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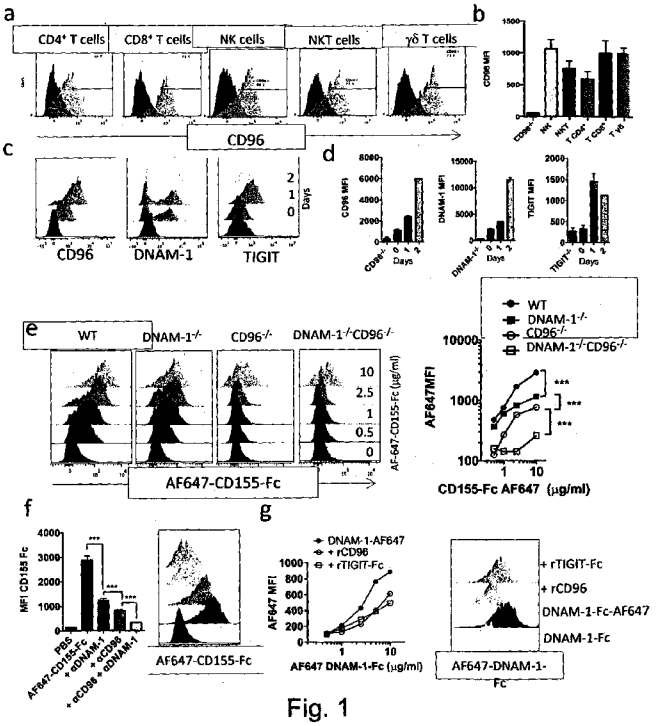

Fig. 1

(57) Abstract: A method of reducing or relieving immune inhibition in a mammal includes the step of at least partly inhibiting or reducing CD96 activity in one or more cells of the mammal to thereby relieve immune inhibition and/or enhance or restore immune surveillance in the mammal. Typically, inhibiting or reducing CD96 activity does not include, or depend upon, killing of CD96-expressing cells in the mammal. The method relieves immune inhibition and/or enhances or restores immune surveillance in the mammal to thereby treat or prevent cancer or cancer metastasis and/or a viral infection in the mammal. Also provided is a method of screening, designing, engineering or otherwise producing a CD96-inhibitory agent that relieves immune inhibition and/or enhances or restores immune surveillance in a mammal. Typically, the CD96-inhibitory agent is an antibody or antibody fragment and the mammal is a human.

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TITLE

IMMUNORECEPTOR MODULATION FOR TREATING CANCER AND VIRAL INFECTIONS

TECHNICAL FIELD

THIS INVENTION relates to the immunoreceptor CD96. More particularly, this invention relates to inhibition of CD96 to thereby enhance the ability of the immune system to target tumours and other diseases or conditions that can evade the immune system.

BACKGROUND

The progression of a productive immune response requires that a number of immunological checkpoints be passed. Passage may require the presence of excitatory co-stimulatory signals or the avoidance of negative or co-inhibitory signals, which act to dampen or terminate immune activity. The immunoglobulin superfamily occupies a central importance in this coordination of immune responses, and the CD28/cytotoxic T-lymphocyte antigen-4 (CTLA-4):B7.1/B7.2 receptor/ligand grouping represents the archetypal example of these immune regulators. In part the role of these checkpoints is to guard against the possibility of unwanted and harmful self-directed activities. While this is a necessary function, aiding in the prevention of autoimmunity, it may act as a barrier to successful immunotherapies aimed at targeting malignant self-cells that largely display the same array of surface molecules as the cells from which they derive. Therapies aimed at overcoming these mechanisms of peripheral tolerance, in particular by blocking the inhibitory checkpoints on T cells, offer the potential to generate antitumor activity, either as monotherapies or in synergism with other therapies that directly or indirectly enhance presentation of tumor epitopes to the immune system. Such anti-T cell checkpoint antibodies are showing promise in early clinical trials of advanced human cancers.

Furthermore, natural killer (NK) cells are innate lymphocytes critical to limit early tumor growth and metastasis [1]. NK cell functions are also regulated by the integration of signals transmitted by a wide range of activating and inhibitory receptors [2]. For example, the recognition of pathogen-derived or stress-induced ligands by activating receptors such as NCRs, NKG2D, or DNAM-1 stimulate NK cells cytotoxicity and the secretion of pro-inflammatory mediators such as interferon gamma (IFN-γ) [3]. In contrast, inhibitory receptors protect target cells from NK cell-

mediated killing [4]. These receptors mostly recognize MHC class I and MHC class I-related molecules and include the KIR (killer cell immunoglobulin-like receptors) and LIR (leukocyte immunoglobulin-like receptors) families, the Ly49 family in mice and the CD94/NKG2 heterodimers in both species.

An emerging group of immunoglobulin superfamily members that interact with ligands of the nectin and nectin-like (necl) family has recently been described to influence NK cell and T cell functions [5]. These include CD226 (DNAM-1) [6], CD96 (TACTILE) [7], TIGIT (T cell immunoglobulin and ITIM domain) [8,9], and CRTAM (class I restricted T cell-associated molecule) [10]. DNAM-1 and TIGIT are the most extensively studied members of this family and they share a common ligand, CD155 (necl-5; PVR) and CD112 (nectin-2; PVRL2) [8,11]. TIGIT also bind an additional ligand CD113 (PVRL3) [8]. The functions of DNAM-1 and TIGIT on NK cells are reportedly counter-balancing [12]. *In vitro,* DNAM-1 potentiates the cytotoxicity of NK cells against a wide range of tumor cells [13,14] and is critical for tumor immunosurveillance *in vivo* [13,15,16]. In contrast, TIGIT bear an ITIM motif and has been proposed prevent self-tissue damage similar to inhibitory Ly49 or KIR interactions with MHC class I [17]. Indeed, engagement of TIGIT by CD155 has been shown to limit IFNγ production and cytotoxicity by NK cells *in vitro* [18,19]. However, the role of TIGIT in NK cell biology relative to the other nectin receptors DNAM-1 and CD96 remains to be assessed *in vivo*.

Despite being cloned 20 years ago [7], little is known about CD96, the other Ig family member that shares CD155 ligand with DNAM-1 and TIGIT [20,21]. In humans, CD96 expression is largely confined to NK cells, CD8 T cells, and CD4 T cells [7]. The major ligand of CD96 is CD155, but CD96 has also been reported to associate with CD111 (nectin-1) and play a role in promoting NK and T cell adhesion [21,22].

SUMMARY

Surprisingly, the present inventors have discovered that CD96 acts as a negative regulator of T cell and NK cell anti-tumor functions. Accordingly, the invention is broadly directed to use of agents that at least partly block or inhibit CD96 to thereby reduce or relieve CD96-mediated immune inhibition to enhance or restore immune surveillance in the mammal. In certain embodiments, this may

facilitate treatment of diseases or conditions responsive at least partial blocking or inhibition of CD96, such as cancers and/or viral infections.

In a first aspect, the invention provides a method of reducing or relieving immune inhibition in a mammal, said method including the step of at least partly inhibiting or reducing CD96 activity in one or more cells of the mammal to thereby relieve immune inhibition and/or enhance or restore immune surveillance in the mammal.

Suitably, the step of inhibiting or reducing CD96 activity in the mammal does not include, or at least depend upon, killing of CD96-expressing cells in the mammal. Preferably, the step of inhibiting or reducing CD96 activity in the mammal includes inhibiting or reducing CD96 binding to CD155 and/or intracellular signaling in one or more cells of the mammal that express CD96.

In one particular embodiment, the step of inhibiting or reducing CD96 activity in the mammal includes increasing or enhancing expression, production and/or secretion of one or more cytokines or chemokines. Preferably, the cytokine is interferon γ (IFN-γ). Typically, the one or more cells of the mammal are T cells, inclusive of $CD4^+$ and $CD8^+$ T cells, γδT cells, NKT cells, and natural killer (NK) cells.

In a preferred embodiment, the method relieves immune inhibition and/or enhances or restores immune surveillance in the mammal to thereby treat or prevent cancer or cancer metastasis in the mammal.

In other embodiments, the method relieves immune inhibition and/or enhances or restores immune surveillance in the mammal to thereby treat or prevent a viral infection in the mammal.

In a second aspect, the invention provides a method of screening, designing, engineering or otherwise producing a CD96-inhibitory agent, said method including the step of determining whether a candidate molecule is capable of at least partly inhibiting or reducing CD96 activity to thereby relieve immune inhibition and/or enhance or restore immune surveillance in a mammal.

In a third aspect, the invention provides a CD96-inhibitory agent screened, designed, engineered or otherwise produced according to the method of the second aspect.

In one embodiment, the CD96-inhibitory agent is an antibody or antibody fragment.

In one particular embodiment, the CD96-inhibitory agent is an anti-cancer agent.

In another particular embodiment, the CD96-inhibitory agent is an anti-viral agent.

In a fourth aspect, the invention provides a CD96-inhibitory agent according to the third aspect for use according to the method of the first aspect.

Suitably, according to the aforementioned aspects the mammal is a human.

Unless the context requires otherwise, the terms "*comprise*", "*comprises*" and "*comprising*", or similar terms are intended to mean a non-exclusive inclusion, such that a recited list of elements or features does not include those stated or listed elements solely, but may include other elements or features that are not listed or stated.

The indefinite articles '*a*' and '*an*' are used here to refer to or encompass singular or plural elements or features and should not be taken as meaning or defining "one" or a "single" element or feature.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: CD96 competes with DNAM-1 for CD155 binding. **a, b** The expression of CD96 was analyzed by flow cytometry on the indicated spleen lymphocyte populations from C57BL/6 WT (light grey) and *CD96*$^{-/-}$ mice (dark grey). The representative FACS Histograms (**a**) and the mean ± SD (**b**) of 3 mice from one representative experiment out of 3 are shown. **c, d** The expression of CD96, DNAM-1 and TIGIT was determined on WT spleen NK cells freshly isolated or activated for 48 hrs with IL-2 (1000 U/ml). **e.** The binding of mouse CD155-Fc coupled with AF-647 to purified NK cells freshly isolated from WT, *CD96*$^{-/-}$, *DNAM-1*$^{-/-}$ or *DNAM-1*$^{-/-}$ *CD96*$^{-/-}$ mice was assessed at the indicated concentrations by flow cytometry. **f.** The binding of CD155-Fc coupled with AF-647 (10 μg/ml) was analyzed on purified WT NK cells in the presence of anti-CD96 and or anti-DNAM-1 mAbs. **g.** The binding of DNAM-1-Fc labeled with AF-647 (0.5-10 μg/ml) at the cell surface of BMDC was analyzed in the presence of 50 μg/ml of control Ig, recombinant CD96 or TIGIT-Fc. **c-g.** The representative FACS Histograms and the mean ± SD of triplicate wells from

one representative experiment out of at least 3 experiments are shown. *** $p<0.001$ Student T test.

Figure 2: CD96 engagement by CD155 regulate NK cell production of IFNγ. CD96 binding to CD155-Fc limits the production of IFN-γ by NK cells induced by exogenous cytokines (**a, b, d**) and NK cell receptors (**c**). **a, b, d.** We analyzed the intracellular production of IFN-γ by freshly purified *CD96*$^{-/-}$, *TIGIT*$^{-/-}$ and *WT* NK cells in the presence or absence of anti-CD96 (50 μg/ml) in response to IL-12 (25-100 pg/ml) and IL-18 (50 ng/ml) using plates coated with or without CD155-Fc (0.5 μg/ well). **c.** We analyzed the intracellular production of IFN-γ by IL-2-activated NK cells from *CD96*$^{-/-}$ and *WT* mice using plates coated with anti-NK1.1 (2.5 μg/ well) and CD155-Fc (0.5 μg/ well). The representative FACS Histograms (**a**) and the mean ± SD of triplicate wells (**b, c, d**) from one representative experiment out of 3 are shown. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, Student T test.

Figure 3: CD96 limits NK cell-dependent tumor immunosurveillance. **a, b.** CD96 and DNAM-1 have an opposite role in the control of B16F10 metastasis. **a.** 2 x 10^5 B16F10 cells were intravenously injected into WT, *CD96*$^{-/-}$, *DNAM-1*$^{-/-}$ and *DNAM-1*$^{-/-}$*CD96*$^{-/-}$ mice and metastatic burden was quantified in the lungs after 14 days. Representative experiment out of 3. **b.** Pictures showing the lung of WT and CD96$^{-/-}$ mice two weeks after the injection of 2 x 10^5 and 5 x 10^5 B16F10 cells. Representative experiment out of two. **c.** CD96 and TIGIT compete with DNAM-1 for the binding of CD155 at the cell surface of B16F10. The binding of DNAM-1-Fc labeled with AF-647 (0.5-20 μg/ml) at the cell surface of B16F10 cells was analyzed in the presence of 50 μg/ml of control Ig, recombinant CD96 or TIGIT-Fc. The FACS histograms and the mean ± SD of triplicate wells from one representative experiment out of 3 are shown. **d.** A 4 hr ^{51}Cr release assay was performed between B16F10 cells and IL-2-activated NK cells from WT, *DNAM-1*$^{-/-}$ and *CD96*$^{-/-}$ mice at the indicated effector target ratios. Solid circles represent WT NK cells, open circles represent CD96$^{-/-}$ NK cells and solid squares represents DNAM-1$^{-/-}$ NK cells. **e-h.** CD96 and DNAM-1 have an opposite role in the immunosurveillance of MCA induced fibrosarcoma mediated by NK cells. **e-h** Groups of 15–30 male, WT, *DNAM-1*$^{-/-}$ and *CD96*$^{-/-}$ and *DNAM-1*$^{-/-}$*CD96*$^{-/-}$ mice were injected with MCA (100 μg/mouse). The survival (**e-g**) and the growth curves of individual mice with sarcoma (**h**) are shown. **f.** WT mice were treated with an anti-CD96, anti-DNAM-1

or anti-CD155 mAbs as defined in the Materials and Methods. g. WT and *CD96*$^{-/-}$ mice were injected with 100 μg MCA and treated with either a control antibody, anti-IFN-γ antibody, or anti-asialoGM1. * $p<0.05$ Mantel-Cox test.

Figure 4: Anti-CD96 mAb has single agent activity and enhances the anti-tumor responses of anti-PD1. C57BL/6 wild type (WT) mice were injected subcutaneously with AT3-OVA^{dim} tumor cells (10^6 cells) and treated on day 16, 20 and 24 with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p) or anti-PD-1 (RMP1-14, 250 μg i.p.). Means ± SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared to cIg alone by Mann-Whitney test).

Figure 5: Anti-CD96 mAb enhances anti-tumor responses generated by Doxorubicin (DOX) chemotherapy. C57BL/6 wild type (WT), DNAM-1$^{-/-}$, and CD96$^{-/-}$ mice were injected subcutaneously with AT3-OVA^{dim} tumor cells (10^6 cells) and treated on day 14 with control PBS or DOX (50 microliters, 2 mM, intratumor). Some groups of WT mice also received on day 12, 14, 18, 21, 24 and 28 intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p) or anti-DNAM-1 (480.1, 250 μg i.p.). Means ± SEM of 5 mice per group (mm^2) are shown.

Figure 6: Enhanced anti-tumor responses of Doxorubicin (DOX) chemotherapy with host CD96 deficiency. C57BL/6 wild type (WT), DNAM-1$^{-/-}$, and CD96$^{-/-}$ mice were injected subcutaneously with AT3-OVA^{dim} tumor cells (10^6 cells) and treated on day 16 with control PBS or DOX (50 microliters, 2 mM, intratumor). Means ± standard errors of 5 mice per group (mm^2) are shown.

Figure 7: Anti-CD96 mAb enhances anti-tumor responses generated by Doxorubicin (DOX) chemotherapy. C57BL/6 wild type (WT) mice were injected subcutaneously with AT3-OVA^{dim} tumor cells (10^6 cells) and treated on day 16 with control PBS or DOX (50 microliters, 2 mM, intratumor). Some groups of WT mice also received on day 16, 20, and 23 intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p). Means ± SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared to cIg alone by Mann-Whitney test).

Figure 8: Early anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 and anti-CTLA-4 mAbs. C57BL/6 wild-type (WT) mice were injected subcutaneously with B16-OVA melanoma cells (10^5 cells) and treated on day 1, 5, and 9 with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-

CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Means ± SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared with anti-CD96 alone, by Mann-Whitney test).

Figure 9: Late anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 mAb. C57BL/6 wild-type (WT) mice were injected subcutaneously with B16-OVA melanoma cells (10^5 cells) and treated on day 16, 20, and 24 with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Means ± SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared with anti-CD96 alone by Mann-Whitney test).

Figure 10: Host CD96 promotes B16F10 lung metastasis. C57BL/6 wild type (WT), DNAM-$1^{-/-}$, $CD96^{-/-}$, and DNAM-$1^{-/-}CD96^{-/-}$ mice were injected intravenously with B16F10 melanoma cells (10^5 cells) and metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 9-17 mice per group are shown (*: $p<0.05$ compared with WT by Mann-Whitney test).

Figure 11: Host CD96 promotes RM-1 lung metastasis. C57BL/6 wild type (WT), DNAM-$1^{-/-}$, $CD96^{-/-}$, and DNAM-$1^{-/-}CD96^{-/-}$ mice were injected intravenous with RM1 prostate carcinoma cells (10^4 cells) and metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 10-15 mice per group are shown (*: $p<0.05$ compared with WT by Mann-Whitney test).

Figure 12: Host CD96 promotes 3LL lung metastasis. C57BL/6 wild type (WT), DNAM-$1^{-/-}$, $CD96^{-/-}$, and DNAM-$1^{-/-}CD96^{-/-}$ mice were injected intravenously with 3LL lung carcinoma cells (10^5 cells) and metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 5 mice per group are shown (*: $p<0.05$ compared with WT by Mann-Whitney test).

Figure 13: Anti-CD96 suppresses B16F10 lung metastasis, alone and in combination with T cell checkpoint blockade. C57BL/6 wild type (WT) mice were injected intravenous with B16F10 melanoma cells (10^5 cells). On day 0 and 3 after tumor inoculation, mice were treated with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-

CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 5 mice per group are shown (*: $p<0.05$ compared with anti-CD96 alone by Mann-Whitney test).

Figure 14: Anti-CD96 suppresses RM-1 lung metastasis, alone and in combination with T cell checkpoint blockade. C57BL/6 wild type (WT) mice were injected intravenous with RM-1 prostate carcinoma cells (10^4 cells). On day 0 and 3 after tumor inoculation, mice were treated with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Means ± SEM of 5 mice per group are shown (*: $p<0.05$ compared with anti-CD96 alone by Mann-Whitney test).

Figure 15: Late anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 mAb. C57BL/6 wild-type (WT) mice were injected subcutaneously with MC38-OVA^{dim} colon adenocarcinoma cells (10^6 cells) and treated on day 14, 18, 22, and 26 with intraperitoneal injections of anti-CD96 mAb (3.3, 250 μg i.p), anti-PD-1 mAb (RMP1-14, 250 μg i.p.), anti-CTLA-4 (UC10-4F10, 250 μg i.p.), anti-CD96/anti-PD-1 mAbs (250 μg i.p each), anti-CD96/anti-CTLA-4 mAbs (250 μg i.p each) or control Ig (cIg) (2A3, 250 μg i.p). Means ± SEM of 5 mice per group (mm^2) are shown (*: $p<0.05$ compared with anti-CD96 alone by Mann-Whitney test).

DETAILED DESCRIPTION

The present invention is at least partly predicated on the unexpected discovery that CD96 is highly expressed by resting NK cells and T cell subsets and competes with DNAM-1 for the binding of CD155 on resting NK cells. Using CD96$^{-/-}$ mice, it is demonstrated that CD96 dampens or suppresses NK cell production of IFN-γ *in vitro* and *in vivo*, through competition with DNAM-1 for CD155 binding and also through a direct inhibition. Furthermore, CD96$^{-/-}$ mice were shown to be more resistant to 3'-methylcholanthrene (MCA)-induced tumor formation as an indicator of carcinogenesis, or B16F10 (melanoma), RM-1 (prostate cancer), 3LL (lung cancer) experimental metastasis. Based on these observations, it

is proposed that CD96 normally acts as a negative regulator of T and NK cell anti-tumor functions, particularly although not exclusively through suppression of IFN-γ production and/or secretion. Accordingly, the invention provides methods of relieving or reducing the negative immunoregulatory function of CD96 to thereby promote or restore immune surveillance, particularly by T cells and NK cells, to thereby treat or prevent cancer, cancer cell metastasis and/or viral infections.

An aspect of the invention therefore provides a method of reducing or relieving immune inhibition in a mammal, said method including the step of at least partly inhibiting or reducing CD96 activity in one or more cells of the mammal to thereby relieve immune inhibition and/or enhance or restore immune surveillance in the mammal.

By "*relieving immune inhibition*" in the context of CD96 is meant at least partly eliminating, removing or overcoming a normal activity or function of CD96 in suppressing or inhibiting one or more immune functions of cells that normally express CD96. Typically, the one or more cells that normally express CD96 are T cells, inclusive of $CD4^+$ and $CD8^+$ T cells, γδT cells, NKT cells, and natural killer (NK) cells. In some embodiments, relieving immune inhibition may include or relate to abrogating peripheral tolerance to foreign pathogens, host cells displaying foreign pathogens (*e.g* displaying foreign pathogen-derived peptides in the context of self-MHC) and/or cancerous cells or tissues of the host.

By "*enhance or restore immune surveillance*" is meant at least partly improving or promoting the ability of one or more elements of the immune system to monitor, detect and/or respond to foreign pathogens, host cells displaying foreign pathogens (*e.g* displaying foreign pathogen-derived peptides in the context of self-MHC) and/or cancerous cells or tissues of the host. Suitably, the elements of the immune system are one or more cells that normally express CD96, such as T cells, inclusive of $CD4^+$ and $CD8^+$ T cells γδT cells, NKT cells and natural killer (NK) cells.

At least partly inhibiting or reducing CD96 activity in one or more cells of the mammal may be performed, facilitated or achieved by administration of a "*CD96-inhibitory agent*" to the mammal. A CD96-inhibitory agent may be any molecule that possesses or displays an ability to at least partly inhibit or reduce a biological activity of CD96. Biological activities of CD96 include one or more of

binding CD155, eliciting intracellular signaling and stimulating or inducing expression and/or secretion of cytokines and/or chemokines. Preferably, the cytokines or chemokines include any pro-inflammatory cytokine or chemokine inclusive of MIP-1α, MIP-1β, RANTES, TNF-α and IFN-γ, although without limitation thereto. Preferably, the cytokine is IFN-γ. Measurement of expression, production and/or secretion of one or more cytokines or chemokines, or nucleic acids encoding same, will be described in more detail hereinafter..

In one embodiment, the CD96-inhibitory agent inhibits, blocks or antagonizes a binding interaction between CD96 and CD155. By way of example only, the CD96-inhibitory agent may bind to an extracellular domain of CD96, or a portion thereof, that is capable of interacting with CD155 (*e.g.* binding CD155 or being bound by CD155) to thereby at least partly inhibit or block CD96 binding to CD155.

In another embodiment, the CD96-inhibitory agent is a molecule that possesses or displays an ability to inhibit or reduce CD96 signaling activity. Inhibition or reduction of CD96 signaling activity may be through inhibiting, blocking or antagonizing a binding interaction with CD155 or may be through blocking CD96-initiated signaling that would normally occur in response to CD155 binding. By way of example, CD96 comprises an immunoreceptor tyrosine-based inhibitory motif (ITIM). ITIMs are structurally defined as 6-amino acid sequences comprising a tyrosine (Y) residue with partly conserved N-terminal (Y-2) and C-terminal (Y+3) residues. A general but non-limiting motif is (S/I/V/LXYXXI/V/L), wherein X is any amino acid. For example, isoform 1 of CD96 comprises the ITIM sequence IKYTCI wherein Y is residue 566.

It has been proposed that when co-aggregated with activating receptors, ITIMs are phosphorylated by Src-family tyrosine kinases, which enables them to recruit Src homology 2 domain-containing phosphatases (PTPases) that antagonize activation signals. Accordingly, in one embodiment the CD96-inhibitory agent possesses or displays an ability to inhibit or reduce CD96 signaling activity mediated by the CD96 ITIM. Preferably, inhibition or reduction of CD96 signaling activity mediated by the CD96 ITIM enables increased or enhanced chemokine and/or cytokine (*e.g* IFN-γ) expression, production and/or secretion by a cell expressing CD96.

The CD96-inhibitory agent may be a protein (inclusive of peptides, antibodies and antibody fragments), a nucleic acid (inclusive of inhibitory RNA molecules such as ribozymes, RNAi, miRNA and siRNA, although without limitation thereto), a lipid, a carbohydrate, a small organic molecule or any combination of these (*e.g* a glycoprotein, a lipoprotein, a peptide-nucleic acid *etc*).

In one particular embodiment, the CD96-inhibitory agent is an antibody or antibody fragment that binds CD96. In one form the antibody binds CD96 and at least partly blocks or inhibits CD96 binding to CD155.

Antibodies may be polyclonal or monoclonal, native or recombinant. Antibody fragments include Fab and Fab'2 fragments, diabodies and single chain antibody fragments (*e.g.* scVs), although without limitation thereto. Antibodies and antibody fragments may be modified so as to be administrable to one species having being produced in, or originating from, another species without eliciting a deleterious immune response to the "foreign" antibody. In the context of humans, this is "humanization" of the antibody produced in, or originating from, another species. Such methods are well known in the art and generally involve recombinant "grafting" of non-human antibody complementarity determining regions (CDRs) onto a human antibody scaffold or backbone.

Suitably, the step of inhibiting or reducing CD96 activity in the mammal does not include killing CD96-expressing cells in the mammal. In this context, "killing" may refer to any antibody-mediated cytotoxic mechanism such as complement-mediated cytolysis and antibody-mediated cell-mediated cytotoxicity (ADCC), the latter typically mediated by natural killer (NK) cells, macrophages, neutrophils and eosinophils. In this regard, it may be advantageous to use antibody fragments lacking an Fc portion or having a mutated Fc portion.

The step of inhibiting or reducing CD96 activity in the mammal may be achieved or facilitated by administering a CD96-inhibitory agent to the mammal.

By "*administering*" is meant the introduction of the CD96-inhibitory agent into the mammal by a particular route. Suitably, a therapeutically effective amount of the CD96-inhibitory agent is administered to the mammal.

The term "*therapeutically effective amount*" describes a quantity of a specified agent sufficient to achieve a desired effect in a mammal being treated with that agent.

Generally, the method of the invention may be useful in reducing or relieving CD96-mediated immune inhibition, suppression or peripheral tolerance. Suitably, the method facilitates treatment or prevention of one or more diseases or conditions that are responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance.

As used herein, "*treating*" or "*treat*" or "*treatment*" refers to a therapeutic intervention that at least party eliminates or ameliorates one or more existing or previously identified symptoms of a disease or condition that is responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance.

As used herein, "*preventing*" or "*prevent*" or "*prevention*" refers to a prophylactic treatment initiated prior to the onset of a symptom of a disease or condition that is responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance, so as to at least partly or temporarily prevent the occurrence of the symptom.

Typically, the disease or condition that is responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance is any disease or condition where enhanced or restored immune surveillance can benefit a subject suffering from the disease or condition. Such diseases and conditions may include those where persistence of the disease or condition can be controlled or suppressed by cell-mediated immunity. Non-limiting examples include cancers and viral infections. Particular viral infections contemplated by the invention include persistent viral infections such as caused by human immunodeficiency virus (HIV), Epstein Barr Virus (EBV), Herpes Simplex Virus (HSV inclusive of HSV1 and HSV2), Human Papillomavirus (HPV), Varicella zoster virus (VSV) and Cytomegalovirus (CMV), although without limitation thereto.

In a preferred embodiment, the method reduces or relieves immune inhibition in a mammal sufficient to treat or prevent cancer or cancer metastasis in the mammal. Suitably, the cancer may be any that is responsive to at least partly blocking CD96-mediated immune inhibition, suppression or peripheral tolerance. Cancers may be in the form of solid tumors, sarcomas, lymphomas, myelomas, carcinomas, melanomas, cytomas and meningiomas, although without limitation thereto. Non-limiting examples of cancers include cancers of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia,

gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, pituitary, parathyroid, prostate, salivary glands, skin, spleen, testis, thyroid, and uterus. Particular non-limiting examples of cancers include colon cancer, lung cancer and prostate cancer. In some embodiments, the cancer is a metastatic cancer, which is capable of migrating to another site, tissue or organ in the body and forming a tumor at that site, tissue or organ. This may occur repeatedly over time. A particularly aggressive metastatic cancer contemplated by the invention is metastatic melanoma.

It will also be appreciated that the method of treatment or prevention of cancer may further include co-administration of one or more other therapeutic agents that facilitate cancer treatment or prevention. By way of example only, these include: chemotherapeutic agents such as paclitaxel, doxorubicin, methotrexate and cisplatin, although without limitation thereto; and/or biotherapeutic agents such as anti-PD-1 antibodies (*e.g.* Nivolumab) and anti-CTLA4 antibodies (*e.g* Ipilimumab), although without limitation thereto. Also contemplated are bi-specific antibodies that bind both CD96 and one or more other molecules including but not limited to PD-1 and CTLA4.

The one or more other agents that facilitate cancer treatment or prevention may be administered in combination with the CD96-inhibitory agent or be administered separately, as is well understood in the art.

In some embodiments, the CD96-inhibitory agent may be formulated alone or together with the one or more other agents is in the form of a pharmaceutical composition.

Suitable dosages of CD96-inhibitory agents, alone or together with other therapeutic agents, for mammalian administration, including human administration, may be readily determined by persons skilled in the art.

Suitably, the pharmaceutical composition comprises an appropriate pharmaceutically-acceptable carrier, diluent or excipient.

