



(51) International Patent Classification:

A61K 38/00 (2006.01) C07K 14/765 (2006.01)

C07K 14/52 (2006.01) C12N 5/0786 (2010.01)

C07K 14/54 (2006.01)

(21) International Application Number:

PCT/US2017/035959

(22) International Filing Date:

05 June 2017 (05.06.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/345,591 03 June 2016 (03.06.2016) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,

SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

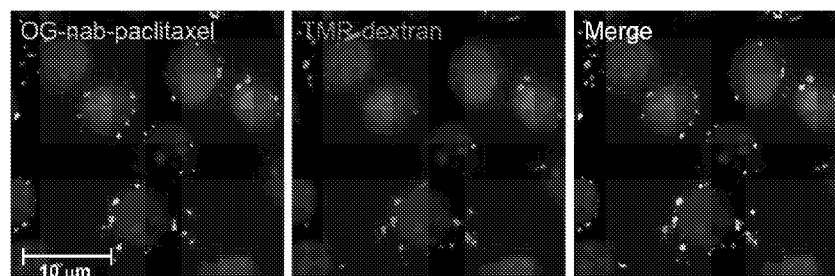
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(54) Title: METHODS AND REAGENTS FOR MODULATING MACROPHAGE PHENOTYPE

A



(57) **Abstract:** The present invention is directed to methods of inducing a phenotypic change in a population of monocytes and/or macrophages. The method includes administering to the population of monocytes and/or macrophages, a macrophage stimulating agent coupled to a carrier molecule, wherein the carrier molecule facilitates macropinocytic uptake of the agent by monocytes and macrophages in the population and is defective in neonatal Fc receptor binding, wherein the administering induces a phenotypic change in the monocytes and macrophages in the population.



METHODS AND REAGENTS FOR MODULATING MACROPHAGE PHENOTYPE

[0001] This application claims the priority benefit of U.S. Provisional Patent Application
5 Serial No. 62/345,591, filed on June 3, 2016, which is hereby incorporated by reference in its
entirety.

FIELD OF THE INVENTION

[0002] The present invention is directed to methods of modifying the phenotype of
10 macrophages for the treatment of cancer, autoimmune disease, and other conditions where such
modification is therapeutic.

BACKGROUND OF THE INVENTION

[0003] Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with a five-year
15 survival rate of less than six percent. A defining feature of PDAC is its prominent desmoplastic
reaction with an extensive leukocytic infiltration that is dominated by macrophages (Kurahara et
al., "Significance of M2-polarized Tumor-associated Macrophage in Pancreatic Cancer," *J. Surg.
Res.* 167:e211-9 (2011)). Depending on signals that prevail within their microenvironment,
macrophages can adopt a variety of functional states. In response to bacterial products such as
20 lipopolysaccharide (LPS) and Th1 cytokines, macrophages become immunostimulatory (type-1
phenotype). Type-1 macrophages are characterized by the expression of high levels of nitric
oxide synthase (INOS), major histocompatibility complex II (MHCII), cluster of differentiation
80 and 86 (CD80, CD86), tumor necrosis alpha (TNF α), and can exert a tumoricidal effect
(Mantovani et al., "Macrophage Polarization: Tumor-associated Macrophages as a Paradigm for
25 Polarized M2 Mononuclear Phagocytes," *Trends Immunol.* 23:549-55 (2002)). By contrast, in
the presence of Th2 cytokines macrophages acquire an alternatively activated state (*i.e.*, a type-2
phenotype) that is immunosuppressive, tumor promoting, and is characterized by the expression
of Arginase 1, cluster of differentiation 206 (CD206) and low levels of MHCII (Mantovani et al.,
"Macrophage Polarization: Tumor-associated Macrophages as a Paradigm for Polarized M2
30 Mononuclear Phagocytes," *Trends Immunol.* 23:549-55 (2002)). While tumor-associated
macrophages (TAMs) in the pancreas exhibit both type-1 and type-2 phenotypes, higher type-
2:type-1 ratios correlate with disease progression and shorter survival in patients (Kurahara et al.,
"Significance of M2-polarized Tumor-associated Macrophage in Pancreatic Cancer," *J. Surg.*

Res. 167:e211-9 (2011); Ino et al., "Immune Cell Infiltration as an Indicator of the Immune Microenvironment of Pancreatic Cancer," *Br. J. Cancer* 108:914-23 (2013)).

[0004] Abraxane (nab-paclitaxel) is a nanoparticle albumin-bound formulation of paclitaxel that, in combination with gemcitabine, is currently the first line treatment for pancreatic cancer (Von Hoff et al., "Increased Survival in Pancreatic Cancer with Nab-paclitaxel Plus Gemcitabine," *N. Engl. J. Med.* 369:1691-703 (2013)). The primary mechanism of antineoplastic activity of paclitaxel is its ability to stabilize microtubules and prevent cell division of rapidly dividing tumor cells. In macrophages paclitaxel can exert cell cycle-independent effects by acting as an LPS mimetic and inducing macrophage type-1 polarization in a Toll-like receptor 4 (TLR4)-dependent manner (Ding et al., "Taxol Shares the Ability of Bacterial Lipopolysaccharide to Induce Tyrosine Phosphorylation of Microtubule-associated Protein Kinase," *J. Immunol.* 151:5596-602 (1993); Ding et al., "Shared Actions of Endotoxin and Taxol on TNF Receptors and TNF Release," *Science* 248:370-2 (1990); Perera et al., "CD11b/CD18 Acts in Concert with CD14 and Toll-like Receptor (TLR) 4 to Elicit Full Lipopolysaccharide and Taxol-inducible Gene Expression," *J. Immunol.* 166:574-81 (2001)). However, it is unknown whether Abraxane can exert any effect on macrophages in the tumor environment or in the context of other diseases.

[0005] The present invention is directed to overcoming these and other deficiencies in the art.

SUMMARY OF THE INVENTION

[0006] A first aspect of the present invention is directed to a method of inducing a phenotypic change in a population of monocytes and/or macrophages. This method includes administering to the population of monocytes and/or macrophages, a macrophage stimulating agent coupled to a carrier molecule, wherein the carrier molecule facilitates macropinocytic uptake of the agent by monocytes and macrophages in the population and is defective in neonatal Fc receptor binding. The administration of the macrophage stimulating agent induces a phenotypic change in the monocytes and macrophages in the population.

[0007] Another aspect of the present invention is directed to a method of treating a tumor in a subject. This method includes selecting a subject having a tumor and administering to the selected subject an anti-tumor agent coupled to a carrier molecule that is defective in neonatal Fc receptor (FcRn) binding, wherein the administering induces a therapeutic tumor cell response in the subject.

[0008] As described herein, the effects of nab-paclitaxel on macrophage polarization were investigated. It is reported that nab-paclitaxel is internalized by macrophages principally

via macropinocytosis and is sufficient to drive macrophage type-1 polarization *in vitro* and *in vivo*. These data reveal a previously unappreciated mechanism of action of nab-paclitaxel in the tumor context and suggest that nab-paclitaxel could co-operate with immunotherapeutic agents to restore immune recognition of tumor cells. However, given FcRn recycling of albumin, nab-paclitaxel is expelled back out of macrophages reducing its type-1 polarizing effects. The present invention solves this unrecognized problem by coupling paclitaxel to a carrier molecule deficient in FcRn binding to facilitate paclitaxel retention in macrophages to increase its type-1 polarizing effects and enhance its overall therapeutic efficacy in not only pancreatic cancer, but other cancers where tumor-associated macrophages harbor a pro-tumor immunosuppressive phenotype. Similarly, coupling other macrophage stimulating agents as described herein to a carrier molecule that facilitates macropinocytic uptake, but is deficient in FcRn binding has broad applicability in modulating macrophage phenotype in a variety of diseases where type-1/type-2 macrophage tropism influence or contribute to the disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] FIGs. 1A-1D demonstrate that macrophages internalize nab-paclitaxel via macropinocytosis. FIG. 1A shows immunofluorescent analysis of RAW 264.7 cells treated for 30 minutes with OG-nab-paclitaxel and TMR-dextran, and FIG. 1B shows cells treated with OG-paclitaxel (left) or OG-nab-paclitaxel (right) and DMSO (vehicle, top) or pretreated with 100 mmol/L EIPA (bottom). FIG. 1C shows cells treated with OG-nab-paclitaxel and PBS (vehicle) or pretreated with anti-CD16/CD32, and FIG. 1D shows cells treated with OG-nab-paclitaxel with DMSO (vehicle) or with BAPTA-AM. All pretreatments were 30 minutes. Macropinocytosis indices are represented graphically in FIGs. 1B–D (right) with bars indicating the SE from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

[0010] FIGs. 2A-2D show that nab-paclitaxel drives M1 macrophage activation *in vitro*. FIG. 2A shows relative gene expression analysis of *Il1 α* , *Il1 β* , *Il6*, *Il12 p40*, and *Tnfa* normalized to *Gapdh* in RAW 264.7 cells treated with vehicle (DMSO), LPS, paclitaxel (ptx), or nab-paclitaxel (nab-ptx). FIG. 2B shows relative gene expression levels of *Il1 α* , *Il1 β* , *Il6*, *Il12 p40*, and *Tnfa* treated with nab-ptx alone or with EIPA, BAPTA-AM, or anti-CD16/32. In FIGs. 2A and 2B, bars represent SE from three independent experiments. FIGs. 2C and 2D show western blot analysis of iNOS expression in RAW 264.7 cells. FIG. 2C shows cells treated with vehicle (DMSO), IFN γ , LPS, ptx or nab-ptx alone, or with IFN γ . FIG. 2D shows cells treated with vehicle (DMSO), IFN γ , nab-ptx, or nab-ptx in combination with IFN γ alone or with EIPA. VINCULIN served as a protein loading control. Numbers below Western blots indicate level of iNOS induction normalized to VINCULIN and relative to IFN γ alone. All western blots were

cropped using ImageJ to show bands of interest and are representative of at least three independent experiments. Graph in FIG. 2D shows relative gene expression of *Inos* normalized to *Gapdh* with bars representing SE from four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ns, not significant

5 **[0011]** FIGs. 3A-3B show nab-paclitaxel induces M1 activation in a TLR4-dependent manner. FIG. 3A shows relative gene expression levels of *Il1 α* , *Il1 β* , *Il6*, *Il12 p40*, and *Tnfa* treated with nab-ptx alone or with 500 nm CLI-095. Bars represent SE from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. FIG. 3B shows western blot analysis of iNOS expression in RAW 264.7 cells treated with vehicle (DMSO), IFN γ , or LPS or nab-ptx alone or with IFN γ or with IFN γ and 25 mmol/L CLI-095.

10 **[0012]** FIGs. 4A-4D show nab-paclitaxel induces M1 activation in pancreatic tumor-associated macrophages *in vivo*. FIG. 4A shows representative hematoxylin and eosin (H&E) and F4/80 immunohistochemistry staining of KPC tumors ten days following implantation into syngeneic mice. Inset shows representative dissected KPC tumor. FIG. 4B shows cryo-
15 immunofluorescent analysis of orthotopic tumors resected 2 weeks after orthotopic implantation and treated *ex vivo* with 100 mg OG-nab-ptx, followed by immunofluorescent staining with anti-F4/80. Data are representative of three independent experiments. FIG. 4C shows quantification of CD80/CD86-positive cells in KPC orthotopic tumors 48 hours after treatment. Left, the CD80+CD86+ cell population was gated on the CD45+F4/80+MHCII+ cell population. Data
20 are representative of four independent experiments. Right, Representative FACS plot of CD80+CD86+ cell population from PBS (vehicle; left) and nab-paclitaxel (nab-ptx; right)-treated mice are shown. FIG. 4D shows quantification of CD80+IL1 α + cells in KPC orthotopic tumors 48 hours after treatment. Left, CD80+IL1 α + cell population was gated on CD45+F4/80+MHCII+ cell population. Right, Representative FACS plot of CD80+ IL1 α + cell
25 population from PBS (vehicle; left) and nab-paclitaxel (nab-ptx; right)-treated mice are shown. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

30 **[0013]** FIG. 5 shows nab-paclitaxel induces M1 cytokine protein expression *in vitro*. Western blot analysis of IL1 α , IL1 β , IL6 and TNF α expression in RAW 264.7 cells treated with vehicle (PBS), LPS or nab-ptx for 8 hours. VINCULIN served as a protein loading control.
Numbers below Western blots indicate level of cytokine induction normalized to VINCULIN and relative to vehicle control.

35 **[0014]** FIG. 6 shows LPS induces M1 cytokine expression in a TLR4-dependent manner. Relative gene expression levels of *Il1 α* , *Il1 β* , *Il6*, *Il12 p40* and *Tnfa* treated with LPS or with LPS and 5 μ M CLI-095. Bars represent standard error from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

[0015] FIGs. 7A-7C shows nab-paclitaxel induces M1 activation in pancreatic tumor-associated macrophages *in vivo*. FIG. 7A shows a representative flow cytometry plot of PE-F4/80-positive cells sorted from KPC orthotopic tumors two weeks after orthotopic implantation and treated *ex vivo* with serum-free medium (vehicle, grey) or 20 µg of OG-nab-ptx (green).
5 Quantification of mean percentage of OG-nab-ptx-positive cells from triplicate samples is shown. Bars depict standard deviation. FIG. 7B shows quantification of total F4/80-positive cells (gated on the CD45-positive population) in KPC orthotopic tumors 48 hours post-treatment. Bars depict standard error from four independent experiments. FIG. 7C shows relative gene expression levels of Il1β in CD45/F4/80 double-positive cells sorted from KPC orthotopic
10 tumors 48 hours post-treatment. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

DETAILED DESCRIPTION OF THE INVENTION

[0016] A first aspect of the present invention is directed to a method of inducing a phenotypic change in a population of monocytes and/or macrophages. The method includes administering to the population of monocytes and/or macrophages, a macrophage stimulating
15 agent coupled to a carrier molecule, where the carrier molecule facilitates macropinocytic uptake of the agent by monocytes and macrophages in the population and is defective in neonatal Fc receptor binding. In accordance with this method, the administered macrophage stimulating agent induces a phenotypic change in the monocytes and macrophages of the population.

[0017] Macrophages have historically been divided into two phenotypically diverse
20 populations, *i.e.*, a macrophage type-1-polarized or “classically activated” population, and a macrophage type-2-polarized or “alternatively activated” population. However, it is well appreciated in the art that a continuum of phenotypes exists between the macrophage type-1-polarized and macrophage type-2-polarized populations (*e.g.*, M2a, M2b, and M2c phenotypes), and in some cases macrophages assume a phenotype that does not fit well within any of these
25 defined phenotypic groups (*see e.g.*, Martinez and Gordon, “The M1 and M2 Paradigm of Macrophage Activation: Time for Reassessment,” *F1000Prime Rep.* 6:13 (2014), which is hereby incorporated by reference in its entirety). The methods described herein are suitable for inducing one or more phenotypic changes in a population of monocytes/macrophages to “tune” or “adjust” a population of macrophages/monocytes to have a desired phenotype, *e.g.*, a type-1 phenotype, a
30 type-2 phenotype, a type-2-sub-phenotype, *i.e.*, an M2a, M2b, or M2c phenotype, or any phenotype that falls within the spectrum of known macrophage phenotypes.

