a protein, and the binding moiety is fused to a portion of the targeting moiety. The location of the targeting moiety can be either in the carboxyl-terminal or amino-terminal end of the construct or in the middle of the fusion protein.

[00422] In some embodiments, a viral-mediated delivery mechanism can also be employed to deliver siRNAs to cells *in vitro* and *in vivo* as described in Xia, H. et al. (2002) *Nat Biotechnol* 20(10): 1006). Plasmid- or viral- mediated delivery mechanisms of shRNA can also be employed to deliver shRNAs to cells *in vitro* and *in vivo* as described in Rubinson, D.A., et al. ((2003) *Nat. Genet.* 33 :401 -406) and Stewart, S.A., et al. ((2003) *RNA* 9:493-501). Alternatively, in other embodiments, a RNAi agent, e.g., a gene silencing- RNAi agent inhibitor of a target gene can also be introduced into cells via the vascular or extravascular circulation, the blood or lymph system, and the cerebrospinal fluid.

[00423] In general, any method of delivering a nucleic acid molecule can be adapted for use with an RNAi interference molecule (see e.g., Akhtar S. and Julian RL. (1992) *Trends Cell. Biol.* 2(5): 139- 144; WO94/02595, which are incorporated herein by reference in their entirety). However, there are three factors that are important to consider in order to successfully deliver an RNAi molecule *in vivo*: (a) biological stability of the RNAi molecule, (2) preventing non-specific effects, and (3) accumulation of the RNAi molecule in the target tissue. The non-specific effects of an RNAi molecule can be minimized by local administration by e.g., direct injection into a tissue including, for example, a tumor or topically administering the molecule.

[00424] Local administration of an RNAi molecule to a treatment site limits the exposure of the e.g., siRNA to systemic tissues and permits a lower dose of the RNAi molecule to be administered. Several studies have shown successful knockdown of gene products when an RNAi molecule is administered locally. For example, intraocular delivery of a VEGF siRNA by intravitreal injection in cynomolgus monkeys (Tolentino, MJ., et al (2004) *Retina* 24: 132-138) and subretinal injections in mice (Reich, SI, et al (2003) *Mol. Vis.* 9:210-216) were both shown to prevent neovascularization in an experimental model of age-related macular degeneration. In addition, direct intratumoral injection of an siRNA in mice reduces tumor

volume (Pille, J., et al (2005) *Mol. Ther.* 1 :267-274) and can prolong survival of tumor-bearing mice (Kim, WJ., et al (2006) *Mol. Ther.* 14:343-350; Li, S., et al (2007) *Mol. Ther.* 15:515-523). RNA interference has also shown success with local delivery to the CNS by direct injection (Dom, G., et al (2004) *Nucleic Acids* 32:e49; Tan, PH., et al (2005) *Gene Ther.* 12:59-66; Makimura, H., et al (2002) *BMC Neurosci.* 3 : 18; Shishkina, GT., et al (2004) *Neuroscience* 129:521 -528; Thakker, ER., et al (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101 : 17270- 17275; Akaneya, Y., et al (2005) *J. Neurophysiol.* 93 :594-602) and to the lungs by intranasal administration (Howard, KA., et al (2006) *Mol. Ther.* 14:476-484; Zhang, X., et al (2004) *J. Biol. Chem.* 279: 10677- 10684; Bitko, V., et al (2005) *Nat. Med.* 11 :50-55).

[00425] For administering an RNAi molecule systemically for the treatment of a disease, the RNAi molecule can be either be modified or alternatively delivered using a drug delivery system; both methods act to prevent the rapid degradation of the RNAi molecule by endo- and exo-nucleases in vivo. Modification of the RNAi molecule or the pharmaceutical carrier can also permit targeting of the RNAi molecule to the target tissue and avoid undesirable off-target effects.

[00426] RNA interference molecules can be modified by chemical conjugation to lipophilic groups such as cholesterol to enhance cellular uptake and prevent degradation. For example, an siRNA directed against ApoB conjugated to a lipophilic cholesterol moiety was injected systemically into mice and resulted in knockdown of apoB mRNA in both the liver and jejunum (Soutschek, I , et al (2004) *Nature* 432: 173- 178). Conjugation of an RNAi molecule to an aptamer has been shown to inhibit tumor growth and mediate tumor regression in a mouse model of prostate cancer (McNamara, JO., et al (2006) *Nat. Biotechnol.* 24: 1005- 1015).

[00427] In an alternative embodiment, the RNAi molecules can be delivered using drug delivery systems such as e.g., a nanoparticle, a dendrimer, a polymer, liposomes, or a cationic delivery system. Positively charged cationic delivery systems facilitate binding of an RNA interference molecule (negatively charged) and also enhance interactions at the negatively charged cell membrane to permit efficient uptake of an siRNA by the cell. Cationic lipids, dendrimers, or polymers can either be bound to an RNA interference molecule, or induced to form a vesicle or micelle (see e.g., Kim SH., et al (2008) *Journal of Controlled Release* 129(2): 107- 116) that encases an RNAi molecule. The formation of vesicles or micelles further prevents degradation of the RNAi molecule when administered systemically. Methods for making and administering cationic-RNAi complexes are well within the abilities of one skilled in the art (see e.g., Sorensen, DR., et al (2003) *J. Mol. Biol* 327:761 -766; Verma,

UN., et al (2003) Clin. Cancer Res. 9: 1291- 1300; Arnold, AS et al (2007) J. Hypertens. 25: 197-205, which are incorporated herein by reference in their entirety).

[00428] Some non-limiting examples of drug delivery systems useful for systemic administration of RNAi include DOTAP (Sorensen, DR., et al (2003), supra; Verma, UN., et al (2003), supra), Oligofectamine, "solid nucleic acid lipid particles" (Zimmermann, TS., et al (2006) Nature 441 : 1 1 1 - 1 14), cardiolipin (Chien, PY., et al (2005) Cancer Gene Ther. 12:321 -328; Pal, A., et al (2005) Int J. Oncol. 26: 1087- 1091), polyethyleneimine (Bonnet ME., et al (2008) Pharm. Res. Aug 16 Epub ahead of print; Aigner, A. (2006) J. Biomed. Biotechnol. 71659), Arg-Gly-Asp (RGD) peptides (Liu, S. (2006) Mol. Pharm. 3 :472-487), and polyamidoamines (Tomalia, DA., et al (2007) Biochem. Soc. Trans. 35:61 -67; Yoo, H., et al (1999) Pharm. Res. 16: 1799- 1804). In some embodiments, an RNAi molecule forms a complex with cyclodextrin for systemic administration. Methods for administration and pharmaceutical compositions of RNAi molecules and cyclodextrins can be found in U.S. Patent No. 7, 427, 605, which is herein incorporated by reference in its entirety. Specific methods for administering an RNAi molecule for the inhibition of angiogenesis can be found in e.g., U.S. Patent Application No. 20080152654, which is herein incorporated by reference in its entirety.

[00429] In some embodiments, the siRNA, dsRNA, or shRNA vector can be administered systemically, such as intravenously, e. g. via central venous catheter (CVC or central venous line or central venous access catheter) placed into a large vein in the neck (internal jugular vein), chest (subclavian vein) or groin (femoral vein). Methods of systemic delivery of siRNA, dsRNA, or shRNA vector are well known in the art, e. g. as described herein and in Gao and Huang, 2008, (Mol. Pharmaceutics, Web publication December 30) and review by Rossi, 2006, Gene Therapy, 13 :583- 584. The siRNA, dsRNA, or shRNA vector can be formulated in various ways, e. g. conjugation of a cholesterol moiety to one of the strands of the siRNA duplex for systemic delivery to the liver and jejunum (Soutschek J. et. al. 2004, Nature, 432: 173-178), complexing of siRNAs to protamine fused with an antibody fragment for receptor-mediated targeting of siRNAs (Song E, et al. 2005, Nat Biotechnol, 23 : 709-717) and the use of a lipid bilayer system by Morrissey et al. 2005 (Nat Biotechnol, , 23 : 1002-1007). The lipid bilayer system produces biopolymers that are in the 120 nanometer diameter size range, and are labeled as SNALPs, for Stable-Nucleic- Acid-Lipid-Particles. The lipid combination protects the siRNAs from serum nucleases and allows cellular endosomal uptake and subsequent cytoplasmic release of the siRNAs (see WO/2006/007712). These references are incorporated by reference in their entirety.

[00430] The dose of the particular RNAi agent will be in an amount necessary to effect RNA interference, e.g., gene silencing of the target gene, thereby leading to a subsequent decrease in the target protein level.

[00431] In another embodiment of the invention, agents which are inhibitors of the target gene or protein are catalytic nucleic acid constructs, such as, for example ribozymes, which are capable of cleaving RNA transcripts and thereby preventing the production of wildtype protein. Ribozymes are targeted to and anneal with a particular sequence by virtue of two regions of sequence complementary to the target flanking the ribozyme catalytic site. After binding, the ribozyme cleaves the target in a site specific manner. The design and testing of ribozymes which specifically recognize and cleave sequences of the gene products described herein can be achieved by techniques well known to those skilled in the art (for example Lleber and Strauss, (1995) Mol Cell Biol 15:540.551, the disclosure of which is incorporated herein by reference).

[00432] The term "vectors" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked; a plasmid is a species of the genus encompassed by "vector". The term "vector" typically refers to a nucleic acid sequence containing an origin of replication and other entities necessary for replication and/or maintenance in a host cell. Vectors capable of directing the expression of genes and/or nucleic acid sequence to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome, and typically comprise entities for stable or transient expression or the encoded DNA. Other expression vectors can be used in the methods as disclosed herein for example, but are not limited to, plasmids, episomes, bacterial artificial chromosomes, yeast artificial chromosomes, bacteriophages or viral vectors, and such vectors can integrate into the host's genome or replicate autonomously in the particular cell. A vector can be a DNA or RNA vector. Other forms of expression vectors known by those skilled in the art which serve the equivalent functions can also be used, for example self replicating extrachromosomal vectors or vectors which integrates into a host genome. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors".

[00433] The term "viral vectors" refers to the use as viruses, or virus-associated vectors as carriers of the nucleic acid construct into the cell. Constructs may be integrated and packaged

into non-replicating, defective viral genomes like Adenovirus, Adeno-associated virus (AAV), or Herpes simplex virus (HSV) or others, including retroviral and lentiviral vectors, for infection or transduction into cells. The vector may or may not be incorporated into the cell's genome. The constructs may include viral sequences for transfection, if desired. Alternatively, the construct may be incorporated into vectors capable of episomal replication, e.g. EPV and EBV vectors.

[00434] As used herein, a "promoter" or "promoter region" or "promoter element" used interchangeably herein, refers to a segment of a nucleic acid sequence, typically but not limited to DNA or RNA or analogues thereof, that controls the transcription of the nucleic acid sequence to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences which modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be cis-acting or may be responsive to trans-acting factors. Promoters, depending upon the nature of the regulation may be constitutive or regulated.

[00435] The term "regulatory sequences" is used interchangeably with "regulatory elements" herein refers to a segment of nucleic acid, typically but not limited to DNA or RNA or analogues thereof, that modulates the transcription of the nucleic acid sequence to which it is operatively linked, and thus act as transcriptional modulators. Regulatory sequences modulate the expression of gene and/or nucleic acid sequence to which they are operatively linked. Regulatory sequences often comprise "regulatory elements" which are nucleic acid sequences that are transcription binding domains and are recognized by the nucleic acid-binding domains of transcriptional proteins and/or transcription factors, repressors or enhancers etc. Typical regulatory sequences include, but are not limited to, transcriptional promoters, inducible promoters and transcriptional elements, an operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences to control the termination of transcription and/or translation. Regulatory sequences can be a single regulatory sequence or multiple regulatory sequences, or modified regulatory sequences or fragments thereof. Modified regulatory sequences are regulatory sequences where the nucleic acid sequence has been changed or modified by some means, for example, but not limited to, mutation, methylation etc.

[00436] The term "operatively linked" as used herein refers to the functional relationship of the nucleic acid sequences with regulatory sequences of nucleotides, such as promoters,

enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of nucleic acid sequences, typically DNA, to a regulatory sequence or promoter region refers to the physical and functional relationship between the DNA and the regulatory sequence or promoter such that the transcription of such DNA is initiated from the regulatory sequence or promoter, by an RNA polymerase that specifically recognizes, binds and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to modify the regulatory sequence for the expression of the nucleic acid or DNA in the cell type for which it is expressed. The desirability of, or need of, such modification may be empirically determined. Enhancers need not be located in close proximity to the coding sequences whose transcription they enhance. Furthermore, a gene transcribed from a promoter regulated in trans by a factor transcribed by a second promoter may be said to be operatively linked to the second promoter. In such a case, transcription of the first gene is said to be operatively linked to the first promoter and is also said to be operatively linked to the second promoter.

[00437] Hence, in certain embodiments the invention involves vectors, e.g. for delivering or introducing in a cell the DNA targeting agent according to the invention as described herein, such as by means of example Cas and/or RNA capable of guiding Cas to a target locus (i.e. guide RNA), but also for propagating these components (e.g. in prokaryotic cells). As used herein, a "vector" is a tool that allows or facilitates the transfer of an entity from one environment to another. It is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Generally, a vector is capable of replication when associated with the proper control elements. In general, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses (AAVs)). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable

of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as "expression vectors." Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

[00438] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (e.g. in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). With regards to recombination and cloning methods, mention is made of U.S. patent application 10/815,730, published September 2, 2004 as US 2004-0171156 A1, the contents of which are herein incorporated by reference in their entirety.

[00439] The vector(s) can include the regulatory element(s), e.g., promoter(s). The vector(s) can comprise Cas encoding sequences, and/or a single, but possibly also can comprise at least 3 or 8 or 16 or 32 or 48 or 50 guide RNA(s) (e.g., sgRNAs) encoding sequences, such as 1-2, 1-3, 1-4 1-5, 3-6, 3-7, 3-8, 3-9, 3-10, 3-8, 3-16, 3-30, 3-32, 3-48, 3-50 RNA(s) (e.g., sgRNAs). In a single vector there can be a promoter for each RNA (e.g., sgRNA), advantageously when there are up to about 16 RNA(s) (e.g., sgRNAs); and, when a single vector provides for more than 16 RNA(s) (e.g., sgRNAs), one or more promoter(s) can drive expression of more than one of the RNA(s) (e.g., sgRNAs), e.g., when there are 32 RNA(s) (e.g., sgRNAs), each promoter can drive expression of two RNA(s) (e.g., sgRNAs), and when there are 48 RNA(s) (e.g., sgRNAs), each promoter can drive expression of three RNA(s) (e.g., sgRNAs). By simple arithmetic and well established cloning protocols and the teachings in this disclosure one skilled in the art can readily practice the invention as to the RNA(s) (e.g., sgRNA(s) for a suitable exemplary vector such as AAV, and a suitable promoter such as the U6 promoter, e.g., U6-sgRNAs. For example, the packaging limit of AAV is -4.7 kb. The length of a single U6-sgRNA (plus restriction sites for cloning) is 361

bp. Therefore, the skilled person can readily fit about 12-16, e.g., 13 U6-sgRNA cassettes in a single vector. This can be assembled by any suitable means, such as a golden gate strategy used for TALE assembly (<http://www.genome-engineering.org/taleffectors/>). The skilled person can also use a tandem guide strategy to increase the number of U6-sgRNAs by approximately 1.5 times, e.g., to increase from 12-16, e.g., 13 to approximately 18-24, e.g., about 19 U6-sgRNAs. Therefore, one skilled in the art can readily reach approximately 18-24, e.g., about 19 promoter-RNAs, e.g., U6-sgRNAs in a single vector, e.g., an AAV vector. A further means for increasing the number of promoters and RNAs, e.g., sgRNA(s) in a vector is to use a single promoter (e.g., U6) to express an array of RNAs, e.g., sgRNAs separated by cleavable sequences. And an even further means for increasing the number of promoter-RNAs, e.g., sgRNAs in a vector, is to express an array of promoter-RNAs, e.g., sgRNAs separated by cleavable sequences in the intron of a coding sequence or gene; and, in this instance it is advantageous to use a polymerase II promoter, which can have increased expression and enable the transcription of long RNA in a tissue specific manner. (see, e.g., nar.oxfordjournals.org/content/34/7/e53. short, www.nature.com/mt/journal/v16/n9/abs/mt2008144a.html). In an advantageous embodiment, AAV may package U6 tandem sgRNA targeting up to about 50 genes. Accordingly, from the knowledge in the art and the teachings in this disclosure the skilled person can readily make and use vector(s), e.g., a single vector, expressing multiple RNAs or guides or sgRNAs under the control or operatively or functionally linked to one or more promoters-especially as to the numbers of RNAs or guides or sgRNAs discussed herein, without any undue experimentation.

[00440] A poly nucleic acid sequence encoding the DNA targeting agent according to the invention as described herein, such as by means of example guide RNA(s), e.g., sgRNA(s) encoding sequences and/or Cas encoding sequences, can be functionally or operatively linked to regulatory element(s) and hence the regulatory element(s) drive expression. The promoter(s) can be constitutive promoter(s) and/or conditional promoter(s) and/or inducible promoter(s) and/or tissue specific promoter(s). The promoter can be selected from the group consisting of RNA polymerases, pol I, pol II, pol III, T7, U6, HI, retroviral Rous sarcoma virus (RSV) LTR promoter, the cytomegalovirus (CMV) promoter, the SV40 promoter, the dihydrofolate reductase promoter, the β -actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 α promoter. An advantageous promoter is the promoter is U6.

[00441] Through this disclosure and the knowledge in the art, the DNA targeting agent as described herein, such as, TALEs, CRISPR-Cas systems, etc., or components thereof or nucleic acid molecules thereof (including, for instance HDR template) or nucleic acid

molecules encoding or providing components thereof may be delivered by a delivery system herein described both generally and in detail.

[00442] Vector delivery, e.g., plasmid, viral delivery: By means of example, the CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can be delivered using any suitable vector, e.g., plasmid or viral vectors, such as adeno associated virus (AAV), lentivirus, adenovirus or other viral vector types, or combinations thereof. The DNA targeting agent as described herein, such as Cas9 and one or more guide RNAs can be packaged into one or more vectors, e.g., plasmid or viral vectors. In some embodiments, the vector, e.g., plasmid or viral vector is delivered to the tissue of interest by, for example, an intramuscular injection, while other times the delivery is via intravenous, transdermal, intranasal, oral, mucosal, or other delivery methods. Such delivery may be either via a single dose, or multiple doses. One skilled in the art understands that the actual dosage to be delivered herein may vary greatly depending upon a variety of factors, such as the vector choice, the target cell, organism, or tissue, the general condition of the subject to be treated, the degree of transformation/modification sought, the administration route, the administration mode, the type of transformation/modification sought, etc.

[00443] Such a dosage may further contain, for example, a carrier (water, saline, ethanol, glycerol, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, etc.), a diluent, a pharmaceutically-acceptable carrier (e.g., phosphate-buffered saline), a pharmaceutically-acceptable excipient, and/or other compounds known in the art. The dosage may further contain one or more pharmaceutically acceptable salts such as, for example, a mineral acid salt such as a hydrochloride, a hydrobromide, a phosphate, a sulfate, etc.; and the salts of organic acids such as acetates, propionates, malonates, benzoates, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, gels or gelling materials, flavorings, colorants, microspheres, polymers, suspension agents, etc. may also be present herein. In addition, one or more other conventional pharmaceutical ingredients, such as preservatives, humectants, suspending agents, surfactants, antioxidants, anticaking agents, fillers, chelating agents, coating agents, chemical stabilizers, etc. may also be present, especially if the dosage form is a reconstitutable form. Suitable exemplary ingredients include microcrystalline cellulose, carboxymethylcellulose sodium, polysorbate 80, phenylethyl alcohol, chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, parachlorophenol, gelatin, albumin and a combination thereof. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S

PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991) which is incorporated by reference herein.

[00444] In an embodiment herein the delivery is via an adenovirus, which may be at a single booster dose containing at least 1×10^5 particles (also referred to as particle units, pu) of adenoviral vector. In an embodiment herein, the dose preferably is at least about 1×10^6 particles (for example, about 1×10^6 - 1×10^{12} particles), more preferably at least about 1×10^7 particles, more preferably at least about 1×10^8 particles (e.g., about 1×10^8 - 1×10^{11} particles or about 1×10^8 - 1×10^{12} particles), and most preferably at least about 1×10^9 particles (e.g., about 1×10^9 - 1×10^{10} particles or about 1×10^9 - 1×10^{12} particles), or even at least about 1×10^{10} particles (e.g., about 1×10^{10} - 1×10^{12} particles) of the adenoviral vector. Alternatively, the dose comprises no more than about 1×10^{14} particles, preferably no more than about 1×10^{13} particles, even more preferably no more than about 1×10^{12} particles, even more preferably no more than about 1×10^{11} particles, and most preferably no more than about 1×10^{10} particles (e.g., no more than about 1×10^9 articles). Thus, the dose may contain a single dose of adenoviral vector with, for example, about 1×10^6 particle units (pu), about 2×10^6 pu, about 4×10^6 pu, about 1×10^7 pu, about 2×10^7 pu, about 4×10^7 pu, about 1×10^8 pu, about 2×10^8 pu, about 4×10^8 pu, about 1×10^9 pu, about 2×10^9 pu, about 4×10^9 pu, about 1×10^{10} pu, about 2×10^{10} pu, about 4×10^{10} pu, about 1×10^{11} pu, about 2×10^{11} pu, about 4×10^{11} pu, about 1×10^{12} pu, about 2×10^{12} pu, or about 4×10^{12} pu of adenoviral vector. See, for example, the adenoviral vectors in U.S. Patent No. 8,454,972 B2 to Nabel, et. al, granted on June 4, 2013; incorporated by reference herein, and the dosages at col 29, lines 36-58 thereof. In an embodiment herein, the adenovirus is delivered via multiple doses.

[00445] In an embodiment herein, the delivery is via an AAV. A therapeutically effective dosage for in vivo delivery of the AAV to a human is believed to be in the range of from about 20 to about 50 ml of saline solution containing from about 1×10^{10} to about 1×10^{10} functional AAV/ml solution. The dosage may be adjusted to balance the therapeutic benefit against any side effects. In an embodiment herein, the AAV dose is generally in the range of concentrations of from about 1×10^5 to 1×10^{50} genomes AAV, from about 1×10^8 to 1×10^{20} genomes AAV, from about 1×10^{10} to about 1×10^{16} genomes, or about 1×10^{11} to about 1×10^{16} genomes AAV. A human dosage may be about 1×10^{13} genomes AAV. Such concentrations may be delivered in from about 0.001 ml to about 100 ml, about 0.05 to about 50 ml, or about 10 to about 25 ml of a carrier solution. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response

curves. See, for example, U.S. Patent No. 8,404,658 B2 to Hajjar, et al, granted on March 26, 2013, at col. 27, lines 45-60.

[00446] In an embodiment herein the delivery is via a plasmid. In such plasmid compositions, the dosage should be a sufficient amount of plasmid to elicit a response. For instance, suitable quantities of plasmid DNA in plasmid compositions can be from about 0.1 to about 2 mg, or from about 1 μ g to about 10 μ g per 70 kg individual. Plasmids of the invention will generally comprise (i) a promoter; (ii) a sequence encoding a DNA targeting agent as described herein, such as a comprising a CRISPR enzyme, operably linked to said promoter; (iii) a selectable marker; (iv) an origin of replication; and (v) a transcription terminator downstream of and operably linked to (ii). The plasmid can also encode the RNA components of a CRISPR complex, but one or more of these may instead be encoded on a different vector.

[00447] The doses herein are based on an average 70 kg individual. The frequency of administration is within the ambit of the medical or veterinary practitioner (e.g., physician, veterinarian), or scientist skilled in the art. It is also noted that mice used in experiments are typically about 20g and from mice experiments one can scale up to a 70 kg individual.

[00448] In some embodiments the RNA molecules of the invention are delivered in liposome or lipofectin formulations and the like and can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Pat. Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference. Delivery systems aimed specifically at the enhanced and improved delivery of siRNA into mammalian cells have been developed, (see, for example, Shen et al FEBS Let. 2003, 539: 111-114; Xia et al, Nat. Biotech. 2002, 20:1006-1010; Reich et al, Mol. Vision. 2003, 9: 210-216; Sorensen et al, J. Mol. Biol. 2003, 327: 761-766; Lewis et al, Nat. Gen. 2002, 32: 107-108 and Simeoni et al, NAR 2003, 31, 11: 2717-2724) and may be applied to the present invention. siRNA has recently been successfully used for inhibition of gene expression in primates (see for example. Tolentino et al., Retina 24(4):660 which may also be applied to the present invention.

[00449] Indeed, RNA delivery is a useful method of *in vivo* delivery. It is possible to deliver the DNA targeting agent as described herein, such as Cas9 and gRNA (and, for instance, HR repair template) into cells using liposomes or particles. Thus delivery of the CRISPR enzyme, such as a Cas9 and/or delivery of the RNAs of the invention may be in RNA form and via microvesicles, liposomes or particles. For example, Cas9 mRNA and gRNA can be packaged into liposomal particles for delivery *in vivo*. Liposomal transfection

reagents such as lipofectamine from Life Technologies and other reagents on the market can effectively deliver RNA molecules into the liver.

[00450] Means of delivery of RNA also preferred include delivery of RNA via nanoparticles (Cho, S., Goldberg, M., Son, S., Xu, Q., Yang, F., Mei, Y., Bogatyrev, S., Langer, R. and Anderson, D., Lipid-like nanoparticles for small interfering RNA delivery to endothelial cells, *Advanced Functional Materials*, 19: 3112-3118, 2010) or exosomes (Schroeder, A., Levins, C, Cortez, C, Langer, R., and Anderson, D., Lipid-based nanotherapeutics for siRNA delivery, *Journal of Internal Medicine*, 267: 9-21, 2010, PMID: 20059641). Indeed, exosomes have been shown to be particularly useful in delivery siRNA, a system with some parallels to the CRISPR system. For instance, El-Andaloussi S, et al. ("Exosome-mediated delivery of siRNA in vitro and in vivo." *Nat Protoc.* 2012 Dec;7(12):21 12-26. doi: 10.1038/nprot.2012.131. Epub 2012 Nov 15.) describe how exosomes are promising tools for drug delivery across different biological barriers and can be harnessed for delivery of siRNA in vitro and in vivo. Their approach is to generate targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. The exosomes are then purified and characterized from transfected cell supernatant, then RNA is loaded into the exosomes. Delivery or administration according to the invention can be performed with exosomes, in particular but not limited to the brain. Vitamin E (α-tocopherol) may be conjugated with CRISPR Cas and delivered to the brain along with high density lipoprotein (HDL), for example in a similar manner as was done by Uno et al. (*HUMAN GENE THERAPY* 22:711-719 (June 2011)) for delivering short-interfering RNA (siRNA) to the brain. Mice were infused via Osmotic minipumps (model 1007D; Alzet, Cupertino, CA) filled with phosphate-buffered saline (PBS) or free TocsiBACE or Toc-siBACE/HDL and connected with Brain Infusion Kit 3 (Alzet). A brain-infusion cannula was placed about 0.5mm posterior to the bregma at midline for infusion into the dorsal third ventricle. Uno et al. found that as little as 3 nmol of Toc-siRNA with HDL could induce a target reduction in comparable degree by the same ICV infusion method. A similar dosage of CRISPR Cas conjugated to α-tocopherol and co-administered with HDL targeted to the brain may be contemplated for humans in the present invention, for example, about 3 nmol to about 3 μmol of CRISPR Cas targeted to the brain may be contemplated. Zou et al. (*HUMAN GENE THERAPY* 22:465-475 (April 2011)) describes a method of lentiviral-mediated delivery of short-hairpin RNAs targeting PKCγ for in vivo gene silencing in the spinal cord of rats. Zou et al. administered about 10 μl of a recombinant lentivirus having a titer of 1 x 10⁹ transducing units (TU)/ml by an intrathecal catheter. A similar

dosage of CRISPR Cas expressed in a lentiviral vector targeted to the brain may be contemplated for humans in the present invention, for example, about 10-50 ml of CRISPR Cas targeted to the brain in a lentivirus having a titer of 1×10^9 transducing units (TU)/ml may be contemplated.

[00451] In terms of local delivery to the brain, this can be achieved in various ways. For instance, material can be delivered intrastrially *e.g.* by injection. Injection can be performed stereotactically via a craniotomy.

[00452] Enhancing NHEJ or HR efficiency is also helpful for delivery. It is preferred that NHEJ efficiency is enhanced by co-expressing end-processing enzymes such as Trex2 (Dumitrache et al. Genetics. 2011 August; 188(4): 787-797). It is preferred that HR efficiency is increased by transiently inhibiting NHEJ machineries such as Ku70 and Ku86. HR efficiency can also be increased by co-expressing prokaryotic or eukaryotic homologous recombination enzymes such as RecBCD, RecA.

Packaging and Promoters generally

[00453] Ways to package nucleic acid molecules, in particular the DNA targeting agent according to the invention as described herein, such as Cas9 coding nucleic acid molecules, *e.g.*, DNA, into vectors, *e.g.*, viral vectors, to mediate genome modification *in vivo* include:

To achieve NHEJ-mediated gene knockout:

Single virus vector:

Vector containing two or more expression cassettes:

Promoter-Cas9 coding nucleic acid molecule -terminator

Promoter-gRNA1 -terminator

Promoter-gRNA2 -terminator

Promoter-gRNA(N)-terminator (up to size limit of vector)

Double virus vector:

Vector 1 containing one expression cassette for driving the expression of Cas9

Promoter-Cas9 coding nucleic acid molecule-terminator

Vector 2 containing one more expression cassettes for driving the expression of one or more guideRNAs

Promoter-gRNA1 -terminator

Promoter-gRNA(N)-terminator (up to size limit of vector)

To mediate homology-directed repair.

In addition to the single and double virus vector approaches described above, an additional vector is used to deliver a homology-direct repair template.

[00454] The promoter used to drive Cas9 coding nucleic acid molecule expression can include:

AAV ITR can serve as a promoter: this is advantageous for eliminating the need for an additional promoter element (which can take up space in the vector). The additional space freed up can be used to drive the expression of additional elements (gRNA, etc.). Also, ITR activity is relatively weaker, so can be used to reduce potential toxicity due to over expression of Cas9.

For ubiquitous expression, can use promoters: CMV, CAG, CBh, PGK, SV40, Ferritin heavy or light chains, etc.

For brain or other CNS expression, can use promoters: Synapsin1 for all neurons, CaMKIIalpha for excitatory neurons, GAD67 or GAD65 or VGAT for GABAergic neurons, etc.

For liver expression, can use Albumin promoter.

For lung expression, can use SP-B.

For endothelial cells, can use ICAM.

For hematopoietic cells can use IFNbeta or CD45.

For Osteoblasts can use OG-2.

[00455] The promoter used to drive guide RNA can include:

Pol III promoters such as U6 or HI

Use of Pol II promoter and intronic cassettes to express gRNA

Adeno associated virus (AAV)

[00456] The DNA targeting agent according to the invention as described herein, such as by means of example Cas9 and one or more guide RNA can be delivered using adeno associated virus (AAV), lentivirus, adenovirus or other plasmid or viral vector types, in particular, using formulations and doses from, for example, US Patents Nos. 8,454,972 (formulations, doses for adenovirus), 8,404,658 (formulations, doses for AAV) and 5,846,946 (formulations, doses for DNA plasmids) and from clinical trials and publications regarding the clinical trials involving lentivirus, AAV and adenovirus. For examples, for AAV, the route of administration, formulation and dose can be as in US Patent No. 8,454,972 and as in clinical trials involving AAV. For Adenovirus, the route of administration, formulation and dose can be as in US Patent No. 8,404,658 and as in clinical trials involving adenovirus. For plasmid delivery, the route of administration, formulation and dose can be as in US Patent No 5,846,946 and as in clinical studies involving plasmids. Doses may be based on or

extrapolated to an average 70 kg individual (*e.g.* a male adult human), and can be adjusted for patients, subjects, mammals of different weight and species. Frequency of administration is within the ambit of the medical or veterinary practitioner (*e.g.*, physician, veterinarian), depending on usual factors including the age, sex, general health, other conditions of the patient or subject and the particular condition or symptoms being addressed. The viral vectors can be injected into the tissue of interest. For cell-type specific genome modification, the expression of the DNA targeting agent according to the invention as described herein, such as by means of example Cas9 can be driven by a cell-type specific promoter. For example, liver-specific expression might use the Albumin promoter and neuron-specific expression (*e.g.* for targeting CNS disorders) might use the Synapsin I promoter.

[00457] In terms of *in vivo* delivery, AAV is advantageous over other viral vectors for a couple of reasons:

Low toxicity (this may be due to the purification method not requiring ultra centrifugation of cell particles that can activate the immune response)

Low probability of causing insertional mutagenesis because it doesn't integrate into the host genome.

[00458] AAV has a packaging limit of 4.5 or 4.75 Kb. This means that for instance Cas9 as well as a promoter and transcription terminator have to be all fit into the same viral vector. Constructs larger than 4.5 or 4.75 Kb will lead to significantly reduced virus production. SpCas9 is quite large, the gene itself is over 4.1 Kb, which makes it difficult for packing into AAV. Therefore embodiments of the invention include utilizing homologs of Cas9 that are shorter. For example:

Species	Cas9 Size
Corynebacter diphtheriae	3252
Eubacterium ventriosum	3321
Streptococcus pasteurianus	3390
Lactobacillus farciminis	3378
Sphaerochaeta globus	3537
Azospirillum B510	3504
Gluconacetobacter diazotrophicus	3150
Neisseria cinerea	3246
Roseburia intestinalis	3420
Parvibaculum lavamentivorans	3111
Staphylococcus aureus	3159
Nitratifactor salsuginis DSM 16511	3396
Campylobacter lari CF89-12	3009
Streptococcus thermophilus LMD-9	3396

[00459] These species are therefore, in general, preferred Cas9 species.

[00460] As to AAV, the AAV can be AAV1, AAV2, AAV5 or any combination thereof. One can select the AAV of the AAV with regard to the cells to be targeted; e.g., one can select AAV serotypes 1, 2, 5 or a hybrid capsid AAV1, AAV2, AAV5 or any combination thereof for targeting brain or neuronal cells; and one can select AAV4 for targeting cardiac tissue. AAV8 is useful for delivery to the liver. The herein promoters and vectors are preferred individually. A tabulation of certain AAV serotypes as to these cells (see Grimm, D. et al, J. Virol. 82: 5887-5911 (2008)) is as follows:

Cell Line	AAV-1	AAV-2	AAV-3	AAV-4	AAV-5	AAV-6	AAV-8	AAV-9
Huh-7	13	100	2.5	0.0	0.1	10	0.7	0.0
HEK293	25	100	2.5	0.1	0.1	5	0.7	0.1
HeLa	3	100	2.0	0.1	6.7	1	0.2	0.1
HepG2	3	100	16.7	0.3	1.7	5	0.3	ND
Hep1A	20	100	0.2	1.0	0.1	1	0.2	0.0
911	17	100	11	0.2	0.1	17	0.1	ND
CHO	100	100	14	1.4	333	50	10	1.0
COS	33	100	33	3.3	5.0	14	2.0	0.5
MeWo	10	100	20	0.3	6.7	10	1.0	0.2
NIH3T3	10	100	2.9	2.9	0.3	10	0.3	ND
A549	14	100	20	ND	0.5	10	0.5	0.1
HT1180	20	100	10	0.1	0.3	33	0.5	0.1
Monocytes	1111	100	ND	ND	125	1429	ND	ND
Immature DC	2500	100	ND	ND	222	2857	ND	ND
Mature DC	2222	100	ND	ND	333	3333	ND	ND

Lentivirus

[00461] Lentiviruses are complex retroviruses that have the ability to infect and express their genes in both mitotic and post-mitotic cells. The most commonly known lentivirus is the human immunodeficiency virus (HIV), which uses the envelope glycoproteins of other viruses to target a broad range of cell types.

[00462] Lentiviruses may be prepared as follows, by means of example for Cas delivery. After cloning pCasESIO (which contains a lentiviral transfer plasmid backbone), HEK293FT at low passage (p=5) were seeded in a T-75 flask to 50% confluence the day before transfection in DMEM with 10% fetal bovine serum and without antibiotics. After 20 hours, media was changed to OptiMEM (serum-free) media and transfection was done 4 hours later. Cells were transfected with 10 µg of lentiviral transfer plasmid (pCasESIO) and the following

packaging plasmids: 5 µg of pMD2.G (VSV-g pseudotype), and 7.5ug of psPAX2 (gag/pol/rev/tat). Transfection was done in 4mL OptiMEM with a cationic lipid delivery agent (50uL Lipofectamine 2000 and 100ul Plus reagent). After 6 hours, the media was changed to antibiotic-free DMEM with 10% fetal bovine serum. These methods use serum during cell culture, but serum-free methods are preferred.

[00463] Lentivirus may be purified as follows. Viral supernatants were harvested after 48 hours. Supernatants were first cleared of debris and filtered through a 0.45µm low protein binding (PVDF) filter. They were then spun in a ultracentrifuge for 2 hours at 24,000 rpm. Viral pellets were resuspended in 50ul of DMEM overnight at 4C. They were then aliquotted and immediately frozen at -80°C.

[00464] In another embodiment, minimal non-primate lentiviral vectors based on the equine infectious anemia virus (EIAV) are also contemplated, especially for ocular gene therapy (see, e.g., Balagaan, J Gene Med 2006; 8: 275 - 285). In another embodiment, RetinoStat®, an equine infectious anemia virus-based lentiviral gene therapy vector that expresses angiostatic proteins endostatin and angiostatin that is delivered via a subretinal injection for the treatment of the web form of age-related macular degeneration is also contemplated (see, e.g., Binley et al., HUMAN GENE THERAPY 23:980-991 (September 2012)) and this vector may be modified for the CRISPR-Cas system of the present invention.

[00465] In another embodiment, self-inactivating lentiviral vectors with an siRNA targeting a common exon shared by HIV tat/rev, a nucleolar-localizing TAR decoy, and an anti-CCR5-specific hammerhead ribozyme (see, e.g., DiGiusto et al. (2010) Sci Transl Med 2:36ra43) may be used/and or adapted to the CRISPR-Cas system of the present invention. A minimum of 2.5×10^6 CD34+ cells per kilogram patient weight may be collected and prestimulated for 16 to 20 hours in X-VIVO 15 medium (Lonza) containing 2 µmol/L-glutamine, stem cell factor (100 ng/ml), Flt-3 ligand (Flt-3L) (100 ng/ml), and thrombopoietin (10 ng/ml) (CellGenix) at a density of 2×10^6 cells/ml. Prestimulated cells may be transduced with lentiviral at a multiplicity of infection of 5 for 16 to 24 hours in 75-cm² tissue culture flasks coated with fibronectin (25 mg/cm²) (RetroNectin, Takara Bio Inc.).

[00466] Lentiviral vectors have been disclosed as in the treatment for Parkinson's Disease, see, e.g., US Patent Publication No. 20120295960 and US Patent Nos. 7303910 and 7351585. Lentiviral vectors have also been disclosed for the treatment of ocular diseases, see e.g., US Patent Publication Nos. 20060281180, 20090007284, US20110117189; US20090017543; US20070054961, US20100317109. Lentiviral vectors have also been disclosed for delivery

to the brain, see, e.g., US Patent Publication Nos. US201 10293571; US201 10293571, US20040013648, US20070025970, US20090111106 and US Patent No. US7259015.

RNA delivery

[00467] RNA delivery: The DNA targeting agent according to the invention as described herein, such as the CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can also be delivered in the form of RNA. Cas9 mRNA can be generated using *in vitro* transcription. For example, Cas9 mRNA can be synthesized using a PCR cassette containing the following elements: T7_promoter-kozak sequence (GCCACC)-Cas9-3' UTR from beta globin-polyA tail (a string of 120 or more adenines). The cassette can be used for transcription by T7 polymerase. Guide RNAs can also be transcribed using *in vitro* transcription from a cassette containing T7_promoter-GG-guide RNA sequence.

[00468] To enhance expression and reduce possible toxicity, the CRISPR enzyme-coding sequence and/or the guide RNA can be modified to include one or more modified nucleoside e.g. using pseudo-U or 5-Methyl-C.

[00469] mRNA delivery methods are especially promising for liver delivery currently.

[00470] Much clinical work on RNA delivery has focused on RNAi or antisense, but these systems can be adapted for delivery of RNA for implementing the present invention. References below to RNAi *etc.* should be read accordingly.

Particle delivery systems and/or formulations;

[00471] Several types of particle delivery systems and/or formulations are known to be useful in a diverse spectrum of biomedical applications. In general, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Particles are further classified according to diameter. Coarse particles cover a range between 2,500 and 10,000 nanometers. Fine particles are sized between 100 and 2,500 nanometers. Ultrafine particles, or nanoparticles, are generally between 1 and 100 nanometers in size. The basis of the 100-nm limit is the fact that novel properties that differentiate particles from the bulk material typically develop at a critical length scale of under 100 nm.

[00472] As used herein, a particle delivery system/formulation is defined as any biological delivery system/formulation which includes a particle in accordance with the present invention. A particle in accordance with the present invention is any entity having a greatest dimension (e.g. diameter) of less than 100 microns ($\mu\pi$). In some embodiments, inventive particles have a greatest dimension of less than 10 $\mu\pi$. In some embodiments, inventive particles have a greatest dimension of less than 2000 nanometers (nm). In some

embodiments, inventive particles have a greatest dimension of less than 1000 nanometers (nm). In some embodiments, inventive particles have a greatest dimension of less than 900 nm, 800 nm, 700 nm, 600 nm, 500 nm, 400 nm, 300 nm, 200 nm, or 100 nm. Typically, inventive particles have a greatest dimension (e.g., diameter) of 500 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 250 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 200 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 150 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 100 nm or less. Smaller particles, e.g., having a greatest dimension of 50 nm or less are used in some embodiments of the invention. In some embodiments, inventive particles have a greatest dimension ranging between 25 nm and 200 nm.

[00473] Particle characterization (including e.g., characterizing morphology, dimension, etc.) is done using a variety of different techniques. Common techniques are electron microscopy (TEM, SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), ultraviolet-visible spectroscopy, dual polarisation interferometry and nuclear magnetic resonance (NMR). Characterization (dimension measurements) may be made as to native particles (i.e., preloading) or after loading of the cargo (herein cargo refers to e.g., one or more components of for instance CRISPR-Cas system e.g., CRISPR enzyme or mRNA or guide RNA, or any combination thereof, and may include additional carriers and/or excipients) to provide particles of an optimal size for delivery for any *in vitro*, *ex vivo* and/or *in vivo* application of the present invention. In certain preferred embodiments, particle dimension (e.g., diameter) characterization is based on measurements using dynamic laser scattering (DLS). Mention is made of US Patent No. 8,709,843; US Patent No. 6,007,845; US Patent No. 5,855,913; US Patent No. 5,985,309; US Patent No. 5,543,158; and the publication by James E. Dahlman and Carmen Barnes et al. Nature Nanotechnology (2014) published online 11 May 2014, doi:10.1038/nano.2014.84, concerning particles, methods of making and using them and measurements thereof.

[00474] Particles delivery systems within the scope of the present invention may be provided in any form, including but not limited to solid, semi-solid, emulsion, or colloidal particles. As such any of the delivery systems described herein, including but not limited to,

e.g., lipid-based systems, liposomes, micelles, microvesicles, exosomes, or gene gun may be provided as particle delivery systems within the scope of the present invention.

Particles

[00475] The DNA targeting agent according to the invention as described herein, such as by means of example CRISPR enzyme mRNA and guide RNA may be delivered simultaneously using particles or lipid envelopes; for instance, CRISPR enzyme and RNA of the invention, e.g., as a complex, can be delivered via a particle as in Dahlman et al, WO20 150894 19 A2 and documents cited therein, such as 7C1 (see, e.g., James E. Dahlman and Carmen Barnes et al. Nature Nanotechnology (2014) published online 11 May 2014, doi:10.1038/nnano.2014.84), e.g., delivery particle comprising lipid or lipidoid and hydrophilic polymer, e.g., cationic lipid and hydrophilic polymer, for instance wherein the cationic lipid comprises 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or 1,2-ditetradecanoyl-sft-glycero-S-phosphocholine (DMPC) and/or wherein the hydrophilic polymer comprises ethylene glycol or polyethylene glycol (PEG); and/or wherein the particle further comprises cholesterol (e.g., particle from formulation 1 = DOTAP 100, DMPC 0, PEG 0, Cholesterol 0; formulation number 2 = DOTAP 90, DMPC 0, PEG 10, Cholesterol 0; formulation number 3 = DOTAP 90, DMPC 0, PEG 5, Cholesterol 5), wherein particles are formed using an efficient, multistep process wherein first, effector protein and RNA are mixed together, e.g., at a 1:1 molar ratio, e.g., at room temperature, e.g., for 30 minutes, e.g., in sterile, nuclease free IX PBS; and separately, DOTAP, DMPC, PEG, and cholesterol as applicable for the formulation are dissolved in alcohol, e.g., 100% ethanol; and, the two solutions are mixed together to form particles containing the complexes).

[00476] For example, Su X, Fricke J, Kavanagh DG, Irvine DJ ("In vitro and in vivo mRNA delivery using lipid-enveloped pH-responsive polymer nanoparticles" Mol Pharm. 2011 Jun 6;8(3):774-87. doi: 10.1021/impl00390w. Epub 2011 Apr 1) describes biodegradable core-shell structured particles with a poly (P-amino ester) (PBAE) core enveloped by a phospholipid bilayer shell. These were developed for in vivo mRNA delivery. The pH-responsive PBAE component was chosen to promote endosome disruption, while the lipid surface layer was selected to minimize toxicity of the polycation core. Such are, therefore, preferred for delivering RNA of the present invention.

[00477] In one embodiment, particles based on self assembling bioadhesive polymers are contemplated, which may be applied to oral delivery of peptides, intravenous delivery of peptides and nasal delivery of peptides, all to the brain. Other embodiments, such as oral

absorption and ocular delivery of hydrophobic drugs are also contemplated. The molecular envelope technology involves an engineered polymer envelope which is protected and delivered to the site of the disease (see, e.g., Mazza, M. et al. ACSNano, 2013. 7(2): 1016-1026; Siew, A., et al. Mol Pharm, 2012. 9(1): 14-28; Lalatsa, A., et al. J Contr Rel, 2012. 161(2):523-36; Lalatsa, A., et al, Mol Pharm, 2012. 9(6): 1665-80; Lalatsa, A., et al. Mol Pharm, 2012. 9(6): 1764-74; Garrett, N.L., et al. J Biophotonics, 2012. 5(5-6):458-68; Garrett, N.L., et al. J Raman Spect, 2012. 43(5):681-688; Ahmad, S., et al. J Royal Soc Interface 2010. 7:S423-33; Uchegbu, I.F. Expert Opin Drug Deliv, 2006. 3(5):629-40; Qu, X., et al. Biomacromolecules, 2006. 7(12):3452-9 and Uchegbu, I.F., et al. Int J Pharm, 2001. 224:185-199). Doses of about 5 mg/kg are contemplated, with single or multiple doses, depending on the target tissue.

[00478] In one embodiment, particles that can deliver DNA targeting agents according to the invention as described herein, such as RNA to a cancer cell to stop tumor growth developed by Dan Anderson's lab at MIT may be used/and or adapted to the CRISPR Cas system according to certain embodiments of the present invention. In particular, the Anderson lab developed fully automated, combinatorial systems for the synthesis, purification, characterization, and formulation of new biomaterials and nanoformulations. See, e.g., Alabi et al, Proc Natl Acad Sci U S A. 2013 Aug 6;110(32): 12881-6; Zhang et al, Adv Mater. 2013 Sep 6;25(33):4641-5; Jiang et al, Nano Lett. 2013 Mar 13;13(3): 1059-64; Karagiannis et al, ACS Nano. 2012 Oct 23;6(10):8484-7; Whitehead et al, ACS Nano. 2012 Aug 28;6(8):6922-9 and Lee et al., Nat Nanotechnol. 2012 Jun 3;7(6):389-93.

[00479] US patent application 20110293703 relates to lipidoid compounds are also particularly useful in the administration of polynucleotides, which may be applied to deliver the DNA targeting agent according to the invention, such as for instance the CRISPR Cas system according to certain embodiments of the present invention. In one aspect, the aminoalcohol lipidoid compounds are combined with an agent to be delivered to a cell or a subject to form microparticles, particles, liposomes, or micelles. The agent to be delivered by the particles, liposomes, or micelles may be in the form of a gas, liquid, or solid, and the agent may be a polynucleotide, protein, peptide, or small molecule. The aminoalcohol lipidoid compounds may be combined with other aminoalcohol lipidoid compounds, polymers (synthetic or natural), surfactants, cholesterol, carbohydrates, proteins, lipids, etc. to form the particles. These particles may then optionally be combined with a pharmaceutical excipient to form a pharmaceutical composition.

[00480] US Patent Publication No. 201 10293703 also provides methods of preparing the aminoalcohol lipidoid compounds. One or more equivalents of an amine are allowed to react with one or more equivalents of an epoxide-terminated compound under suitable conditions to form an aminoalcohol lipidoid compound of the present invention. In certain embodiments, all the amino groups of the amine are fully reacted with the epoxide-terminated compound to form tertiary amines. In other embodiments, all the amino groups of the amine are not fully reacted with the epoxide-terminated compound to form tertiary amines thereby resulting in primary or secondary amines in the aminoalcohol lipidoid compound. These primary or secondary amines are left as is or may be reacted with another electrophile such as a different epoxide-terminated compound. As will be appreciated by one skilled in the art, reacting an amine with less than excess of epoxide-terminated compound will result in a plurality of different aminoalcohol lipidoid compounds with various numbers of tails. Certain amines may be fully functionalized with two epoxide-derived compound tails while other molecules will not be completely functionalized with epoxide-derived compound tails. For example, a diamine or polyamine may include one, two, three, or four epoxide-derived compound tails off the various amino moieties of the molecule resulting in primary, secondary, and tertiary amines. In certain embodiments, all the amino groups are not fully functionalized. In certain embodiments, two of the same types of epoxide-terminated compounds are used. In other embodiments, two or more different epoxide-terminated compounds are used. The synthesis of the aminoalcohol lipidoid compounds is performed with or without solvent, and the synthesis may be performed at higher temperatures ranging from 30-100 °C, preferably at approximately 50-90 °C. The prepared aminoalcohol lipidoid compounds may be optionally purified. For example, the mixture of aminoalcohol lipidoid compounds may be purified to yield an aminoalcohol lipidoid compound with a particular number of epoxide-derived compound tails. Or the mixture may be purified to yield a particular stereo- or regioisomer. The aminoalcohol lipidoid compounds may also be alkylated using an alkyl halide (e.g., methyl iodide) or other alkylating agent, and/or they may be acylated.

[00481] US Patent Publication No. 201 10293703 also provides libraries of aminoalcohol lipidoid compounds prepared by the inventive methods. These aminoalcohol lipidoid compounds may be prepared and/or screened using high-throughput techniques involving liquid handlers, robots, microtiter plates, computers, etc. In certain embodiments, the aminoalcohol lipidoid compounds are screened for their ability to transfect polynucleotides or other agents (e.g., proteins, peptides, small molecules) into the cell.

[00482] US Patent Publication No. 20130302401 relates to a class of poly(beta-amino alcohols) (PBAAAs) has been prepared using combinatorial polymerization. The inventive PBAAAs may be used in biotechnology and biomedical applications as coatings (such as coatings of films or multilayer films for medical devices or implants), additives, materials, excipients, non-biofouling agents, micropatterning agents, and cellular encapsulation agents. When used as surface coatings, these PBAAAs elicited different levels of inflammation, both in vitro and in vivo, depending on their chemical structures. The large chemical diversity of this class of materials allowed us to identify polymer coatings that inhibit macrophage activation in vitro. Furthermore, these coatings reduce the recruitment of inflammatory cells, and reduce fibrosis, following the subcutaneous implantation of carboxylated polystyrene microparticles. These polymers may be used to form polyelectrolyte complex capsules for cell encapsulation. The invention may also have many other biological applications such as antimicrobial coatings, DNA or siRNA delivery, and stem cell tissue engineering. The teachings of US Patent Publication No. 20130302401 may be applied to the DNA targeting agent according to the invention, such as for instance the CRISPR Cas system according to certain embodiments of the present invention.

[00483] In another embodiment, lipid particles (LNPs) are contemplated. An antitransferrin small interfering RNA has been encapsulated in lipid particles and delivered to humans (see, e.g., Coelho et al, N Engl J Med 2013;369:819-29), and such a system may be adapted and applied to the CRISPR Cas system of the present invention. Doses of about 0.01 to about 1 mg per kg of body weight administered intravenously are contemplated. Medications to reduce the risk of infusion-related reactions are contemplated, such as dexamethasone, acetaminophen, diphenhydramine or cetirizine, and ranitidine are contemplated. Multiple doses of about 0.3 mg per kilogram every 4 weeks for five doses are also contemplated.

[00484] LNPs have been shown to be highly effective in delivering siRNAs to the liver (see, e.g., Taberero et al., Cancer Discovery, April 2013, Vol. 3, No. 4, pages 363-470) and are therefore contemplated for delivering RNA encoding CRISPR Cas to the liver. A dosage of about four doses of 6 mg/kg of the LNP every two weeks may be contemplated. Taberero et al. demonstrated that tumor regression was observed after the first 2 cycles of LNPs dosed at 0.7 mg/kg, and by the end of 6 cycles the patient had achieved a partial response with complete regression of the lymph node metastasis and substantial shrinkage of the liver tumors. A complete response was obtained after 40 doses in this patient, who has remained in remission and completed treatment after receiving doses over 26 months. Two patients with

RCC and extrahepatic sites of disease including kidney, lung, and lymph nodes that were progressing following prior therapy with VEGF pathway inhibitors had stable disease at all sites for approximately 8 to 12 months, and a patient with PNET and liver metastases continued on the extension study for 18 months (36 doses) with stable disease.

[00485] However, the charge of the LNP must be taken into consideration. As cationic lipids combined with negatively charged lipids to induce nonbilayer structures that facilitate intracellular delivery. Because charged LNPs are rapidly cleared from circulation following intravenous injection, ionizable cationic lipids with pKa values below 7 were developed (see, e.g., Rosin et al, *Molecular Therapy*, vol. 19, no. 12, pages 1286-2200, Dec. 2011). Negatively charged polymers such as RNA may be loaded into LNPs at low pH values (e.g., pH 4) where the ionizable lipids display a positive charge. However, at physiological pH values, the LNPs exhibit a low surface charge compatible with longer circulation times. Four species of ionizable cationic lipids have been focused upon, namely 1,2-dilinoyleoyl-3-dimethylammonium-propane (DLinDAP), 1,2-dilinoyleoxy-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoyleoxy-keto-N,N-dimethyl-3-aminopropane (DLinKDMA), and 1,2-dilinoyleoyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA). It has been shown that LNP siRNA systems containing these lipids exhibit remarkably different gene silencing properties in hepatocytes in vivo, with potencies varying according to the series DLinKC2-DMA>DLinKDMA>DLinDMA»DLinDAP employing a Factor VII gene silencing model (see, e.g., Rosin et al, *Molecular Therapy*, vol. 19, no. 12, pages 1286-2200, Dec. 2011). A dosage of 1 µg/ml of LNP or by means of example CRISPR-Cas RNA in or associated with the LNP may be contemplated, especially for a formulation containing DLinKC2-DMA.