Preferably, the pharmaceutically-acceptable carrier, diluent or excipient is suitable for administration to mammals, and more preferably, to humans.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, diluents and excipients well known in the art may be used. These carriers, diluents and excipients may be selected from a group including sugars,

starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates, and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. NJ USA, 1991).

Any safe route of administration may be employed for providing a subject with compositions comprising the CD96-inhibitory agent. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal, and the like may be employed.

A further aspect of the invention provides a method of screening, designing, engineering or otherwise producing a CD96-inhibitory agent, said method including the step of determining whether a candidate molecule is capable of at least partly inhibiting or reducing CD96 activity to thereby relieve immune inhibition and/or enhance or restore immune surveillance in a mammal.

The invention also provides a CD96-inhibitory agent screened, designed, engineered or otherwise produced according to the aforementioned aspect.

The candidate molecule may be a protein (inclusive of peptides, antibodies and antibody fragments), a nucleic acid (inclusive of inhibitory RNA molecules such as ribozymes, RNAi, miRNA and siRNA, although without limitation thereto), a lipid, a carbohydrate, a small organic molecule or any combination of these (*e.g* a glycoprotein, a lipoprotein, a peptide-nucleic acid *etc*).

In some embodiments, the candidate modulator may be rationally designed or engineered *de novo* based on desired or predicted structural characteristics or features that indicate the candidate modulator could block or inhibit one or more biological activities of CD96, such as CD155 binding, intracellular signaling and/or IFN-γ production and/or secretion. In other embodiments, the candidate modulator may be identified by screening a library of molecules without initial selection based on desired or predicted structural characteristics or features that indicate the candidate modulator could block or inhibit one or more biological activities of

CD96. Such libraries may comprise randomly generated or directed libraries of proteins, peptides, nucleic acids, recombinant antibodies or antibody fragments (*e.g.* phage display libraries), carbohydrates and/or lipids, libraries of naturally-occurring molecules and/or combinatorial libraries of synthetic organic molecules.

Non-limiting examples of techniques applicable to the design and/or screening of candidate modulators may employ X-ray crystallography, NMR spectroscopy, computer assisted screening of structural databases, computer-assisted modelling or biochemical or biophysical techniques which detect molecular binding interactions, as are well known in the art.

Biophysical and biochemical techniques which identify molecular interactions include competitive radioligand binding assays, co-immunoprecipitation, fluorescence-based assays including fluorescence resonance energy transfer (FRET) binding assays, electrophysiology, analytical ultracentrifugation, label transfer, chemical cross-linking, mass spectroscopy, microcalorimetry, surface plasmon resonance and optical biosensor-based methods, such as provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) Biochemical techniques such as two-hybrid and phage display screening methods are provided in Chapter 19 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997).

Accordingly, an initial step of the method may include identifying a plurality of candidate molecules that are selected according to broad structural and/or functional attributes, such as an ability to bind CD96.

The method may include a further step that measures or detects a change in one or more biological activities of CD96 in response to the candidate molecule(s). These may include CD155 binding, intracellular signaling, cytokine and/or chemokine production or secretion and/or protection from tumor challenge in an *in vivo* model.

Inhibition of CD155 binding to CD96 by a candidate molecule may be determined by any of several techniques known in the art including competitive radioligand binding assays, surface plasmon resonance (*e.g.* BIACore™ analysis), co-immunoprecipitation and fluorescence-based analysis of the ability of a candidate inhibitor to block CD155 binding to CD96 (such as by flow cytometry where CD155 is labeled with a fluorophore). Non-limiting examples of fluorophores include fluorescein isothiocyanate (FITC), allophycocyanin (APC), fluoroscein derivatives

such as FAM and ROX, Texas Red, tetramethylrhodamine isothiocyanate (TRITL), R-Phycoerythrin (RPE), Alexa and Bodipy fluorophores, although without limitation thereto.

Alternatively, this fluorescence-based analysis could include FRET analysis (*e.g.* one protein coupled to a donor fluorophore, the other coupled to an acceptor fluorophore), although without limitation thereto.

In some embodiments, intracellular signaling may be measured directly at the level of CD96, such as by measuring recruitment of SH2 domain-containing PTPases by CD96 expressed by NK cells, or T cell subsets. A candidate molecule of the invention suitably prevents or reduces recruitment of SH2 domain-containing PTPases by CD96 in the presence of CD155. According to this embodiment, the candidate molecule may at least partly inhibit or prevent binding between CD96 and CD155, thereby at least partly inhibiting or preventing intracellular signaling by CD96, and/or at least partly inhibit or prevent intracellular signaling by CD96 despite CD155 binding.

In other embodiments, an effect of a candidate molecule on CD96 may be determined by measuring expression, production and/or secretion of one or more cytokines or chemokines by cells expressing CD96. Generally, changes in cytokine or chemokine expression production and/or secretion may be measured at the level of gene expression (such as by RT-PCR of cytokine mRNA), measurement of cytokine or chemokine protein located intracellularly (*e.g* by immunocytochemistry using cytokine- or chemokine-specific antibodies) and/or measurement of secreted cytokines or chemokines such as by flow cytometric Cytokine Bead Array (such as commercially available from BD Biosciences), by ELISA using cytokine- or chemokine-specific antibodies and by bioassays that use cytokine- or chemokine-responsive cell lines to measure cytokines and/or chemokines secreted into cell supernatants. The cytokine may be any pro-inflammatory cytokine or chemokine inclusive of MIP-1α, MIP-1β, RANTES, TNF-α and IFN-γ, although without limitation thereto. Preferably, the cytokine is IFN-γ.

Preferably, the CD96-inhibitory effect of a candidate molecule may be determined using an *in vivo* tumor challenge model. For example, a mouse model using CD96-expressing mice may be used to determine the ability of a candidate molecule to inhibit or prevent tumor formation and/or growth in response to an administered carcinogenic agent such as methycholanthrene (MCA). In another

example, a mouse model using CD96-expressing mice may be used to determine the ability of a candidate molecule to inhibit or prevent tumor formation and/or growth in response to administration of tumor cells such as melanomas, colon adenocarcinomas, prostate carcinomas and mammary carcinomas, although without limitation thereto. Other mouse models may utilize mice that are predisposed to spontaneously forming tumors including but not limited to MMTV-polyoma, MT mammary cancer, DMBA/TPA induced skin cancer, p53 loss lymphoma/sarcoma and TRAMP Tg prostate cancer.

It will be understood that the method of this aspect may be performed iteratively, whereby multiple rounds of screening, design, and biological testing are performed. This may include where a candidate molecule is structurally modified before each round, thereby enabling "fine-tuning" of the candidate molecule.

It will also be appreciated that the method may be performed in a "high throughput", "automated" or "semi-automated" manner, particularly during early stages of candidate molecule identification and selection.

In a preferred embodiment, the candidate molecule is an antibody or antibody fragment. As hereinbefore described, antibodies may be polyclonal or monoclonal, native or recombinant. Antibody fragments include Fab and Fab'2 fragments, diabodies and single chain antibody fragments (*e.g.* scVs), although without limitation thereto. Well-known protocols applicable to antibody production, selection, purification and use may be found, for example, in Chapter 2 of Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY (John Wiley & Sons NY, 1991-1994) and Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbor, Cold Spring Harbor Laboratory, 1988, which are both herein incorporated by reference.

Polyclonal antibodies may be prepared for example by injecting CD96 or a fragment (*e.g* a peptide) thereof into a production species, which may include mice or rabbits, to obtain polyclonal antisera. Methods of producing polyclonal antibodies are well known to those skilled in the art. Exemplary protocols that may be used are described for example in Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY, *supra*, and in Harlow & Lane, 1988, *supra*.

Monoclonal antibodies may be produced using the standard method as for example, described in an article by Köhler & Milstein, 1975, Nature **256**, 495, which is herein incorporated by reference, or by more recent modifications thereof as for

example, described in Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY, *supra* by immortalizing spleen or other antibody producing cells derived from a production species which has been inoculated with one or more of the isolated proteins, fragments, variants or derivatives of the invention. Suitably, the antibody or antibody fragment is suitable for administration to a human. In this context, as hereinbefore described the antibody or antibody fragment may be a "humanized" form of an antibody or antibody fragment produced in, or originating from, another species. Such methods are well known in the art and generally involve recombinant "grafting" of non-human antibody complementarity determining regions (CDRs) onto a human antibody scaffold or backbone.

In a preferred embodiment, the antibody or antibody fragment does not kill CD96-expressing cells upon administration to a human. In this context, "killing" may refer to any antibody-mediated cytotoxic mechanism such as complement-mediated cytolysis and antibody-mediated cell-mediated cytotoxicity (ADCC), the latter typically mediated by natural killer (NK) cells, macrophages, neutrophils and eosinophils. In this regard, it may be advantageous to use antibody fragments lacking an Fc portion or having a human Fc portion (*e.g* a humanized antibody).

A CD96-inhibitory agent screened, designed, engineered or otherwise produced according to the aforementioned aspect may be used according to the method of the first aspect (*e.g* as an anti-cancer agent and/or an anti-viral agent), preferably in the form of a pharmaceutical composition as hereinbefore described.

So that the invention may be readily understood and put into practical effect, reference is made to the following non-limiting examples.

EXAMPLES

Example 1

CD96 binding to CD155 and the effects of CD96 inhibition and knockout in mouse tumor models

Materials and Methods

Mice

Wild Type C57BL/6 mice were purchased from the Walter and Eliza Hall Institute for Medical Research or ARC Animal Resource Centre. C57BL/6 $CD96^{-/-}$ mice were generated by Dr. Marco Colonna and Dr. Susan Gilfillan at the

Washington University School of Medicine (St Louis, MO, USA) as follows. A targeting construct designed to replace exons 1 and 2 of CD96, including the start site, with a MC1-neor gene flanked by loxP sites was electroporated into E14.1 (129P2/OlaHsd) embryonic stem cells (Fig. S1). Chimeras transmitting the targeted allele were obtained from two clones following injection into C57BL/6 blastocysts. Mice carrying the targeted allele were bred to C57BL/6 mice expressing a *Cre* transgene under the CMV promoter to delete the MC1-neor gene (Schwenk et al., 1995). The CD96 deletion was backcrossed onto a C57BL/6 background, facilitated by a genome-wide screening of polymorphic microsatellite markers at 10-centiMorgan intervals at each generation. $CD96^{+/-}$ >99% C57BL/6 mice were intercrossed to generate the $CD96^{-/-}$ mice. $DNAM\text{-}1^{-/-}$ mice have already been described. $DNAM\text{-}1^{-/-}$ $CD96^{-/-}$ were generated by intercrossing $CD96^{-/-}$ with $DNAM\text{-}1^{-/-}$ mice. Mice were bred and used between the ages of 6-14 weeks. All experiments were approved by animal ethics committee.

Cell Culture

B16F10, RM-1, 3LL, AT3, MC38 and YAC-1 cell lines were grown in complete RPMI Medium (Gibco, Invitrogen,) i.e supplemented with 10% FCS (Thermo Scientific), L-Glutamine (Gibco), Non-Essential Amino Acids, Sodium Pyruvate, HEPES (Gibco) and Penicillin/Streptomycin (Gibco), at 37 ^{0}C in 5% CO_2. For cytotoxicity assays and IL-12/IL-18 titration experiments, primary NK cells were harvested from the spleen, sorted using a mouse NK cell isolation kit (Miltenyi Biotec) and AutoMACS (Miltenyi Biotec), and subsequently cultured for 5 days in RPMI Medium supplemented with 10% FCS, L-Glutamine, Penicillin/Streptomycin, Non-Essential Amino Acids (Gibco), Sodium Pyruvate (Gibco), HEPES (Gibco), β-2-mercaptoethanol (Calbiochem), and 1000 IU/ml recombinant human IL-2 (Chiron Corporation). All cells were incubated at 37°C in 5% CO_2.

In vivo LPS challenges

LPS (from *E. Coli* 0127:B8, Sigma) suspended in PBS was injected intraperitoneally into mice at the described doses. For survival curves, mice were checked hourly for symptoms of sepsis. Serum from these mice was taken at various time points by retro-orbital or cardiac bleeding for cytokine analysis. Spleens were

also taken from mice at various time points to analyse receptor and ligand expression, and intracellular IFN-γ expression from NK cells.

In vivo tumor challenges

Mouse B16F10 or B16-OVA melanomas, RM-1 prostate carcinoma, 3LL lung carcinoma, MC38-OVA^{dim} colon adenocarcinoma or AT3-OVA^{dim} mammary carcinoma, were injected into WT, DNAM-1$^{-/-}$, CD96$^{-/-}$, or DNAM-1$^{-/-}$CD96$^{-/-}$ mice subcutaneously or intravenously at the indicated doses and monitored for solid tumor growth or metastasis, respectively. Treatments were administered as indicated in the Figure legends. To monitor solid tumor growth, the area of the ensuing tumor was calculated by taking the length and width of palpable tumors by calipers and plotted against time. To monitor metastasis formation, 14 days after cells were injected, lungs were harvested, placed in Bouin's fixative, and metastases were counted using a dissection microscope.

MCA-induced fibrosarcoma

WT, DNAM-1$^{-/-}$, CD96$^{-/-}$ and DNAM-1$^{-/-}$CD96$^{-/-}$ mice were injected subcutaneously on the right flank with various doses of MCA (5-400 μg, e.g. 100 μg MCA) and were monitored over time for fibrosarcoma formation. In addition, some mice were treated with control antibody, depleted of NK cells by treatment with anti-asialoGM1 (Wako Chemicals; 100 μg injected i.p. at day -1, 0 and then weekly until week 8), neutralized for IFN-γ (H22, 250 μg injected i.p. at day -1, 0 and then weekly until week 8), for CD155, for DNAM-1 or CD96.

Dendritic cells (BMDC): NK cell coculture assays

BMDC were generated as described previously. Briefly, we harvested bone marrow cells from the femur and tibia of mice and cultured them in DMEM supplemented with 10% FCS, L-Glutamine, Penicillin/Streptomycin, Non-Essential Amino Acids, Sodium Pyruvate, β-2-mercaptoethanol and 250 ng/ml GM-CSF (eBioscience) for 6 days. WT or CD96$^{-/-}$ NK cells were harvested from the spleens and FACS sorted to purity by staining with NK1.1 (PK136) and TCRβ (H57-597) and CD3 (17A2) antibodies. NK cells were harvested on the day of the assay. For assay set up, 5 x 10^4 BMDM were plated in 96 well U bottom plates. NK cells were

then added to the BMDM at varying titrations (2:1, 1:1, 0.5:1, and 0.25:1). BMDM only and NK only were always included in the assay as controls. Once all cells were plated, each well was filled with the appropriate amount of media to yield equivalent volumes between wells. 100 ng/ml of LPS was then added to the wells for 2 h, followed by 5 mM purified ATP (Sigma) for 30 mins. This was performed at 37°C in 5% CO_2. LPS only and ATP only controls were also included in the assay as controls. After 30 mins with ATP, supernatants were harvested and stored at -20°C until analysed.

^{51}Cr Cytotoxicity Assays

Standard ^{51}Cr cytotoxicity assays were used to analyse the ability of WT and $CD96^{-/-}$ NK cells to kill targets. Briefly 20,000 targets labeled with 100 μCi of ^{51}Cr were added to V bottom plates and NK cells were then added to the targets at defined effector to target ratios. After 4 h at 37°C in 5% CO_2, supernatants were harvested, and the level of ^{51}Cr was quantified by a gamma counter (Wallac Wizard). Percentage specific killing was determined using the formula (Sample Cr release-Spontaneous Cr release)/(Total Cr release-Spontaneous Cr release) x 100.

Cytokine Detection

All cytokine detection in serum or supernatants except IL-18 was achieved by utilising Cytometric Bead Array (CBA) technology (BD Biosciences). Acquisition was completed using a Canto II or LSRII Flow Cytometric Analyser (BD Biosciences). Analysis was performed using the FCAP array software. IL-18 was detected by an ELISA according to manufacturer's instructions (MRL). For intracellular cytokine detection, isolated lymphocytes were obtained from the liver, stained for surface markers, fixed and permeabilised (BD Biosciences), and stained with an anti-IFN-γ antibody (XMG1.2).

Flow Cytometry Analysis and Sorting

Analysis of Immune Cell Homeostasis and CD96/CD155 expression: Various organs (lymph node, lung, spleen, bone marrow, and liver) were processed into single lymphocyte suspensions that included red blood cell lysis. Between 1 x 10^6 and 5 x 10^6 cells were initially subject to incubation with 2.4G2 to block non-specific

Fc antibody binding before specific antibodies were utilised. To analyse NK cell homeostasis and IFN-γ production, the following antibodies were used: anti mouse- NK1.1, -TCRβ, -CD27 (LG.7F9), -CD11b (M1/70), and -IFN-γ. For T cells: anti mouse- TCRβ, -CD8 (53-6.7), and -CD4 (RM4-5). For B cells: anti mouse -B220 (RA3-6B2), -CD19 (1D3). For NKT cells: mouse CD1d tetramer loaded with α-galactosylceramide (kindly provided by Professor Dale Godfrey, University of Melbourne), anti mouse- TCRβ or –CD3, -CD4, and –NK1.1. For macrophages: anti mouse- F4/80 (BM8) and –CD11b. For neutrophils: anti mouse- Ly6G (1A8) and –CD11b. For conventional DC: anti mouse- MHC II (M5/114.15.2) and –CD11c (N418). For γδ T cells: anti mouse -γδ TCR (GL3) and -CD3. To analyse CD96 and CD155 expression, the specific cell type of interest was gated upon using the above antibody cocktails along with anti mouse- CD96 (3.3.3) or anti mouse- CD155 (4.24.3). Acquisition was performed using an LSR II, or Canto II flow cytometric analyser (BD Biosciences). Analysis was achieved using Flowjo (Treestar).

Cell Sorting

Naïve NK cells and macrophages from the spleen were prepared and stained for as described above. These cells were then sorted to purity using an Aria II FACS sorter (BD Biosciences).

Statistical Analysis

Statistical analysis was achieved using Graphpad Prism Software. Data was considered to be statistically significant where the p value was equal to or less than 0.05. Statistical tests used were the unpaired Student's t test, Mann Whitney t test, and the Mantel-Cox test for survival. The appropriate test used is defined in the Figure legends.

Results

CD96 competes with DNAM-1 for CD155 binding (Figure 1) and CD96 engagement by CD155 down-regulates NK cell production of IFNγ (Figure 2). CD96 limits NK cell-dependent tumor immunosurveillance in MCA-treated mice and promotes experimental B16F10 lung metastasis (Figure 3).

The data in Figure 4 show that anti-CD96 mAb has single agent activity (i.e without anti-PD1 treatment) while also enhancing the anti-tumor responses of anti-PD1. Anti-CD96 mAb treatment also enhances anti-tumor responses generated by Doxorubicin (DOX) chemotherapy (Figures 5 & 7) which is consistent with Figure 6 where enhanced anti-tumor responses to Doxorubicin (DOX) chemotherapy were observed in host with CD96 deficiency. Referring to Figures 8 & 9, given early or late, anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 and anti-CTLA-4 mAbs and shows a particularly strong synergy with anti-PD-1.

The effect of CD96 in promotion of tumour metastasis was also investigated. In Figure 10, regulation of B16F10 lung metastasis was investigated in C57BL/6 wild type (WT), DNAM-$1^{-/-}$, $CD96^{-/-}$, and DNAM-$1^{-/-}CD96^{-/-}$ mice In Figure 11, host CD96 promoted RM-1 lung metastasis and in Figure 12, host CD96 promoted 3LL lung metastasis Figure 13 shows that anti-CD96 mAb suppresses B16F10 lung metastasis, alone and in combination with a T cell checkpoint blockade. In Figure 14, anti-CD96 mAb suppresses RM-1 lung metastasis, alone and in combination with T cell checkpoint blockade. In Figure 15, anti-CD96 mAb enhances anti-tumor responses generated by anti-PD-1 and anti-CTLA-4 mAbs against MC38 colon tumors and shows a particularly strong synergy with anti-PD-1.

Example 2

Screening assays for identifying anti-CD96 antibodies

Introduction

The following assays may be used to identify antibodies useful in the invention. The first assay would be used to identify human antibodies capable of blocking or inhibiting binding between human CD96 and human CD155. The second assay may be used to test whether or not the identified antibodies cause antibody-dependent cell-mediated cytotoxicity (ADCC). The third assay can then be applied to lead candidates and involves determining whether or not a human CD96 antibody can modulate human lymphocyte effector function.

Materials and Methods

Assay 1: CD96 binding to CD155

The ability of candidate anti-CD96 antibodies to prevent the binding of CD155 to the cell surface of CD96 expressing cells (such as NK cells) will be tested

as follows. Recombinant Human CD155 fused to the C terminal Fc region of human IgG1 (such as CD155-Fc available from Sino Biological) will be labeled with a fluorophore such as Alexa Fluor 647 (AF647) using Zenon Human IgG Labeling kit (Molecular Probe) accordingly to the manufacturer's instructions. NK cells or other CD96-expressing cells freshly isolated from the peripheral blood of healthy donors will be incubated with AF647 labeled CD155-Fc in the presence of anti-CD96 or control Ig at different concentrations (The cells will be harvested and the cell surface binding of AF647-CD155-Fc will be tested by flow cytometry). Antibodies that prevent binding of CD155 cells to CD96-expressing cells will be identified by their ability to block binding of CD155-Fc to CD96-expressing cells.

Assay 2: ADCC assay

The survival of immune cells (such as NK cells and/or T cells) in the presence of anti-CD96 antibodies will be analyzed as follows. The peripheral blood immune cells from healthy donors will be isolated by Ficoll gradient separation. Immune cells will be plated in 96 well plates in the presence of human IL-2 at an appropriate dosage and increasing concentrations of anti-CD96 mAbs. The survival as well as the percentages of CD96 expressing cells (such as NK cells and/or T cells) will be analyzed over time by flow cytometry. A non-limiting example of a suitable commercially available kit for this assay is the Annexin V Apoptosis Detection Kit.

Assay 3: Assay for modulation of human leukocyte effector function by human CD96 antibodies

Fresh blood samples will be collected from healthy donors. Peripheral blood mononuclear cells (PBMC) will be prepared on a Ficoll–Paque density gradient by centrifugation. Highly pure CD3-CD56+ NK cells will be obtained from PBMC by magnetically activated cell sorting. To analyze the ability of CD96 to impact human NK cell production of IFN-γ, 96 well U bottom plates will be coated overnight at 4°C with recombinant Human CD155-Fc chimera (Sino Biological Inc.; 0.5 μg/ well) or with non-relevant human IgG1 antibodies. Freshly purified human NK cells will then be plated in complete RMPI media supplemented with Human IL-12 and IL-18 for 24 h and the intracellular content and the level of IFN-γ in the supernatant will be analysed in the different cultures. Alternatively, human NK cells will be stimulated

for 24 h in wells coated with anti-NKG2D, anti-NKp46, anti-NKp30 or anti-CD16 antibodies to analyze the ability of CD96 signalling to interact with other NK cells receptors. The anti-human CD96 antibodies to be tested or control antibodies will be added to the cultures prior to the cytokines or antibodies above to confirm the ability of these test anti-human CD96 antibodies to enhance the IFNγ production of the human NK cells. Statistical increases in IFNγ production above the control would be considered significant.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

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20. *Fuchs, A., Cella, M., Kondo, T. & Colonna, M. Paradoxic inhibition of human natural interferon-producing cells by the activating receptor NKp44. Blood* ***106****, 2076-2082 (2005).*

21. *Seth, S., et al. The murine pan T cell marker CD96 is an adhesion receptor for CD155 and nectin-1. Biochemical and biophysical research communications* ***364****, 959-965 (2007).*

22. *Fuchs, A., Cella, M., Giurisato, E., Shaw, A.S. & Colonna, M. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). J Immunol* ***172****, 3994-3998 (2004).*

CLAIMS

1. A method of reducing or relieving immune inhibition in a mammal, said method including the step of at least partly inhibiting or reducing CD96 activity in one or more cells of the mammal to thereby relieve immune inhibition and/or enhance or restore immune surveillance in the mammal.

2. The method of Claim 1, wherein the step of at least partly inhibiting or reducing CD96 activity in the mammal does not include, or depend upon, killing of CD96-expressing cells in the mammal.

3. The method of Claim 1 or Claim 2, wherein the step of at least partly inhibiting or reducing CD96 activity in the mammal includes administering a CD96-inhibitory agent to the mammal.

4. The method of Claim 3, wherein the CD96-inhibitory agent at least partly blocks or inhibits CD96 binding to CD155 and/or intracellular signaling by CD96.

5. The method of Claim 4, wherein the CD96-inhibitory agent is an anti-CD96 antibody or antibody fragment.

6. The method of any preceding claim which includes administering one or more other therapeutic agents.

7. The method of Claim 6, wherein the one or more other therapeutic agents include a chemotherapeutic agent and one or more antibodies or antibody fragments that bind PD1 and/or CTLA4.

8. The method of any preceding claim, which increases or enhances cytokine and/or chemokine expression and/or secretion by one or more cells in the mammal.

9. The method of Claim 8, wherein the cytokine and/or chemokines include MIP-1α, MIP-1β, RANTES, TNF-α and IFN-γ,

10. The method of Claim 9, wherein the cytokine is interferon γ (IFN-γ).

11. The method of Claim 8, Claim 9 or Claim 10, wherein the one or more cells are T cells, inclusive of $CD4^+$ and $CD8^+$ T cells, γδ T cells and NK T cells and natural killer (NK) cells.

12. The method of any preceding claim, which treats or prevents cancer or cancer metastasis in the mammal.

13. The method of any preceding claim, which treats or prevents a viral infection in the mammal.

14. The method of any preceding claim, wherein the mammal is a human.

15. A method of screening, designing, engineering or otherwise producing a CD96-inhibitory agent, said method including the step of determining whether a candidate molecule is capable of at least partly inhibiting or reducing CD96 activity to thereby relieve immune inhibition and/or enhance or restore immune surveillance in a mammal.

16. The method of Claim 15, wherein the CD96-inhibitory agent is an antibody or antibody fragment.

17. The method of Claim 15 or Claim 16, wherein the CD96-inhibitory agent is an anti-cancer agent.

18. The method of Claim 15, Claim 16 or Claim 17, wherein the CD96-inhibitory agent is an anti-viral agent.

19. The method of any one of Claims 15-18, wherein the mammal is a human.

20. A CD96-inhibitory agent screened, designed, engineered or otherwise produced according to the method of any one of Claims 15-19.