[0018] Macrophages exhibiting a type-1 phenotype are pro-inflammatory, and are capable of either direct (pathogen pattern recognition receptors) or indirect (Fc receptors, complement receptors) recognition of pathogens and tumor antigens (*i.e.*, they exhibit anti-tumor

activity). Type-1 macrophages produce reactive oxygen species and secrete pro-inflammatory cytokines and chemokines, such as, for example, but without limitation, TNF α , IL-1, IL-6, IL-15, IL-18, IL-23, and iNOS. Type-1 macrophages also express high levels of MHC, costimulatory molecules, and FC γ R. The type-1 phenotype is triggered by GM-CSF and further stimulated by interferon- γ (IFN- γ), bacterial lipopolysaccharide (LPS), or tumor necrosis factor α (TNF α), and is mediated by several signal transduction pathways involving signal transducer and activator of transcription (STAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), and mitogen-activated protein kinases (MAPK). These events enhance the production of agents such as the reactive oxygen species and nitric oxide (NO) and promote subsequent inflammatory immune responses by increasing antigen presentation capacity and inducing the Th1 immunity through the production of cytokines such as IL-12.

[0019] In contrast, macrophages exhibiting a type-2 phenotype are often characterized as being anti-inflammatory and immunosuppressive as they suppress T-cell responses and are involved in the Th2-type immune response. The type-2 macrophage phenotype facilitates tissue repair, wound healing, and is profibrotic. Type-2 macrophages often undesirably infiltrate and surround tumors, where they provide an immunosuppressive microenvironment that promotes rather than suppresses tumor progression. Type-2 macrophages are characterized by high surface expression of IL-4R, Fc ϵ R, Dectin-1, CD136, CD206, and CD209A. Type-2 macrophages include IL-4/IL-13-stimulated macrophages, IL-10-induced macrophages, and immune complex-triggered macrophages.

[0020] As used herein, a “phenotypic change” encompasses an observable or detectable change in a characteristic, property, attribute, or function of the macrophages/monocytes in the population. For example, phenotypic characteristics/properties/functions of macrophages/monocytes that can be modified or modulated in accordance with the methods of the present invention include, without limitation, pro-inflammatory activity, anti-inflammatory activity, immunogenic activity, tolerogenic activity, tissue damaging activity, tissue healing activity, cytotoxic activity, migratory activity, bone-resorbing activity, angiogenic activity, anti-angiogenic activity, suppressor activity, antigen presenting activity, or phagocytic activity. A phenotypic change in the monocytes and macrophages may be observed or detected in any of a number of ways. For example, a phenotypic change may be observed or detected either by performing a test, observation, or measurement on the macrophages/monocytes themselves or by performing a test, observation, or measurement, on other cells, tissues, organs, etc., that may be affected by the monocytes/macrophages, or by performing a test, observation, or measurement on a subject that contains the phenotypically modified macrophages.

[0021] Phenotypic change or modulation can be assessed by detecting or measuring, for example, (i) a change in the expression of one or more genes (*e.g.*, cytokines, inflammatory mediators, etc.); (ii) the change in secretion of one or more molecules (*e.g.*, cytokines, inflammatory mediators, etc.); (iii) an increase or decrease in migration to one or more sites in the body; (iv) a change in the ability to cause an alteration in one or more phenotypic characteristics or phenotypes of another macrophage-related cell or ability to cause an alteration in one or more phenotypic characteristics or phenotypes of a non-macrophage-related cell.

[0022] Methods for observing, detecting, and measuring these phenotypic changes are known in the art and described herein. For example, gene expression profiles can be assessed at the RNA level using cDNA or oligonucleotide microarray analysis, Northern blots, RT-PCR, sequencing, etc. Protein expression can be measured using, for example, immunoblotting, immunohistochemistry, protein microarrays, etc. Various cell-based assays and animal models readily known and utilized in the art can also be used.

[0023] As described herein, a “macrophage stimulating agent” can be, for example, a small molecule, a peptide, an oligopeptide, a polypeptide, a protein, an antibody, a synthetic binding molecule, an aptamer, an RNA molecule, a DNA molecule, an oligomer, a polymer, a lipid, or a liposome. In accordance with the methods described herein, the macrophage stimulating agent is delivered to the macrophage via a carrier that facilitates macropinocytic delivery of the agent. Accordingly, the macrophage stimulating agent is one that induces a phenotypic change in monocytes and/or macrophage via an endosome or intracellular signaling pathway. Exemplary classes of molecules that may act as macrophage stimulating agents of the present invention include, without limitation, cytokines, chemokines, pattern recognition receptor ligands, hormones, adrenergic and cholinergic agonists, fatty acids, phospholipids, immunoglobulins or portions thereof, Fc domains of immunoglobulins, lipopolysaccharides (LPS), toll-like receptor (TLR) ligands, histamines, and peroxisome proliferator-activated receptor ligands.

[0024] In one embodiment, it is desirable to induce type-1-phenotypic characteristics in a population of monocytes and/or macrophages comprising non-type-1 macrophages, *e.g.*, a population of type-2 macrophages/monocytes or a heterogenous population of type-2 and type-1 macrophages/monocytes. In accordance with this embodiment, a macrophage type-1 stimulating agent coupled to a carrier molecule as described herein is administered to the population of macrophages/monocytes. Suitable macrophage type-1 stimulating agents include, without limitation, paclitaxel, colony stimulating factor-1 (CSF-1) receptor antagonists, IL-10 receptor antagonists, a Toll-like receptor (TLR)-2 agonist, a TLR-3 agonist, a TLR-4 agonist, a TLR-7 agonist, a TLR-8 agonist, and a TLR-9 agonist.

[0025] In one embodiment, the macrophage type-1 stimulating agent is paclitaxel.

Paclitaxel is a plant derived alkaloid in the taxane family of neoplastic drugs. Paclitaxel mimics LPS and induces the production of IL-12 in macrophages (Mullins et al., "Paclitaxel Enhances Macrophage IL-12 Production in Tumor-bearing Hosts Through Nitric Oxide," *J. Immunol.*

5 162(11):6811-8 (1999), which is hereby incorporated by reference in its entirety). Derivatives of paclitaxel that similarly induce IL-12 production in macrophages are also suitable for use in accordance with the methods disclosed herein.

[0026] In another embodiment, the macrophage type-1 stimulating agent is a CSF-1 receptor antagonist. Suitable CSF-1 receptor antagonists include, without limitation ABT-869

10 (Guo et al., "Inhibition of Phosphorylation of the Colony-Stimulating Factor-1 Receptor (c-Fms) Tyrosine Kinase in Transfected Cells by ABT-869 and Other Tyrosine Kinase Inhibitors," *Mol. Cancer. Ther.* 5(4):1007-1012 (2006), which is hereby incorporated by reference in its entirety), imatinib (Guo et al., "Inhibition of Phosphorylation of the Colony-Stimulating Factor-1 Receptor (c-Fms) Tyrosine Kinase in Transfected Cells by ABT-869 and Other Tyrosine Kinase

15 Inhibitors," *Mol. Cancer. Ther.* 5(4):1007-1012 (2006), which is hereby incorporated by reference in its entirety), PLX3397 (Mok et al., "Inhibition of CSF1 Receptor Improves the Anti-tumor Efficacy of Adoptive Cell Transfer Immunotherapy," *Cancer Res.* 74(1):153-161 (2014), which is hereby incorporated by reference in its entirety), PLX5622 (Dagher et al., "Colony-stimulating Factor 1 Receptor Inhibition Prevents Microglial Plaque Association and Improves

20 Cognition in 3xTg-AD Mice," *J. Neuroinflamm.* 12:139 (2015), which is hereby incorporated by reference in its entirety), DCC-3014 (Deciphera Pharmaceuticals), BLZ945 (Krauser et al., "Phenotypic and Metabolic Investigation of a CSF-1R Kinase Receptor Inhibitor (BLZ945) and its Pharmacologically Active Metabolite," *Xenobiotica* 45(2):107-123 (2015), which is hereby incorporated by reference in its entirety), and GW2580 (Olmos-Alonso et al., "Pharmacological

25 Targeting of CSF1R Inhibits Microglial Proliferation and Prevents the Progression of Alzheimer's-like Pathology," *Brain* 139:891-907 (2016), which is hereby incorporated by reference in its entirety).

[0027] In another embodiment, the macrophage type-1 stimulating agent is an IL-10 receptor antagonist. Suitable IL-10 receptor antagonists include, without limitation peptide

30 antagonists as described in Naiyer et al., "Identification and Characterization of a Human IL-10 Receptor Antagonist," *Hum. Immunol.* 74(1):28-31 (2013), which is hereby incorporated by reference in its entirety, and IL-10 receptor antagonistic antibodies as described in U.S. Patent No. 7,553,932 to Von Herrath et al., which is hereby incorporated by reference in its entirety.

[0028] In another embodiment, the macrophage type-1 stimulating agent is a TLR

35 agonist, *i.e.*, a TLR2, TLR3, TLR4, TLR7, TLR8, or TLR9 agonist. Suitable TLR-2 agonists for

use in the methods described herein include Pam3CSK4, a synthetic triacylated lipoprotein, and lipoteichoic acid (LTA) (Brandt et al., "TLR2 Ligands Induce NF- κ B Activation from Endosomal Compartments of Human Monocytes" *PLoS One* 8(12):e80743, which is hereby incorporated by reference in its entirety).

- 5 **[0029]** A suitable TLR-3 agonist includes, without limitation, polyinosinic:polycytidylic acid (poly I:C) (Smole et al., "Delivery System for the Enhanced Efficiency of Immunostimulatory Nucleic Acids," *Innate Immun.* 19(1):53-65 (2013), which is hereby incorporated by reference in its entirety).

- 10 **[0030]** Suitable TLR-4 agonists include, without limitation, MPL (Engel et al., "The Pharmacokinetics of Toll-like Receptor Agonists and the Impact on the Immune System," *Expert Rev. Clin. Pharmacol.* 4(2):275-289 (2011), which is hereby incorporated by reference in its entirety), Glucopyranosyl Lipid-A (Matzner et al., "Perioperative treatment with the new synthetic TLR-4 agonist GLA-SE reduces cancer metastasis without adverse effects," *Int. J. Cancer* 138(7):1754-64 (2016), which is hereby incorporated by reference in its entirety), and
- 15 Immunomax® (Ghochikyan et al., "Targeting TLR-4 with a novel pharmaceutical grade plant derived agonist, Immunomax®, as a therapeutic strategy for metastatic breast cancer," *J. Trans. Med.* 12:322 (2014), which is hereby incorporated by reference in its entirety)

- 20 **[0031]** Suitable TLR-7 agonists include, without limitation, uridine/guanidine-rich single-stranded RNA (Engel et al., "The Pharmacokinetics of Toll-like Receptor Agonists and the Impact on the Immune System," *Expert Rev. Clin. Pharmacol.* 4(2):275-289 (2011), which is hereby incorporated by reference in its entirety), 852A (Dudek et al., "First in Human Phase I Trial of 852A, a Novel Systemic Toll-like Receptor 7 Agonist, to Activate Innate Immune Responses in Patients With Advanced Cancer," *Clin. Cancer Res.* 13(23):7119-7125 (2007), which is hereby incorporated by reference in its entirety), resiquimod (Chang et al., "Topical
- 25 resiquimod Promotes Priming of CTL to Parenteral Antigens," *Vaccine* 27(42):5791-5799 (2009), which is hereby incorporated by reference in its entirety), imidazoquinolines (Itoh et al., "The Clathrin-mediated Endocytic Pathway Participates in dsRNA-induced IFN-beta Production," *J. Immunol.* 181:5522-9 (2008), which is hereby incorporated by reference in its entirety), ANA975 (Fletcher et al., "Masked oral Prodrugs of Toll-like Receptor 7 Agonists: a
- 30 New Approach for the Treatment of Infectious Disease," *Curr. Opin. Investig. Drugs* 7(8):702-708 (2006), which is hereby incorporated by reference in its entirety), and imiquimod (Engel et al., "The Pharmacokinetics of Toll-like Receptor Agonists and the Impact on the Immune System," *Expert Rev. Clin. Pharmacol.* 4(2):275-289 (2011), which is hereby incorporated by reference in its entirety).

[0032] Suitable TLR-8 agonists include, without limitation, resiquimod (Chang et al., "Topical resiquimod Promotes Priming of CTL to Parenteral Antigens," *Vaccine* 27(42):5791-5799 (2009), which is hereby incorporated by reference in its entirety), and imidazoquinolines (Itoh et al., "The Clathrin-mediated Endocytic Pathway Participates in dsRNA-induced IFN-beta Production," *J. Immunol.* 181:5522-9 (2008), which is hereby incorporated by reference in its entirety),

[0033] Suitable TLR-9 agonists include, without limitation, CpG-ODN (Yao et al., "Late Endosome/Lysosome-localized Rab7b Suppresses TLR-9-initiated Proinflammatory Cytokine and Type I IFN Production in Macrophages," *J. Immunol.* 183:1751-8 (2009), which is hereby incorporated by reference in its entirety). Specific CpG-ODNs suitable for use are described in Engel et al., "The Pharmacokinetics of Toll-like Receptor Agonists and the Impact on the Immune System," *Expert Rev. Clin. Pharmacol.* 4(2):275-289 (2011), which is hereby incorporated by reference in its entirety.

[0034] In accordance with this aspect of the disclosure, administering a macrophage type-1 stimulating agent coupled to a carrier molecule as described herein induces one or more macrophage type-1 phenotypic changes, characteristics, or properties in the macrophages or monocytes of the population. As noted above, these phenotypic changes can be monitored by changes in expression or secretion of one more chemokines, cytokines, or other molecules that typify the macrophage type-1 phenotype. Exemplary markers of the macrophage type-1 phenotype that may be induced and can be monitored include, without limitation, expression, secretion, and/or activity of IL-1 α , IL-1 β , IL-6, iNOS, IFN γ , IL-12, and TNF α . Accordingly, an increase in the expression or secretion of any one or more of these markers serves to indicate a change in the macrophage phenotype to a type-1 phenotype. Alternatively, the desired phenotypic changes in the population of macrophages/monocytes administered the macrophage type-1 stimulating agent can be monitored by assessing decreases in the expression or secretion of chemokines, cytokines, and other proteins that exemplify the macrophage type-2 phenotype (*e.g.*, IL-10, TGF- β , MRC1, TGM2, CD23, CCL22, and Arginase 1). Methods of detecting and quantifying mRNA and protein expression, protein secretion, or protein activity in cells to monitor phenotypic changes are well known in the art.

[0035] In another embodiment, it is desirable to induce macrophage type-2-phenotypic characteristics in a population of monocytes and/or macrophages comprising non-type-2 macrophages, *e.g.*, a population of type-1 macrophages/monocytes or a heterogenous population of type-1 and type-2 macrophages/monocytes. In accordance with this embodiment, a macrophage type-2 stimulating agent coupled to a carrier molecule as described herein is administered to the population. Suitable macrophage type-2 stimulating agents include, without

limitation, IL-33, IL-4 receptor agonists, glucocorticoids, IL-10 receptor agonists, and IL-1 receptor agonists.