[00486] Preparation of LNPs and the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas encapsulation may be used/and or adapted from Rosin et al, *Molecular Therapy*, vol. 19, no. 12, pages 1286-2200, Dec. 2011). The cationic lipids 1,2-dilinoyleoyl-3-dimethylammonium-propane (DLinDAP), 1,2-dilinoyleoxy-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoyleoxyketo-N,N-dimethyl-3-aminopropane (DLinK-DMA), 1,2-dilinoyleoyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA), (3-o-[2"-(methoxypolyethyleneglycol 2000) succinoyl]-1,2-dimyristoyl-sn-glycol (PEG-S-DMG), and R-3-[(ro-methoxy-poly(ethylene glycol)2000) carbamoyl]-1,2-dimyristyloxylpropyl-3 -amine (PEG-C-DOMG) may be provided by Tekmira Pharmaceuticals (Vancouver, Canada) or synthesized. Cholesterol may be purchased from Sigma (St Louis, MO). The specific CRISPR Cas RNA may be encapsulated in LNPs containing DLinDAP, DLinDMA, DLinK-DMA, and DLinKC2-DMA (cationic

lipid:DSPC:CHOL: PEGS-DMG or PEG-C-DOMG at 40:10:40:10 molar ratios). When required, 0.2% SP-DiOC18 (Invitrogen, Burlington, Canada) may be incorporated to assess cellular uptake, intracellular delivery, and biodistribution. Encapsulation may be performed by dissolving lipid mixtures comprised of cationic lipid:DSPC:cholesterol:PEG-c-DOMG (40:10:40:10 molar ratio) in ethanol to a final lipid concentration of 10 mmol/l. This ethanol solution of lipid may be added drop-wise to 50 mmol/l citrate, pH 4.0 to form multilamellar vesicles to produce a final concentration of 30% ethanol vol/vol. Large unilamellar vesicles may be formed following extrusion of multilamellar vesicles through two stacked 80 nm Nuclepore polycarbonate filters using the Extruder (Northern Lipids, Vancouver, Canada). Encapsulation may be achieved by adding RNA dissolved at 2 mg/ml in 50 mmol/l citrate, pH 4.0 containing 30% ethanol vol/vol drop-wise to extruded preformed large unilamellar vesicles and incubation at 31 °C for 30 minutes with constant mixing to a final RNA/lipid weight ratio of 0.06/1 wt/wt. Removal of ethanol and neutralization of formulation buffer were performed by dialysis against phosphate-buffered saline (PBS), pH 7.4 for 16 hours using Spectra/Por 2 regenerated cellulose dialysis membranes. Particle size distribution may be determined by dynamic light scattering using a NICOMP 370 particle sizer, the vesicle/intensity modes, and Gaussian fitting (Nicomp Particle Sizing, Santa Barbara, CA). The particle size for all three LNP systems may be ~70 nm in diameter. RNA encapsulation efficiency may be determined by removal of free RNA using VivaPureD MiniH columns (Sartorius Stedim Biotech) from samples collected before and after dialysis. The encapsulated RNA may be extracted from the eluted particles and quantified at 260 nm. RNA to lipid ratio was determined by measurement of cholesterol content in vesicles using the Cholesterol E enzymatic assay from Wako Chemicals USA (Richmond, VA). In conjunction with the herein discussion of LNPs and PEG lipids, PEGylated liposomes or LNPs are likewise suitable for delivery of a CRISPR-Cas system or components thereof.

[00487] Preparation of large LNPs may be used/and or adapted from Rosin et al, *Molecular Therapy*, vol. 19, no. 12, pages 1286-2200, Dec. 2011. A lipid premix solution (20.4 mg/ml total lipid concentration) may be prepared in ethanol containing DLinKC2-DMA, DSPC, and cholesterol at 50:10:38.5 molar ratios. Sodium acetate may be added to the lipid premix at a molar ratio of 0.75:1 (sodium acetate:DLinKC2-DMA). The lipids may be subsequently hydrated by combining the mixture with 1.85 volumes of citrate buffer (10 mmol/l, pH 3.0) with vigorous stirring, resulting in spontaneous liposome formation in aqueous buffer containing 35% ethanol. The liposome solution may be incubated at 37 °C to allow for time-dependent increase in particle size. Aliquots may be removed at various times

during incubation to investigate changes in liposome size by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). Once the desired particle size is achieved, an aqueous PEG lipid solution (stock = 10 mg/ml PEG-DMG in 35% (vol/vol) ethanol) may be added to the liposome mixture to yield a final PEG molar concentration of 3.5% of total lipid. Upon addition of PEG-lipids, the liposomes should their size, effectively quenching further growth. RNA may then be added to the empty liposomes at an RNA to total lipid ratio of approximately 1:10 (wt:wt), followed by incubation for 30 minutes at 37 °C to form loaded LNPs. The mixture may be subsequently dialyzed overnight in PBS and filtered with a 0.45-µm syringe filter.

[00488] Spherical Nucleic Acid (SNA™) constructs and other particles (particularly gold particles) are also contemplated as a means to deliver the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR-Cas system to intended targets. Significant data show that AuraSense Therapeutics' Spherical Nucleic Acid (SNA™) constructs, based upon nucleic acid-functionalized gold particles, are useful.

[00489] Literature that may be employed in conjunction with herein teachings include: Cutler et al, J. Am. Chem. Soc. 2011 133:9254-9257, Hao et al, Small. 2011 7:3158-3162, Zhang et al, ACS Nano. 2011 5:6962-6970, Cutler et al, J. Am. Chem. Soc. 2012 134:1376-1391, Young et al., Nano Lett. 2012 12:3867-71, Zheng et al, Proc. Natl. Acad. Sci. USA. 2012 109:11975-80, Mirkin, Nanomedicine 2012 7:635-638 Zhang et al, J. Am. Chem. Soc. 2012 134:16488-1691, Weintraub, Nature 2013 495:S14-S16, Choi et al, Proc. Natl. Acad. Sci. USA. 2013 110(19):7625-7630, Jensen et al, Sci. Transl. Med. 5, 209ra152 (2013) and Mirkin, et al., Small, 10:186-192.

[00490] Self-assembling particles with RNA may be constructed with polyethyleneimine (PEI) that is PEGylated with an Arg-Gly-Asp (RGD) peptide ligand attached at the distal end of the polyethylene glycol (PEG). This system has been used, for example, as a means to target tumor neovasculature expressing integrins and deliver siRNA inhibiting vascular endothelial growth factor receptor-2 (VEGF R2) expression and thereby achieve tumor angiogenesis (see, e.g., Schiffelers et al, Nucleic Acids Research, 2004, Vol. 32, No. 19). Nanoplexes may be prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. A dosage of

about 100 to 200 mg of CRISPR Cas is envisioned for delivery in the self-assembling particles of Schiffelers et al.

[00491] The nanoplexes of Bartlett et al. (PNAS, September 25, 2007, vol. 104, no. 39) may also be applied to the present invention. The nanoplexes of Bartlett et al. are prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. The DOTA-siRNA of Bartlett et al. was synthesized as follows: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono(N-hydroxysuccinimide ester) (DOTA-NHS ester) was ordered from Macrocyclics (Dallas, TX). The amine modified RNA sense strand with a 100-fold molar excess of DOTA-NHS-ester in carbonate buffer (pH 9) was added to a microcentrifuge tube. The contents were reacted by stirring for 4 h at room temperature. The DOTA-RNA sense conjugate was ethanol-precipitated, resuspended in water, and annealed to the unmodified antisense strand to yield DOTA-siRNA. All liquids were pretreated with Chelex-100 (Bio-Rad, Hercules, CA) to remove trace metal contaminants. Tf-targeted and nontargeted siRNA particles may be formed by using cyclodextrin-containing polycations. Typically, particles were formed in water at a charge ratio of 3 (+/-) and an siRNA concentration of 0.5 g/liter. One percent of the adamantane-PEG molecules on the surface of the targeted particles were modified with Tf (adamantane-PEG-Tf). The particles were suspended in a 5% (wt/vol) glucose carrier solution for injection.

[00492] Davis et al. (Nature, Vol 464, 15 April 2010) conducts a RNA clinical trial that uses a targeted particle-delivery system (clinical trial registration number NCT00689065). Patients with solid cancers refractory to standard-of-care therapies are administered doses of targeted particles on days 1, 3, 8 and 10 of a 21-day cycle by a 30-min intravenous infusion. The particles consist of a synthetic delivery system containing: (1) a linear, cyclodextrin-based polymer (CDP), (2) a human transferrin protein (TF) targeting ligand displayed on the exterior of the particle to engage TF receptors (TFR) on the surface of the cancer cells, (3) a hydrophilic polymer (polyethylene glycol (PEG) used to promote particle stability in biological fluids), and (4) siRNA designed to reduce the expression of the RRM2 (sequence used in the clinic was previously denoted siR2B+5). The TFR has long been known to be upregulated in malignant cells, and RRM2 is an established anti-cancer target. These particles (clinical version denoted as CALAA-01) have been shown to be well tolerated in multi-

dosing studies in non-human primates. Although a single patient with chronic myeloid leukaemia has been administered siRNA by liposomal delivery, Davis et al.'s clinical trial is the initial human trial to systemically deliver siRNA with a targeted delivery system and to treat patients with solid cancer. To ascertain whether the targeted delivery system can provide effective delivery of functional siRNA to human tumours, Davis et al. investigated biopsies from three patients from three different dosing cohorts; patients A, B and C, all of whom had metastatic melanoma and received CALAA-01 doses of 18, 24 and 30 mg m⁻² siRNA, respectively. Similar doses may also be contemplated for the CRISPR Cas system of the present invention. The delivery of the invention may be achieved with particles containing a linear, cyclodextrin-based polymer (CDP), a human transferrin protein (TF) targeting ligand displayed on the exterior of the particle to engage TF receptors (TFR) on the surface of the cancer cells and/or a hydrophilic polymer (for example, polyethylene glycol (PEG) used to promote particle stability in biological fluids).

[00493] In terms of this invention, it is preferred to have one or more components of the DNA targeting agent according to the invention as described herein, such as by means of example the CRISPR complex, e.g., CRISPR enzyme or mRNA or guide RNA delivered using particles or lipid envelopes. Other delivery systems or vectors may be used in conjunction with the particle aspects of the invention.

[00494] In general, a "nanoparticle" refers to any particle having a diameter of less than 1000 nm. In certain preferred embodiments, nanoparticles of the invention have a greatest dimension (e.g., diameter) of 500 nm or less. In other preferred embodiments, nanoparticles of the invention have a greatest dimension ranging between 25 nm and 200 nm. In other preferred embodiments, nanoparticles of the invention have a greatest dimension of 100 nm or less. In other preferred embodiments, particles of the invention have a greatest dimension ranging between 35 nm and 60 nm. In other preferred embodiments, the particles of the invention are not nanoparticles.

[00495] Particles encompassed in the present invention may be provided in different forms, e.g., as solid particles (e.g., metal such as silver, gold, iron, titanium), non-metal, lipid-based solids, polymers), suspensions of particles, or combinations thereof. Metal, dielectric, and semiconductor particles may be prepared, as well as hybrid structures (e.g., core-shell particles). Particles made of semiconducting material may also be labeled quantum dots if they are small enough (typically sub 10 nm) that quantization of electronic energy levels occurs. Such nanoscale particles are used in biomedical applications as drug carriers or imaging agents and may be adapted for similar purposes in the present invention.

[00496] Semi-solid and soft particles have been manufactured, and are within the scope of the present invention. A prototype particle of semi-solid nature is the liposome. Various types of liposome particles are currently used clinically as delivery systems for anticancer drugs and vaccines. Particles with one half hydrophilic and the other half hydrophobic are termed Janus particles and are particularly effective for stabilizing emulsions. They can self-assemble at water/oil interfaces and act as solid surfactants.

[00497] US Patent No. 8,709,843, incorporated herein by reference, provides a drug delivery system for targeted delivery of therapeutic agent-containing particles to tissues, cells, and intracellular compartments. The invention provides targeted particles comprising polymer conjugated to a surfactant, hydrophilic polymer or lipid. US Patent No. 6,007,845, incorporated herein by reference, provides particles which have a core of a multiblock copolymer formed by covalently linking a multifunctional compound with one or more hydrophobic polymers and one or more hydrophilic polymers, and contain a biologically active material. US Patent No. 5,855,913, incorporated herein by reference, provides a particulate composition having aerodynamically light particles having a tap density of less than 0.4 g/cm³ with a mean diameter of between 5 μm and 30 μm, incorporating a surfactant on the surface thereof for drug delivery to the pulmonary system. US Patent No. 5,985,309, incorporated herein by reference, provides particles incorporating a surfactant and/or a hydrophilic or hydrophobic complex of a positively or negatively charged therapeutic or diagnostic agent and a charged molecule of opposite charge for delivery to the pulmonary system. US Patent No. 5,543,158, incorporated herein by reference, provides biodegradable injectable particles having a biodegradable solid core containing a biologically active material and poly(alkylene glycol) moieties on the surface. WO2012135025 (also published as US20120251560), incorporated herein by reference, describes conjugated polyethyleneimine (PEI) polymers and conjugated aza-macrocycles (collectively referred to as "conjugated lipomer" or "lipomers"). In certain embodiments, it can be envisioned that such conjugated lipomers can be used in the context of the CRISPR-Cas system to achieve *in vitro*, *ex vivo* and *in vivo* genomic perturbations to modify gene expression, including modulation of protein expression.

[00498] In one embodiment, the particle may be epoxide-modified lipid-polymer, advantageously 7C1 (see, e.g., James E. Dahlman and Carmen Barnes et al. *Nature Nanotechnology* (2014) published online 11 May 2014, doi:10.1038/nnano.2014.84). 7C1 was synthesized by reacting C15 epoxide-terminated lipids with PEI600 at a 14:1 molar ratio,

and was formulated with C14PEG2000 to produce particles (diameter between 35 and 60 nm) that were stable in PBS solution for at least 40 days.

[00499] An epoxide-modified lipid-polymer may be utilized to deliver the CRISPR-Cas system of the present invention to pulmonary, cardiovascular or renal cells, however, one of skill in the art may adapt the system to deliver to other target organs. Dosage ranging from about 0.05 to about 0.6 mg/kg are envisioned. Dosages over several days or weeks are also envisioned, with a total dosage of about 2 mg/kg.

Exosomes

[00500] Exosomes are endogenous nano-vesicles that transport RNAs and proteins, and which can deliver RNA to the brain and other target organs. To reduce immunogenicity, Alvarez-Erviti et al. (2011, Nat Biotechnol 29: 341) used self-derived dendritic cells for exosome production. Targeting to the brain was achieved by engineering the dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG peptide. Purified exosomes were loaded with exogenous RNA by electroporation. Intravenously injected RVG-targeted exosomes delivered GAPDH siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a specific gene knockdown. Pre-exposure to RVG exosomes did not attenuate knockdown, and non-specific uptake in other tissues was not observed. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA (60%) and protein (62%) knockdown of BACE1, a therapeutic target in Alzheimer's disease.

[00501] To obtain a pool of immunologically inert exosomes, Alvarez-Erviti et al. harvested bone marrow from inbred C57BL/6 mice with a homogenous major histocompatibility complex (MHC) haplotype. As immature dendritic cells produce large quantities of exosomes devoid of T-cell activators such as MHC-II and CD86, Alvarez-Erviti et al. selected for dendritic cells with granulocyte/macrophage-colony stimulating factor (GM-CSF) for 7 d. Exosomes were purified from the culture supernatant the following day using well-established ultracentrifugation protocols. The exosomes produced were physically homogenous, with a size distribution peaking at 80 nm in diameter as determined by particle tracking analysis (NTA) and electron microscopy. Alvarez-Erviti et al. obtained 6-12 µg of exosomes (measured based on protein concentration) per 10⁶ cells.

[00502] Next, Alvarez-Erviti et al. investigated the possibility of loading modified exosomes with exogenous cargoes using electroporation protocols adapted for nanoscale applications. As electroporation for membrane particles at the nanometer scale is not well-

characterized, nonspecific Cy5-labeled RNA was used for the empirical optimization of the electroporation protocol. The amount of encapsulated RNA was assayed after ultracentrifugation and lysis of exosomes. Electroporation at 400 V and 125 μ F resulted in the greatest retention of RNA and was used for all subsequent experiments.

[00503] Alvarez-Erviti et al. administered 150 μ g of each BACE1 siRNA encapsulated in 150 μ g of RVG exosomes to normal C57BL/6 mice and compared the knockdown efficiency to four controls: untreated mice, mice injected with RVG exosomes only, mice injected with BACE1 siRNA complexed to an *in vivo* cationic liposome reagent and mice injected with BACE1 siRNA complexed to RVG-9R, the RVG peptide conjugated to 9 D-arginines that electrostatically binds to the siRNA. Cortical tissue samples were analyzed 3 d after administration and a significant protein knockdown (45%, $P < 0.05$, versus 62%, $P < 0.01$) in both siRNA-RVG-9R-treated and siRNARVG exosome-treated mice was observed, resulting from a significant decrease in BACE1 mRNA levels (66% [+ or -] 15%, $P < 0.001$ and 61% [+ or -] 13% respectively, $P < 0.01$). Moreover, Applicants demonstrated a significant decrease (55%, $P < 0.05$) in the total [beta]-amyloid 1-42 levels, a main component of the amyloid plaques in Alzheimer's pathology, in the RVG-exosome-treated animals. The decrease observed was greater than the β -amyloid 1-40 decrease demonstrated in normal mice after intraventricular injection of BACE1 inhibitors. Alvarez-Erviti et al. carried out 5'-rapid amplification of cDNA ends (RACE) on BACE1 cleavage product, which provided evidence of RNAi-mediated knockdown by the siRNA.

[00504] Finally, Alvarez-Erviti et al. investigated whether RNA-RVG exosomes induced immune responses *in vivo* by assessing IL-6, IP-10, TNF α and IFN- α serum concentrations. Following exosome treatment, nonsignificant changes in all cytokines were registered similar to siRNA-transfection reagent treatment in contrast to siRNA-RVG-9R, which potently stimulated IL-6 secretion, confirming the immunologically inert profile of the exosome treatment. Given that exosomes encapsulate only 20% of siRNA, delivery with RVG-exosome appears to be more efficient than RVG-9R delivery as comparable mRNA knockdown and greater protein knockdown was achieved with fivefold less siRNA without the corresponding level of immune stimulation. This experiment demonstrated the therapeutic potential of RVG-exosome technology, which is potentially suited for long-term silencing of genes related to neurodegenerative diseases. The exosome delivery system of Alvarez-Erviti et al. may be applied to deliver the the DNA targeting agent according to the invention as described herein, such as by means of example the CRISPR-Cas system of the present invention to therapeutic targets, especially neurodegenerative diseases. A dosage of about

100 to 1000 mg of CRISPR Cas encapsulated in about 100 to 1000 mg of RVG exosomes may be contemplated for the present invention.

[00505] El-Andaloussi et al. (Nature Protocols 7,21 12-2126(2012)) discloses how exosomes derived from cultured cells can be harnessed for delivery of RNA in vitro and in vivo. This protocol first describes the generation of targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. Next, El-Andaloussi et al. explain how to purify and characterize exosomes from transfected cell supernatant. Next, El-Andaloussi et al. detail crucial steps for loading RNA into exosomes. Finally, El-Andaloussi et al. outline how to use exosomes to efficiently deliver RNA in vitro and in vivo in mouse brain. Examples of anticipated results in which exosome-mediated RNA delivery is evaluated by functional assays and imaging are also provided. The entire protocol takes ~3 weeks. Delivery or administration according to the invention may be performed using exosomes produced from self-derived dendritic cells. From the herein teachings, this can be employed in the practice of the invention.

[00506] In another embodiment, the plasma exosomes of Wahlgren et al. (Nucleic Acids Research, 2012, Vol. 40, No. 17 e130) are contemplated. Exosomes are nano-sized vesicles (30-90nm in size) produced by many cell types, including dendritic cells (DC), B cells, T cells, mast cells, epithelial cells and tumor cells. These vesicles are formed by inward budding of late endosomes and are then released to the extracellular environment upon fusion with the plasma membrane. Because exosomes naturally carry RNA between cells, this property may be useful in gene therapy, and from this disclosure can be employed in the practice of the instant invention.

[00507] Exosomes from plasma can be prepared by centrifugation of buffy coat at 900g for 20 min to isolate the plasma followed by harvesting cell supernatants, centrifuging at 300g for 10 min to eliminate cells and at 16 500g for 30 min followed by filtration through a 0.22 mm filter. Exosomes are pelleted by ultracentrifugation at 120 000g for 70 min. Chemical transfection of siRNA into exosomes is carried out according to the manufacturer's instructions in RNAi Human/Mouse Starter Kit (Quiagen, Hilden, Germany). siRNA is added to 100 ml PBS at a final concentration of 2 mmol/ml. After adding HiPerFect transfection reagent, the mixture is incubated for 10 min at RT. In order to remove the excess of micelles, the exosomes are re-isolated using aldehyde/sulfate latex beads. The chemical transfection of CRISPR Cas into exosomes may be conducted similarly to siRNA. The exosomes may be co-cultured with monocytes and lymphocytes isolated from the peripheral blood of healthy donors. Therefore, it may be contemplated that exosomes containing the DNA targeting agent

according to the invention as described herein, such as by means of example CRISPR Cas may be introduced to monocytes and lymphocytes of and autologously reintroduced into a human. Accordingly, delivery or administration according to the invention may be performed using plasma exosomes.

Liposomes

[00508] Delivery or administration according to the invention can be performed with liposomes. Liposomes are spherical vesicle structures composed of a uni- or multilamellar lipid bilayer surrounding internal aqueous compartments and a relatively impermeable outer lipophilic phospholipid bilayer. Liposomes have gained considerable attention as drug delivery carriers because they are biocompatible, nontoxic, can deliver both hydrophilic and lipophilic drug molecules, protect their cargo from degradation by plasma enzymes, and transport their load across biological membranes and the blood brain barrier (BBB) (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi: 10.1155/2011/469679 for review).

[00509] Liposomes can be made from several different types of lipids; however, phospholipids are most commonly used to generate liposomes as drug carriers. Although liposome formation is spontaneous when a lipid film is mixed with an aqueous solution, it can also be expedited by applying force in the form of shaking by using a homogenizer, sonicator, or an extrusion apparatus (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi: 10.1155/2011/469679 for review).

[00510] Several other additives may be added to liposomes in order to modify their structure and properties. For instance, either cholesterol or sphingomyelin may be added to the liposomal mixture in order to help stabilize the liposomal structure and to prevent the leakage of the liposomal inner cargo. Further, liposomes are prepared from hydrogenated egg phosphatidylcholine or egg phosphatidylcholine, cholesterol, and dicetyl phosphate, and their mean vesicle sizes were adjusted to about 50 and 100 nm. (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi: 10.1155/2011/469679 for review).

[00511] A liposome formulation may be mainly comprised of natural phospholipids and lipids such as 1,2-distearoyl-sn-glycero-3-phosphatidyl choline (DSPC), sphingomyelin, egg phosphatidylcholines and monosialoganglioside. Since this formulation is made up of phospholipids only, liposomal formulations have encountered many challenges, one of the ones being the instability in plasma. Several attempts to overcome these challenges have been

made, specifically in the manipulation of the lipid membrane. One of these attempts focused on the manipulation of cholesterol. Addition of cholesterol to conventional formulations reduces rapid release of the encapsulated bioactive compound into the plasma or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) increases the stability (see, e.g., Spuch and Navarro, *Journal of Drug Delivery*, vol. 2011, Article ID 469679, 12 pages, 2011. doi: 10.1155/2011/469679 for review).

[00512] In a particularly advantageous embodiment, Trojan Horse liposomes (also known as Molecular Trojan Horses) are desirable and protocols may be found at cshprotocols.cshlp.org/content/2010/4/pdb.prot5407.1.long. These particles allow delivery of a transgene to the entire brain after an intravascular injection. Without being bound by limitation, it is believed that neutral lipid particles with specific antibodies conjugated to surface allow crossing of the blood brain barrier via endocytosis. Applicant postulates utilizing Trojan Horse Liposomes to deliver the the DNA targeting agent according to the invention as described herein, such as by means of example the CRISPR family of nucleases to the brain via an intravascular injection, which would allow whole brain transgenic animals without the need for embryonic manipulation. About 1-5 g of DNA or RNA may be contemplated for in vivo administration in liposomes.

[00513] In another embodiment, the the DNA targeting agent according to the invention as described herein, such as by means of example the CRISPR Cas system may be administered in liposomes, such as a stable nucleic-acid-lipid particle (SNALP) (see, e.g., Morrissey et al, *Nature Biotechnology*, Vol. 23, No. 8, August 2005). Daily intravenous injections of about 1, 3 or 5 mg/kg/day of a specific CRISPR Cas targeted in a SNALP are contemplated. The daily treatment may be over about three days and then weekly for about five weeks. In another embodiment, a specific CRISPR Cas encapsulated SNALP) administered by intravenous injection to at doses of about 1 or 2.5 mg/kg are also contemplated (see, e.g., Zimmerman et al, *Nature Letters*, Vol. 441, 4 May 2006). The SNALP formulation may contain the lipids 3-N-[(w-methoxy poly(ethylene glycol) 2000) carbamoyl] -1,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio (see, e.g., Zimmerman et al, *Nature Letters*, Vol. 441, 4 May 2006).

[00514] In another embodiment, stable nucleic-acid-lipid particles (SNALPs) have proven to be effective delivery molecules to highly vascularized HepG2-derived liver tumors but not in poorly vascularized HCT-116 derived liver tumors (see, e.g., Li, *Gene Therapy* (2012) 19, 775-780). The SNALP liposomes may be prepared by formulating D-Lin-DMA and PEG-C-

DMA with distearoylphosphatidylcholine (DSPC), Cholesterol and siRNA using a 25:1 lipid/siRNA ratio and a 48/40/10/2 molar ratio of Cholesterol/D-Lin-DMA/DSPC/PEG-C-DMA. The resulted SNALP liposomes are about 80-100 nm in size.

[00515] In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich, St Louis, MO, USA), dipalmitoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA), 3-N-[(w-methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxypropylamine, and cationic 1,2-dilinoleyloxy-3-N,Ndimethylaminopropane (see, e.g., Geisbert et al, Lancet 2010; 375: 1896-905). A dosage of about 2 mg/kg total CRISPR Cas per dose administered as, for example, a bolus intravenous infusion may be contemplated.

[00516] In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids Inc.), PEG-cDMA, and 1,2-dilinoleyloxy-3-(N;N-dimethyl)aminopropane (DLinDMA) (see, e.g., Judge, J. Clin. Invest. 119:661-673 (2009)). Formulations used for in vivo studies may comprise a final lipid/RNA mass ratio of about 9:1.

[00517] The safety profile of RNAi nanomedicines has been reviewed by Barros and Gollob of Alnylam Pharmaceuticals (see, e.g., Advanced Drug Delivery Reviews 64 (2012) 1730-1737). The stable nucleic acid lipid particle (SNALP) is comprised of four different lipids — an ionizable lipid (DLinDMA) that is cationic at low pH, a neutral helper lipid, cholesterol, and a diffusible polyethylene glycol (PEG)-lipid. The particle is approximately 80 nm in diameter and is charge-neutral at physiologic pH. During formulation, the ionizable lipid serves to condense lipid with the anionic RNA during particle formation. When positively charged under increasingly acidic endosomal conditions, the ionizable lipid also mediates the fusion of SNALP with the endosomal membrane enabling release of RNA into the cytoplasm. The PEG-lipid stabilizes the particle and reduces aggregation during formulation, and subsequently provides a neutral hydrophilic exterior that improves pharmacokinetic properties.

[00518] To date, two clinical programs have been initiated using SNALP formulations with RNA. Tekmira Pharmaceuticals recently completed a phase I single-dose study of SNALP-ApoB in adult volunteers with elevated LDL cholesterol. ApoB is predominantly expressed in the liver and jejunum and is essential for the assembly and secretion of VLDL and LDL. Seventeen subjects received a single dose of SNALP-ApoB (dose escalation across 7 dose levels). There was no evidence of liver toxicity (anticipated as the potential dose-limiting toxicity based on preclinical studies). One (of two) subjects at the highest dose

experienced flu-like symptoms consistent with immune system stimulation, and the decision was made to conclude the trial.

[00519] Alnylam Pharmaceuticals has similarly advanced ALN-TTR01, which employs the SNALP technology described above and targets hepatocyte production of both mutant and wild-type TTR to treat TTR amyloidosis (ATTR). Three ATTR syndromes have been described: familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC) — both caused by autosomal dominant mutations in TTR; and senile systemic amyloidosis (SSA) cause by wildtype TTR. A placebo-controlled, single dose-escalation phase I trial of ALN-TTR01 was recently completed in patients with ATTR. ALN-TTR01 was administered as a 15-minute IV infusion to 31 patients (23 with study drug and 8 with placebo) within a dose range of 0.01 to 1.0 mg/kg (based on siRNA). Treatment was well tolerated with no significant increases in liver function tests. Infusion-related reactions were noted in 3 of 23 patients at >0.4 mg/kg; all responded to slowing of the infusion rate and all continued on study. Minimal and transient elevations of serum cytokines IL-6, IP-10 and IL-1ra were noted in two patients at the highest dose of 1 mg/kg (as anticipated from preclinical and NHP studies). Lowering of serum TTR, the expected pharmacodynamics effect of ALN-TTR01, was observed at 1 mg/kg.

[00520] In yet another embodiment, a SNALP may be made by solubilizing a cationic lipid, DSPC, cholesterol and PEG-lipid e.g., in ethanol, e.g., at a molar ratio of 40:10:40:10, respectively (see, Semple et al, Nature Nanotechnology, Volume 28 Number 2 February 2010, pp. 172-177). The lipid mixture was added to an aqueous buffer (50 mM citrate, pH 4) with mixing to a final ethanol and lipid concentration of 30% (vol/vol) and 6.1 mg/ml, respectively, and allowed to equilibrate at 22 °C for 2 min before extrusion. The hydrated lipids were extruded through two stacked 80 nm pore-sized filters (Nuclepore) at 22 °C using a Lipex Extruder (Northern Lipids) until a vesicle diameter of 70-90 nm, as determined by dynamic light scattering analysis, was obtained. This generally required 1-3 passes. The siRNA (solubilized in a 50 mM citrate, pH 4 aqueous solution containing 30% ethanol) was added to the pre-equilibrated (35 °C) vesicles at a rate of ~5 ml/min with mixing. After a final target siRNA/lipid ratio of 0.06 (wt/wt) was reached, the mixture was incubated for a further 30 min at 35 °C to allow vesicle reorganization and encapsulation of the siRNA. The ethanol was then removed and the external buffer replaced with PBS (155 mM NaCl, 3 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.5) by either dialysis or tangential flow diafiltration. siRNA were encapsulated in SNALP using a controlled step-wise dilution method process. The lipid constituents of KC2-SNALP were DLin-KC2-DMA (cationic lipid),

dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids), synthetic cholesterol (Sigma) and PEG-C-DMA used at a molar ratio of 57.1:7.1:34.3:1.4. Upon formation of the loaded particles, SNALP were dialyzed against PBS and filter sterilized through a 0.2 μm filter before use. Mean particle sizes were 75-85 nm and 90-95% of the siRNA was encapsulated within the lipid particles. The final siRNA/lipid ratio in formulations used for in vivo testing was -0.15 (wt/wt). LNP-siRNA systems containing Factor VII siRNA were diluted to the appropriate concentrations in sterile PBS immediately before use and the formulations were administered intravenously through the lateral tail vein in a total volume of 10 ml/kg. This method and these delivery systems may be extrapolated to the CRISPR Cas system of the present invention.

Other Lipids

[00521] Other cationic lipids, such as amino lipid 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA) may be utilized to encapsulate the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas or components thereof or nucleic acid molecule(s) coding therefor e.g., similar to SiRNA (see, e.g., Jayaraman, *Angew. Chem. Int. Ed.* 2012, 51, 8529 -8533), and hence may be employed in the practice of the invention. A preformed vesicle with the following lipid composition may be contemplated: amino lipid, distearoylphosphatidylcholine (DSPC), cholesterol and (R)-2,3-bis(octadecyloxy) propyl-1-(methoxy poly(ethylene glycol)2000)propylcarbamate (PEG-lipid) in the molar ratio 40/10/40/10, respectively, and a FVII siRNA/total lipid ratio of approximately 0.05 (w/w). To ensure a narrow particle size distribution in the range of 70-90 nm and a low polydispersity index of 0.11 ± 0.04 (n=56), the particles may be extruded up to three times through 80 nm membranes prior to adding the CRISPR Cas RNA. Particles containing the highly potent amino lipid 16 may be used, in which the molar ratio of the four lipid components 16, DSPC, cholesterol and PEG-lipid (50/10/38.5/1.5) which may be further optimized to enhance in vivo activity.

[00522] Michael S D Kormann et al. ("Expression of therapeutic proteins after delivery of chemically modified mRNA in mice: *Nature Biotechnology*, Volume:29, Pages: 154-157 (2011)) describes the use of lipid envelopes to deliver RNA. Use of lipid envelopes is also preferred in the present invention.

[00523] In another embodiment, lipids may be formulated with the CRISPR Cas system of the present invention to form lipid particles (LNPs). Lipids include, but are not limited to, DLin-KC2-DMA4, CI2-200 and colipids disteroylphosphatidyl choline, cholesterol, and

PEG-DMG may be formulated with CRISPR Cas instead of siRNA (see, e.g., Novobrantseva, *Molecular Therapy-Nucleic Acids* (2012) 1, e4; doi:10.1038/mtna.2011.3) using a spontaneous vesicle formation procedure. The component molar ratio may be about 50/10/38.5/1.5 (DLin-KC2-DMA or C12-200/disteroylphosphatidyl choline/cholesterol/PEG-DMG). The final lipid:siRNA weight ratio may be -12:1 and 9:1 in the case of DLin-KC2-DMA and C12-200 lipid particles (LNPs), respectively. The formulations may have mean particle diameters of -80 nm with >90% entrapment efficiency. A 3 mg/kg dose may be contemplated.

[00524] Tekmira has a portfolio of approximately 95 patent families, in the U.S. and abroad, that are directed to various aspects of LNPs and LNP formulations (see, e.g., U.S. Pat. Nos. 7,982,027; 7,799,565; 8,058,069; 8,283,333; 7,901,708; 7,745,651; 7,803,397; 8,101,741; 8,188,263; 7,915,399; 8,236,943 and 7,838,658 and European Pat. Nos 1766035; 1519714; 1781593 and 1664316), all of which may be used and/or adapted to the present invention.

[00525] The the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system or components thereof or nucleic acid molecule(s) coding therefor may be delivered encapsulated in PLGA Microspheres such as that further described in US published applications 20130252281 and 20130245107 and 20130244279 (assigned to Moderna Therapeutics) which relate to aspects of formulation of compositions comprising modified nucleic acid molecules which may encode a protein, a protein precursor, or a partially or fully processed form of the protein or a protein precursor. The formulation may have a molar ratio 50:10:38.5:1.5-3.0 (cationic lipid: fusogenic lipid:cholesterol:PEG lipid). The PEG lipid may be selected from, but is not limited to PEG-c-DOMG, PEG-DMG. The fusogenic lipid may be DSPC. See also, Schrum et al, *Delivery and Formulation of Engineered Nucleic Acids*, US published application 20120251618.

[00526] Nanomerics' technology addresses bioavailability challenges for a broad range of therapeutics, including low molecular weight hydrophobic drugs, peptides, and nucleic acid based therapeutics (plasmid, siRNA, miRNA). Specific administration routes for which the technology has demonstrated clear advantages include the oral route, transport across the blood-brain-barrier, delivery to solid tumours, as well as to the eye. See, e.g., Mazza et al, 2013, *ACS Nano*. 2013 Feb 26;7(2):1016-26; Uchegbu and Siew, 2013, *J Pharm Sci*. 102(2):305-10 and Lalatsa et al, 2012, *J Control Release*. 2012 Jul 20; 161(2):523-36.

[00527] US Patent Publication No. 20050019923 describes cationic dendrimers for delivering bioactive molecules, such as polynucleotide molecules, peptides and polypeptides

and/or pharmaceutical agents, to a mammalian body. The dendrimers are suitable for targeting the delivery of the bioactive molecules to, for example, the liver, spleen, lung, kidney or heart (or even the brain). Dendrimers are synthetic 3-dimensional macromolecules that are prepared in a step-wise fashion from simple branched monomer units, the nature and functionality of which can be easily controlled and varied. Dendrimers are synthesised from the repeated addition of building blocks to a multifunctional core (divergent approach to synthesis), or towards a multifunctional core (convergent approach to synthesis) and each addition of a 3-dimensional shell of building blocks leads to the formation of a higher generation of the dendrimers. Polypropylenimine dendrimers start from a diaminobutane core to which is added twice the number of amino groups by a double Michael addition of acrylonitrile to the primary amines followed by the hydrogenation of the nitriles. This results in a doubling of the amino groups. Polypropylenimine dendrimers contain 100% protonable nitrogens and up to 64 terminal amino groups (generation 5, DAB 64). Protonable groups are usually amine groups which are able to accept protons at neutral pH. The use of dendrimers as gene delivery agents has largely focused on the use of the polyamidoamine, and phosphorous containing compounds with a mixture of amine/amide or N~P(O)S as the conjugating units respectively with no work being reported on the use of the lower generation polypropylenimine dendrimers for gene delivery. Polypropylenimine dendrimers have also been studied as pH sensitive controlled release systems for drug delivery and for their encapsulation of guest molecules when chemically modified by peripheral amino acid groups. The cytotoxicity and interaction of polypropylenimine dendrimers with DNA as well as the transfection efficacy of DAB 64 has also been studied.

[00528] US Patent Publication No. 20050019923 is based upon the observation that, contrary to earlier reports, cationic dendrimers, such as polypropylenimine dendrimers, display suitable properties, such as specific targeting and low toxicity, for use in the targeted delivery of bioactive molecules, such as genetic material. In addition, derivatives of the cationic dendrimer also display suitable properties for the targeted delivery of bioactive molecules. See also, Bioactive Polymers, US published application 20080267903, which discloses "Various polymers, including cationic polyamine polymers and dendrimeric polymers, are shown to possess anti-proliferative activity, and may therefore be useful for treatment of disorders characterised by undesirable cellular proliferation such as neoplasms and tumours, inflammatory disorders (including autoimmune disorders), psoriasis and atherosclerosis. The polymers may be used alone as active agents, or as delivery vehicles for other therapeutic agents, such as drug molecules or nucleic acids for gene therapy. In such

cases, the polymers' own intrinsic anti-tumour activity may complement the activity of the agent to be delivered." The disclosures of these patent publications may be employed in conjunction with herein teachings for delivery of CRISPR Cas system(s) or component(s) thereof or nucleic acid molecule(s) coding therefor.

Supercharged proteins

[00529] Supercharged proteins are a class of engineered or naturally occurring proteins with unusually high positive or negative net theoretical charge and may be employed in delivery of the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system(s) or component(s) thereof or nucleic acid molecule(s) coding therefor. Both supernegatively and superpositively charged proteins exhibit a remarkable ability to withstand thermally or chemically induced aggregation. Superpositively charged proteins are also able to penetrate mammalian cells. Associating cargo with these proteins, such as plasmid DNA, RNA, or other proteins, can enable the functional delivery of these macromolecules into mammalian cells both in vitro and in vivo. David Liu's lab reported the creation and characterization of supercharged proteins in 2007 (Lawrence et al, 2007, Journal of the American Chemical Society 129, 10110-10112).

[00530] The nonviral delivery of RNA and plasmid DNA into mammalian cells are valuable both for research and therapeutic applications (Akinc et al., 2010, Nat. Biotech. 26, 561-569). Purified +36 GFP protein (or other superpositively charged protein) is mixed with RNAs in the appropriate serum-free media and allowed to complex prior addition to cells. Inclusion of serum at this stage inhibits formation of the supercharged protein-RNA complexes and reduces the effectiveness of the treatment. The following protocol has been found to be effective for a variety of cell lines (McNaughton et al, 2009, Proc. Natl. Acad. Sci. USA 106, 6111-6116) (However, pilot experiments varying the dose of protein and RNA should be performed to optimize the procedure for specific cell lines): (1) One day before treatment, plate 1×10^5 cells per well in a 48-well plate. (2) On the day of treatment, dilute purified +36 GFP protein in serumfree media to a final concentration 200nM. Add RNA to a final concentration of 50nM. Vortex to mix and incubate at room temperature for 10min. (3) During incubation, aspirate media from cells and wash once with PBS. (4) Following incubation of +36 GFP and RNA, add the protein-RNA complexes to cells. (5) Incubate cells with complexes at 37 °C for 4h. (6) Following incubation, aspirate the media and wash three times with 20 U/mL heparin PBS. Incubate cells with serum-containing media

for a further 48h or longer depending upon the assay for activity. (7) Analyze cells by immunoblot, qPCR, phenotypic assay, or other appropriate method.

[00531] David Liu's lab has further found +36 GFP to be an effective plasmid delivery reagent in a range of cells. As plasmid DNA is a larger cargo than siRNA, proportionately more +36 GFP protein is required to effectively complex plasmids. For effective plasmid delivery Applicants have developed a variant of +36 GFP bearing a C-terminal HA2 peptide tag, a known endosome-disrupting peptide derived from the influenza virus hemagglutinin protein. The following protocol has been effective in a variety of cells, but as above it is advised that plasmid DNA and supercharged protein doses be optimized for specific cell lines and delivery applications: (1) One day before treatment, plate 1×10^5 per well in a 48-well plate. (2) On the day of treatment, dilute purified p36 GFP protein in serumfree media to a final concentration 2 mM. Add 1mg of plasmid DNA. Vortex to mix and incubate at room temperature for 10min. (3) During incubation, aspirate media from cells and wash once with PBS. (4) Following incubation of p36 GFP and plasmid DNA, gently add the protein-DNA complexes to cells. (5) Incubate cells with complexes at 37 C for 4h. (6) Following incubation, aspirate the media and wash with PBS. Incubate cells in serum-containing media and incubate for a further 24-48h. (7) Analyze plasmid delivery (e.g., by plasmid-driven gene expression) as appropriate. See also, e.g., McNaughton et al, Proc. Natl. Acad. Sci. USA 106, 6111-6116 (2009); Cronican et al, ACS Chemical Biology 5, 747-752 (2010); Cronican et al, Chemistry & Biology 18, 833-838 (2011); Thompson et al, Methods in Enzymology 503, 293-319 (2012); Thompson, D.B., et al, Chemistry & Biology 19 (7), 831-843 (2012). The methods of the super charged proteins may be used and/or adapted for delivery of the CRISPR Cas system of the present invention. These systems of Dr. Lui and documents herein in conjunction with herein teachints can be employed in the delivery of the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system(s) or component(s) thereof or nucleic acid molecule(s) coding therefor.

Cell Penetrating Peptides (CPPs)

[00532] In yet another embodiment, cell penetrating peptides (CPPs) are contemplated for the delivery of the the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system. CPPs are short peptides that facilitate cellular uptake of various molecular cargo (from nanosize particles to small chemical molecules and large fragments of DNA). The term "cargo" as used herein includes but is not limited to the group consisting of therapeutic agents, diagnostic probes, peptides, nucleic

acids, antisense oligonucleotides, plasmids, proteins, particles, liposomes, chromophores, small molecules and radioactive materials. In aspects of the invention, the cargo may also comprise any component of the the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system or the entire functional CRISPR Cas system. Aspects of the present invention further provide methods for delivering a desired cargo into a subject comprising: (a) preparing a complex comprising the cell penetrating peptide of the present invention and a desired cargo, and (b) orally, intraarticularly, intraperitoneally, intrathecally, intrarterially, intranasally, intraparenchymally, subcutaneously, intramuscularly, intravenously, dermally, intrarectally, or topically administering the complex to a subject. The cargo is associated with the peptides either through chemical linkage via covalent bonds or through non-covalent interactions.

[00533] The function of the CPPs are to deliver the cargo into cells, a process that commonly occurs through endocytosis with the cargo delivered to the endosomes of living mammalian cells. Cell-penetrating peptides are of different sizes, amino acid sequences, and charges but all CPPs have one distinct characteristic, which is the ability to translocate the plasma membrane and facilitate the delivery of various molecular cargoes to the cytoplasm or an organelle. CPP translocation may be classified into three main entry mechanisms: direct penetration in the membrane, endocytosis-mediated entry, and translocation through the formation of a transitory structure. CPPs have found numerous applications in medicine as drug delivery agents in the treatment of different diseases including cancer and virus inhibitors, as well as contrast agents for cell labeling. Examples of the latter include acting as a carrier for GFP, MRI contrast agents, or quantum dots. CPPs hold great potential as *in vitro* and *in vivo* delivery vectors for use in research and medicine. CPPs typically have an amino acid composition that either contains a high relative abundance of positively charged amino acids such as lysine or arginine or has sequences that contain an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids. These two types of structures are referred to as polycationic or amphipathic, respectively. A third class of CPPs are the hydrophobic peptides, containing only apolar residues, with low net charge or have hydrophobic amino acid groups that are crucial for cellular uptake. One of the initial CPPs discovered was the trans-activating transcriptional activator (Tat) from Human Immunodeficiency Virus 1 (HIV-1) which was found to be efficiently taken up from the surrounding media by numerous cell types in culture. Since then, the number of known CPPs has expanded considerably and small molecule synthetic analogues with more effective

protein transduction properties have been generated. CPPs include but are not limited to Penetratin, Tat (48-60), Transportan, and (R-Ahx-R4) (Ahx=aminohexanoyl).

[00534] US Patent 8,372,951, provides a CPP derived from eosinophil cationic protein (ECP) which exhibits highly cell-penetrating efficiency and low toxicity. Aspects of delivering the CPP with its cargo into a vertebrate subject are also provided. Further aspects of CPPs and their delivery are described in U. S. patents 8,575,305; 8,614,194 and 8,044,019. CPPs can be used to deliver the CRISPR-Cas system or components thereof. That CPPs can be employed to deliver the CRISPR-Cas system or components thereof is also provided in the manuscript "Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA", by Suresh Ramakrishna, Abu-Bonsrah Kwaku Dad, Jagadish Beloor, et al. Genome Res. 2014 Apr 2. [Epub ahead of print], incorporated by reference in its entirety, wherein it is demonstrated that treatment with CPP-conjugated recombinant Cas9 protein and CPP-complexed guide RNAs lead to endogenous gene disruptions in human cell lines. In the paper the Cas9 protein was conjugated to CPP via a thioether bond, whereas the guide RNA was complexed with CPP, forming condensed, positively charged particles. It was shown that simultaneous and sequential treatment of human cells, including embryonic stem cells, dermal fibroblasts, HEK293T cells, HeLa cells, and embryonic carcinoma cells, with the modified Cas9 and guide RNA led to efficient gene disruptions with reduced off-target mutations relative to plasmid transfections.

Implantable devices

[00535] In another embodiment, implantable devices are also contemplated for delivery of the the DNA targeting agent according to the invention as described herein, such as by means of example the CRISPR Cas system or component(s) thereof or nucleic acid molecule(s) coding therefor. For example, US Patent Publication 20110195123 discloses an implantable medical device which elutes a drug locally and in prolonged period is provided, including several types of such a device, the treatment modes of implementation and methods of implantation. The device comprising of polymeric substrate, such as a matrix for example, that is used as the device body, and drugs, and in some cases additional scaffolding materials, such as metals or additional polymers, and materials to enhance visibility and imaging. An implantable delivery device can be advantageous in providing release locally and over a prolonged period, where drug is released directly to the extracellular matrix (ECM) of the diseased area such as tumor, inflammation, degeneration or for symptomatic objectives, or to injured smooth muscle cells, or for prevention. One kind of drug is RNA, as disclosed above,

and this system may be used/and or adapted to the the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system of the present invention. The modes of implantation in some embodiments are existing implantation procedures that are developed and used today for other treatments, including brachytherapy and needle biopsy. In such cases the dimensions of the new implant described in this invention are similar to the original implant. Typically a few devices are implanted during the same treatment procedure.

[00536] As described in US Patent Publication 201 10 195 123, there is provided a drug delivery implantable or insertable system, including systems applicable to a cavity such as the abdominal cavity and/or any other type of administration in which the drug delivery system is not anchored or attached, comprising a biostable and/or degradable and/or bioabsorbable polymeric substrate, which may for example optionally be a matrix. It should be noted that the term "insertion" also includes implantation. The drug delivery system is preferably implemented as a "Loder" as described in US Patent Publication 201 10 195 123.

[00537] The polymer or plurality of polymers are biocompatible, incorporating an agent and/or plurality of agents, enabling the release of agent at a controlled rate, wherein the total volume of the polymeric substrate, such as a matrix for example, in some embodiments is optionally and preferably no greater than a maximum volume that permits a therapeutic level of the agent to be reached. As a non-limiting example, such a volume is preferably within the range of 0.1 m³ to 1000 mm³, as required by the volume for the agent load. The Loder may optionally be larger, for example when incorporated with a device whose size is determined by functionality, for example and without limitation, a knee joint, an intra-uterine or cervical ring and the like.

[00538] The drug delivery system (for delivering the composition) is designed in some embodiments to preferably employ degradable polymers, wherein the main release mechanism is bulk erosion; or in some embodiments, non degradable, or slowly degraded polymers are used, wherein the main release mechanism is diffusion rather than bulk erosion, so that the outer part functions as membrane, and its internal part functions as a drug reservoir, which practically is not affected by the surroundings for an extended period (for example from about a week to about a few months). Combinations of different polymers with different release mechanisms may also optionally be used. The concentration gradient at the surface is preferably maintained effectively constant during a significant period of the total drug releasing period, and therefore the diffusion rate is effectively constant (termed "zero mode" diffusion). By the term "constant" it is meant a diffusion rate that is preferably

maintained above the lower threshold of therapeutic effectiveness, but which may still optionally feature an initial burst and/or may fluctuate, for example increasing and decreasing to a certain degree. The diffusion rate is preferably so maintained for a prolonged period, and it can be considered constant to a certain level to optimize the therapeutically effective period, for example the effective silencing period.

[00539] The drug delivery system optionally and preferably is designed to shield the nucleotide based therapeutic agent from degradation, whether chemical in nature or due to attack from enzymes and other factors in the body of the subject.

[00540] The drug delivery system as described in US Patent Publication 201 10 195 123 is optionally associated with sensing and/or activation appliances that are operated at and/or after implantation of the device, by non and/or minimally invasive methods of activation and/or acceleration/deceleration, for example optionally including but not limited to thermal heating and cooling, laser beams, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices.

[00541] According to some embodiments of US Patent Publication 201 10 195 123, the site for local delivery may optionally include target sites characterized by high abnormal proliferation of cells, and suppressed apoptosis, including tumors, active and or chronic inflammation and infection including autoimmune diseases states, degenerating tissue including muscle and nervous tissue, chronic pain, degenerative sites, and location of bone fractures and other wound locations for enhancement of regeneration of tissue, and injured cardiac, smooth and striated muscle.

[00542] The site for implantation of the composition, or target site, preferably features a radius, area and/or volume that is sufficiently small for targeted local delivery. For example, the target site optionally has a diameter in a range of from about 0.1 mm to about 5 cm.

[00543] The location of the target site is preferably selected for maximum therapeutic efficacy. For example, the composition of the drug delivery system (optionally with a device for implantation as described above) is optionally and preferably implanted within or in the proximity of a tumor environment, or the blood supply associated thereof.

[00544] For example the composition (optionally with the device) is optionally implanted within or in the proximity to pancreas, prostate, breast, liver, via the nipple, within the vascular system and so forth.

[00545] The target location is optionally selected from the group consisting of (as non-limiting examples only, as optionally any site within the body may be suitable for implanting a Loder): 1. brain at degenerative sites like in Parkinson or Alzheimer disease at the basal

ganglia, white and gray matter; 2. spine as in the case of amyotrophic lateral sclerosis (ALS); 3. uterine cervix to prevent HPV infection; 4. active and chronic inflammatory joints; 5. dermis as in the case of psoriasis; 6. sympathetic and sensoric nervous sites for analgesic effect; 7. Intra osseous implantation; 8. acute and chronic infection sites; 9. Intra vaginal; 10. Inner ear-auditory system, labyrinth of the inner ear, vestibular system; 11. Intra tracheal; 12. Intra-cardiac; coronary, epicardiac; 13. urinary bladder; 14. biliary system; 15. parenchymal tissue including and not limited to the kidney, liver, spleen; 16. lymph nodes; 17. salivary glands; 18. dental gums; 19. Intra-articular (into joints); 20. Intra-ocular; 21. Brain tissue; 22. Brain ventricles; 23. Cavities, including abdominal cavity (for example but without limitation, for ovary cancer); 24. Intra esophageal and 25. Intra rectal.

[00546] Optionally insertion of the system (for example a device containing the composition) is associated with injection of material to the ECM at the target site and the vicinity of that site to affect local pH and/or temperature and/or other biological factors affecting the diffusion of the drug and/or drug kinetics in the ECM, of the target site and the vicinity of such a site.

[00547] Optionally, according to some embodiments, the release of said agent could be associated with sensing and/or activation appliances that are operated prior and/or at and/or after insertion, by non and/or minimally invasive and/or else methods of activation and/or acceleration/deceleration, including laser beam, radiation, thermal heating and cooling, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices, and chemical activators.

[00548] According to other embodiments of US Patent Publication 20110195123, the drug preferably comprises a RNA, for example for localized cancer cases in breast, pancreas, brain, kidney, bladder, lung, and prostate as described below. Although exemplified with RNAi, many drugs are applicable to be encapsulated in Loder, and can be used in association with this invention, as long as such drugs can be encapsulated with the Loder substrate, such as a matrix for example, and this system may be used and/or adapted to deliver the CRISPR Cas system of the present invention.

[00549] As another example of a specific application, neuro and muscular degenerative diseases develop due to abnormal gene expression. Local delivery of RNAs may have therapeutic properties for interfering with such abnormal gene expression. Local delivery of anti apoptotic, anti inflammatory and anti degenerative drugs including small drugs and macromolecules may also optionally be therapeutic. In such cases the Loder is applied for prolonged release at constant rate and/or through a dedicated device that is implanted

separately. All of this may be used and/or adapted to the the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system of the present invention.

[00550] As yet another example of a specific application, psychiatric and cognitive disorders are treated with gene modifiers. Gene knockdown is a treatment option. Loders locally delivering agents to central nervous system sites are therapeutic options for psychiatric and cognitive disorders including but not limited to psychosis, bi-polar diseases, neurotic disorders and behavioral maladies. The Loders could also deliver locally drugs including small drugs and macromolecules upon implantation at specific brain sites. All of this may be used and/or adapted to the CRISPR Cas system of the present invention.

[00551] As another example of a specific application, silencing of innate and/or adaptive immune mediators at local sites enables the prevention of organ transplant rejection. Local delivery of RNAs and immunomodulating reagents with the Loder implanted into the transplanted organ and/or the implanted site renders local immune suppression by repelling immune cells such as CD8 activated against the transplanted organ. All of this may be used/and or adapted to the the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system of the present invention.

[00552] As another example of a specific application, vascular growth factors including VEGFs and angiogenin and others are essential for neovascularization. Local delivery of the factors, peptides, peptidomimetics, or suppressing their repressors is an important therapeutic modality; silencing the repressors and local delivery of the factors, peptides, macromolecules and small drugs stimulating angiogenesis with the Loder is therapeutic for peripheral, systemic and cardiac vascular disease.