21. The CD96-inhibitory agent of Claim 20 for use according to the method of any one of Claims 1-14.

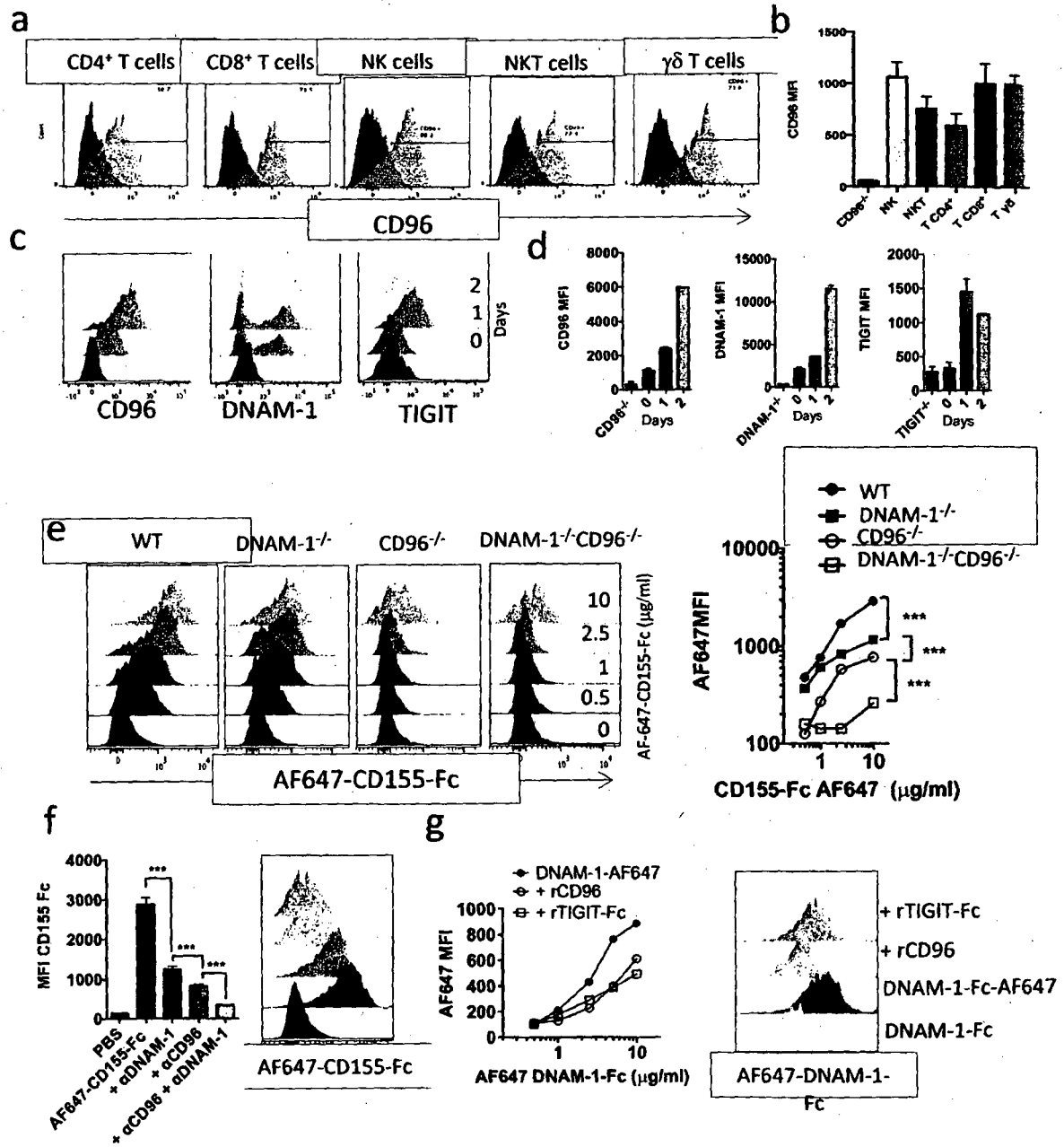

a
CD4+ T cells
CD8+ T cells
NK cells
NKT cells
γδ T cells
CD96
b
CD96 MFI
c
CD96
DNAM-1
TIGIT
Days
d
CD96 MFI
DNAM-1 MFI
TIGIT MFI
Days
e
WT
DNAM-1-/-
CD96-/-
DNAM-1-/-CD96-/-
AF647-CD155-Fc
AF-647-CD155-Fc (μg/ml)
AF647MFI
CD155-Fc AF647 (μg/ml)
f
MFI CD155 Fc
PBS
AF647-CD155-Fc
+ αDNAM-1
+ αCD96
+ αCD96 + αDNAM-1
AF647-CD155-Fc
g
DNAM-1-AF647
+ rCD96
+ rTIGIT-Fc
AF647 MFI
AF647 DNAM-1-Fc (μg/ml)
+ rTIGIT-Fc
+ rCD96
DNAM-1-Fc-AF647
DNAM-1-Fc
AF647-DNAM-1-Fc

Fig. 1

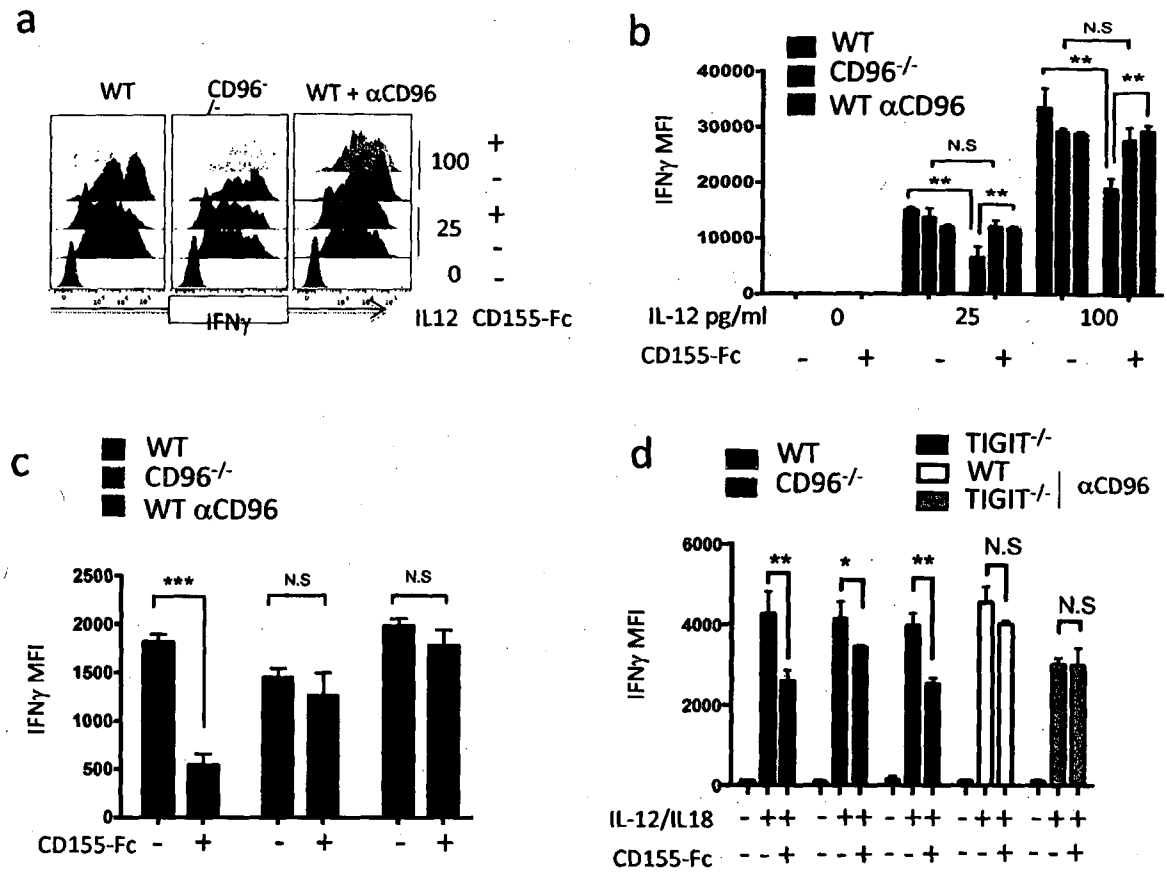

a
WT
CD96-/-
WT + αCD96
100
25
0
+
-
IFNγ
IL12 CD155-Fc
b
WT
CD96-/-
WT αCD96
IFNγ MFI
40000
30000
20000
10000
0
N.S
**
IL-12 pg/ml 0 25 100
CD155-Fc - + - + - +
c
WT
CD96-/-
WT αCD96
IFNγ MFI
2500
2000
1500
1000
500
0
***
N.S
CD155-Fc - + - + - +
d
WT
CD96-/-
TIGIT-/-
WT
TIGIT-/-
αCD96
IFNγ MFI
6000
4000
2000
0
**
*
IL-12/IL18 - ++ - ++ - ++ - ++ - ++
CD155-Fc - - + - - + - - + - - + - - +

Fig. 2

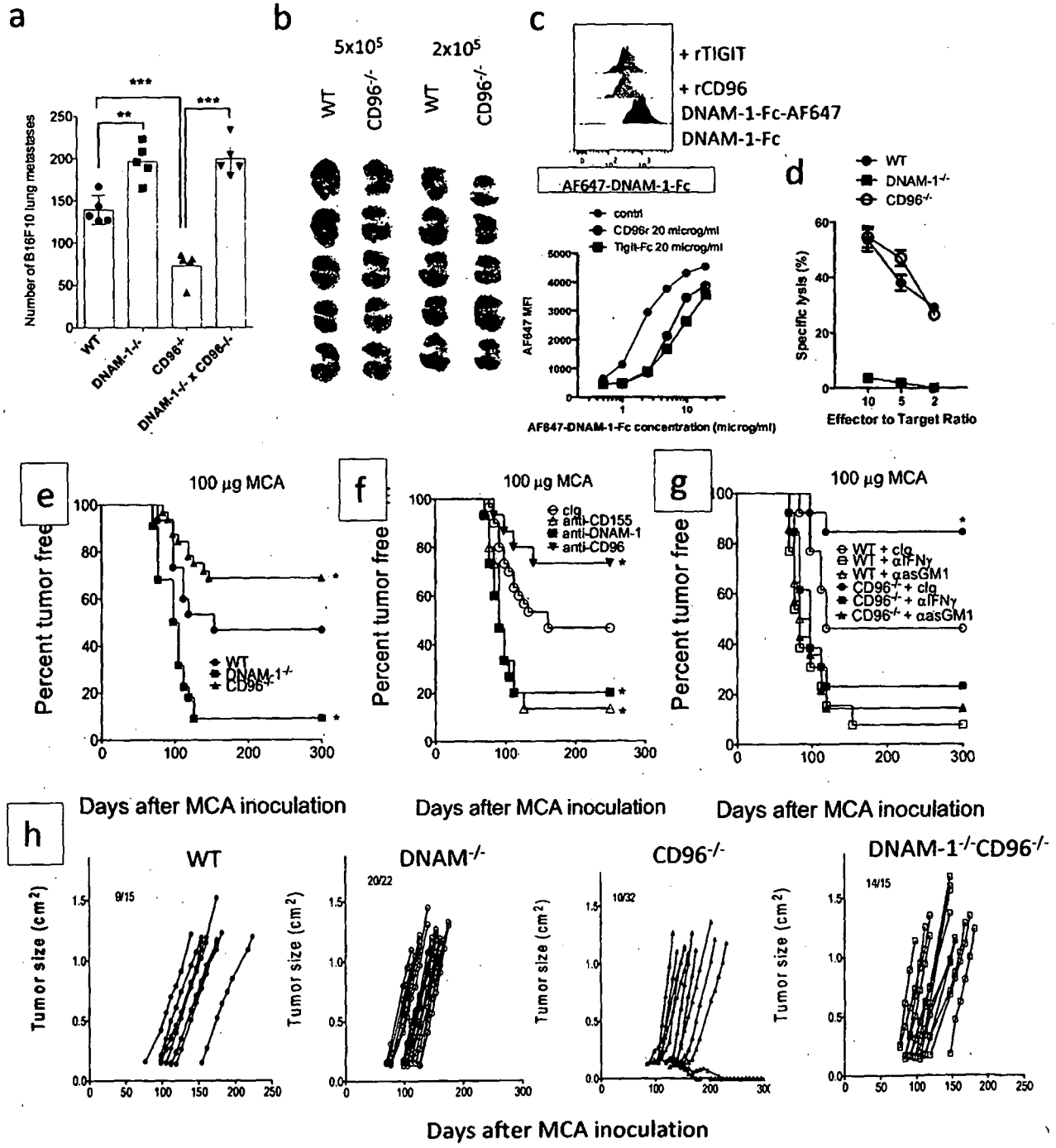

a
Number of B16F10 lung metastases
WT
DNAM-1-/-
CD96-/-
DNAM-1-/- x CD96-/-
b
5x10^5
2x10^5
WT
CD96-/-
c
+ rTIGIT
+ rCD96
DNAM-1-Fc-AF647
DNAM-1-Fc
AF647-DNAM-1-Fc
contrl
CD96r 20 microg/ml
Tigit-Fc 20 microg/ml
AF647 MFI
AF647-DNAM-1-Fc concentration (microg/ml)
d
WT
DNAM-1-/-
CD96-/-
Specific lysis (%)
Effector to Target Ratio
e
100 µg MCA
Percent tumor free
WT
DNAM-1-/-
CD96-/-
Days after MCA inoculation
f
100 µg MCA
Percent tumor free
clg
anti-CD155
anti-DNAM-1
anti-CD96
Days after MCA inoculation
g
100 µg MCA
Percent tumor free
WT + clg
WT + αIFNγ
WT + αasGM1
CD96-/- + clg
CD96-/- + αIFNγ
CD96-/- + αasGM1
Days after MCA inoculation
h
WT
9/15
DNAM-/-
20/22
CD96-/-
10/32
DNAM-1-/-CD96-/-
14/15
Tumor size (cm2)
Days after MCA inoculation

Fig. 3

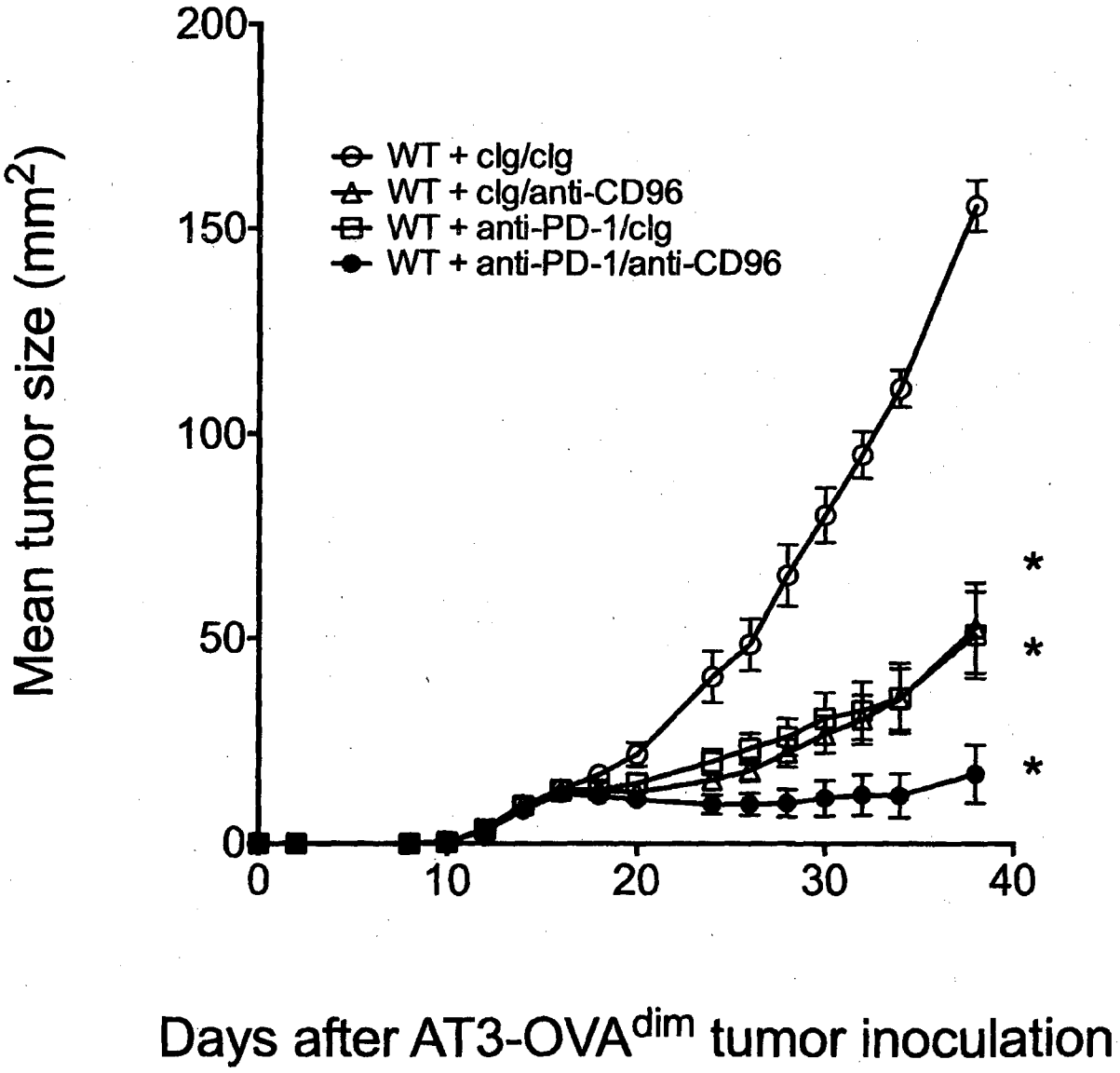

200
150
100
50
0
0
10
20
30
40
Mean tumor size (mm2)
WT + clg/clg
WT + clg/anti-CD96
WT + anti-PD-1/clg
WT + anti-PD-1/anti-CD96
*
*
*
Days after AT3-OVAdim tumor inoculation

Fig. 4

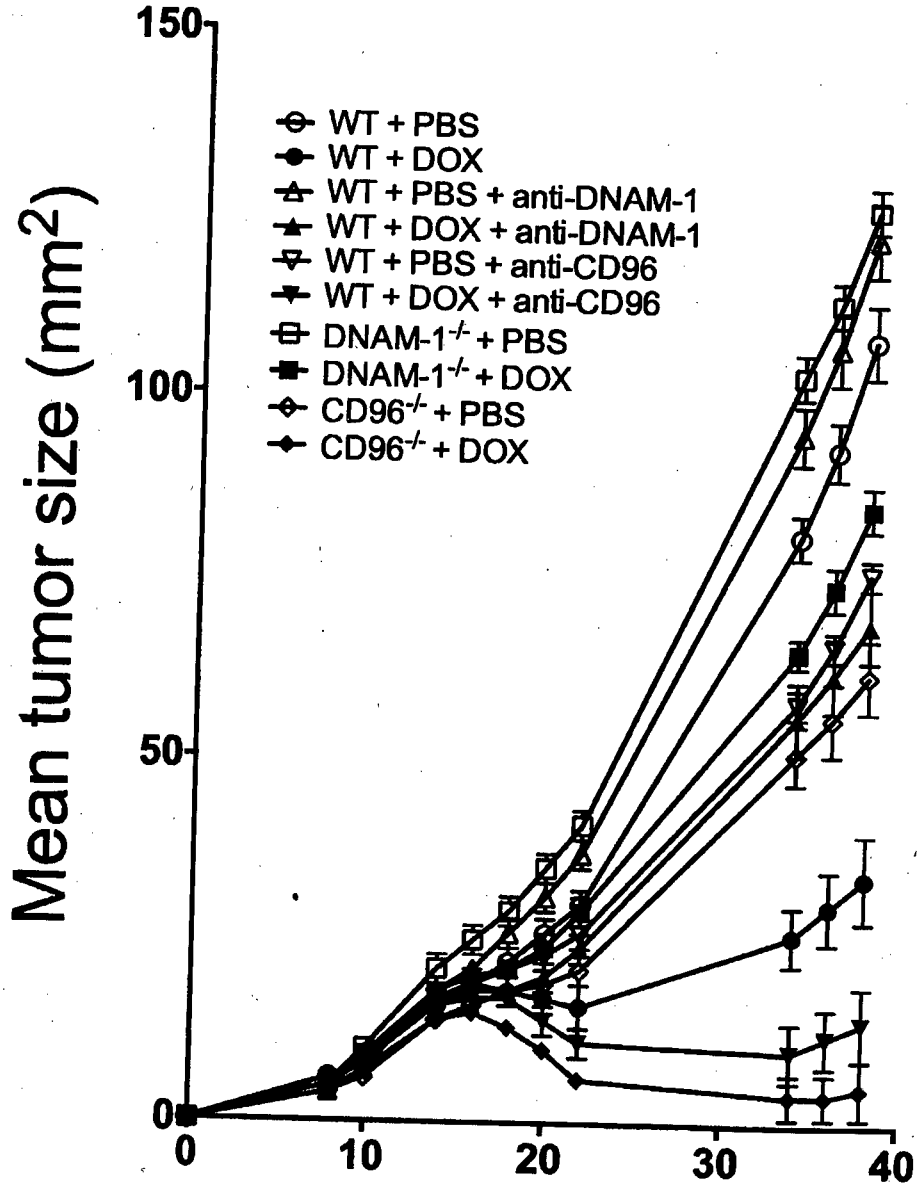

150
100
50
0
0
10
20
30
40
Mean tumor size (mm2)
WT + PBS
WT + DOX
WT + PBS + anti-DNAM-1
WT + DOX + anti-DNAM-1
WT + PBS + anti-CD96
WT + DOX + anti-CD96
DNAM-1-/- + PBS
DNAM-1-/- + DOX
CD96-/- + PBS
CD96-/- + DOX
Days after AT3-OVAdim tumor inoculation

Fig. 5

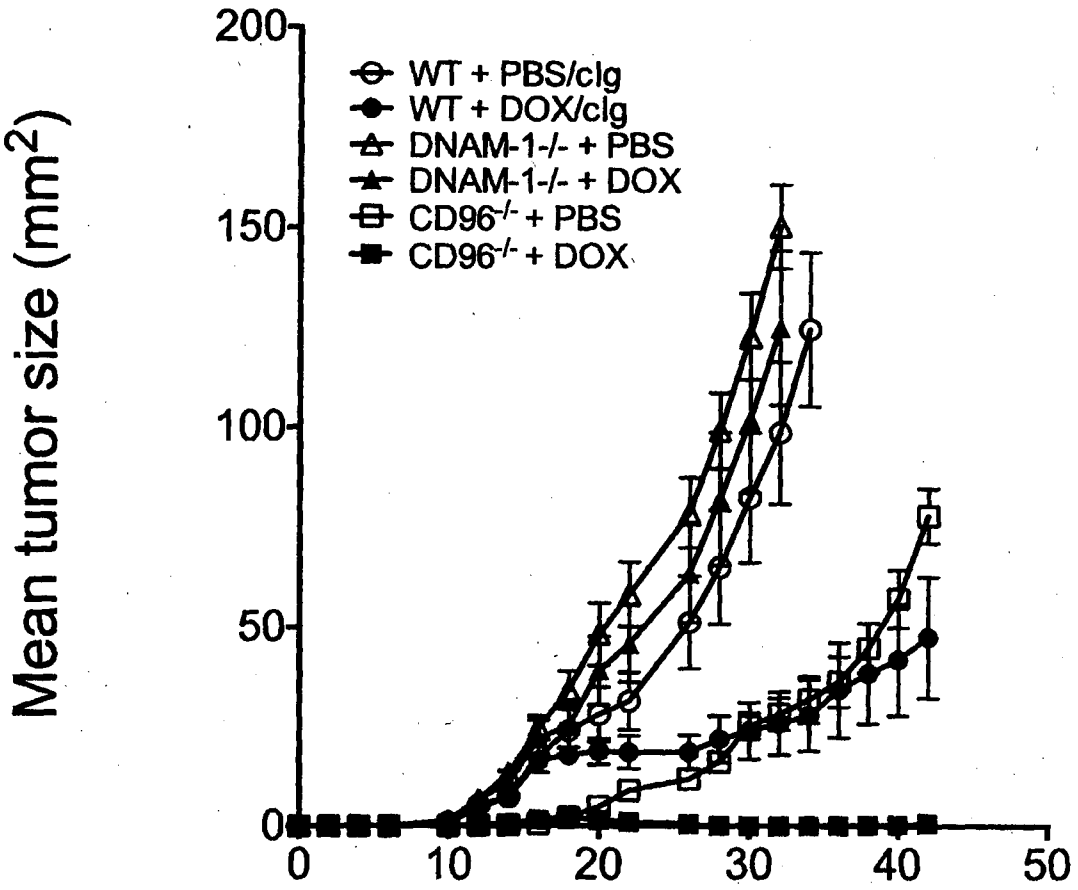

200
150
100
50
0
0
10
20
30
40
50
WT + PBS/clg
WT + DOX/clg
DNAM-1-/- + PBS
DNAM-1-/- + DOX
CD96-/- + PBS
CD96-/- + DOX
Mean tumor size (mm2)
Days after AT3-OVAdim tumor inoculation

Fig. 6

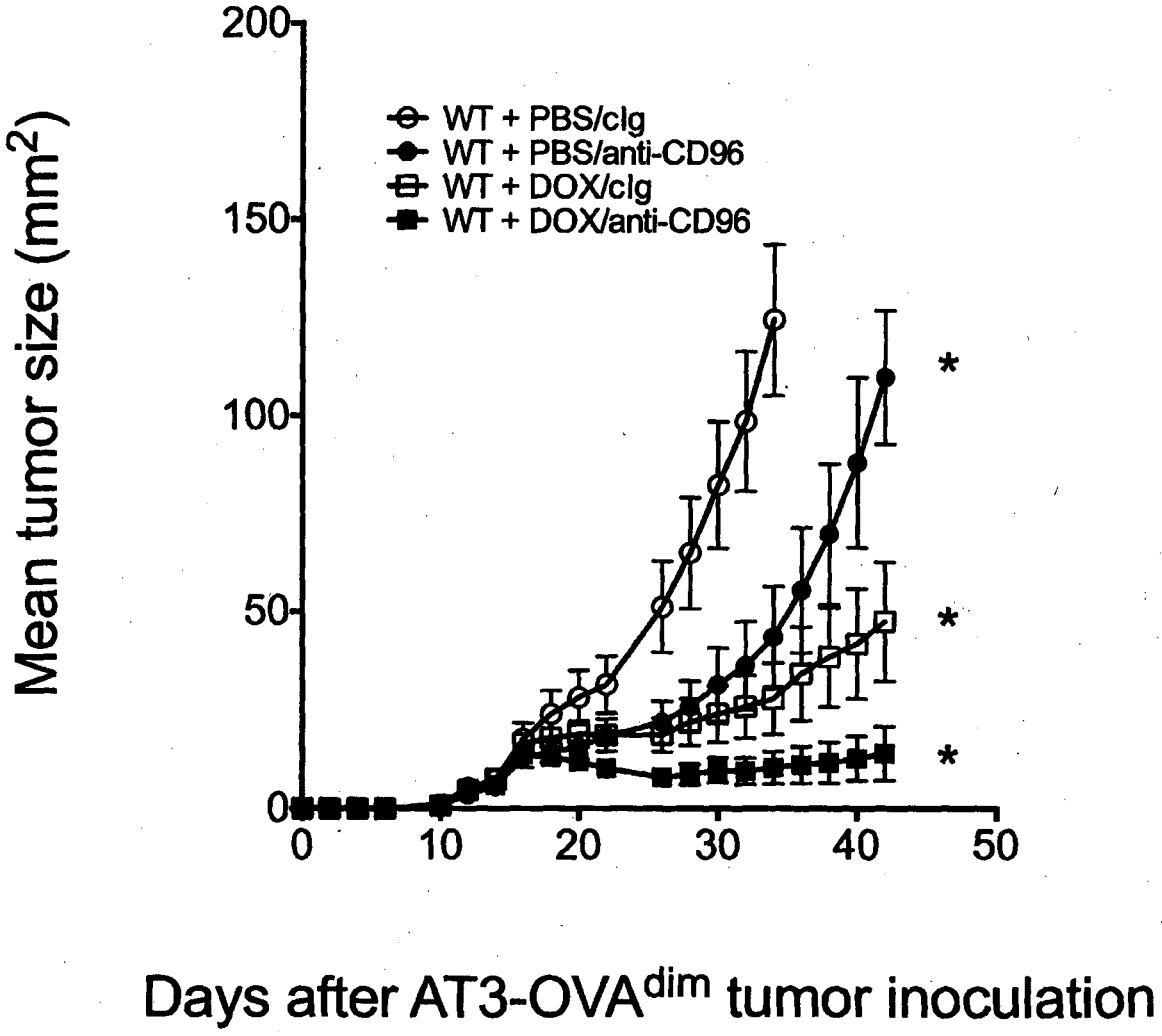

200
150
100
50
0
Mean tumor size (mm2)
WT + PBS/clg
WT + PBS/anti-CD96
WT + DOX/clg
WT + DOX/anti-CD96
*
*
*
0
10
20
30
40
50
Days after AT3-OVAdim tumor inoculation

Fig. 7

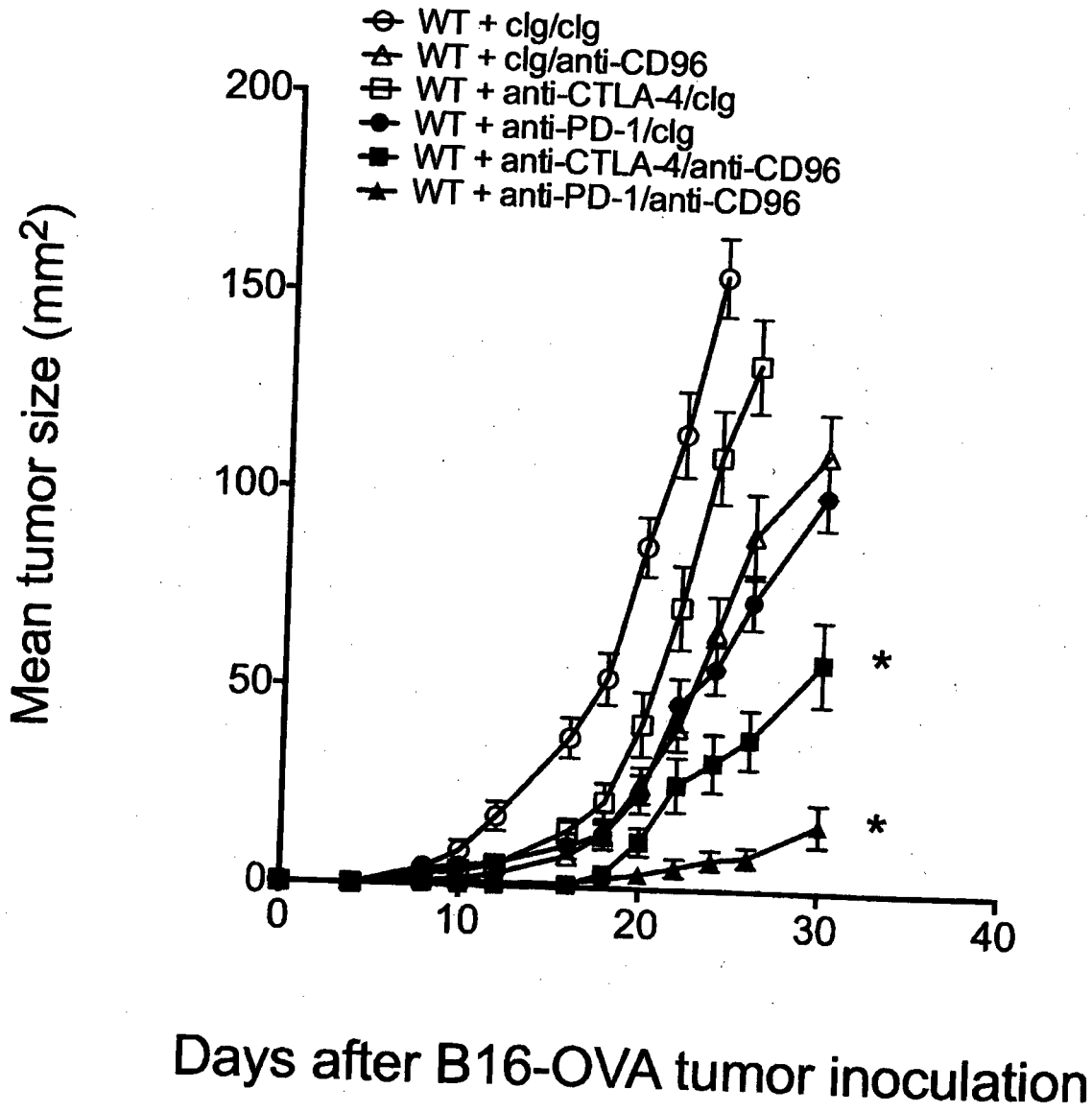

WT + clg/clg
WT + clg/anti-CD96
WT + anti-CTLA-4/clg
WT + anti-PD-1/clg
WT + anti-CTLA-4/anti-CD96
WT + anti-PD-1/anti-CD96
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
*
*
Days after B16-OVA tumor inoculation