[0036] Suitable IL-4 receptor agonists include, without limitation, mutant IL-4 proteins. Exemplary mutant IL-4 proteins include, but are not limited to those described in U.S. Patent No. 5,723,118 to Sebald, which is hereby incorporated by reference in its entirety.

[0037] Glucocorticoids are a class of corticosteroids, which are well known in the art and suitable for inducing a macrophage type-2 phenotype. Exemplary glucocorticoids for use in the present invention include, without limitation, cortisol, cortisone, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone, beclomethasone, fludrocortisone, deoxycorticosterone, and aldosterone.

[0038] IL-10 receptor agonists are also capable of inducing a macrophage type-2 phenotype in accordance with the methods described herein. Suitable IL-10 receptor agonists include, without limitation, mutant IL-10 proteins as described in U.S. Patent No. 7,749,490 to Sommer et al., which is hereby incorporated by reference in its entirety.

[0039] IL-1 receptor agonists are also capable of inducing a macrophage type-2 phenotype in accordance with the methods described herein. Suitable IL-1 receptor agonists include, without limitation, IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β , and IL-36 γ (Palomo et al., "The Interleukin (IL)-1 Cytokine Family- Balance Between Agonists and Antagonists in Inflammatory Diseases," *Cytokine* 76(1):25-37 (2015), which is hereby incorporated by reference in its entirety).

[0040] In accordance with this aspect of the disclosure, administering a macrophage type-2 stimulating agent coupled to a carrier molecule as described herein induces one or more type-2 phenotypic changes, characteristics, or properties in the macrophages or monocytes of the population. As noted above, these phenotypic changes can be monitored by changes in expression or secretion of one more chemokines, cytokines, or other molecules that typify the macrophage type-2-phenotype or any of the macrophage type-2-phenotype subtypes. Exemplary markers of the macrophage type-2 phenotype include, without limitation, IL-10, TGF- β , MRC1, TGM2, CD23, CCL22, and Arginase 1. Alternatively, the desired phenotypic change(s) in the population of macrophages/monocytes administered the macrophage type-2 stimulating agent are monitored by assessing decreases in the expression or secretion of chemokines, cytokines, and other proteins that exemplify the macrophage type-1 phenotype, e.g., IL-1, IL-6, IL-12, IL-23, and TNF α .

[0041] The macrophage type-1 or type-2 stimulating agents described herein are coupled to a carrier molecule for administration in accordance with the methods of the present invention.

As noted above, the carrier molecule is a molecule that facilitates macropinocytic uptake of the

macrophage stimulating agent by macrophages/monocytes in a population. In addition, the carrier molecule is a molecule that is defective in neonatal Fc receptor binding.

[0042] The neonatal Fc receptor (FcRn) is an immunoglobulin G (IgG) receptor as well as a major albumin receptor that is expressed by a variety of cell types in the body, including macrophages and more variably tumor cells. In addition to regulating the internalization of albumin and IgG molecules, FcRn recycles both molecules into the extracellular space.

Therefore, in accordance with the methods disclosed herein, where it is desirable to deliver a macrophage stimulating agent to macrophages where the agent is retained inside of the macrophage for purposes of inducing a phenotypic change in the macrophage, the carrier molecule delivering the stimulating agent is one that is deficient or defective in FcRn binding.

[0043] In one embodiment, the carrier molecule that facilitates macropinocytic uptake of the macrophage stimulating agent and is defective or deficient in FcRn binding is a variant human albumin protein or fragment thereof. As used herein, the term “variant” albumin means a polypeptide derived from a parent albumin that differs from the parent albumin by one or more amino acid alterations, *i.e.*, a substitution, insertion, and/or deletion, at one or more amino acid residue positions.

[0044] As used herein, “albumin” refers to a protein having the same, or very similar three dimensional structure as human serum albumin (HSA) and having a long plasma half-life. Human serum albumin (HSA) is a highly soluble globular protein of M_r 65K and consists of 585 amino acids. HSA is the most abundant protein in the plasma and accounts for 70-80% of the colloid osmotic pressure of human plasma. The amino acid sequence of HSA contains a total of 17 disulphide bridges, one free thiol (Cys 34), and a single tryptophan (Trp 214). In its natural form it is not glycosylated. The amino acid sequence of HSA is shown as SEQ ID NO: 1 below.

SEQ ID NO: 1

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25  Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu
    1          5          10          15

30  Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln
    20          25          30

35  Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
    35          40          45

    Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
        50          55          60
40  Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
    65          70          75          80

    Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
        85          90          95

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Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr
340 345 350

-14-

Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu
 355 360 365
 5

Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro
 370 375 380
 10

Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu
 385 390 395 400
 15

Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro
 405 410 415
 20

Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys
 420 425 430
 25

Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys
 435 440 445
 30

Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His
 450 455 460
 35

Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser
 465 470 475 480
 40

Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr
 485 490 495
 45

Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp
 500 505 510
 50

Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala
 515 520 525
 55

Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu
 530 535 540
 60

Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys
 545 550 555 560
 65

Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val
 565 570 575
 70

Ala Ala Ser Gln Ala Ala Leu Gly Leu
 580 585

[0045] A number of variant human albumin proteins having reduced FcRn binding or that are deficient in FcRn binding that are suitable for use in the methods described herein have

been described in the art. Variant human albumin proteins or fragments thereof having reduced or deficient FcRn include, without limitation, those having one or more amino acid modifications at one or more amino acid positions involved in mediating FcRn binding. These amino acid positions include, without limitation, those corresponding to positions 464, 494, 495, 496, 499, 500, 510, 535, 536, 537, 538, and 573 of SEQ ID NO: 1. Exemplary variant human albumin proteins or fragments thereof comprise an amino acid sequence of SEQ ID NO: 1, or a peptide fragment thereof, with one or more amino acid modifications selected from the group consisting of D494N, D494Q, D494A, E495Q, E495A, T496A, P499A, K536A, P537A, K538A, K500A, K573STOP, H464Q, H510Q, and H535Q.

[0046] Additional exemplary variant albumin proteins include those comprising mutations at the C-terminus of albumin, including K574, Q580, N111, and D108 (*see* U.S. patent No. 8,822,417 to Andersen et al., which is hereby incorporated by reference in its entirety), and variants comprising mutations at position E492 as described by U.S. Patent Application Publication No. 2016/0009787 to Sleep et al., which is hereby incorporated by reference in its entirety. Other variant albumin proteins or fragments thereof that have reduced or defective binding to FcRn and are suitable for use in the methods described herein are disclosed in U.S. Patent No. 8,314,156 to Desai et al., U.S. Patent Application Publication No. 2012/0220530 to Plumridge et al., U.S. Patent Application Publication No. 2016/0009787 to Sleep et al., U.S. Patent Application Publication No. 2015/0210752 to Cameron et al., WO2011/051489 to Plumridge et al., WO2012/150319 to Andersen et al., U.S. Patent Application Publication No. 2012/0076728 to Wu et al., and U.S. Patent Application Publication No. 2014/0315816 to Andersen et al., which are hereby incorporated by reference in their entirety.

[0047] In another embodiment, the carrier molecule that facilitates macropinocytic uptake of the macrophage stimulating agent and is defective or deficient in FcRn binding is a variant human immunoglobulin G (IgG) protein or fragment thereof. As used herein, a “variant” human IgG protein or fragment encompasses a polypeptide derived from a parent IgG that differs from the parent IgG by one or more amino acid alterations, *i.e.*, substitution, insertion, and/or deletion, at one or more positions. FcRn binds with high affinity to the heavy chain constant regions (CH2 and CH3) of the Fc-region of an IgG. Accordingly, variants of IgG with reduced FcRn binding preferably contain one or more amino acid alterations within the CH2 and/or CH3 region of the Fc portion.

[0048] Variant human immunoglobulin G proteins or fragments thereof that are defective in FcRn binding and suitable for use in accordance with the methods described herein have been described in the art. Exemplary variant IgG proteins include those having an amino acid substitution as one or more amino acid positions selected from 252, 255, 282, 309, and 434

(numbering of the IgG heavy chain residues is according to the Kabat EU index numbering system; Kabat et al., "Sequences of Proteins of Immunological Interest" NIH Publication No 91-3242 (1991), which is hereby incorporated by reference in its entirety). By way of example, IgG proteins comprising at least one mutation at M252T, R255D, V282R, V282D, V282E, V282K, L309R, L309D, L309K, L309E, or N434L of the Fc portion of IgG1 are suitable for use in the methods described herein (*see* U.S. Patent Application Publication No. 2007/0148164 to Farrington et al., which is hereby incorporated by reference in its entirety). Other suitable variant IgG proteins include, without limitation, those described in U.S. Patent No. 8,802,820 to Chamberlain et al., and WO2016/071377 to Schlothauer et al., which are hereby incorporated by reference in their entirety.

[0049] Modulating or modifying macrophage/monocyte phenotype as described herein is carried out for the purpose of treating, preventing, or slowing the progression of a disease or condition that is caused or exacerbated, at least in part, by macrophages exhibiting one or more undesirable phenotypic characteristics or phenotypes. For example, inflammatory diseases and conditions, including but not limited to macular degeneration, atherosclerosis, osteoporosis, immune inflammation, non-immune inflammation, renal inflammation, tuberculosis, multiple sclerosis, arthritis, chronic obstructive pulmonary disease (COPD), and Alzheimer's disease, involve the undesired actions of type-1 macrophages. Employing the methods of the present invention to induce a macrophage type-2 phenotypic change in the type-1 pro-inflammatory macrophages that are involved in or contributing to these disease processes alleviates one or more symptoms or causes of the disease. Accordingly, in one embodiment, the administering is carried out *in vivo* or *ex vivo* as described *infra* to a population of type-1 macrophages in or from a subject having an inflammatory or autoimmune condition, including, but not limited to any of those enumerated above. Administering a type-2 macrophage stimulating agent to a population of type-1 macrophages in this context will induce a type-2 phenotypic change, thereby reducing the undesired actions of the type-1 macrophages associated with the disease.

[0050] Modulating or modifying macrophage/monocyte phenotype is also therapeutically beneficial in context of treating various forms of cancer. Recent studies indicate that tumor-associated macrophages (TAMs) exhibit a macrophage type-2-like phenotype. These type-2 macrophages are important tumor-infiltrating cells and play pivotal roles in tumor growth and metastasis. In most solid tumors, the existence of TAMs is advantageous for tumor growth and metastasis. These TAMs produce interleukin IL-10 and transforming growth factor (TGF) β to suppress general antitumor immune responses. Meanwhile, TAMs promote tumor neo-angiogenesis by the secretion of pro-angiogenic factors and define the invasive

microenvironment to facilitate tumor metastasis and dissemination. Therefore, employing the methods of the present invention to induce a type-1 phenotypic change in the TAMs to enhance anti-tumor immunity will significantly alter the progression of the cancer. Cancers that typically have a type-2 macrophage-related component include, without limitation, pancreatic cancer, breast cancer, and non-small cell lung cancer. Accordingly, in one embodiment, administering a macrophage stimulating agent, e.g., a macrophage type-1 stimulating agent, is carried out *in vivo* or *ex vivo* as described in more detail below, to a population of type-2 macrophages in or from a subject having cancer, where the cancer is characterized as having an immunosuppressive microenvironment. As noted above, such cancers include, without limitation pancreatic cancer, breast cancer, and non-small cell lung cancer.

[0051] In one embodiment of this aspect of the invention, the administration of a macrophage type-1 stimulating agent is combined with a T-cell immunotherapy to boost the patient's anti-tumor immune response. Suitable T-cell immunotherapies include, without limitation, adoptive T-cell therapy, tumor-infiltrating lymphocyte therapy, chimeric antigen receptor (CAR) T-cell therapy, and antigen-specific T-cell receptor transduced T-cell therapy.

[0052] Accordingly, in practicing the methods described herein, the administering step can be carried out *in vitro*, *ex vivo*, or *in vivo*. For example, in certain embodiments, it is desirable to obtain a population of macrophages/monocytes from a subject, administer the macrophage stimulating agent to the population of macrophages/monocytes *ex vivo* under conditions suitable for one or more phenotypic changes to occur, and then transplant, inject, or implant the phenotypically modified macrophages into the subject in need thereof (*see e.g.*, Ferenback and Kluth, "Macrophage Cell Therapy in Renal Disease," *Semin. Nephrol.* 30(3): 345-53 (2010), and Rybalko et al., "The Development of Macrophage-Mediated Cell Therapy to Improve Skeletal Muscle Function after Injury," *PLoS One* 10(12): e0145550 (2015), which are hereby incorporated by reference in their entirety).

[0053] In another embodiment, the macrophage stimulating agent can be administered *in vivo* to a subject in need thereof. Such administration can be carried out systemically. By way of example, suitable modes of systemic administration include, without limitation, orally, topically, transdermally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intranasal instillation, by intracavitary or intravesical instillation, intraocularly, intra-arterially, intralesionally, or by application to mucous membranes. Alternatively, the *in vivo* administration can be carried out via local administration. Suitable modes of local administration include, without limitation, catheterization, implantation, direct injection, dermal/transdermal application, stenting, ear/eye drops, or portal vein administration to relevant tissues, or any other local administration technique, method or procedure, as is generally

known in the art. In the context of treating cancer, for example, local administration to the tumor site can be carried out to selectively modify or induce a type-1-macrophage phenotype in macrophages of the tumor microenvironment.

[0054] In accordance with this and all other aspects of the present invention, a “subject”, “patient”, or “individual” that is amendable to treatment using the methods described herein includes any animal. In one embodiment, the subject is a mammal. In another embodiment, the subject is a human.

[0055] Another aspect of the present invention is directed to compositions comprising the macrophage stimulating reagent coupled to a carrier molecule, where the carrier molecule is taken up by macrophages and/or monocytes via macropinocytosis and is defective in neonatal Fc receptor binding. Suitable macrophage type-1 and type-2 stimulating reagents are described *supra*. Suitable carrier molecules include modified or variant human albumin proteins and peptides, where said modification or variant is one that reduces or eliminates albumin binding to FcRn. Suitable modified albumin proteins and peptides are disclosed *supra*. Other suitable carrier molecules include modified or variant IgG proteins or peptides, where said modification or variant is one that reduces or eliminates IgG binding affinity to FcRn. Suitable IgG variants are disclosed *supra*.

[0056] Pharmaceutical compositions containing the macrophage stimulating agents coupled to the a carrier molecule having reduced or deficient FcRN binding affinity of the present invention can be formulated as necessary according to the intended route of administration. For example, for compositions administered orally, the macrophage stimulating agent couple to the carrier molecule is mixed with an inert diluent, or with an assimilable edible carrier, or it may be enclosed in hard or soft shell capsules, or it may be compressed into tablets, or they may be incorporated directly with the food of the diet. Agents of the present invention may also be administered in a time release manner incorporated within such devices as time-release capsules or nanotubes. Such devices afford flexibility relative to time and dosage. For oral therapeutic administration, the agents of the present invention may be formulated with excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, and the like. Such compositions and preparations should contain at least 0.1% of the agent, although lower concentrations may be effective and indeed optimal. The percentage of the agent in these compositions may, of course, be varied and may conveniently be between about 2% to about 60% of the weight of the unit. The amount of an agent of the present invention in such therapeutically useful compositions is such that a suitable dosage will be obtained.