[00553] The method of insertion, such as implantation, may optionally already be used for other types of tissue implantation and/or for insertions and/or for sampling tissues, optionally without modifications, or alternatively optionally only with non-major modifications in such methods. Such methods optionally include but are not limited to brachytherapy methods, biopsy, endoscopy with and/or without ultrasound, such as ERCP, stereotactic methods into the brain tissue, Laparoscopy, including implantation with a laparoscope into joints, abdominal organs, the bladder wall and body cavities.

[00554] Implantable device technology herein discussed can be employed with herein teachings and hence by this disclosure and the knowledge in the art, the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR-Cas

system or components thereof or nucleic acid molecules thereof or encoding or providing components may be delivered via an implantable device.

[00555] The present application also contemplates an inducible CRISPR Cas system. Reference is made to international patent application Serial No. PCT/US13/51418 filed July 21, 2013, which published as WO2014/018423 on January 30, 2014.

[00556] In one aspect the invention provides a DNA targeting agent according to the invention as described herein, such as by means of example a non-naturally occurring or engineered CRISPR Cas system which may comprise at least one switch wherein the activity of said CRISPR Cas system is controlled by contact with at least one inducer energy source as to the switch. In an embodiment of the invention the control as to the at least one switch or the activity of said CRISPR Cas system may be activated, enhanced, terminated or repressed. The contact with the at least one inducer energy source may result in a first effect and a second effect.

[00557] The first effect may be one or more of nuclear import, nuclear export, recruitment of a secondary component (such as an effector molecule), conformational change (of protein, DNA or RNA), cleavage, release of cargo (such as a caged molecule or a co-factor), association or dissociation. The second effect may be one or more of activation, enhancement, termination or repression of the control as to the at least one switch or the activity of said the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system. In one embodiment the first effect and the second effect may occur in a cascade.

[00558] The invention comprehends that the inducer energy source may be heat, ultrasound, electromagnetic energy or chemical. In a preferred embodiment of the invention, the inducer energy source may be an antibiotic, a small molecule, a hormone, a hormone derivative, a steroid or a steroid derivative. In a more preferred embodiment, the inducer energy source maybe abscisic acid (ABA), doxycycline (DOX), cumate, rapamycin, 4-hydroxytamoxifen (4OHT), estrogen or ecdysone.

[00559] The invention provides that the at least one switch may be selected from the group consisting of antibiotic based inducible systems, electromagnetic energy based inducible systems, small molecule based inducible systems, nuclear receptor based inducible systems and hormone based inducible systems. In a more preferred embodiment the at least one switch may be selected from the group consisting of tetracycline (Tet)/DOX inducible systems, light inducible systems, ABA inducible systems, cumate repressor/operator systems,

40HT/estrogen inducible systems, ecdysone-based inducible systems and FKBP12/FRAP (FKBP12-rapamycin complex) inducible systems.

[00560] In one aspect of the invention the inducer energy source is electromagnetic energy. The electromagnetic energy may be a component of visible light having a wavelength in the range of 450nm-700nm. In a preferred embodiment the component of visible light may have a wavelength in the range of 450nm-500nm and may be blue light. The blue light may have an intensity of at least 0.2mW/cm², or more preferably at least 4mW/cm². In another embodiment, the component of visible light may have a wavelength in the range of 620-700nm and is red light.

[00561] In a further aspect, the invention provides a method of controlling a the DNA targeting agent according to the invention as described herein, such as by means of example a non-naturally occurring or engineered CRISPR Cas system, comprising providing said CRISPR Cas system comprising at least one switch wherein the activity of said CRISPR Cas system is controlled by contact with at least one inducer energy source as to the switch.

[00562] In an embodiment of the invention, the invention provides methods wherein the control as to the at least one switch or the activity of said the DNA targeting agent according to the invention as described herein, such as by means of example CRISPR Cas system may be activated, enhanced, terminated or repressed. The contact with the at least one inducer energy source may result in a first effect and a second effect. The first effect may be one or more of nuclear import, nuclear export, recruitment of a secondary component (such as an effector molecule), conformational change (of protein, DNA or RNA), cleavage, release of cargo (such as a caged molecule or a co-factor), association or dissociation. The second effect may be one or more of activation, enhancement, termination or repression of the control as to the at least one switch or the activity of said CRISPR Cas system. In one embodiment the first effect and the second effect may occur in a cascade.

[00563] The invention comprehends that the inducer energy source may be heat, ultrasound, electromagnetic energy or chemical. In a preferred embodiment of the invention, the inducer energy source may be an antibiotic, a small molecule, a hormone, a hormone derivative, a steroid or a steroid derivative. In a more preferred embodiment, the inducer energy source maybe abscisic acid (ABA), doxycycline (DOX), cumate, rapamycin, 4-hydroxytamoxifen (40HT), estrogen or ecdysone. The invention provides that the at least one switch may be selected from the group consisting of antibiotic based inducible systems, electromagnetic energy based inducible systems, small molecule based inducible systems, nuclear receptor based inducible systems and hormone based inducible systems. In a more

preferred embodiment the at least one switch may be selected from the group consisting of tetracycline (Tet)/DOX inducible systems, light inducible systems, ABA inducible systems, cumate repressor/operator systems, 40HT/estrogen inducible systems, ecdysone-based inducible systems and FKBP12/FRAP (FKBP12-rapamycin complex) inducible systems.

[00564] In one aspect of the methods of the invention the inducer energy source is electromagnetic energy. The electromagnetic energy may be a component of visible light having a wavelength in the range of 450nm-700nm. In a preferred embodiment the component of visible light may have a wavelength in the range of 450nm-500nm and may be blue light. The blue light may have an intensity of at least 0.2mW/cm², or more preferably at least 4mW/cm². In another embodiment, the component of visible light may have a wavelength in the range of 620-700nm and is red light.

[00565] In another preferred embodiment of the invention, the inducible effector may be a Light Inducible Transcriptional Effector (LITE). The modularity of the LITE system allows for any number of effector domains to be employed for transcriptional modulation. In yet another preferred embodiment of the invention, the inducible effector may be a chemical. The invention also contemplates an inducible multiplex genome engineering using CRISPR (clustered regularly interspaced short palindromic repeats)/Cas systems.

Self-inactivating systems

[00566] Once all copies of a gene in the genome of a cell have been edited, continued CRISPR/Cas9 expression in that cell is no longer necessary. Indeed, sustained expression would be undesirable in case of off-target effects at unintended genomic sites, *etc.* Thus time-limited expression would be useful. Inducible expression offers one approach, but in addition Applicants have engineered a Self-Inactivating CRISPR-Cas9 system that relies on the use of a non-coding guide target sequence within the CRISPR vector itself. Thus, after expression begins, the CRISPR system will lead to its own destruction, but before destruction is complete it will have time to edit the genomic copies of the target gene (which, with a normal point mutation in a diploid cell, requires at most two edits). Simply, the self inactivating CRISPR-Cas system includes additional RNA (i.e., guide RNA) that targets the coding sequence for the CRISPR enzyme itself or that targets one or more non-coding guide target sequences complementary to unique sequences present in one or more of the following:

- (a) within the promoter driving expression of the non-coding RNA elements,
- (b) within the promoter driving expression of the Cas9 gene,
- (c) within 100bp of the ATG translational start codon in the Cas9 coding sequence,

(d) within the inverted terminal repeat (iTR) of a viral delivery vector, e.g., in the AAV genome.

[00567] Furthermore, that RNA can be delivered via a vector, e.g., a separate vector or the same vector that is encoding the CRISPR complex. When provided by a separate vector, the CRISPR RNA that targets Cas expression can be administered sequentially or simultaneously. When administered sequentially, the CRISPR RNA that targets Cas expression is to be delivered after the CRISPR RNA that is intended for e.g. gene editing or gene engineering. This period may be a period of minutes (e.g. 5 minutes, 10 minutes, 20 minutes, 30 minutes, 45 minutes, 60 minutes). This period may be a period of hours (e.g. 2 hours, 4 hours, 6 hours, 8 hours, 12 hours, 24 hours). This period may be a period of days (e.g. 2 days, 3 days, 4 days, 7 days). This period may be a period of weeks (e.g. 2 weeks, 3 weeks, 4 weeks). This period may be a period of months (e.g. 2 months, 4 months, 8 months, 12 months). This period may be a period of years (2 years, 3 years, 4 years). In this fashion, the Cas enzyme associates with a first gRNA/chiRNA capable of hybridizing to a first target, such as a genomic locus or loci of interest and undertakes the function(s) desired of the CRISPR-Cas system (e.g., gene engineering); and subsequently the Cas enzyme may then associate with the second gRNA/chiRNA capable of hybridizing to the sequence comprising at least part of the Cas or CRISPR cassette. Where the gRNA/chiRNA targets the sequences encoding expression of the Cas protein, the enzyme becomes impeded and the system becomes self inactivating. In the same manner, CRISPR RNA that targets Cas expression applied via, for example liposome, lipofection, nanoparticles, microvesicles as explained herein, may be administered sequentially or simultaneously. Similarly, self-inactivation may be used for inactivation of one or more guide RNA used to target one or more targets.

[00568] In some aspects, a single gRNA is provided that is capable of hybridization to a sequence downstream of a CRISPR enzyme start codon, whereby after a period of time there is a loss of the CRISPR enzyme expression. In some aspects, one or more gRNA(s) are provided that are capable of hybridization to one or more coding or non-coding regions of the polynucleotide encoding the CRISPR-Cas system, whereby after a period of time there is a inactivation of one or more, or in some cases all, of the CRISPR-Cas system. In some aspects of the system, and not to be limited by theory, the cell may comprise a plurality of CRISPR-Cas complexes, wherein a first subset of CRISPR complexes comprise a first chiRNA capable of targeting a genomic locus or loci to be edited, and a second subset of CRISPR complexes comprise at least one second chiRNA capable of targeting the polynucleotide encoding the CRISPR-Cas system, wherein the first subset of CRISPR-Cas

complexes mediate editing of the targeted genomic locus or loci and the second subset of CRISPR complexes eventually inactivate the CRISPR-Cas system, thereby inactivating further CRISPR-Cas expression in the cell.

[00569] Thus the invention provides a CRISPR-Cas system comprising one or more vectors for delivery to a eukaryotic cell, wherein the vector(s) encode(s): (i) a CRISPR enzyme; (ii) a first guide RNA capable of hybridizing to a target sequence in the cell; (iii) a second guide RNA capable of hybridizing to one or more target sequence(s) in the vector which encodes the CRISPR enzyme; (iv) at least one tracr mate sequence; and (v) at least one tracr sequence. The first and second complexes can use the same tracr and tracr mate, thus differing only by the guide sequence, wherein, when expressed within the cell: the first guide RNA directs sequence-specific binding of a first CRISPR complex to the target sequence in the cell; the second guide RNA directs sequence-specific binding of a second CRISPR complex to the target sequence in the vector which encodes the CRISPR enzyme; the CRISPR complexes comprise (a) a tracr mate sequence hybridised to a tracr sequence and (b) a CRISPR enzyme bound to a guide RNA, such that a guide RNA can hybridize to its target sequence; and the second CRISPR complex inactivates the CRISPR-Cas system to prevent continued expression of the CRISPR enzyme by the cell.

[00570] Further characteristics of the vector(s), the encoded enzyme, the guide sequences, *etc.* are disclosed elsewhere herein. For instance, one or both of the guide sequence(s) can be part of a chiRNA sequence which provides the guide, tracr mate and tracr sequences within a single RNA, such that the system can encode (i) a CRISPR enzyme; (ii) a first chiRNA comprising a sequence capable of hybridizing to a first target sequence in the cell, a first tracr mate sequence, and a first tracr sequence; (iii) a second guide RNA capable of hybridizing to the vector which encodes the CRISPR enzyme, a second tracr mate sequence, and a second tracr sequence. Similarly, the enzyme can include one or more NLS, *etc.*

[00571] The various coding sequences (CRISPR enzyme, guide RNAs, tracr and tracr mate) can be included on a single vector or on multiple vectors. For instance, it is possible to encode the enzyme on one vector and the various RNA sequences on another vector, or to encode the enzyme and one chiRNA on one vector, and the remaining chiRNA on another vector, or any other permutation. In general, a system using a total of one or two different vectors is preferred.

[00572] Where multiple vectors are used, it is possible to deliver them in unequal numbers, and ideally with an excess of a vector which encodes the first guide RNA relative to

the second guide RNA, thereby assisting in delaying final inactivation of the CRISPR system until genome editing has had a chance to occur.

[00573] The first guide RNA can target any target sequence of interest within a genome, as described elsewhere herein. The second guide RNA targets a sequence within the vector which encodes the CRISPR Cas9 enzyme, and thereby inactivates the enzyme's expression from that vector. Thus the target sequence in the vector must be capable of inactivating expression. Suitable target sequences can be, for instance, near to or within the translational start codon for the Cas9 coding sequence, in a non-coding sequence in the promoter driving expression of the non-coding RNA elements, within the promoter driving expression of the Cas9 gene, within 100bp of the ATG translational start codon in the Cas9 coding sequence, and/or within the inverted terminal repeat (iTR) of a viral delivery vector, e.g., in the AAV genome. A double stranded break near this region can induce a frame shift in the Cas9 coding sequence, causing a loss of protein expression. An alternative target sequence for the "self-inactivating" guide RNA would aim to edit/inactivate regulatory regions/sequences needed for the expression of the CRISPR-Cas9 system or for the stability of the vector. For instance, if the promoter for the Cas9 coding sequence is disrupted then transcription can be inhibited or prevented. Similarly, if a vector includes sequences for replication, maintenance or stability then it is possible to target these. For instance, in a AAV vector a useful target sequence is within the iTR. Other useful sequences to target can be promoter sequences, polyadenylation sites, etc.

[00574] Furthermore, if the guide RNAs are expressed in array format, the "self-inactivating" guide RNAs that target both promoters simultaneously will result in the excision of the intervening nucleotides from within the CRISPR-Cas expression construct, effectively leading to its complete inactivation. Similarly, excision of the intervening nucleotides will result where the guide RNAs target both ITRs, or targets two or more other CRISPR-Cas components simultaneously. Self-inactivation as explained herein is applicable, in general, with CRISPR-Cas9 systems in order to provide regulation of the CRISPR-Cas9. For example, self-inactivation as explained herein may be applied to the CRISPR repair of mutations, for example expansion disorders, as explained herein. As a result of this self-inactivation, CRISPR repair is only transiently active.

[00575] Addition of non-targeting nucleotides to the 5' end (e.g. 1 - 10 nucleotides, preferably 1 - 5 nucleotides) of the "self-inactivating" guide RNA can be used to delay its processing and/or modify its efficiency as a means of ensuring editing at the targeted genomic locus prior to CRISPR-Cas9 shutdown.

[00576] In one aspect of the self-inactivating AAV-CRISPR-Cas9 system, plasmids that co-express one or more sgRNA targeting genomic sequences of interest (e.g. 1-2, 1-5, 1-10, 1-15, 1-20, 1-30) may be established with "self-inactivating" sgRNAs that target an SpCas9 sequence at or near the engineered ATG start site (e.g. within 5 nucleotides, within 15 nucleotides, within 30 nucleotides, within 50 nucleotides, within 100 nucleotides). A regulatory sequence in the U6 promoter region can also be targeted with an sgRNA. The U6-driven sgRNAs may be designed in an array format such that multiple sgRNA sequences can be simultaneously released. When first delivered into target tissue/cells (left cell) sgRNAs begin to accumulate while Cas9 levels rise in the nucleus. Cas9 complexes with all of the sgRNAs to mediate genome editing and self-inactivation of the CRISPR-Cas9 plasmids.

[00577] One aspect of a self-inactivating CRISPR-Cas9 system is expression of singly or in tandem array format from 1 up to 4 or more different guide sequences; e.g. up to about 20 or about 30 guides sequences. Each individual self inactivating guide sequence may target a different target. Such may be processed from, e.g. one chimeric pol3 transcript. Pol3 promoters such as U6 or HI promoters may be used. Pol2 promoters such as those mentioned throughout herein. Inverted terminal repeat (iTR) sequences may flank the Pol3 promoter - sgRNA(s)-Pol2 promoter- Cas9.

[00578] One aspect of a chimeric, tandem array transcript is that one or more guide(s) edit the one or more target(s) while one or more self inactivating guides inactivate the CRISPR/Cas9 system. Thus, for example, the described CRISPR-Cas9 system for repairing expansion disorders may be directly combined with the self-inactivating CRISPR-Cas9 system described herein. Such a system may, for example, have two guides directed to the target region for repair as well as at least a third guide directed to self-inactivation of the CRISPR-Cas9. Reference is made to Application Ser. No. PCT/US2014/069897, entitled "Compositions And Methods Of Use Of Crispr-Cas Systems In Nucleotide Repeat Disorders," published Dec. 12, 2014 as WO/2015/089351.

[00579] It will be appreciated that administration of therapeutic entities in accordance with the invention will be administered with suitable carriers, excipients, and other agents that are incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (15th ed, Mack Publishing Company, Easton, PA (1975)), particularly Chapter 87 by Blaug, Seymour, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as Lipofectin™), DNA conjugates, anhydrous

absorption pastes, oil-in- water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in treatments and therapies in accordance with the present invention, provided that the active ingredient in the formulation is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. *See also* Baldrick P. "Pharmaceutical excipient development: the need for preclinical guidance." *Regul. Toxicol Pharmacol.* 32(2):210-8 (2000), Wang W. "Lyophilization and development of solid protein pharmaceuticals." *Int. J. Pharm.* 203(1-2): 1-60 (2000), Charman WN "Lipids, lipophilic drugs, and oral drug delivery-some emerging concepts." *J Pharm Sci.* 89(8):967-78 (2000), Powell *et al.* "Compendium of excipients for parenteral formulations" *PDA J Pharm Sci Technol.* 52:238-311 (1998) and the citations therein for additional information related to formulations, excipients and carriers well known to pharmaceutical chemists.

[00580] Therapeutic formulations of the invention, which include a T cell modulating agent, are used to treat or alleviate a symptom associated with an immune-related disorder or an aberrant immune response. The present invention also provides methods of treating or alleviating a symptom associated with an immune-related disorder or an aberrant immune response. A therapeutic regimen is carried out by identifying a subject, *e.g.*, a human patient suffering from (or at risk of developing) an immune-related disorder or aberrant immune response, using standard methods. For example, T cell modulating agents are useful therapeutic tools in the treatment of cancers.

[00581] A therapeutically effective amount of a T cell modulating agent relates generally to the amount needed to achieve a therapeutic objective. The amount required to be administered will furthermore depend on the specificity of the T cell modulating agent for its specific target, and will also depend on the rate at which an administered T cell modulating agent is depleted from the free volume other subject to which it is administered. The T cell modulating agent may be administered *in vivo* or *ex vivo* as described herein.

[00582] T cell modulating agents can be administered for the treatment of a variety of diseases and disorders in the form of pharmaceutical compositions. Principles and considerations involved in preparing such compositions, as well as guidance in the choice of components are provided, for example, in Remington: The Science And Practice Of Pharmacy 19th ed. (Alfonso R. Gennaro, et al., editors) Mack Pub. Co., Easton, Pa.: 1995; Drug Absorption Enhancement: Concepts, Possibilities, Limitations, And Trends, Harwood

Academic Publishers, Langhorne, Pa., 1994; and Peptide And Protein Drug Delivery (Advances In Parenteral Sciences, Vol. 4), 1991, M. Dekker, New York.

[00583] Where polypeptide-based T cell modulating agents are used, the smallest fragment that specifically binds to the target and retains therapeutic function is preferred. Such fragments can be synthesized chemically and/or produced by recombinant DNA technology. (See, e.g., Marasco et al., Proc. Natl. Acad. Sci. USA, 90: 7889-7893 (1993)). The formulation can also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the composition can comprise an agent that enhances its function, such as, for example, a cytotoxic agent, cytokine, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

[00584] Therapy or treatment according to the invention may be performed alone or in conjunction with another therapy, and may be provided at home, the doctor's office, a clinic, a hospital's outpatient department, or a hospital. Treatment generally begins at a hospital so that the doctor can observe the therapy's effects closely and make any adjustments that are needed. The duration of the therapy depends on the age and condition of the patient, the stage of the a cardiovascular disease, and how the patient responds to the treatment. Additionally, a person having a greater risk of developing a cardiovascular disease (e.g., a person who is genetically predisposed) may receive prophylactic treatment to inhibit or delay symptoms of the disease.

[00585] The medicaments of the invention are prepared in a manner known to those skilled in the art, for example, by means of conventional dissolving, lyophilizing, mixing, granulating or confectioning processes. Methods well known in the art for making formulations are found, for example, in Remington: The Science and Practice of Pharmacy, 20th ed., ed. A. R. Gennaro, 2000, Lippincott Williams & Wilkins, Philadelphia, and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York.

[00586] Administration of medicaments of the invention may be by any suitable means that results in a compound concentration that is effective for treating or inhibiting (e.g., by delaying) the development of a cardiovascular disease. The compound is admixed with a suitable carrier substance, e.g., a pharmaceutically acceptable excipient that preserves the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable excipient is physiological saline. The suitable carrier substance

is generally present in an amount of 1-95% by weight of the total weight of the medicament. The medicament may be provided in a dosage form that is suitable for oral, rectal, intravenous, intramuscular, subcutaneous, inhalation, nasal, topical or transdermal, vaginal, or ophthalmic administration. Thus, the medicament may be in form of, e.g., tablets, capsules, pills, powders, granulates, suspensions, emulsions, solutions, gels including hydrogels, pastes, ointments, creams, plasters, drenches, delivery devices, suppositories, enemas, injectables, implants, sprays, or aerosols.

[00587] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989) (Sambrook, Fritsch and Maniatis); MOLECULAR CLONING: A LABORATORY MANUAL, 4th edition (2012) (Green and Sambrook); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (1987) (F. M. Ausubel, et al. eds.); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); PCR 2: A PRACTICAL APPROACH (1995) (M.J. MacPherson, B.D. Hames and G.R. Taylor eds.); ANTIBODIES, A LABORATORY MANUAL (1988) (Harlow and Lane, eds.); ANTIBODIES A LABORATORY MANUAL, 2nd edition (2013) (E.A. Greenfield ed.); and ANIMAL CELL CULTURE (1987) (R.I. Freshney, ed.).

[00588] The practice of the present invention employs, unless otherwise indicated, conventional techniques for generation of genetically modified mice. See Marten H. Hofker and Jan van Deursen, TRANSGENIC MOUSE METHODS AND PROTOCOLS, 2nd edition (2011).

[00589] This invention is further illustrated by the following examples which should not be construed as limiting. It is understood that the foregoing description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those of skill in the art, may be made without departing from the spirit and scope of the present invention. Further, all patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by

virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the dates or contents of these documents.

[00590] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined in the appended claims.

[00591] The present invention will be further illustrated in the following Examples which are given for illustration purposes only and are not intended to limit the invention in any way.

Examples

[00592] IL-27 is a member of the IL-12 family of cytokines that is produced by antigen presenting cells. IL-27 was initially found to promote a Type I pro-inflammatory response; however, emerging evidence suggests that this cytokine plays an important role in the resolution of tissue inflammation (Yoshida, H. & Hunter, C. A. (2015) *Annual review of immunology* **33**, 417-443). IL-27 administration *in vivo* suppresses the development of effector T cells and inhibits the development of autoimmunity. In contrast, IL-27ra (WSX-1) deficient mice exhibit increased inflammation during *Toxoplasma gondii* infection and exhibit exacerbated central nervous system autoimmunity (Awasthi, A. *et al.* 2007; Hirahara, K. *et al.* 2012; Villarino, A. *et al.* 2003). Indeed, it has been shown that IL-27 induces IL-10-secreting Type I regulatory (Tr1) cells that are immune suppressive (Awasthi, A. *et al.* 2007). Moreover, it has been shown that IL-27 induces Tim-3, which has been shown to cooperate with PD-1 in exhausted T cells (Zhu, C. *et al.* 2015; Sakuishi, et al, 2011). Together these observations raised the possibility that IL-27 may also induce the expression of additional co-inhibitory receptors that cooperate to promote T cell dysfunction.

[00593] Data provided herein shows that IL-27 signaling drives the expression of a gene module that includes not only Tim-3, but also LAG-3 and Tigit, molecules that have been previously associated with T cell dysfunction. The inventors identified a large overlap in the IL-27-induced transcriptome and the gene signatures that define dysfunctional T cells in chronic viral infection and cancer. Further, the inventors identified a panel of novel candidate molecules that are induced by IL-27, are associated with T-cell dysfunction, and can be modulated to improve effector T cell responses *in vivo*. These data define a new role for IL-27 signaling in an inhibitory gene module that sets the stage for the development of a

dysfunctional phenotype in T cells and further provide a means by which to identify novel and potentially synergistic targets for therapeutic application in chronic disease settings.

[00594] The inventors further realised that modulation of genes or gene products comprised by the gene signatures as taught herein in isolated immune cells can modulate the properties of the cells and thereby provide for advantageous effects, such as increasing or decreasing dysfunctional phenotype of the immune cells, or rendering the immune cells more resistant or more sensitive to becoming dysfunctional, or increasing or decreasing activated phenotype of the immune cells, or rendering the immune cells more resistant or more sensitive to becoming activated. Such modulation can be of value *inter alia* in therapeutic applications, such as for example but without limitation in *ex vivo* or allogeneic therapies involving immune cells, such as T cells, such as CD8⁺ T cells, e.g., CAR-T therapies.

Example 1: Experimental Results

IL-27 induces a co-expressed set of co-inhibitory receptors associated with T cell dysfunction on CD4 and CD8 T cells.

[00595] Recent studies have demonstrated that IL-27 induces the expression of co-inhibitory cell-surface receptors, such as Tim-3 and PD-L1, on CD4⁺ and CD8⁺ T cells (Hirahara et al, (2012) *Immunity* 36, 1017-30; Zhu et al, (2015) *Nature communications* 6, 6072). Together with evidence supporting a key role for IL-27 in driving resolution of tissue inflammation (e.g., Awasthi et al., (2007) *Nature immunology* 8, 1380-1389; Hirahara et al, (2012) *Immunity* 36, 1017-30; Stumhofer et al, (2007) *Nature immunology* 8, 1363-1371; Fitzgerald et al, (2007b) *Nature immunology* 8, 1372-1379), Applicants hypothesized that IL-27 might induce expression of additional co-inhibitory receptors in T cells. Accordingly, it was examined whether activation of naive CD4 and CD8 T cells in the presence of IL-27 induced additional co-inhibitory molecules.

[00596] Indeed, Applicants found that besides Tim-3 (Havcr2) IL-27 induced at both the mRNA and protein level two additional co-inhibitory molecules associated with T cell dysfunction, Lag-3 and TIGIT (**Figure 1A, B**), on CD4⁺ and CD8⁺ T cells. Expression of all three co-inhibitory molecules (Tim-3, Lag-3 and TIGIT) was reduced in IL-27R-deficient T cells, further confirming the importance of IL-27 in driving their expression. Interestingly, while the induction of Tim-3, Lag-3, and TIGIT *in vitro* was largely dependent on IL-27, PD-1 (Pcdcl) expression was not affected by IL-27 (**Figure 1A, B**).

[00597] At a population level, co-inhibitory receptors are often co-expressed on dysfunctional T cells *in vivo*, where the accumulation of co-inhibitory receptor expression has

been shown to correlate directly with the degree of dysfunction (Wherry, E.J. and Kurachi, M. (2015) *Nature Reviews Immunology* 15, 486-499). However, it has not been clear to what extent co-inhibitory receptors are co-expressed at the single cell level. Applicants recently showed with single cell RNA-Seq the co-expression of a module of co-inhibitory receptors (including Tim-3, TIGIT, PDI, Lag-3 and CTLA-4) in CD8⁺ TILs from human melanoma tumors (Tirosh et al., (2016) *Science* 352, 189-196); however, assessing the functional state of human cells *in vivo* is challenging. Applicants therefore analyzed single cell RNAseq profiles of 516 CD8 TILs from B16F10 melanoma (Singer *et al.*, companion manuscript) and indeed found that PD-1, Lag3, Tim-3, CTLA-4, 41BB and TIGIT strongly co-vary across single cells, such that cells co-express their transcripts (**Figure 1C**).

[00598] The observed induction of multiple known co-inhibitory receptors by IL-27 suggested the possibility of shared regulatory elements and co-variant expression on T cells. Indeed, co-inhibitory receptors are often co-expressed on dysfunctional T cells *in vivo* where the accumulation of co-inhibitory receptor expression has been shown to be proportional to the severity of dysfunctional phenotype. The co-expressed set of co-inhibitory genes is also apparent at the protein level in CD4⁺ and CD8⁺ TILs, as assessed by single-cell mass cytometry (CyTOF, Bendall et al., (2011) *Science* 332, 687-696; Newell et al, (2012) *Immunity* 36, 142-152). This technology allows for simultaneous analysis of the expression of up to 30 molecules on a single cell. Applicants developed a custom CyTOF panel that included 15 antibodies against known co-stimulatory and co-inhibitory cell-surface receptors, as well as lineage-defining cell surface markers (**Table 15; Figure ID**), and used it to analyze TILs isolated from B16F10 melanoma tumors from WT and IL-27R knockout mice.

Table 15

Gene	Log2FC
PD-1	1.7176
Tim-3	1.4176
LAG-3	1.4758
TIGIT	1.4958
CTLA4	1.7676
GITR	1.7476
CD160	1.4388
BTLA	1.5376
LILRB4	1.5288
ICOS	1.6068
A-1BB	1.6076
OX40	1.7076
SLAMF6	1.6776
CD226	1.6888
HVEM	1.7576
Thy1.2	1.5668
CD8a	1.6408
CD4	1.4288
IFN γ	1.5976
TNFA	1.4888

[00599] Four co-inhibitory receptors (PD-1, LAG3, TIM-3, and TIGIT) had tightly correlated expression on CD8⁺ and CD4⁺ TILs. PD-1, TIM-3, and LAG-3 showed the highest degree of correlation, particularly on CD8⁺ TILs (**Figure 1C** and **Figure 1D** for CD4⁺ TILs). K-means clustering of the cells following visualization with a non-linear embedding of the protein expression profiles using t-stochastic neighborhood embedding (t-SNE (Maaten L, (2008) *Journal of Machine Learning Research*, 2579-2605), **Example 2: Methods**) showed two discrete groups of CD8⁺ TILs, described herein as clusters 1 and 2. The co-inhibitory receptor quartet (PD-1, LAG3, TIM-3, and TIGIT) was mainly expressed in cells in cluster 1 (**Figure 1E**, **Figure 1F,G,H**). Additional co-inhibitory receptors, including CD160, CTLA-4, and LILRB4 were expressed on smaller sub-sets of cells within cluster 1 (**Figure 1I**). Some known co-stimulatory molecules, particularly those of the TNF-receptor family, such as 4-1BB, OX-40, and GITR, were also co-expressed with the co-inhibitory receptors on cells within cluster 1 (**Figure 1I**). In contrast, other co-stimulatory molecules such as ICOS and CD226 were more comparably expressed on cells in both cluster 1 and cluster 2 (**Figure 1I**). Thus, cluster 1 is highly enriched for CD8⁺ T cells that express multiple co-inhibitory receptors together with co-stimulatory receptors of the TNF-receptor family.

[00600] Notably, cluster 1 was relatively depleted of cells from IL-27ra KO CD8⁺ TILs compared to WT (**Figure 1J**, hyperG p-value= 5e-23), suggesting that in the absence of IL-27 signaling there are far fewer CD8⁺ TILs with co-expressed co-inhibitory receptors. Applicants further confirmed the reduced proportion of cells that express PD-1, TIM-3, LAG3, TIGIT, and IL-10 on CD8⁺ TILs isolated from IL-27ra KO mice by flow cytometry

(**Figure IK**) and in several replicate CyTOF experiments (**Figure 1L**). Thus, IL-27 signaling is a key driver of an inhibitory gene module that includes co-inhibitory receptors and IL-10 which are strongly co-expressed *in vivo*. Of note and in contrast to our *in vitro* data, Applicants saw that PD-1 expression is dependent on IL-27R signaling *in vivo*. Together, these data indicate that cluster 1 is highly enriched for cells that express co-inhibitory receptors and found that the TILs identified as cluster 1 is significantly decreased in the absence of IL-27 signaling. This significant reduction of CD8 T cells expressing PD-1, Tim-3, LAG-3, and TIGIT was confirmed in IL-27ra^{-/-} mice using conventional flow cytometry. Together these data indicate that IL-27 signaling is a key driver of a module of co-inhibitory receptors that exhibit a high degree of co-variance *in vivo*.

IL-27 driven inhibitory molecules dissect cluster 1-CD8 TILs

[00601] The identification of additional co-inhibitory molecules dependent upon IL-27R signaling permitted the further dissection of the subpopulation of cluster 1-CD8 TILs based on their function. IL-27R signaling dependent population was overlapped with PD-1 expressing cells. While PD-1 is important for exhaustion (Wherry, E. J., (2011) *Nature immunology* 12, 492-499), it is also expressed on activated T cells. IL-27 does not induce PD-1 directly. Each inhibitory molecule had a different pattern of expression within cluster 1-CD8 T cells, for example PD-1 high CD8 cells produce more IFN γ than PD-1 middle cells, but PD-1 high Tim-3 high CD8 T cells produce less TNF α than PD-1 high Tim-3 low cells do. This correlation is further emphasized for PD-1 high Tim-3 high Tigit⁺ cells. Thus, the accumulation of multiple inhibitory molecules rather than the intensity of a single one on the same cell leads to is a better predictor for stepwise decrease of effector cytokines from CD8 TILs. In general, cluster-2-CD8 TILs, which is enriched for IL27ra KO derived cells, showed a stronger effector function than cluster-1. However, the expression of some co-stimulatory molecules was observed, including 4-1BB co-expressed with several inhibitory molecules; PD-1, Tim-3, Tigit, and CD160 in a part of cluster-1-CD8 TILs where it might represent counter regulation of exhaustion pressures under tumor microenvironment. On the other hand, IL-10 producing cells are also higher in the exhausted phenotype of CD8 T cells in an IL-27R signal dependent manner. IL-10 has been reported to be immunosuppressive in the context of tumor immunity (Hisada, M. *et al*, (2004) *Cancer research* 64, 1152-1156). Within the tumor microenvironment, IL-27 signature drives inhibitory molecules that augment deficit of effector cytokines from CD8 T cells. At the same time they are producing IL-10 and further exacerbating the circumstance of immune suppressive environment.

IL-27 induces a gene module that is present in other dysfunctional T cells and includes novel cell-surface molecules

[00602] The importance of IL-27 signaling for driving known co-inhibitory receptors both *in vitro* and *in vivo*, prompted the inventors to examine whether IL-27 may drive additional and yet unknown molecules that have regulatory function. To examine whether IL-27 may also induce the expression of additional novel inhibitory molecules that could regulate anti-tumor immunity, Applicants used transcriptional profiling to identify a signature of IL-27 dependent genes in wild-type and IL-27ra^{-/-} T cells after stimulation with or without IL-27 at time points selected for optimal expression of known co-inhibitory receptors (Tim-3, Lag-3, and Tigit) on CD4⁺ and CD8⁺ T cells. Applicants first measured a 445 gene transcriptional signature (measured by nCounter, **Example 2: Methods, Table 16**) in WT and IL-27ra KO CD4⁺ and CD8⁺ T cells at 6 times point along a 96 hour time course after activation in the presence or absence of IL-27.

1700097N02Rik	Ccn1	Daxx	Hif1a	Irf5	Ncoal	Rgs16	Tap1
2310031A07Rik	Ccr1	Ddr1	Hip1r	Irf7	Nfatc1	Rgs8	Tbx21
2900064A13Rik	Ccr2	Dntt	Hlx	Irf8	Nfatc2	Rora	Tcf4
5830405N20Rik	Ccr4	Dpp4	Hprt	Irf9	NFE2	Rorc	Tcf7
6330442E10Rik	Ccr5	Drd1	Hsbp1	Isg20	Nfe2l2	Rpp14	Tgfb1
Abcg2	Ccr6	dsc2	Htrl1a	Itch	Nfil3	Runx1	Tgfb3
AchE	Ccr8	EBi3	Icos	Itga3	Nfkbi	Runx2	Tgfb3
Actin	Ccr9	Egr2	Id2	Itgb1	Nfkbi	Runx3	Tgfb3
Acvr1b	Cd160	Eif3e	Id3	Jak3	Nkg7	Rxra	Tgif1
Acvr2a	Cd2	Eif3h	Ier3	Jun	Nmdar1	Sap30	Tgm2
adam8	Cd200	Elk3	Ifi35	Jup	Notch1	Sema4d	Tigit
Adrb2	Cd226	Emp1	Ifih1	Kat2b	Notch2	Sema7a	Timp2
Aes	Cd247	Eomes	Ifit1	Katna1	Nr3c1	Serpina1	TLE1
Ahr	Cd24a	Ercc5	Ifitm2	Khdrbs1	Nudt4	Serpina1b	TLE2
Aim1	Cd274	Errfi1	Ifng	Klf10	Oas2	serpina9b	TLE3
alox5	Cd28	ETS1	Ifngr2	Klf3	p28	Serpine1	TLE4
Anxa4	Cd36	Etv6	Ifnra1	Klf6	Pbx3	Serpine2	Tmed7
Api5	Cd39	Fas	Ifnra2	Klf7	Pcbp2	Sertad1	Tmem119
Aqp3	Cd4	Fasl	Igfbp4	Klf9	Pdcd1	Sesn3	Tmem126a
Arg1	Cd44	Fasn	Ikzf3	Klrd1	Pdcd11	Sgk1	TNFA
Arhgef3	Cd51	FGL2	Ikzf4	Klrg1	Pdcd1lg2	Sgta	Tnfrsf12a
Arid5a	Cd70	Fip1l1	Il10	L1CAM	Pdpm	SIM1	Tnfrsf13b
Arl5a	Cd74	Fli1	Il3	Lad1	Peci	SIM2	Tnfrsf25
Armex2	Cd80	Flna	Il6st	Lag3	Peli2	Skap2	Tnfrsf4
Arnt1	Cd83	Flot1	Il10ra	Lamp2	Phlda1	Ski	Tnfrsf11
Arnt2	Cd86	Foxf1	Il12rb1	Lef1	Plac8	Slamf7	Tnfrsf8
Arntl	Cd9	Foxm1	Il12rb2	Lgals3bp	Plagl1	Slc1a4	Tnfrsf9
Atf4	CEACAM1	Foxo1	Il15ra	Lif	Plek	Slc2a1	Tnip2
B4galt1	Cebpb	Foxp1	Il17a	Lilrb4	Plekhf2	Slc6a4	Tob
Bat3	Chat	Foxp3	Il17f	Litaf	Pmepa1	Slc6a6	Toso
Batf	Chd7	Frmd4b	Il17ra	Lmnb1	Pml	Slc7a3	Tox2
Batf2	ChRM1	Fzd7	Il1r1	LPXN	Pomc	Smad2	Tph1

Batf3	ChRM3	GABRA1	Il1r2	LRMP	Pou2af1	Smad3	Traf3
BC021614	ChRM5	Gad1	Il1rl1	LMipl	Prcl	Smad4	Tratl
Bel1ib	ChRNA10	Gap43	Il1rn	Lspl	Prdml	Smad7	Trim24
Bcl2	ChRNA4	Gapdh	1121	Ltf	Prfl	Smarca4	Trim25
Bcl211	ChRNA9	Gata3	1121r	Ly6c2	Prickle 1	Smox	Trim30
Bcl211.1	ClirNB2	Gem	1122	Maf	Prkca	socs2	Trpsl
Bcl3	ClirNB4	Gfil	1123	Maff	Prkd3	Socs3	Tsc22d3
Bcl6	Clcfl	GIMAP5	1123r	Maob	Prnp	Sppl	Tubb5
Beta Actin	Cmtm6	Gjal	1124	Map3k5	Procr	Spry1	Tyh
BHLHE40	CMTM6	Glipr1	I127ra	Max	Prrxl	Srxnl	Ube3a
Bmprla	Comt	GMFG	I12ra	Mbnl3	Psmb9	Stard10	Ubiadl
Calca	CREBZF	gngl 1	I12rb	Med24	Pstpipl	Stat1	Vav3
Candl	Csf2	Golga3	113	Mgll	Ptprj	Stat2	Vax2
Casp1	Csnklal	Gpl30	1133	Mina	Ptprk	Stat3	Xbpl
Casp3	Ctla2A	Gpr56	1135	Mklnl	Pxf/Pexl9	Stat4	Xrcc5
Casp4	Ctla2b	Gpr65	114	Mtl	Pycrl	Stat5	ZBTB32
Casp6	CTLA4	Grail	I14ra	Mt2	Rab33a	Stat5a	Zeb1
cbl-b	Ctsw	Grn	IL6	Mta3	rab37	Stat5b	Zfp161
ccdc64	Cxcl10	Gusb	I16st	Mxil	Rad51apl	Stat6	Zfp238
Cell	Cxcl3	Gzma	I17r	Mycll	Rasg φ 1	Sufu	Zfp281
Ccl2	Cxcr3	Gzmd	119	Myd88	Rbpj	Sult2bl	Zfp410
Ccl20	Cxcr4	Gzmg	Inhba	Myst4	Rel	Tacl	
Ccl4	Cxcr5	H2-Q10	Irf1	Nampt	Rela	Tacr1	
Ccl5	Cxcr6	Havcr2	M4	Ncfl	Rfk	Tal2	

[00603] Optimal expression of these co-inhibitory receptors (Tim-3, Lag-3, and Tigit) was observed at 96 hours for CD4⁺ and 72 hours for CD8⁺ T cells (**Figure 6A,B**). Applicants then undertook whole genome mRNA profiling of CD4⁺ and CD8⁺ T cells in the presence of IL-27 at these corresponding timepoints. Applicants identified 1,392 genes that were differentially expressed between WT CD4⁺ T cells stimulated in the presence or absence of IL-27 (Fold change > 2 and FDR < 0.2) and depended on IL-27 signaling based on IL-27ra KO CD4⁺ T cells. A subset of 118 differentially expressed genes were annotated as cell surface receptors or cytokines. Importantly, several genes known to encode molecules that have been previously shown to have an inhibitory effect on T cells such as, Tim3, Lag3, Inhba, Alcam, CTLA2A as well as, cytokines such as IL10 were among the 118 genes. The subset (**Figure 6C, Figure 6D**) of 118 genes that encode cell surface receptors or cytokines, also included Tim3, Lag3, TIGIT, and IL10. Importantly, CD4⁺ and CD8⁺ T cells showed a similar pattern of differential gene expression (**Figure 6C, E, F**).

[00604] Strikingly, there is a highly significant overlap between the IL-27-driven gene signature and gene signatures for other T cell states associated with dysfunction, including cancer, chronic viral infection, anergy, and tolerance (**Figure 6G, H, I, J**). Specifically, Applicants found a significant overlap with each of the following signatures: **(1)** a gene signature for dysfunctional T cells in cancer (Singer *et al.*, companion manuscript) defined by

comparing PD-1⁺Tim-3⁺ CD8⁺ (DP) TILs (representative of cluster 1 in **Figure IE**), which contain CD8⁺ T cells that exhibit a severe dysfunctional phenotype, to that of PD-1⁻Tim-3⁻ CD8⁺ (DN) TILs (representative of cluster 2 in **Figure IE**), which preferentially contain CD8⁺ T cells that have preserved effector function (Sakuishi et al, (2010) *The Journal of experimental medicine* 207, 2187-2194); (2) a gene signature for dysfunctional T cells from chronic viral infection, from previously published profiles (Doering et al, (2012) *Immunity* 37, 1130-1144) from virus-specific CD8⁺ T cells isolated from mice infected with either the chronic clone 13 strain or the acute Armstrong strain of LCMV; (3) T cell anergy (Safford et al, (2005) *Nature immunology* 6, 472-480); and (4) induced T cell tolerance with either antigen-specific (Burton et al., (2014) *Nature communications* 5, 4741) or non-specific (anti-CD3 antibody) (Mayo et al., (2016) *Brain, A journal of Neurology*, Advance Access doi:10.1093/brain/aww113, 1-19) stimulation. This overall significant overlap (**Figure 6G**), suggests that IL-27 may impact T cell function through one gene module across multiple states of T cell non-responsiveness. In particular, the IL-27-induced co-inhibitory receptors Tim-3, TIGIT, and Lag-3 were shared across at least four of the 5 analyzed signatures. Indeed, blockade of each of these molecules has already been shown to inhibit T cell exhaustion and promote anti-tumor and anti-viral immunity (Johnston et al, (2014) *Cancer cell* 26, 923-937; Woo et al., (2012) *Cancer research* 72, 917-927; Sakuishi et al., (2010) *The Journal of experimental medicine* 207, 2187-2194; Jin et al, (2010) *Proc Natl Acad Sci US A* 107, 14733-14738; and Blackburn et al, (2009) *Nature immunology* 10, 29-37.

[00605] More specifically, a gene signature for dysfunctional T cells in cancer was generated by comparing the gene expression of PD-1⁺Tim-3⁺ CD8⁺ TILs (representative of cluster 1), which contains CD8⁺ T cells with severe exhausted phenotype, to that of PD-1⁻Tim-3⁻ CD8⁺ TILs (representative of cluster 2), which contains CD8⁺ T cells that retain good effector function (Sakuishi, et al., (2011) *Trends in immunology* 32, 345-349). Gene signatures for exhausted T cells were further generated in the chronic LCMV model from publically available gene expression data by comparing virus-specific CD8⁺ T cells from clone13 LCMV infection to virus-specific CD8⁺ T cells from Armstrong LCMV infection (Harker, J. A., et al, (2013) *Immunity* 39, 548-559). The IL-27-induced module of surface receptors/cytokines was then compared with the signatures for dysfunctional T cells from cancer and chronic viral infection and significant overlap was observed in the number of surface receptors/cytokines across the different data sets. Importantly, it was found that the IL-27 induced co-inhibitory receptors Tim-3 (HAVCR2), Tigit and Lag-3 were shared among the three data sets, supporting the association of IL-27-driven genes to dysfunctional T cell

states *in vivo*. The entire IL-27-induced gene signature was further found to overlap significantly with the gene signatures for dysfunctional T cells from cancer and chronic virus infection as well as other states of T cell non-responsiveness such as anergy and tolerance (p-value<0.01). Of note, several survival factors including IL-21, IL-2Ra, I16st and IL-7R and activation markers were also found as shared genes, indicating that the IL-27-driven gene module is not merely a collection of co-inhibitory molecules that restrain activated T cells but also factors that regulate the survival of cells in tissue. Together these data strongly point to a key role for IL-27 in driving molecular programs that dampen effector T cell function.

Procr and Pdpn are novel co-inhibitory receptors induced by IL-27

[00606] Among the 118 surface molecules and cytokines induced by IL-27 (**Figure 6C**), some molecules were also highly expressed in specific settings (**Figure 6G**), such as in cancer or in chronic viral infection (**Figure 6K**), allowing stratification of molecules for additional investigation, based on their uniqueness to specific settings. In particular, two of the IL27-induced surface molecules, Procr (protein C receptor) and Pdpn (podoplanin) were highly expressed in the setting of cancer T cell dysfunction compared to other states of T cell non-responsiveness (**Figure 6K**). Applicants confirmed that activation of naive CD4⁺ and CD8⁺ T cells *in vitro* in the presence of IL-27 induced the expression of both Procr and Pdpn as determined by qPCR and flow cytometry (**Figure 6L**). Furthermore, both Procr and Pdpn were co-expressed with PD-1 and Tim-3 on CD8⁺ TILs and their expression was lost in the absence of IL-27 receptor signaling (**Figure 6M**).

[00607] Procr is a cell surface receptor known to be expressed on both vascular endothelial cells and tumor cells, where it regulates endothelial cell function and tumor cell migration and invasion, respectively (Mohan Rao et al, (2014) *Blood* 124, 1553-1562). In the lymphocyte compartment, Procr is expressed on CD4⁺ T cells, particularly Th17 cells (Yosef et al, (2013) *Nature* 496, 461-468), where it is in co-variance with the regulatory module (Gaublomme et al, (2015) *Cell* 163, 6, p1400-1412); however its function on CD8⁺ T cells has not been previously explored. Procr⁺ CD8⁺ TILs exhibit a dysfunctional phenotype, producing less TNF α and IL-2 and more IL-10 than Procr⁻ CD8⁺ TILs (**Figure 6N**).

[00608] To examine the role of Procr in regulating effector CD8⁺ T cell function, Applicants used a Procr hypomorph (Procr^{h/h}) mouse strain (Castellino et al, (2002) *Thrombosis and haemostasis* 88, 462-472). B16F10 melanoma cells were implanted into Procr^{h/h} mice and striking inhibition of tumor growth was observed (**Figure 7A**). Importantly, CD8⁺ TILs from Procr^{h/h} mice exhibited enhanced TNF α production, corresponding to enhanced tumor immunity but did not show a significant difference in the expression of other

cytokines, including IL-2, IFN- γ and IL-10 (**Figure 7B**). Moreover, Procr^{d/d} TILs exhibited a striking decrease in the frequency of CD8⁺ T cells expressing high levels of Tim-3 and PD-1, suggesting that Procr signaling on CD8⁺ T cells promotes severe dysfunctional phenotype and loss of Procr in the host partially reverses this (**Figure 7C**).

[00609] Another cell surface molecule Podoplanin (Pdpn) is expressed on several tumor types, in which it has a role in lymphovascular invasion and metastasis (Wicki et al, (2006) *Cancer cell* 9, 261-272). More recently, it was reported that Pdpn is expressed in effector CD4⁺ T cells where it functions to limit T cell survival in inflamed tissues in an autoimmune setting (Peters et al, (2015) *The Journal of clinical investigation* 125, 129-140); however, whether Pdpn has a role in tumor-induced CD8⁺ T cell dysfunction is not known. The current data indicate that Pdpn is specifically expressed on CD8⁺Tim-3⁺PD-1⁺ TILs and marks a population which still has pro-inflammatory cytokine production, but already start producing IL-10. (**Figure 10A**).

[00610] To analyze the functional role of Pdpn in anti-tumor immunity, Applicants used T-cell specific Pdpn conditional knock-out mice (Pdpn cKO). Mice with Pdpn-deficient T cells showed a significant delay in growth of B16F10 melanoma compared to control mice (**Figure 11A**) and Pdpn-deficient CD8⁺ TILs exhibited enhanced IL-2 and TNF α production but no significant difference in IFN- γ and IL-10 production (**Figure 11B**). Consistent with these data, lack of Pdpn on T cells was also associated with a decrease in the frequency of CD8⁺ TILs expressing high levels of Tim-3 and PD-1, indicating reduced accumulation of T cells with a severe dysfunctional T cell phenotype (Sakuishi et al, (2010) *The Journal of experimental medicine* 207, 2187-2194) (**Figure 11C**). Moreover, Pdpn-deficient PD-1⁺Tim-3⁺ CD8⁺ TILs had higher expression of IL-7Ra when compared to wild type, as was previously shown (Peters et al, (2015) *The Journal of clinical investigation* 125, 129-140), indicating that Pdpn may contribute to T cell dysfunction by limiting the survival of CD8⁺ TILs in the tumor microenvironment (**Figure 10B**).

[00611] CD8⁺ T cells exhibit an exhausted phenotype within the tumor microenvironment, and express multiple co-inhibitory receptors on their surface. Here it is shown that the IL-27 signaling pathway induces multiple known, as well as several heretofore unknown receptors with co-inhibitory function on naive CD8⁺ T cells. By using global gene expression data and computational approaches to compare the IL-27-driven gene signature to the gene signature of dysfunctional T cells in two chronic disease states, Applicants identified an "inhibitory module" induced by IL-27 that includes known co-inhibitory receptors (Tim-3, Lag-3, TIGIT), along with 37 novel cell-surface molecules and cytokines. It is shown herein that two

of these novel molecules have co-inhibitory function *in vivo*. These data indicate that IL-27 signaling induces a complex repertoire of inhibitory receptors, each of which can contribute to the exhausted state, thus setting the stage for the development of a dysfunctional effector T cell phenotype.

[00612] The inventors further applied this computational approach including gene signatures from several T cell impairment states, such as anergic CD4 T cells, tolerized CD4 T cells following chronic stimulation with subcutaneous antigen, and anti-CD3 stimulated IL-10 producing Foxp3⁻ CD4 T (Tr1) cells compared with to IL-10 non-producing Foxp3⁻ CD4 T cells following nasal tolerance. This approach increased the number of candidates represent regulatory state of IL-27 signature to a total of 57 molecules. Of note, known co-inhibitory molecules; LAG-3, Tim-3, and Tigit were still highly shared genes among data sets, indicating that the IL-27 signature has the potential to introduce general gene module of T cell impairment states.

[00613] The inventors identified 2 of the molecules, Pdpn and Procr, as co-inhibitory receptors that suppress tumor immunity and promote a dysfunctional phenotype in TILs cells. It was previously reported that Pdpn regulates IL-7R expression on T cells, which is important for long-term T cell survival (Peters et al, 2015). Studies suggested that exhausted CD8 T cells have a defect in their survival and IL-7R expression, whereas IL-7 antagonized inhibitory networks and promote survival of CD8 T cells (Lang, K. S. et al. (2005) European journal of immunology 35, 738-745; Pellegrini, M. et al. (2009) Nature medicine 15, 528-536).. In the current tumor model, loss of Pdpn resulted in recovery of IL-7R expression on PD-1⁺Tim-3⁺ CD8 T cells. This indicates that there may be antagonism between PDPN and IL-7R expression and therefore affecting IL-7 responsiveness and survival of exhausted T cells.

[00614] Lack of Procr signaling had strong impact on losing PD-1⁺Tim-3⁺ CD8 TILs and facilitating tumor immunity. Although the role of Procr on CD8 T cells still needs further analysis, the inventors also found that with mutations of Procr resulted in a loss of the exhausted CD8 T cell phenotype in the chronic model of LCMV infection mice.

[00615] The strategy of global screening analysis of IL-27R signaling identified novel biomarkers in the field of T cell exhaustion that facilitated dissection of this functional state and can also be useful for prognosis prediction before and after check-point therapy. Thus, targeting Pdpn and Procr for enhanced tumor immunity has been validated as a potential new check-point therapy.

Prdml partially regulates the IL-27-driven gene module

[00616] Given the observation that individual cells co-express multiple co-inhibitory molecules, many of which are induced by a common stimulus, IL-27, Applicants hypothesized that a common regulator downstream of IL-27 signaling controls this module. Several lines of evidence supported a role for the transcription factor Prdml as a common regulator. First, Prdml can be induced by IL-27 and is known to regulate IL-10 production in T cells (Newmann et al, (2014) *The Journal of experimental medicine* 211, 1807-1819). Second, 80% of the genes within the IL-27-driven inhibitory gene module have evidence for binding by Prdml in their promoter regions based on ChIP-Seq data from CD8⁺ T cells (Shin et al, (2013) *Immunity* 39, 661-675) (**Example 2: Methods and Resources**). Third, the ChIP-Seq evidence was further extended into a validated network model by *in vitro* functional testing based on gene expression profiles from naive CD8⁺ T cells from WT and Prdml -deficient mice stimulated with IL-27. Thus, Prdml binds and functionally regulates multiple cell surface molecules and cytokines in the IL-27 driven inhibitory gene module including Tim-3, Tigit, and Lag3 (**Figure 12A**). Finally, Prdml was not only induced by IL-27 in CD8⁺ cells *in vitro* but also expressed at higher levels by dysfunctional Tim-3⁻PD-1⁺ (SP) and Tim-3⁺PD-1⁺ (DP) CD8⁺ TILs compared to Tim-3⁻ PD-1⁻ CD8⁺ (DN) TILs that maintain effector function (**Figure 12B**).