Fig. 8

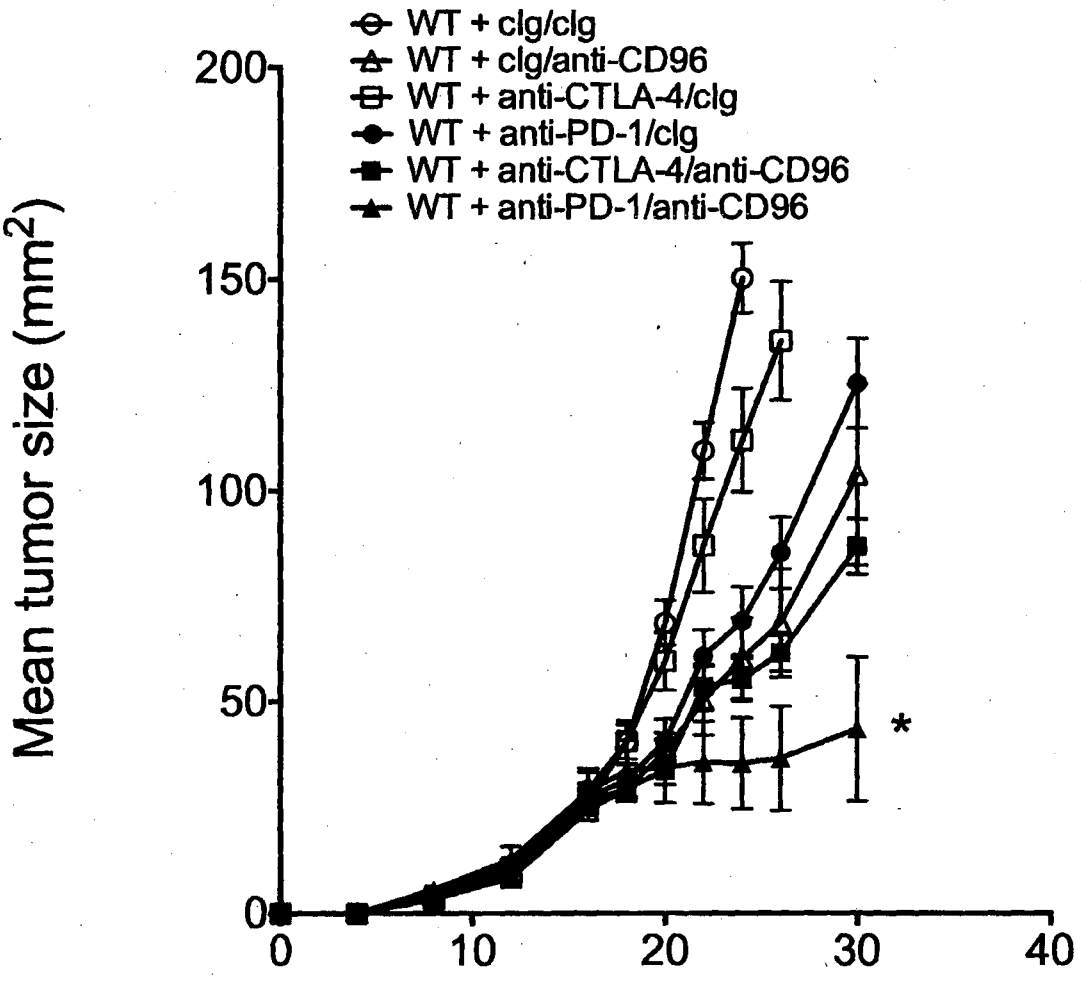

WT + clg/clg
WT + clg/anti-CD96
WT + anti-CTLA-4/clg
WT + anti-PD-1/clg
WT + anti-CTLA-4/anti-CD96
WT + anti-PD-1/anti-CD96
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
*
Days after B16-OVA tumor inoculation

Fig. 9

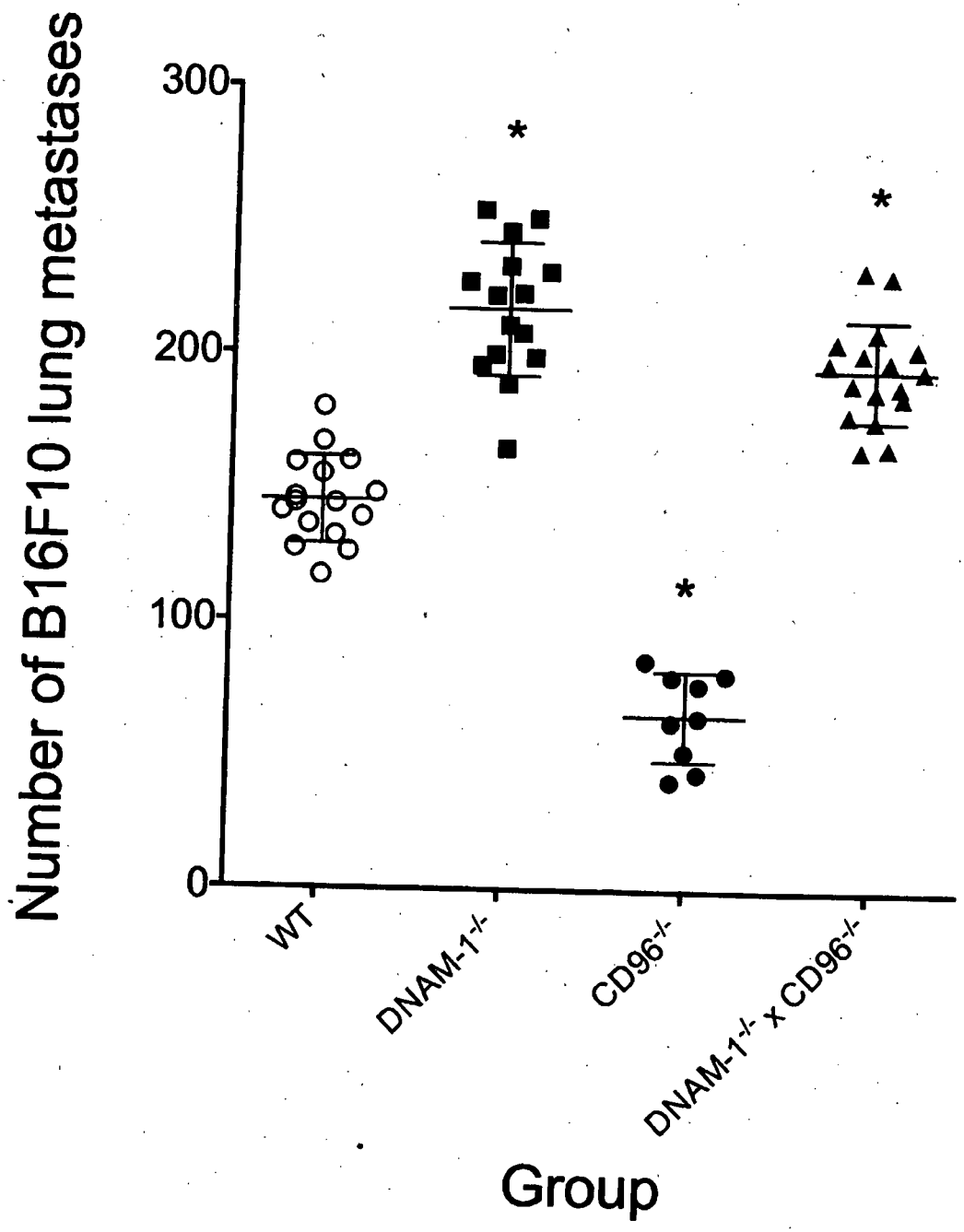

Number of B16F10 lung metastases
300
200
100
0
WT
DNAM-1-/-
CD96-/-
DNAM-1-/- x CD96-/-
Group

Fig. 10

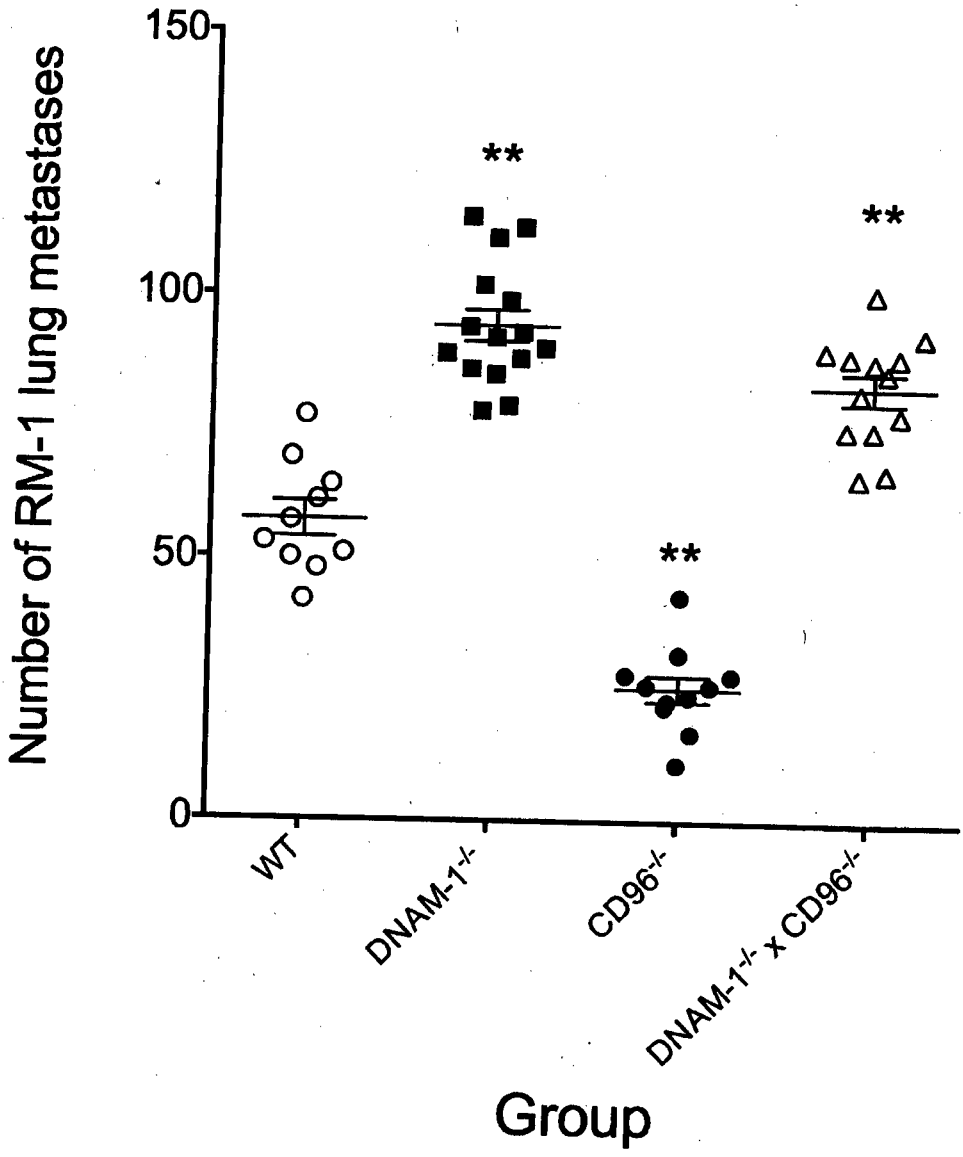

Number of RM-1 lung metastases
150
100
50
0
**
**
**
WT
DNAM-1-/-
CD96-/-
DNAM-1-/- x CD96-/-
Group

Fig. 11

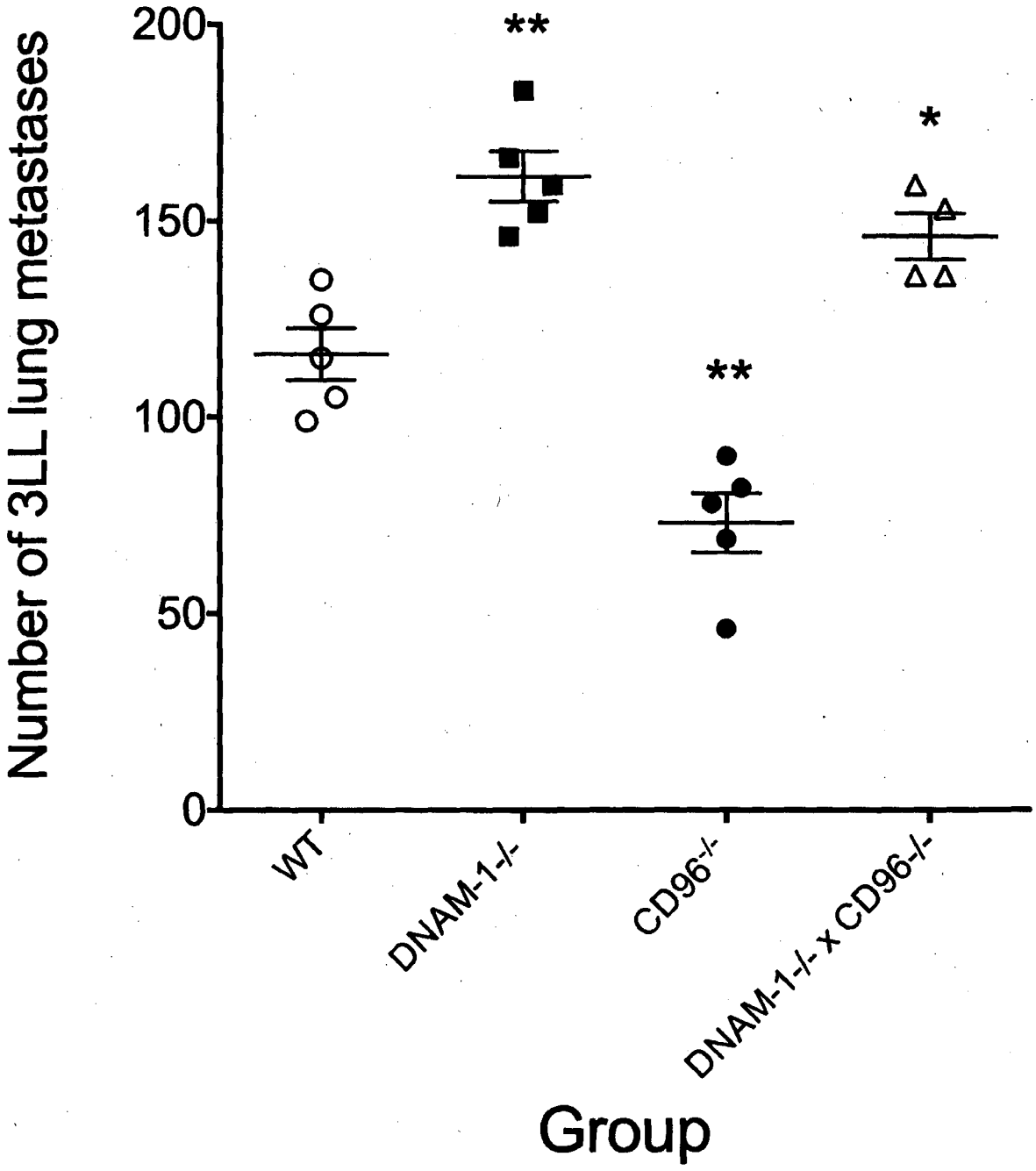

Number of 3LL lung metastases
200
150
100
50
0
**
**
*
WT
DNAM-1-/-
CD96-/-
DNAM-1-/- x CD96-/-
Group

Fig. 12

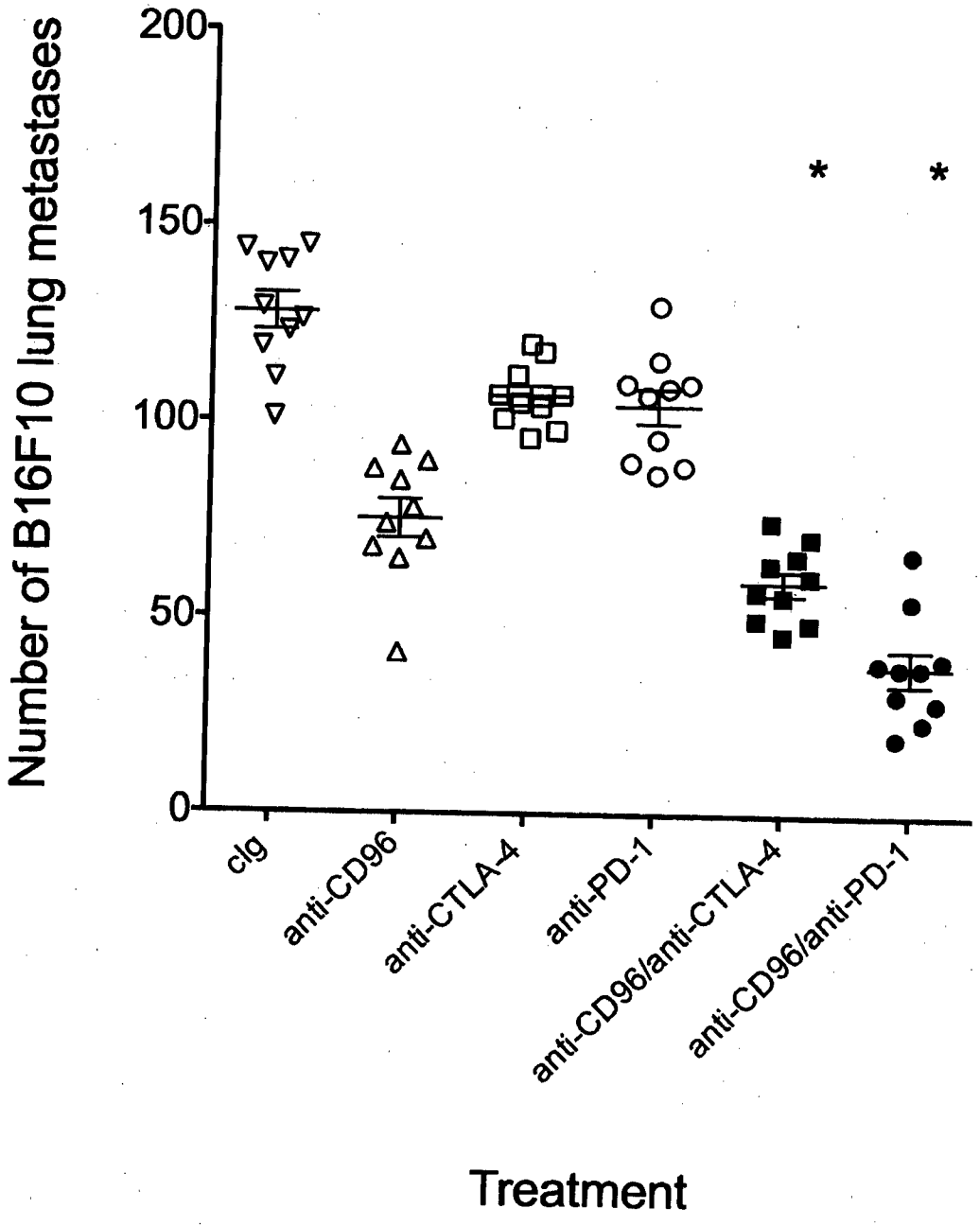

Number of B16F10 lung metastases
200
150
100
50
0
*
*
clg
anti-CD96
anti-CTLA-4
anti-PD-1
anti-CD96/anti-CTLA-4
anti-CD96/anti-PD-1
Treatment

Fig. 13

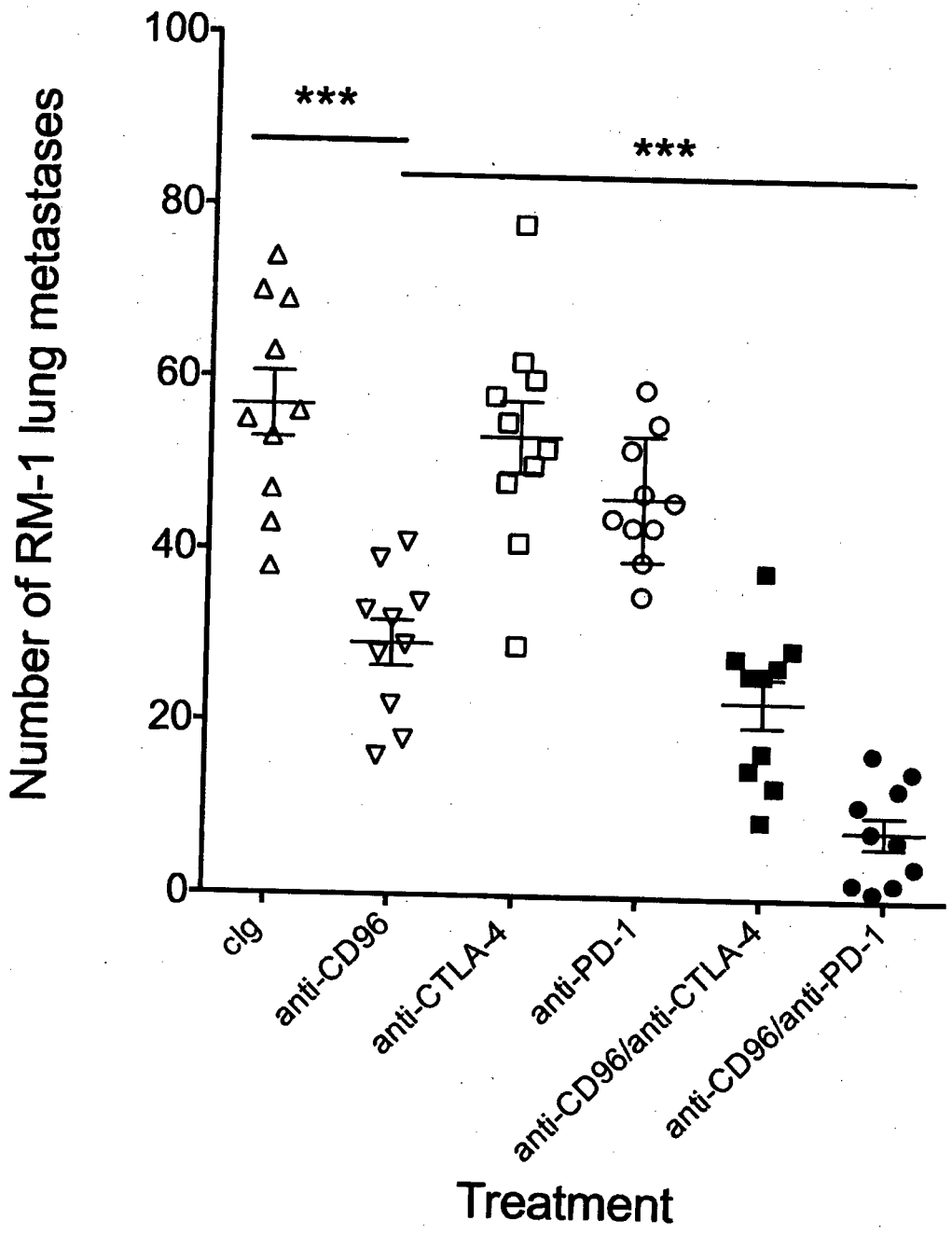

Number of RM-1 lung metastases
100
80
60
40
20
0
***
***
clg
anti-CD96
anti-CTLA-4
anti-PD-1
anti-CD96/anti-CTLA-4
anti-CD96/anti-PD-1
Treatment

Fig. 14

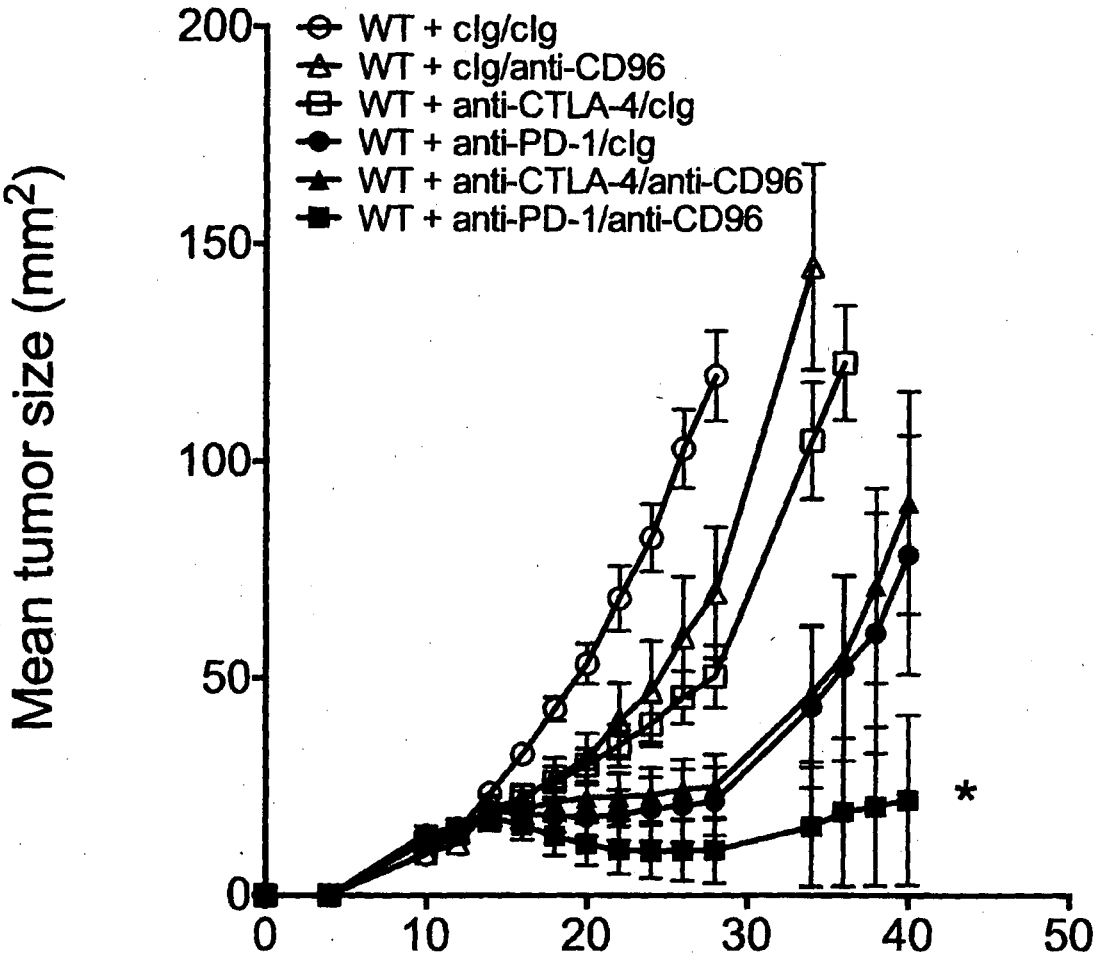

200
150
100
50
0
0
10
20
30
40
50
Mean tumor size (mm2)
WT + clg/clg
WT + clg/anti-CD96
WT + anti-CTLA-4/clg
WT + anti-PD-1/clg
WT + anti-CTLA-4/anti-CD96
WT + anti-PD-1/anti-CD96
*
Days after MC38-OVAdim tumor inoculation

Fig. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2013/001132

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/28 (2006.01) A61K 39/395 (2006.01) A61P 37/04 (2006.01) A61P 35/00 (2006.01) A61P 31/12 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WPI, EPODOC; Keywords: CD96, Tactile, anti-CD96, Reduc+, Inhibit+, Decreas+, Prevent+, Diminish+, Suppress+, Block+, Antagonis+, Antibod+, Immunoglob+, SCFV, FAB??_Fragment, siRNA, miRNA, RNAi

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 December 2013	03 December 2013
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au Facsimile No.: +61 2 6283 7999	Monica Graham AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262833179

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2013/001132**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOHSENI NODEHI, S. et al. "Enhanced ADCC activity of affinity maturated and Fc-engineered mini-antibodies directed against the AML stem cell antigen CD96." PLoS One. 2012;7(8):e42426. Epub 2012 Aug 3. (see abstract and page 9 left column last paragraph – right column first paragraph)	20 and 21
X	WO 2008/073316 A2 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 19 June 2008 (see Figure 2, paragraphs [0023], [0044], [0082] and claim 15)	20 and 21
X	GRAMATZKI, M. et al. "Antibodies TC-12 ("unique") and TH-111 (CD96) characterize T-cell acute lymphoblastic leukemia and a subgroup of acute myeloid leukemia." Exp Hematol. 1998 Dec;26(13):1209-14. (see whole document)	20 and 21
X	WO 2011/057216 A1 (THE PENNSYLVANIA STATE RESEARCH FOUNDATION) 12 May 2011 (see page 17 last paragraph – page 18 first paragraph, Figure 9 and claim 36)	20 and 21
X	BARTH, B.M. et al. "Targeted indocyanine-green-loaded calcium phosphosilicate nanoparticles for in vivo photodynamic therapy of leukemia." ACS Nano. 2011 Jul 26;5(7):5325-37. Epub 2011 Jul 8. (see whole document in particular page 5330 right column paragraph 2 and Figure 6)	20 and 21
X	WO 2009/126688 A2 (GENENTECH, INC) 15 October 2009 (see page 7 lines 22-27, Example 2, in particular page 108 lines 7-10 and claims 22 and 23)	20 and 21
X	ZENG, J.M. et al. "Human CD96 gene cloning, expression and identification". Nan Fang Yi Ke Da Xue Xue Bao. 2011 Jun;31(7):1232-5. *Abstract only (see abstract)	20 and 21
X	GONG, J. et al. "Establishment of an enzyme-linked immunosorbent assay system for determining soluble CD96 and its application in the measurement of sCD96 in patients with viral hepatitis B and hepatic cirrhosis." Clin Exp Immunol. 2009 Feb;155(2):207-15. Epub 2008 Nov 24. (see abstract and page 209 right column last paragraph)	20 and 21
X	FUCHS, A. et al. "Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155)." J Immunol. 2004 Apr 1;172(7):3994-8. (see page 3995 left column, paragraph 2 and 6; page 3996 left column paragraph 3 and Figure 2)	20 and 21
X	SETH, S. et al. "The murine pan T cell marker CD96 is an adhesion receptor for CD155 and nectin-1." Biochem Biophys Res Commun. 2007 Dec 28;364(4):959-65. Epub 2007 Oct 29. (see Abstract, Materials and Methods, Page 964 left column and Figure 3)	20 and 21
X	LARSEN, H. et al. "Nonviral transfection of leukemic primary cells and cells lines by siRNA-a direct comparison between Nucleofection and Accell delivery." Exp Hematol. 2011 Nov;39(11):1081-9. Epub 2011 Aug 18. (see Abstract and Materials and methods)	20 and 21
A	CHAN, C.J. "Receptors that interact with nectin and nectin-like proteins in the immunosurveillance and immunotherapy of cancer." Curr Opin Immunol. 2012 Apr;24(2):246-51.Epub 2012 Jan 28. (see whole document)	1-19