[0057] When the macrophage stimulating agents of the present invention are administered parenterally, solutions or suspensions of the agent can be prepared in water suitably

mixed with a surfactant such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solution, and glycols, such as propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0058] Pharmaceutical formulations suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

[0059] When it is desirable to deliver the agents of the present invention systemically, they may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[0060] Intraperitoneal or intrathecal administration of the agents of the present invention can also be achieved using infusion pump devices. Such devices allow continuous infusion of desired compounds avoiding multiple injections and multiple manipulations.

[0061] Compositions containing a macrophage stimulating agent coupled to an appropriate carrier molecule of the present invention may also be formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0062] Effective doses of the compositions of the present invention, for the therapeutic treatment (*e.g.*, therapeutic treatment of inflammatory conditions or cancer) vary depending upon many different factors, including type and stage of the disease, means of administration, target site, physiological state of the patient, other medications or therapies administered, and physical state of the patient relative to other medical complications. Treatment dosages need to be titrated to optimize safety and efficacy.

[0063] Another aspect of the present invention is directed to a method of treating a tumor in a subject. This method involves selecting a subject having a tumor and administering to the selected subject an anti-tumor agent coupled to a carrier molecule that is defective in neonatal Fc receptor (FcRn) binding, wherein the administering induces a therapeutic tumor cell response in the subject.

[0064] In accordance with this embodiment, the tumor is characterized by tumor cells expressing FcRn. Exemplary tumor types that exhibit FcRn expression include, without limitation, tumors of the lung, colon, pancreas, and glioblastoma multiforme. As noted above, the FcRn binds to and actively recycles albumin and IgG molecules. Therefore, the activity of an anti-tumor cell agent that is coupled to an albumin carrier molecule (such as nanoparticle albumin bound paclitaxel) or fused to an IgG will be enhanced if coupled to a variant albumin protein or variant IgG that is defective or deficient in FcRn binding so as to prevent or reduce FcRn mediate recycling of the anti-tumor agent out of the cell. For example, nanoparticle albumin bound paclitaxel (nab-paclitaxel, trade name Abraxane) is a human albumin bound formulation of paclitaxel that is approved by the FDA as a first line therapy for advanced pancreatic cancer. Paclitaxel's primary mechanism of anti-neoplastic activity is its ability to stabilize microtubules and prevent cell division of rapidly dividing tumor cells. However, some KRas-mutant pancreatic cancer cells express FcRn. Therefore, these cells escape the cytotoxic effects of nab-paclitaxel, resulting in decreased tumor cell killing. Accordingly, in one embodiment, a subject having pancreatic cancer, in particular pancreatic ductal adenocarcinoma (PDAC), is selected and administered an anti-tumor agent that is coupled to a carrier molecule defective in FcRn binding.

[0065] In one embodiment, the method of treating a tumor in a subject as described herein further involves selecting a subject suitable for treatment by detecting the presence or absence of FcRn expression in the tumor cells. Subjects having a tumor, where the tumor cells are positive for FcRn expression would benefit from treatment with an anti-tumor agent that is coupled to a carrier molecule defective in FcRn binding. Methods and modes of administering agents of the present invention are described *supra*.

[0066] As used herein, the term "anti-tumor agent" encompasses any reagent that acts directly on or in a tumor cell to inhibit tumor cell division and/or cause tumor cell death. These agents include, without limitation, chemotherapeutic agents such as, alkylating agents (*e.g.*, chlorambucil, cyclophosphamide, CCNU, melphalan, procarbazine, thiotepa, BCNU, and busulfan), antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, and 5-fluorouracil), anthracyclines (daunorubicin, doxorubicin, idarubicin, epirubicin, and mitoxantrone), antitumor antibiotics (*e.g.*, bleomycin, monoclonal antibodies (*e.g.*, Alemtuzumab, Bevacizumab,

Cetuximab, Gemtuzumab, Ibritumomab, Panitumumab, Rituximab, Tositumomab, and Trastuxmab), platinum (e.g., cisplatin and oxaliplatin) or plant alkaloids (e.g., topoisomerase inhibitors, vinca alkaloids, taxanes (e.g. paclitaxel), and epipodophyllotoxins). As indicated above, coupling an anti-tumor cell agent to a carrier molecule that is defective or deficient in FcRn binding (e.g., variant albumin or variant IgG proteins or fragments thereof) will enhance the activity of the reagent on the target tumor cells to induce a therapeutic tumor cell response, i.e., inhibition of tumor cell division or induction of tumor cell death.

EXAMPLES

[0067] The following examples are provided to illustrate embodiments of the present invention but they are by no means intended to limit its scope.

Material and Methods for Examples

[0068] **Cell lines and cell culture treatments.** All cells were maintained under 5% CO₂ at 37 °C and cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), penicillin and streptomycin (Gibco), and 25 mmol/L HEPES (Gibco). Mouse RAW 264.7 cells were obtained from the American Type Culture Collection (2014, ATCC TIB-71). Mouse KPC cells were a kind gift from Dr. R.H. Vonderheide (2013) and generated as previously described (Lo et al., “Tumor-Promoting Desmoplasia Is Disrupted by Depleting FAP-Expressing Stromal Cells,” *Cancer Res.* 75:2800-10 (2015), which is hereby incorporated by reference in its entirety). Cell lines were not authenticated and were tested for mycoplasma contamination every 3 months by DAPI stain of cells grown for 1 week in the absence of antibiotics. KPC and RAW 264.7 were propagated for 2 to 3 passages (approximately 1 week) prior to use in all experiments and were kept in culture for no longer than 1 month. Recombinant mouse interferon gamma IFN γ was purchased from R&D Systems (CN 485 MI/CF), LPS (L2630), and paclitaxel (T7402) were purchased from Sigma-Aldrich, CLI-095 was purchased from Invivogen (TLRL-CLI95), BAPTA-AM was purchased from Life Technologies (B6769), and 5-(N-ethyl-N-isopropyl) amiloride (EIPA) was purchased from Invitrogen Molecular Probes (e-3111). Reagents were used at concentrations of 5 ng/mL (IFN γ), 20 ng/mL (LPS), 10 mmol/L (paclitaxel), 500 nmol/L (CLI-095), 50 mmol/L (BAPTA-AM), and 50 mmol/L (EIPA) unless indicated otherwise. Tetramethylrhodamine (TMR)-labeled dextran was purchased from Invitrogen (D1822). Nab-paclitaxel (Abraxane; Abraxis BioScience) pharmaceutical grade powder was purchased through the NYU Langone Medical Center pharmacy and used at a concentration of 10 mmol/L for *in vitro* experiments.

[0069] Antibodies. Antibodies used for western blotting are as follows: rabbit anti-IL1 α , rabbit anti-IL1 β (Abcam ab9722), rabbit anti-IL6 (Novus Biologicals NB600-1131), rabbit anti-iNOS (Cell Signaling Technology, CST 13120), rabbit anti-TNF α (Abcam ab9739), and mouse anti-VINCULIN (Sigma V9131). F4/80 antibody used for immunofluorescence and

5 immunohistochemistry was purchased from eBiosciences (rat anti-mouse F4/80, clone BM8, ref 14-480182). CD16/CD32 antibody was purchased from BD Biosciences (rat anti-mouse-CD16/CD32, clone 2.4G2) and used at a concentration of 25 mg/mL. Monoclonal antibodies used for flow cytometry were all purchased from Biolegend unless indicated otherwise: anti-CD45 (Brilliant Violet 421-anti-mouse-CD45, clone 30-F11), anti-F4/80 (PE-anti-mouse-F4/80, clone BM8 or APC-anti-mouse-F4/80, clone BM8), anti-MHCII (FITC-anti-mouse-I-A/I-E, clone M5/114.15.2, BD Biosciences), anti-CD80 (PE/Cy7-anti-mouse-CD80, clone 16-10A1), anti-CD86 (PerCP-anti-mouse-CD86, clone GL-1), and anti-IL1 α (PE-anti-mouse-IL1 α , clone ALF-161).

[0070] Western blot analysis. Western blot analyses were initiated 24 hours (iNOS) or 15 8 hours (IL1 α , IL1 β , IL6, and TNF α) following cell culture treatments. Total cell lysates were harvested in sample buffer (40 mmol/L Tris, pH 6.8, 1% SDS, 5% beta-mercaptoethanol, 7.5% glycerol, and 0.01% bromophenol blue) and incubated at 99°C for 5 minutes. Cell lysates (1/10 of total volume) were loaded onto 10% SDS-Polyacrylamide gels for electrophoresis and transferred onto nitrocellulose membranes for 1 hour at 100 volts at 4°C. Membranes were 20 blocked for 1 hour at room temperature in 5% BSA (Sigma-Aldrich) diluted in PBS containing 0.1% Tween 20 prior to immunodetection.

[0071] Gene expression analysis. For *in vitro* qPCR analysis, total RNA was harvested 2 hours (IL1 α , IL1 β , IL6, TNF α , and iNOS) or 8 hours (IL12 β) after cell culture treatments, using the QIAGEN RNA extraction Kit (QIAGEN). For *in vivo* gene expression analysis, the 25 CD45 F4/80 double-positive population was sorted directly into TRIzol LS Reagent (ThermoFisher Scientific; CN 10296) from dissociated orthotopic tumors 48 hours after treatment, and total RNA was purified according to the manufacturer's protocol. Purified RNA (1 mg) was reverse-transcribed using the QuantiTect Reverse Transcription Kit (QIAGEN), and 1 of 10 of cDNA mixture (*in vitro* experiments) or 1 of 3 of cDNA mixture (*in vivo* experiments) was used 30 for qPCR reaction. Primers were obtained from Integrated DNA Technologies (IDT) with the following sequences (5'-3'): *Il1 α* (Forward) AATCAAGATGGCCAAAGTTCC (SEQ ID NO: 2), (Reverse) ATTCAGAGAGAGATGGTCAATGG (SEQ ID NO: 3); *Il1 β* (Forward) GCAACTGTTCTGAAGTCAACT (SEQ ID NO: 4), (Reverse) ATCTTTTGGGGTCCGTCAACT (SEQ ID NO: 5); *Il6* (Forward) TAGTCCTTCCTA- 35 CCCCAATTTC (SEQ ID NO: 6), (Reverse) TTGGTCCTTAGCCACTCCTTC (SEQ ID NO: 7).

7); *Inos* (Forward) GTTCTCAGCCCAACAATACAAGA (SEQ ID NO: 8), (Reverse) GTGGACGGGTCGATCTCAC (SEQ ID NO: 9); *Thfa* (Forward) CTGTAGCCCACGTCGTAGC (SEQ ID NO: 10), (Reverse) TTGAGATCCATGCCGTTG (SEQ ID NO: 11); *Il12b* (Il12 p40) (Forward) TGGTTTGCCATCGTTTTGCTG (SEQ ID NO: 12), (Reverse) ACAGGTGA-GGTTCACTGTTTCT (SEQ ID NO: 13); *Gapdh* (Forward) CACGGCAAATTCAACGGCACAGTC (SEQ ID NO: 14), (Reverse) ACCCGTTTGGCTCCACCCTTCA (SEQ ID NO: 15); *Rps29* GTCTGATCCGCAAATACGGG (SEQ ID NO: 16) (Forward), AGCCTATGTCCTTC-GCGTACT (SEQ ID NO: 17) (Reverse).

[0072] Synthesis of Oregon green-labeled paclitaxel and nab-paclitaxel. Albumin-bound Oregon Green 488-labeled paclitaxel (OG-nab-paclitaxel) was prepared by coupling Oregon Green 488-labeled paclitaxel (OG-paclitaxel; Thermo Fisher Scientific, CN P22310) to human serum albumin (Sigma-Aldrich; CN 05420). Briefly, 0.9 mg OG-paclitaxel was dissolved in 64.8 mL chloroform and 7.2 mL ethanol and added to 18 mg human serum albumin dissolved in 3.6 mL water with vigorous stirring. The suspension was sonicated at 40% amplitude for 4 cycles of 1 minute each with 30-second intervals. The organic solvent was then evaporated using rotavapor at 40°C. The resulting suspension was distributed equally into glass vials and lyophilized to obtain dry OG-nab-paclitaxel amenable to long-term storage at -20°C.

[0073] Macropinocytosis assays. Macropinocytosis assays, image acquisition, image processing tools, and macropinosome quantification were performed as previously described (Commisso et al., "Determining the Macropinocytic Index of Cells Through a Quantitative Image-based Assay," *Nat. Protoc.* 9:182-92 (2014), which is hereby incorporated by reference in its entirety). For *ex vivo* nab-paclitaxel uptake assays, orthotopic tumors were cut into approximately 3 mm x 3 mm sections and incubated for 20 minutes at 37°C in serum-free medium containing Oregon Green-labeled nab-paclitaxel (0.5 mg/mL). Tumor sections were then washed once with PBS at room temperature and immediately embedded in Tissue-Tek OCT medium (VWR) and fixed for 1 hour on dry ice. Fixed tumor sections were stored at -80°C until cryosectioned and mounted on coverslips to be stained for immunofluorescence.

[0074] Immunohistochemistry. Immunohistochemistry on tumor sections was performed as previously described (Pylayeva-Gupta et al., "Oncogenic Kras-induced GM-CSF Production Promotes the Development of Pancreatic Neoplasia," *Cancer Cell* 21:836-47 (2012), which is hereby incorporated by reference in its entirety).

[0075] Cell sorting and flow cytometry. Staining for cell sorting and flow cytometry analysis was performed by incubating single-cell suspensions with primary fluorochrome-labeled antibodies on ice for 30 minutes. For cell sorting, cells were washed once with FACS buffer

(PBS supplemented with 1% FBS) and immediately sorted on an SY2300 cell sorter (Sony). For flow cytometry, cells were fixed in 3.7% formaldehyde diluted in FACS buffer overnight at 4°C prior to analysis. For IL1 α staining, cells were permeabilized and fixed with FoxP3/Transcription Factor Staining Buffer Set (eBioscience CN00-5523) for 45 minutes at 4°C and stained overnight at 4°C. All samples were washed once with FACS buffer prior to analysis on an LSRII UV flow cytometer (BD Biosciences). Collected data were analyzed using FlowJo data analysis software (V10).

[0076] Animal studies. All animals used in this study were 8-week-old C57/BL6 female mice purchased from Charles River Laboratories. For orthotopic implantations, 1×10^5 KPC cells were resuspended in 20 μ L cold PBS and mixed with 20 μ L Matrigel (Corning; ref 254348) prior to injection into the pancreas of syngeneic mice. After 2 weeks, orthotopically implanted mice were treated intraperitoneally with gemcitabine (120 mg/kg, Gemzar, Eli Lilly) and nab-paclitaxel (120 mg/kg, Abraxane; Abraxis Bioscience) resuspended in sterile PBS. Both drugs were purchased through the NYU Langone Medical Center pharmacy. All animal care and procedures were approved by the Institutional Animal Care and Use Committee at the NYU School of Medicine.