[00617] Applicants thus hypothesized that Prdml plays a role in CD8⁺ T cells *in vivo* in regulating the expression of members of the co-inhibitory gene module and in anti-tumor immunity. To test this, Applicants examined mice with a T cell specific deletion of Prdml (Prdml cKO) and found that Prdml -deficient CD8⁺ TILs expressed lower levels of multiple co-inhibitory receptors including Tim-3, PD-1, TIGIT, Lag3, and Procr, but not Pdpn (**Figure 12C**). However, despite the overall decreased expression of co-inhibitory receptors in Prdml cKO mice, there was no difference in the growth of B16F10 melanoma as compared to wild type controls (**Figure 12D**). Thus, the reduction of co-inhibitory receptor expression in Prdml cKO mice was not sufficient to completely reverse the dysfunctional phenotype and recover effector T cell responses to promote anti-tumor immunity.

c-Maf plays an alternate role for regulating co-inhibitory molecules

[00618] Since regulatory networks are often dense and inter-connected across multiple, partially redundant regulators (Novershtern et al, (2011) *Cell* 144, 296-309; Yosef et al, (2013) *Nature* 496, 461-468), Applicants explored whether other transcriptional regulator(s) may also mediate expression of the co-inhibitory receptor module and could compensate *in vivo* for the lack of Prdml. Applicants analyzed gene expression in CD8⁺ TILs from Prdml cKO mice using a custom code set of 397 genes representing both the IL-27-driven gene

signature (245 genes) and the dysfunctional CD8⁺ TIL gene signature (245 genes) (**Example 2: Methods, Table 17**). In addition to the expected reduction in the expression of multiple co-inhibitory, including PD-1, Tim-3, Lag3, and Tigit in Prdml deficient CD8⁺ TILs relative to wild type T cells (**Figure 13A**), only a few genes were consistently induced, including one transcription factor, c-Maf.

Tr1 and Cancer		Tr1 not in cancer		Cancer not in Tr1		Other	House keeping
SPP1	KLHL6	CEBPB	GATM	EPAS1	ZFP362	CD94	Tubb5
GZME	ST6GAL1	JUN	P4HA1	PBX3	RAI1	RankL	hprt
KLRE1	PARP9	HLX	ACADL	ARNT2	SLC39A8	CD160	actin
GZMD	CXCR4	FOSL2	SLC7A3	MDFIC	HEMGN	CD200	gapdh
IL1R2	CXCL10	IRF8	FZD7	UHRF2	TNFSF8	CD152	
GSTM5	SOCS1	STAT1	IER3	CDKN2B	PKD1	CD226	
CALCB	EPCAM	KLF7	IL12RB2	TRPS1	WDR59	CD279 (PD-1)	
GZMC	SOCS3	ATF6	LGALS3	ETV5	STIM2	ICOS	
MT2	GATSL3	GTF3C5	NFIL3	PABPC1L	GSTK1	TNFRSF14	
MT1	IGTP	NFE2L2	PSTPIP1	NCOR2	GYPC	TNFRSF18	
MYO10	CDK5R1	NFYB	ALCAM	GZMD	MAPK1IP1	TNFRSF9	
PENK	DAXX	TLE6	LILRB4	GZMF	TOX	BTLA	
SPATS2	IFI47	ZKSCAN6	BCL2L11	GLDC	SPRY2	NR3C1	
SERPINE2	IRF6	MAFF	GZMA	SERPINEB9B	REM2	TIGIT	
SRXN1	TOP1MT	STAT3	IL10RA	SPIN4	NR4A2	FoxP3	
SDCBP2	DHCR24	BATF	IL2RB	OSGIN1	ELK3	KLRA3	
PRF1	STAT5A	HIF1A	KLRC1	TMPRSS6	BHLHE40	IFNg	
ENO3	BC006779	IRF4	PTPN1	IGSF5.PC P4	NFATC1	TNFa	
SYTL3	MTAP	STAT4	IL12RB1	TMEM171	PDCD1	IL2	
FILIP1	EGR3	FLI1	SIGIRR	GABRR1	PKD2	WSX1	
AKR1B8	FAM26F	RUNX1	BCL2	OSBPL3	NRN1	IL23R	
OCIAD2	STYK1	GATA3	IL21R	CD244	DUSP4	CCR4	
RBPJ	DUSP16	IRF1	SEMA7A	CCRL2	PLSCR1	CCR5	
ADAM9	SEMA4C	IRF9	IL21	LTF	SLC16A11	CCR8	
BNIP3	C10L3	BCL3	CCR5	NSL1	SLC22A15	CCR7	
EMILIN2	ITIH5	ETV6	CTLA2A	GZMG	RAPH1	CXCR3	
GEM	PHACTR2	ID2	CTLA2B	GPR56	GPD2	CCL4	
CDK6	TG	AHR	IL10	RASD2	ATP2B2	CCL5	

ANXA2	CSF1	ARID5A	SERPINF1	RIPPLY3	NCAM1	Runx2
CCNB1	PADI2	BATF3	DDR1	TMEM119	SLC16A4	comes
PRDM1	CREB3L2	CHD7	SEMA4D	DEPDC1A	SERPINB6A	rorc
LITAF	TWSG1	MYST4	SERPINB1A	ALOX5	FASL	rora
ABCB9	SERPINA3G	SAP30	IFITM3	MSRB3	UBASH3B	Foxo3a
SLC39A14	PTER	CREM	MYD88	MGAT3	TNFRSF4	Tcf4
ZBTB32	COPZ2	PML	IL17RA	ARF2	DUSP3	Tcfe2a
BC068157	SERPINB9	ATF3	IL6ST	KLHL30	AFF3	Tcf7L1
GALC	SERPINB6B	ETS1	SGK1	RXRA	LEF1	Tcf7L2
AA467197	TBX21	NOTCH1	LAMP2	TCF7	IL1RL2	axin2
EXO1	CASP4	SERTAD1		RERE		cysltr1
DENND3	IL2RA	GFI1		SSBP2		cysltr2
SLC2A3	NDRG1	JUNB		PPP1R13B		cysltr3
LPXN	TMCC3	KLF6		ZSCAN12		IL33
MXI1	TRPC1	MLLT6		IKBKB		bcl6
WDFY1	LANCL3	SP4		TCF12		bcl6b
KLF10	GSTT3	TULP4		FOXP1		
PLEKHF1	SRGAP3	IRF7		FOXO1		
PPP1R3B	FAM176B	ZFP281		YEATS2		
CTSD	SH3BGR	SELP		TLE4		
PKP2	TMEM49	SERPINB5		ZEB1		
HAVCR2	KLRD1	TMEM35		PHC2		
ADAM8	LPAR3	CH25H		ZFP1		
IGF2BP2	CIAPIN1	RAB33A		RGS10		
PIWIL2	PMEPA1	TGM1		LOC100048338.PDLI M1		
DAPL1	ST3GAL6	LAG3		PIK3R5		
EMB	SELL	ERO1L		TLR1		
PDE4B	GBP2	GBE1		FGF13		
ID3	PLTP	GSN		IL6RA		
AB124611	SEMA4F	H2-O10		ARHGEF3		
IL7R	CAMKK2	HOPX		RECK		
SLAMF6	THA1	PYGL		PRICKLE1		
SEMA4B	FAM65B	SELENBP1		ITGB7		
SMAD3	GPR114	GZMB		RASA3		
GPR18	SH3BP5	<i>nupin</i>		CD7		

ENC1	AOP9	KLRK1		IFIT1	
KBTBD8	SATB1	NKG7		IFIT3	
AS3MT	LPIN1	PLAC8		PIM2	
PGS1	SNHG7	ACVRL1		ARHGEF1 8	
EGLN3	PDPN	DNTT		CHD3	
RTP4	PROCR	TGFB3		DGKA	

[00619] c-Maf is a transcription factor, which regulates IL-10 expression (Apetoh et al, 2010), is induced by IL-27 (Awasthi et al, (2007) *Nature immunology* 8, 1380-1389), and was reported to drive expression of co-inhibitory molecules (Giordano et al, (2015) *EMBO J* 34, 2042-2058). Since Prdml is also reported to regulate IL-10 expression, Applicants hypothesized that compensatory up-regulation of c-Maf could explain the lack of anti-tumor immunity observed in Prdml cKO mice. Supporting this hypothesis, many of the genes in the IL-27-driven inhibitory gene module have a binding motif and a reported binding event for c-Maf within their promoter regions (Ciofani et al, (2012) *Cell* 151, 289-303).

[00620] Indeed, similar to CD8⁺ TILs from Prdml cKO mice, CD8⁺ TILs from c-Maf cKO exhibited a decrease in the expression of multiple co-inhibitory receptors, including PD-1, Tim-3, Lag3, and Tigit (**Figure 13B**). However, each of the two transcription factors impacted the expression of the various co-inhibitory receptors only partially (**Figure 13C**). As in the Prdml cKO mice, c-Maf cKO mice did not show any significant difference in growth of B16F10 melanoma as compared to controls (**Figure 13D**). Notably, Prdml expression in c-Maf cKO derived TILs cells was similar to that in wild type TILs. Thus, Prdml is available to drive expression of the inhibitory gene module in the absence of c-Maf.

Prdml together with c-Maf regulates co-inhibitory receptor expression and anti-tumor immunity

[00621] The analysis indicated that each of Prdml and c-Maf contributes to the regulation of co-inhibitory receptor expression. To address the possibility that the two factors act cooperatively to regulate co-inhibitory receptor expression, Applicants generated a new network model for both factors (**Figure 14A**). Applicants revised the model originally developed for Prdml (**Figure 12A**) to incorporate regulation by c-Maf based on previously published c-Maf ChIP data (Ciofani et al, (2012) *Cell* 151, 289-303) and c-Maf functional targets defined as genes differentially expressed in wild type versus c-Maf cKO CD8⁺ T cell activated *in vitro* in the presence of IL-27. The expanded network model suggested that Prdml and c-Maf bind a large number of shared targets (**Figure 14A**, grey arrows), but those shared bound genes are **not** affected in either individual (single) knockout. This is consistent,

among other possibilities, with cooperative ("AND") regulation (Capaldi et al, (2008) *Nat Genet* 40, 1300-1306). Furthermore, except for Procr and Tim3, other key module genes (TIGIT, LAG3, IL10, PDPN) are affected only by one of the two factors, even though they are bound by the other, further supporting a non-linear interaction between the two factors.

[00622] To test this, Applicants generated mice with a T cell specific deletion in both Prdml and c-Maf (Prdml/c-Maf cDKO). Applicants implanted B16F10 melanoma in Prdml/c-Maf cDKO mice and examined the expression of the co-inhibitory gene module and effector cytokine production in CD8⁺ TILs. CD8⁺ TILs from Prdml/c-Maf cDKO mice exhibited a near absence of PD-1, Tim-3, Lag3, Tigit, Pdpn, and Procr expression (**Figure 14B**), indicating that Prdml and c-Maf functionally co-operate to regulate the expression of co-inhibitory molecules in CD8⁺ TILs. Importantly, Prdml/c-Maf-deficient CD8⁺ TILs had enhanced IL-2 and TNF α production and markedly reduced IL-10 production (**Figure 14C**). Finally, in striking contrast to each single knockout strain, Prdml/c-Maf cDKO mice showed a significant delay in the growth of B16F10 melanoma as compared to controls (**Figure 14D**). Collectively, the data show Prdml/c-Maf cDKO CD8⁺ TILs exhibit loss of co-inhibitory receptor expression and retain effector function.

[00623] Applicant also assessed the functional state of the Prdml/c-Maf cDKO CD8⁺ TILs, in the context of gene expression signatures developed for T cell dysfunction (Singer et al., companion manuscript) for dysfunction and effector-like states. Applicants performed RNA-seq on CD8⁺ TILs from Prdml/c-Maf cDKO and CD8⁺ TILs from wild type mice and identified 940 differentially expressed (adj. P. value<0.05, likelihood ratio test and FDR correction). The gene expression pattern of cDKO CD8⁺ TILs strongly overlapped with that of CD8⁺ Tim-3⁻PD-1⁻ TILs as well as effector/memory cells from naive tumor-free mice (p-value = 2.834e-07 and 0.008, respectively, one-sample Kolmogorov-Smirnov test; **Figure 14E** and **Figure 15A,B**). There was strong evidence for activity of the Foxo1 transcription factor in the cDKO cells including enrichment of genes with Foxo1 binding events (Liao et al., (2014) *Bioinformatics* 26, 2347-2348). Among the genes up-regulated in cDKO compared to WT (P= 1.486e-100, Fisher exact test), induction of the Foxo1 transcript itself, and induction of multiple Foxo1 downstream targets (Ness Michelini et al, (2013) *The Journal of experimental medicine* 210, 1189-1200), including the transcription factors Lef1, Bach2, Klf2 and Tcf7, as well as downstream targets of Tcf7 (e.g., Ccr7, Sell, and Tnfrsf8 (CD30)) (Zhou et al, (2010) *Immunity* 33, 229-240) were upregulated in cDKO. The up-regulated genes were also enriched for targets of Myc (Kidder et al, (2008) *PLoS One* 3, e3932) and Stat3 (Kwon et al, (2009) *Immunity* 31, 941-952), although only Myc was also

transcriptionally up-regulated. Importantly, Foxo1, Tcf7, and Myc are also up-regulated in CD8⁺ Tim-3⁺PD-1⁺ (DN) TILs compared to dysfunctional PD-1⁺Tim-3⁺ TILs (DP) (**Figure 15C**). Overall, loss of c-Maf and Prdm-1 preferentially induces a population akin to the DN population, which shares features with both activated effector CD8⁺ and memory T cells (**Figure 14E**).

Discussion

[00624] IL-27 signaling on naive T cells induces IL-10, and blocks Th1, Th2 and Th17 differentiation. In an immune suppressive environment, IL-27 up-regulates inhibitory receptors and therefore marks them as dysfunctional. Co-inhibitory receptors play a crucial role in immune regulation and their dysregulated expression contributes to the dysfunctional T cell state in chronic disease conditions. Here, Applicants identify that the immunoregulatory cytokine IL-27 drives a co-inhibitory gene module that includes several known co-inhibitory receptors, including Tim-3, Lag-3, and TIGIT, in addition to the anti-inflammatory cytokine IL-10, and that this gene module strongly overlaps with multiple signatures of dysfunctional or tolerant T cell states. The module includes additional surface receptors that are co-regulated with known co-inhibitory receptors, including Procr and Pdpr, which Applicants show act as novel co-inhibitory receptors that cooperate with other inhibitory receptors to induce T cell dysfunction in the tumor microenvironment. Applicants further identified c-Maf and Prdm1 as key transcriptional regulators downstream of IL-27 that drive the inhibitory gene module. Our data thus provide a framework for understanding the underlying organizational principles by which co-inhibitory molecules are co-expressed and co-regulated in dysfunctional T cells.

[00625] Although IL-27 was initially described to have pro-inflammatory properties, its role as a potent immunoregulatory cytokine has come to the forefront in recent years (Awasthi et al, 2007; Fitzgerald et al., 2007b; Hirahara et al., 2012; Stumhofer et al, 2007). IL-27 has been shown to block the differentiation of Th17 cells (Fitzgerald et al, 2007a), and to promote the differentiation of both natural Tregs that specifically suppress Type 1 immunity (Hall et al, 2012) and IL-10 producing regulatory Treg cells (Awasthi et al, 2007). Our studies uncover a new mechanism, by which IL-27 inhibits effector T cells through the up-regulation of multiple co-inhibitory receptors on effector T cells, thereby priming them for the development of dysfunctional phenotype.

[00626] The IL-27 induced gene module not only includes co-inhibitory receptors but also several co-stimulatory molecules from the TNF-receptor family (4-1BB, OX-40 and GITR). The co-membership of co-inhibitory and co-stimulatory receptors in the IL-27 module

provides a rationale for considering the combination of checkpoint receptor blockade with agonists that target TNF-receptor family co-stimulatory receptors. Such a combination could function synergistically by abrogating inhibitory signals (*e.g.*, via blockade of PD-1 signaling), while enhancing co-stimulatory signals (*e.g.*, via activating OX-40) to expand clonotypes that are otherwise inhibited in the tumor microenvironment.

[00627] It was recently shown that IL-35, which shares the Ebi3 chain with IL-27, is produced by intratumoral CD4⁺Foxp3⁺Tregs and that IL-35 promotes co-inhibitory receptor expression on CD8⁺T cells (Turnis et al, 2016). Treg-specific deletion of Ebi3 resulted in a reduction in tumor growth and a loss of dysfunctional CD8⁺T cell phenotype. It is possible that IL-35 and IL-27 may synergize to dampen anti-tumor immunity by promotion of co-inhibitory receptor expression and T cell dysfunction in the tumor microenvironment.

[00628] The induction of multiple co-inhibitory receptors on the same cell suggests that individual molecules could either potentially regulate distinct aspects of T cell dysfunction, or that signals from multiple molecules could combine additively or non-linearly to enhance the response. Similar to our previous results for CD4⁺T cells (Peters et al., 2015), Pdpn may regulate T cell survival through inhibition of IL-7Ra expression on CD8⁺T cells. Indeed, previous studies have shown that dysfunctional CD8⁺T cells have defects in their survival and IL-7Ra expression (Lang et al., 2005; Pellegrini et al, 2009). In contrast, Procr may preferentially modulate proinflammatory cytokine production. In fact, this property underlies the therapeutic use of Activated protein C, a Procr ligand, to induce protease activated receptor-1 driven NF- κ B suppression in acute and chronic inflammatory conditions (Mohan Rao et al, 2014).

[00629] Applicants identified two transcription factors, Prdml and c-Maf, which co-regulate the expression of the IL-27 module. Prdml and c-Maf expression is increased by IL-27R signaling and both are implicated in IL-10 production. CD8⁺T cells deficient in either transcription factor exhibited decreased expression of multiple co-inhibitory receptors in the IL-27R dependent gene expression module, but for effective anti-tumor immunity, both had to be deleted together from CD8⁺TILs. Thus, a partial down-regulation of co-inhibitory receptors is not always sufficient to restore effective T cell responses, due to alternative compensatory mechanisms. This has been borne out in a recent study where anti-PD-1 non-responsiveness was due to increased expression of Tim-3 in CD8⁺TILs (Koyama et al, 2016). Interestingly, the transcriptional signature of TILs from mice deficient for both Prdml and c-Maf significantly overlapped that of Tim-3⁻PD-1⁻ DN TILs, suggesting that Prdml and c-Maf DKO cells resemble cells that normally exist *in vivo*.

[00630] The *in vitro* defined IL-27 module did not include PD-1; however PD-1 expression was dependent on IL-27R signaling *in vivo*. PD-1 expression was partially reduced in both Prdml cKO and c-Maf cKO CD8⁺ TILs, and nearly lost in Prdml/c-Maf cDKO, further supporting the dependence of PD-1 expression on IL-27R signaling *in vivo*. Further analysis for the upstream transcriptional network of Prdml and c-Maf may provide additional clues as to why PD-1 expression depends on IL-27R induction *in vivo* but not *in vitro*. More generally, the presence of multiple, complex and possibly synergistic inputs into infiltrating T cells in the tumor microenvironment could explain why Applicants cannot fully replicate *in vitro* the IL-27 circuit that is present *in vivo*.

[00631] In conclusion, the data adds to the mechanisms by which IL-27 signaling can suppress immune responses. IL-27 acts on naive T cells to induce IL-10 producing Tr1 cells (Awasthi et al, 2007; Stumhofer et al., 2007) and inhibit Th17 differentiation (Batten et al, 2008; Murugaiyan et al., 2009). It acts on Treg to specialize them for suppression of Type 1 immunity. Applicants now show that IL-27 can promote co-inhibitory receptor expression on effector T cells and target them for T cell dysfunction. Our identification of the IL-27-driven gene module further provides a tool with which to identify novel molecules that may play an important role in promoting T cell dysfunction and uncover co-stimulatory molecules that might work together with the co-inhibitory molecules to antagonize T cell dysfunction. The elucidation of the IL-27 driven inhibitory gene module broadens the potential repertoire of therapeutic targets and a molecular basis for understanding the pathways that lead to the dysfunctional T cell state that could constitute mechanisms of resistance to current checkpoint blockade therapies.

Example 2: Methods

[00632] **Mice:** C57BL/6 wild-type (WT), IL-27ra KO (WSX-1^{-/-}), and Prdml M1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). c-Maf fl/fl, Pdpn fl/fl mice and Procr delta/delta mice were previously described (Castellino et al, 2002; Peters et al, 2015; Wende et al, 2012). Pdpn fl/fl mice were initially obtained from Christopher Buckley (University of Birmingham, Birmingham, UK) and crossed to CD4Cre mice to obtain conditional CD4 and CD8 T cell gene knock-out mice. CD4Cre mice were purchased from Taconic (Hudson, NY). Prdml fl/fl and c-Maf fl/fl mice were crossed to CD4Cre mice to generate doubly deficient T cell conditional knockout mice. All experiments were performed in accordance to the guidelines outlined by the Harvard Medical Area Standing Committee on Animals (Boston, MA).

[00633] *Flow cytometry:* Single cell suspensions were stained with antibodies against CD4 (RM4-5), CD8 (53-6.7), PD-1 (RMP1-30), Lag-3 (C9B7W), TIGIT (GIGD7), and Tim-3 (5D12), Procr (eBio1560), and Pdpn (8.1.1.) and were obtained from BioLegend (San Diego, CA). Fixable viability dye eF506 (eBioscience) was used to exclude dead cells. For intracytoplasmic cytokine staining, cells were stimulated with (PMA) (50ng/ml, Sigma-Aldrich, MO), ionomycin (μ g/ml, Sigma-Aldrich, MO). Permeabilized cells were then stained with antibodies against IL-2, TNF- α , IFN- γ or IL-10. All data were collected on a BD LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

[00634] *In vitro T-cell differentiation:* CD4⁺ and CD8⁺ T cells were purified from spleen and lymph nodes using anti-CD4 microbeads (Miltenyi Biotech) then stained in PBS with 0.5% BSA for 15 min on ice with anti-CD4, anti-CD8, anti-CD62L, and anti-CD44 antibodies (all from Biolegend, CA). Naive CD4⁺ or CD8⁺ CD62L^{high}CD44^{low} T cells were sorted using the BD FACSAria cell sorter. Sorted cells were activated with plate bound anti-CD3 (2 μ g/ml for CD4 and μ g/ml for CD8) and anti-CD28 (μ g/ml) in the presence of rmlL-27 (25ng/ml) (eBioscience). Cells were harvested at various time points for RNA, intracellular cytokine staining, and flow cytometry.

[00635] *Real-time PCR:* Total RNA was extracted using RNeasy columns (Qiagen). Reverse transcription of mRNA was performed in a thermal cycler (Bio-Rad) using iScriptTM cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed in the Vii7TM Real-Time PCR system (Applied Biosystems) using the primers for Taqman gene expression (Applied Biosystems). Data was normalized to the expression of ACTB.

[00636] *Nanostring RNA analysis:*

[00637] **Expression profiling along a time course *in vitro*.** Naive CD4⁺ and CD8⁺ T cells isolated from WT and IL-27ra KO mice were activated *in vitro* with IL-27 stimulation. Cells were collected at 0, 12, 24, 48, 72 and 96 hours and analyzed in 3 replicates, using a custom nanostring code-set containing probes for regulatory genes on T cells (TableS2). Expression values were normalized by first adjusting each sample based on its relative value to all samples. This was followed by subtracting the calculated background (mean.2sd) from each sample with additional normalization by housekeeping geometric mean, where housekeeping genes were defined as: Hprt, Gapdh, Actin and Tubb5.

[00638] **Expression profiling of TILs.** Applicants analyzed gene expression in CD8⁺ TILs from Prdml or c-Maf cKO mice bearing B16F10 melanoma collected on day 14 after tumor implantation, using a custom code set of 397 genes representing both the IL-27-driven gene signature (245 genes) and the dysfunctional CD8⁺ TIL gene signature (245 genes)

(Table 17). Expression values were normalized as described above. Differentially expressed genes were defined using the function that fits multiple linear models from the Bioconductor package limma in R (Smyth, 2004) with p-value<0.05.

[00639] *Microarray and data analysis:* Naive CD4⁺ and CD8⁺ T cells were isolated from WT or IL-27ra KO mice, and differentiated *in vitro* with or without IL-27. Cells were collected at 72 hours for CD8⁺ and 96 hours for CD4⁺ and Affymetrix GeneChip Mouse Genome 430 2.0 Arrays were used to measure the resulting mRNA levels at these time points. Individual .CEL files were RMA normalized and merged to an expression matrix using the ExpressionFileCreator of GenePattern with default parameters (Reich et al., 2006). Gene-specific intensities were then computed by taking for each gene *j* and sample *i* the maximal probe value observed for that gene. Samples were then transferred to log-space by taking log₂(intensity).

[00640] Differentially expressed genes were annotated as genes with fold-change>2 and FDR-corrected ANOVA <0.2 computed between the CD4 with or without IL-27 stimulation (CD4⁺ IL27 and ThO) subpopulations. A list of 972 cell surface/cytokines genes of interest that include: cytokines, adhesion, aggregation, chemotaxis and other cell surface molecules (**Table 18**) was composed using GO annotation in Biomart.

DDR1	PAM	BMP10	EGFR	HYAL5	THBS1	IL9	CKLF
CX3CL1	CXCL10	FCER1A	EMR4	UMODL1	ARSA	RPL13A	GRAMD2
PLAT	CORIN	KLRC3	KLRC1	CD40	ADAM2	IL25	CXCL5
LAMP1	MPP3	EFNA5	SCNN1A	RTN4RL2	ENTPD6	CMTM5	PPBP
KIT	SCNN1G	LDLR	ACE2	ADAM6A	CD164	TNFSF14	PF4
LPL	FGFBP1	BACE1	FCGR4	ITGA1	CD320	CLCF1	CXCL3
SDC1	HCST	ABCA1	PEAR1	LY6E	ENTPD5	IL31	CXCL15
PEBP1	PDGFC	FGFR3	CR2	HEG1	CD248	TNFSF13B	CXCL1
SLC2A4	ITGAX	ADAM17	CD8A	FZD10	CAP1	CER1	CXCL2
VCAM1	TLR2	NR3C1	H2-K1	GPR116	AMN	FAM3B	CXCL11
FGG	ACVR2B	PDGFRA	GREM1	UNC5D	SIVA1	BMP8B	CCL26
CD24A	CD163	EPHA4	ITGAL	CHRNA7	TRAF3	BMP8A	TRPM4
SCARB1	CD3G	ICOS	WNT1	GPR174	MS4A6B	IL1F8	ARHGEF5
HSPA5	CD37	TGFA	AQP4	WNT4	CD47	IL1F9	RETNLG
CD9	ICOSL	L1CAM	KLRA8	KCNH5	ABCB1A	IL1F6	RPS19
CD34	TNFRSF11A	NCAM1	H2-AB1	TNN	IGLL1	IL1F5	FLT1
ADAM9	CD96	ITGA3	BMPR1A	LAYN	CD160	IL1F10	MYO9B
CD83	ENPP1	CRHR2	CD74	DLK1	IDO1	CCL17	CALCA
HMGB1	FZD4	TGFBR2	H2-D1	LRP1	PROCR	NAMPT	PTPRO
USP14	TNFRSF13	TNFRSF22	ANXA5	TRPV1	CD2AP	IL12B	RAC1

	C						
IL2RG	PDPN	SEMA7A	SLAMF1	NTRK1	IL18R1	IL22	CXADR
CD81	CTLA4	ITGAM	PTPRJ	CD226	TNFRSF8	ILTIFB	PRKCA
MIF	STAB2	CDH5	AIPL1	GHSR	IL8RB	IL1 1	SYK
LAMP2	SPAM1	GABBR1	TGFBR3	LBP	CXCR5	GRN	SLC37A4
MCAM	CLEC2D	MSR1	CEP290	ITGAE	TNFRSF10 B	IFNB 1	AMICA1
HSP90AB1	SELL	WNT3A	KLHL20	IFNGR1	ABCG2	GM12597	NCKAP1L
M6PR	C3AR1	ACVR1B	NFAM1	CDH1	ICAM4	IFNA14	TGFB2
CD82	IL1R2	TNFRSF23	HMMR	C1QBP	ENTPD1	IFNA9	EDN3
BGN	FLT3	CD3E	PSEN2	ADAM3	GYPE	IFNA12	EDN2
GABARAP LI	ISG20	FLT3L	AMOT	THBD	CD99L2	IFNA13	S100A8
CRYAB	LGALS1	FCER2A	IFNG	IL7R	PRR3	GM13280	S100A9
AIMP1	CD93	PTPRC	KLRB 1F	CD53	SLC3A2	IFNA2	CSF3R
PDIA4	TNF	SPN	PTPRR	FCGR3	CAST	IFNAB	CXCR2
EPCAM	CCR1	PLAU	CD72	ENPEP	NT5E	GM13271	ITGA9
SFRP1	BMPR2	CLEC2I	CD209B	HYAL2	PGP	GM13283	PDE4B
PLA2G1B	CD4	C5AR1	CD8B1	CXCR4	CCND2	GM13290	PDE4D
AMBP	PROM1	CD200R1	KLRA7	ICAM2	CD3EAP	TNFSF1 1	COROIA
SERPINE2	WNT6	SCN5A	CD209A	OCN	SCOI	GM13289	LYST
BACE2	CD7	CCR5	ROB04	NCOR2	DARC	GM13272	SBDS
PTPRU	CD274	IL6RA	ALCAM	IL 1R1	SLC44A1	IFNZ	CCR2
APOH	RTN4R	GLRA1	SLC1A3	CD1D1	PTGFRN	GM13276	GAS6
DPP4	CD22	TACR1	HSPD1	LY9	NRP	GM13277	HRH1
PLG	GAB2	CD40LG	RYK	CD68	LSM1	GM13278	NUP85
ATP5B	TREML2	CCR8	GPM6A	GPR65	CTSD	GM13275	EDNRB
ADAM8	P2RX7	TNFRSF18	HSP90AA1	MUC1	PRNP	GM13279	ROCK1
CLPTM1	CSF1R	GP1BA	AGRN	NDP	GSS	GM13285	MSN
LY6D	TMX3	IL1RL1	LRPAP1	B2M	PTPRCAP	GM13287	EZR
TRPV2	RC3H2	ART1	GPR125	PTGER2	CD2BP2	GM13288	OLR1
HSPA2	PSEN1	IL15RA	BOC	LY75	PVRL3	IFNA7	FERMT3
LPAR1	TNFRSF4	ITGA6	ITGB 1	RAPSN	CD200	IFNA1 1	TNIP1
PDGFRB	CD86	GLRB	PCSK6	KDR	CD302	IFNA6	GCNT1
LY6A	KLRB 1B	CR1L	ACE	AOC3	IFNAR1	IFNA5	PODXL2
TMEM123	DCBLD2	WNT5B	ENOX2	KCNE2	TLR1	IFNA4	LEP
CD 14	EPHA5	ACHE	ROBO1	IL27RA	CD5L	IFNA1	SELPLG
ENG	SELP	ADIPOQ	CD48	KLRB1C	CCR6	IFNE	GOLPH3
H13	LPAR2	EBAG9	MUC3	PDCD1	PEMT	CMTM2A	CHST4
TNFRSF1 A	CFTR	IRAK1BP1	ITGB4	EPHB4	LAP3	CMTM6	STKIO
CAV2	GPR84	TLR3	DSCAML1	SCUBE1	PLXNC1	CMTM7	FN1
F3	APP	CNTNAP2	FZD9	IL4	SCARB2	CMTM2B	IGHG2C
TGFB3	HSPA8	CXCR6	SHH	VPREB1	IL13RA1	CMTM8	PLA2G2A
RAMP1	TGFB 1	EPS8	GHRHR	CASR	SP1	CMTM3	REG4
SERPINF2	ARNT2	CD3D	CD80	LAG3	CD151	CMTM4	F11

FLOT2	KCNE1	KCND2	CCRL2	CXCR3	TNFSF10	C1QTNF4	PTGDS
TRPV4	IL17RB	CD84	TNFRSF9	CD70	IGSF8	SCGB3A1	KLKB1
IL2RB	IL2RA	TNFSF9	GPRC6A	CD244	TIGIT	IL16	OLFM4
CXCL12	CLEC7A	FZD5	KLRA1	CX3CR1	LILRB4	IL17D	BGLAP2
SLC11A2	HPN	HSPB1	FGB	PLA2R1	Gene	SCG2	SPOCK3
CD28	CD247	FPR2	ADAMIO	FGF22	PDCD ILG 2	GDF10	C8G
CD276	CHRN2	AGTRAP	NRXN1	KLRB1A	CTOA2B	GDF2	SERPINC1
CALR	KLRA5	TFRC	REEP4	IL6	CTOA2A	PGLYRP1	OLFM1
CD79B	ART2B	MME	CD69	GABRR1	IL12A	CCL20	CTRB1
SDC4	IL13	FZD1	XPOT	KLRC2	SPP1	INHBA	OGN
PVRL2	GDI2	GFRA2	RPS6KB1	PDGFB	TNFSF18	IL34	C1QTNF7
TJP1	SEMA4D	GYP A	CD99	MRC1	OSM	AREG	GPLD1
ITGA2B	NTRK2	IL1RN	ADCYAP1 R1	IL21R	LIF	TNFSF12	EGF
APOA4	FGFR2	HNRNPU	PAQR3	KISS1R	BMP3	BC096441	IL18BP
PLA2G5	WNT7B	PAQR4	HFE2	KLRK1	WNT2	TNFSF13	UCMA
SYNJ2BP	IL17RA	IDE	AQP11	ITGA2	SLURP1	GDF9	CFI
FCGR1	VEGFA	THY1	HYAL4	CD33	PRL7D1	IL5	MMP1A
GPR97	MFGE8	RALA	RSP02	LY6F	GDF1	THPO	MMP8
VLDLR	TIMP2	CD36	CNRIP1	CD19	CRLF1	CSF2	APOC2
GHR	IL4RA	TNFRSF13 B	GUCY2G	ITGB2	IL17F	IL3	IAPP
ADA	MRC2	MS4A1	SULF2	FSHR	GDF15	BMP4	PTPRG
B4GALT1	GPC4	ERP44	CD200R3	FUT4	IL17C	CCL24	C8B
EPHB6	TRPC1	ITGA5	ULBP1	TDGF1	GDF7	IL2	GIF
NRP1	GPR56	PTPRK	SCARA5	FOLR1	GDF6	IL21	COL6A2
TRIP10	ITGA4	SLC34A1	ANXA9	LRP6	GDF5	FGF2	C8A
CAR4	PTPN11	SORT1	P2RY12	SFRP4	TSLP	CSF3	SERPINE3
TLR4	ITGAV	WNT7A	MUC16	EMR1	GDF3	IL24	MYOC
STX2	CHRNA4	CLIC4	APOE	CTSL	IFNL2	IL20	ADAMTS20
IL12RB1	CIITA	ADRB1	INTO	STX4A	IFNL3	IL19	F7
RAMP2	TREM2	PDIA3	NR4A2	HAVCR2	IL23A	IL10	FGF10
THSD1	PTPRT	PGRMC1	CD38	AMELX	CCL1	BMP5	CTS7
IL15	IL6ST	CCR7	ECE1	FOLR2	GREM2		SERPINB10
FCERIG	PECAMI	2-Sep	FERMT2	FGF8	IL1A	CCL21C	CTSB
HBEGF	IL18RAP	SLC46A2	ATPIF1	NOTCH2	BMP15	CCL27A	WNT9A
CD5	KLRA2	JAM3	ISLR2	CD6	IL1B	CXCL13	NEPN
GPR124	H2-M3	NID2	CNTN2	SLC6A1	IFNK	GM21541	POMC
ITGA7	CLEC5A	CDH13	P2RX2	ADAMTS7	IL27	GM13304	APOD
CD97	ANPEP	ABCG1	GRIA1	CD27	BMP7	GM13306	PRL3D1
TSPAN32	HHIP	S1PR1	H2-AA	TNFRSF14	GDF11	CCL28	CEL
CAV3	CDON	TRPC4	CD200R4	PLAUR	EBI3	CCL21B	COL25A1
SCNN1B	KCNJ3	AXL	VWF	TREML1	GPI1	GM10591	PRL3B1
HSPA9	MS4A2	BMP2	FCGR2B	GPIHBP1	IL7	GM2564	BCHE

FURIN	ITGB3	ATP6AP2	ACVRL1	GPR160	TNFSF15	CCL27B	CNTF
TNFRSF12A	CD46	IFITM3	SCUBE3	TMEM102	LEFTY2	CCL19	CEACAM10
GPR162	CEACAM2	CD44	TLN1	SLAMF7	LEFTY1	CCL21A	SMPD1
ASTN1	GRIN2A	PVR	TNR	ERP29	TNFSF8	XCL1	HPX
NOTCH1	TRPM8	1600029D21RIK	SULF1	AAMP	CTF1	CXCL16	WFDC1
CXCL9	IL5RA	PDLIM2	IRAK2	NLGN2	CTF2	CCL2	TFF2
CAPN5	IL12RB2	ICAM1	GRK5	PTPN3	CSF1	CCL7	FBLN1
TIRAP	TMC1	IGF2R	WNT5A	BTLA	IL18	CCL11	SERPINI2
CD59A	SLC6A2	IFITM1	RTN4RL1	BSG	BMP6	CCL12	TFF1
PDGFA	CYSLTR2	FGA	NOTCH4	PPFIA2	IL17B	CCL8	SEZ6
LPAR3	CD1D2	REEP2	F2R	PKD2L1	KITL	CCL5	FBLN5
ITGB7	NLGN1	CST8	ACVR2A	VTCN1	LTB	CCL9	ADCYAP1
CD55	CCR4	ANGPTL3	PCSK9	ROB02	IL33	CCL6	F5
CD2	IL17A	PSTPIP1	P4HB	KLRE1	LTA	CCL3	CNP
TEK	GPR98	SLIT2	IL10RA	CD52	MSTN	CCL4	
FASL	STRC	BCAM	GRIN1	MSLN	BMP1	CXCL14	
SERPINA5	SELE	KCNMA1	RGMA	KLRD1	EDN1	CCL25	
CHRNA1	TNFSF4	BST2	58304UNO6RIK	FAS	NODAL	CCL22	

[00641] *Signature analysis of other dysfunctional states:* For viral exhaustion: Microarray dataset (Doering et al, 2012) was downloaded, followed by RMA. A signature of viral exhaustion was defined as the genes that are differentially expressed between chronic and acute viral infection on day 15 and day 30. Genes were ranked based on a t -test statistic and fold change, each gene rank was then adjusted for multiple hypotheses testing using false discovery rate (FDR). A threshold of fold change >1.1 and FDR <0.2 was applied.

[00642] For anergy: Data ((Safford et al, 2005), Table 1) were downloaded. 90 genes were reported as upregulated in T cells stimulated in conditions that promote versus inhibit anergy.

[00643] For antigen-specific tolerance: Data (Burton et al, 2014) were downloaded. Two groups were defined, group 1 that includes the PBS and 0.008 μg treated samples (treatment number 1) versus group 2 - 80 μg (treatment number 5 and 6). After Log₂ transformation and quantile normalization, the Limma package was used to estimate the fold changes and standard errors by fitting a linear model for each gene for the assessment of differential expression. Genes with p value < 0.05 were selected: 1,845 genes were upregulated of which 88 were defined as cytokine and cell surface molecules (Davis and Meltzer, 2007; Smyth, 2004, 2005).

[00644] For antigen non-specific tolerance: Data was downloaded from (Mayo et al, 2016). Robust Multi-array Average (RMA) and quantile normalization were applied for background correction and normalization using the ExpressionFileCreator module of GenePatterns. Differentially expressed genes were defined using signal-to-noise ratio (SNR), following FDR correction. Differentially expressed genes were identified as genes having a $FDR < 0.2$ between mRNA expression profiles of naive $CD4^+$ or $CD4^+$ GFP/IL-10⁺ T-cells isolated from the spleen or cLNs of B6N0DF1 ^{IL1 α :GFP} mice following nasal treatment with anti-CD3 which attenuates the of progressive phase of EAE.

[00645] For cancer: Data was obtained from (Singer *et al.*, companion manuscript). Briefly, mRNA samples from $CD8^+Tim3^-PD1^-$ (DN) TILs, $CD8^+Tim3^-PD1^+$ (SP), and $CD8^+Tim3^+PD1^+$ (DP) TILs were measured using Affimetrix GeneChip Mouse Genome 430 2.0 Arrays, expression values were RMA normalized, corrected for batch effects using COMBAT (Johnson et al, 2007) and gene-specific intensities were then computed by using the maximal prob intensity per gene, values were transferred to log-space by taking $\log_2(\text{intensity})$. Differentially expressed genes were defined as genes with either an FDR-corrected t-test p-value smaller or equal to 0.2 computed between the DN and DP subpopulations and a fold-change of at least 1.5 between the two subpopulations.

[00646] *RNA expression profiling of tumor infiltrating cells:* Tumor infiltrating $CD8^+$ T cells were isolated from WT or IL-27ra KO tumor bearing mice via FACS sorting on a FACSAria (BD Biosciences). Tumor infiltrating $CD8^+$ T cells were processed using an adaptation of the SMART-Seq 2 protocol (Tirosh et al, 2016) (Shekhar et al. 2016 in press), using 5 μ L of lysate from bulk $CD8^+$ T cells as the input for each sample during RNA cleanup via SPRI beads (-2,000 cells lysed on average in RLT).

[00647] RNAseq reads were aligned using Tophat (Trapnell et al, 2009) (mm9) and RSEM-based quantification (Li and Dewey, 2011) using known transcripts (mm9), followed by further processing using the Bioconductor package DESeq in R (Anders and Huber, 2010). The data was normalized using TMM normalization. The TMM method estimates scale factors between samples that can be incorporated into currently used statistical methods for DE analysis. Post-processing and statistical analysis was carried out in R (Li and Dewey, 2011). Differentially expressed genes were defined using the differential expression pipeline on the raw counts with a single call to the function DESeq (adjusted p value < 0.1). Heatmap figures were generated using pheatmap package (Kolde, 2015).

[00648] *Network construction:* Networks were generated using Cytoscape version 3.2.1 (Lopes et al, 2010). The network model is based on coupling *in vitro* gene expression data of

naive CD8⁺ T cells from KO (Prdml or c-Maf) and WT controls stimulated in the presence of IL-27 and previously published ChIPseq data for that specific regulator. More specifically, samples were analyzed using a custom code set of 397 genes representing both the IL-27-driven gene signature (245 genes) and the dysfunctional CD8⁺ TIL gene signature (245 genes) (Table 17). Differentially expressed genes between WT control and KO were defined using the function that fits multiple linear models from the Bioconductor package limma in R (Smyth, 2004) with p-value<0.05. For the ChIP-Seq evidence Applicants used published Prdml (Shin et al., 2013) and c-Maf (Ciofani et al, 2012) published binding events dataset. In the network presentation, Applicants selected the 61 genes that are part of the IL-27 inhibitory module (Figure 6G).

[00649] *Single-cell RNA-Seq:* Briefly, tumor infiltrating lymphocytes from B16 melanomas were sorted into 96-well plates with 5 μ l lysis buffer comprised of Buffer TCL (Qiagen) plus 1% 2-mercaptoethanol (Sigma). Plates were then spun down for one minute at 3000rpm and immediately frozen at -80°C. Cells were thawed and RNA was isolated with 2.2x RNAClean SPRI beads (Beckman Coulter Genomics) without final elution (Shalek et al, 2013). The beads were then air-dried and processed immediately for cDNA synthesis. Samples were then processed using the Smart-seq2 protocol (Picelli et al, 2014), with minor modifications applied to the reverse transcription (RT) step. This was followed by making 25 μ l reaction mix for each PCR and performed 21 cycles for cDNA amplification. Then, using 0.25 ng cDNA of each cell and 1/4 of the standard Illumina NexteraXT reaction volume in both the tagmentation and final PCR amplification steps. Finally, plates were pooled to 384 single-cell libraries, and sequenced 50 x 25 paired-end reads using a single kit on the NextSeq500 5 instrument.

[00650] *Single-cell analysis:* Briefly, paired reads were mapped to mouse annotation mmlO using Bowtie (Langmead et al, 2009) (allowing a maximum of one mismatch in seed alignment, and suppressing reads that had more than 10 valid alignments) and TPMs were computed using RSEM (Li and Dewey, 2011), and log₂(TPM+1) values were used for subsequent analyses.

[00651] *Tumor Experiments:* 5 x 10⁵ B16F10 melanoma cells (ATCC) were implanted into the right flank of C57BL/6 mice. Tumor size was measured in two dimensions using a caliper. TILs were isolated by dissociating tumor tissue in the presence of 2.5 mg/ml collagenase D for 20 min before centrifugation on a discontinuous Percoll gradient (GE Healthcare). Isolated cells were then used in various assays of T cell function.

[00652] **CyTOF analysis:** Antibodies were labeled using MaxPar® Metal Labeling Kits (DVS) by The Longwood Medical Area CyTOF Antibody Resource and Core. In some experiments, TILs were enriched using Dynabeads FlowComp Mouse Pan T (CD90.2) Kit (Invitrogen). Cells were washed and resuspended in CyTOF PBS (PBS + 0.05% sodium azide + 0.5% BSA) and stained with the cocktail of antibodies against cell-surface molecules for 30 min. Cells were washed again and resuspended in CyTOF PBS with 4% paraformaldehyde. After 10 min fixation, cells were washed and stained with Cell-ID intercalators (DVS) overnight. Before analysis, cells were resuspended in water with beads and loaded to the CyTOF® Mass Cytometer (DVS). CyTOF data were recorded in dual-count according to Fluidigm's recommended settings and the analysis was done on the fly.

[00653] To obtain clusters of cells similar in their protein expression patterns, cells were clustered using k-means algorithm. Optimal cluster number was estimated using the within groups sum of squared error (SSE) plot followed by gap statistics with bootstrapping and first SE max method. These methods suggested 9 clusters as optimal in the multidimensional space. Applying k-means clustering with (k=9) on our CyTOF data, resulted in clear distinction between cluster 1 and cluster 2 of the CD8⁺ cells. This separation could be further visualized by two-dimensional non-linear embedding of the protein expression profiles using t-stochastic neighborhood embedding (t-SNE (Maaten L, 2008)). The t-SNE plot can then be overlaid by k-means clustering results to reflect a non-biased approach to the clusters or with intensity of the different markers.

Example 3: CD39 regulates dysfunction in CD8⁺ TILs and marks a novel population with an altered functional phenotype

[00654] CD39 (also known as ectonucleoside triphosphate diphosphohydrolase-1) is encoded by the gene *ENTPD1*. It is a cell surface protein with an extracellular catalytic site that catalyzes the hydrolysis of various P2 receptor ligands, including ATP, ADP, UTP and other phosphate containing molecules. The enzymatic activities of CD39, in conjunction with CD73, play a role in calibrating the duration, magnitude, and chemical nature of purinergic signals delivered to immune cells. As disclosed herein, CD39 and up-regulation of *ENTPD1* is associated with several dysfunctional T cell states.

[00655] Applicants postulated that CD39 (i.e. *ENTPD1*) may be involved in regulating CD8⁺ T cell dysfunction. Applicants can validate that CD39 performs important functions for inducing T cell dysfunction, and more specifically, can determine whether modulating CD39 in T cells provides an enhanced immune response in cancer.

[00656] In a certain example, Applicants characterize CD39 expression and its associated function in CD8⁺ WT tumor-bearing mice. TILs (tumor infiltrating lymphocytes) are isolated from the mice and expression is determined. Cells may be sorted and sequenced in bulk or single cells may be sequenced. CD39 may be expressed on a subpopulation of CD8 T cells having a signature of dysfunction as described herein or a signature of dysfunction previously described (Singer et al, Cell, Vol 166, Issue 6, p1500-1511.e9, 8 September 2016). The dysfunctional subpopulation may be found in TILs, but not in tumor draining lymph node.

[00657] In a certain example, cytokine expression in CD39-expressing CD8⁺ TILs is examined to determine whether the CD39 expression correlates with CD8⁺ T cell function. This result may determine whether CD39 CD8 TILs are not only poorly functional as measured by a dysfunctional signature, but they may also actively produce suppressive cytokines and contribute to suppression locally in the tumor microenvironment. Suppressive cytokines may include, but are not limited to IL-10.

[00658] Applicants can determine whether CD39 is a regulator of the suppressive function of dysfunctional CD8⁺ T cells in cancer. In a certain example, CD39 WT or knockout CD8⁺ T cells are assessed for their ability to influence effector T cell proliferation using a suppression assay, such that CD39^{-/-} TILs fail to suppress effector T cell proliferation compared to WT dysfunctional TILs.

[00659] In a certain example, to directly analyze the functional role of CD39 in regulating CD8⁺ T cell dysfunction, a lentiviral CRISPR/cas9 targeting approach is used to knockout CD39 in T cells. In a certain example, naive transgenic pmel CD8⁺ T cells are used. Control or CD39 CRISPR lentiviruses are transduced into CD8⁺ T cells isolated from PMEL transgenic mice in which all T cells have a single tumor antigen specific TCR with specificity for the mouse homologue of the human premelanosome protein. PMEL CD8⁺ T cells are normally ineffective at controlling growth of B16F10 melanoma tumors, such that perturbations that promote tumor clearance can be readily discerned. Control or CD39-targeted (deleted, i.e., CD39^{-/-}) pmel CD8⁺ T cells are activated and equal numbers of cells are transferred into WT mice with established B16F10 melanoma tumor. Mice are then followed for tumor growth. Efficiency of CD39 deletion may be determined by quantitative real time PCR. The transfer of CD39^{-/-} pmel CD8⁺ T cells is expected to significantly delay tumor growth in WT mice.

[00660] Upon transfer into WT hosts, CD39^{-/-} pmel CD8⁺ T cells may produce a higher percent of poly-functional IL-2 and IFN γ -producing cells, consistent with a less dysfunctional phenotype compared to control WT pmel CD8⁺ T cells. Accordingly, the

transfer of CD39^{-/-} pmel CD8⁺ T cells may delay tumor growth in WT mice. These data may support a role for CD39 as a regulator of the CD8⁺ T cell dysfunction program that contributes to poor tumor control.

[00661] In a certain example, Applicants can further demonstrate that tumor growth is significantly reduced or abolished in CD39^{-/-} KO mice, and that splenic CD39^{-/-} CD8⁺ T cells from CD39^{-/-} KO mice harboring a tumor has a reduction in tumor size when transferred into tumor harboring wild type animals. In particular, WT or CD39^{-/-} mice are implanted with B16-F10 tumor subcutaneously. At day 18, CD8 and CD4 T cells are isolated from the spleens of WT and CD39^{-/-} mice and transferred into WT host mice which are subsequently injected with B16-F10 tumor subcutaneously. Tumor growth is then followed.

[00662] A CRISPR/cas9 targeting approach is also used to knockout CD39 followed by RNA-seq to determine gene networks regulated by CD39.

[00663] Turning to Figure 17, Applicants show that CD39 is co-expressed with PD-1+Tim3+ CD8 T cells and blocking antibody slightly suppress tumor growth (B16 melanoma).

Example 4: Therapeutic modulation of CD39

[00664] In a certain example, modulation of CD39 is used in the treatment of cancer in a patient in need thereof. In a certain example, Applicants modulate expression or activity of CD39 in autologous T cells obtained from a patient in need thereof to perform adoptive cell transfer. The autologous T cells may be made resistant to exhaustion or exhausted T cells are activated by knockdown or knockout of expression or activity of CD39. Additionally, activity or expression of CD39 is modulated in CAR T cells. T cells may be modulated *ex vivo* and transferred to a patient by any method described herein.

[00665] In a certain example, Applicants target dysfunctional CD8⁺ T cells *in vivo* in a patient in need thereof suffering from cancer, such that T cells expressing CD39 are targeted with a therapeutic composition with specific affinity for CD39. The therapeutic composition may be an antibody, such as but not limited to an antibody drug conjugate. Effective tumor control may be provided by removing dysfunctional T cells in the tumor microenvironment, thus enhancing immunity and decreasing suppression.

Example 5: Experimental procedures for verifying activity of CD39

Mice

[00666] 6-8 week old female Balb/c, C57BL/6, pmel transgenic, and OTI transgenic mice are purchased from the Jackson Laboratory.

Tumor Experiments

[00667] B16F10 (5×10^5) are implanted subcutaneously into the right flank. Tumor size was measured in two dimensions by caliper and is expressed as the product of two perpendicular diameters. For adoptive transfer tumor experiments, tumor cells are implanted five days prior to intravenous injection of T cells. Naive ($CD8^+CD62L^+CD44^{10}$) T cells from PMEL (for crispr/cas9 targeting experiments) are isolated by cell sorting (BDFACS Aria) and activated by $2 \mu\text{g/ml}$ each of plate-bound anti-CD3 and anti-CD28 antibodies for 48 hours, rested for 3 days, and then reactivated with $1 \mu\text{g/ml}$ of anti-CD3 and anti-CD28 antibodies for 2 days prior to transfer into recipient mice. Retroviral and lentiviral infections of primary T cells are optimized and experiments are performed as described herein. Briefly, retrovirus is used to spin-infect T cells one day after activation and lentivirus is used to infect T cells twice, at 16 hours prior to activation and at 4 hours post activation. Targeting efficiency of retrovirus is determined by measuring GFP expression; whereas effective CRISPR/cas9-mediated deletion of the target gene using lentivirus is determined by qPCR.

Isolation of Tumor Infiltrating Lymphocytes.

[00668] Tumor infiltrating lymphocytes are isolated by dissociating tumor tissue in the presence of collagenase D (2.5 mg/ml) for 20 min prior to centrifugation on a discontinuous Percoll gradient (GE Healthcare). Isolated cells are then used in various assays of T cell function. Cells are cultured in DMEM supplemented with 10% (vol/vol) FCS, $50 \mu\text{M}$ 2-mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids, L-glutamine and 100 U/ml penicillin and $100 \mu\text{g/ml}$ streptomycin.

Flow Cytometry

[00669] Single cell suspensions are stained with antibodies against surface molecules. CD4 (RM4-5), CD8 (53-6.7), and PD-1 (RMP1-30) antibodies are purchased from BioLegend. Tim-3 (5D12) antibody is generated in house. Fixable viability dye eF506 (eBioscience) is used to exclude dead cells. For intra-cytoplasmic cytokine staining, cells are stimulated with 12-myristate 13-acetate (PMA) (50 ng/ml , Sigma-Aldrich, MO), ionomycin ($1 \mu\text{g/ml}$, Sigma-Aldrich, MO) in the presence of Brefeldin A (Golgiplug, BD Bioscience) for four hours prior to staining with antibodies against surface proteins followed by fixation and permeabilization and staining with antibodies against IL-2 (JES6-5H4), TNF- α (MP6-XT22), IFN- γ (XMG-1.2) (eBioscience), and Granzyme B (GBII) (Biolegend). For measurement of intracellular zinc, cells are stained with $1 \mu\text{M}$ Zinpyr-1 (Sigma) in PBS for 20 min at 37°C , washed with media, followed by regular surface staining. All data are collected on a BD LsrII (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Generation of Lentiviral constructs using CRISPR/CAS9 targeting.