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2013/001132**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STANIETSKY, N & MANDELBOIM, O. "Paired NK cell receptors controlling NK cytotoxicity." FEBS Lett. 2010 Dec 15;584(24):4895-900. Epub 2010 Sep 7. (see whole document)	1-19
A	FUCHS, A & COLONNA, M. "The role of NK cell recognition of nectin and nectin-like proteins in tumor immunosurveillance." Semin Cancer Biol. 2006 Oct;16(5):359-66. Epub 2006 Jul 7. (see whole document)	1-19

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2013/001132**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2008/073316 A2	19 Jun 2008	None	
WO 2011/057216 A1	12 May 2011	None	
WO 2009/126688 A2	15 Oct 2009	None	

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 105636983 A
(43)申请公布日 2016.06.01

(21)申请号 201380079711.6
(22)申请日 2013.10.03
(30)优先权数据
2013903189 2013.08.22 AU
(85)PCT国际申请进入国家阶段日
2016.03.21
(86)PCT国际申请的申请数据
PCT/AU2013/001132 2013.10.03
(87)PCT国际申请的公布数据
WO2015/024042 EN 2015.02.26
(71)申请人 昆士兰医学研究所理事会
地址 澳大利亚昆士兰州
(72)发明人 M·斯迈思
(74)专利代理机构 北京戈程知识产权代理有限公司 11314
代理人 程伟 韩文华
(51)Int.Cl.
C07K 16/28(2006.01)
A61K 39/395(2006.01)
A61P 37/04(2006.01)
A61P 35/00(2006.01)
A61P 31/12(2006.01)

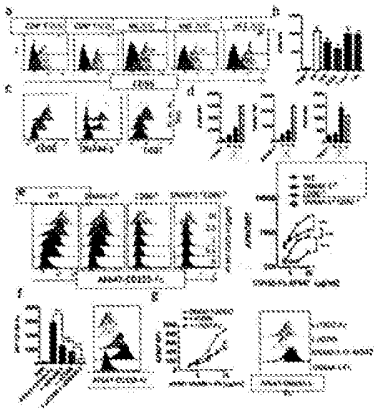
权利要求书1页 说明书16页 附图15页

(54)发明名称

用于治疗癌症和病毒感染的免疫受体调节

(57)摘要

一种在哺乳动物中减少或缓解免疫抑制的方法，包括在一种或更多种哺乳动物的细胞中至少部分抑制或减少CD96活性的步骤，从而在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视。典型地，抑制或减少CD96活性不包括或不依赖杀伤哺乳动物中表达CD96的细胞。所述方法在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视，从而在哺乳动物中治疗或预防癌症或癌症转移和/或病毒感染。还提供了一种筛选、设计、工程化或者生产CD96抑制试剂的方法，所述CD96抑制试剂在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视。典型地，CD96抑制试剂是抗体或抗体片段并且哺乳动物是人类。



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1.一种在哺乳动物中减少或缓解免疫抑制的方法，所述方法包括在一种或更多种哺乳动物的细胞中至少部分抑制或减少CD96活性的步骤，从而在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视。

2.根据权利要求1所述的方法，其中在哺乳动物中至少部分抑制或减少CD96活性的步骤不包括或不依赖杀伤哺乳动物中表达CD96的细胞。

3.根据权利要求1或2所述的方法，其中在哺乳动物中至少部分抑制或减少CD96活性的步骤包括对哺乳动物施用CD96抑制试剂。

4.根据权利要求3所述的方法，其中所述CD96抑制试剂至少部分封闭或抑制CD96结合CD155和/或CD96的细胞内信号传导。

5.根据权利要求4所述的方法，其中所述CD96抑制试剂是抗CD96抗体或抗体片段。

6.根据前述任一项权利要求所述的方法，其包括施用一种或更多种其它的治疗试剂。

7.根据权利要求6所述的方法，其中所述一种或更多种其它的治疗试剂包括化疗试剂和一种或更多种结合PD1和/或CTLA4的抗体或抗体片段。

8.根据前述任一项权利要求所述的方法，其增加或提高在哺乳动物中一种或更多种细胞的细胞因子和/或趋化因子表达和/或分泌。

9.根据权利要求8所述的方法，其中所述细胞因子和/或趋化因子包括MIP-1α、MIP-1β、RANTES、TNF-α和IFN-γ。

10.根据权利要求9所述的方法，其中所述细胞因子是干扰素γ(IFN-γ)。

11.根据权利要求8、权利要求9或权利要求10所述的方法，其中一种或更多种细胞是T细胞，包括$CD4^+$和$CD8^+$T细胞、γδT细胞、NK T细胞、以及自然杀伤(NK)细胞。

12.根据前述任一项权利要求所述的方法，其在哺乳动物中治疗或预防癌症或癌症转移。

13.根据前述任一项权利要求所述的方法，其在哺乳动物中治疗或预防病毒感染。

14.根据前述任一项权利要求所述的方法，其中所述哺乳动物是人类。

15.一种筛选、设计、工程化或者生产CD96抑制试剂的方法，所述方法包括测定候选分子是否能够至少部分抑制或减少CD96活性、从而在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视的步骤。

16.根据权利要求15所述的方法，其中所述CD96抑制试剂是抗体或抗体片段。

17.根据权利要求15或权利要求16所述的方法，其中所述CD96抑制试剂是抗癌症试剂。

18.根据权利要求15、权利要求16或权利要求17所述的方法，其中所述CD96抑制试剂是抗病毒试剂。

19.根据权利要求15-18中任一项所述的方法，其中所述哺乳动物是人类。

20.一种CD96抑制试剂，其根据权利要求15-19中任一项所述的方法筛选、设计、工程化或者生产。

21.根据权利要求20所述的CD96抑制试剂用于根据权利要求1-14中任一项所述的方法中的用途。

用于治疗癌症和病毒感染的免疫受体调节

技术领域

[0001] 本发明涉及免疫受体CD96。更具体地，本发明涉及CD96的抑制，从而提高免疫系统靶向肿瘤和其它能逃逸免疫系统的疾病或病症的能力。

背景技术

[0002] 有效免疫应答的进展需要通过若干免疫的检查点。通路可能需要兴奋性共刺激信号的存在，或者负面的或共抑制信号的逃避，负面的或共抑制信号作用于降低或终止免疫活性。免疫球蛋白超家族在这种免疫应答的协调中占据着核心重要地位，并且CD28/细胞毒T淋巴细胞抗原-4(CTLA-4):B7.1/B7.2受体/配体组合代表了这些免疫调节因子的原型实例。这些检查点的部分作用是监视不需要的和有害的自主(self-directed)活性的可能性。虽然这是一种必要的功能，有助于自体免疫的预防，但是它可能充当旨在靶向恶性的自体细胞的成功的免疫治疗的障碍，其中恶性的自体细胞主要显示与它们所衍生来自的细胞相同的表面分子阵列。旨在克服这些外周耐受机制的治疗，特别是通过封闭在T细胞上抑制性检查点，提供了产生抗肿瘤活性的潜力，或者作为单一治疗或与其它治疗协同作用，直接或间接的提高肿瘤表位提呈至免疫系统。此种抗T细胞检查点的抗体在晚期人类癌症的早期临床试验中显示出前景。

[0003] 此外，自然杀伤(NK)细胞是限制早期肿瘤生长和转移的关键的先天(innate)淋巴细胞[1]。NK细胞功能同样是通过广泛的激活和抑制受体的信号传递的整合来调节[2]。例如，病原体来源的或压力诱导的配体被激活受体如NCRs、NKG2D、或DNAM-1识别，刺激NK细胞细胞毒性和促炎介质的分泌，如干扰素γ(IFN-γ)[3]。相反地，抑制性受体保护靶细胞免受NK细胞介导的杀伤[4]。这些受体主要地识别MHC I类和MHC I类相关分子，以及包括KIR(杀伤细胞免疫球蛋白样受体)和LIR(白细胞免疫球蛋白样受体)家族，小鼠中Ly49家族和在两物种中的CD94/NKG2异二聚体。

[0004] 最近已描述了免疫球蛋白超家族成员的新出现的组与粘连蛋白和粘连蛋白样(necl)家族的配体相互作用，影响NK细胞和T细胞功能[5]。这些包括CD226(DNAM-1)[6]、CD96(TACTILE)[7]、TIGIT(T细胞免疫球蛋白和ITIM结构域)[8,9]、和CRTAM(I类限制性T细胞相关分子)[10]。DNAM-1和TIGIT是该家族最广泛研究的成员，并且它们共享共同的配体，CD 155(necl-5;PVR)和CD112(粘连蛋白-2;PVRL2)[8,11]。TIGIT还结合额外的配体CD113(PVRL3)[8]。据报道，在NK细胞上DNAM-1和TIGIT的功能是平衡的(counter-balancing)[12]。在体外，DNAM-1加强NK细胞对广泛的肿瘤细胞的细胞毒性[13,14]，并且在体内对肿瘤的免疫监视至关重要[13,15,16]。相反地，TIGIT携带ITIM基序，并且已经提出与抑制性Ly49或KIR与MHC I类分子的相互作用相似地防止自体组织损伤[17]。确实，在体外CD155结合(engagement)TIGIT已经显示了限制IFNγ产生和NK细胞的细胞毒性[18,19]。然而在体内，NK细胞生物学中TIGIT的作用相对于其它粘连蛋白受体DNAM-1和CD96仍有待评估。

[0005] 尽管在20年前已被克隆[7]，关于CD96，与DNAM-1和TIGIT共享CD155配体的其它Ig家族成员，所知甚少[20,21]。在人类中，CD96表达主要局限于NK细胞、CD8T细胞、和CD4T细胞[7]。

CD96的主要配体是CD155，但是还有报道CD96与CD111(粘连蛋白-1)有关，并且在促进NK和T细胞粘附中起作用[21]，[22]。

发明内容

[0006] 出人意料地，本发明人已经发现了CD96充当T细胞和NK细胞抗肿瘤功能的负调节因子。相应地，本发明广泛涉及试剂的用途，所述试剂至少部分封闭或抑制CD96，从而减少或缓解CD96介导的免疫抑制，在哺乳动物中提高或恢复免疫监视。在某些实施方案中，这可促进至少对CD96部分封闭或抑制应答的疾病或病症的治疗，例如癌症和/或病毒感染。

[0007] 在第一方面中，本发明提供了一种在哺乳动物中减少或缓解免疫抑制的方法，所述方法包括在一种或更多种哺乳动物的细胞中至少部分抑制或减少CD96活性的步骤，从而在哺乳动物中缓解免疫抑制以及或提高或恢复免疫监视。

[0008] 适当地，在哺乳动物中抑制或减少CD96活性的步骤不包括或至少不依赖于杀伤哺乳动物中表达CD96的细胞。优选地，在哺乳动物中抑制或减少CD96活性的步骤包括在一种或更多种表达CD96的哺乳动物的细胞中抑制或减少CD96结合CD155和/或细胞内信号传导。

[0009] 在一个具体的实施方案中，在哺乳动物中抑制或减少CD96活性的步骤包括增加或提高一种或更多种细胞因子或趋化因子的表达、产生和/或分泌。优选地，所述细胞因子是干扰素γ(IFN-γ)。典型地，一种或更多种哺乳动物的细胞是T细胞，包括$CD4^+$和$CD8^+$T细胞、γδT细胞、NKT细胞、以及自然杀伤(NK)细胞。

[0010] 在一个优选的实施方案中，所述方法在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视，从而在哺乳动物中治疗或预防癌症或癌症转移。

[0011] 在其它实施方案中，所述方法在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视，从而在哺乳动物中治疗或预防病毒感染。

[0012] 在第二方面中，本发明提供了一种筛选、设计、工程化或者生产CD96抑制试剂的方法，所述方法包括测定候选分子是否能够至少部分抑制或减少CD96活性、从而在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视的步骤。

[0013] 在第三方面中，本发明提供了一种根据第二方面的方法筛选、设计、工程化或者生产的CD96抑制试剂。

[0014] 在一个实施方案中，所述CD96抑制试剂是抗体或抗体片段。

[0015] 在一个具体地实施方案中，所述CD96抑制试剂是一种抗癌症试剂。

[0016] 在另一个具体地实施方案中，所述CD96抑制试剂是一种抗病毒试剂。

[0017] 在第四方面中，本发明提供了根据本发明第三方面的CD96抑制试剂，其用于根据本发明第一方面的方法。

[0018] 适当地，根据上述的方面，所述哺乳动物是人类。

[0019] 除非文中另有要求，术语“包括(comprise)”、“包含(comprises)”和“含有(comprising)”、或类似术语旨在表示非排他性的包括，如此要素和特征的列举不仅包括阐明的或列举的要素，但可能包括其它未列举或阐明的要素或特征。

[0020] 本文所使用的不定冠词“一个(a)”和“一种(an)”指的是包含单数或复数要素或特征，并且不应作为表示或定义为“单一(one)”或“单独(single)”要素或特征。

附图说明

[0021] 图1:CD96与DNAM-1竞争结合CD155。a、b通过流式细胞术分析所示的来自C57BL/6WT(浅灰)和CD96$^{-/-}$小鼠(深灰)的脾淋巴细胞群的CD96的表达。显示来自3个实验中的一个代表性实验的3只小鼠的代表性FACS柱状图(a)和平均值±SD(b)。c、d在新鲜分离的或使用IL-2(1000U/ml)激活48小时的野生型脾NK细胞上测定CD96、DNAM-1和TIGIT的表达。e.通过流式细胞术,在所示的浓度评估偶联有AF-647的小鼠CD155-Fc与新鲜分离自WT、CD96$^{-/-}$、DNAM-1$^{-/-}$或DNAM-1$^{-/-}$CD96$^{-/-}$小鼠的纯化的NK细胞的结合。f.在存在抗CD96和或抗DNAM-1mAb时,在纯化的WT NK细胞上分析偶联有AF-647(10μg/ml)的CD155-Fc的结合。g.在存在50μg/ml的对照Ig、重组CD96或TIGIT-Fc时,分析在BMDC的细胞表面上标记有AF-647(0.5-10μg/ml)的DNAM-1-Fc的结合。c-g.显示来自至少3个实验中的一个代表性实验的一式三份的孔的代表性FACS柱状图和平均值±SD。***p<0.001,T检验(Student T text)。

[0022] 图2:CD155结合CD96调节NK细胞IFNγ的产生。CD96结合CD155-Fc限制通过外源性细胞因子(a、b、d)和NK细胞受体(c)诱导的NK细胞IFN-γ的产生。a、b、d.使用包被有或没有CD155-Fc(0.5μg/孔)的板,我们分析了在存在或不存在抗CD96抗体(50μ/ml)时,新鲜纯化的CD96$^{-/-}$、TIGIT$^{-/-}$和WT NK细胞对IL-12(25-100pg/ml)和IL-18(50ng/ml)应答细胞内产生的IFN-γ。c.使用包被有抗NK-1.1(2.5μg/孔)和CD155-Fc(0.5μg/孔)的板,我们分析了被IL-2激活的来自CD96$^{-/-}$和WT小鼠的NK细胞细胞内产生的IFN-γ。显示来自3个实验中的一个代表性实验的一式三份的孔的代表性FACS柱状图(a)和平均值±SD(b、c、d)。*p<0.05、**p<0.01、***p<0.001,T检验。

[0023] 图3:CD96限制依赖NK细胞的肿瘤免疫监视。a、b.CD96和DNAM-1在B16F10转移的控制中具有相反的作用。a.2x10^{5}B16F10细胞静脉注射至WT、CD96$^{-/-}$、DNAM-1$^{-/-}$和DNAM-1$^{-/-}$CD96$^{-/-}$小鼠,并且在14天后定量在肺中的转移负荷。3个实验中的代表性实验。b.图片显示在注射2x10^{5}和5x10^{5}B16F10细胞两周后WT和CD96$^{-/-}$小鼠的肺。两个实验中的代表性实验。c.在B16F10细胞表面CD96和TIGIT与DNAM-1竞争结合CD155。存在50μg/ml的对照Ig、重组CD96或TIGIT-Fc时,分析在B16F10细胞的细胞表面上标记有AF-647(0.5-20μg/ml)的DNAM-1-Fc的结合。显示来自3个实验中的一个代表性实验的一式三份的孔的代表性FACS柱状图和平均值±SD。d.以所示的效靶比,在B16F10细胞和来自WT、DNAM-1$^{-/-}$和CD96$^{-/-}$小鼠的IL-2激活的NK细胞之间进行4小时的^{51}Cr释放试验。实心圆代表WT NK细胞,空心圆代表CD96$^{-/-}$NK细胞以及实心方块代表DNAM-1$^{-/-}$NK细胞。e-h.在NK细胞介导的MCA诱导的纤维肉瘤的免疫监视中,CD96和DNAM-1具有相反的作用。e-h使用MCA(100μg/小鼠)注射15-30只雄性WT、DNAM-1$^{-/-}$和CD96$^{-/-}$和DNAM-1$^{-/-}$CD96$^{-/-}$小鼠的组。显示具有肉瘤的个体小鼠的生存(e-g)和生长曲线(h)。f.使用如材料和方法中定义的抗CD96、抗DNAM-1或者抗CD155mAb处理WT小鼠。g.使用100μg MCA注射WT和CD96$^{-/-}$小鼠,并且使用对照抗体、抗IFN-γ抗体、或者抗去唾液酸(asialo)GM1处理。*p<0.05Mantel-Cox检验。

[0024] 图4:抗CD96mAb具有单独试剂活性并且提高抗PD1的抗肿瘤应答。使用AT3-OVA^{di}m肿瘤细胞(10^{6}细胞)皮下注射C57BL/6野生型(WT)小鼠,并且在第16、20和24天使用抗CD96mAb(3.3,250μg i.p.)或者抗PD-1(RMP1-14,250μg,i.p.)腹腔注射处理。显示每组5只小鼠的平均值±SEM(mm^2)(*:p<0.05通过Mann-Whitney检验与单独cIg对比)。

[0025] 图5:抗CD96mAb提高通过阿霉素(DOX)化疗产生的抗肿瘤应答。使用AT3-OVA^{dim}肿瘤细胞(10^6细胞)皮下注射C57BL/6野生型(WT)、DNAM-$1^{-/-}$和CD$96^{-/-}$小鼠,并且在第14天使用对照PBS或者DOX(50微升,2mM,瘤内)处理。WT小鼠的一些组还在第12、14、18、21、24和28天接受抗CD96mAb(3.3,250μg,i.p.)或者抗DNAM-1(480.1,250μg,i.p.)的腹腔注射。显示每组5只小鼠的平均值±SEM(mm^2)。

[0026] 图6:宿主CD96缺陷提高阿霉素(DOX)化疗的抗肿瘤应答。使用AT3-OVA^{dim}肿瘤细胞(10^6细胞)皮下注射C57BL/6野生型(WT)、DNAM-$1^{-/-}$和CD$96^{-/-}$小鼠,并且在第16天使用对照PBS或者DOX(50微升,2mM,瘤内)处理。显示每组5只小鼠的平均值±标准误(mm^2)。

[0027] 图7:抗CD96mAb提高由阿霉素(DOX)化疗产生的抗肿瘤应答。使用AT3-OVA^{dim}肿瘤细胞(10^6细胞)皮下注射C57BL/6野生型(WT)小鼠,并且在第16天使用对照PBS或者DOX(50微升,2mM,瘤内)处理。WT小鼠的一些组还在第16、20和23天接受抗CD96mAb(3.3,250μg,i.p.)的腹腔注射。显示每组5只小鼠的平均值±SEM(mm^2)(*:$p<0.05$通过Mann-Whitney检验与单独cIg对比)。

[0028] 图8:早期抗CD96mAb提高由抗PD-1和抗CTLA-4mAb产生的抗肿瘤应答。使用B16-OVA黑色素瘤细胞(10^5细胞)皮下注射C57BL/6野生型(WT)小鼠,并且在第1、5和9天使用抗CD96mAB(3.3,250μg,i.p.)、抗PD-1mAb(RMP1-14,250μg,i.p.)、抗CTLA-4(UC10-4F10,250μg,i.p.)、抗D96/抗PD-l mAb(每个250μg,i.p.)、抗CD96/抗CTLA-4mAb(每个250μg,i.p.)或者对照Ig(cIg)(2A3,250μg,i.p.)的腹腔注射处理。显示每组5只小鼠的平均值±SEM(mm^2)(*:$p<0.05$通过Mann-Whitney检验与单独抗CD96对比)。

[0029] 图9:后期抗CD96mAb提高由抗PD-1mAb产生的抗肿瘤应答。使用B16-OVA黑色素瘤细胞(10^5细胞)皮下注射C57BL/6野生型(WT)小鼠,并且在第16、20和24天使用抗CD96mAb(3.3,250μg,i.p.)、抗PD-1mAb(RMP1-14,250μg,i.p.)、抗CTLA-4(UC10-4F10,250μg,i.p.)、抗CD96/抗PD-l mAb(每个250μg,i.p.)、抗CD96/抗CTLA-4mAb(每个,250μg,i.p.)或者对照Ig(cIg)(2A3,250μg,i.p.)的腹腔注射处理。显示每组5只小鼠的平均值±SEM(mm^2)(*:$p<0.05$通过Mann-Whitney检验与单独抗CD96对比)。

[0030] 图10:宿主CD96促进B16F10肺转移。使用B16F10黑色素瘤细胞(10^5细胞)静脉注射C57BL/6野生型(WT)、DNAM-$1^{-/-}$、CD$96^{-/-}$和DNAM-$1^{-/-}$CD$96^{-/-}$小鼠,并且在14天后通过在肺表面计数克隆定量在肺中的转移负荷。显示每组9-17只小鼠的平均值±SEM(*:$p<0.05$通过Mann-Whitney检验与WT对比)。

[0031] 图11:宿主CD96促进RM-1肺转移。使用RM1前列腺癌细胞(10^4细胞)静脉注射C57BL/6野生型(WT)、DNAM-$1^{-/-}$、CD$96^{-/-}$、DNAM-$1^{-/-}$CD$96^{-/-}$小鼠,并且在14天后通过在肺表面计数克隆定量在肺中的转移负荷。显示每组10-15只小鼠的平均值±SEM(*:$p<0.05$通过Mann-Whitney检验与WT对比)。

[0032] 图12:宿主CD96促进3LL肺转移。使用3LL肺癌细胞(10^5细胞)静脉注射C57BL/6野生型(WT)、DNAM-$1^{-/-}$、CD$96^{-/-}$、DNAM-$1^{-/-}$CD$96^{-/-}$小鼠,并且在14天后通过在肺表面计数克隆定量在肺中的转移负荷。显示每组5只小鼠的平均值±SEM(*:$p<0.05$通过Mann-Whitney检验与WT对比)。

[0033] 图13:抗CD96单独和与T细胞检查点封闭结合抑制B16F10肺转移。使用B16F10黑色素瘤细胞(10^5细胞)静脉注射C57BL/6野生型(WT)小鼠。在肿瘤接种后第0和第3天,使用抗

CD96mAb(3.3,250μg,i.p.)、抗PD-1mAb(RMP1-14,250μg,i.p.)、抗CTLA-4(UC10-4F10,250μg,i.p.)、抗CD96/抗PD-1 mAb(每个,250μg,i.p.)、抗CD96/抗CTLA-4mAb(每个,250μg,i.p.)或者对照Ig(cIg)(2A3,250μg,i.p.)的腹腔注射处理。并且在14天后通过在肺表面计数克隆定量在肺中的转移负荷。显示每组5只小鼠的平均值±SEM(*:p<0.05通过Mann-Whitney检验与单独抗CD96对比)。

[0034] 图14:抗CD96单独和与T细胞检查点封闭结合抑制RM-1肺转移。使用RM-1前列腺癌细胞(10^4细胞)静脉注射C57BL/6野生型(WT)小鼠。在肿瘤接种后第0和第3天,使用抗CD96mAb(3.3,250μg,i.p.)、抗PD-1mAb(RMP1-14,250μg,i.p.)、抗CTLA-4(UC10-4F10,250μg,i.p.)、抗CD96/抗PD-1 mAb(每个250μg,i.p.)、抗CD96/抗CTLA-4mAb(每个250μg,i.p.)或者对照Ig(cIg)(2A3,250μg,i.p.)的腹腔注射处理。并且在14天后通过在肺表面计数克隆定量在肺中的转移负荷。显示每组5只小鼠的平均值±SEM(*:p<0.05通过Mann-Whitney检验与单独抗CD96对比)。

[0035] 图15:后期的抗CD96mAb提高由抗PD-1mAb产生的抗肿瘤应答。使用MC38-OVA^{dim}结肠腺癌细胞(10^6细胞)皮下注射C57BL/6野生型(WT)小鼠,并且在第14、18、22和26天使用抗CD96mAb(3.3,250μg,i.p.)、抗PD-1mAb(RMP1-14,250μg,i.p.)、抗CTLA-4(UC10-4F10,250μg,i.p.)、抗CD96/抗PD-1 mAb(每个250μg,i.p.)、抗CD96/抗CTLA-4mAb(每个250μg,i.p.)或者对照Ig(cIg)(2A3,250μg,i.p.)的腹腔注射处理。显示每组5只小鼠的平均值±SEM(mm^2)(*:p<0.05通过Mann-Whitney检验与单独抗CD96对比)。

具体实施方式

[0036] 本发明至少部分地基于意外的发现:即CD96被静息NK细胞和T细胞亚群高表达,并且与DNAM-1竞争结合于在静息NK细胞上的CD155。使用$CD96^{-/-}$小鼠,证明CD96通过与DNAM-1竞争结合CD155以及还通过直接的抑制,在体外或体内降低或抑制NK细胞产生的IFN-γ。此外$CD96^{-/-}$小鼠显示对作为致癌作用的指标的3'-甲基胆蒽(MCA)诱导的肿瘤形成、或者B16F10(黑色素瘤)、RM-1(前列腺癌)、3LL(肺癌)实验性转移更有抵抗力。基于这些观察,提出CD96通常充当T细胞和NK细胞的抗肿瘤功能的负调节因子,特别是虽然非唯一地通过IFN-γ产生和/或分泌的抑制。相应地,本发明提供了缓解或减少CD96的负免疫调节功能的方法,从而促进或恢复特别是通过T细胞和NK细胞的免疫监视,从而治疗或预防癌症、癌症细胞转移和/或病毒感染。

[0037] 因此,本发明的一个方面提供了一种在哺乳动物中减少或缓解免疫抑制的方法,所述方法包括在一种或更多种哺乳动物的细胞中至少部分抑制或减少CD96活性的步骤,从而在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视。

[0038] 在CD96的上下文中"缓解免疫抑制"表示以抑制或抑制一种或更多种通常表达CD96的细胞的免疫功能,至少部分消除、去除或克服CD96的正常活性或功能。典型地,一种或更多种通常表达CD96的细胞是T细胞,包括$CD4^+$和$CD8^+$T细胞、γδT细胞、NKT细胞、以及自然杀伤(NK)细胞。在一些实施方案中,缓解免疫抑制可能包括或涉及废除对外来病原体、显示外来病原体的宿主细胞(例如在自体MHC中显示外来病原体衍生的多肽)和/或宿主的癌细胞或组织的外周耐受。

[0039] "提高或恢复免疫监视"表示至少部分提高或促进一种或更多种免疫系统的要素

的能力以对外来病原体、显示外来病原体的宿主细胞(例如在自体MHC中显示外来病原体衍生的多肽)和/或宿主的癌细胞或组织监测、检测和/或应答。适当地,免疫系统的要素是一种或更多种通常表达CD96的细胞,例如T细胞,包括$CD4^+$和$CD8^+$T细胞、γδT细胞、NKT细胞、以及自然杀伤(NK)细胞。

[0040] 在一种或更多种哺乳动物的细胞中至少部分抑制或减少CD96的活性可以通过对哺乳动物施用"CD96抑制试剂"来执行、促进或实现。CD96抑制试剂可以是具有或显示至少部分抑制或减少CD96的生物学活性的能力的任何分子。CD96的生物学活性包括结合CD155、诱发细胞内信号传导和刺激或诱导细胞因子和/或趋化因子的表达和/或分泌中的一种或更多种。优选地,所述细胞因子或趋化因子包括任何促炎细胞因子或趋化因子,包括MIP-1α、MIP-1β、RANTES、TNF-α和IFN-γ,而不仅限于此。优选地,所述细胞因子是IFN-γ。一种或更多种细胞因子或趋化因子的表达、产生和/或分泌的测量,或者其编码核苷酸的测量将在下文中详细描述。