[0077] Statistical analyses. Statistical comparisons were evaluated by the Student t test. All P values stated are intentionally presented as uncorrected for multiple comparisons, as one planned comparison was conducted for each experimental condition tested (i.e., multiple comparisons were not performed).

Example 1 – Macrophages Internalized Nab-Paclitaxel Via Macropinocytosis

[0078] Macrophages undergo constitutive macropinocytosis, a form of nonselective fluid-phase endocytosis that, together with receptor-activated phagocytosis, regulates antigen sampling and scavenging of invading pathogens (Sallusto et al., “Dendritic Cells Use Macropinocytosis and the Mannose Receptor to Concentrate Macromolecules in the Major Histocompatibility Complex Class II Compartment: Downregulation by Cytokines and Bacterial Products,” *J. Exp. Med.* 182:389-400 (1995), which is hereby incorporated by reference in its entirety). Macropinocytosis is also an established route of internalization of many nanoparticle formulations (Lim et al., “Macropinocytosis: An Endocytic Pathway for Internalising Large Gulps,” *Immunol. Cell Biol.* 89:836-43 (2011), which is hereby incorporated by reference in its entirety). Given that macrophages constitute a dominant immune infiltrate in PDAC, the extent and mode of nab-paclitaxel internalization by macrophages was assessed. To this end, the macrophage cell line RAW 264.7 was incubated with high molecular weight TMR-labeled dextran (TMR-dextran), an established marker of macropinosomes, and Oregon Green-labeled

nab-paclitaxel (OG-nab-paclitaxel). OG-nab-paclitaxel localized predominantly to dextran-positive macropinosomes (Fig. 1A). Furthermore, OG-nab-paclitaxel internalization was reduced following treatment with the macropinocytosis inhibitor 5-[N-ethyl-N-isopropyl] amiloride (EIPA), indicating that nab-paclitaxel internalization depended on a macropinocytotic uptake mechanism (Fig. 1B). To determine the contribution of phagocytosis to nab-paclitaxel internalization, the requirement of Fc receptors for uptake was tested because, unlike other major phagocytic receptor families, Fc receptors are constitutively active in macrophages (Ravetch JV, "Fc Receptors: Rubor Redux," *Cell* 78:553-60 (1994), which is hereby incorporated by reference in its entirety). Inhibition of Fc receptors I and III with blocking antibodies to CD16 and CD32 (Indik et al., "The Molecular Dissection of Fc Gamma Receptor Mediated Phagocytosis," *Blood* 86:4389-99 (1995), which is hereby incorporated by reference in its entirety) did not affect the internalization of OG-nab-paclitaxel (Fig. 1C). Moreover, depletion of intracellular calcium, required for constitutive macropinocytosis but not Fc receptor-dependent phagocytosis in macrophages (Di Virgilio et al., "Fc Receptor-mediated Phagocytosis Occurs in Macrophages at Exceedingly Low Cytosolic Ca²⁺ Levels," *J. Cell Biol.* 106:657-66 (1988); Jongstra-Bilen et al., "Fcgamma-receptors Induce Mac-1 (CD11b/CD18) Mobilization and Accumulation in the Phagocytic Cup for Optimal Phagocytosis," *J. Biol. Chem.* 278:45720-9 (2003), which are hereby incorporated by reference in their entirety), effectively blunted OG-nab-paclitaxel uptake (Fig. 1D). Together, these data demonstrate that nab-paclitaxel is internalized by macrophages predominantly via macropinocytosis.

Example 2 - Macropinocytosis of Nab-Paclitaxel Drove M1 Macrophage Activation *In Vitro* Via TLR4

[0079] Consistent with recent findings by Tanei and colleagues (Tanei et al., "Redirecting Transport of Nanoparticle Albumin-Bound Paclitaxel to Macrophages Enhances Therapeutic Efficacy against Liver Metastases," *Cancer Res.* 76:429-39 (2016), which is hereby incorporated by reference in its entirety), significant cytotoxic effects of nab-paclitaxel on macrophages was not observed at concentrations required for its internalization. Therefore, the capacity of nab-paclitaxel to induce macrophage type-1 (M1) activation was assessed. Because paclitaxel promotes M1 polarization by acting as an LPS mimetic (Zimmer et al., "Paclitaxel Binding to Human and Murine MD-2," *J. Biol. Chem.* 283:27916-26 (2008), which is hereby incorporated by reference in its entirety), the effect of nab-paclitaxel treatment on the induction of a panel of LPS-inducible cytokines was evaluated. Treatment of RAW 264.7 cells with nab-paclitaxel was sufficient to induce the gene expression of *Il1α*, *Il1β*, *Il6*, *Il12b* (*Il12 p40*), and *Tnfa* in a similar manner to free paclitaxel (Fig. 2A). Nab-paclitaxel also increased IL1α, IL13,

IL6, and TNF α protein expression to a similar extent as LPS (FIG. 5). Nab-paclitaxel-dependent M1 cytokine expression was inhibited by EIPA and BAPTA-AM, but not by blocking antibodies to the Fc I and III receptors, demonstrating that macropinocytosis was required for its LPS mimetic effects (Fig. 2B). The tumor cell inhibitory function of M1 macrophages is partially attributed to the upregulation of inducible nitric oxide synthase (iNOS), an enzyme that catalyzes the production of tumor cell cytotoxic nitric oxide from L-Arginine (Stuehr et al., "Nitric Oxide. A Macrophage Product Responsible for Cytostasis and Respiratory Inhibition in Tumor Target Cells," *J. Exp. Med.* 169:1543-55 (1989), which is hereby incorporated by reference in its entirety). It has been shown that LPS synergizes with IFN γ to induce iNOS protein expression in macrophages (Lorsbach et al., "Expression of the Nitric Oxide Synthase Gene in Mouse Macrophages Activated for Tumor Cell Killing. Molecular Basis for the Synergy Between Interferon-gamma and Lipopolysaccharide," *J. Biol. Chem.* 268:1908-13 (1993); Manthey et al., "Taxol Provides a Second Signal for Murine Macrophage Tumoricidal Activity," *J. Immunol.* 152:825-31 (1994), which are hereby incorporated by reference in their entirety). Similarly, nab-paclitaxel treatment induced robust iNOS expression in the presence of IFN γ (Fig. 2C). Nab-paclitaxel-driven iNOS gene and protein expression was partially inhibited by EIPA (Fig. 2D), further supporting the idea that macropinocytosis of nab-paclitaxel contributes to its M1 polarizing effects. Paclitaxel induces macrophage activation via binding to myeloid differentiation factor 2 (MD2), an adaptor protein to TLR4 (Zimmer et al., "Paclitaxel Binding to Human and Murine MD-2," *J. Biol. Chem.* 283:27916-26 (2008), which is hereby incorporated by reference in its entirety). Indeed, it was found that the TLR4 inhibitor CLI-095 abrogated nab-paclitaxel and LPS-dependent *Il1 α* , *Il1 β* , *Il6*, *Il12 β* , and *Tnf α* gene expression (Fig. 3A and FIG. 6) and iNOS protein expression (Fig. 3B). These results indicate that nab-paclitaxel signals through the TLR4 receptor.

Example 3 - Nab-paclitaxel Induces Type-1 Macrophage Activation in Pancreatic Tumor-Associated Macrophages *In Vivo*

[0080] Next, whether nab-paclitaxel could induce M1 polarization of macrophages in the pancreatic tumor microenvironment was determined. To this end, an orthotopic model of PDAC was employed in which cells isolated from primary pancreas tumors of Kras^{LSL-G12D/+}, Trp53^{LSL-R172H/+}, Pdx1-Cre (KPC) transgenic mice were surgically implanted into the pancreas of immunocompetent syngeneic wild-type animals. In this model, the cells formed tumors within 2 weeks after implantation and exhibited an extensive F4/80⁺ macrophage infiltrate (Fig. 4A). To determine whether TAMs can internalize nab-paclitaxel, pancreatic tumors were incubated with OG-nab-paclitaxel and found that F4/80⁺ cells within the tumor took up nab-paclitaxel (Fig. 4B).

Moreover, 83% of CD45⁺/F4/80⁺ cells isolated from KPC orthotopic tumors by FACS internalized OG-nab-paclitaxel following ex vivo treatment (FIG. 7A). To determine whether nab-paclitaxel internalization induces M1 polarization of pancreatic tumor-associated macrophages *in vivo*, orthotopic tumor-bearing mice were treated with a single therapeutic dose of nab-paclitaxel, alone or in combination with gemcitabine, and analyzed for the functional activation of macrophages within the tumor 48 hours after treatment (Fig. 4C). Optimal macrophage activation by immunogenic stimuli requires both the upregulation of antigen-presenting MHCII molecules and the induction of T-cell costimulatory molecules CD80 and CD86 at the cell surface (Subauste et al., "Role of CD80 (B7.1) and CD86 (B7.2) in the Immune Response to an Intracellular Pathogen," *J. Immunol.* 160:1831-40 (1998), which is hereby incorporated by reference in its entirety). Analysis of the MHCII⁺CD80⁺CD86⁺ cell population by flow cytometry revealed that single-agent nab-paclitaxel was sufficient to increase the MHCII⁺CD80⁺CD86⁺ macrophage population within the tumors compared with vehicle-treated mice, with no change in total macrophage numbers across treatment cohorts (Fig. 4C and FIG. 7B). Nab-paclitaxel treatment also induced an increase in IL1 α protein expression in the tumor-associated M1 population compared with vehicle-treated mice (Fig. 4D). Gene expression analysis of the total macrophage population also revealed a 2-fold increase in IL1 β expression following treatment with nab-paclitaxel and gemcitabine (FIG. 7C). Together, these data demonstrate that internalization of nab-paclitaxel by pancreatic tumor-associated macrophages can drive them toward an M1 activation state *in vivo*.

Discussion of Examples 1-3

[0081] Taxanes, and in particular paclitaxel, represent an important class of antitumor agents that have proven to be effective in the treatment of a number of solid malignancies. The albumin-bound form of paclitaxel, nab-paclitaxel, has fewer side effects, shows increased tumor cell cytotoxicity, and patients have higher overall response rates, compared with equal doses of solvent-based paclitaxel in breast, non-small cell lung (NSCLC), and pancreatic cancers (Kundrandra and Niu, "Albumin-bound Paclitaxel in Solid Tumors: Clinical Development and Future Directions," *Drug Des Devel Ther* 9:3767-77 (2015), which is hereby incorporated by reference in its entirety). The improved tumor response to nab-paclitaxel has been attributed to elevated intratumoral concentrations mediated by binding of albumin to endothelial 60-kDa glycoprotein receptor (gp60; Desai et al., "Increased Antitumor Activity, Intratumor Paclitaxel Concentrations, and Endothelial Cell Transport of Cremophor-free, Albumin-bound Paclitaxel, ABI-007, Compared With Cremophor-based Paclitaxel," *Clin Cancer Res* 12: 1317-24 (2006), which is hereby incorporated by reference in its entirety.), thereby facilitating vascular

transcytosis. It has been proposed that the antitumor activity of nab-paclitaxel might be attributed to its binding to SPARC, a cell surface receptor with sequence homology to gp60 that is expressed on multiple tumor cell types (Desai et al., “Improved Effectiveness of Nanoparticle Albumin-bound (nab) Paclitaxel Versus Polysorbate-based Docetaxel in Multiple Xenografts as a Function of HER2 and SPARC Status,” *Anticancer Drugs* 19:899–909 (2008); Desai et al., “SPARC Expression Correlates With Tumor Response to Albumin-bound Paclitaxel in Head and Neck Cancer Patients,” *Transl Oncol* 2:59–64 (2009), which are hereby incorporated by reference in their entirety). The findings reported herein suggest that nab-paclitaxel-dependent tumor cell killing may be additionally mediated via its immunostimulatory effects on TAMs.

The potential relevance of this tumor cell–extrinsic mechanism in the context of PDAC is supported by the observation that paclitaxel provides limited clinical benefit despite exhibiting comparable effects on microtubule function to nab-paclitaxel in tumor cells (Awasthi et al., “Comparative Benefits of Nab-paclitaxel Over Gemcitabine or Polysorbate-based Docetaxel in Experimental Pancreatic Cancer,” *Carcinogenesis* 34:2361–9 (2013), which is hereby incorporated by reference in its entirety). In murine and human studies of PDAC, nab-paclitaxel more effectively reduces stromal density relative to solvent-based taxanes (Awasthi et al., “Comparative Benefits of Nab-paclitaxel over Gemcitabine or Polysorbate-based Docetaxel in Experimental Pancreatic Cancer,” *Carcinogenesis* 34:2361–9 (2013); Alvarez et al., “Stromal Disrupting Effects of Nab-paclitaxel in Pancreatic Cancer,” *BrJ Cancer* 109:926–33 (2013); Von Hoff et al., “Gemcitabine Plus Nab-paclitaxel is an Active Regimen in Patients With Advanced Pancreatic Cancer: a Phase I/II Trial,” *J Clin Oncol* 29:4548–54 (2011); DeNardo et al., “CD4(+) T Cells Regulate Pulmonary Metastasis of Mammary Carcinomas by Enhancing Protumor Properties of Macrophages,” *Cancer Cell* 16:91–102 (2009), which are hereby incorporated by reference in their entirety). As the activation of pancreatic stellate cells is influenced by M2 macrophages (Neesse et al., “SPARC Independent Drug Delivery and Antitumour Effects of Nab-paclitaxel in Genetically Engineered Mice,” *Gut* 63:974–83 (2014), which is hereby incorporated by reference in its entirety), the stromal-depleting consequences of nab-paclitaxel treatment on the tumor stroma could reflect its effect on macrophage M1 polarization. Both breast cancer and NSCLC, malignancies for which nab-paclitaxel is a standard treatment regimen (Kundranda and Niu, “Albumin-bound Paclitaxel in Solid Tumors: Clinical Development and Future Directions,” *Drug Des Devel Ther* 9:3767–77 (2015), which is hereby incorporated by reference in its entirety), have extensive immunosuppressive macrophage infiltrates (Shi et al., “Fibrogenesis in Pancreatic Cancer is a Dynamic Process Regulated by Macrophage-Stellate Cell Interaction,” *Lab Invest* 94:409–21 (2014); Tang, “Tumor-associated Macrophages as Potential Diagnostic and Prognostic Biomarkers in Breast Cancer,” *Cancer Lett*

332:3–10 (2013), which are hereby incorporated by reference in their entirety). Thus, the proposed mode of action of nab-paclitaxel in promoting macrophage activation might be of broad relevance to tumor sites in which the drug shows therapeutic benefits.