[00670] The initial guide sequences are selected based on the exon structure of target genes (i.e. ENTPD1) and ranked by the repertoire of potential off-target sites to select designs that minimize the possibility of off-target cleavage. The guides are then cloned into CRISPR-Cas9 vectors via golden-gate cloning as described previously (Cong et al, 2013, Science 339, 819-823). The vector used is a lenti-viral vector, pCKO_2, bearing mammalian-codon-optimized SaCas9 linked to puromycin selection cassette (Ran et al, 2015, Nature 520, 186-191; Shalem et al, 2014, Science 343, 84-87), and an sgRNA-expression cassette that is modified to enhance RNA expression. The constructs are sequence verified and tested to screen for the efficiency against ENTPD1 using a mouse T-lymphocyte cell line, EL4 (ATCC) before moving on to lentiviral production. To quantify the genomic modification induced by the CRISPR-Cas9 system, genomic DNA is extracted using QuickExtract Solution (Epicentre), as described previously (Cong et al, 2013, *supra*). Indel formation is measured by either SURVEYOR nuclease assay (IDT DNA) or targeted deep sequencing as described previously (Cong et al, 2013, *supra*). Briefly, the genomic region around the CRISPR-Cas9 targeting site (i.e. ENTPD1) is amplified, and then subject to either SURVEYOR nuclease digestion following re-annealing or re-amplified to add on Illumina P5/P7 adapters with barcodes for deep-sequencing analysis using the MiSeq sequencing system (Illumina).

[00671] After screening of guides in cell lines, the top-ranked guides based on their targeting efficiency for ENTPD1 are used for viral production. 293FT cells (Thermo Fisher) are maintained as recommended by the manufacturer in 150mm plates. For each transfection, 10µg of pVSVG envelope plasmid, 15µg of pDelta packaging plasmids, and 20µg of pCKO_2 vector carrying the construct of interest are used. The transfection is either carried out using lipofectamine 2000 (Thermo Fisher) following the manufacturer's recommendations, or with PEI, where 5:1 ratio of PEI solution is added to the DNA mixture, and incubated for 5 minutes before adding the final complex onto cells. After incubation for 16 hours, 20 mL of fresh warm media is applied to replace the old growth media. Virus is harvested between 48h and 72h post transfection by taking the supernatant and pelleting cell debris via centrifugation. The viral particles are then filtered through a 0.45µm filtration system (Millipore), and then either directly used as purified supernatant, or concentrated further with 15-mL Amicon concentrator (Millipore). Lentiviral vectors are titered by real-time qPCR using a customized probe against the transgene.

[00672] For all primary T-cell experiments, the efficacy of the CRISPR-Cas9 lentiviral vectors is first tested by transducing *in vitro* primary mouse T-cell culture, followed by cleavage measurement and qPCR detection of target gene knock-down. The most efficient viral constructs are then used for downstream experiments.

[00673] The invention is further described by the following numbered paragraphs:

1. A method of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, or any combination thereof.

2. The method of paragraph 1, wherein the T-cell dysfunction is T-cell exhaustion.

3. The method of paragraph 2, wherein the modulation of T-cell exhaustion comprises a decrease in the exhausted T-cell phenotype, such that functional T-cell activity is increased.

4. The method of paragraph 1, wherein the selected target gene or gene product or a combination thereof is/are identified as participating in the inhibition of functional T-cell activity.

5. The method of paragraph 4, wherein the modulating agent inhibits the expression, activity and/or function of the selected target gene or gene product or combination thereof.

6. The method of paragraph 1, wherein the selected target gene or combination of target genes is/are identified as participating in the promotion of functional T-cell activity.

7. The method of paragraph 6, wherein the modulating agent promotes or activates the expression, activity and/or function of the selected target gene or gene product or combination thereof.

8. The method of paragraph 1, comprising contacting the dysfunctional T-cell with modulating agents that modulate the expression, activity and/or function of at least two target genes or gene products selected from the target genes listed in Table 1, Table 2, or any combination thereof.

9. The method of paragraph 1, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

10. The method of paragraph 1, further comprising contacting the dysfunctional T-cell with an agent or treatment selected from the group consisting of a PD-1 inhibitor, a

CTLA4 inhibitor, chemotherapy, radiation therapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-IBB, GITR, CD226, KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and/or SLAMF7.

11. A method of treating a condition involving or characterized by the presence of T cells exhibiting a dysfunctional or exhausted phenotype, the method comprising administering an amount of a modulating agent effective to modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, or any combination thereof.

12. The method of paragraph 11, wherein the condition is cancer or a persistent infection.

13. The method of paragraph 11, wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

14. The method of paragraph 13, wherein the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

15. The method of paragraph 11 wherein a selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

16. The method of paragraph 15, wherein the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

17. The method of paragraph 11, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

18. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising a first modulating agent and a second modulating agent that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, or any combination thereof.

19. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising a first modulating agent that inhibits the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, or any combination thereof and a second modulating agent that promotes the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, or any combination thereof.

20. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising a modulating agent that modulates the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, or any combination thereof and an agent selected from the group consisting of a PD-1 inhibitor, a CTLA4 inhibitor, chemotherapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-1BB, GITR, CD226, KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and/or SLAMF7.

21. The composition of any one of paragraphs 18-20 wherein the T cell dysfunction comprises T cell exhaustion.

22. The composition of any one of paragraphs 18-21 wherein the T cell exhaustion occurs in an individual with cancer or a persistent infection.

23. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising an inhibitor of the expression and/or activity of PDPN and an inhibitor of the expression and/or activity of PROCR.

24. A pharmaceutical composition for modulating T cell dysfunction comprising:
(a) an inhibitor of the expression and/or activity of PDPN and an inhibitor of the expression and/or activity of PROCR; and
(b) an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of TIGIT, LAG3, LILRB4, and KLRC1; and/or an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

25. A pharmaceutical composition for modulating an IL-27-regulated co-inhibitory module comprising:

(a) an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM and KLRC1; and

(b) an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

26. The pharmaceutical composition of any one of paragraphs 23-25, further comprising an inhibitor of the expression and/or activity of TIM-3.

27. The pharmaceutical composition of any one of paragraphs 23-25, further comprising an inhibitor of the expression and/or activity of PD-1.

28. The pharmaceutical composition of any one of paragraphs 23-25, further comprising an inhibitor of the expression and/or activity of CTLA4.

29. The pharmaceutical composition of any one of paragraphs 23-25, further comprising an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of PD-1 .

30. The pharmaceutical composition of any one of paragraphs 23-25, further comprising an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of CTLA4.

31. The pharmaceutical composition of any one of paragraphs 23-25, further comprising an inhibitor of the expression and/or activity of CTLA4 and an inhibitor of the expression and/or activity of PD-1 .

32. The pharmaceutical composition of any one of paragraphs 23-25, further comprising an inhibitor of the expression and/or activity of TIM-3, an inhibitor of the expression and/or activity of CTLA4 and an inhibitor of the expression and/or activity of PD-1.

33. The pharmaceutical composition of any one of paragraphs 23-32, wherein the inhibitors and activators are selected from an antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog.

34. The pharmaceutical composition of paragraph 33, wherein the antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog is selected from the group consisting of: an anti-CTLA4 antibody, an anti-PD-1 antibody, or aPDL-1 antagonist.

35. A method of modulating an IL-27-regulated co-inhibitory module in a subject in need thereof, the method comprising administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of PDPN and an inhibitor of the expression and/or activity of PROCR.

36. A method of modulating an IL-27-regulated co-inhibitory module in a subject in need thereof, the method comprising:

(a) administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of PDPN, and an inhibitor of the expression and/or activity of PROCR; and

(b) administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of an inhibitor of the expression and/or activity of TIGIT, LAG3, LILRB4, and KLRC1; and/or an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

37. A method of modulating an IL-27-regulated co-inhibitory module in a subject in need thereof, the method comprising:

(a) administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM, and KLRC1; and

(b) administering a pharmaceutical composition comprising an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

38. The method of any one of paragraphs 35-37, further comprising an inhibitor of the expression and/or activity of TIM-3.

39. The method of any one of paragraphs 35-37, further comprising an inhibitor of the expression and/or activity of PD-1.

40. The method of any one of paragraphs 35-37, further comprising an inhibitor of the expression and/or activity of CTLA4.

41. The method of any one of paragraphs 35-37, further comprising an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of PD-1.

42. The method of any one of paragraphs 35-37, further comprising an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of CTLA4.

43. The method of any one of paragraphs 35-37, further comprising an inhibitor of the expression and/or activity of CTLA4 and an inhibitor of the expression and/or activity of PD-1.

44. The method of any one of paragraphs 35-37, further comprising an inhibitor of the expression and/or activity of TIM-3, an inhibitor of the expression and/or activity of PD-1, and an inhibitor of the expression and/or activity of CTLA4.

45. The method of any one of paragraphs 35-44, wherein the inhibitors and activators are selected from an antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog.

46. The method of paragraph 45, wherein the antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog is selected from the group consisting of: an anti-CTLA4 antibody, an anti-PD-1 antibody, and a PDL-1 antagonist.

47. The method of any one of paragraphs 35-46, wherein the subject in need thereof has a disease or disorder characterized by T-cell exhaustion.

48. The method of any one of paragraphs 35-46, wherein the subject in need thereof is diagnosed as having a cancer or tumor.

49. The method of any one of paragraphs 35-46, wherein the subject in need thereof is diagnosed as having a persistent infection.

50. A method of modulating T cell dysfunction, the method comprising contacting a dysfunctional T cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the group consisting of: BTLA, TIGIT, HAVCR2 (TIM-3), LAG3, PDPN, IL10RA, IL1R2, PROCR, LILRB4, KLRC1, KLRC2, KLRE1, TNFSF9 (4-1BB), KLRK1, IL12RB1, IL1R1, AND SLAMF7.

51. The method of paragraph 50, wherein the T cell dysfunction is T cell exhaustion.

52. The method of paragraph 51, wherein the modulation of T cell exhaustion comprises a decrease in the exhausted T cell phenotype, such that T cell activation is increased.

53. The method of paragraph 51, wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

54. The method of paragraph 53, wherein the modulating agent promotes the expression, activity and/or function of the target gene or gene product or combination thereof.

55. The method of paragraph 51, wherein the selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

56. The method of paragraph 55, wherein the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

57. The method of paragraph 50, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

58. A method of treating a condition involving or characterized by the presence of T cells exhibiting an exhausted phenotype, the method comprising administering an amount of a modulating agent effective to modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the group consisting of: BTLA, TIGIT, HAVCR2 (TIM-3), LAG3, PDPN, IL10RA, IL1R2, PROCR, LILRB4, KLRC 1, KLRC2, KLRE1, TNFSF9 (4-1BB), KLRK1, IL12RB 1, IL1R1, AND SLAMF7.

59. The method of paragraph 58 wherein the condition is cancer or a persistent infection.

60. The method of paragraph 58 wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

61. The method of paragraph 60 wherein the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

62. The method of paragraph 58 wherein the selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

63. The method of paragraph 62 wherein the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

64. The method of paragraph 58 wherein the agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

65. A method of determining the presence of T cells exhibiting an exhausted phenotype, the method comprising detecting, in a sample comprising T cells, a level of expression, activity and/or function of one or more genes or expression products thereof selected from the target genes listed in Table 1, Table 2 or any combination thereof, and comparing the detected level to a reference, wherein a difference in the detected level relative to the reference indicates the presence of T cells exhibiting an exhausted phenotype.

66. The method of paragraph 65 wherein the sample is from an individual with cancer or a persistent infection.

67. A method of treating a disease or disorder characterized by aberrant or unwanted T-cell functional activity in a subject in need thereof, the method comprising administering a therapeutically effective amount of a modulating agent effective to modulate

the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, or any combination thereof.

68. The method of paragraph 67, wherein the disease or disorder is an autoimmune disease or graft vs. host disease.

69. The method of paragraph 67, wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

70. The method of paragraph 69, wherein the modulating agent promotes the expression, activity and/or function of the target gene or gene product or combination thereof.

71. The method of paragraph 67, wherein the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

72. The method of paragraph 67, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

73. A method of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 5.

74. A method of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 6.

75. A method of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 7.

76. A method of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 8.

77. A method of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 9.

78. The method of any one of paragraphs 73-77, wherein the T-cell dysfunction is T-cell exhaustion.

79. The method of paragraph 78, wherein the modulation of T-cell exhaustion comprises a decrease in the exhausted T-cell phenotype, such that functional T-cell activity is increased.

80. The method of any one of paragraphs 73-77, wherein the selected target gene or gene product or a combination thereof is/are identified as participating in the inhibition of functional T-cell activity.

81. The method of paragraph 80, wherein the modulating agent inhibits the expression, activity and/or function of the selected target gene or gene product or combination thereof.

82. The method of any one of paragraphs 73-77, wherein the selected target gene or combination of target genes is/are identified as participating in the promotion of functional T-cell activity.

83. The method of paragraph 82, wherein the modulating agent promotes or activates the expression, activity and/or function of the selected target gene or gene product or combination thereof.

84. The method of any one of paragraphs 73-77, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

85. The method of any one of paragraphs 73-77, further comprising contacting the dysfunctional T-cell with an agent or treatment selected from the group consisting of a PD-1 inhibitor, a CTLA4 inhibitor, chemotherapy, radiation therapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-1BB, GITR, CD226, KLRC2, KLRE1, KLRK1, IL12RB 1, IL1R1, and/or SLAMF7.

86. A method of treating a condition involving or characterized by the presence of T cells exhibiting a dysfunctional or exhausted phenotype, the method comprising administering an amount of a modulating agent effective to modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9.

87. The method of paragraph 86, wherein the condition is cancer or a persistent infection.

88. The method of paragraph 87, wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

89. The method of paragraph 88, wherein the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

90. The method of paragraph 86, wherein a selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

91. The method of paragraph 90, wherein the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

92. The method of paragraph 86, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

93. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising at least one modulating agent that modulates the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, Table 11, Table 12, or Table 13.

94. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising a first modulating agent that inhibits the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, Table 11, Table 12, or Table 13 and a second modulating agent that promotes the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, Table 9, Table 10, Table 11, Table 12, or Table 13.

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* * *

[00674] Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the above paragraphs is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

WHAT IS CLAIMED IS:

1. An isolated immune cell modified to comprise an altered expression or activity of, or modified to comprise an agent capable of inducibly altering expression or activity of, one or more of protein C receptor (PROCR), PRDMI and c-MAF, and Podoplanin (PDPN).

2. The isolated immune cell according to claim 1, wherein the immune cell is a T cell, preferably a CD8+ T cell.

3. The isolated immune cell according to any one of claims 1 to 2, wherein the immune cell displays tumor specificity.

4. The isolated immune cell according to claim 3, wherein the immune cell has been isolated from a tumor of a subject, preferably wherein the immune cell is a tumor infiltrating lymphocyte.

5. The isolated immune cell according to claim 3, wherein the immune cell comprises a tumor-specific chimeric antigen receptor (CAR).

6. The isolated immune cell according to any one of claims 1 to 5, modified to comprise downregulated or abolished expression or activity of PROCR, PRDMI and c-MAF, or PDPN.

7. The isolated immune cell according to claim 5, wherein the endogenous PROCR, PRDMI and c-MAF, or PDPN gene has been modified, whereby the cell comprises downregulated or abolished expression or activity of PROCR, PRDMI and c-MAF, or PDPN.

8. The isolated immune cell according to claim 7, wherein the endogenous PROCR, PRDMI and c-MAF, or PDPN genes has been modified using a nuclease.

9. The isolated immune cell according to claim 8, wherein the nuclease comprises (i) a DNA-binding portion configured to specifically bind to the endogenous PROCR, PRDMI and c-MAF, or PDPN genes and (ii) a DNA cleavage portion.

10. The isolated immune cell according to claim 9, wherein the DNA-binding portion comprises:

a zinc finger protein or DNA-binding domain thereof, a transcription activator-like effector (TALE) protein or DNA-binding domain thereof, or an RNA-guided protein or DNA-binding domain thereof;

a Cas protein modified to eliminate its nuclease activity; or

a DNA-binding domain of a Cas protein.

11. The isolated immune cell according to any one of claims 9 to 10, wherein the DNA cleavage portion comprises FokI or variant thereof or DNA cleavage domain of FokI or variant thereof.

12. The isolated immune cell according to claim 9, wherein the nuclease is an RNA-guided nuclease, such as a Cas protein.

13. The isolated immune cell according to claim 6, wherein the cell comprises a protein comprising a DNA-binding portion configured to specifically bind to the endogenous PROCR, PRDM1 and c-MAF, or PDPN genes.

14. The isolated immune cell according to claim 13, wherein the protein is a heterologous repressor protein capable of repressing the transcription of the endogenous PROCR, PRDM1 and c-MAF, or PDPN genes.

15. The isolated immune cell according to claim 14, wherein the heterologous repressor protein comprises at least a DNA-binding portion configured to specifically bind to the endogenous PROCR, PRDM1 and c-MAF, or PDPN genes, preferably to the endogenous PROCR, PRDM1 and c-MAF, or PDPN gene promoter.

16. The isolated immune cell according to any one of claims 14 or 15, wherein the heterologous repressor protein comprises (i) a DNA-binding portion configured to specifically bind to the endogenous PDPN gene, preferably to the endogenous PDPN gene promoter, and (ii) a transcription repression portion.

17. The isolated immune cell according to claims 15 or 16, wherein the DNA-binding portion comprises:

a zinc finger protein or DNA-binding domain thereof, a transcription activator-like effector (TALE) protein or DNA-binding domain thereof, or an RNA-guided protein or DNA-binding domain thereof;

a Cas protein modified to eliminate its nuclease activity; or

a DNA-binding domain of a Cas protein.

18. The isolated immune cell according to any one of claims 1 to 17, further modified to comprise:

(a) an altered expression or activity of PDPN;

(b) an altered expression or activity of PRDM1 and c-MAF;

(c) an altered expression or activity of PROCR;

(d) an altered expression or activity of any one or more of PD1, CTLA4, TIGIT, TIM3, LAG3, and PDL1;

- (e) an altered expression or activity of any one or more of TIGIT, LAG3, LILRB4, and KLRC1;
- (f) an altered expression or activity of any one or more of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7;
- (g) an altered expression or activity of any one or more of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM and KLRC1;
- (h) an altered expression or activity of any one or more of BTLA, TIGIT, HAVCR2 (TIM-3), LAG3, PDPN, ILIORA, IL1R2, PROCR, LILRB4, KLRC1, KLRC2, KLRE1, TNFSF9 (4-1BB), KLRK1, IL12RB1, IL1R1, and SLAMF7;
- (i) an agent capable of inducibly altering expression or activity of PDPN;
- (j) an agent capable of inducibly altering expression or activity of PRDM1 and c-MAF;
- (k) an agent capable of inducibly altering expression or activity of PROCR;
- (l) an agent capable of inducibly altering expression or activity of any one or more of PD1, CTLA4, TIGIT, TIM3, LAG3, and PDL1;
- (m) an agent capable of inducibly altering expression or activity of any one or more of TIGIT, LAG3, LILRB4, and KLRC1;
- (n) an agent capable of inducibly altering expression or activity of any one or more of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7;
- (o) an agent capable of inducibly altering expression or activity of any one or more of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM and KLRC1; or
- (p) an agent capable of inducibly altering expression or activity of any one or more of BTLA, TIGIT, HAVCR2 (TIM-3), LAG3, PDPN, ILIORA, IL1R2, PROCR, LILRB4, KLRC1, KLRC2, KLRE1, TNFSF9 (4-1BB), KLRK1, IL12RB1, IL1R1, or SLAMF7.

19. A cell population of immune cells as defined in any one of claims 1-18.

20. A method for generating the modified immune cell as defined in any one of claims 1 to 18, the method comprising (i) providing an isolated immune cell, and (ii) modifying said isolated immune cell such as to comprise an altered expression or activity of PDPN, PROCR, or PRDM1 and c-MAF.

21. A method for generating the modified immune cell as defined in any one of claims 1 to 18, the method comprising (i) providing an isolated immune cell, and (ii)

modifying said isolated immune cell such as to comprise an agent capable of inducibly altering expression or activity of PDPN, PROCR, or PRDM1 and c-MAF.

22. The method according to any one of claims 20 or 21, wherein the step of providing the isolated immune cell comprises providing the immune cell isolated from a subject, or isolating the immune cell from a subject.

23. The method according to claim 68, wherein the immune cell isolated from the subject expresses PDPN, PROCR, and/or PRDM1 and c-MAF, wherein the immune cell isolated from the subject is dysfunctional or is not dysfunctional, or wherein the immune cell isolated from the subject expresses a signature of dysfunction as defined in any one of claims 34 to 38.

24. The method of any one of claims 20 to 23, further comprising the step of expanding the isolated immune cell prior to and/or subsequent to the modification.

25. A pharmaceutical composition comprising the isolated immune cell according to any one of claims 1 to 18, or the cell population according to claim 19.

26. The isolated immune cell according to any one of claims 1 to 18, or the cell population according to claim 19, for use in therapy, wherein therapy comprises immunotherapy or adoptive immunotherapy, preferably immunotherapy or adoptive immunotherapy of a proliferative disease, such as a tumor or cancer, or a chronic infection, such as a chronic viral infection.

27. The isolated immune cell or cell population for use according to claim 26 in a subject, wherein the subject has been determined to comprise immune cells which:

- (a) express PDPN, PROCR, and/or PRDM1 and c-MAF;
- (b) are dysfunctional, or are not dysfunctional; or
- (c) express a signature of dysfunction as defined in any one of claims 34

to 38.

28. A method of treating a subject in need thereof, preferably a subject in need of immunotherapy or adoptive immunotherapy, more preferably immunotherapy or adoptive immunotherapy of a proliferative disease, such as a tumor or cancer, or a chronic infection, such as a chronic viral infection, comprising administering to said subject the isolated immune cell according to any one of claims 1 to 8, or the cell population according to claim 19.

29. The method according to claim 28, further comprising administering to said subject one or more other active pharmaceutical ingredient, preferably wherein said one or more other active pharmaceutical ingredient is useful in immunotherapy or adoptive

immunotherapy, or wherein said one or more other active pharmaceutical ingredient is useful in the treatment of a proliferative disease, such as a tumor or cancer, or a chronic infection, such as a chronic viral infection, wherein the one or more other active pharmaceutical ingredient is:

(a) an agonist of a cell molecule, such as a cell surface molecule, which when activated is capable of upregulating immune response, such as one or more of an agonist of 4-1BB, an agonist of OX40, an agonist of GITR, an agonist of STING, an agonist of TLR, and an agonist of BTLA; and/or

(b) an inhibitor of a cell molecule, such as a cell surface molecule, which when not inhibited is capable of downregulating immune response, such as a checkpoint inhibitor, or such as one or more of an antagonist of PD1, an antagonist of CTLA4, an antagonist of BTLA, an antagonist of TIGIT, an antagonist of TIM3, an antagonist of LAG3, an antagonist of VISTA, an antagonist of LILRB4, an antagonist of CD160, an antagonist of CD274, and an antagonist of IDO.

30. The method according to any one of claims 28 to 29, wherein the subject has been determined to comprise immune cells which:

(a) express PDPN, PROCR, and/or PRDM1 and c-MAF;

(b) are dysfunctional, or are not dysfunctional; or

(c) express a signature of dysfunction as defined in any one of claims 34 to 38.

31. A method of treating a subject in need thereof, preferably a subject in need of immunotherapy or adoptive immunotherapy, more preferably immunotherapy or adoptive immunotherapy of a proliferative disease, such as a tumor or cancer, or a chronic infection, such as a chronic viral infection, comprising:

(a) providing an isolated immune cell from the subject, or isolating an immune cell from a subject;

(b) modifying said isolated immune cell such as to comprise an altered expression or activity of PDPN, PROCR, and/or PRDM1 and c-MAF, or modifying said isolated immune cell such as to comprise an agent capable of inducibly altering expression or activity of PDPN, PROCR, and/or PRDM1 and c-MAF; and

(c) reintroducing the modified isolated immune cell to the subject.

32. The method according to claim 31, wherein the immune cell isolated from the subject:

(a) expresses PDPN, PROCR, and/or PRDM1 and c-MAF;

- (b) is dysfunctional or is not dysfunctional; or
 (c) expresses a signature of dysfunction as defined in any one of claims 34

to 38.

33. The method of any one of claims 31 or 32, further comprising the step of expanding the isolated immune cell prior to and/or subsequent to the modification, and before reintroduction to the subject.

34. A method of detecting dysfunctional immune cells comprising detection of a gene expression signature comprising one or more markers selected from the group consisting of Abcal, Adam8, Adam9, Alcam, Ccl5, Ccl9, Ccl9, Ccl9, Ccr2, Ccr5, Cd68, Cd93, Cxcl10, Cysltr2, Ddrl, Entpd1, Entpd1, Epcam, Gabarapl1, Gcntl, Gpr65, Havcr2, Ifitm1, Ifitm3, 1110, IllOra, I112rb1, I113ral, Illrl, Illr2, 1121, I12ra, I12rb, 1133, I16st, Inhba, Isg20, Klrc2, Klrc2, Klrc2, Klrc2, Klrc2, Klrc2, Klrd1, Klrkl, Lag3, Lamp2, Lpar3, Ly75, Ly75, Nampt, Olfml, Pdpn, Pglyrpl, Procr, Pstipl, Ptpn3, Sdcl, Sdc4, Selp, Sema7a, Slamf7, Sppl, Tgfb3, Tigit, Tnfrsf8, Tnfsf9, Vldlr, Bst2, Btla, Cell, Ccr4, Cd226, Cd401g, Cd83, Cd8a, Csf2, Cxcl13, Cxcr4, Ifitm3, Isg20, Lap3, Lif, Serpincl, Timp2, Tnfsf11, Acvrl1, Ada, Are, Bmp2, Bmprla, ccl22, Ccr6, Ccr8, Cdl60, Cd200r4, Cd24a, Cd70, Cd74, Cmtm7, Csf1, Ctla2a, Ctla2b, Ctsd, Ctsl, Dkl1, Enpep, Enpp1, Eps8, F2r, Fgf2, Flt31, H2-Abl, Hspbl, Ifngr1, I112rb2, 1118, I118rl, I118rap, 112, 1124, I127ra, 114, I14ra, I17r, Itga4, Itga7, Itga9, Klrc1, Klrel, Lpar2, Lta, Ly6a, Ly6e, Nlgn2, Nrpl, Flt31, H2-Ab2, Hspb2, Ifngr2, I112rb3, 1119, I118r2, I118rap, 1146, 1168, I127ra, 115, Smpdl, Tgdb3, Tirap, Tnfrsf13c, Tnfrsf23, Tnfsf10, Tnfsf4, Trem12, Trpcl, Trpm4, Tspan32, and Xcll; or selected from the group consisting of ABCA1, ADAM8, ADAM9, ALCAM, CCL5, CCL15, CCL23, CCL15-CCL14, CCR2, CCR2, CD68, CD93, CXCL10, CYSLTR2, DDR1, ENTPD1, EPCAM, GABARAPL1, GCNT1, GPR65, HAVCR2, IFITM1, IFITM1, IL10, IL10RA, IL12RB1, IL13RA1, IL1R1, IL1R2, IL21, IL2RA, IL2RB, IL33, IL6ST, INHBA, ISG20, KLRC4-KLRK1, KLRC4, KLRC1, KLRC3, KLRC2, KLRD1, KLRK1, LAG3, LAMP2, LPAR3, LY75-CD302, LY75, NAMPT, OLFM1, PDPN, PGLYRP1, PROCR, PSTPIP1, PTPN3, SDC1, SDC4, SELP, SEMA7A, SLAMF7, SPP1, TGFB3, TIGIT, TNFRSF8, TNFSF9, VLDLR, BST2, BTLA, CCL1, CCR4, CD226, CD40LG, CD83, CD8A, CSF2, CXCL13, CXCR4, IFITM1, ISG20, LAP3, LIF, SERPINC1, TIMP2, TNFSF11, ACVRL1, ADA, BMPR1A, CCR5, CD160, CD24, CMTM7, CSF1, CTSD, CTSL1, CYSLTR2, ENPP1, EPS 8, F2R, FLT3LG, HSPB1, IFNGR1, IL18, IL18R1, IL18RAP, IL24, IL24, IL27RA, IL27RA, IL4R, IL7R, ITGA4, ITGA7, LY6E, NLGN2, NRP1, OSM, PDE4B, PEAR1,

PLXNC1, PRNP, PRNP, PRNP, PTPRJ, SIPRI, SDC1, SELL, SEMA4D, SERPINE2, SERPINE2, SMPD1, TIRAP, TNFSF10, TRPC1, TRPM4, and XCL1.

35. A method of detecting dysfunctional immune cells comprising detection of a gene expression signature comprising one or more markers selected from the group consisting of ABCA1, ADAM8, ADAM9, ALCAM, CCL5, CCL9, CCR2, CCR5, CD68, CD93, CTLA2A, CXCL10, CYSLTR2, ENTPD1, EPCAM, GABARAPL1, GCNT1, GPR65, HAVCR2, IFITM1, IFITM3, IL10IL10RA, IL12RB1, IL13RA1, IL1R1, IL1R2, IL21, IL2RA, IL2RB, IL33, IL6ST, INHBA, ISG20, KLRC2, KLRD1, KLRE1, KLRK1, LAG3, LAMP2, LILRB4, LPAR3, LY75, NAMPT, OLFM1, PDPN, PGLYRP1, PROCR, PSTPIP1, PTPN3, SDC1, SDC4, SELP, SEMA7A, SLAMF7, SPP1, TGFB3, TIGIT, TNFRSF8, TNFSF9, and VLDLR.

36. A method of detecting dysfunctional immune cells comprising detection of a gene expression signature comprising one or more markers selected from the group consisting of IL33, KLRC2, KLRD1, KLRE1, OLFM1, PDPN, PTPN3, SDC1, TNFSF9, VLDLR, PROCR, GABARAPL1, SPP1, ADAM8, LPAR3, CCL9, CXCL10, CCR2, IL10RA, IL2RB, CD68, KLRK1, IL12RB2, IL6ST, IL7R, INHBA, ISG20, LAMP2, LY75, NAMPT, SIPRI, IL21, IL13RA1, TIGIT, CCR5, ALCAM, HAVCR2, LAG3, IL1R2, CYSLTR2, ENTPD1, GCNT1, IFITM3, IL2RA, PGLYRP1, CD93, ADAM9, LILRB4, IL-10, CTLA2A, and GPR65.

37. The method of claims 34-36, wherein the gene expression signature comprises at least three markers, or at least four markers, or at least five markers, or six or more markers, such as wherein the signature consists of two markers, three markers, four markers, or five markers.

38. The method of claims 34-36, wherein the gene expression signature comprises two or more markers, and wherein:

- (a) one of said two or more markers is PDPN;
- (b) one of said two or more markers is PROCR; or
- (c) two of said two or more markers are PDPN and PROCR.

39. A method of isolating a dysfunctional immune cell comprising binding of an affinity ligand to a signature gene as defined in any one of claims 34 to 38, wherein the signature gene is expressed on the surface of the immune cell.

40. A kit of parts comprising means for detection of the signature of dysfunction as defined in any one of claims 34 to 38.

41. A method of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination thereof.

42. The method of claim 41, wherein the T-cell dysfunction is T-cell exhaustion.

43. The method of claim 42, wherein the modulation of T-cell exhaustion comprises a decrease in the exhausted T-cell phenotype, such that functional T-cell activity is increased.

44. The method of claim 41, wherein the selected target gene or gene product or a combination thereof is/are identified as participating in the inhibition of functional T-cell activity.

45. The method of claim 44, wherein the modulating agent inhibits the expression, activity and/or function of the selected target gene or gene product or combination thereof.

46. The method of claim 41, wherein the selected target gene or combination of target genes is/are identified as participating in the promotion of functional T-cell activity.

47. The method of claim 46, wherein the modulating agent promotes or activates the expression, activity and/or function of the selected target gene or gene product or combination thereof.

48. The method of claim 41, comprising contacting the dysfunctional T-cell with modulating agents that modulate the expression, activity and/or function of at least two target genes or gene products selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination thereof.

49. The method of claim 41, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

50. The method of claim 41, further comprising contacting the dysfunctional T-cell with an agent or treatment selected from the group consisting of a PD-1 inhibitor, a CTLA4 inhibitor, chemotherapy, radiation therapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-1BB, GITR, CD226, KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and/or SLAMF7.

51. A method of treating a condition involving or characterized by the presence of T cells exhibiting a dysfunctional or exhausted phenotype, the method comprising administering an amount of a modulating agent effective to modulate the expression, activity

and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination thereof.

52. The method of claim 51, wherein the condition is cancer or a persistent infection.

53. The method of claim 51, wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

54. The method of claim 53, wherein the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

55. The method of claim 51, wherein a selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

56. The method of claim 55, wherein the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

57. The method of claim 51, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

58. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising at least one modulating agent that modulates the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination thereof.

59. The pharmaceutical composition of claim 58, wherein the composition comprises at least two modulating agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination thereof.

60. The pharmaceutical composition of claim 58, wherein the composition comprises an agonist of OX-40, 4-1BB, GITR, CD226, KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and/or SLAMF7.

61. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising a first modulating agent that inhibits the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination

thereof and a second modulating agent that promotes the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination thereof.

62. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising a modulating agent that modulates the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination thereof and an agent selected from the group consisting of a PD-1 inhibitor, a CTLA4 inhibitor, chemotherapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-1BB, GITR, CD226, KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and/or SLAMF7.

63. The pharmaceutical composition of any one of claims 58-62 wherein the T cell dysfunction comprises T cell exhaustion.

64. The pharmaceutical composition of any one of claims 58-63 wherein the T cell exhaustion occurs in an individual with cancer or a persistent infection.

65. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising an inhibitor of the expression, activity, and/or function of PDPN and an inhibitor of the expression, activity, and/or function of PROCR.

66. The pharmaceutical composition of claim 65, further comprising an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of TIGIT, LAG3, LILRB4, and KLRC1; and/or an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

67. A pharmaceutical composition for modulating an IL-27-regulated co-inhibitory module comprising:

(a) an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM and KLRC1; and

(b) an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

68. The pharmaceutical composition of any one of claims 65-67, further comprising an inhibitor of the expression and/or activity of TIM-3; an inhibitor of the

expression and/or activity of PD-1; an inhibitor of the expression and/or activity of CTLA4; an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of PD-1; an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of CTLA4; an inhibitor of the expression and/or activity of CTLA4 and an inhibitor of the expression and/or activity of PD-1; or an inhibitor of the expression and/or activity of TIM-3, an inhibitor of the expression and/or activity of CTLA4 and an inhibitor of the expression and/or activity of PD-1.

69. The pharmaceutical composition of any one of claims 65-68, wherein the inhibitors and activators are selected from an antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog.

70. The pharmaceutical composition of claim 69, wherein the antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog is selected from the group consisting of: an anti-CTLA4 antibody, an anti-PD-1 antibody, or aPDL-1 antagonist.

71. A method of modulating an IL-27-regulated co-inhibitory module in a subject in need thereof, the method comprising administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of PDPN and an inhibitor of the expression and/or activity of PROCR.

72. The method of claim 71, further comprising administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of an inhibitor of the expression and/or activity of TIGIT, LAG3, LILRB4, and KLRC1; and/or an activator of the expression and/or activity of at least one of the molecules selected from the group consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

73. A method of modulating an IL-27-regulated co-inhibitory module in a subject in need thereof, the method comprising:

(a) administering a pharmaceutical composition comprising an inhibitor of the expression and/or activity of at least one of the molecules selected from the group consisting of PDPN, PROCR, TIGIT, LAG3, LILRB4, ALCAM, and KLRC1; and

(b) administering a pharmaceutical composition comprising an activator the expression and/or activity of at least one of the molecules selected from the group

consisting of CD226, OX-40, GITR, TNFSF9 (4-1BB), KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and SLAMF7.

74. The method of any one of claims 71-73, further comprising administering an inhibitor of the expression and/or activity of TIM-3; an inhibitor of the expression and/or activity of PD-1; an inhibitor of the expression and/or activity of CTLA4; an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of PD-1; an inhibitor of the expression and/or activity of TIM-3 and an inhibitor of the expression and/or activity of CTLA4; an inhibitor of the expression and/or activity of CTLA4 and an inhibitor of the expression and/or activity of PD-1; an inhibitor of the expression and/or activity of PD-1, and an inhibitor of the expression and/or activity of CTLA4.

75. The method of any one of claims 71-74, wherein the inhibitors and activators are selected from an antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog.

76. The method of claim 75, wherein the antibody or antigen binding fragment thereof, a small molecule compound, a protein or peptide molecule, a DNA or RNA aptamer, an antisense or siRNA molecule, and a structural analog is selected from the group consisting of: an anti-CTLA4 antibody, an anti-PD-1 antibody, and a PDL-1 antagonist.

77. The method of any one of claims 71-76, wherein the subject in need thereof has a disease or disorder characterized by T-cell exhaustion.

78. The method of any one of claims 71-77, wherein the subject in need thereof is diagnosed as having a cancer or tumor.

79. The method of any one of claims 71-77, wherein the subject in need thereof is diagnosed as having a persistent infection.

80. A method of modulating T cell dysfunction, the method comprising contacting a dysfunctional T cell with a modulating agent or agents that modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the group consisting of: BTLA, TIGIT, HAVCR2 (TIM-3), LAG3, PDPN, IL10RA, IL1R2, PROCR, LILRB4, KLRC1, KLRC2, KLRE1, TNFSF9 (4-1BB), KLRK1, IL12RB1, IL1R1, and SLAMF7.

81. The method of claim 80, wherein the T cell dysfunction is T cell exhaustion.

82. The method of claim 81, wherein the modulation of T cell exhaustion comprises a decrease in the exhausted T cell phenotype, such that T cell activation is increased.

83. The method of claim 81, wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

84. The method of claim 83, wherein the modulating agent promotes the expression, activity and/or function of the target gene or gene product or combination thereof.

85. The method of claim 81, wherein the selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

86. The method of claim 85, wherein the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

87. The method of claim 80, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

88. A method of treating a condition involving or characterized by the presence of T cells exhibiting an exhausted phenotype, the method comprising administering an amount of a modulating agent effective to modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the group consisting of: BTLA, TIGIT, HAVCR2 (TIM-3), LAG3, PDPN, IL10RA, IL1R2, PROCR, LILRB4, KLRC 1, KLRC2, KLRE1, TNFSF9 (4-1BB), KLRK1, IL12RB 1, IL1R1, and SLAMF7.

89. The method of claim 88 wherein the condition is cancer or a persistent infection.

90. The method of claim 88 wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

91. The method of claim 90 wherein the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

92. The method of claim 88 wherein the selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

93. The method of claim 92 wherein the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

94. The method of claim 88 wherein the agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

95. A method of determining the presence of T cells exhibiting an exhausted phenotype, the method comprising detecting, in a sample comprising T cells, a level of expression, activity and/or function of one or more genes or expression products thereof

selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination thereof, and comparing the detected level to a reference, wherein a difference in the detected level relative to the reference indicates the presence of T cells exhibiting an exhausted phenotype.

96. The method of claim 95 wherein the sample is from an individual with cancer or a persistent infection.

97. A method of treating a disease or disorder characterized by aberrant or unwanted T-cell functional activity in a subject in need thereof, the method comprising administering a therapeutically effective amount of a modulating agent effective to modulate the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 1, Table 2, Table 10, Table 11, Table 12, Table 13, and any combination thereof.

98. The method of claim 97, wherein the disease or disorder is an autoimmune disease or graft vs. host disease.

99. The method of claim 97, wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

100. The method of claim 99, wherein the modulating agent promotes the expression, activity and/or function of the target gene or gene product or combination thereof.

101. The method of claim 97, wherein the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

102. The method of claim 97, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

103. A method of modulating T-cell dysfunction, the method comprising contacting a dysfunctional T-cell with a modulating agent or agents that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9.

104. The method of any one of claims 103, wherein the T-cell dysfunction is T-cell exhaustion.

105. The method of claim 104, wherein the modulation of T-cell exhaustion comprises a decrease in the exhausted T-cell phenotype, such that functional T-cell activity is increased.

106. The method of any one of claims 103-105, wherein the selected target gene or gene product or a combination thereof is/are identified as participating in the inhibition of functional T-cell activity.

107. The method of claim 103, wherein the modulating agent inhibits the expression, activity and/or function of the selected target gene or gene product or combination thereof.

108. The method of any one of claims 103-105, wherein the selected target gene or combination of target genes is/are identified as participating in the promotion of functional T-cell activity.

109. The method of claim 103, wherein the modulating agent promotes or activates the expression, activity and/or function of the selected target gene or gene product or combination thereof.

110. The method of any one of claims 103-105, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

111. The method of any one of claims 103-105, further comprising contacting the dysfunctional T-cell with an agent or treatment selected from the group consisting of a PD-1 inhibitor, a CTLA4 inhibitor, chemotherapy, radiation therapy, a Braf inhibitor, a MEK inhibitor, a Sting agonist, a TLR agonist, an IDO inhibitor, and an agonist for OX-40, 4-1BB, GITR, CD226, KLRC2, KLRE1, KLRK1, IL12RB1, IL1R1, and/or SLAMF7.

112. The method of claim 103, wherein the condition is cancer or a persistent infection.

113. The method of claim 112, wherein the selected target gene or combination of target genes is/are identified as participating in the inhibition of T cell activation.

114. The method of claim 113, wherein the modulating agent inhibits the expression, activity and/or function of the target gene or gene product or combination thereof.

115. The method of claim 112, wherein a selected target gene or combination of target genes is/are identified as participating in the promotion of T cell activation.

116. The method of claim 115, wherein the modulating agent promotes or activates the expression, activity and/or function of the target gene or gene product or combination thereof.

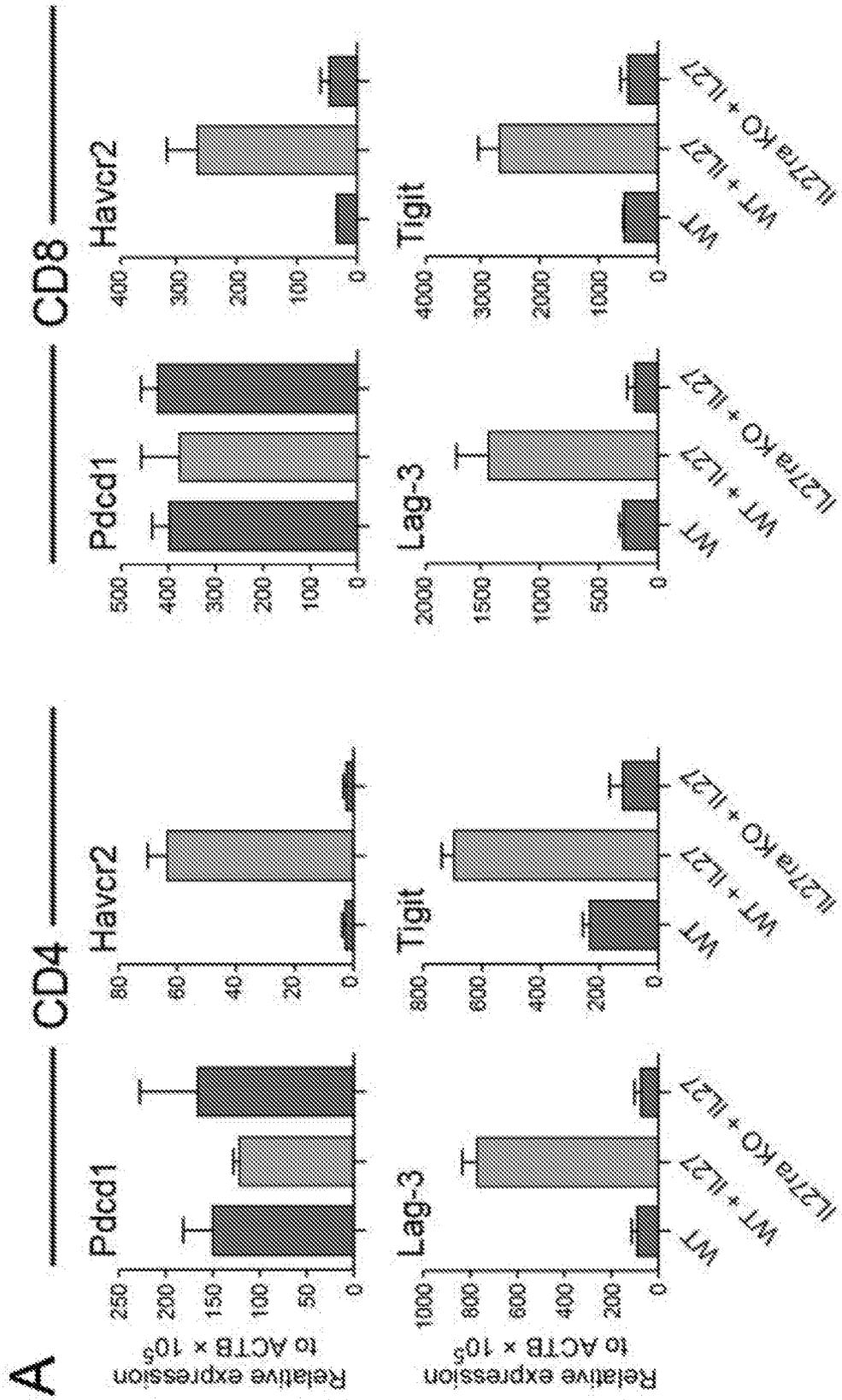
117. The method of claim 112, wherein the modulating agent comprises a peptide agent, polypeptide agent, a soluble variant of a membrane-associated polypeptide, antibody agent, a nucleic acid agent, a nucleic acid ligand, or a small molecule agent.

118. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising at least one modulating agent that modulates the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9.

119. The pharmaceutical composition of claim 118, wherein the composition comprises at least two modulating agents that modulate the expression, activity and/or function of two or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9.

120. A pharmaceutical composition for modulating T cell dysfunction, the composition comprising a first modulating agent that inhibits the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9 and a second modulating agent that promotes the expression, activity and/or function of one or more target genes or gene products thereof selected from the target genes listed in Table 5, Table 6, Table 7, Table 8, or Table 9.

FIG. 1A



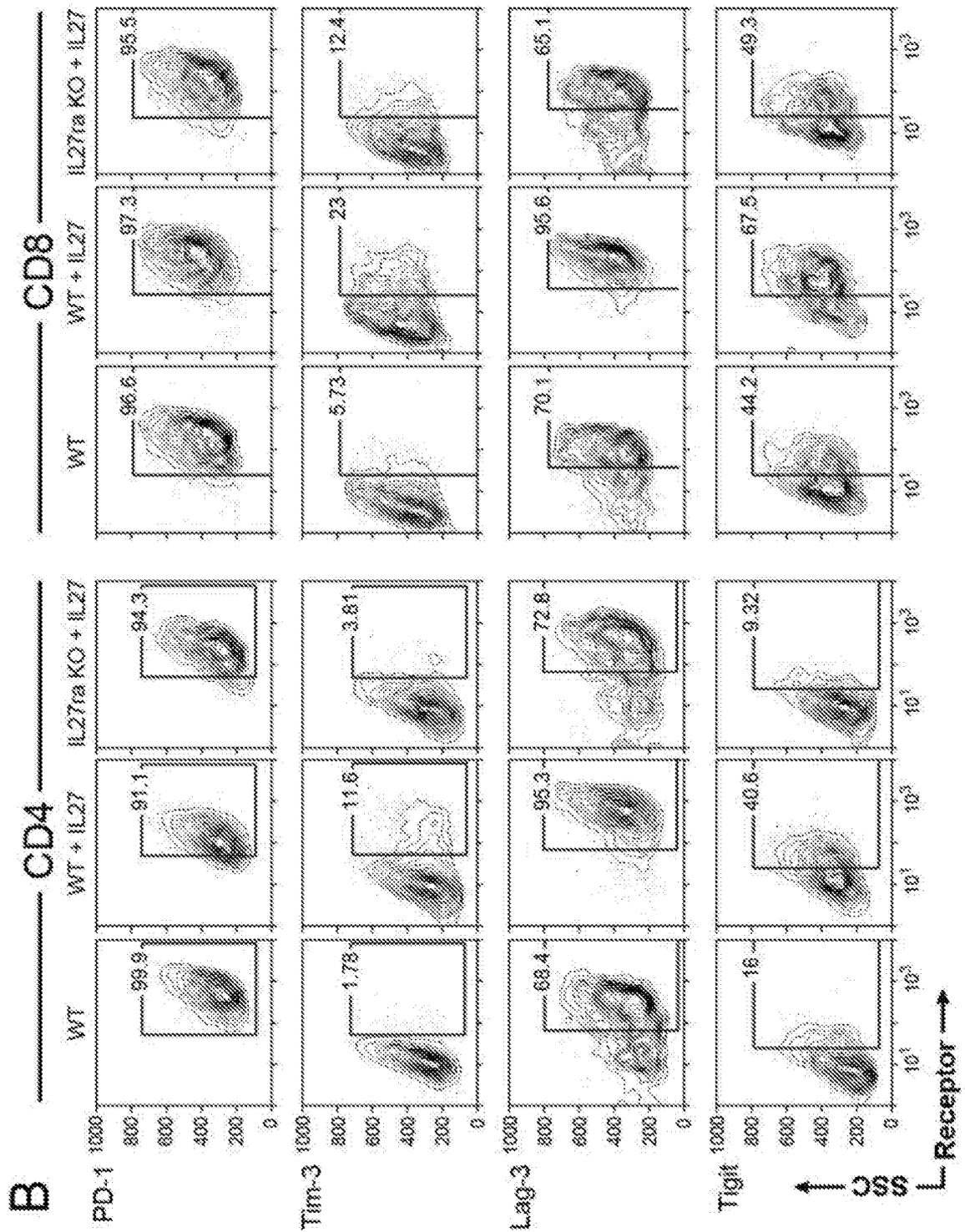
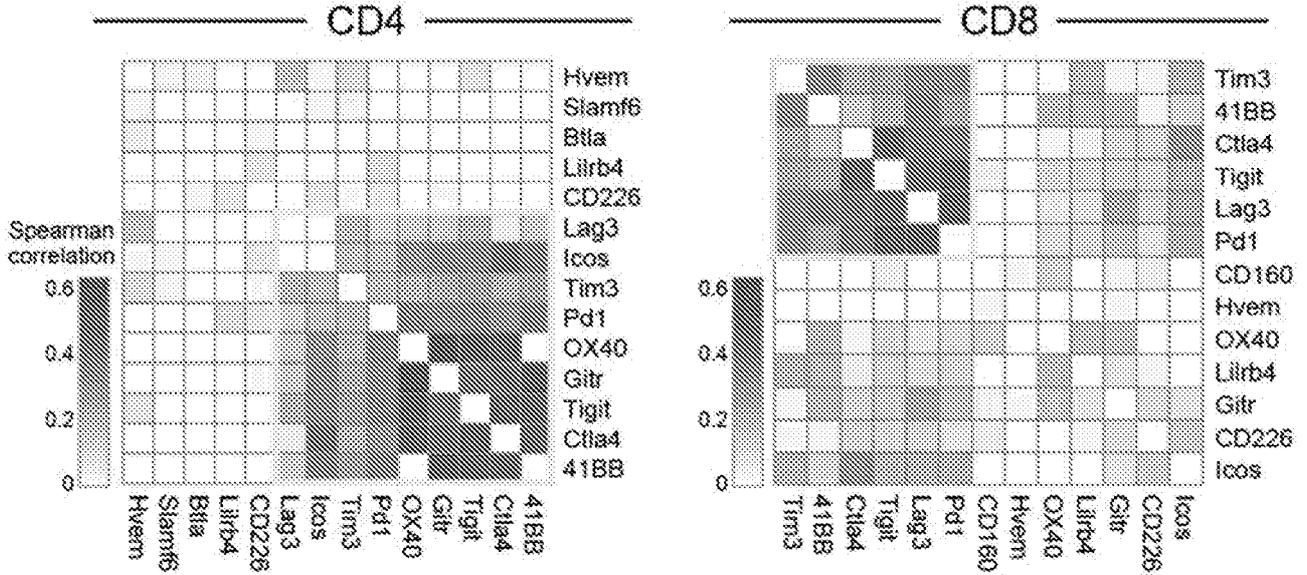