[0041] 在一个实施方案中,所述CD96抑制试剂抑制、封闭或拮抗CD96和CD 155间的结合相互作用。仅作为实施例,CD96抑制试剂可以结合至CD96的细胞外结构域、或者其部分,该细胞外结构域、或者其部分能够与CD155相互作用(例如结合CD155或被CD155结合)从而至少部分抑制或封闭CD96结合于CD155。

[0042] 在另一个实施方案中,所述CD96抑制试剂是具有或显示抑制或减少CD96信号传导活性的能力的分子。CD96的信号传导活性的抑制或减少可以通过抑制、封闭或拮抗与CD155的结合相互作用或者可通过封闭CD96启动的信号传导,该信号传导通常在对CD155结合的应答时发生。作为实施例,CD96包含基于免疫受体酪氨酸的抑制性基序(ITIM)。ITIM是结构定义为包含酪氨酸(Y)残基,伴有部分保守的N-端(Y-2)和C-端(Y+3)残基的6氨基酸序列。基序一般但不限于是(S/I/V/LXYXXI/V/L),其中X是任意氨基酸。例如CD96的亚型1包含ITIM序列IKYTCI,其中Y是残基566。

[0043] 已经提出了当与激活受体共同聚合时,ITIM被Src家族酪氨酸激酶磷酸化,使得它们募集含有Src同源2结构域的拮抗激活信号的磷酸酶(PTPases)。相应地,在一个实施方案中,所述CD96抑制试剂具有或显示抑制或减少通过CD96ITIM介导的CD96信号传导活性的能力。优选地,抑制或减少通过CD96ITIM介导的CD96信号传导活性能够增加或提高表达CD96的细胞的趋化因子和/或细胞因子(例如IFN-γ)表达、产生和/或分泌。

[0044] 所述CD96抑制试剂可以是一种蛋白(包括多肽、抗体或抗体片段)、核酸(包括抑制性RNA分子,例如核酶、RNAi、miRNA和siRNA,而不仅限于此)、脂质、碳水化合物、有机小分子或者这些的任意组合(例如糖蛋白、脂蛋白、多肽核酸等)。

[0045] 在一个具体的实施方案中,所述CD96抑制试剂是结合CD96的抗体或抗体片段。在一种形式中抗体结合CD96并且至少部分封闭或抑制CD96结合于CD155。

[0046] 抗体可以是多克隆的或者单克隆的,天然的或者重组的。抗体片段包括Fab和Fab'2片段,双体和单链抗体片段(例如scVs),而不仅限于此。抗体和抗体片段可以经修饰,以便在以下情况中是可施用的:生产或者起源自一个物种,而在另一物种对"外来"抗体不引起有害免疫应答。在人类中,这是已在其它物种中生产或者起源自其它物种的抗体的"人源化"。现有技术公知该方法,并且一般涉及重组"接枝"非人类抗体互补决定区(CDRs)至人类抗体支架或骨架。

[0047] 适当地，在哺乳动物中抑制或减少CD96活性的步骤不包括在哺乳动物中杀伤表达CD96的细胞。在本文中，“杀伤”可指任何抗体介导的细胞毒机制，如补体介导的细胞裂解和抗体介导的细胞介导的细胞毒作用(ADCC)，后者典型地通过自然杀伤(NK)细胞、巨噬细胞、中性粒细胞和嗜酸性粒细胞介导。就这一点而言，使用缺少Fc部分或具有突变的Fc部分的抗体片段可能是有利的。

[0048] 在哺乳动物中抑制或减少CD96活性的步骤可通过对哺乳动物施用CD96抑制试剂实现或促进。

[0049] “施用”表示通过具体的途径将CD96抑制试剂引入至哺乳动物。适当地，CD96抑制试剂的治疗有效量施用于哺乳动物。

[0050] 术语“治疗有效量”描述了在用该试剂治疗的哺乳动物中，足以实现期望的效果的指定试剂的量。

[0051] 一般地，本发明的方法在减少或缓解CD96介导的免疫抑制、抑制或外周耐受中是有用的。适当地，所述方法促进治疗或预防一种或更多种疾病或病症，所述疾病或病症对至少部分封闭CD96介导的免疫抑制、抑制或外周耐受是应答的。

[0052] 如本文所使用的“治疗(treating)”或者“治疗(treat)”或者“治疗(treatment)”指的是一种治疗性干预，治疗性干预至少部分消除或者改善了一种或更多种存在的或之前鉴定的疾病或病症的症状，疾病或病症对至少部分封闭CD96介导的免疫抑制、抑制或外周耐受是应答的。

[0053] 如本文所使用的“预防(preventing)”或“预防(prevent)”或“预防(prevention)”指的是一种在疾病或病症的症状开始之前启动的预防性处理，所述疾病或病症对至少部分封闭CD96介导的免疫抑制、抑制或外周耐受是应答的，以便至少部分或暂时预防症状的出现。

[0054] 典型地，对至少部分封闭CD96介导的免疫抑制、抑制或外周耐受是应答的疾病或病症是提高或恢复免疫监视能够使患有疾病或病症的受试者受益的任何疾病或病症。该疾病和病症可能包括那些能够被细胞介导的免疫所控制或抑制的持久性疾病或病症。非限制性的实施例包括癌症和病毒感染。具体地，本发明所涉及的病毒感染包括持久的病毒感染，例如由人类免疫缺陷病毒(HIV)、EB病毒(EBV)、单纯疱疹病毒(HSV包括HSV1和HSV2)、人乳头瘤病毒(HPV)、水痘带状疱疹病毒(VSV)以及巨细胞病毒(CMV)，而不仅限于此。

[0055] 在一个优选的实施方案中，所述方法在哺乳动物中减少或缓解免疫抑制，足以在哺乳动物中治疗或预防癌症或癌症转移。适当地，癌症可以是任何对至少部分封闭CD96介导的免疫抑制、抑制或外周耐受应答的癌症。癌症可以是实体肿瘤、肉瘤、淋巴瘤、骨髓瘤、癌(carcinomas)、黑色素瘤、细胞瘤和脑膜瘤的形式，而不仅限于此。癌症的非限制性的实施例包括肾上腺、膀胱、骨、骨髓、脑、乳腺、宫颈、胆囊、神经节、胃肠道、心脏、肾脏、肝脏、肺、肌肉、卵巢、胰腺、脑垂体、甲状旁腺、前列腺、唾液腺、皮肤、脾、睾丸、甲状腺以及子宫的癌症。具体地，癌症的非限制性实施例包括结肠癌、肺癌和前列腺癌。在一些实施方案中，癌症是转移性癌症，其有能力迁移到机体内的另一位点、组织或器官，并且在该位点形成肿瘤。这可以随时间重复发生。本发明所涉及的一个具体地侵袭性转移性癌症是转移性黑色素瘤。

[0056] 还应当意识到癌症的治疗或预防的方法可以进一步包括与一种或更多种其它治

疗试剂的共同施用，所述治疗试剂促进癌症治疗或预防。仅作为实施例，这些包括：化疗试剂，例如紫杉醇、阿霉素、甲氨蝶呤和顺铂，而不仅限于此；和/或生物治疗试剂，例如抗PD-1抗体(例如Nivolumab)和抗CTLA4抗体(例如Ipilimumab)，而不仅限于此。还预期的是结合CD96和一种或更多种其它分子(包括但不限于PD-1和CTLA4)的双特异抗体。

[0057] 如现有技术公知地，一种或更多种促进癌症治疗或预防的其它试剂可以与CD96抑制试剂结合施用，或者分别地施用。

[0058] 在一些实施方案中，所述CD96抑制试剂可以是单独制剂，或者是与一种或更多种其它试剂以药物组合物的形式的制剂。

[0059] 本领域技术人员容易确定施用于哺乳动物，包括人类施用的CD96抑制试剂的合适的剂量(单独或与其它治疗试剂共同)。

[0060] 适当地，药物组合物包含合适地药学可接受的载体、稀释剂或者赋形剂。

[0061] 优选地，药学可接受的载体、稀释剂或者赋形剂是适合施用于哺乳动物的，并且更优选地，施用于人类。

[0062] “药学可接受的载体、稀释剂或者赋形剂”表示可以在系统施用中安全地使用的固体或液体填充物、稀释剂或封装物质。根据具体的施用途径，可以使用在现有技术中公知的各种载体、稀释剂或者赋形剂。这些载体、稀释剂或者赋形剂可选自：糖、淀粉、纤维素及其衍生物、麦芽、明胶、滑石、硫酸钙、植物油、合成油、多元醇、海藻酸、磷酸缓冲溶液、乳化剂、等渗盐水和盐(例如，包括盐酸盐、溴化物和硫酸盐的无机酸盐，例如乙酸盐、丙酸盐和丙二酸酯的有机酸)以及无致热原的水。

[0063] 描述药学可接受的载体、稀释剂或者赋形剂的有用的参考文献为雷明顿药物科学(Remington's Pharmaceutical Sciences)(马克出版有限公司(Mack Publishing Co.)NJ USA,1991)。

[0064] 可以利用任何安全的施用途径对受试者提供包含CD96抑制试剂的组合物。例如可以利用口服、直肠、胃肠外、舌下、口腔、静脉内、关节内、肌内、真皮内、皮下、吸入、眼内、腹腔内、脑室内、透皮等。

[0065] 本发明的进一步的方面提供了一种筛选、设计、工程化或者生产CD96抑制试剂的方法，所述方法包括测定候选分子是否能够至少部分抑制或减少CD96活性、从而在哺乳动物中缓解免疫抑制和/或提高或恢复免疫监视的步骤。

[0066] 本发明还提供了一种根据前述方面筛选、设计、工程化或者生产的CD96抑制试剂。

[0067] 候选分子可以是蛋白(包括多肽、抗体和抗体片段)、核酸(包括不限于抑制性RNA分子，例如核酶、RNAi、miRNA和siRNA)、脂质、碳水化合物、有机小分子或者这些的任意组合(例如糖蛋白、脂蛋白、多肽核酸等)。

[0068] 在一些实施方案中，候选调节因子可以是基于期望的或预测的结构特点或特征从头合理设计的或者经工程化的，这些结构特点或特征表明该候选调节因子能够封闭或者抑制一种或更多种CD96的生物学活性，例如结合CD155、细胞内信号传导和/或IFN-γ的生产和/或分泌。在其它实施方案中，候选调节因子可以通过筛选分子库鉴定，而没有基于期需或预测的结构特点或特征的初始选择，这些结构特点或特征表明该候选调节因子能够封闭或者抑制一种或更多种CD96的生物学活性。该库可包括蛋白、肽、核酸、重组抗体或抗体片段(例如噬菌体展示文库)、碳水化合物和/或脂的随机地生成的或者定向的库(directed

libraries),天然产生的分子的库和/或合成的有机分子的组合库。

[0069] 如现有技术公知,适用于候选调节因子的设计和/或筛选的技术的非限制性实施例可以利用检测分子结合相互作用的X射线晶体学、NMR光谱学、计算机辅助筛选结构数据库、计算机辅助建模或生物化学或生物物理技术。

[0070] 鉴定分子相互作用的生物物理和生物化学技术包括竞争放射配体结合试验、免疫共沉淀、基于荧光的试验,包括荧光共振能量转移(FRET)结合试验、电生理学、分析超速离心、标签转移、化学交联、质谱、微量热法、表面等离子体共振和基于光学生物传感器的方法,例如在CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds.Coligan等人(John Wiley& Sons,1997)中的20章提供。生物化学技术,例如双杂交和噬菌体展示筛选方法在CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds.Coligan等人(John Wiley&Sons,1997)中的19章提供。

[0071] 相应地,所述方法的初始步骤可包括鉴定多个候选分子,这些候选分子根据广泛的结构和/或功能属性(例如结合CD96的能力)所选择。

[0072] 所述方法可能进一步包括测量或检测对候选分子应答的一种或更多种CD96的生物学活性变化的步骤。这些可能包括CD155结合、细胞内信号传导、细胞因子和/或趋化因子产生或分泌和/或在体内模型中对肿瘤挑战的保护。

[0073] 候选分子抑制CD155结合CD96可以通过现有技术已知的数个技术测定,包括竞争放射性配体结合试验、表面等离子体共振(例如BIACore™分析)、免疫共沉淀和候选抑制因子封闭CD155结合CD96能力的基于荧光的分析(例如通过流式细胞术,其中CD155标记有荧光团)。荧光团的非限制性实施例包括异硫氰酸荧光素(FITC)、别藻蓝蛋白(APC)、荧光素衍生物例如FAM和ROX、德克萨斯红(Texas Red)、异硫氰酸四甲基若丹明(tetramethylrhodamine isothiocyanate,TRITL)、藻红蛋白(R-Phycoerythrin,RPE)、Alexa和氟硼二吡咯(Bodipy)荧光素。

[0074] 或者,该基于荧光的分析包括FRET分析(例如一种蛋白偶联于供体荧光团,另一种偶联于受体荧光团),而不仅限于此。

[0075] 在一些实施方案中,细胞内信号传导可以在CD96水平直接测量,例如通过测量NK细胞或T细胞亚群表达的CD96的含SH2结构域的PTPases的募集。本发明的候选分子适当地防止或减少存在CD155时,CD96募集的含SH2结构域的PTPases。根据该实施方案,候选分子可至少部分抑制或防止CD96和CD155之间的结合,从而至少部分抑制或防止通过CD96的细胞内信号传导,和/或至少部分抑制或防止尽管结合CD155的通过CD96的细胞内信号传导。

[0076] 在其它实施方案中,候选分子对CD96的作用可以通过测量表达CD96的细胞所表达、产生和/或分泌的一种或更多种细胞因子或趋化因子来测定。一般地,细胞因子或趋化因子表达产生和/或分泌的变化可以在基因表达的水平测量(例如通过细胞因子mRNA的RT-PCR),位于细胞内的细胞因子或趋化因子蛋白的测量(例如通过使用细胞因子或趋化因子特异的抗体的免疫细胞化学)和/或例如通过流式细胞术细胞因子微球阵列术(Cytokine Bead Array)(例如来自BD Biosciences商业提供)测量分泌的细胞因子或趋化因子,通过使用细胞因子或趋化因子特异的抗体的ELISA和通过使用细胞因子或趋化因子应答细胞系的生物测定来测量分泌至细胞上清中的细胞因子和/或趋化因子。这些细胞因子可以是任何促炎细胞因子或趋化因子,包括MIP-1α、MIP-1β、RANTES、TNF-α和IFN-γ,而不仅限于此。

优选地，所述细胞因子是IFN-γ。

[0077] 优选地，候选分子的CD96抑制作用可以使用体内肿瘤挑战模型测定。例如，使用表达CD96的小鼠的鼠模型可以用于测定候选分子抑制或防止对施用致癌试剂(例如甲基胆蒽(MCA))应答的肿瘤形成和/或生长的能力。在另一个实施例中，使用表达CD96的小鼠的鼠模型用于测定候选分子抑制或防止对肿瘤细胞(例如黑色素瘤、结肠腺癌、前列腺癌和乳腺癌，而不仅限于此)应答的肿瘤形成和/或生长的能力。其它鼠模型可利用倾向于自发形成肿瘤的小鼠，肿瘤包括但不限于MMTV-多瘤、MT乳腺癌、DMBA/TPA诱导的皮肤癌、p53缺失的淋巴瘤/肉瘤以及TRAMP Tg前列腺癌。

[0078] 可以理解，在这个方面的方法可以通过实施多轮筛选、设计和生物学测试反复的实施。这可以包括候选分子在每一轮之前经结构修饰的情况，从而使候选分子“微调(fine-tuning)”。

[0079] 应当意识到所述方法可以以“高通量”、“自动化”或“半自动化”的方式实施，具体地，在候选分子鉴定和选择的早期阶段。

[0080] 在一个优选的实施方案中，所述候选分子是抗体或抗体片段。如上文中所述，抗体可以是多克隆的或者单克隆的，天然的或者重组的。抗体片段包括Fab和Fab'2片段，双体或单链抗体片段(例如scVs)，而不仅限于此。可以查询适用于抗体生产、选择、纯化和使用的公知的方案例如在Coligan等人CURRENT PROTOCOLS IN IMMUNOLOGY(John Wiley&Sons NY,1991-1994)的2章中和Harlow,E.&Lane,D.Antibodies:A Laboratory Manual,Cold Spring Harbor,Cold Spring Harbor Laboratory,1988,二者均通过参考文献包含在本文中。

[0081] 例如可以通过注射CD96或者其片段(例如多肽)至生产物种以获得多克隆血清从而制备多克隆抗体，生产物种包括小鼠或兔。本领域技术人员公知生产多克隆抗体的方法。可使用的示范步骤例如在Coligan等人CURRENT PROTOCOLS IN IMMUNOLOGY，上文，以及在Harlow&Lane,1988，上文中描述。

[0082] 可以使用标准方法生产单克隆抗体，例如包含在本文中参考文献的Köhler & Milstein,1975,Nature 256,495文章中所描述，或者通过更多的其最近修订版本，例如在Coligan等人CURRENT PROTOCOLS IN IMMUNOLOGY，上文中所描述，使用衍生于生产物种的永生化脾或其它抗体生产细胞，所述生产物种已使用本发明的一种或更多种分离的蛋白、片段、变体或衍生物接种。适当地，所述抗体或抗体片段适合用于施用给人类。在本文中，如前所述，抗体或抗体片段可以是生产自或者起源于另一物种的抗体或抗体片段的“人源化”形式。该方法在现有技术中公知，并且一般涉及重组“接枝”非人类抗体互补决定区(CDRs)至人类抗体支架或骨架。

[0083] 在一个优选的实施方案中，在施用于人类时，抗体或抗体片段不杀伤表达CD96的细胞。在本文中，“杀伤”可指抗体介导的细胞毒机制，如补体介导的细胞裂解和抗体介导的细胞介导的细胞毒作用(ADCC)，后者典型地通过自然杀伤(NK)细胞、巨噬细胞、中性粒细胞和嗜酸性粒细胞介导。就这一点而言，可能有利地使用缺少Fc部分或具有突变的Fc部分的抗体片段(例如人源化抗体)。

[0084] 根据前述方面筛选、设计、工程化或者生产的CD96抑制试剂可以用于根据第一方面的方法中(例如作为抗癌症试剂和/或抗病毒试剂)，优选地，以如前所述的药物组合物的

形式。

[0085] 因此，参考以下非限制性的实施例，本发明可很容易理解，并且付诸实际作用。

[0086] 实施例

[0087] 实施例1

[0088] CD96结合CD155以及在小鼠肿瘤模型中CD96抑制和敲除的作用

[0089] 材料和方法

[0090] 小鼠

[0091] 野生型C57BL/6小鼠购自沃尔特和伊莱扎霍尔医学研究所(Walter and Eliza Hall Institute for Medical Research)或ARC动物资源中心。C57BL/6CD96$^{-/-}$小鼠由Marco Colonna博士和Susan Gilfillan博士在华盛顿大学医学院(St Louis,MO,USA)如下产生。设计靶向构建体用两侧为loxP位点的MC1-neor基因替换CD96的外显子1和2(包括起始位点)，电穿孔至E1 4.1(129P2/O1aHsd)胚胎干细胞中(图S1)。注射至C57BL/6囊胚后从两个克隆获得递送靶向等位基因的嵌合体。携带靶向等位基因的小鼠繁殖成在CMV启动子下表达Cre转基因的C57BL/6小鼠，从而删除MC1-neor基因(Schwenk等人,1995)。在每一代，通过10厘摩间隔的多态性微卫星标记的全基因组筛选帮助下，CD96删除回交C57BL/6背景。CD96$^{+/-}$>99％C57BL/6小鼠杂交生成CD96$^{-/-}$小鼠。已经描述DNAM-1$^{-/-}$小鼠。DNAM-1$^{-/-}$CD96$^{-/-}$通过CD96$^{-/-}$和DNAM-1$^{-/-}$小鼠杂交生成。饲养小鼠并在6-14周龄使用。所有实验经动物伦理委员会的批准。

[0092] 细胞培养

[0093] B16F10、RM-1、3LL、AT3、MC38和YAC-1细胞系在完全RPMI培养基(Gibco，Invitrogen,)中，即添加有10％FCS(Thermo Scientific)、L-谷氨酰胺(Gibco)、非必需氨基酸、丙酮酸钠、HEPES(Gibco)以及青霉素/链霉素(Gibco)，在37℃5％的CO_2中生长。对于细胞毒性试验和IL-12/IL-18滴定实验，从脾收集原代NK细胞，使用小鼠NK细胞分离试剂盒(Miltenyi Biotec)和AutoMACS(Miltenyi Biotec)分选，并且随后在添加有10％FCS、L-谷氨酰胺、青霉素/链霉素、非必需氨基酸(Gibco)、丙酮酸钠(Gibco)、HEPES(Gibco)、β-2-巯基乙醇(Calbiochem)、以及1000IU/ml重组人类IL-2(Chiron Corporation)的RPMI培养基中培养5天。所有细胞在37℃5％的CO_2中孵育。

[0094] 在体内LPS挑战

[0095] 悬浮于PBS中的LPS(来自E.Coli 0127:B8,Sigma)以所述剂量腹腔注射至小鼠。对于生存曲线，每小时地检查小鼠脓毒症(sepsis)的症状。通过眼球(retro-orbital)或心脏采血，在不同时间点取来自这些小鼠的血清用于细胞因子的分析。还在不同时间点取脾以分析来自NK细胞的受体和配体的表达，以及细胞内IFN-γ的表达。

[0096] 在体内肿瘤挑战

[0097] 小鼠B16F10或B16-OVA黑色素瘤、RM-1前列腺癌、3LL肺癌、MC38-OVA^{dim}结肠癌或AT3-OVA^{dim}乳腺癌分别以所示剂量皮下或静脉注射至WT、DNAM-1$^{-/-}$、CD96$^{-/-}$、或者DNAM-1$^{-/-}$CD96$^{-/-}$小鼠，并且监测实体瘤生长或转移。如图例中所示施用治疗。为监测实体瘤生长，通过卡尺测量明显的肿瘤长度和宽度计算确实的(ensuing)肿瘤的面积并针对时间制图。为监测转移的形成，细胞注射14天后，收集肺，放置在Bouin's固定剂中，并且使用解剖显微镜计数转移。

[0098] MCA诱导纤维肉瘤

[0099] WT、DNAM-1$^{-/-}$、CD96$^{-/-}$和DNAM-1$^{-/-}$CD96$^{-/-}$小鼠在右侧肋腹皮下注射不同剂量的MCA(5-400μg,例如100μg MCA),并且随时间监测纤维肉瘤形成。此外,一些小鼠用对照抗体处理,通过使用抗去唾液酸(asialo)GM1(Wako化学品;在-1、0天以及随后每周直至第八周i.p.注射100μg)耗尽NK细胞,中和IFN-γ(H22,在-1、0天以及随后每周直至第八周i.p.注射250μg),中和CD155,中和DNAM-1或者CD96。

[0100] 树突状细胞(BMDC):NK细胞共培养试验

[0101] 如前述产生BMDC。简要地,我们从小鼠的股骨和胫骨收集骨髓细胞,并且在添加有10%FCS、L-谷氨酰胺、青霉素/链霉素、非必需氨基酸、丙酮酸钠、β-2-巯基乙醇和250ng/ml GM-CSF(eBioscience)的DMEM中培养6天。从脾收集WT或者CD96$^{-/-}$NK细胞,并且FACS分选以通过NK1.1(PK136)和TCRβ(H57-597)和CD3(17A2)抗体染色纯化。在试验当天收集NK细胞。对于试验设置,5x 10^4BMDM接种于96孔U底板。接着以不同的滴度(2:1、1:1、0.5:1、和0.25:1)加入NK细胞至BMDM。试验中始终包括仅BMDM和仅NK作为对照。所有细胞一经接种,每孔注入适量的培养基,以使各孔之间体积相等。接着加入100ng/ml的LPS至孔内持续2h,接着加入5mM纯化的ATP(Sigma)持续30分钟。在37℃5%CO_2中执行。试验中还包括仅LPS和仅ATP对照作为对照。在使用ATP 30分钟后,收集上清并存储于-20℃直至分析。

[0102] ^{51}Cr细胞毒性试验

[0103] 使用标准的^{51}Cr细胞毒性试验以分析WT和CD96$^{-/-}$NK细胞杀伤靶标的能力。简要地,标记有100μCi的^{51}Cr的20,000靶标加入V底板,并且接着以确定的效靶比添加NK细胞至靶标。在37℃5%CO_2中4小时后,收集上清,并且通过γ计数器(Wallac Wizard)定量^{51}Cr的水平。使用公式(样本Cr释放-自发Cr释放)/(总Cr释放-自发Cr释放)x 100来确定特异性杀伤百分比。

[0104] 细胞因子检测

[0105] 在血清或上清中除IL-18以外的所有细胞因子的检测通过使用细胞微球阵列(Cytometric Bead Array,CBA)技术(BD Biosciences)实现。使用Canto II或者LSR II流式细胞术分析仪(BD Biosciences)完成采集(acquisition)。使用FCAP阵列软件执行分析。根据生产商说明书(MRL)通过ELISA检测IL-18。对于细胞内细胞因子检测,从肝脏获得分离的淋巴细胞,表面标记物染色,固定并使渗透(permeabilised)(BD Biosciences),并且用抗IFN-γ抗体(XMG1.2)染色。

[0106] 流式细胞术分析和分选

[0107] 免疫细胞内稳态和CD96/CD155表达的分析:不同的器官(淋巴结、肺、脾、骨髓和肝脏)被加工成单淋巴细胞悬液,悬液中包括红细胞裂解。起初1x 10^6和5x 10^6之间的细胞经过与2.4G2的孵育,用以在利用特异性抗体之前封闭非特异Fc抗体结合。为分析NK细胞内稳态和IFN-γ产生,使用以下抗体:抗小鼠-NK1.1、-TCRβ、-CD27(LG.7F9)、-CD1 1b(M1/70)、以及-IFN-γ。对于T细胞:抗小鼠-TCRβ、-CD8(53-6.7)和-CD4(RM4-5)。对于B细胞抗小鼠-B220(RA3-6B2)、-CD19(1D3)。对于NKT细胞:负载有α-半乳糖神经酰胺(a-galactosylceramide)的小鼠CD1d四聚体(由Dale Godfrey教授友善提供,墨尔本大学)、抗小鼠-TCRβ或者-CD3、-CD4、和-NK1.1。对于巨噬细胞:抗小鼠-F4/80(BM8)和-CD11b。对于中性粒细胞:抗小鼠-Ly6G(1A8)和-CD11b。对于传统DC:抗小鼠-MHC II(M5/114.15.2)和-

CD11c(N418)。对于γδT细胞:抗小鼠-γδTCR(GL3)和-CD3。为分析CD96和CD 155表达,通过使用的以上抗体混合物(cocktails)连同抗小鼠-CD96(3.3.3)或者抗小鼠-CD 155(4.24.3)对感兴趣的特异细胞类型设门(gated)。使用LSRII或者Canto II流式细胞术分析仪(BD Biosciences)完成采集。使用Flowjo(Treestar)实现分析。

[0108] 细胞分选

[0109] 如上所述准备和染色来自脾的天然NK细胞和巨噬细胞。这些细胞接着使用Aria II FACS分选仪(BD Biosciences)分选以纯化。

[0110] 统计学分析

[0111] 使用Graphpad Prism软件实现统计学分析。当p值等于或者小于0.05时认为是统计上显着的。所使用的统计学检验为非配对t检验(unpaired Student's t test)、Mann-Whitney t检验、和用于生存的Mantel-Cox检验。如图例中确定所使用的合适的检验。

[0112] 结果

[0113] CD96与DNAM-1竞争结合CD 155(图1),以及CD155结合CD96下调NK细胞产生的IFNγ(图2)。在MCA处理的小鼠中,CD96限制依赖NK细胞的肿瘤免疫监视,并且促进实验性B16F10肺转移(图3)。

[0114] 图4中的数据显示抗CD96mAb具有单独试剂活性(即无抗PD1处理),同时还提高抗PD1的抗肿瘤应答。与图6相一致,抗CD96mAb处理还提高由阿霉素(DOX)化疗产生的抗肿瘤应答(图5和7),图6中在CD96缺陷宿主中观察到对阿霉素(DOX)化疗提高的抗肿瘤应答。关于图8和9,指定的早期或者后期抗CD96mAb提高由抗PD-1和抗CTLA-4mAb产生的抗肿瘤应答,并且特别地显示与抗PD-1的强协同作用。

[0115] 还研究了CD96在肿瘤转移的促进中的作用。在图10中,在C57BL/6野生型(WT)、DNAM-$1^{-/-}$、$CD96^{-/-}$和DNAM-$1^{-/-}CD96^{-/-}$小鼠中研究B16F10肺转移的调节。在图11中,宿主CD96促进RM-1肺转移,在图12中,宿主CD96促进3LL肺转移,图13显示抗CD96mAb单独和与T细胞检查点封闭的结合抑制B16F10肺转移。在图14中,抗CD96mAb单独和与T细胞检查点封闭的结合抑制RM-1肺转移。在图15中,抗CD96mAb提高由抗PD-1和抗CTLA-4mAb所产生的针对MC38结肠肿瘤的抗肿瘤应答,并且特别地显示与抗PD-1的协同作用。

[0116] 实施例2

[0117] 用于鉴定抗CD96抗体的筛选试验

[0118] 前言

[0119] 以下试验可用于鉴定本发明中有用的抗体。第一个试验可用于鉴定能够封闭或抑制人类CD96和人类CD155之间的结合的人类抗体。第二个试验可用于检验所鉴定的抗体是否引起抗体依赖的细胞介导的细胞毒作用(ADCC)。第三个试验接着能应用于指导候选并且涉及测定人类CD96抗体是否能够调节人类淋巴细胞效应功能。