[0082] Paclitaxel promotes M1 polarization via direct binding to MD2, an extracellular adaptor protein of TLR4 (Zimmer et al., “Paclitaxel Binding to Human and Murine MD-2,” *J Biol Chem* 283:27916–26 (2008), which is hereby incorporated by reference in its entirety). Upon activation, TLR4 is rapidly internalized into endosomes and engages downstream signaling pathways via endocytic shuttling (Ma et al., “The M1 Form of Tumor-associated Macrophages in Non-small Cell Lung Cancer is Positively Associated With Survival Time,” *BMC Cancer* 10:112 (2010), which is hereby incorporated by reference in its entirety). TLR4 internalization and trafficking is required for efficient LPS-dependent TLR4 signal propagation (Ma et al., “The M1 Form of Tumor-associated Macrophages in Non-small Cell Lung Cancer is Positively Associated With Survival Time,” *BMC Cancer* 10:112 (2010), which is hereby incorporated by reference in its entirety). Although macropinosomes and endosomes are formed as distinct vesicular entities, they fuse in the course of their intracellular trafficking (Latz et al., “Lipopolysaccharide Rapidly Traffics to and From the Golgi Apparatus With the Toll-like Receptor 4-MD-2-CD14 Complex in a Process That is Distinct From the Initiation of Signal transduction,” *J Biol Chem* 277:47834–43 (2002), which is hereby incorporated by reference in its entirety). It is therefore plausible that the macropinocytic uptake of nab-paclitaxel enables it to act on endosomal TLR4 complexes. Whether this mode of activation provides the means to increase the local effective concentration of paclitaxel, and/or induce a different signaling repertoire, remains to be established. Inflammatory stimuli, including IFN γ and TNF α , induce a shift from phagocytosis to macropinocytosis for the internalization of pathogens by macrophages (Racoosin and Swanson, “Macropinosome Maturation and Fusion With Tubular Lysosomes in Macrophages,” *J Cell Biol* 121:1011–20 (1993), which is hereby incorporated by reference in its entirety). Nab-paclitaxel-mediated M1 induction may therefore result in positive feedback signaling, promoting further uptake of drug and enhancing its M1-activating effects in both an autocrine and paracrine fashion. Together, these data support the hypothesis that macropinocytosis of the albumin formulation of paclitaxel promotes its M1-polarizing effects and may account for its elevated activity over solvent-based formulations of paclitaxel. One study suggests that nab-paclitaxel treatment may induce tumor cell toxicity via its internalization and subsequent release by macrophages in the tumor microenvironment (Tanei et al., “Redirecting Transport of Nanoparticle Albumin-bound Paclitaxel to Macrophages Enhances Therapeutic Efficacy Against Liver Metastases,” *Cancer Res* 76:429–39 (2016), which is hereby incorporated by reference in its entirety). However, because a direct uptake of nab-paclitaxel by macrophages was not

formally demonstrated in that study, the relevance of this proposed mechanism to our findings that nab-paclitaxel mediates M1 activation cannot be ascertained.

[0083] Single-agent immunotherapies designed to activate cytotoxic T cells have shown little benefit in PDAC, despite showing efficacy in many solid tumors (Bosadasgupta and Pieters, “Inflammatory Stimuli Reprogram Macrophage Phagocytosis to Macropinocytosis for the Rapid Elimination of Pathogens,” *PLoS Pathog* 10:e1003879 (2014); Brahmer et al., “Safety and Activity of Anti-PD-L1 Antibody in Patients With Advanced Cancer,” *N Engl J Med* 366:2455–65 (2012); Le et al., “Evaluation of Ipilimumab in Combination With Allogeneic Pancreatic Tumor Cells Transfected With a GM-CSF Gene in Previously Treated Pancreatic Cancer,” *J Immunother* 36:382–9 (2013), which are hereby incorporated by reference in their entirety). Given that pancreatic tumor-associated macrophages can contribute to an immunosuppressive microenvironment by inhibiting cytotoxic T-cell function (Royal et al., “Phase 2 Trial of Single Agent Ipilimumab (anti-CTLA-4) for Locally Advanced or Metastatic Pancreatic Adenocarcinoma,” *J Immunother* 33:828–33 (2010), which is hereby incorporated by reference in its entirety), immune recognition may be improved by combining nab-paclitaxel with T-cell immunotherapies. Considering the highly immunocompromised microenvironment of PDAC, however, it is likely that more potent M1 agonists may be required to restore immune surveillance. Several macrophage-activating immunotherapies are in clinical trials for the treatment of a variety of malignancies, including blocking antibodies to colony stimulating factor 1 (De Palma and Lewis, “Macrophage regulation of tumor responses to anticancer therapies,” *Cancer Cell* 23:277–86 (2013), which is hereby incorporated by reference in its entirety); TLR-activating agents PAM3CSK4 (Pyonteck et al., “CSF-1R Inhibition Alters Macrophage Polarization and Blocks Glioma Progression,” *Nat Med* 19:1264–72 (2013), which is hereby incorporated by reference in its entirety) and Poly I:C (Vogelpoel et al., “Fc gamma Receptor-TLR Cross-talk Elicits Pro-inflammatory Cytokine Production by Human M2 Macrophages,” *Nat Commun* 5:5444 (2014), which is hereby incorporated by reference in its entirety); and blocking antibodies to IL10 (Cui et al., “Identification and Characterization of Poly(I:C)-induced Molecular Responses Attenuated by Nicotine in Mouse Macrophages,” *Mol Pharmacol* 83:61–72 (2013), which is hereby incorporated by reference in its entirety). It is possible that coupling such agents to albumin nanoparticles, in particular, modified albumin nanoparticles having reduced or defective FcRN binding affinity, may improve their delivery to macrophages in the tumor microenvironment and more efficiently restore immune recognition. Employing albumin nanoparticles, in particular modified albumin nanoparticles having reduced or defective FcRN binding affinity, as vehicles for macrophage-activating agents may therefore serve broad applicability in a variety of tumor types exhibiting extensive M2 infiltration.

[0084] Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims

5 which follow.

WHAT IS CLAIMED IS:

1. A method of inducing a phenotypic change in a population of monocytes and/or macrophages, said method comprising:

administering to the population of monocytes and/or macrophages, a macrophage stimulating agent coupled to a carrier molecule, wherein said carrier molecule facilitates macropinocytic uptake of said agent by monocytes and macrophages in the population and is defective in neonatal Fc receptor binding, and wherein said administering induces a phenotypic change in the monocytes and macrophages in the population.

2. The method of claim 1, wherein the macrophage stimulating agent is a macrophage type-1 stimulating agent selected from the group consisting of paclitaxel, a colony stimulating factor -1 (CSF-1) receptor antagonist, an IL-10 receptor antagonist, a Toll-like receptor (TLR)-2 agonist, a TLR-3 agonist, a TLR-4 agonist, a TLR-7 agonist, a TLR-8 agonist, and a TLR-9 agonist.

3. The method of claim 2, wherein said administering induces the expression of one or more macrophage type-1 phenotypic markers selected from the group consisting of IL-1 α , IL-1 β , IL-6, iNOS, IFN γ , and TNF α in macrophages of the population.

4. The method of claim 2, wherein said population of monocytes and/or macrophages comprises type-2 macrophages.

5. The method of claim 2, wherein the population of monocytes and/or macrophages comprises tumor-associated macrophages.

6. The method of claim 2, wherein said administering is carried out *in vivo* to a subject having pancreatic cancer, breast cancer, or non-small cell lung carcinoma.

7. The method of claim 1, wherein the macrophage stimulating agent is a macrophage type-2 stimulating agent selected from the group consisting of IL-33, IL-4 receptor agonists, glucocorticoids, IL-10 receptor agonist, IL-1 receptor agonist.

8. The method of claim 7, wherein said administering induces the expression of one or more macrophage type-2 phenotypic markers selected from the group consisting of IL-10, TGF- β , MRC1, TGM2, CD23, and CCL22 in macrophages of the population

5 9. The method of claim 7, wherein the population of monocytes and/or macrophages comprises type-1 macrophages.

10 10. The method of claim 7, wherein said administering is carried out *in vivo* to a subject having an inflammatory or autoimmune condition.

11. The method of claim 7, wherein said administering is carried out *in vivo* to a subject having Alzheimer's disease.

15 12. The method of claim 1, wherein the carrier molecule is a variant human albumin protein or fragment thereof.

20 13. The method of claim 12, wherein the variant human albumin protein or fragment thereof comprises one or more amino acid modifications at one or more amino acid positions corresponding to 464, 494, 495, 496, 499, 500, 510, 535, 536, 537, 538, and 573 of SEQ ID NO: 1.

25 14. The method of claim 13, wherein the variant human albumin protein or fragment thereof comprises one or more amino acid modifications selected from the group consisting of D494N, D494Q, D494A, E495Q, E495A, T496A, P499A, K536A, P537A, K538A, K500A, K573STOP, H464Q, H510Q, and H535Q.

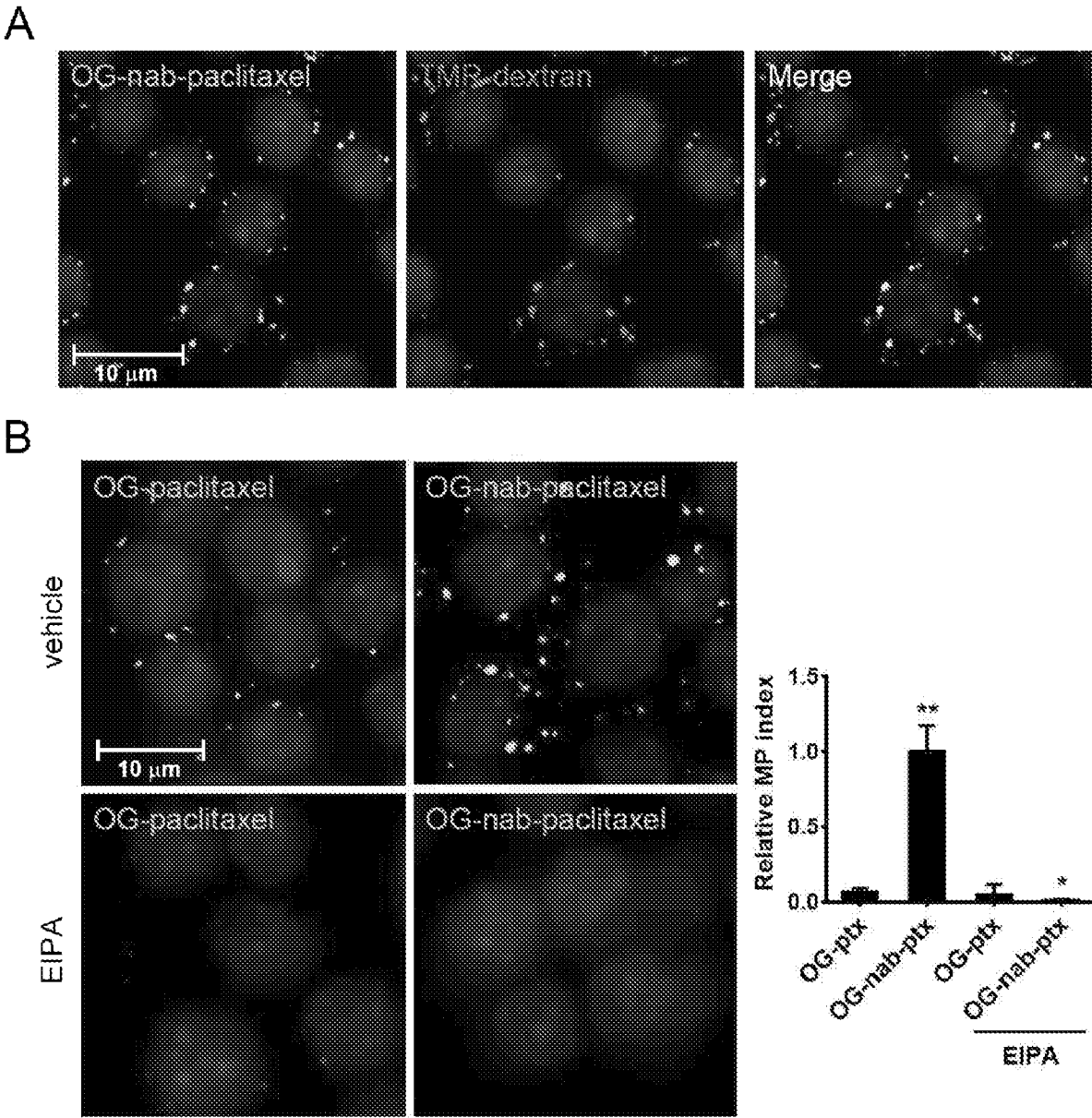
15. The method of claim 2, wherein the macrophage type-1 stimulating agent is paclitaxel and the carrier molecule is a variant human albumin protein or fragment thereof.

30 16. The method of claim 1, wherein the carrier molecule defective in FcRn binding is a variant immunoglobulin G protein or fragment thereof.

17. A method of treating a tumor in a subject comprising:
selecting a subject having a tumor and

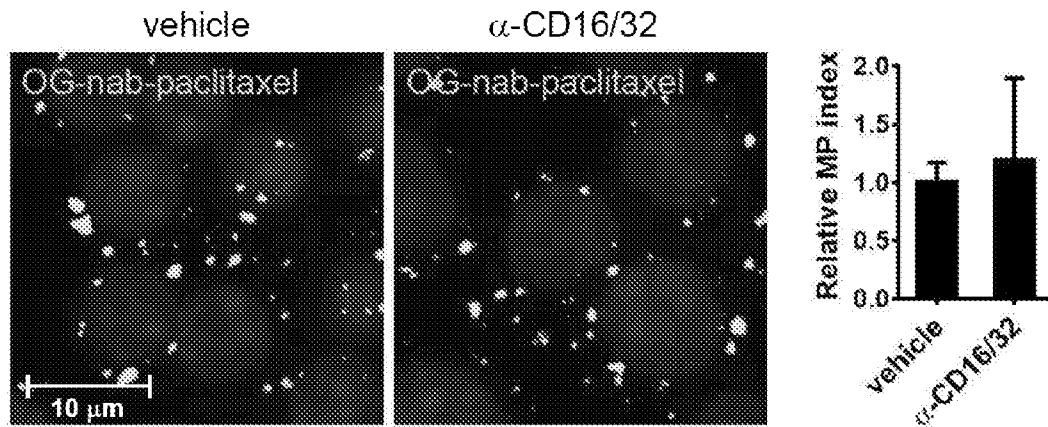
administering to the selected subject an anti-tumor agent coupled to a carrier molecule that is defective in neonatal Fc receptor (FcRn) binding, wherein said administering induces a therapeutic tumor cell response in the subject.

- 5 18. The method of claim 17, wherein the tumor is characterized by tumor cells expressing FcRn.
19. The method of claim 17, wherein the anti-tumor agent is a chemotherapeutic agent.
- 10 20. The method of claim 17, wherein the chemotherapeutic agent is a taxane.
21. The method of claim 20, wherein the taxane is paclitaxel.
- 15 22. The method of claim 17, wherein the carrier molecule is a variant human albumin protein or fragment thereof.
23. The method of claim 22, wherein the variant human albumin protein or fragment thereof comprises one or more amino acid modifications at one or more amino acid positions corresponding to 464, 494, 495, 496, 499, 500, 510, 535, 536, 537, 538, and 573 of SEQ ID NO: 1.
- 20 24. The method of claim 23, wherein the variant human albumin protein or fragment thereof comprises one or more amino acid modification selected from the group consisting of D494N, D494Q, D494A, E495Q, E495A, T496A, P499A, K536A, P537A, K538A, K500A, K573STOP, H464Q, H510Q, and H535Q.
- 25 25. The method of claim 17, wherein the anti-tumor agent is paclitaxel and the carrier molecule is a variant human albumin protein or fragment thereof.
- 30 26. The method of claim 17, wherein the carrier molecule defective in FcRn binding is a variant immunoglobulin G protein or fragment thereof.