FIG. 1B

FIG. 1C-D

C

WT single cell RNAseq



D

WT CyTOF

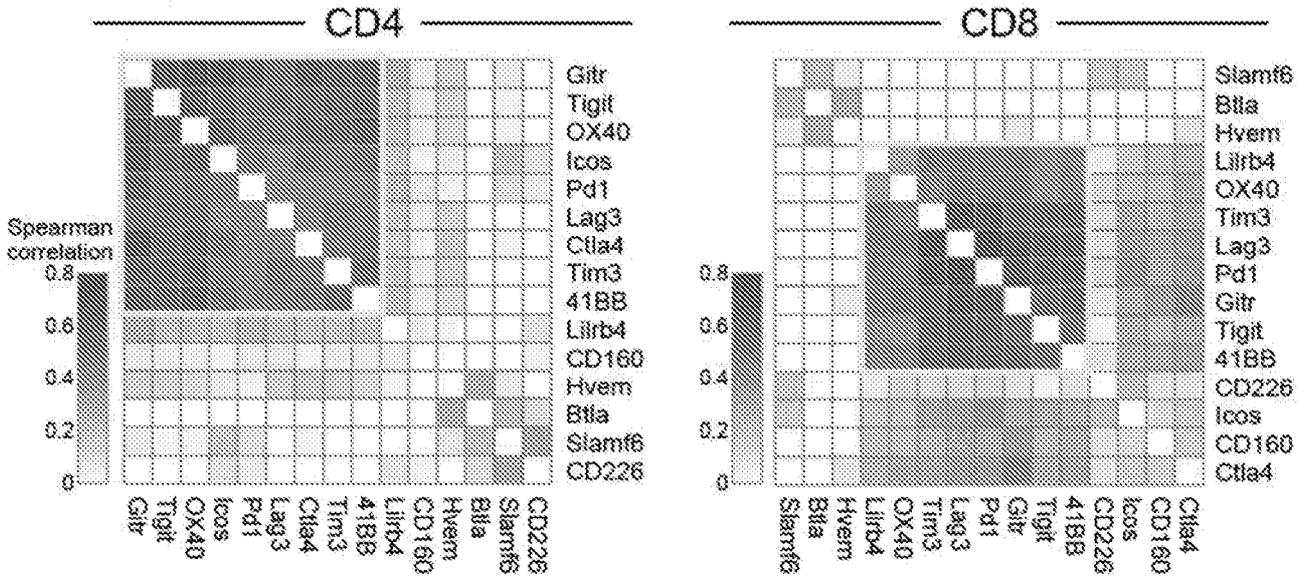


FIG. 1E

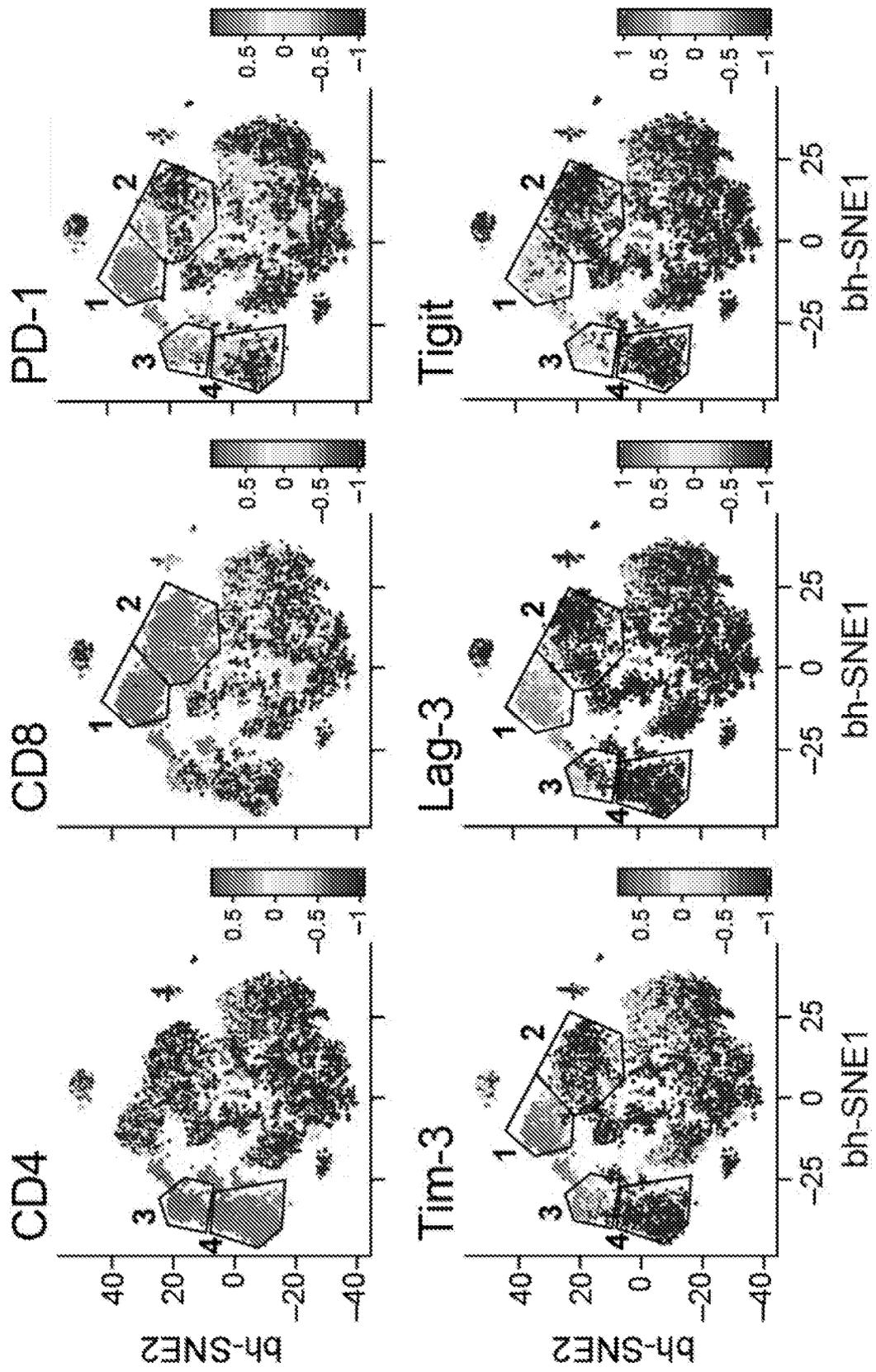
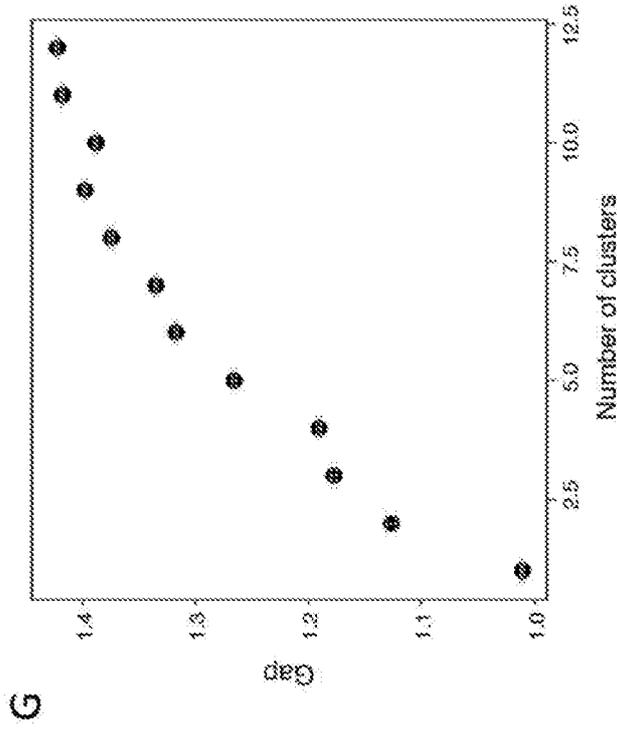
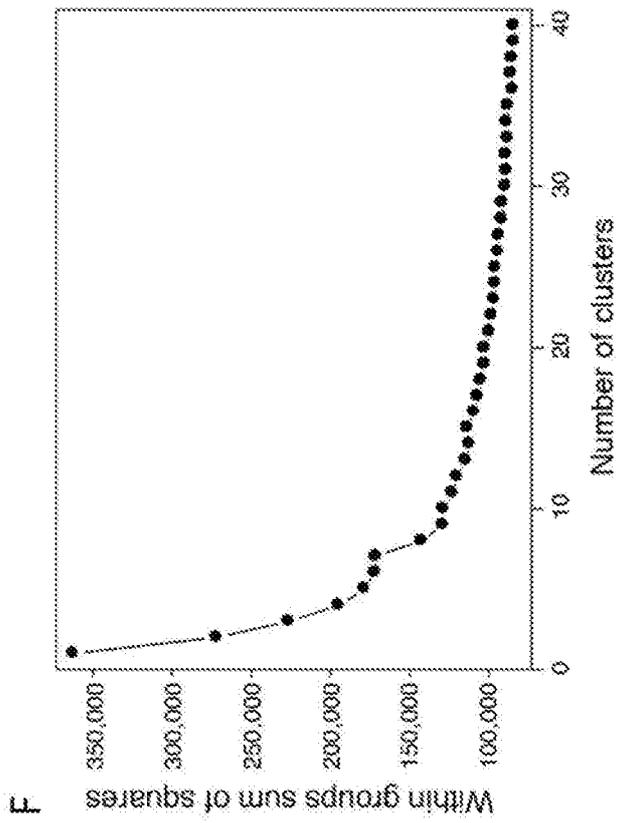


FIG. 1F-G



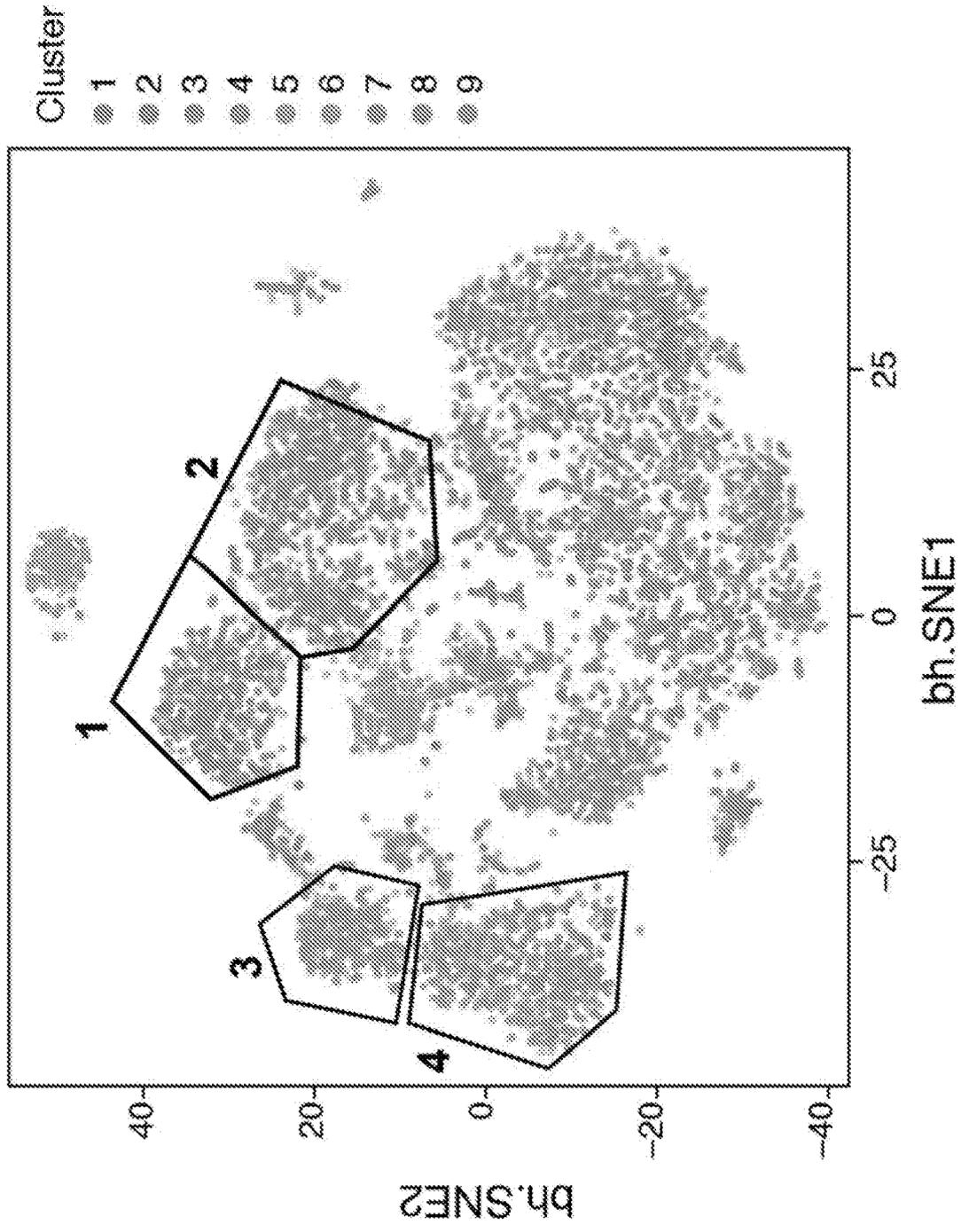


FIG. 1H

FIG. 1I

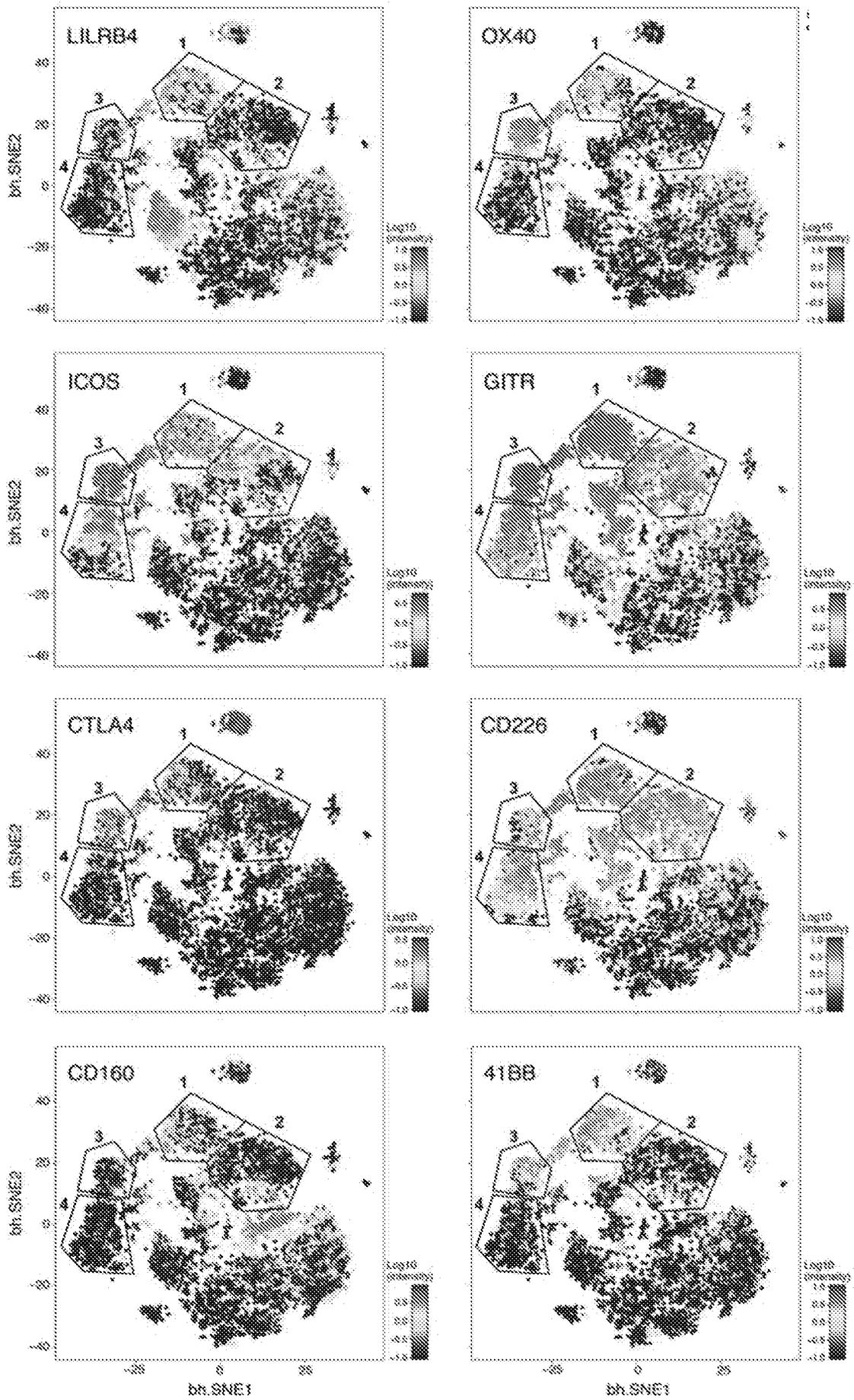


FIG. 1J

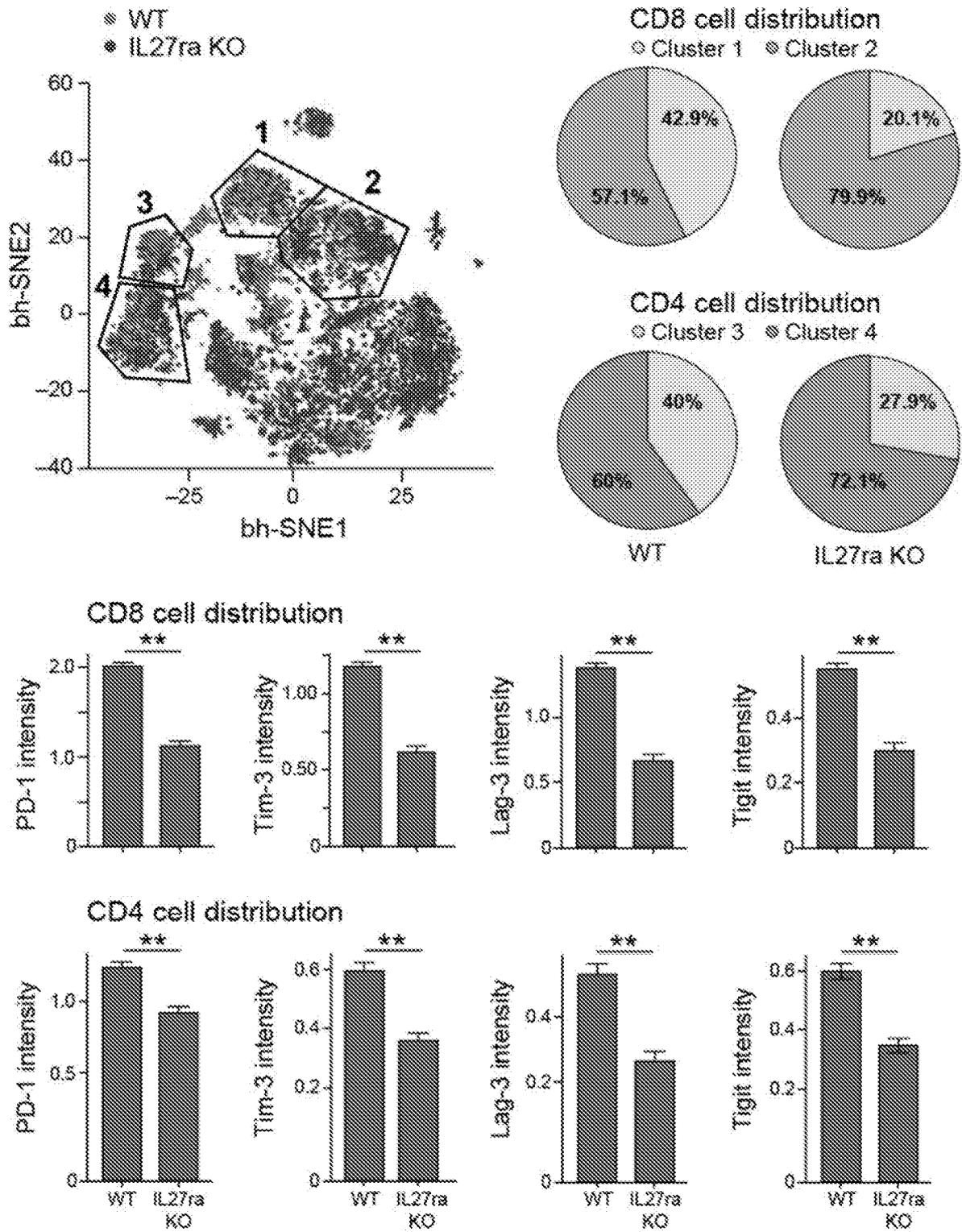


FIG. 1K

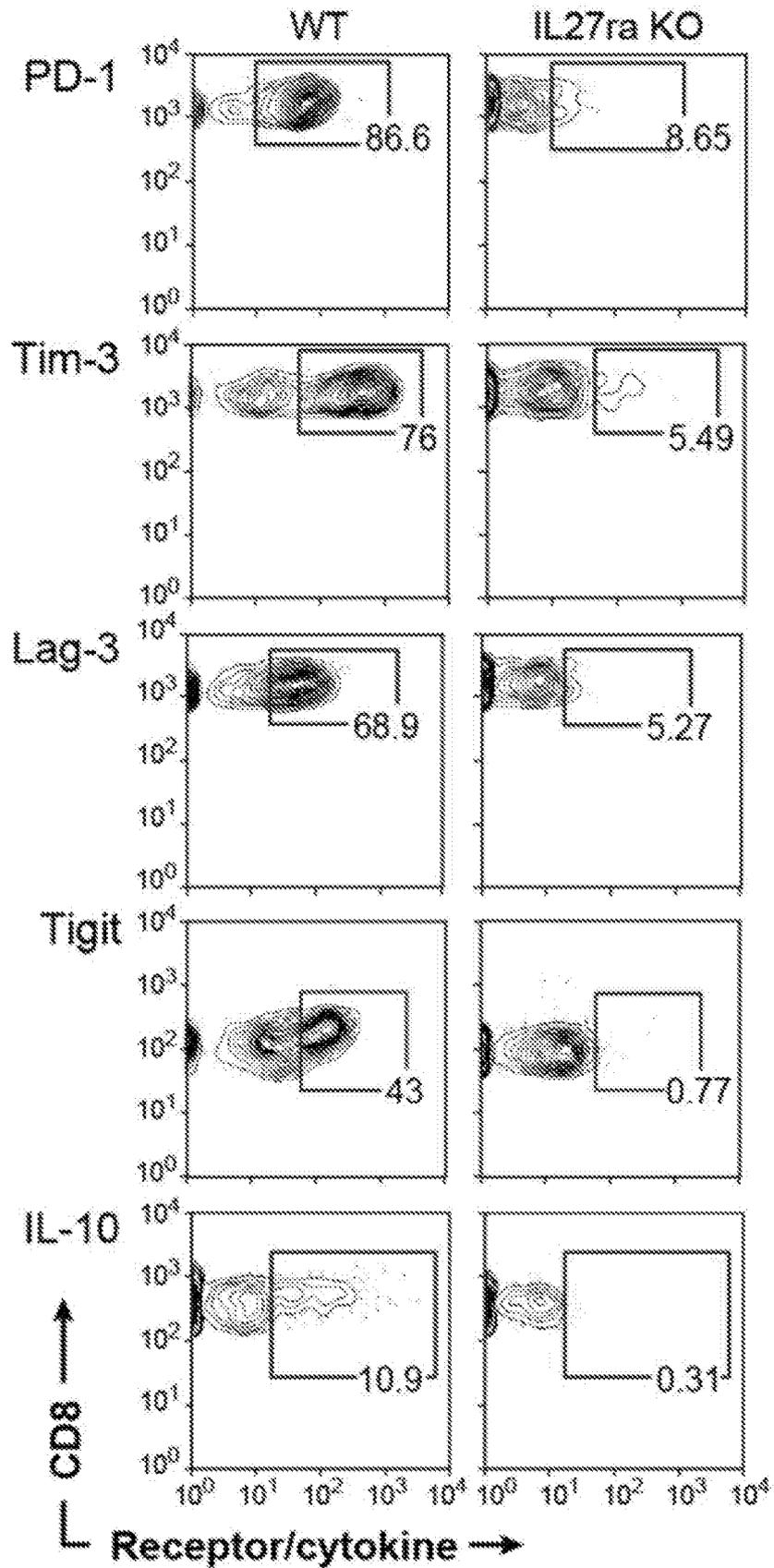


FIG. 1L

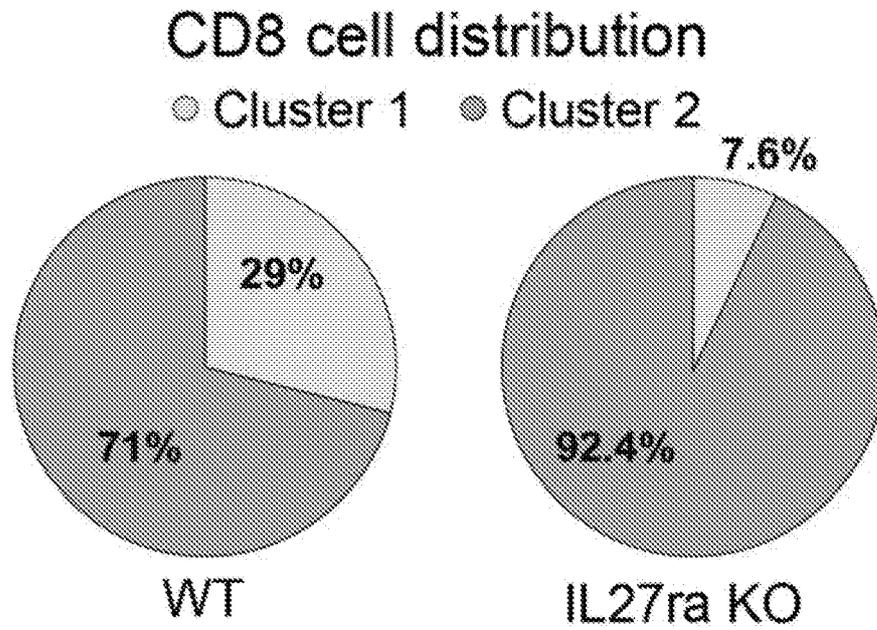


FIG. 1M

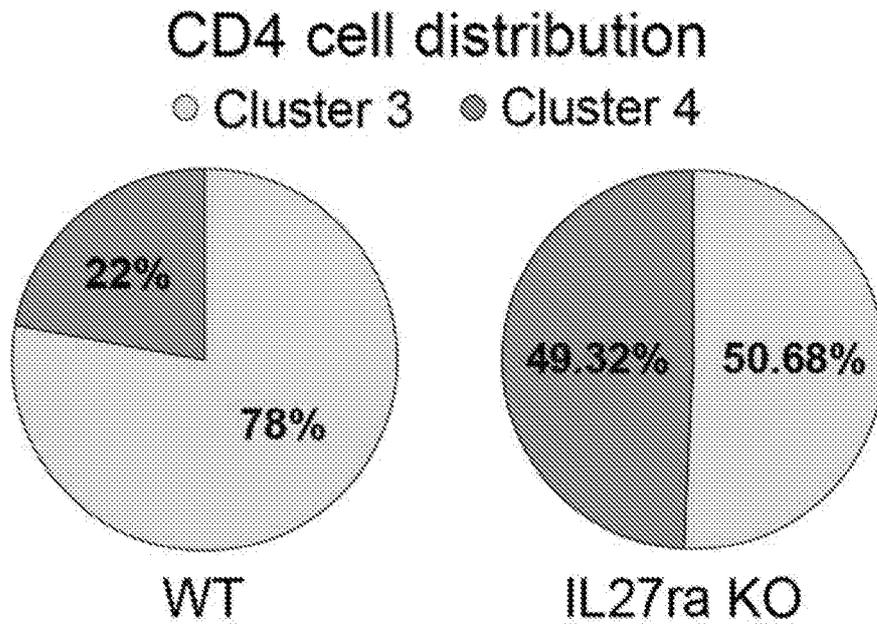
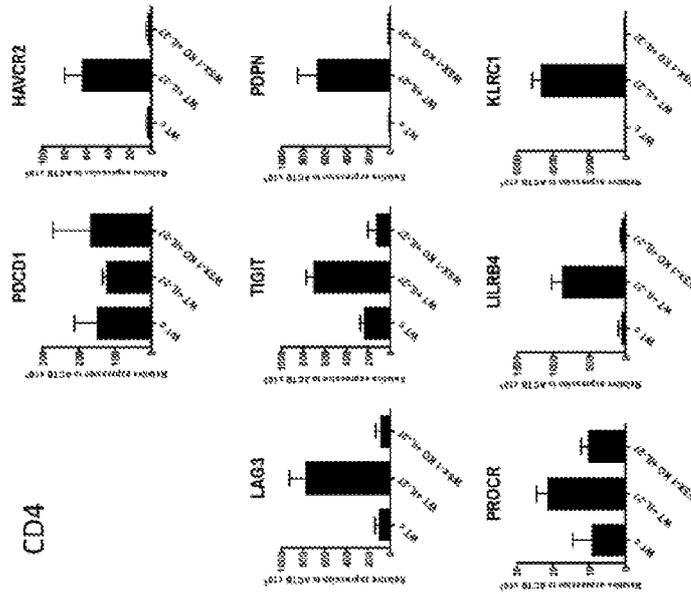


FIG. 2A-B

a)



b)

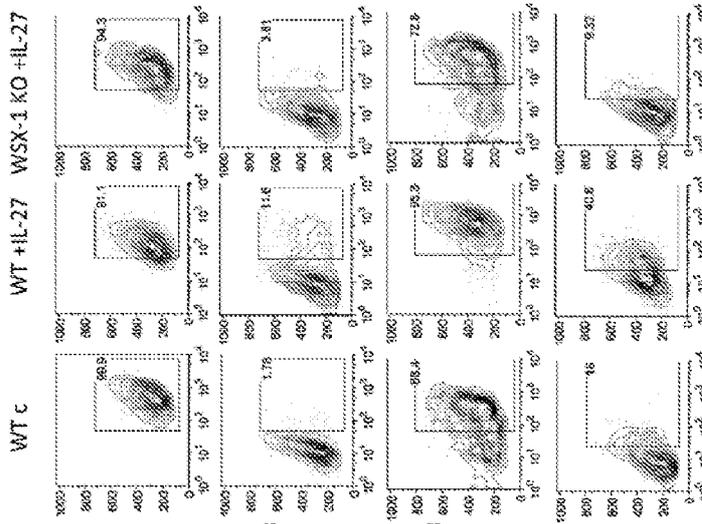
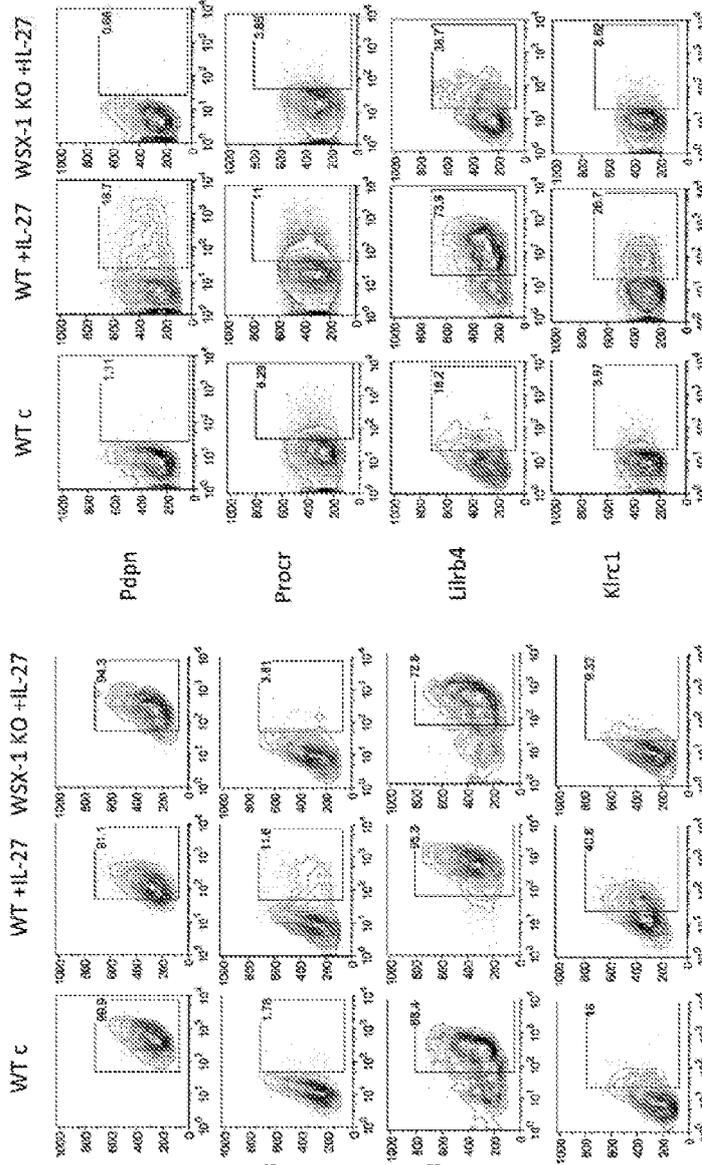
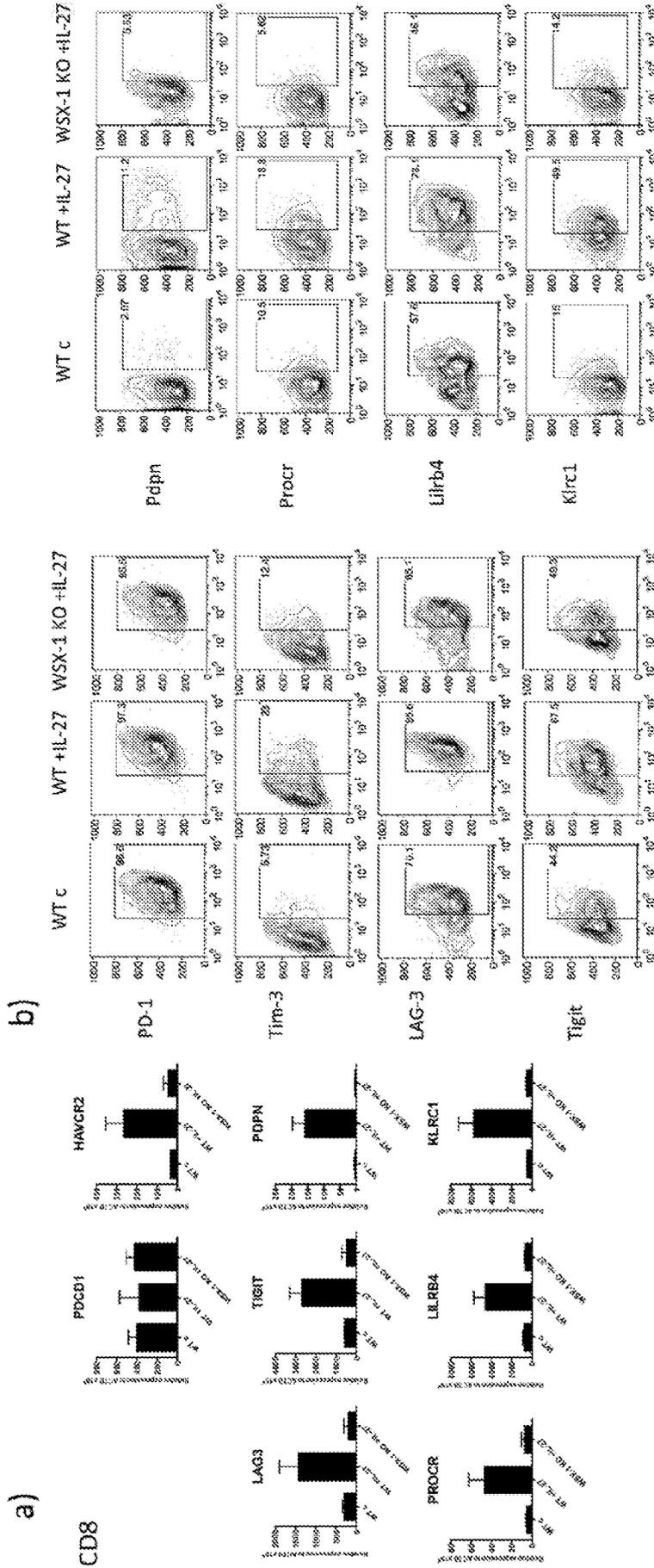


FIG. 3A-B



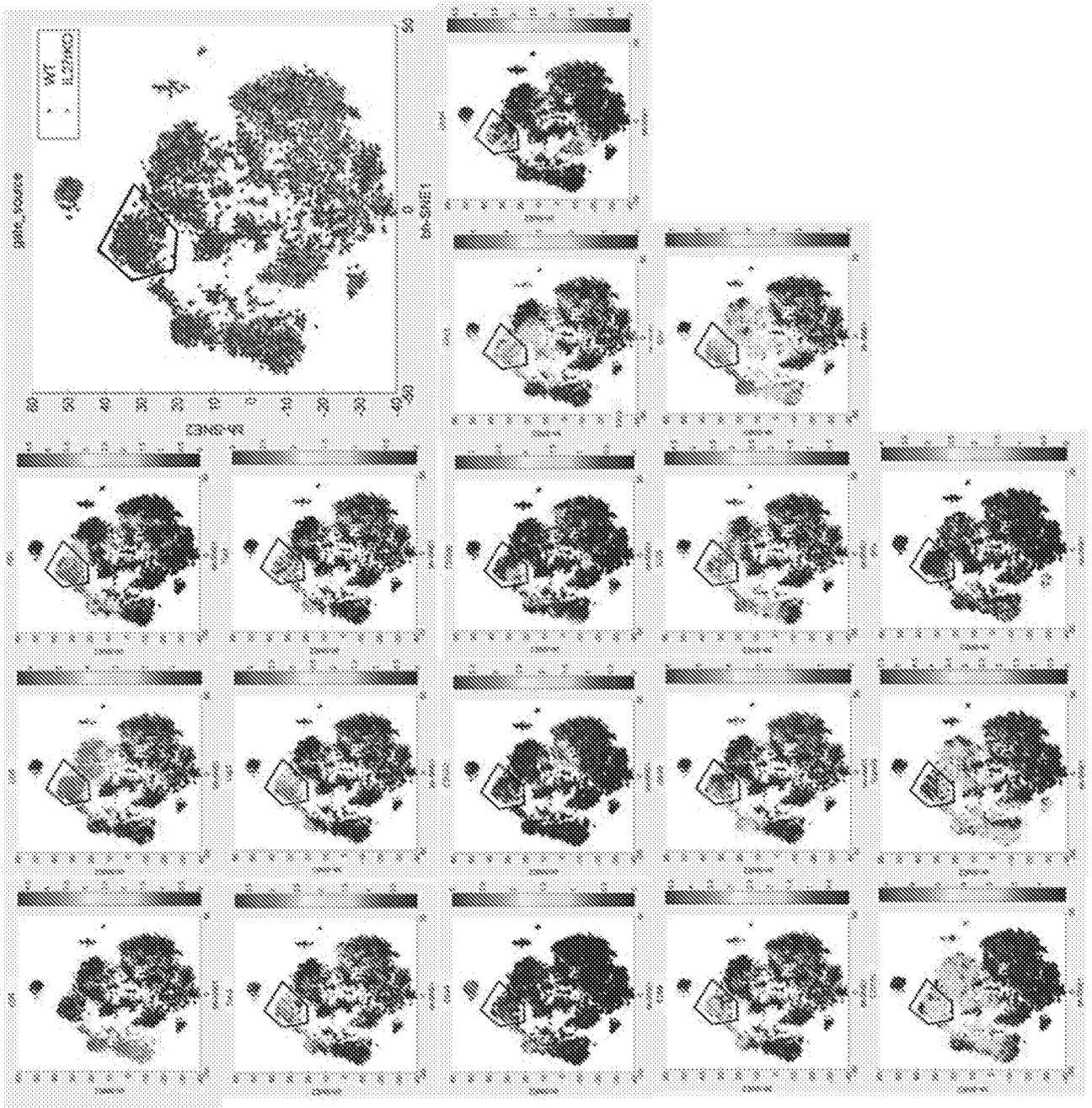


FIG. 4

FIG. 5

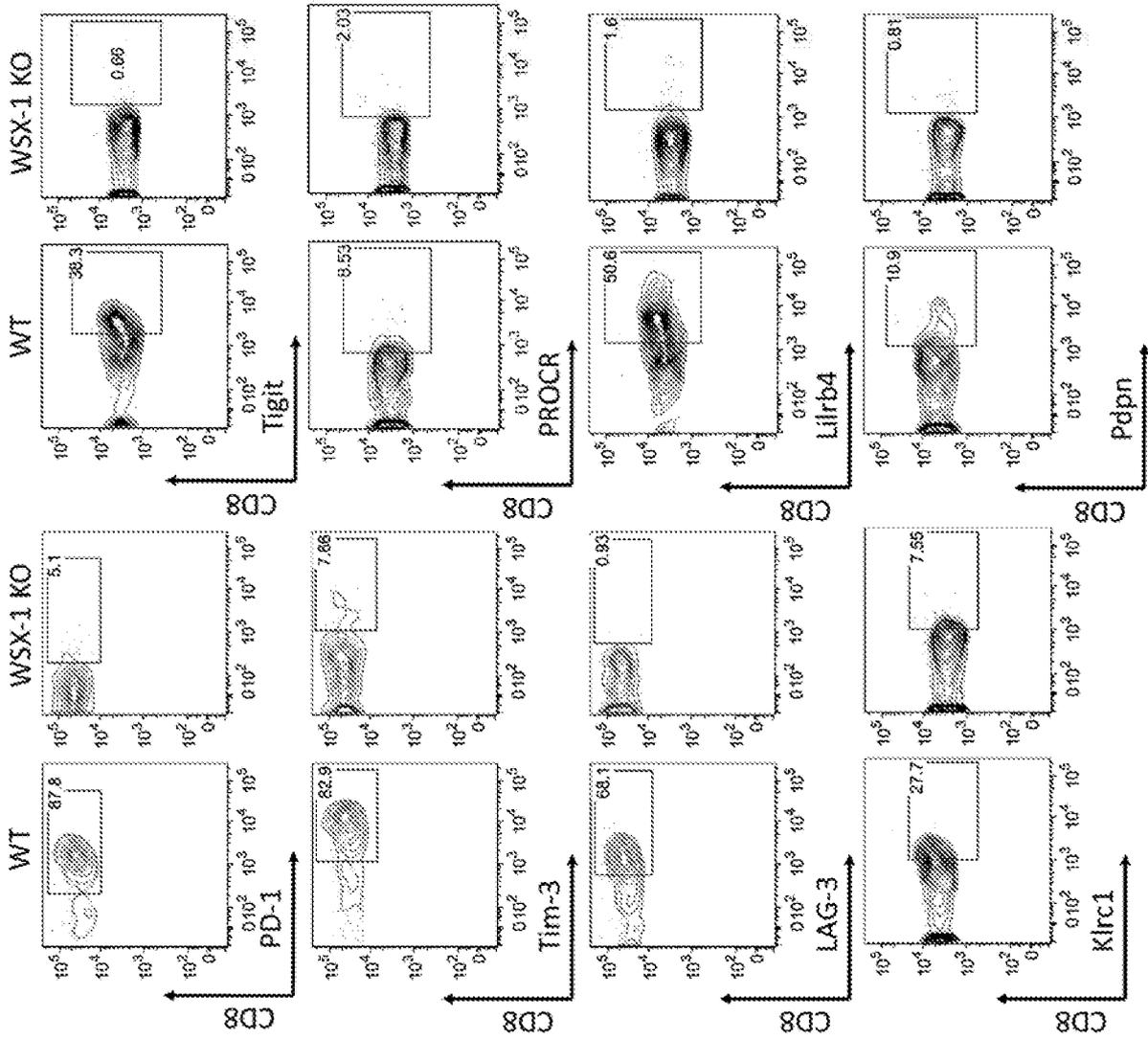


FIG. 6A-B

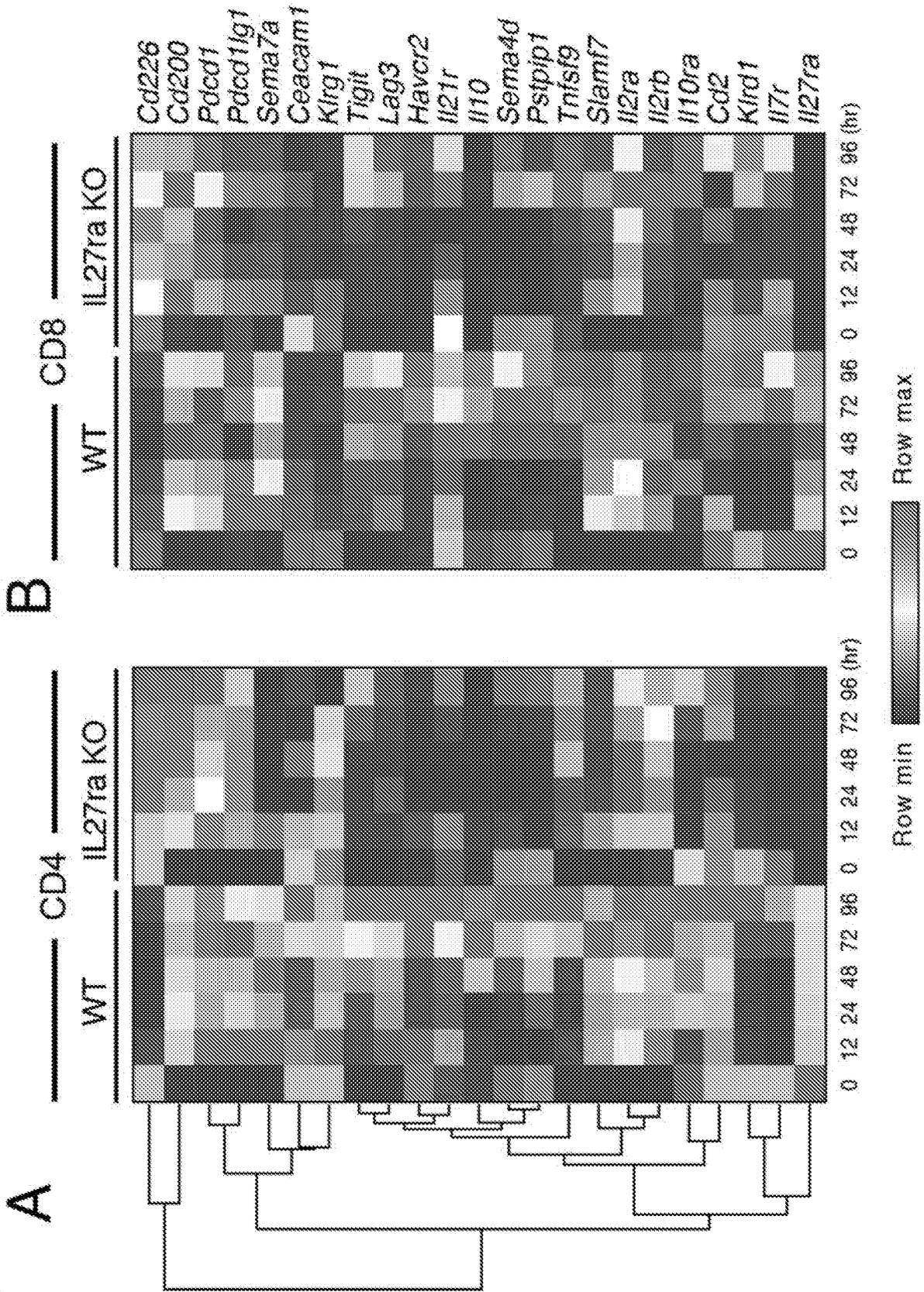


FIG. 6C

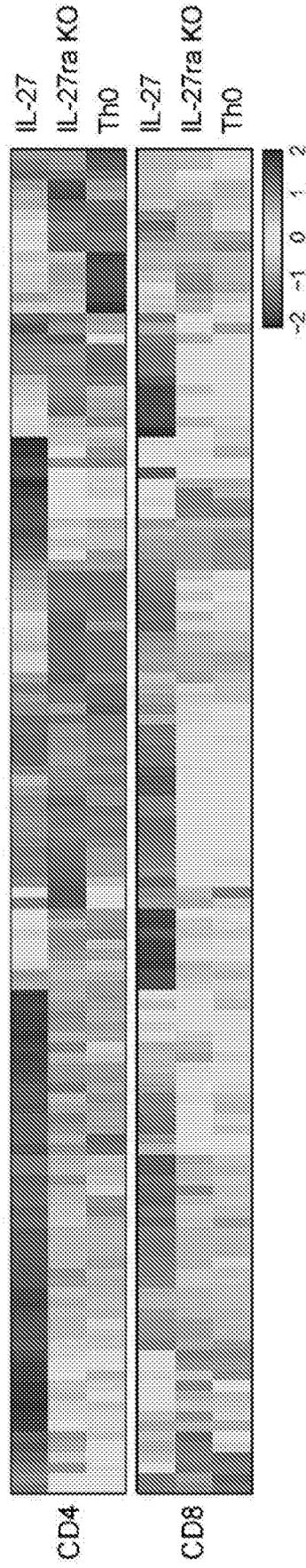


FIG. 6D

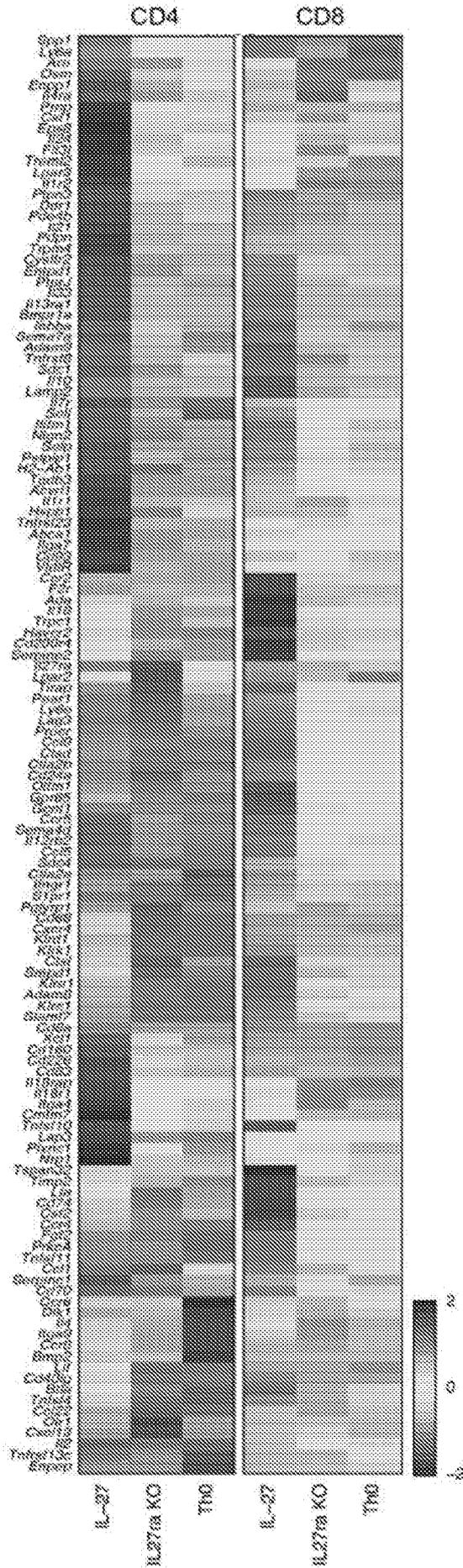


FIG. 6E-F

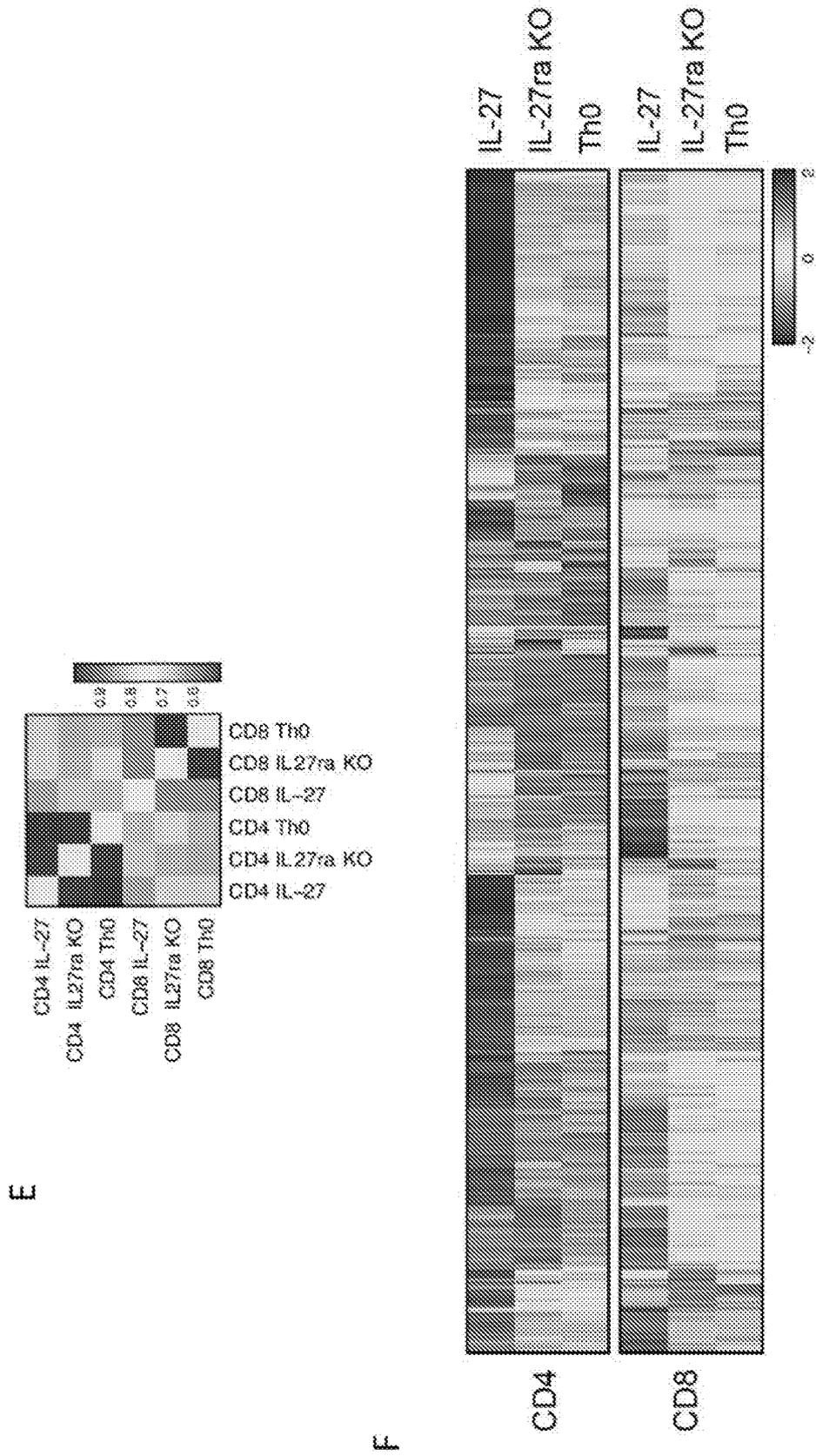


FIG. 6H

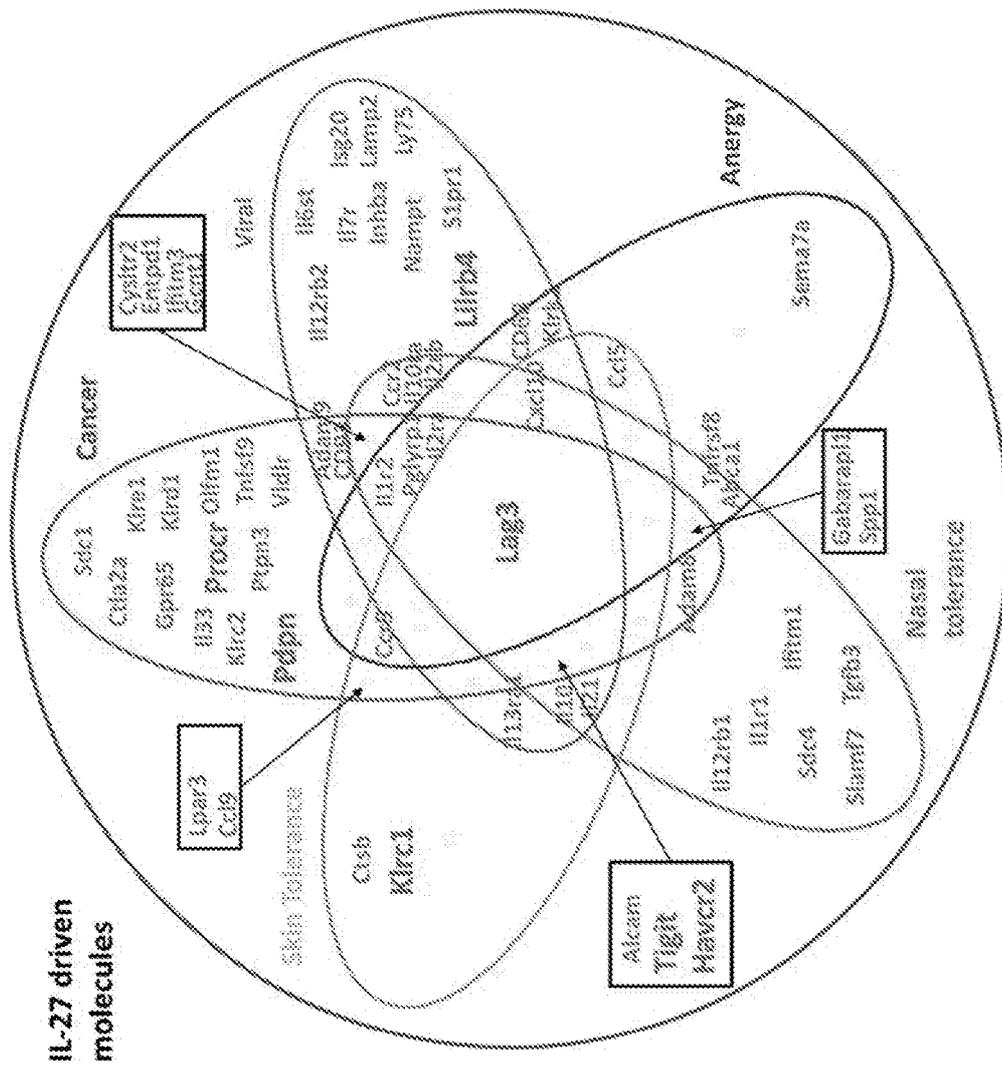


FIG. 6I-J

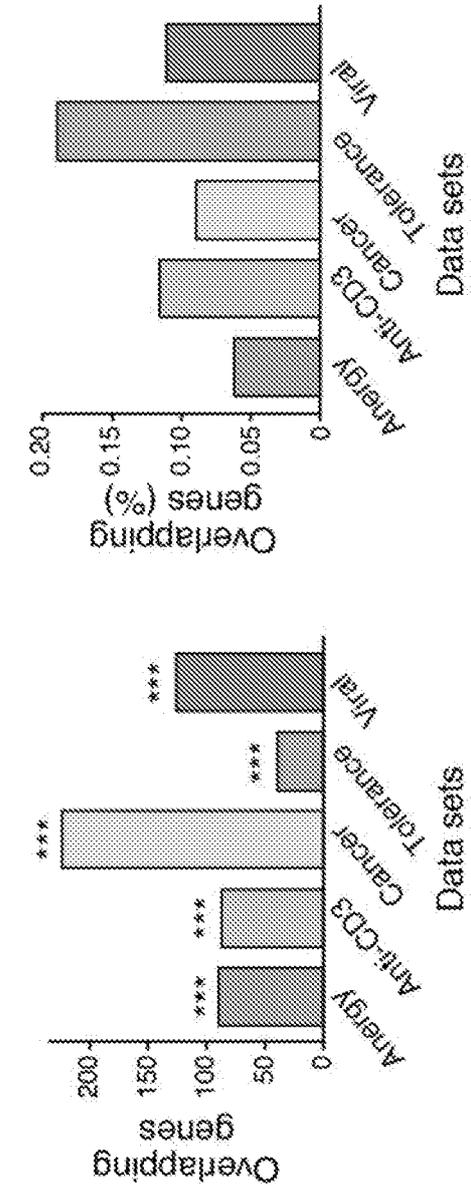
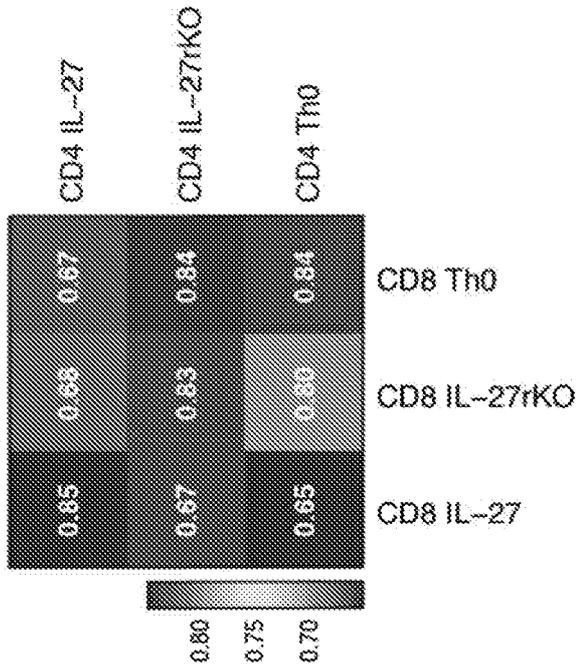