[0120] 材料和方法

[0121] 试验1:CD96结合CD155

[0122] 用以防止CD155结合于表达CD96的细胞(例如NK细胞)的细胞表面的候选抗CD96抗体的能力将按以下检验。融合于人类IgG1的C端Fc区域的重组人类CD155(例如可从Sino Biological获得的CD155-Fc)将使用荧光团标记,例如Alexa Fluor 647(AF647),根据生产商说明书,使用Zenon人类IgG标记试剂盒(Molecular Probe)。从健康供体的外周血新鲜分

离的NK细胞或者其它表达CD96的细胞将在不同浓度的抗CD96抗体或者对照Ig存在时，与AF647标记的CD155-Fc孵育(将收集细胞并且通过流式细胞术检验细胞表面AF647-CD155-Fc的结合)。通过其封闭CD 155-Fc结合于表达CD96的细胞的能力鉴定防止CD155细胞结合于表达CD96的细胞的抗体。

[0123] 试验2:ADCC试验

[0124] 在存在抗CD96抗体时，免疫细胞(例如NK细胞和/或T细胞)的生存将按以下分析。来自健康供体的外周血免疫细胞将通过Ficoll梯度分离而分离。免疫细胞将铺板于存在合适剂量的人类IL-2以及递增浓度的抗CD96mAb的96孔板中。表达CD96的细胞(例如NK细胞和/或T细胞)的存活和百分比将通过流式细胞术随时间分析。用于该试验的合适的商业可提供的试剂盒的非限制性实施例为Annexin V凋亡检测试剂盒。

[0125] 试验3:人类CD96抗体的人类白细胞效应功能的调节试验

[0126] 从健康供体收集新鲜血液样本。外周血单个核细胞(PBMC)将通过以Ficoll-Paque密度梯度离心制备。通过磁性激活细胞分选，从PBMC中获得高纯度CD3-CD56+NK细胞。为分析CD96影响人类NK细胞产生IFN-γ的能力，96孔U底板将用重组人类CD155-Fc嵌合体(Sinb Biological股份有限公司;0.5μg/孔)或者用无关人类IgG1抗体在4℃包被过夜。新鲜纯化的人类NK细胞接着将铺板于添加有人类IL-12和IL-18的RMPI介质中24小时，并且在不同的培养中分析细胞内含物和上清中IFN-γ的水平。或者，人类NK细胞将在包被有抗NKG2D、抗NKp46、抗NKp30或者抗CD16抗体的孔中刺激24小时，用以分析CD96信号传导与其它NK细胞受体相互作用的能力。将待检验的抗人类CD96抗体或对照抗体在上述细胞因子或抗体之前加入培养，以确认这些检验的抗人类CD96抗体提高人类NK细胞产生的IFNγ的能力。在IFNγ产生中高于对照的统计学增加将被认为是显著的。

[0127] 整个说明书的目的是描述本发明的优选实施方案，而不限制本发明到任何一个实施方案或特征的特定集合。因此，鉴于本公开内容，本领域技术人员将理解在示例的具体实施方案中可以进行各种修改和改变而不脱离本发明的范围。

[0128] 本文提及的所有计算机程序、算法、专利和科学文献以参考文献的方式并入本文。

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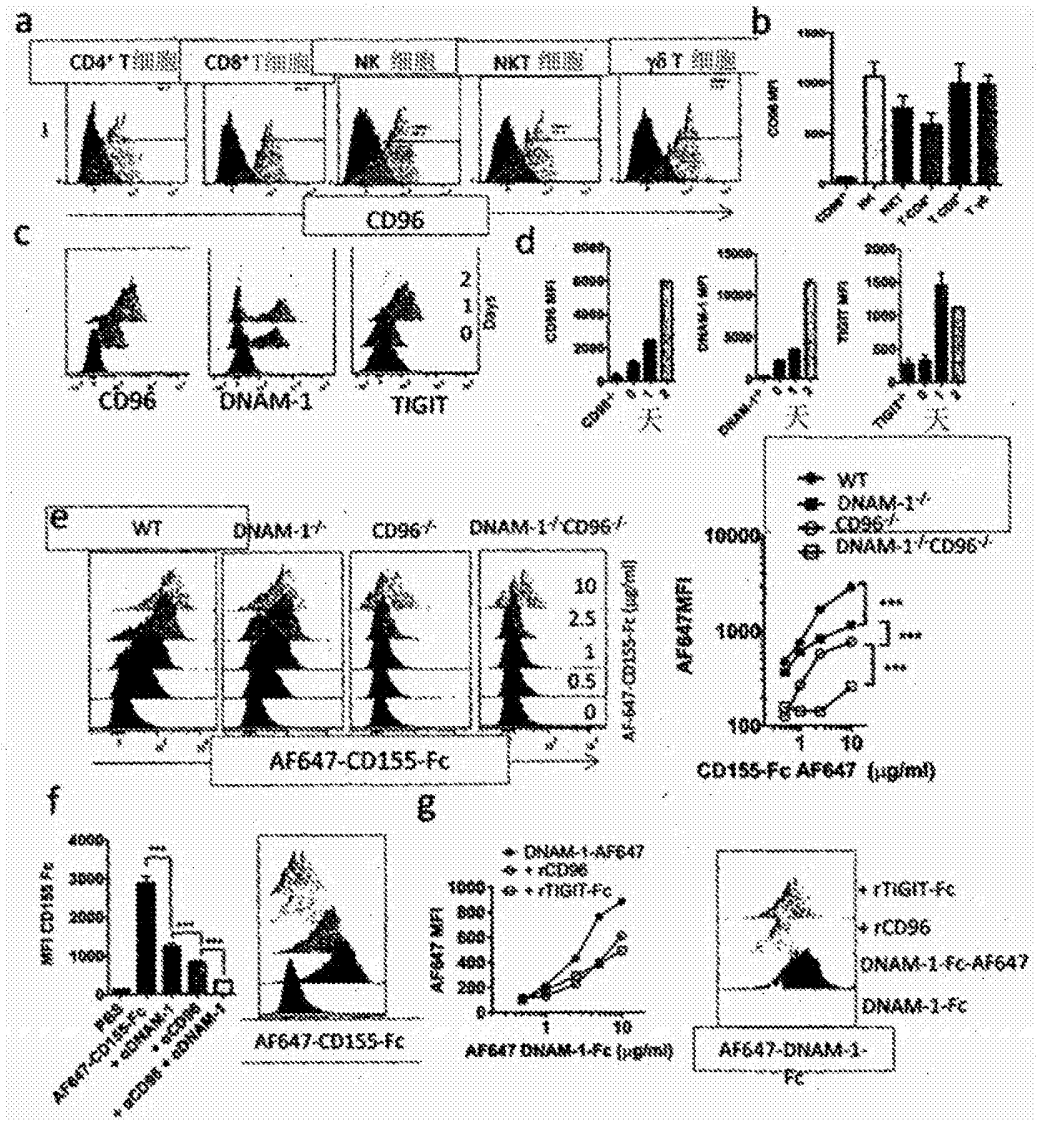

a
CD4+ T 细胞
CD8+ T 细胞
NK 细胞
NKT 细胞
γδ T 细胞
CD96
b
c
CD96
DNAM-1
TIGIT
d
天
e
WT
DNAM-1-/-
CD96-/-
DNAM-1-/-CD96-/-
AF647-CD155-Fc
AF647MFI
CD155-Fc AF647 (μg/ml)
f
PBS
AF647-CD155-Fc
+ αDNAM-1
+ αCD96
+ αCD96 + αDNAM-1
AF647-CD155-Fc
g
DNAM-1-AF647
+ rCD96
+ rTIGIT-Fc
AF647 MFI
AF647 DNAM-1-Fc (μg/ml)
+ rTIGIT-Fc
+ rCD96
DNAM-1-Fc-AF647
DNAM-1-Fc
AF647-DNAM-1-Fc

图1

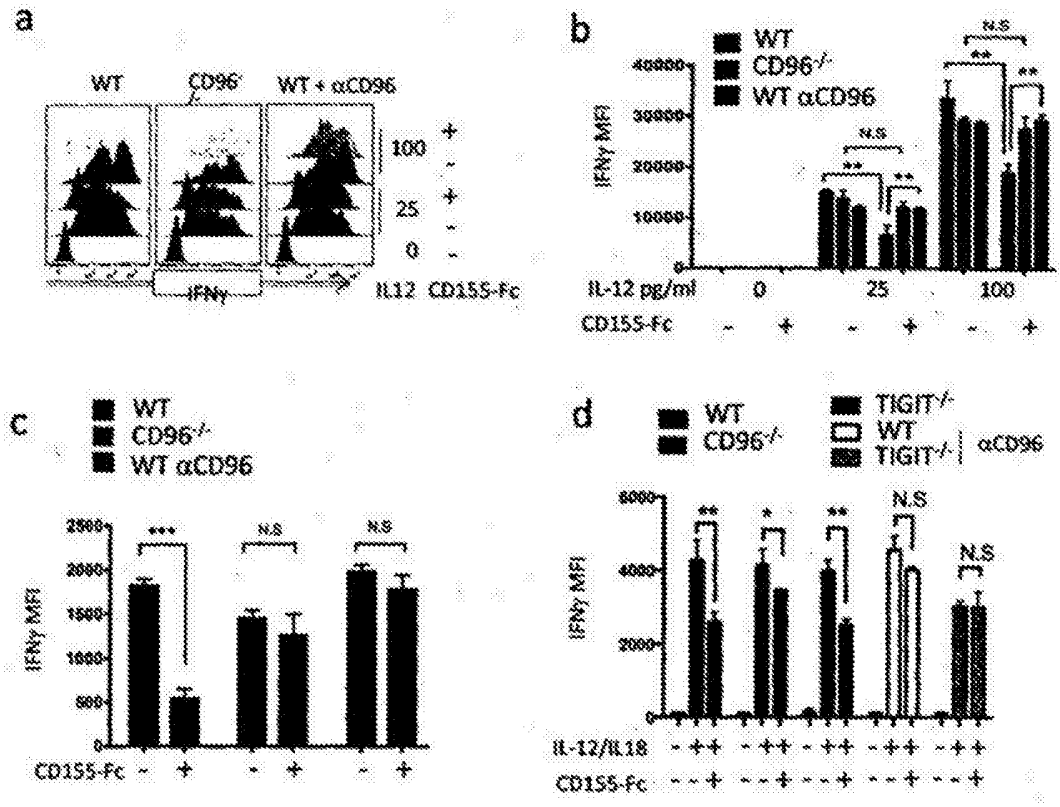

a
WT
CD96-/-
WT + αCD96
100
25
0
IFNγ
IL12 CD155-Fc
b
WT
CD96-/-
WT αCD96
IFNγ MFI
IL-12 pg/ml
CD155-Fc
N.S
c
WT
CD96-/-
WT αCD96
IFNγ MFI
CD155-Fc
d
WT
CD96-/-
TIGIT-/-
WT
TIGIT-/-
αCD96
IFNγ MFI
IL-12/IL18
CD155-Fc

图2

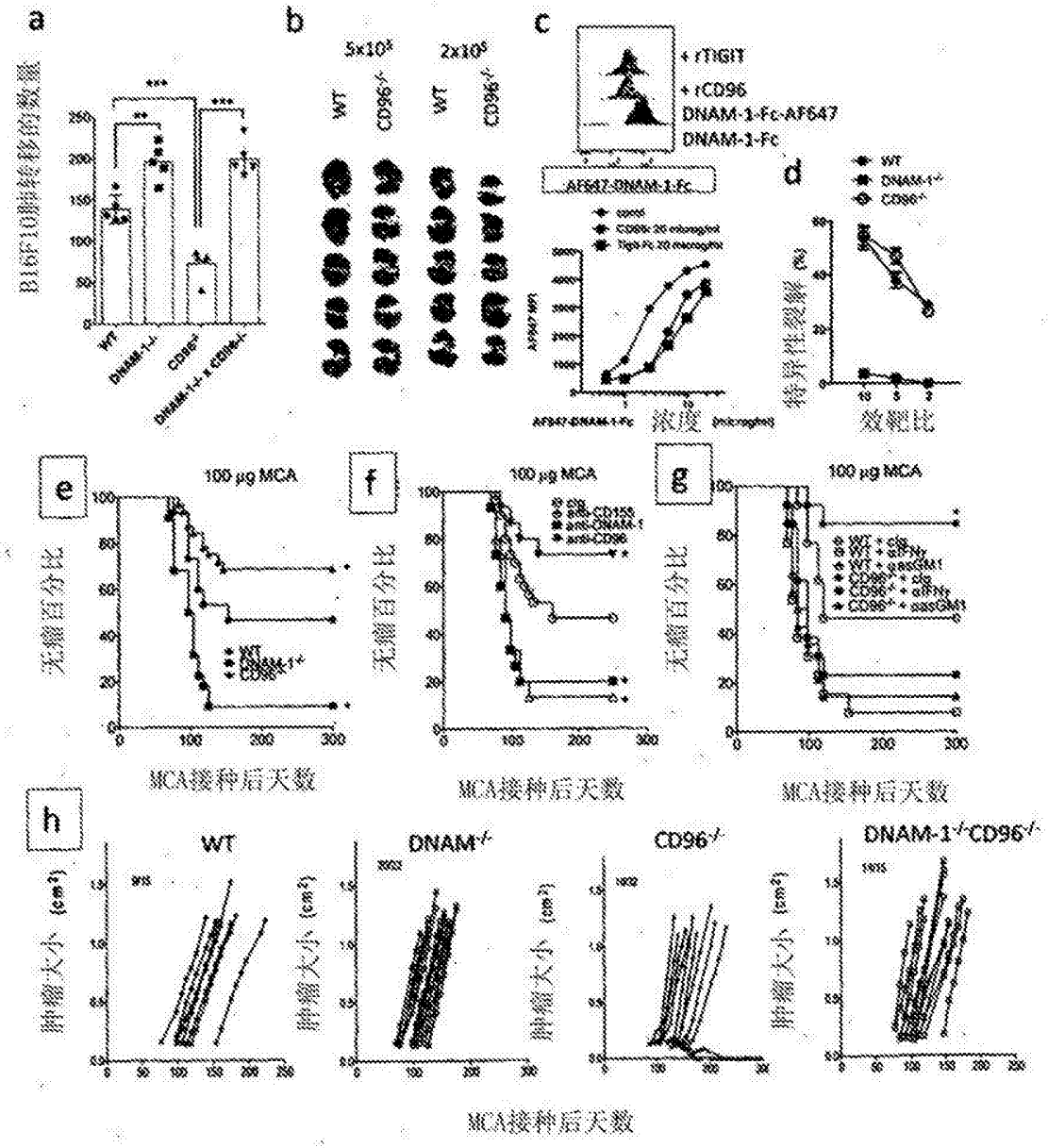

a
b
c
d
e
f
g
h
WT
CD96-/-
DNAM-1-/-
DNAM-1-/-CD96-/-
+ rTIGIT
+ rCD96
DNAM-1-Fc-AF647
DNAM-1-Fc
浓度
效靶比
特异性裂解
100 μg MCA
无瘤百分比
MCA接种后天数
肿瘤大小

图3

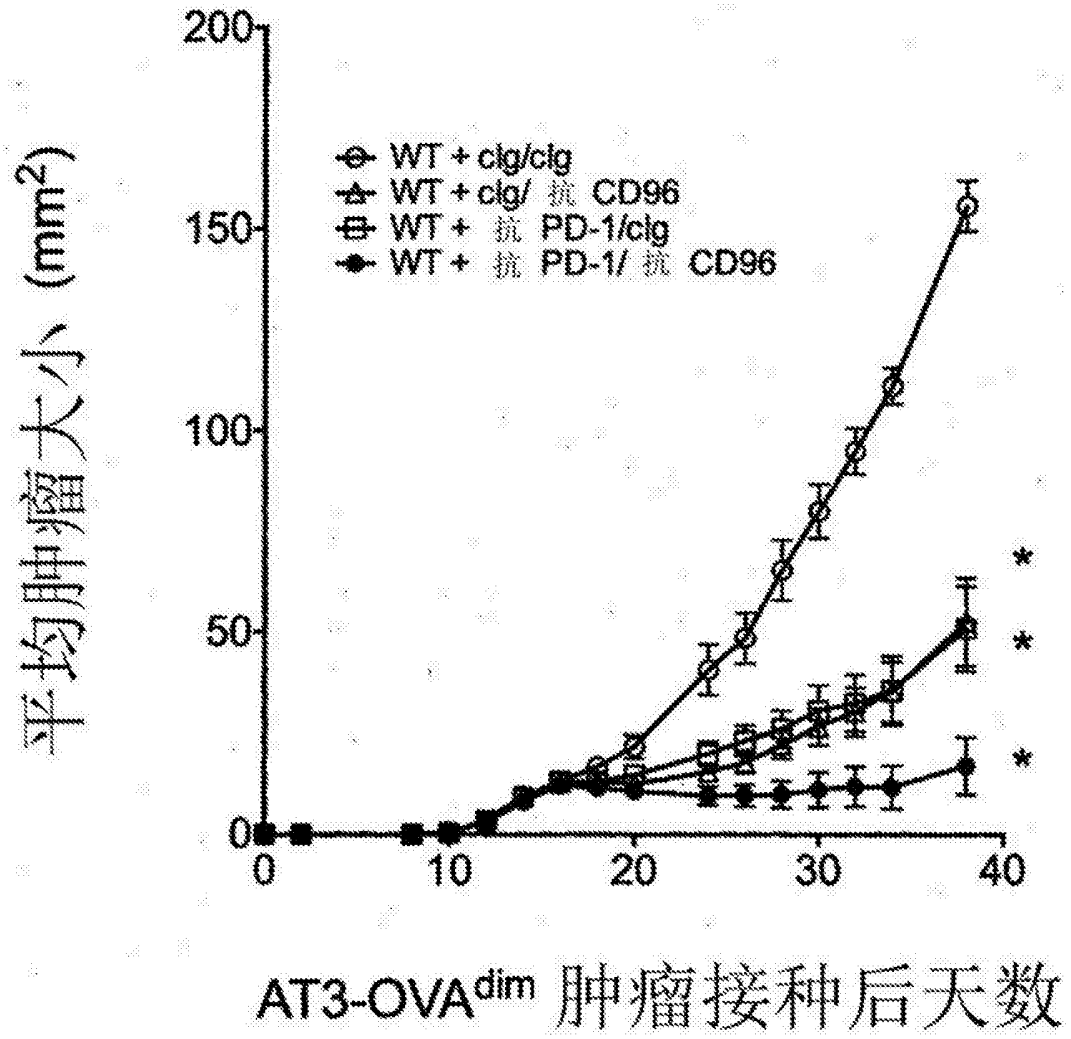

200
150
100
50
0
0
10
20
30
40
WT + clg/clg
WT + clg/ 抗 CD96
WT + 抗 PD-1/clg
WT + 抗 PD-1/ 抗 CD96
*
*
*
平均肿瘤大小 (mm2)
AT3-OVAdim 肿瘤接种后天数

图4

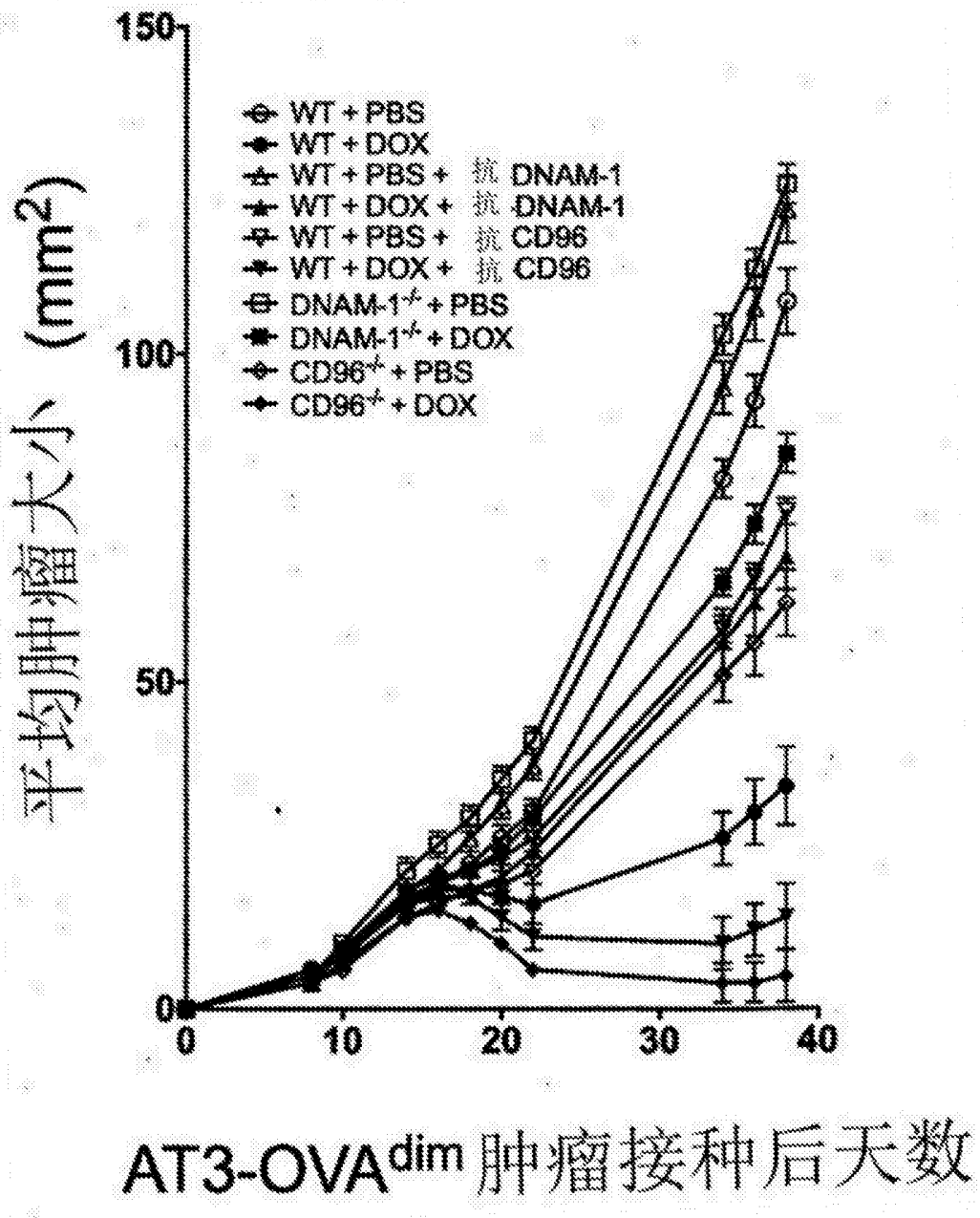

150
100
50
0
0
10
20
30
40
WT + PBS
WT + DOX
WT + PBS + 抗 DNAM-1
WT + DOX + 抗 DNAM-1
WT + PBS + 抗 CD96
WT + DOX + 抗 CD96
DNAM-1-/- + PBS
DNAM-1-/- + DOX
CD96-/- + PBS
CD96-/- + DOX
平均肿瘤大小 (mm2)
AT3-OVAdim 肿瘤接种后天数

图5

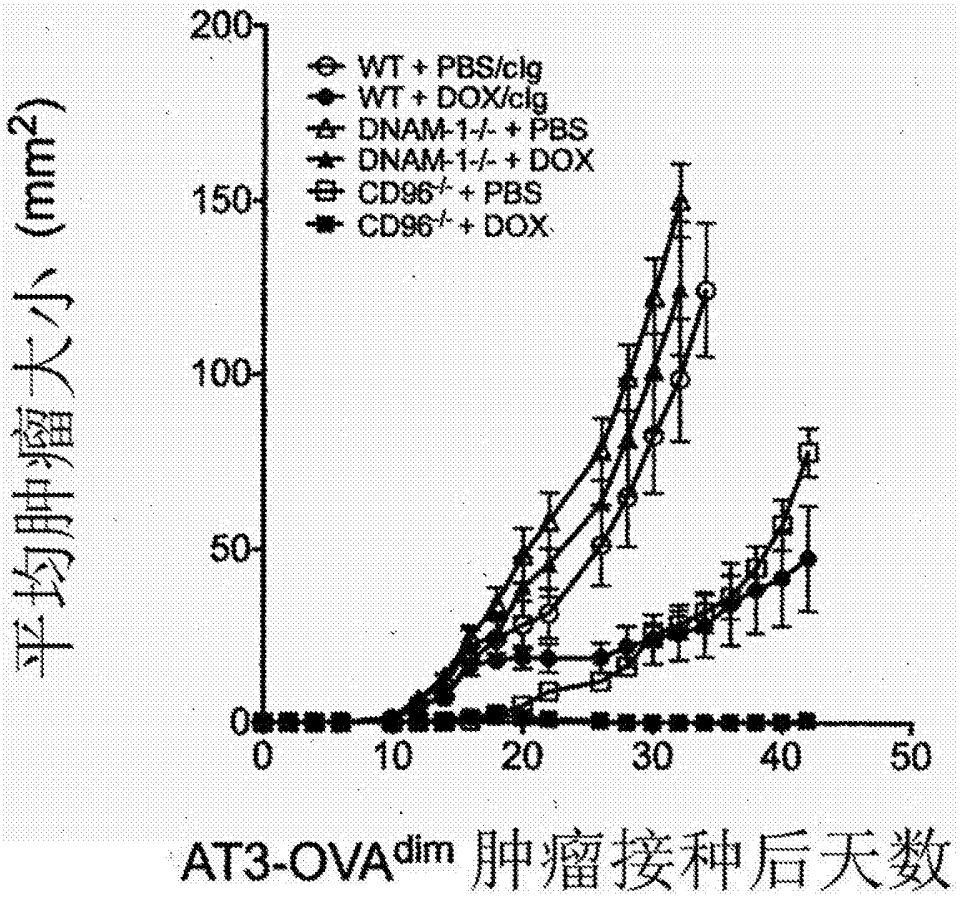

200
150
100
50
0
0
10
20
30
40
50
WT + PBS/clg
WT + DOX/clg
DNAM-1-/- + PBS
DNAM-1-/- + DOX
CD96-/- + PBS
CD96-/- + DOX
平均肿瘤大小 (mm2)
AT3-OVAdim 肿瘤接种后天数

图6

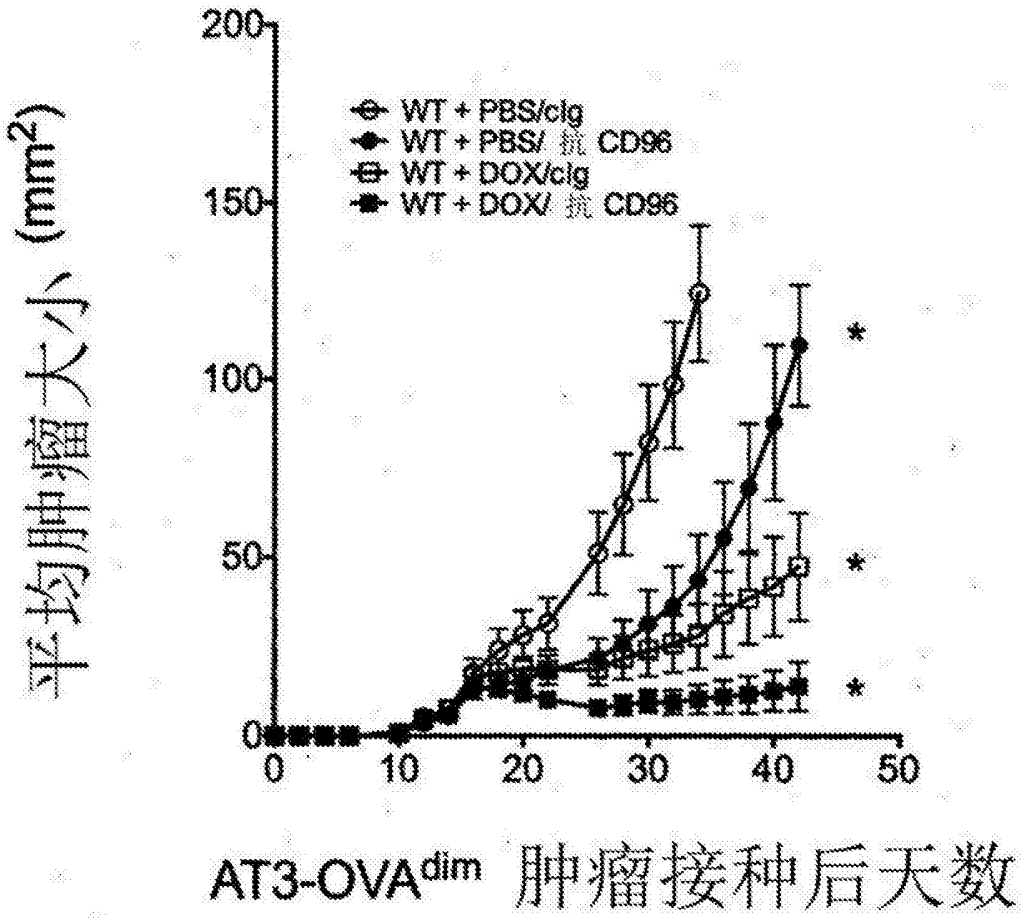

200
150
100
50
0
0
10
20
30
40
50
WT + PBS/clg
WT + PBS/ 抗 CD96
WT + DOX/clg
WT + DOX/ 抗 CD96
*
*
*
平均肿瘤大小 (mm2)
AT3-OVAdim 肿瘤接种后天数