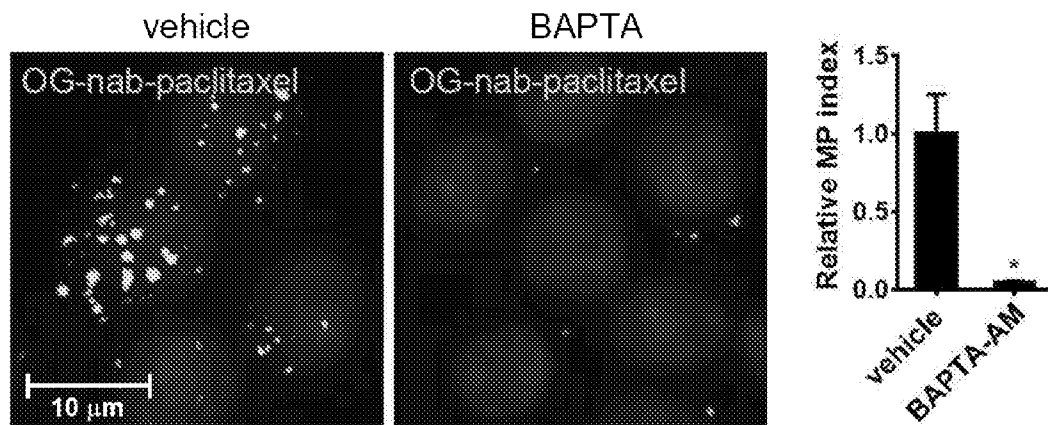


FIGs. 1A-1B

C



D



FIGs. 1C-1D

A

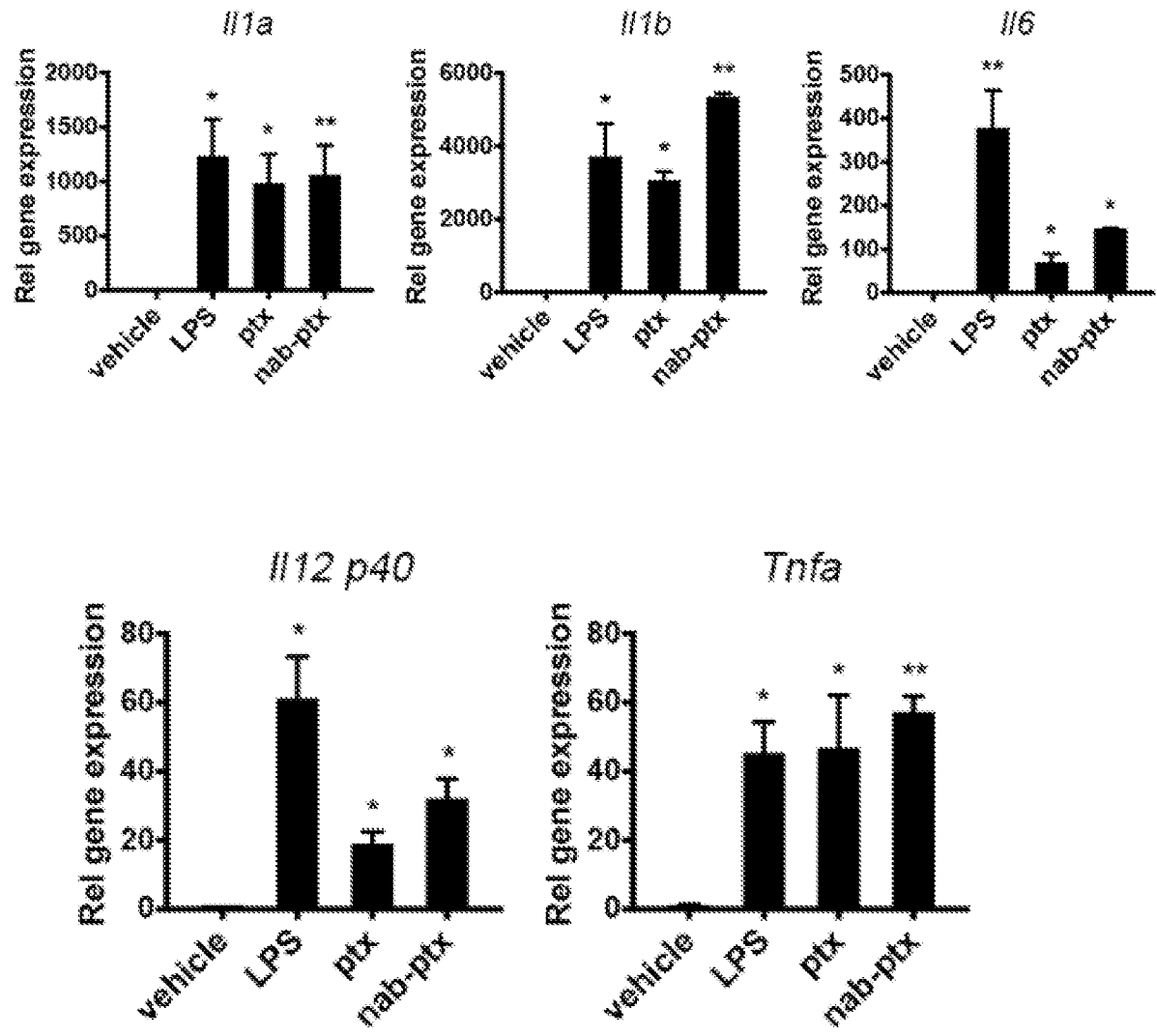


FIG. 2A

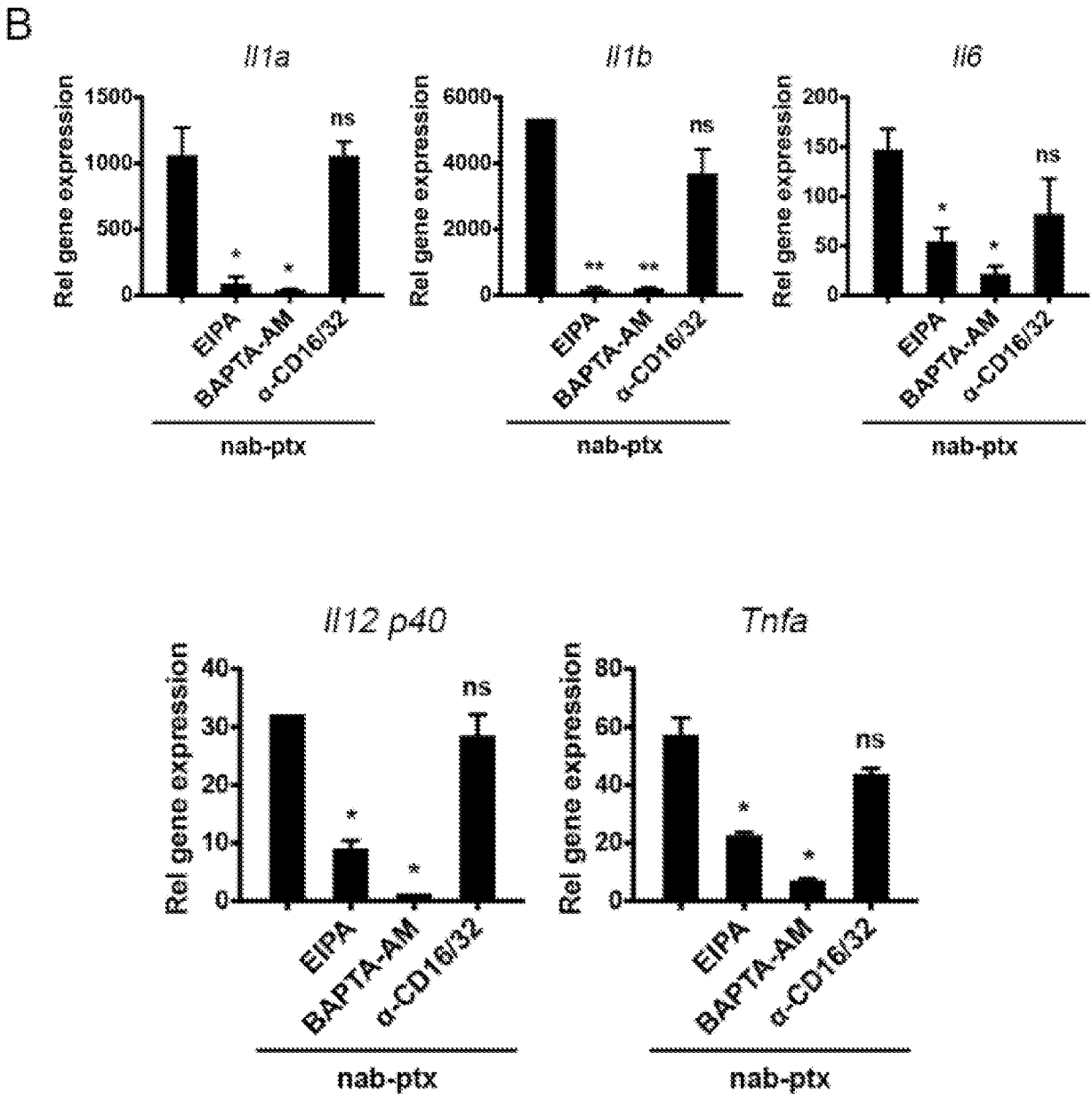
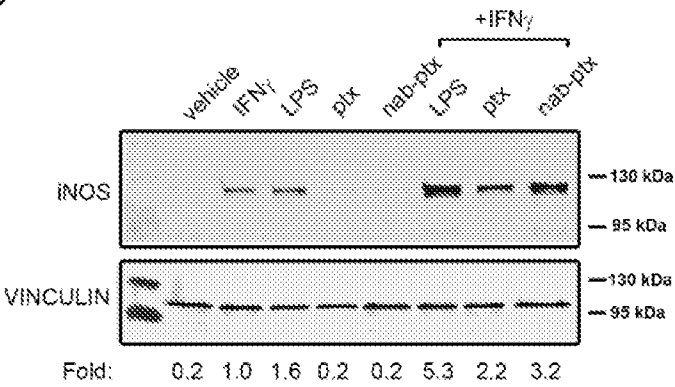
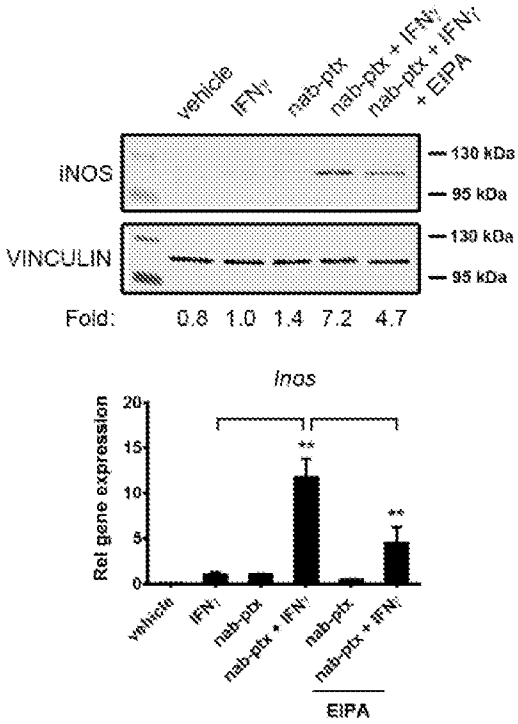