FIG. 6K

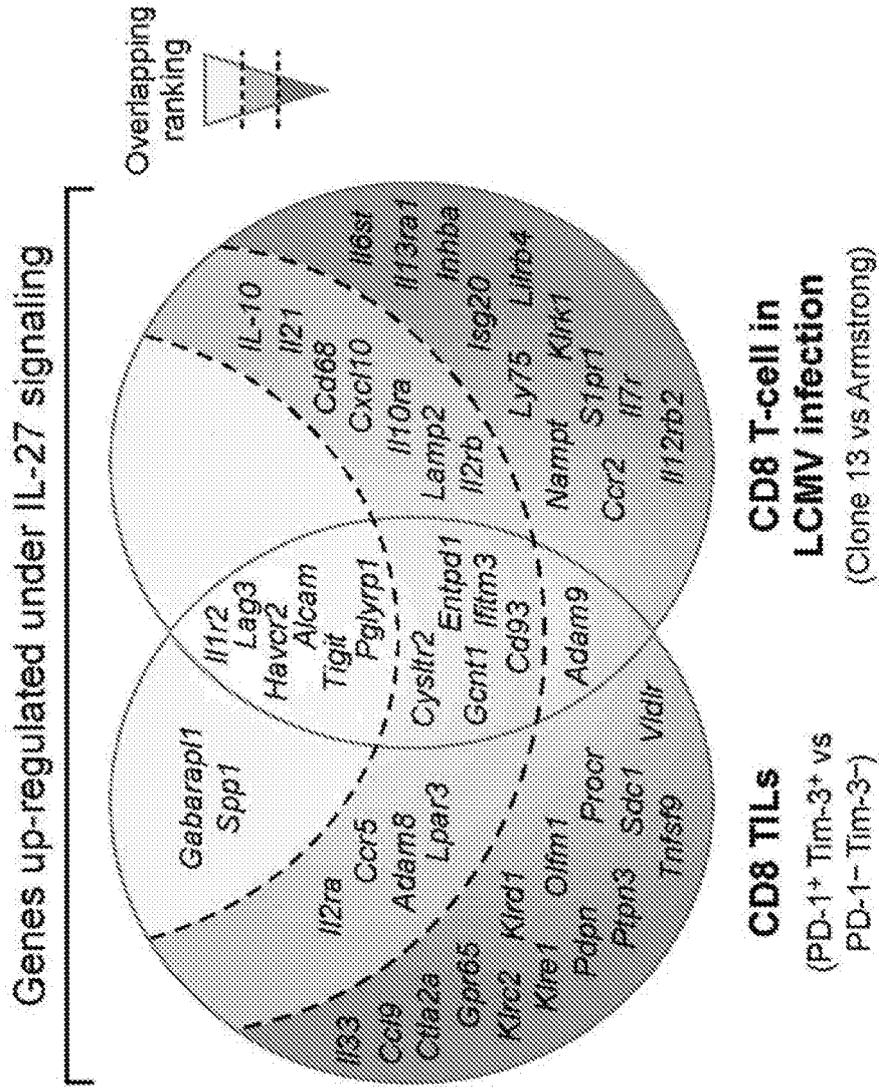


FIG. 6L

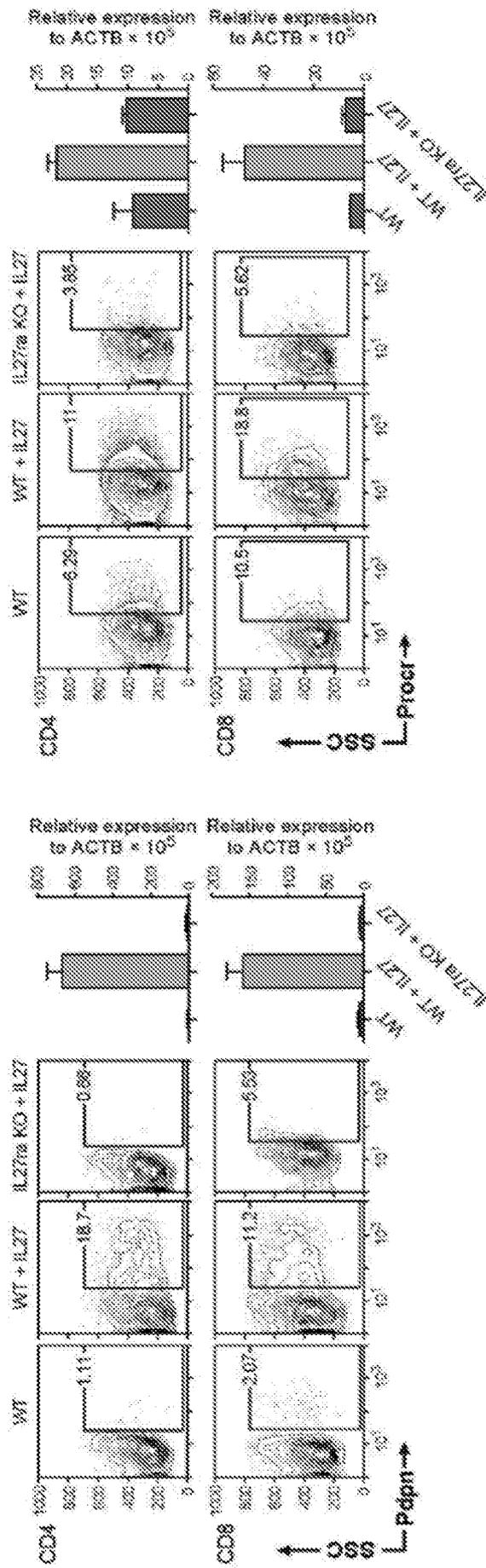


FIG. 6M

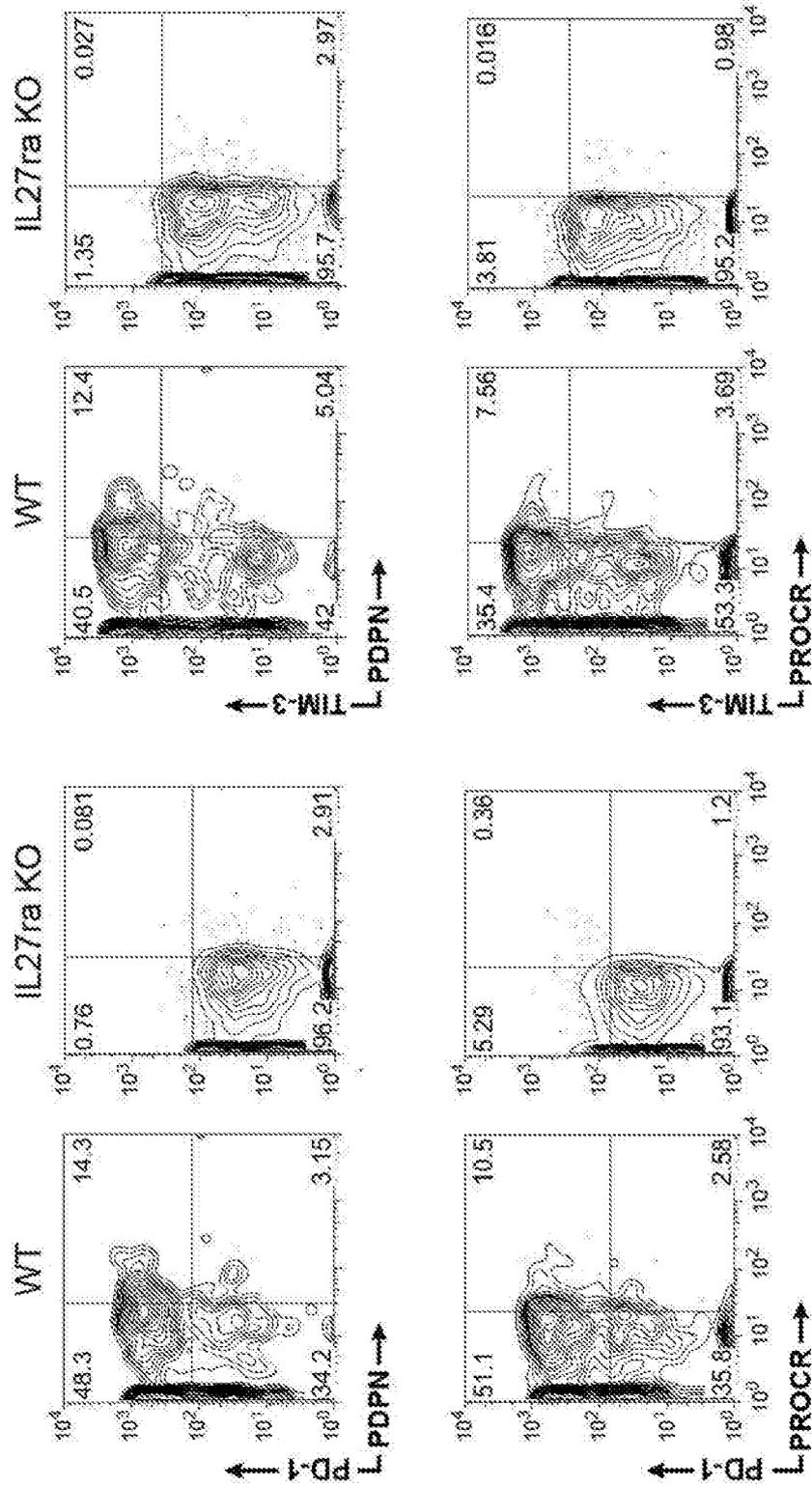


FIG. 6N

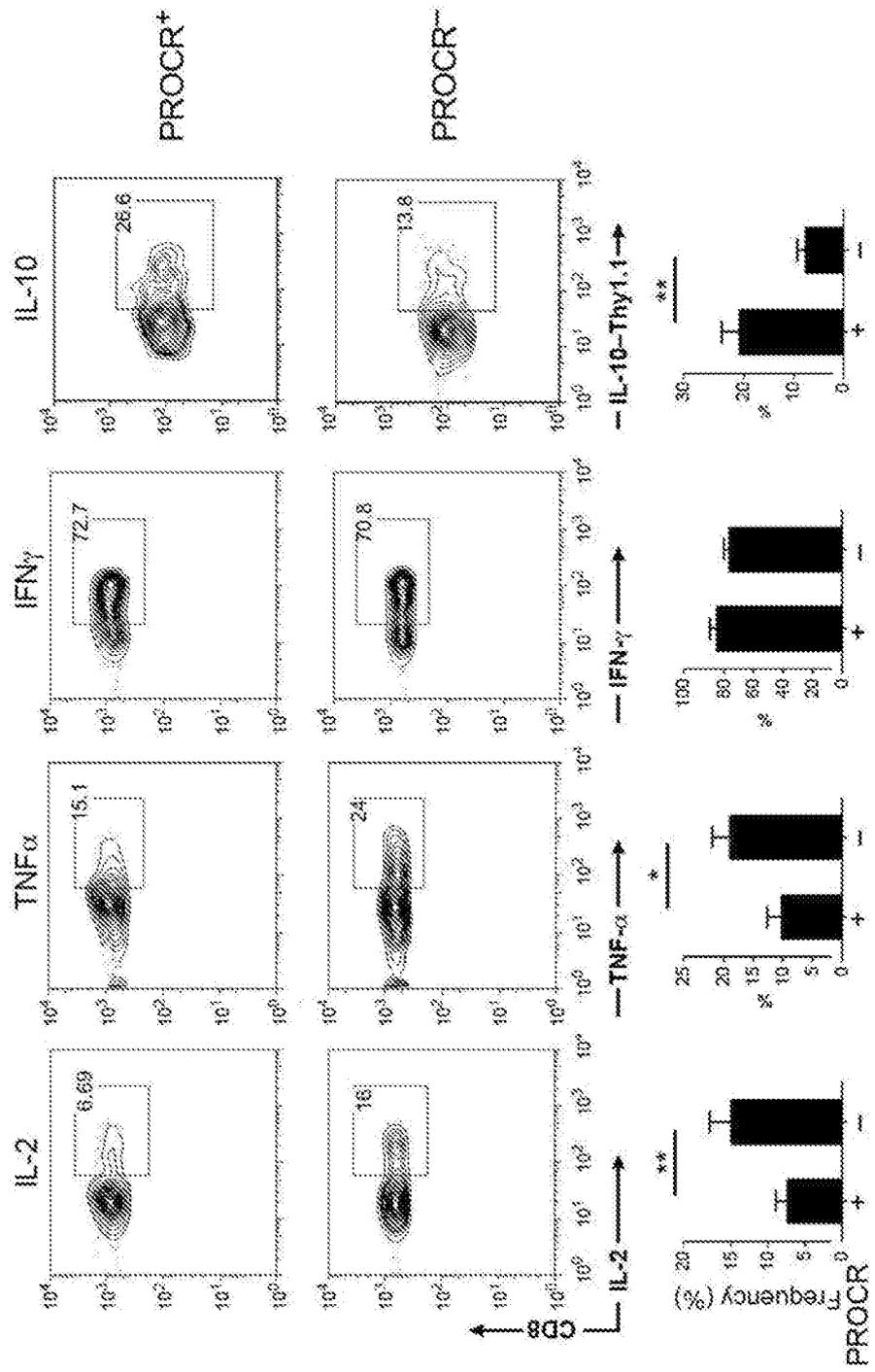
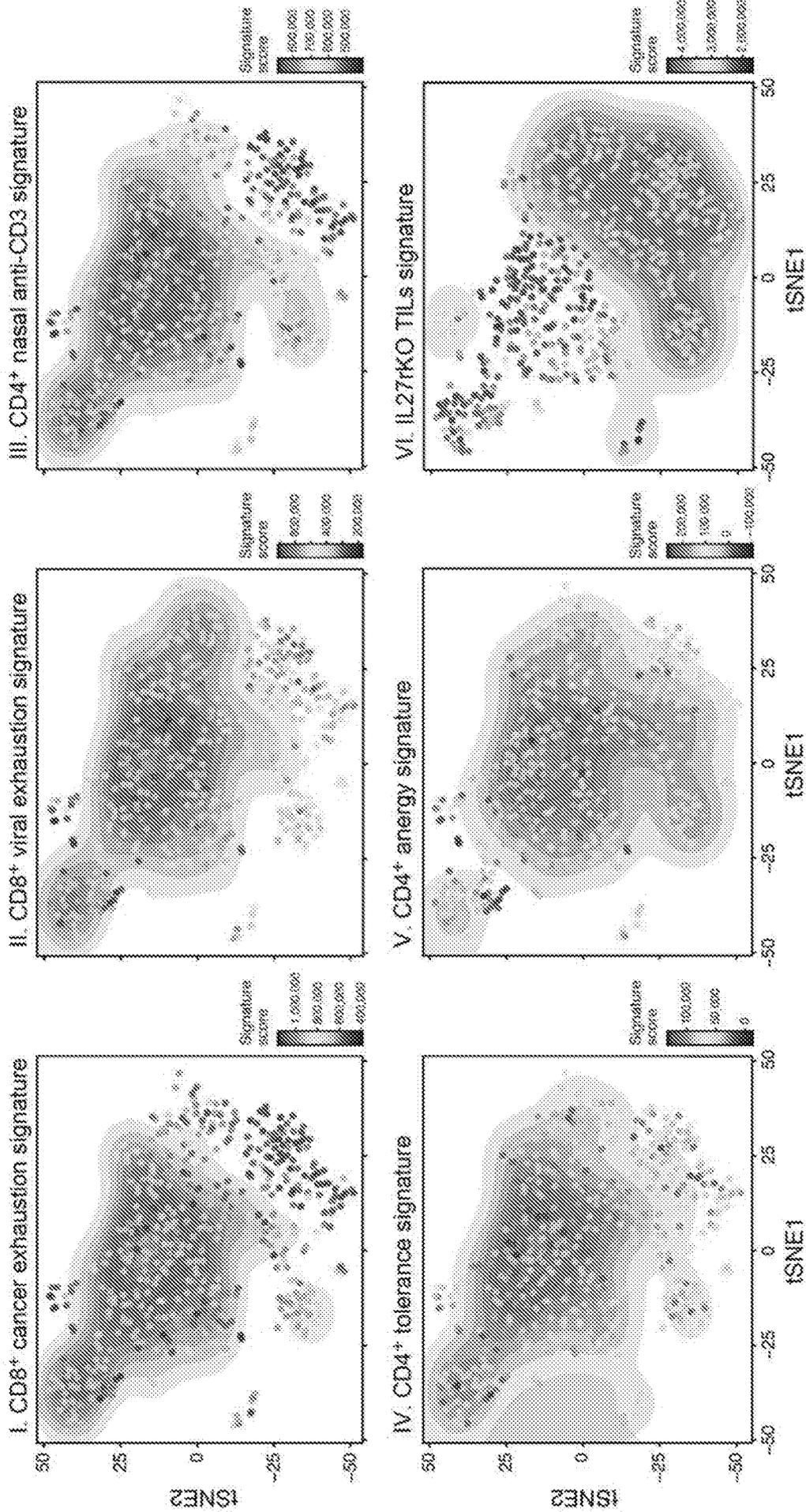


FIG. 60



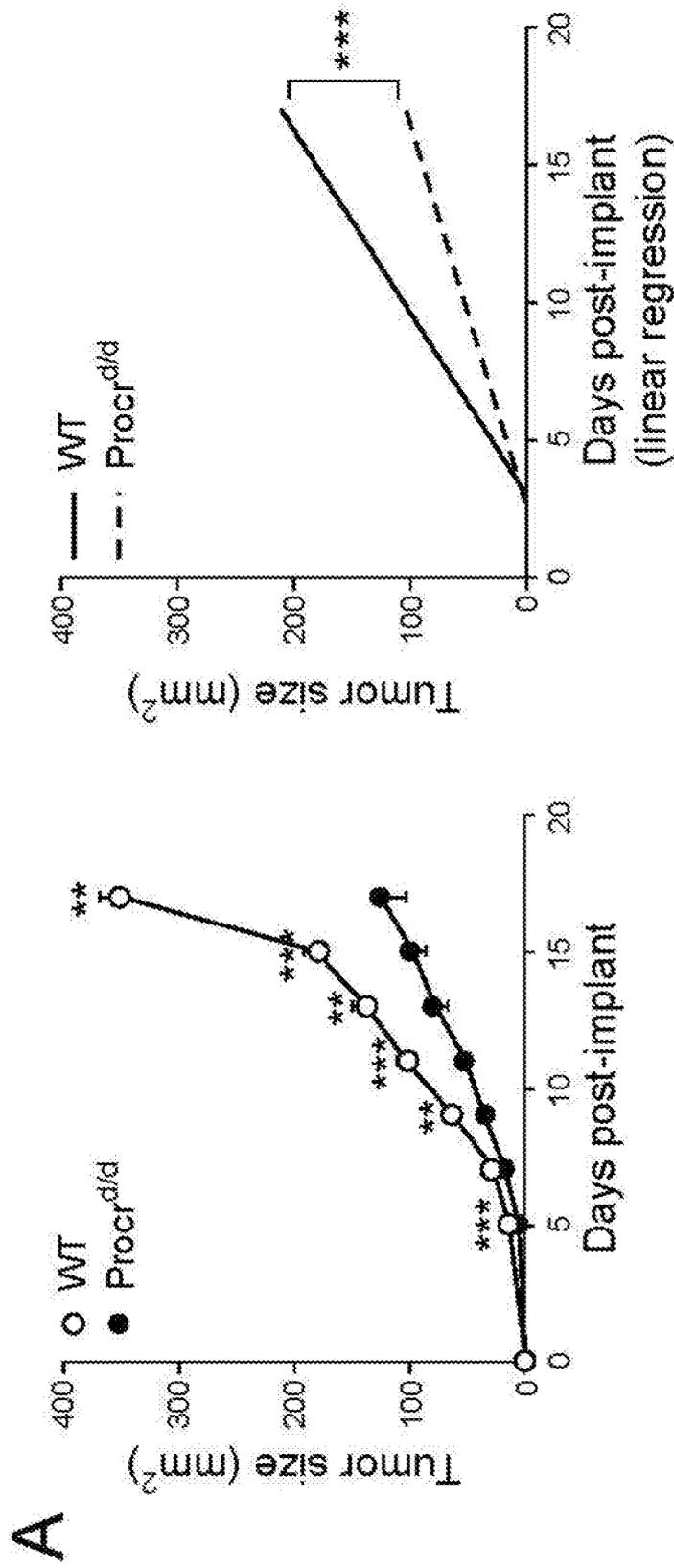


FIG. 7A

FIG. 7B

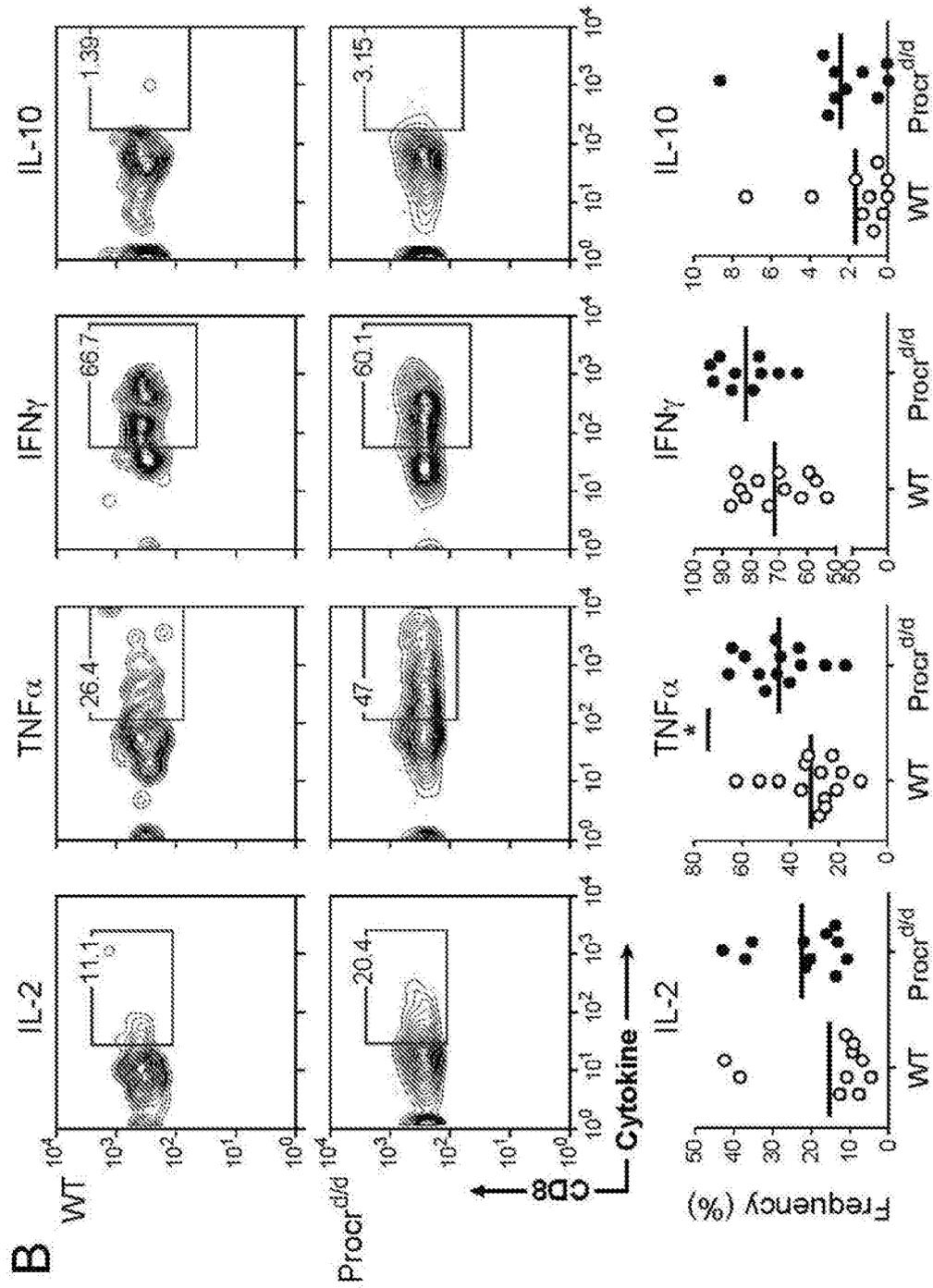


FIG. 7C

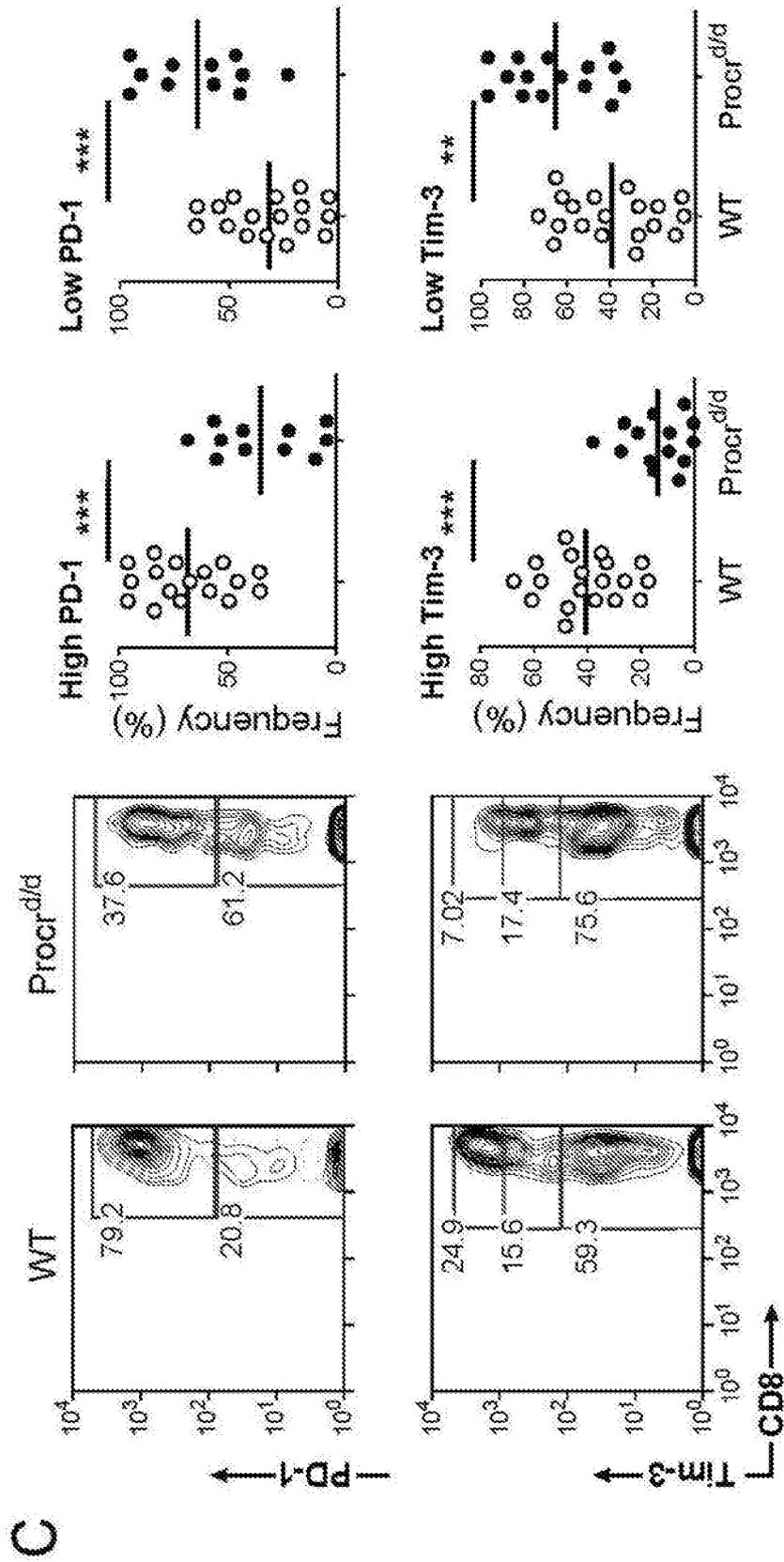
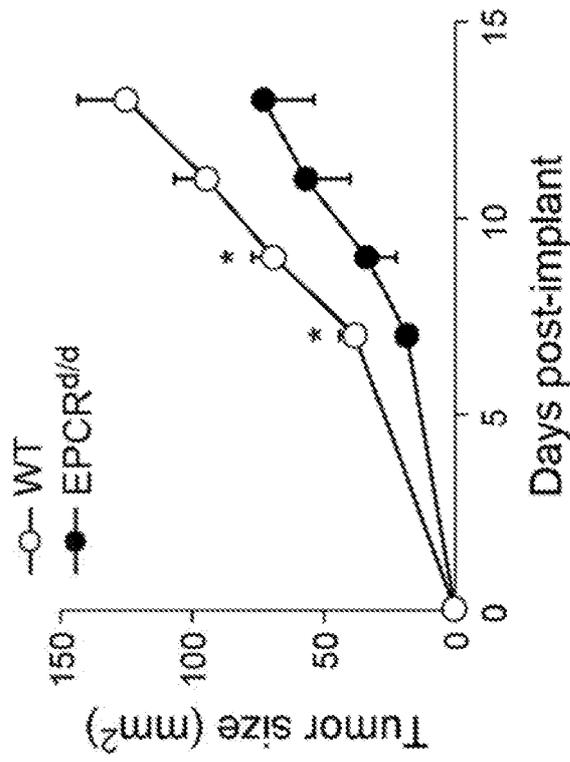


FIG. 7D-E

D



E

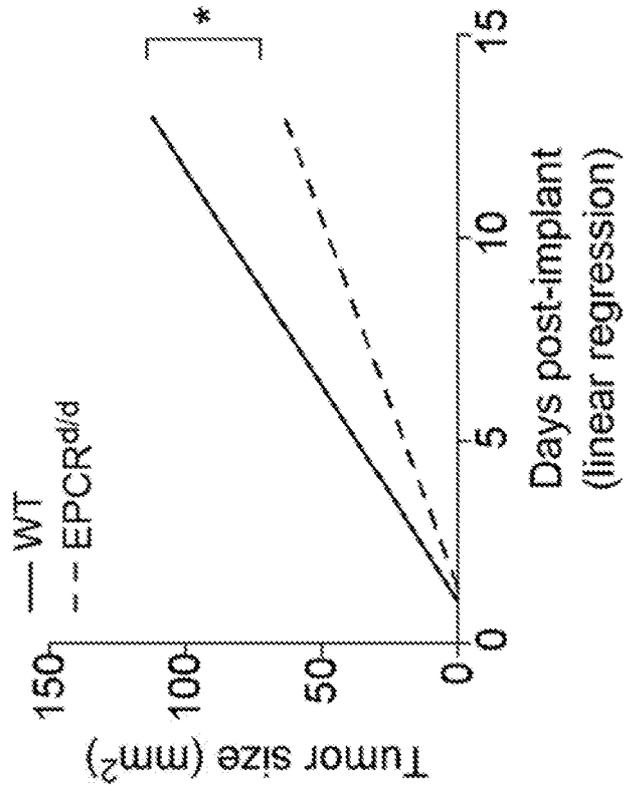


FIG. 8

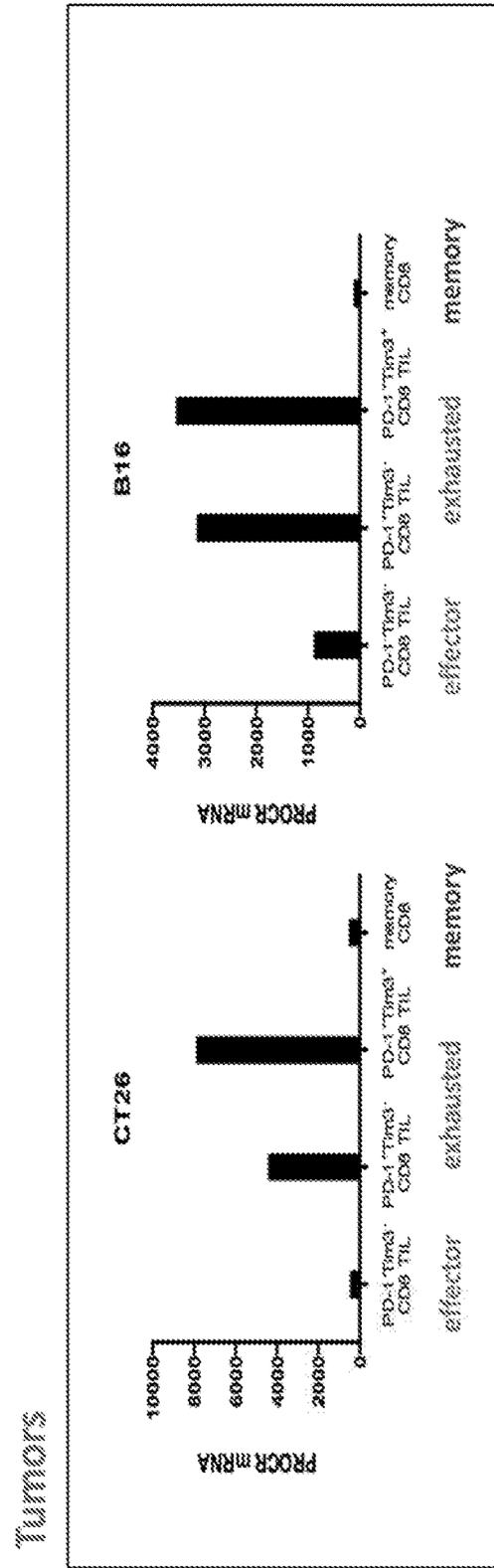


FIG. 9

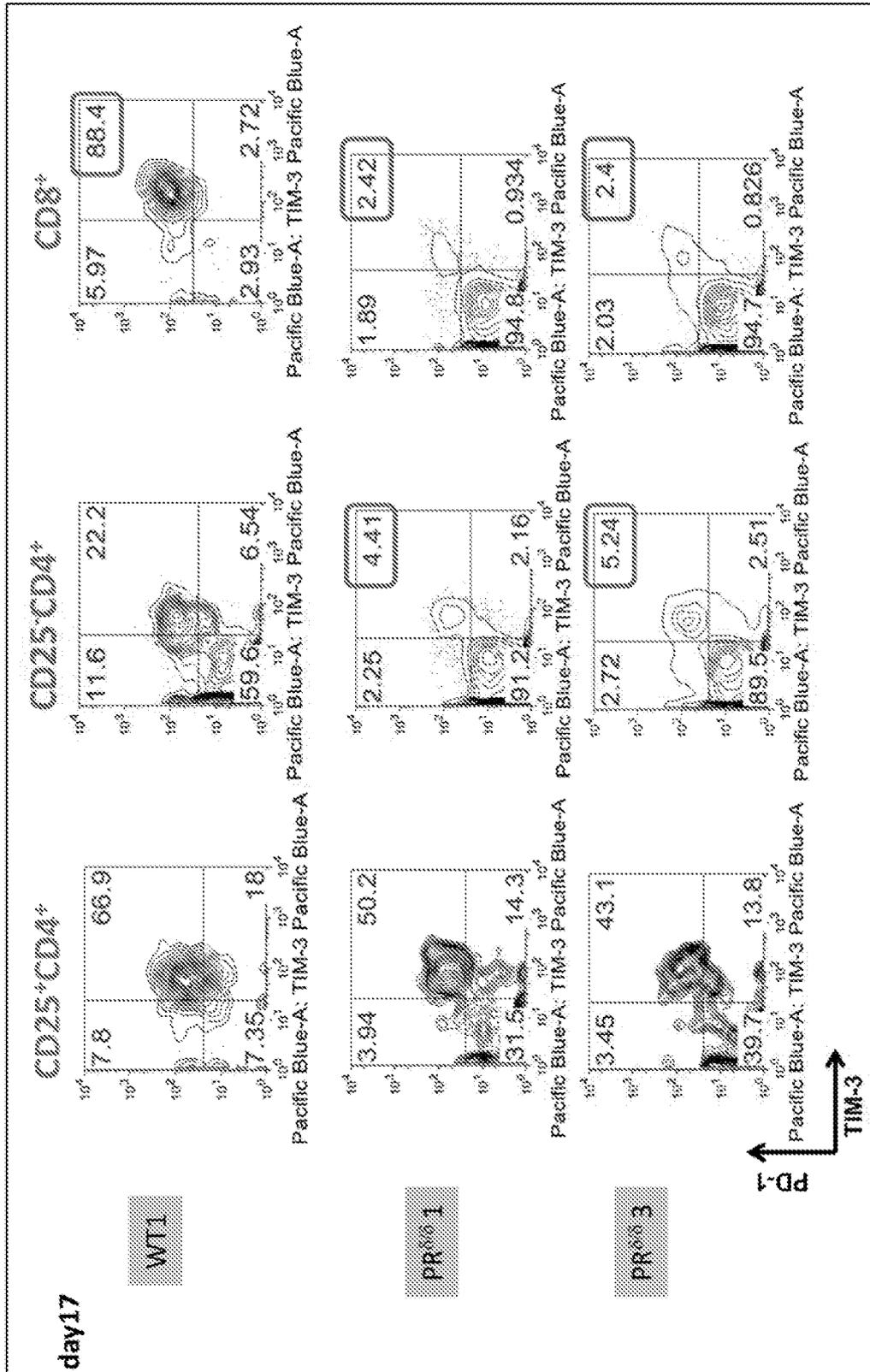


FIG. 10A-B

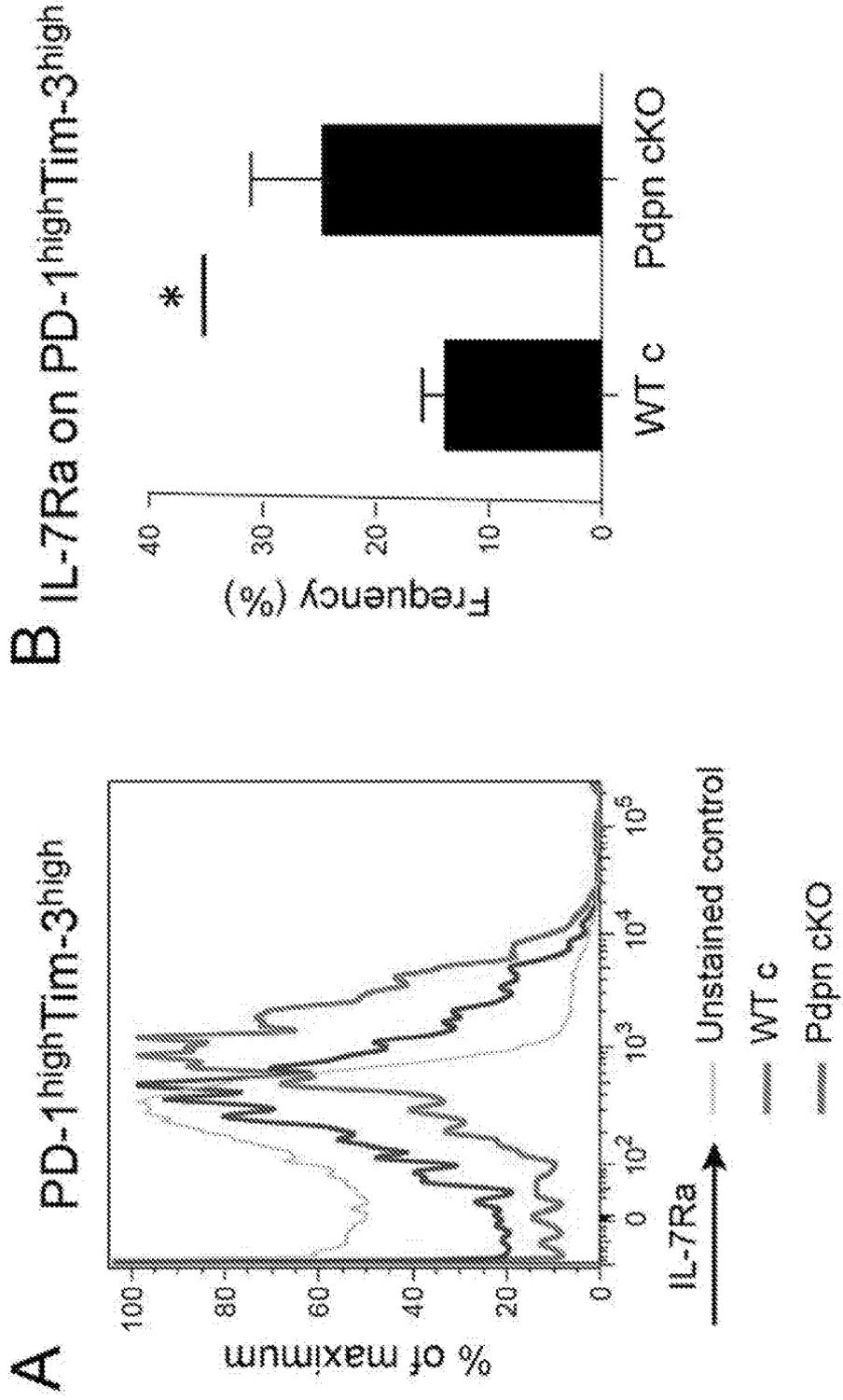
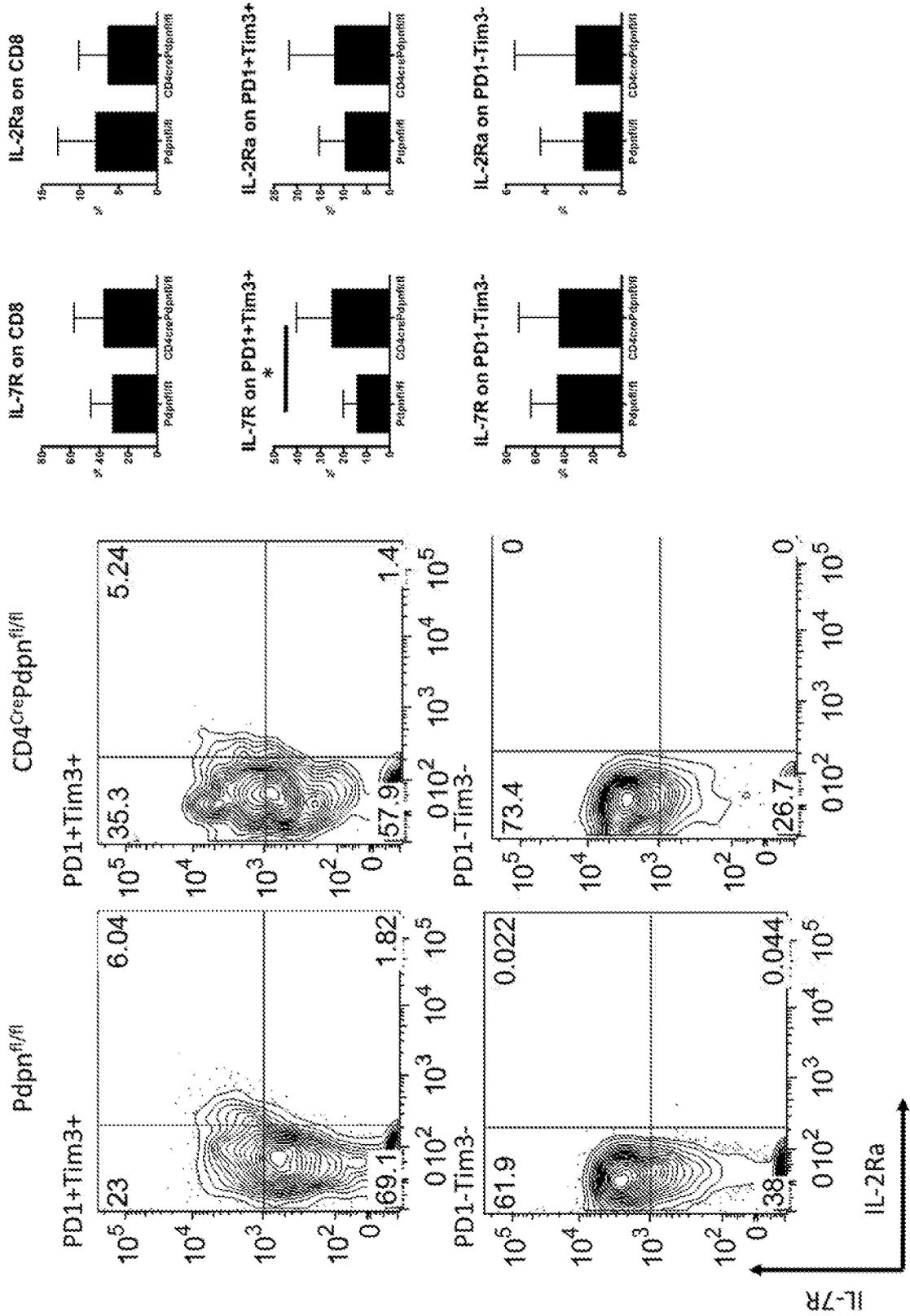