图7

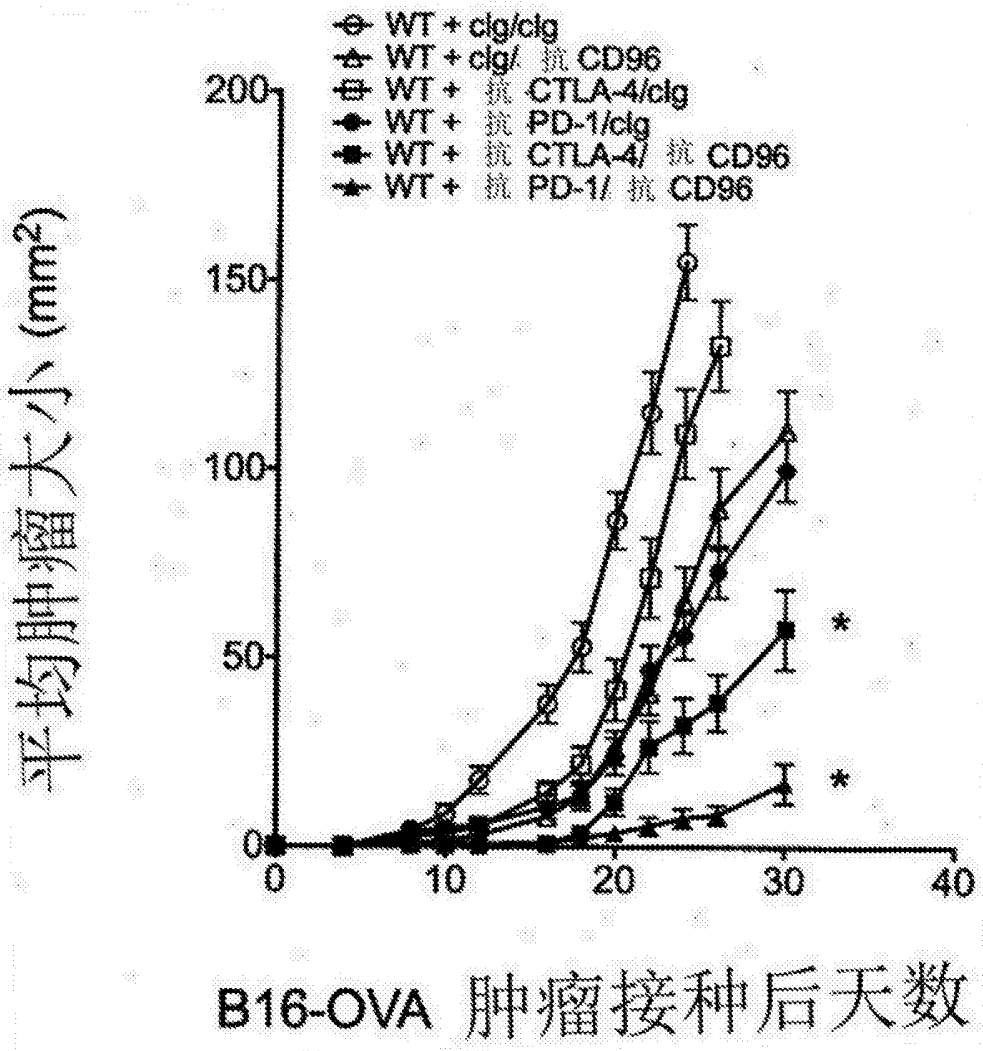

WT + clg/clg
WT + clg/ 抗 CD96
WT + 抗 CTLA-4/clg
WT + 抗 PD-1/clg
WT + 抗 CTLA-4/ 抗 CD96
WT + 抗 PD-1/ 抗 CD96
平均肿瘤大小 (mm²)
200
150
100
50
0
0
10
20
30
40
*
*
B16-OVA 肿瘤接种后天数

图8

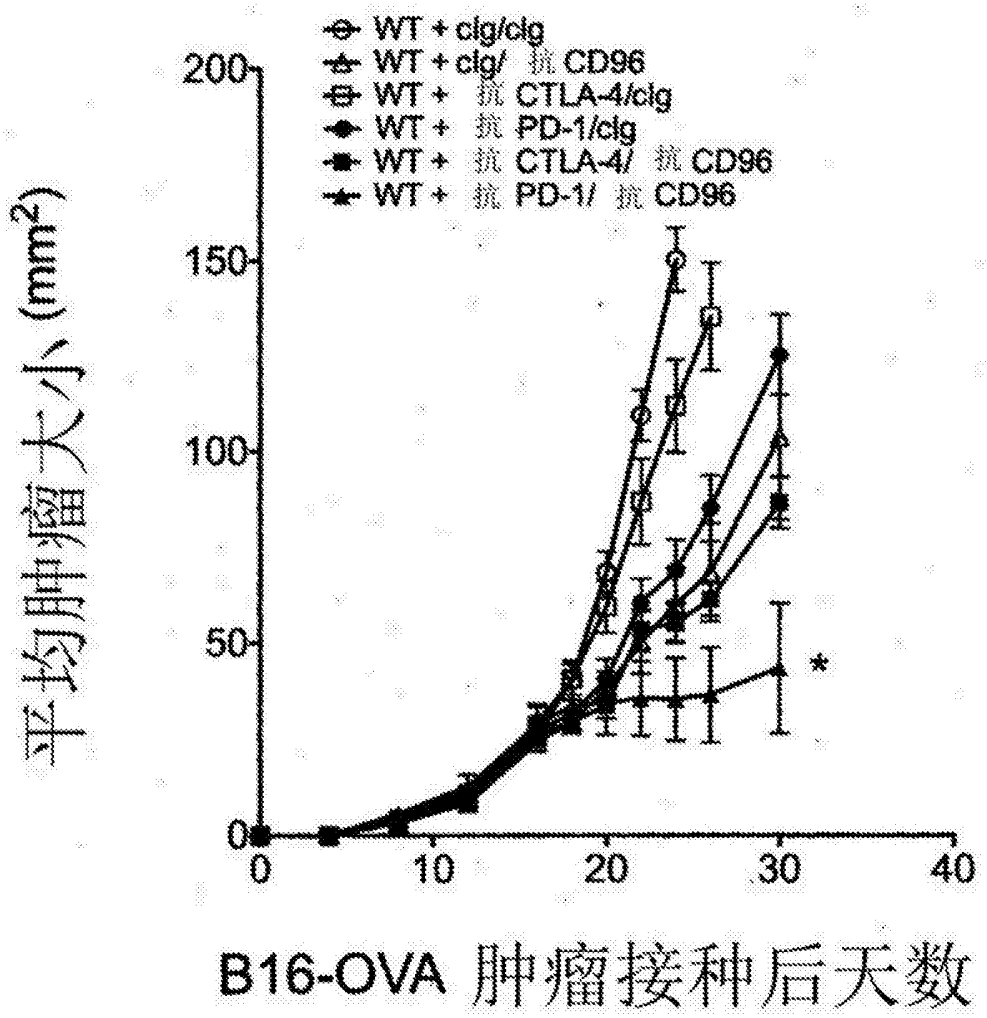
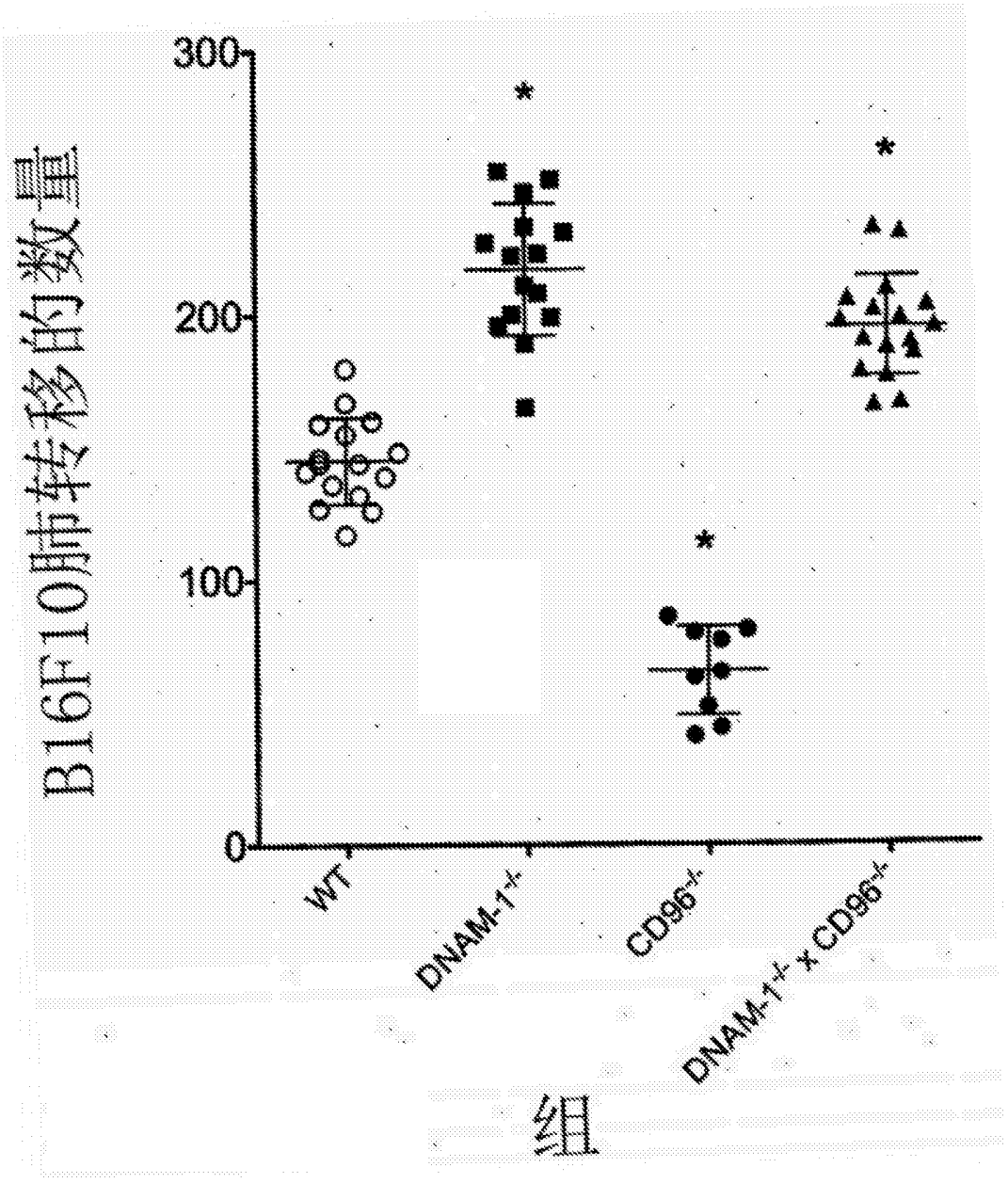

WT + clg/clg
WT + clg/ 抗 CD96
WT + 抗 CTLA-4/clg
WT + 抗 PD-1/clg
WT + 抗 CTLA-4/ 抗 CD96
WT + 抗 PD-1/ 抗 CD96
200
150
100
50
0
0
10
20
30
40
*
平均肿瘤大小 (mm2)
B16-OVA 肿瘤接种后天数

图9



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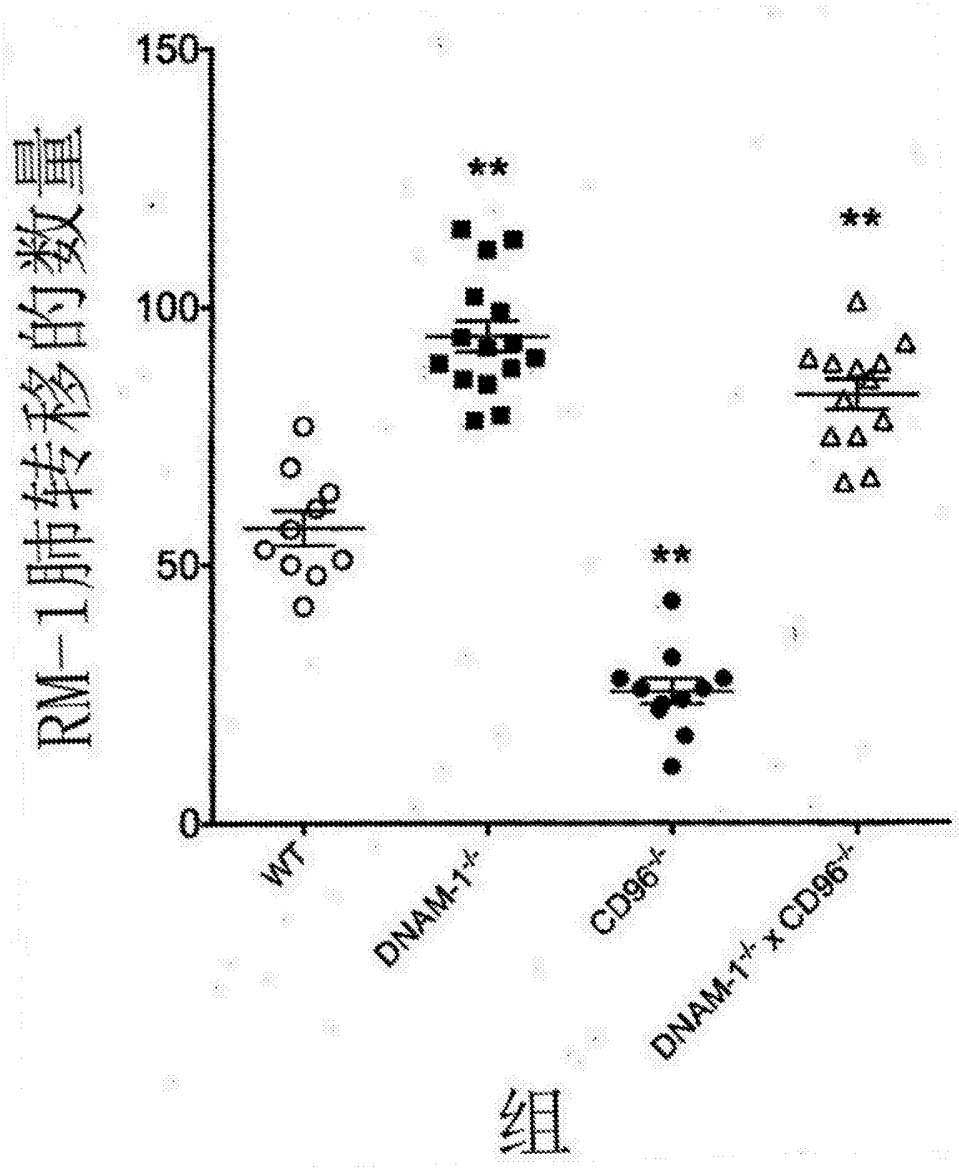

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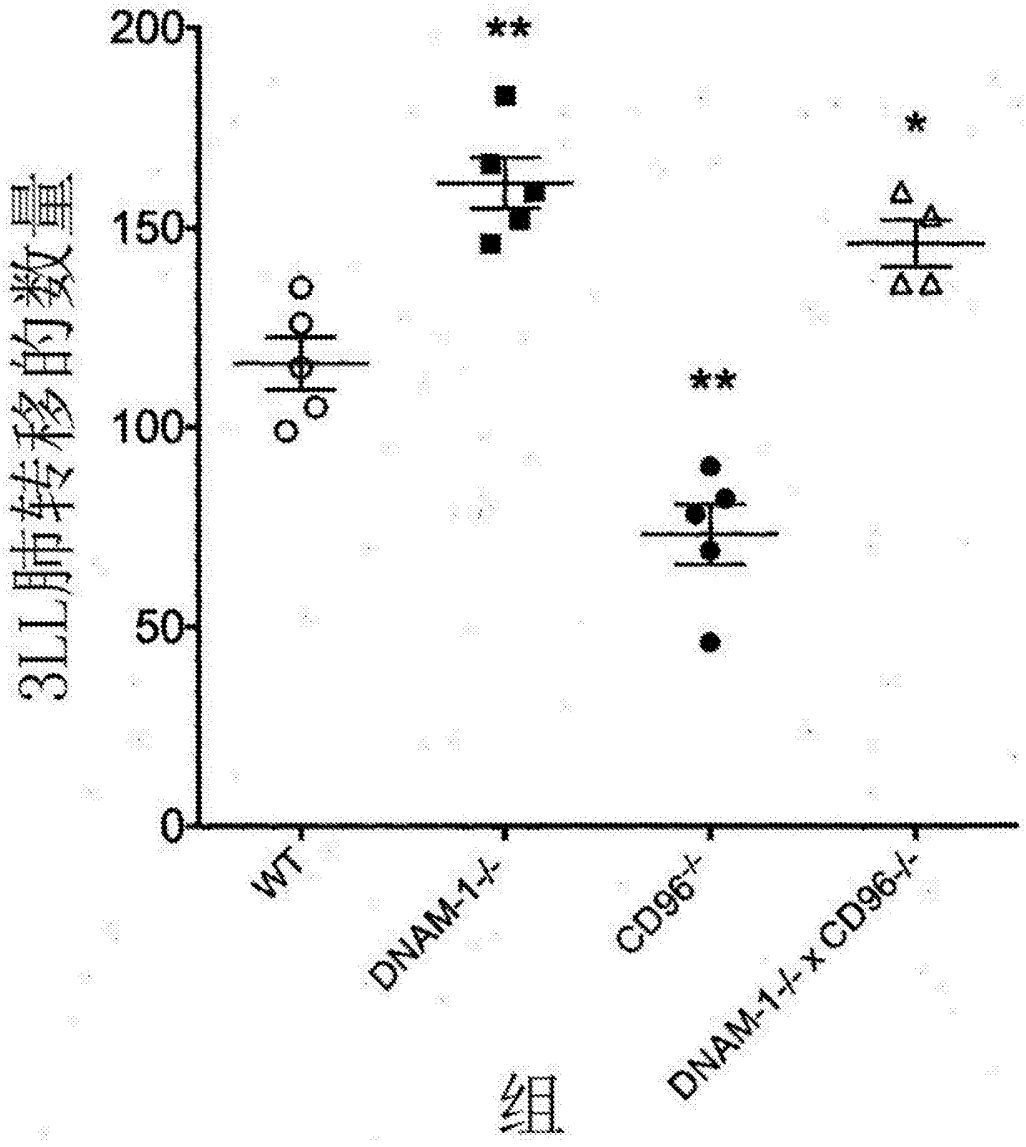

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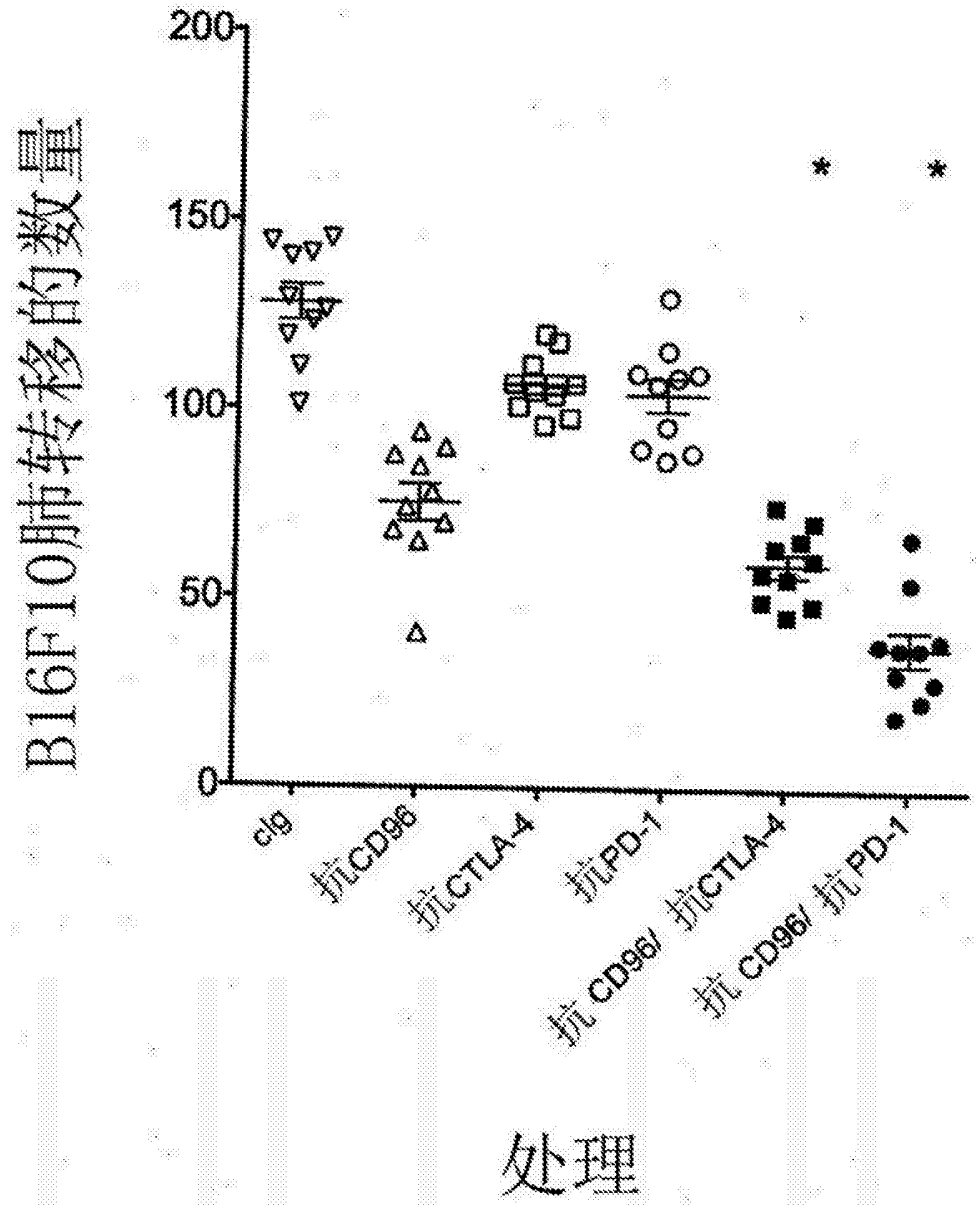
B16F10肺转移的数量
200
150
100
50
0
cIg
抗CD96
抗CTLA-4
抗PD-1
抗 CD96/ 抗CTLA-4
抗 CD96/ 抗 PD-1
处理

图13

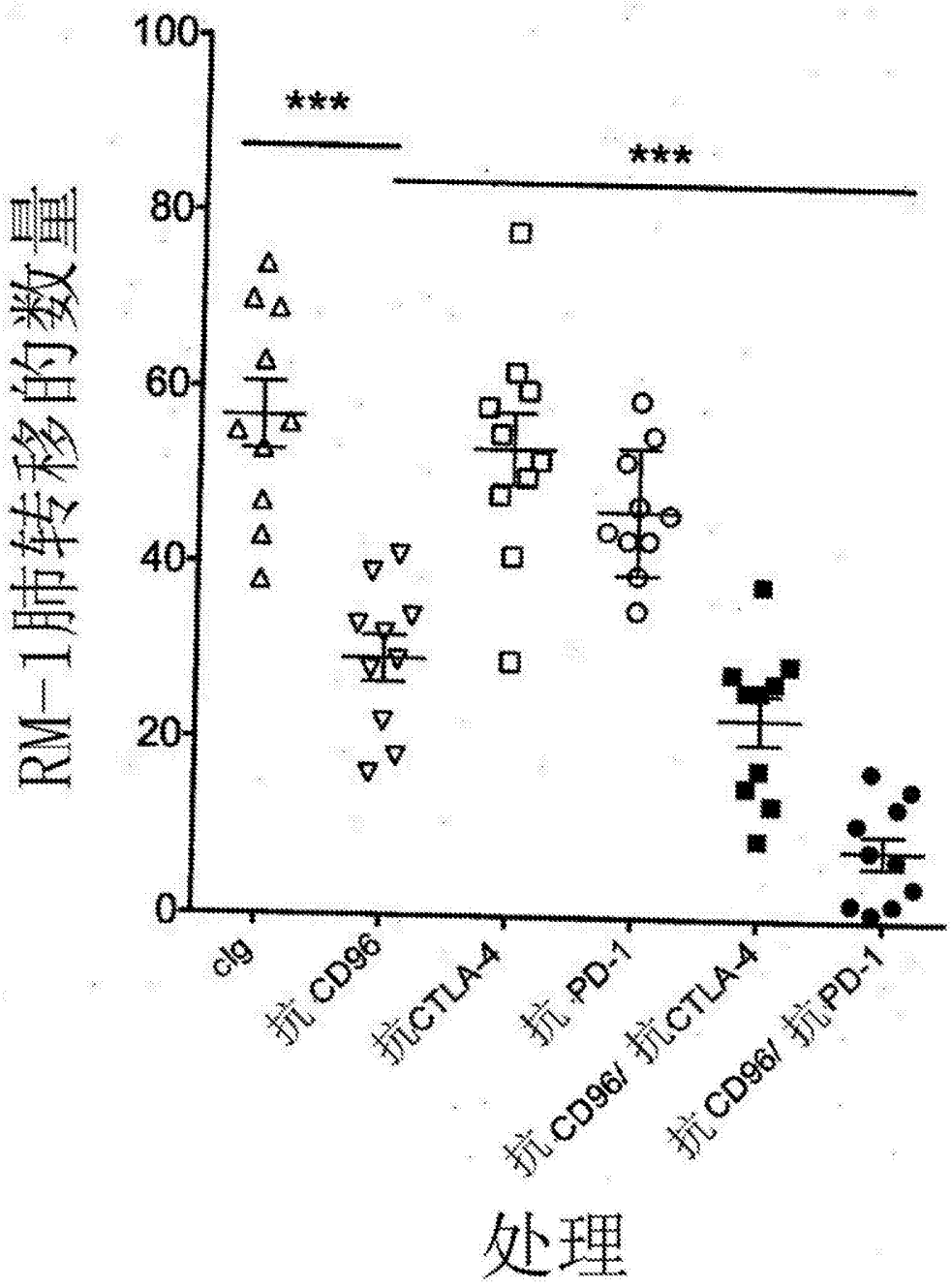
RM-1肺转移的数量
100
80
60
40
20
0
***
***
clg
抗CD96
抗CTLA-4
抗PD-1
抗CD96/抗CTLA-4
抗CD96/抗PD-1
处理

图14

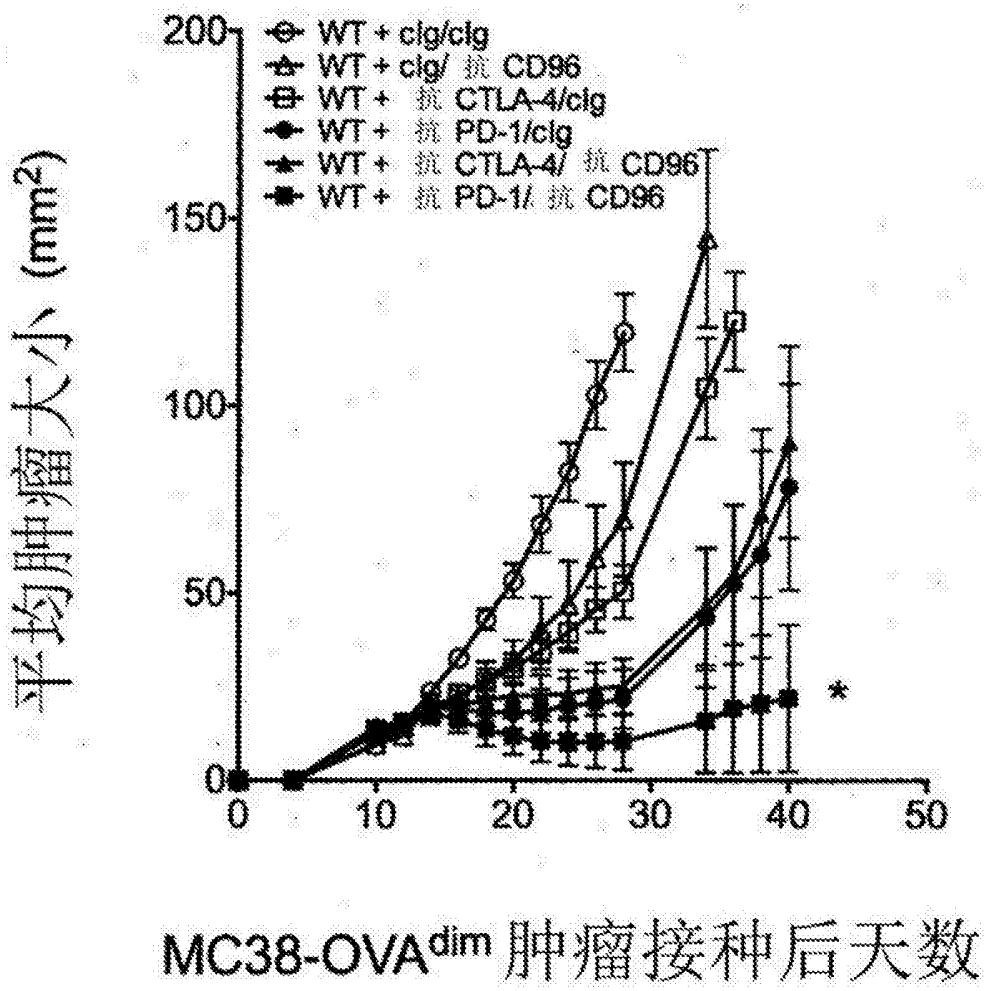
200
150
100
50
0
0
10
20
30
40
50
WT + clg/clg
WT + clg/ 抗 CD96
WT + 抗 CTLA-4/clg
WT + 抗 PD-1/clg
WT + 抗 CTLA-4/ 抗 CD96
WT + 抗 PD-1/ 抗 CD96
*
平均肿瘤大小 (mm2)
MC38-OVAdim 肿瘤接种后天数

图15