FIG. 10C



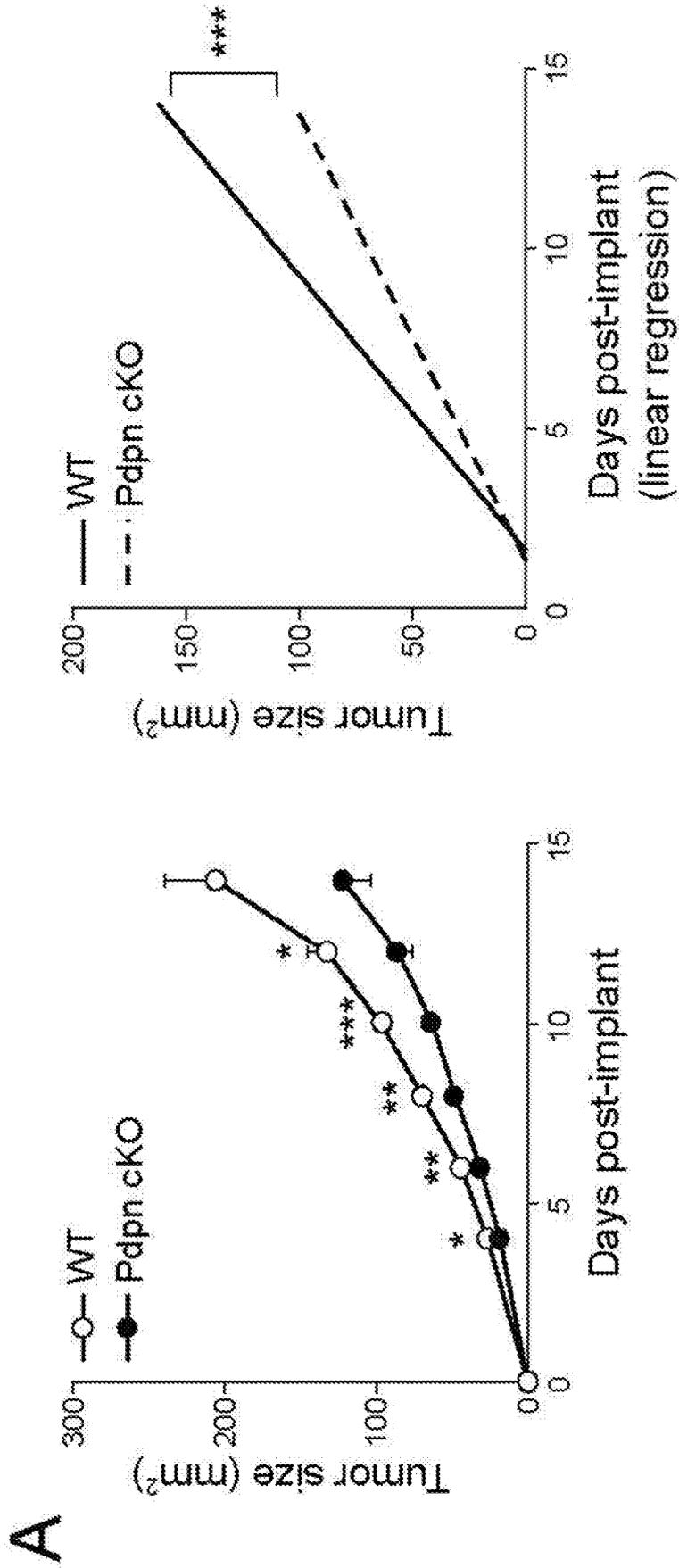


FIG. 11A

FIG. 11B

B

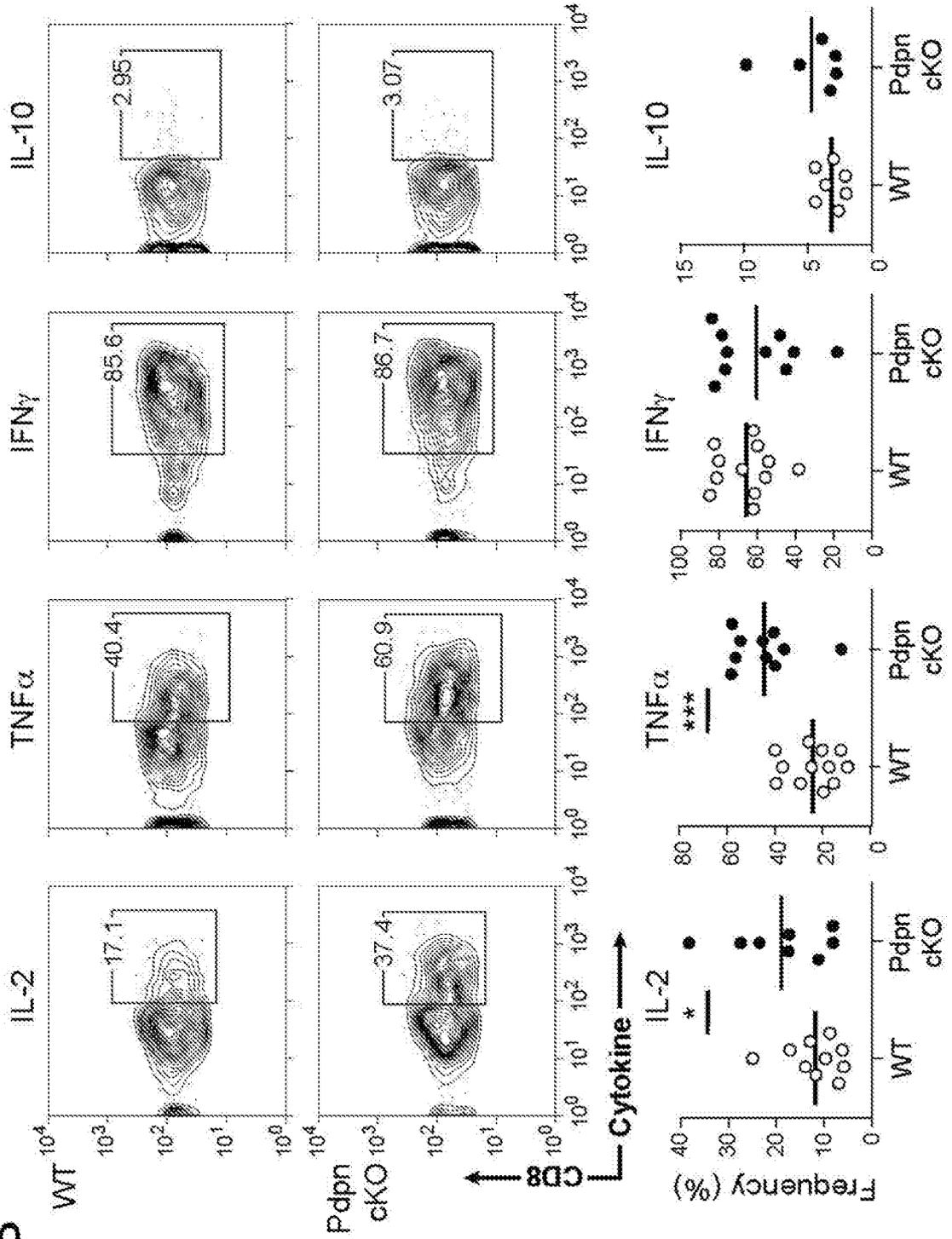


FIG. 11C

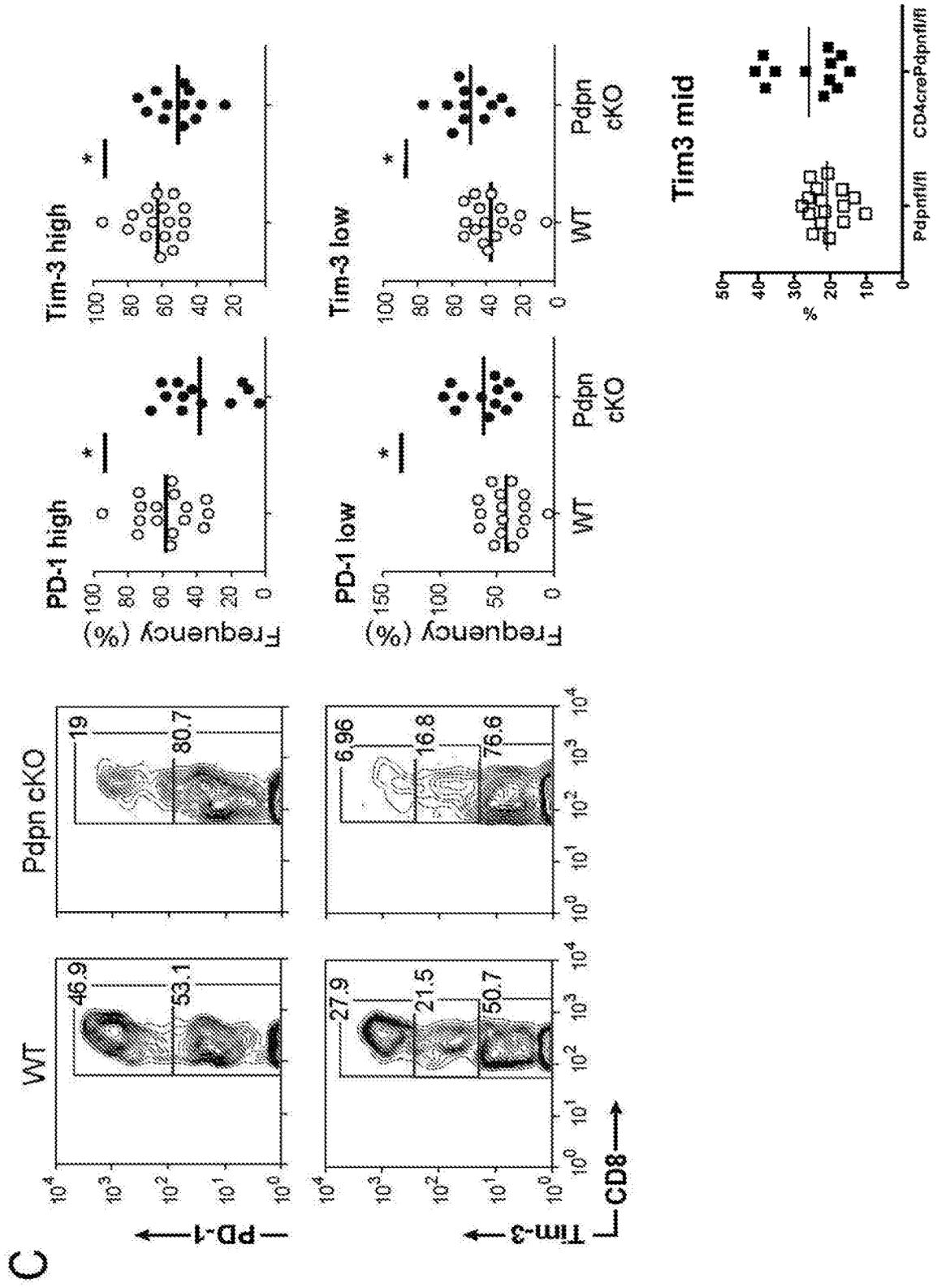


FIG. 12A-B

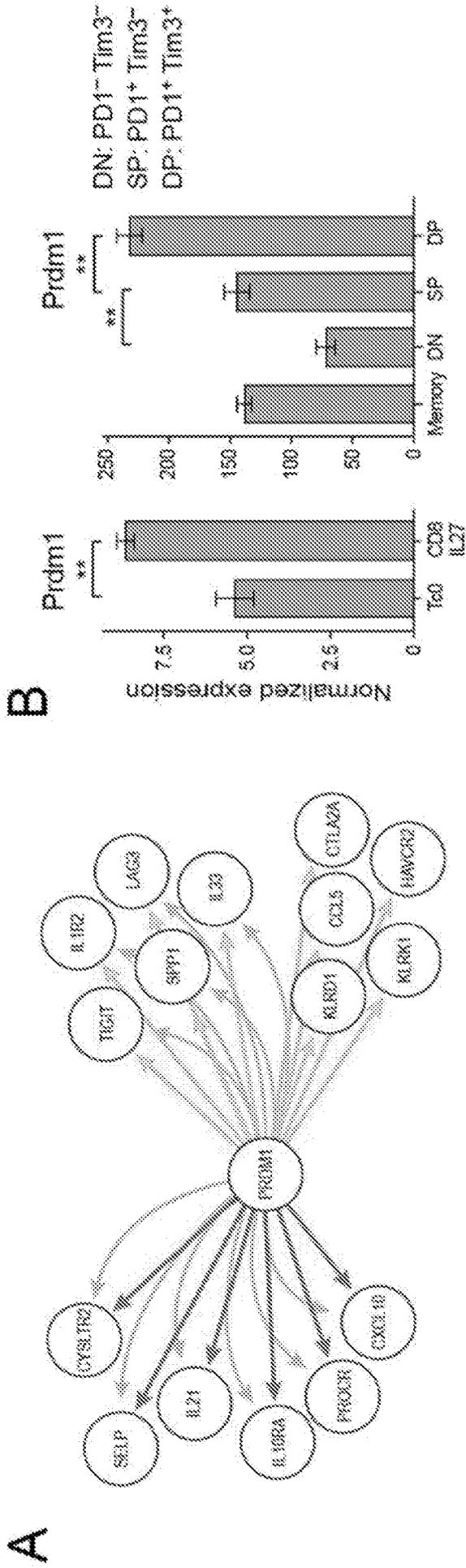
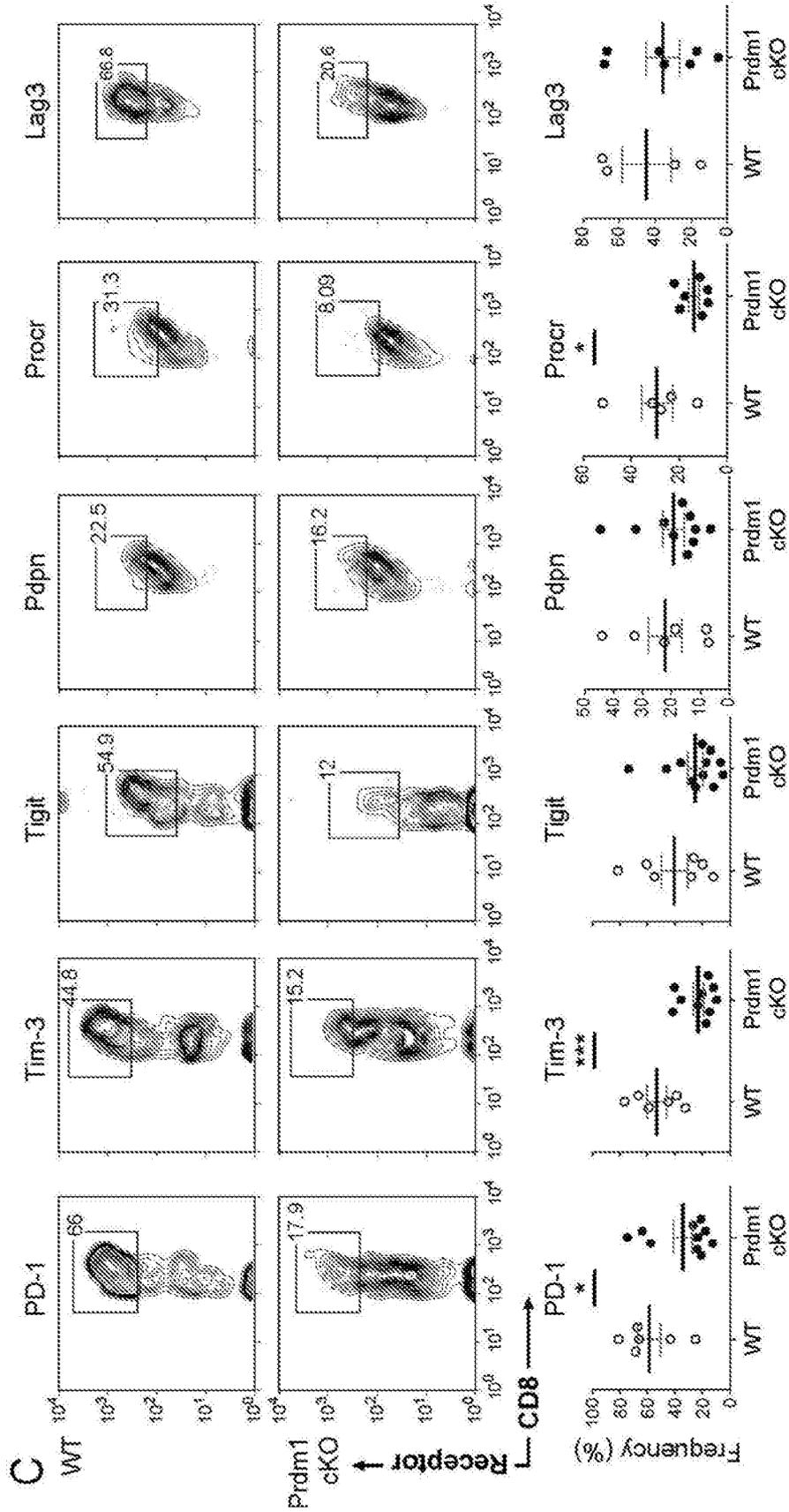


FIG. 12C



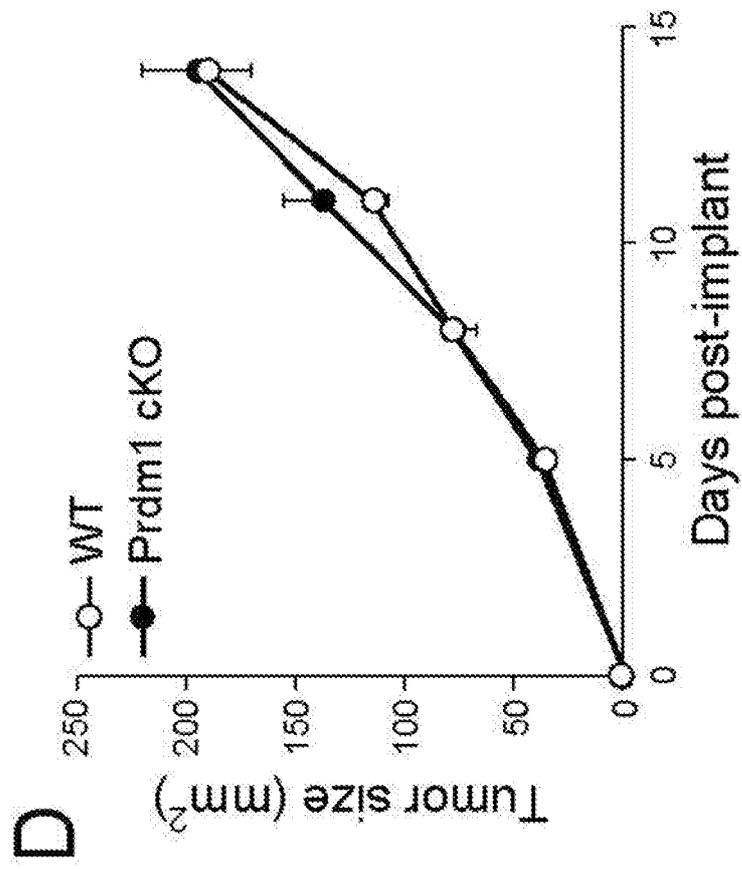


FIG. 12D

A

FIG. 13A

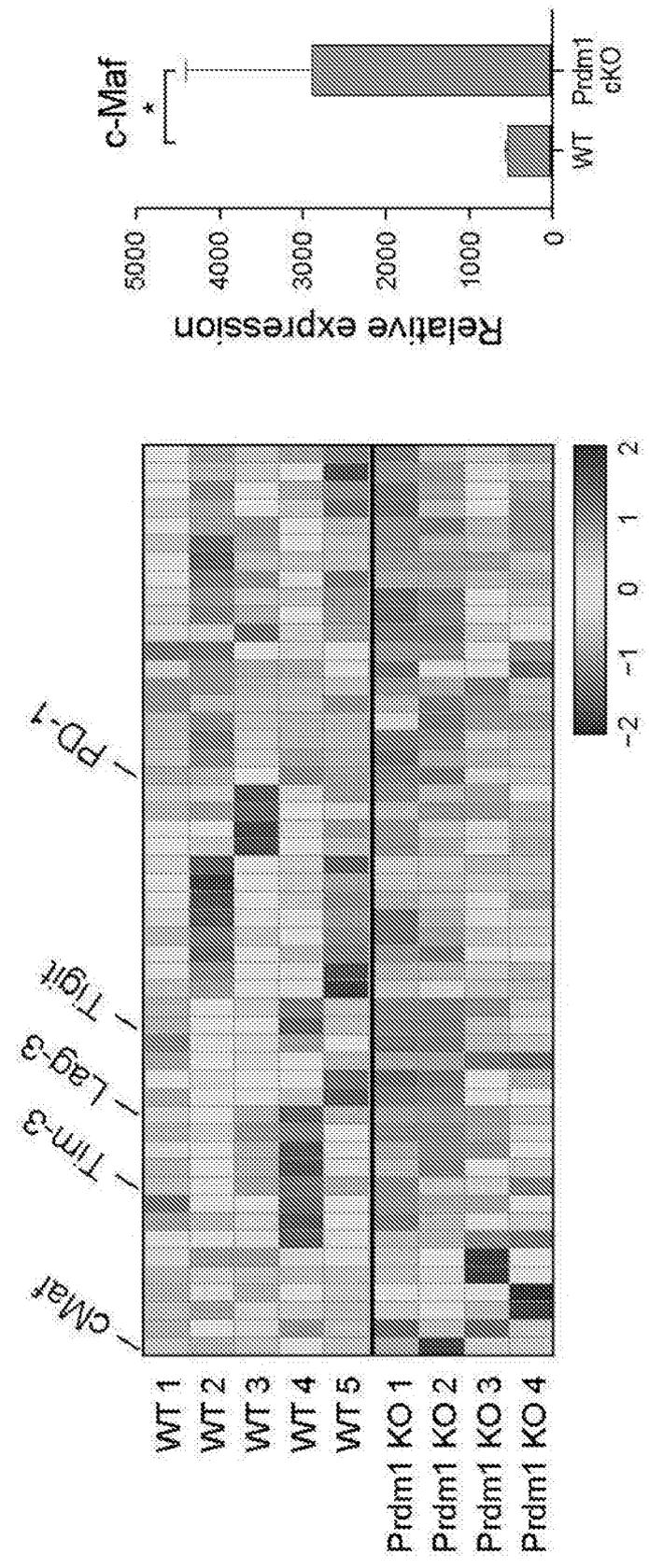


FIG. 13B

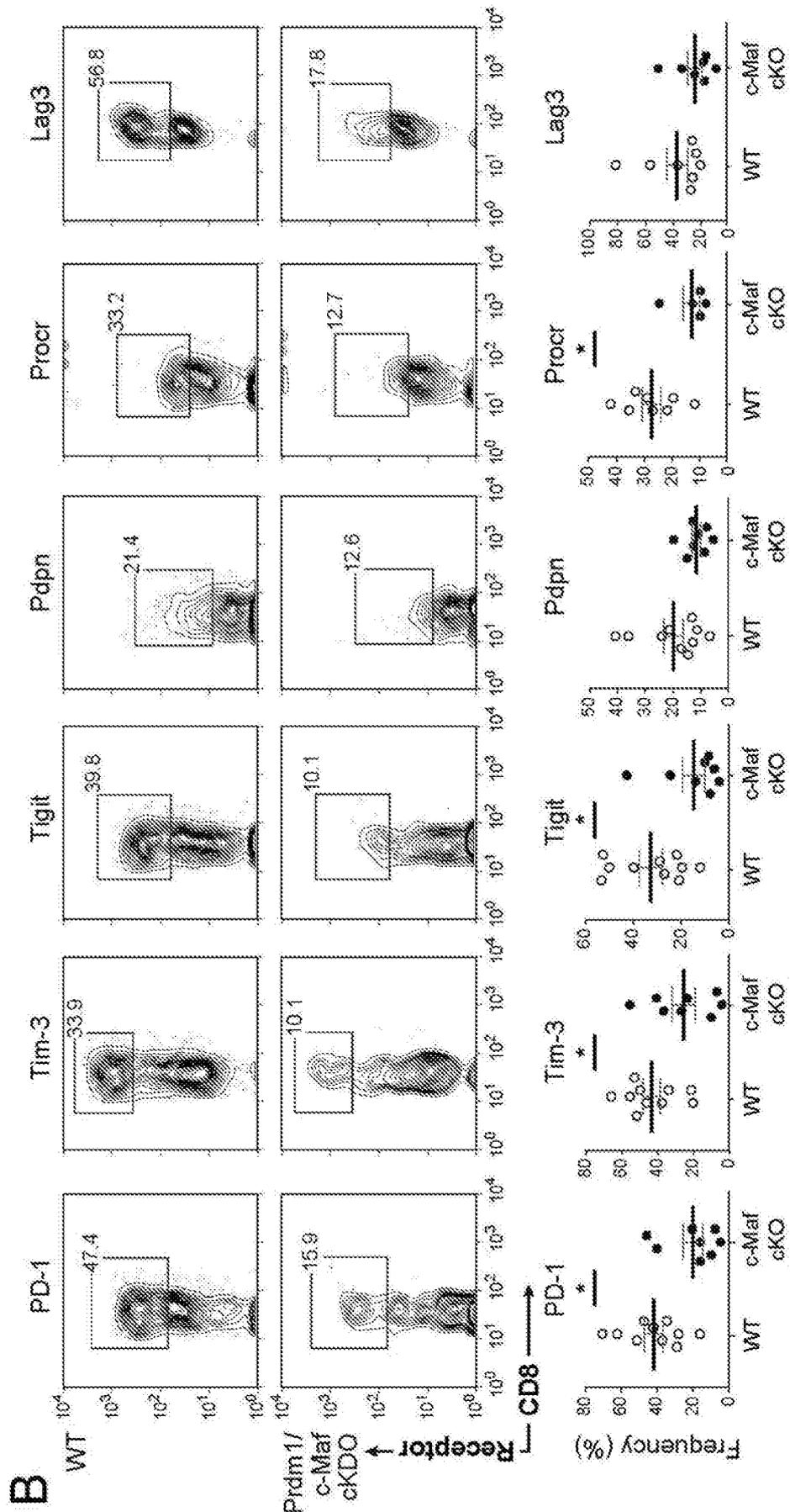


FIG. 13C-D

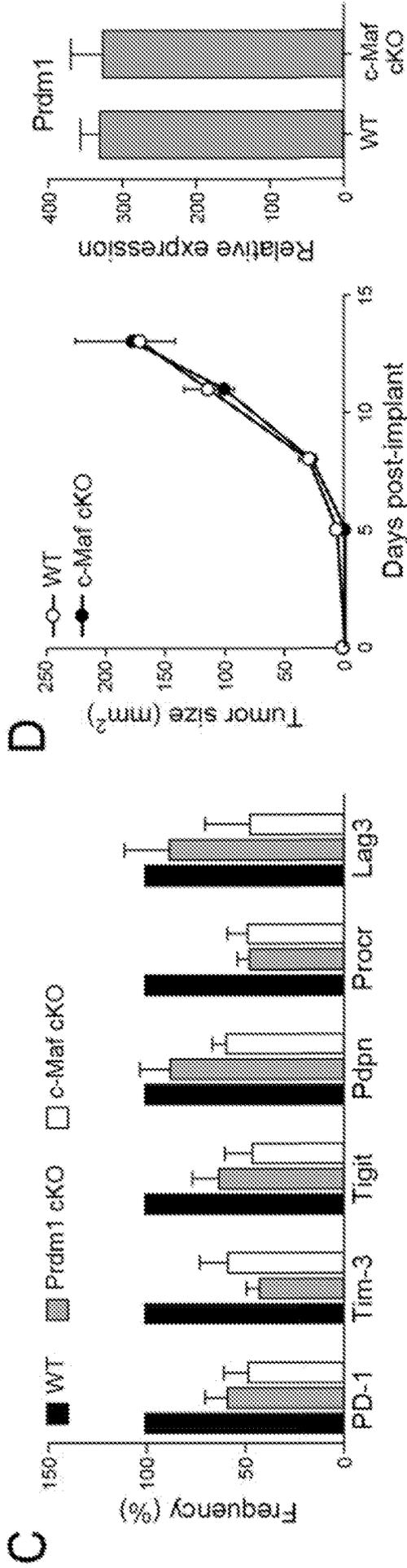


FIG. 14C-D

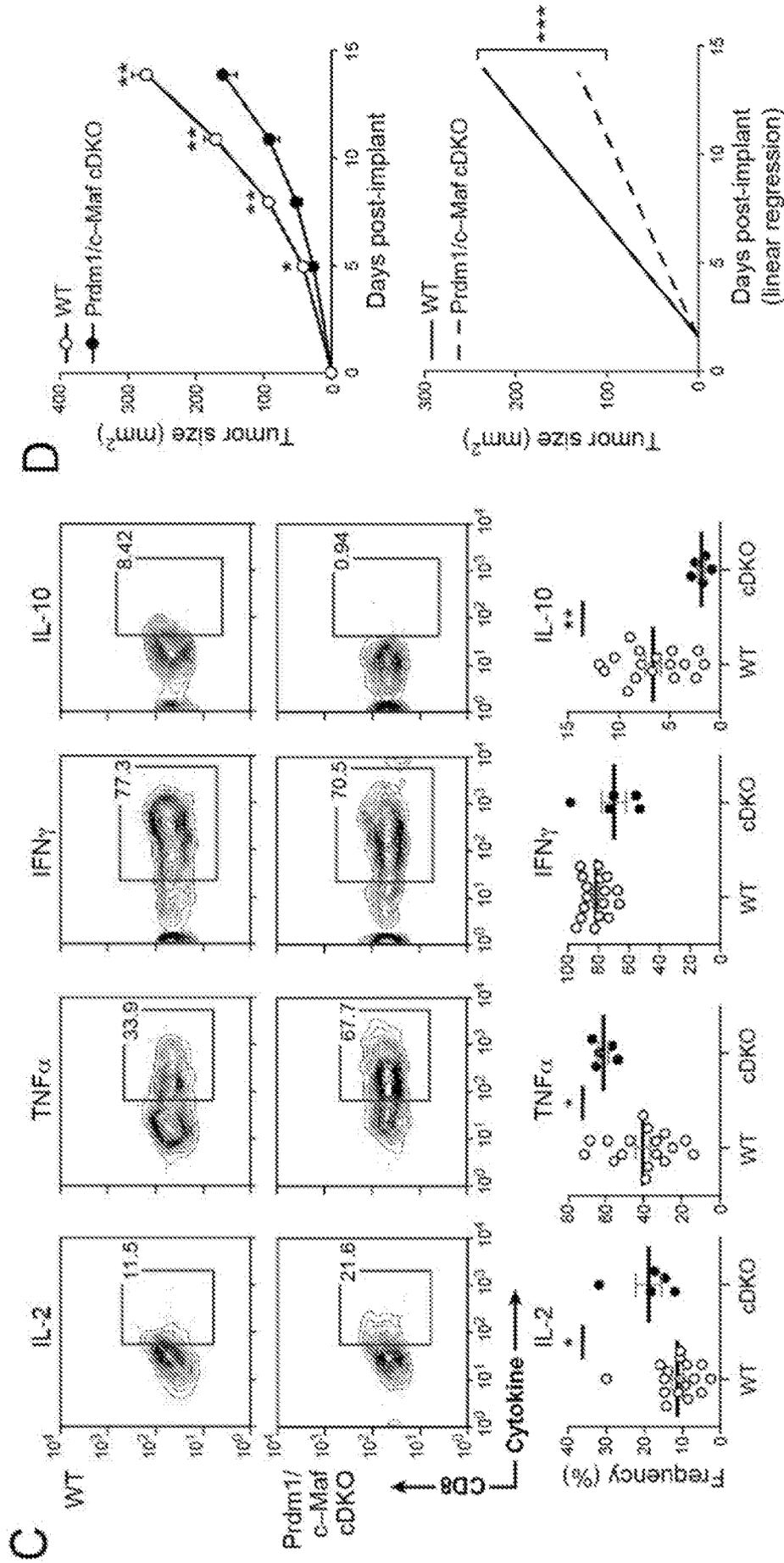


FIG. 14E

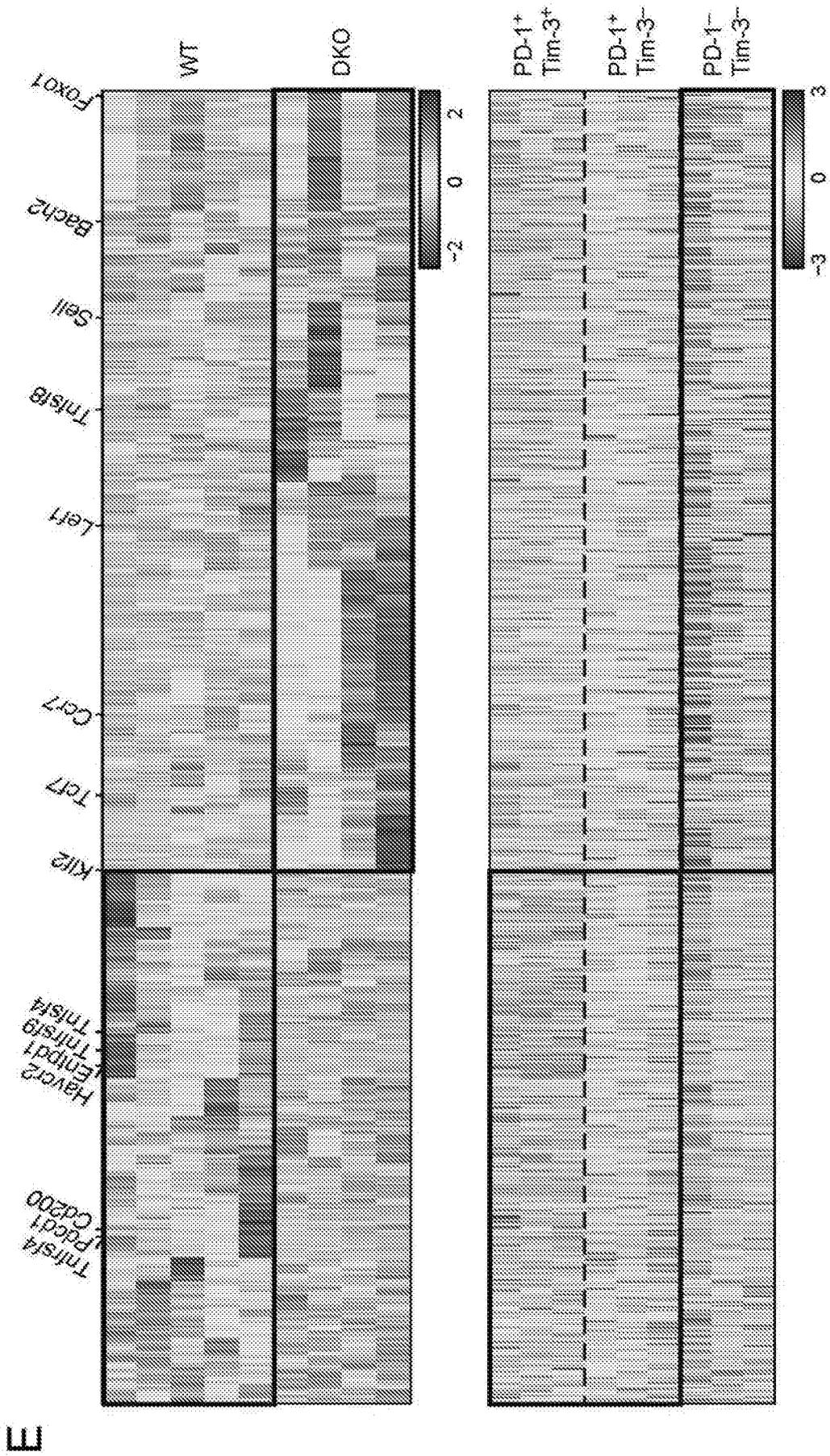
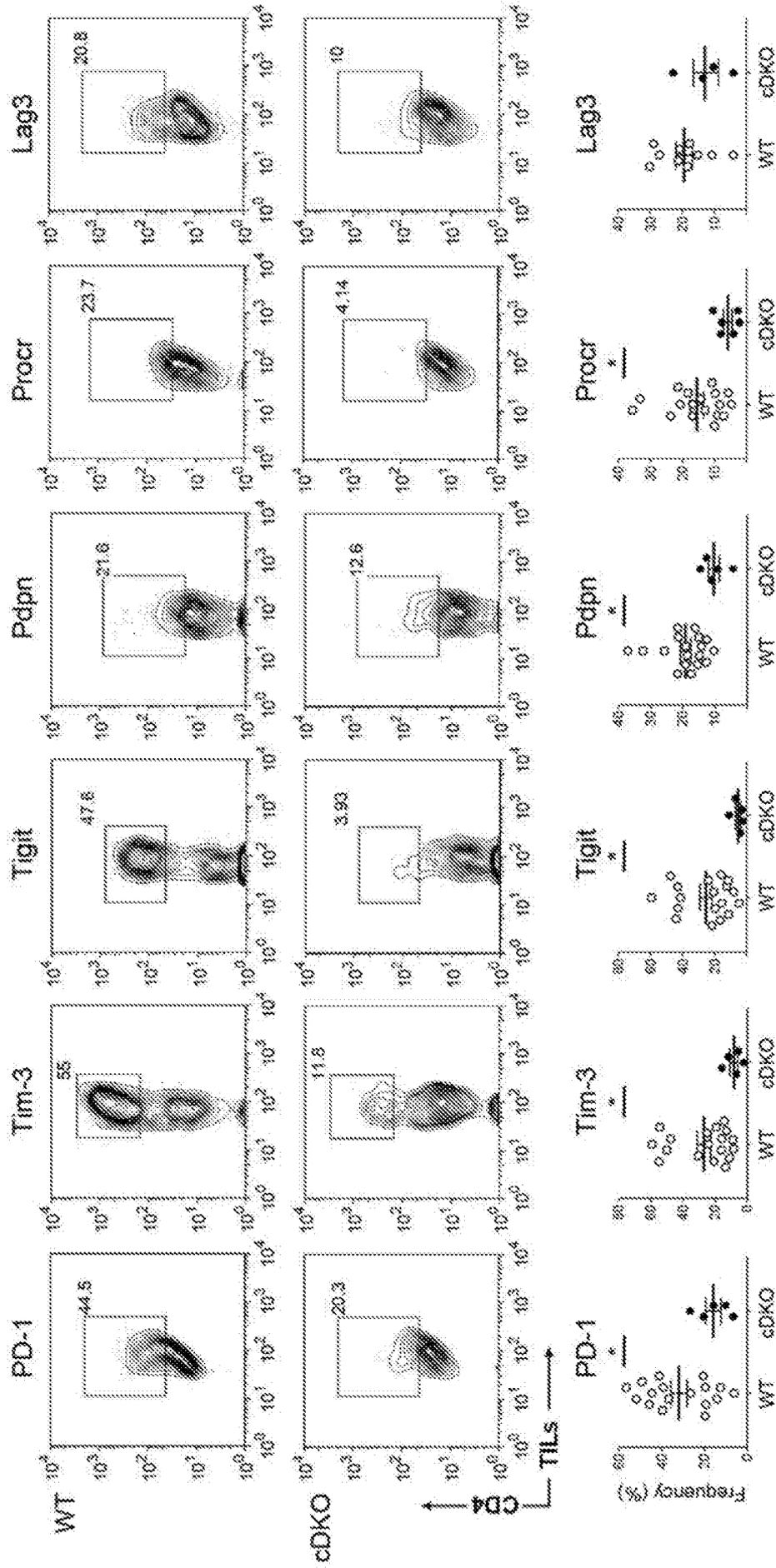


FIG. 14F



* p < 0.05

FIG. 14G

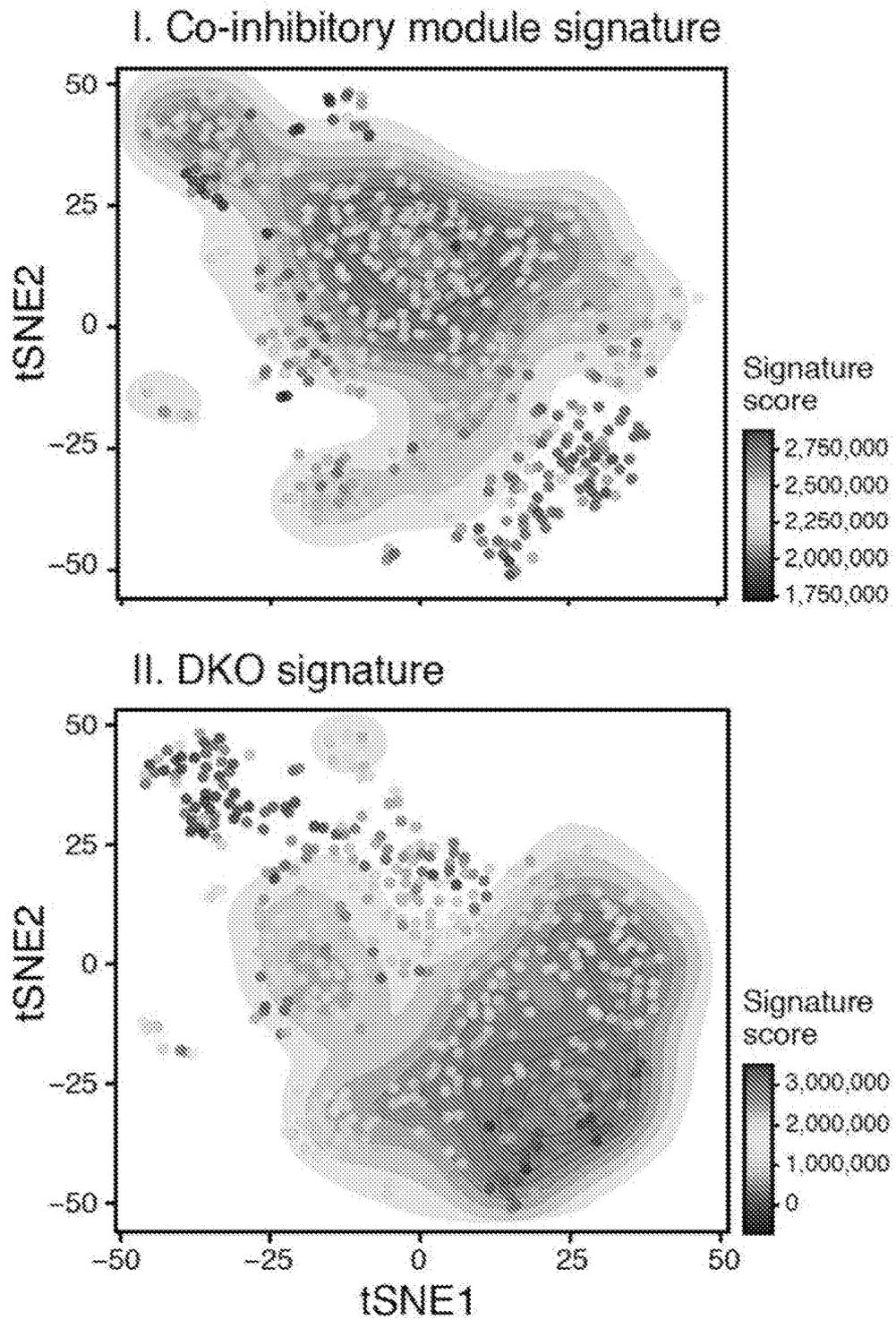


FIG. 15A

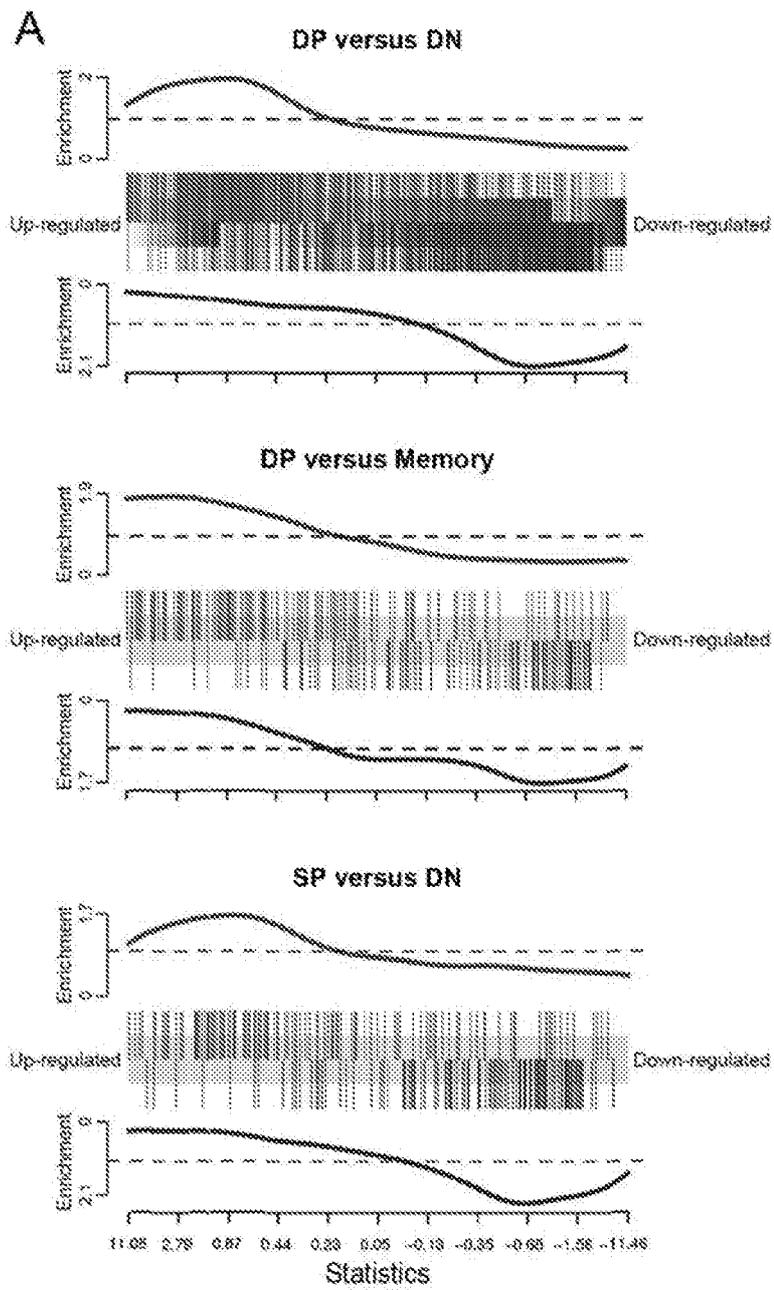


FIG. 15B-C

B

	One-sample Kolmogorov- Smirnov test	Mean-rank gene set test (Wilcoxon GST)	Hyper- geometric	Competitive gene set test for inter- gene correlation
DP versus DN up-regulated genes	2.00×10^{-7}	9.76×10^{-5}	1.98×10^{-43}	0.08
DP versus DN down-regulated genes	2.00×10^{-16}	0.67	1.61×10^{-64}	0.046
SP versus DN up-regulated genes	0.32	0.25	1.80×10^{-10}	0.26
SP versus DN down-regulated genes	0.81	0.84	9.54×10^{-17}	0.067
DP versus memory up-regulated genes	0.008	0.003	7.66×10^{-8}	0.14
DP versus memory down-regulated genes	2.00×10^{-10}	0.99	1.82×10^{-15}	0.39

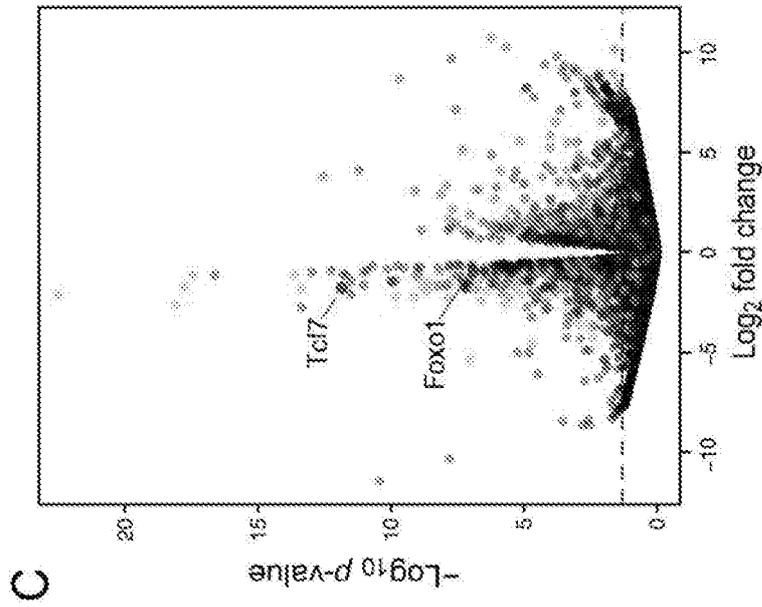


FIG. 16

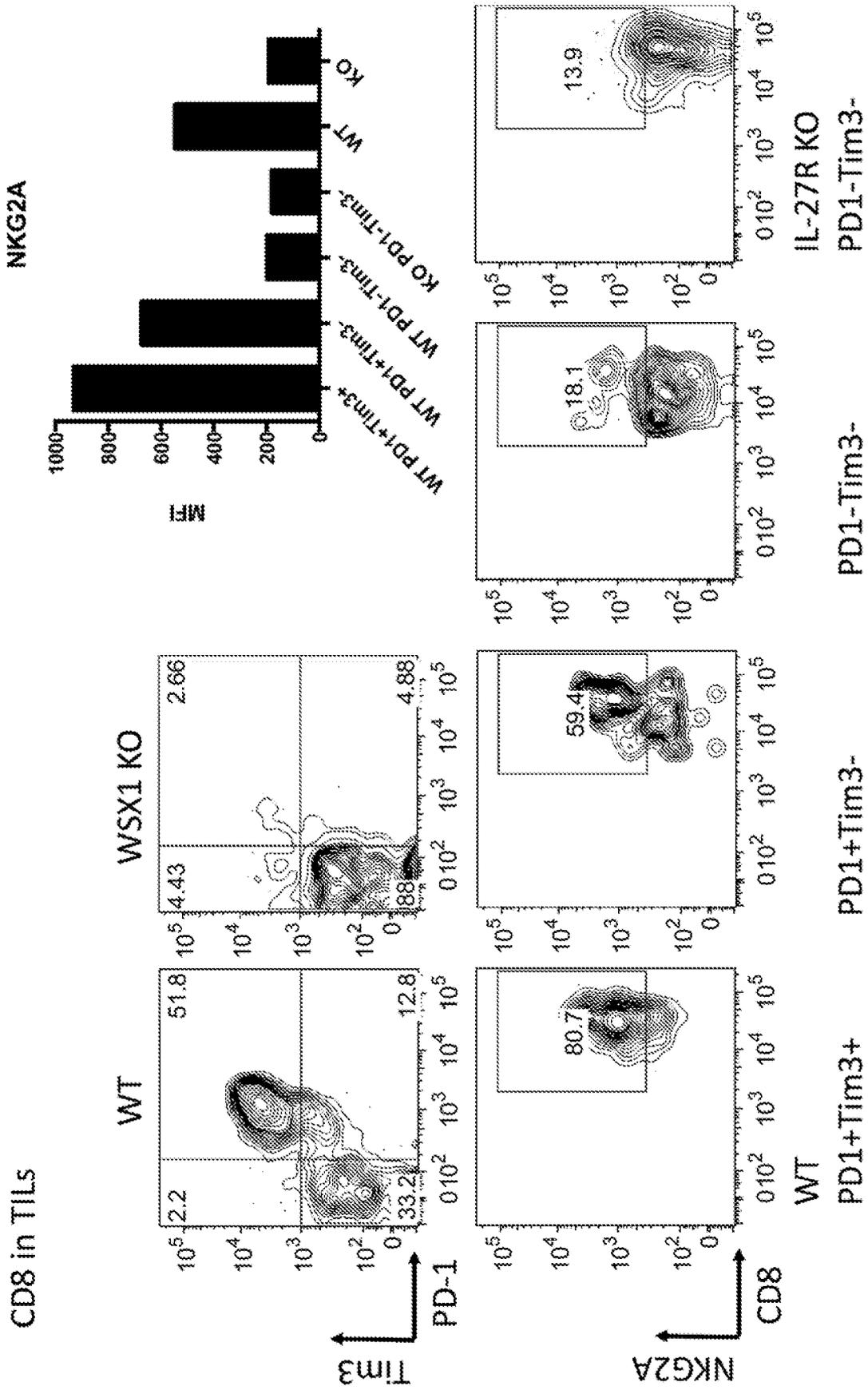


FIG. 17

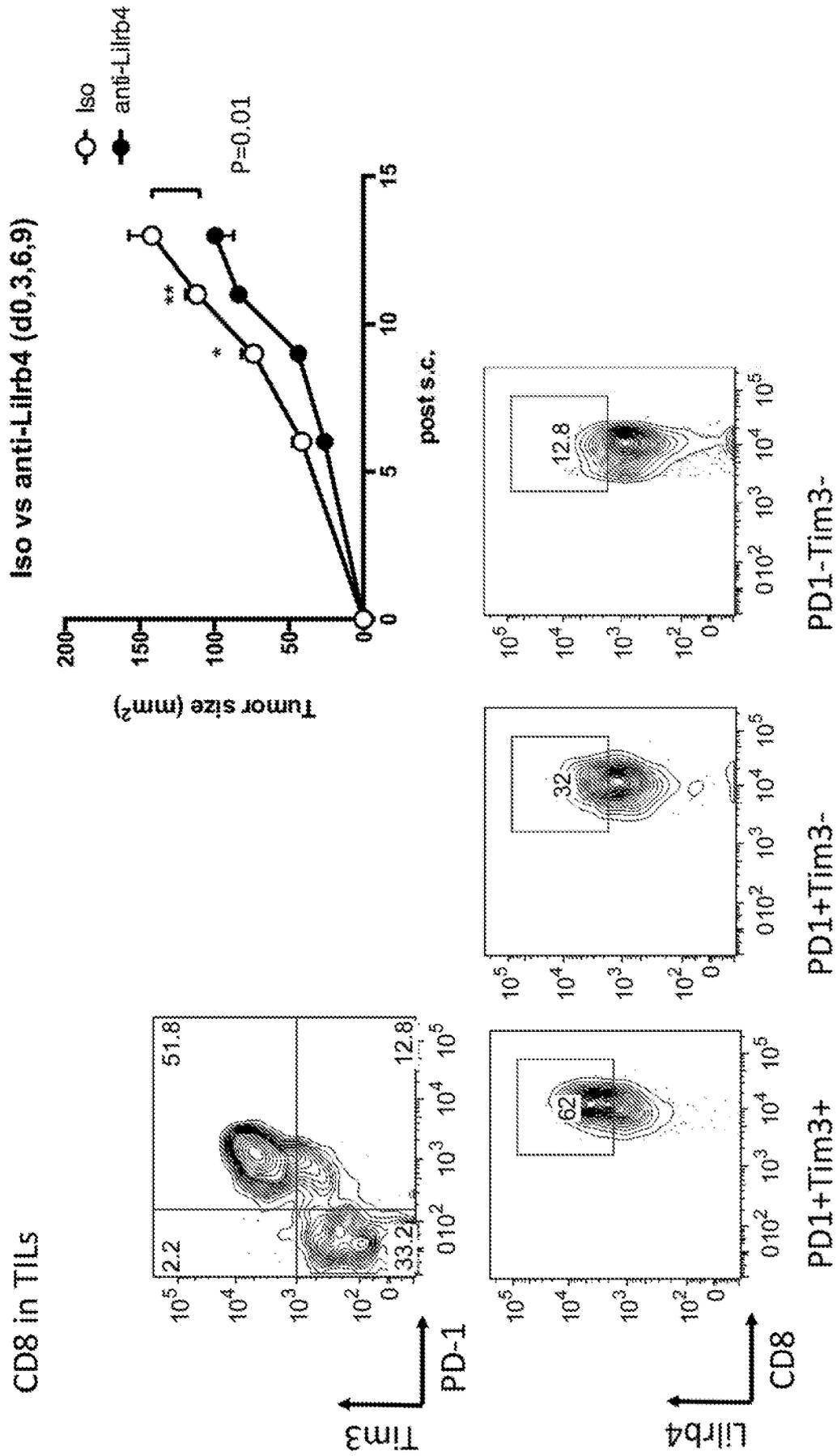


FIG. 18

Lack of Cys1tr2(LT2) is enhanced tumor growth (B16 melanoma)

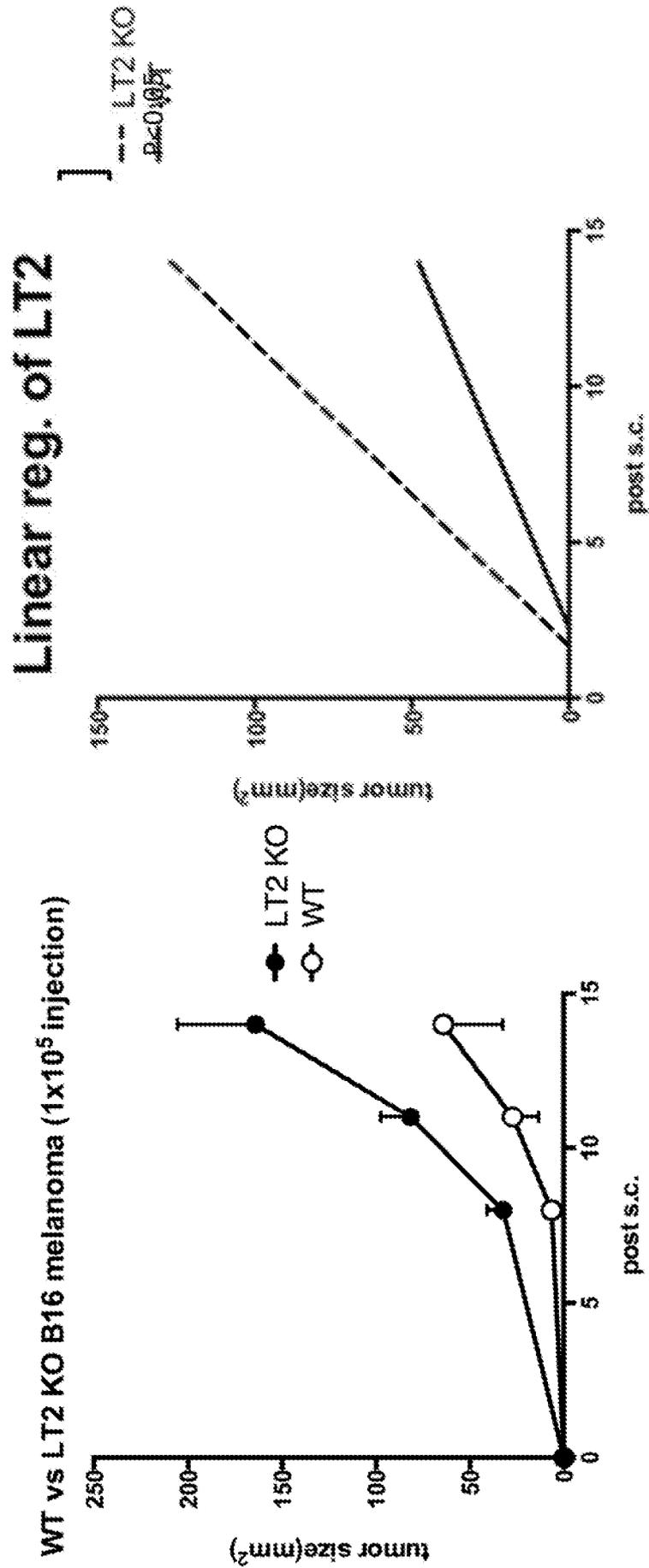


FIG. 19

Lack of Cysltr2(LT2) CD8 TILs shows less IL-2 production

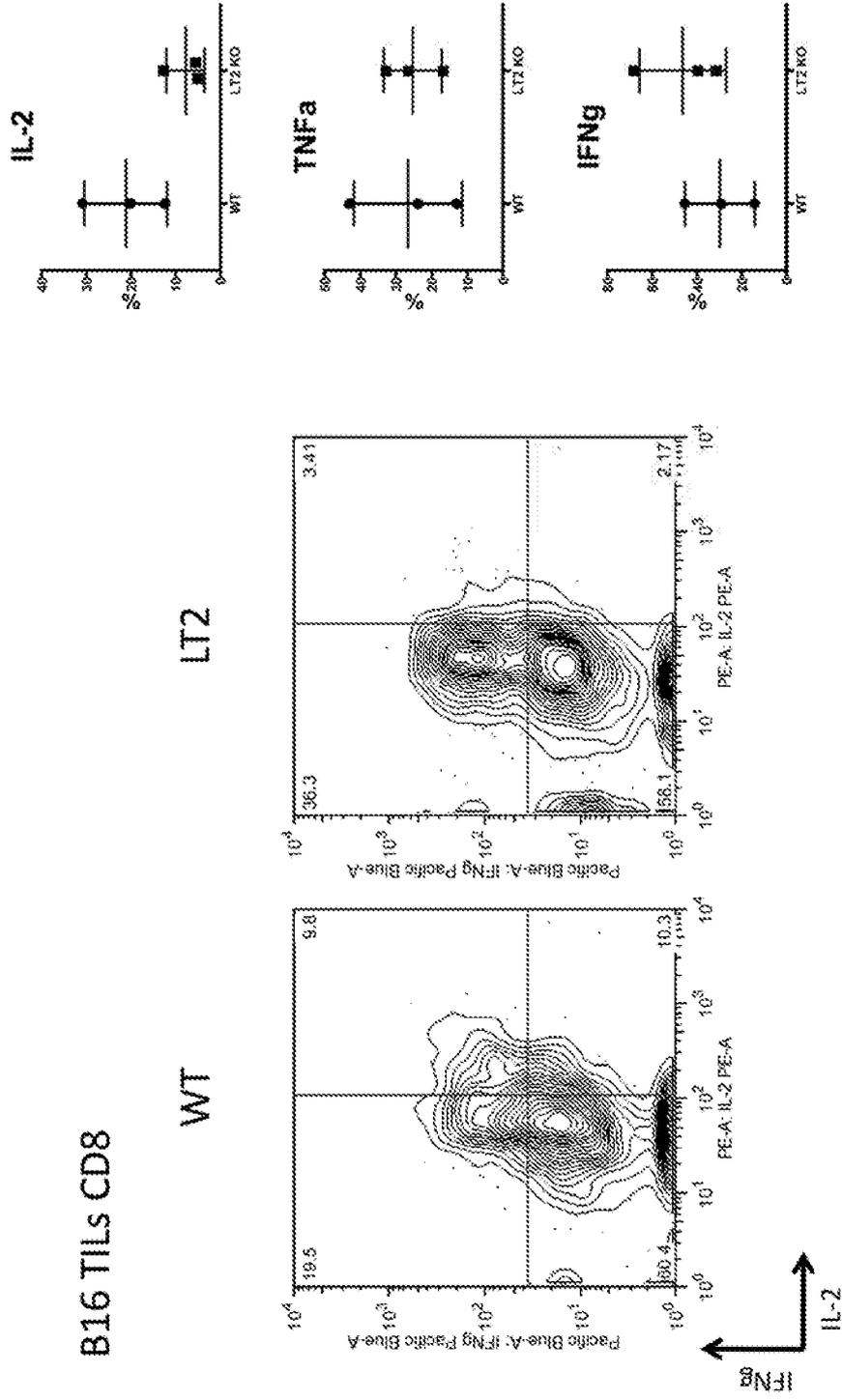


FIG. 20