FIG. 2B

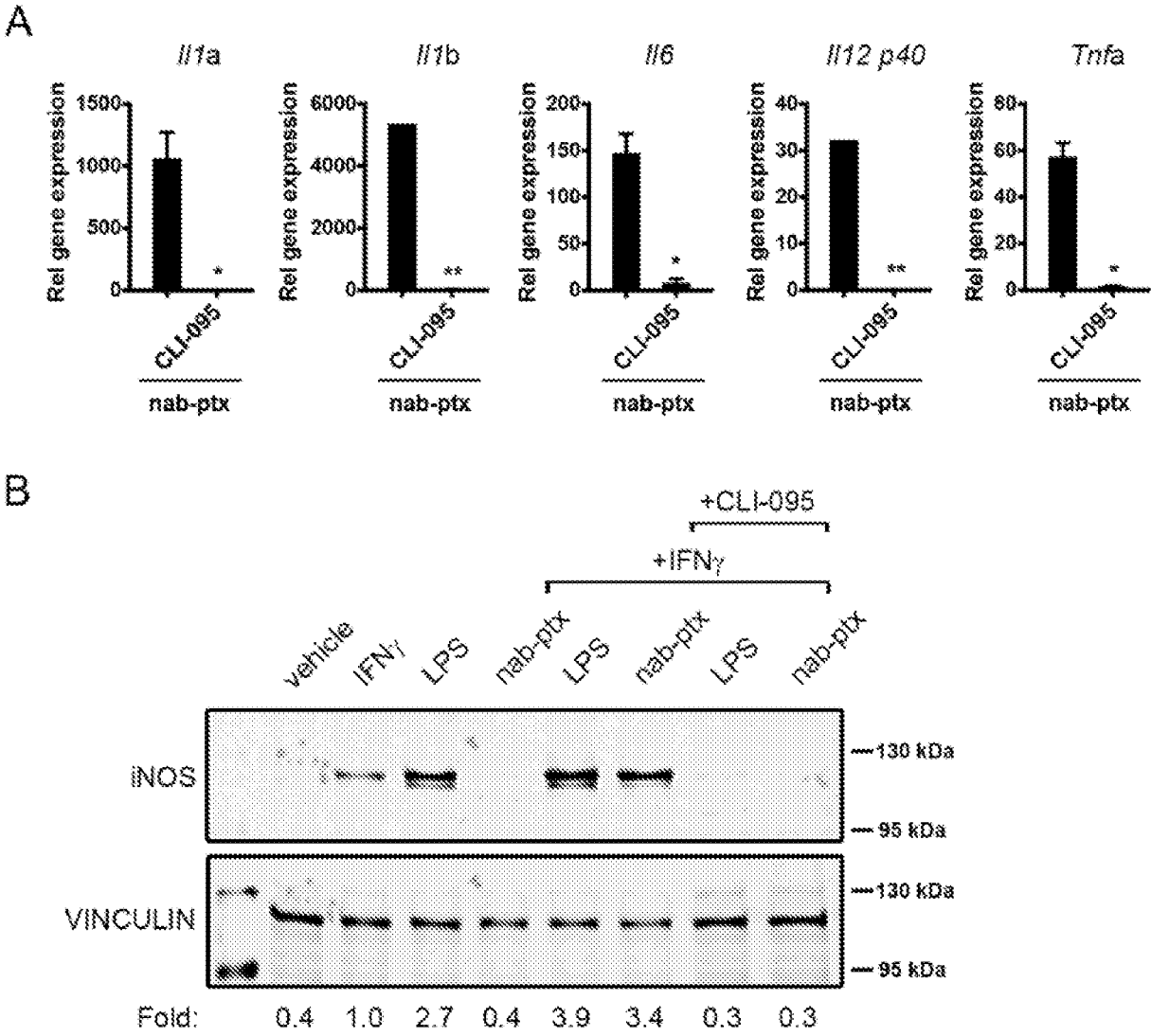
C



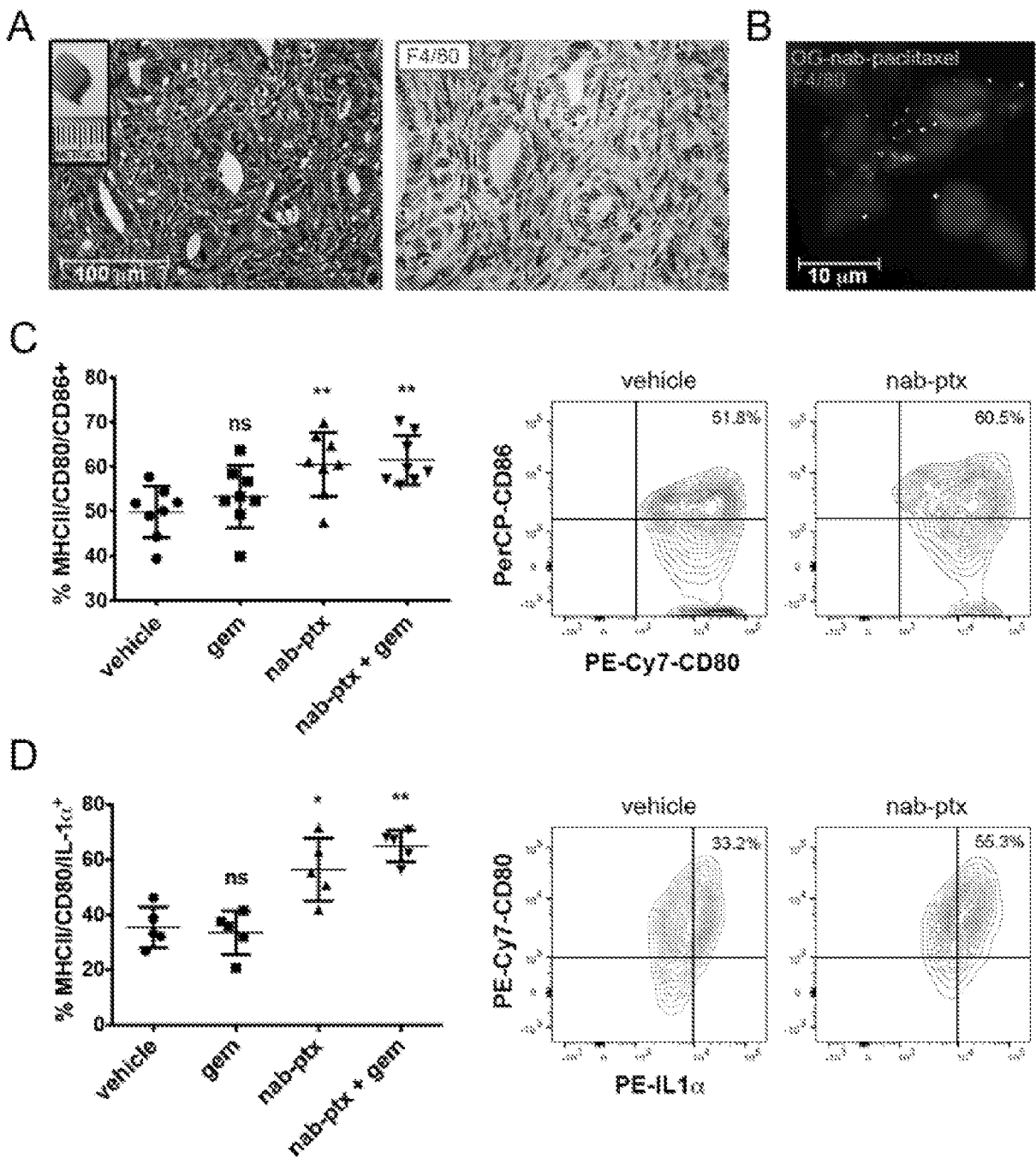
D



FIGs. 2C-2D



FIGs. 3A-3B



FIGs. 4A-4D

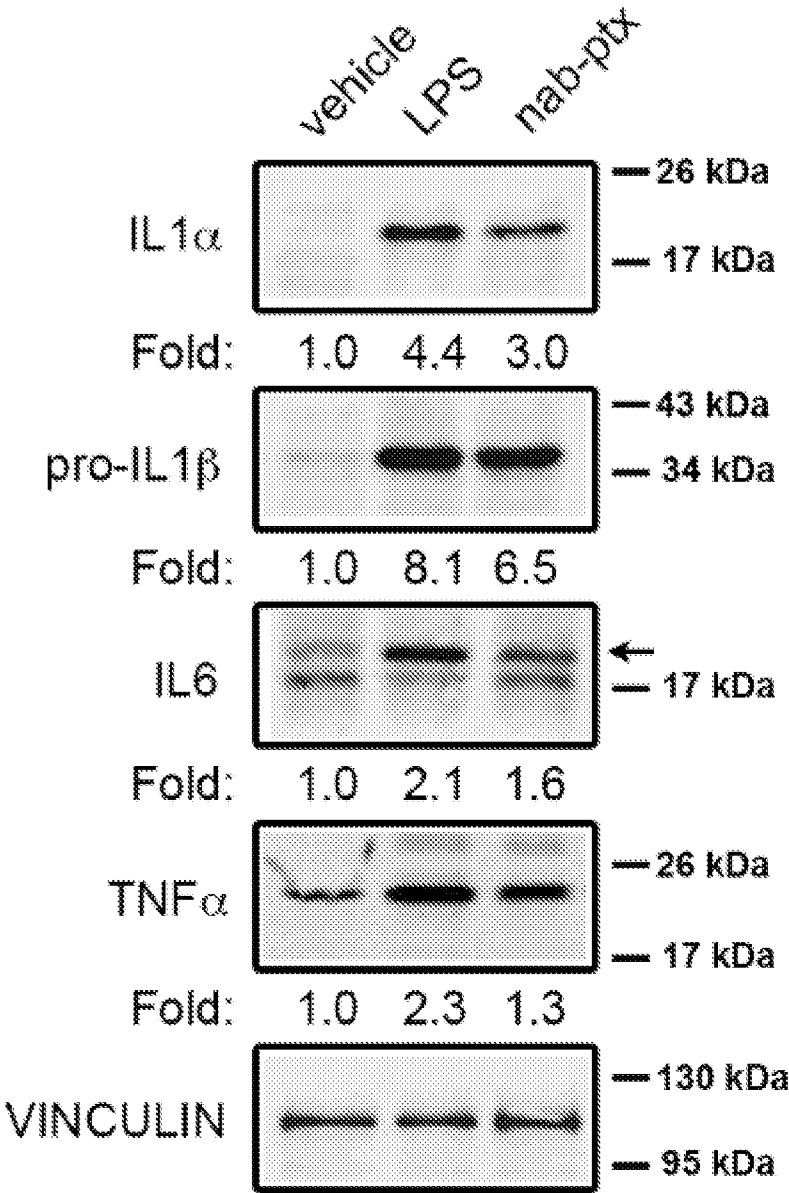


FIG. 5

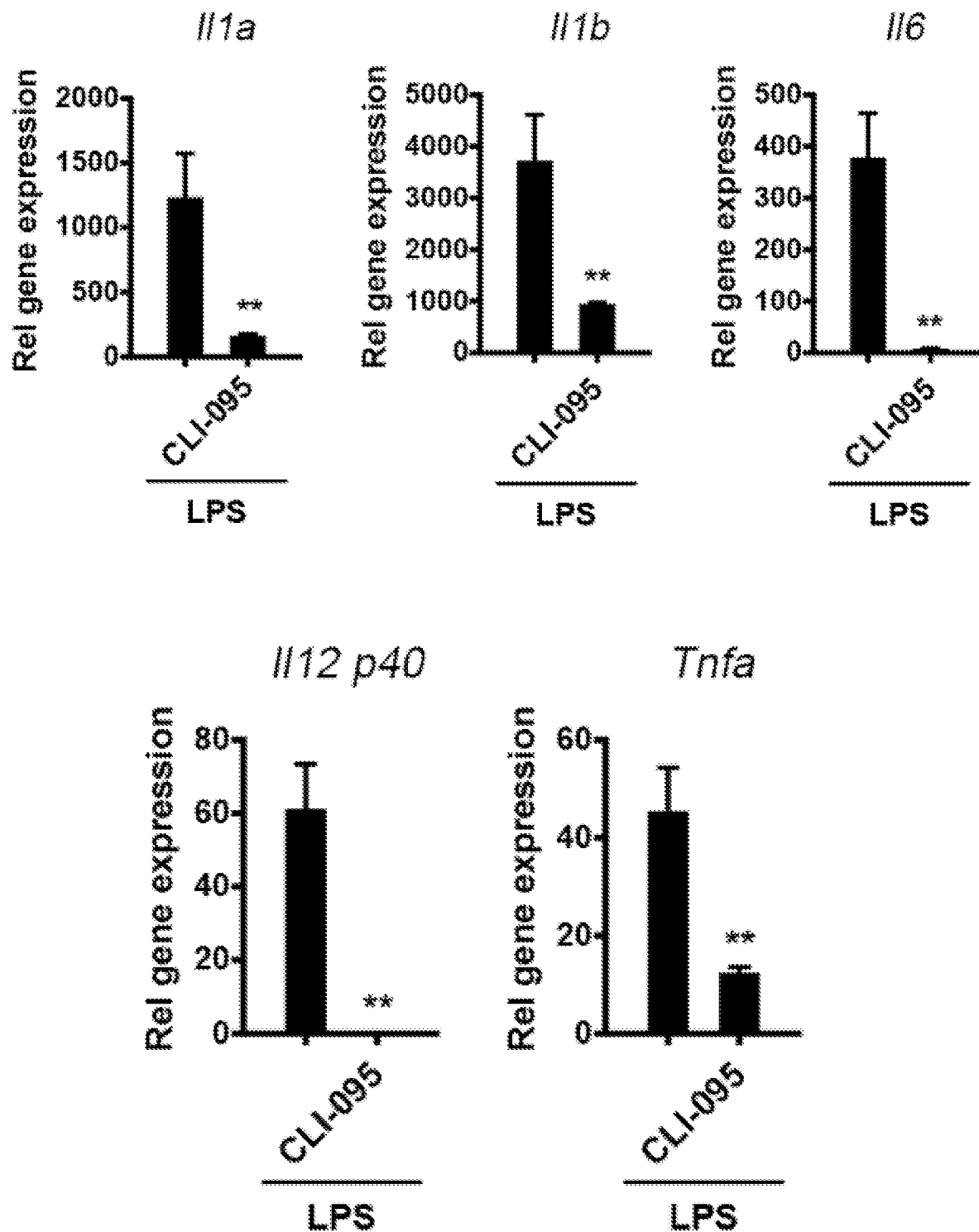
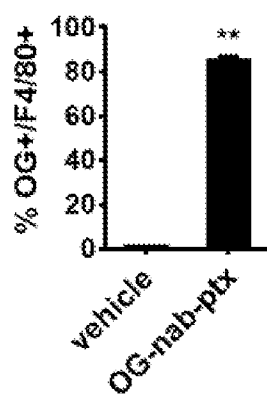
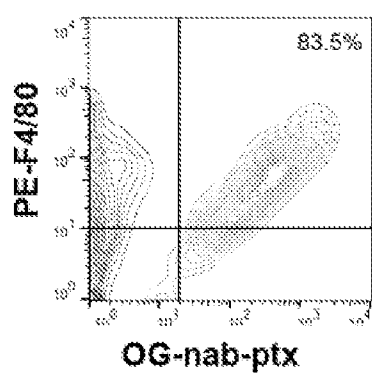
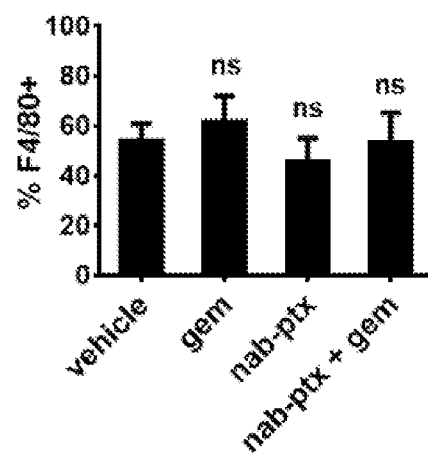


FIG. 6

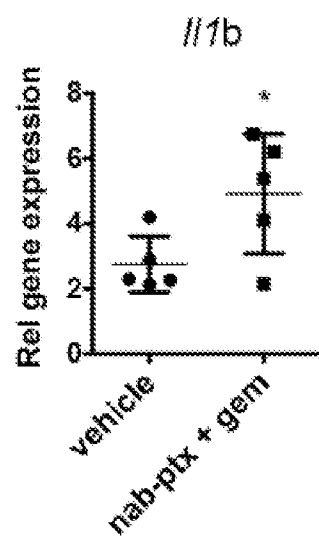
A



B



C



FIGs. 7A-7C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/35959

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/00; C07K 14/52, 14/54, 14/765; C12N 5/0786 (2017.01)

CPC - A61K 38/00; C12N 5/0645; C07K 14/52, 14/54, 14/765; G01N 33/5055

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)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012/0122207 A1 (SIEWEKE) May 17, 2012; abstract; paragraphs [0015], [0041], [0053]; claim 1	1-16
A	US 2016/0075763 A1 (NOVOZYMES BIOPHARM DK A/S) March 17, 2016; paragraphs [0042], [0085], [0236]	1-16
A	US 2015/0086603 A1 (ABBOTT CARDIOVASCULAR SYSTEMS INC.) March 26, 2015; abstract; paragraph [0098]	1-16
A	SOCKOLOSKY et al. 'The Neonatal Fc Receptor, FcRn, As A Target For Drug Delivery And Therapy' Adv Drug Deliv Rev. August 30, 2015, Vol 91, pp 1-40; abstract; page 5, second paragraph. DOI:10.1016/j.addr.2015.02.005.	1-16

☐ Further documents are listed in the continuation of Box C.☐ 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

06 October 2017 (06.10.2017)

Date of mailing of the international search report

31 OCT 2017

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/35959

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-Please See Supplemental Page-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-16

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US17/35959

-***-Continued from Box No. III Observations where unity of invention is lacking: -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, Claims 1-16 are directed toward a method of inducing a phenotypic change in a population of monocytes and/or macrophages, said method comprising: administering a macrophage stimulating agent coupled to a carrier molecule.

Group II, Claims 17-26 are directed toward a method of treating a tumor in a subject comprising administering to the subject an anti-tumor agent coupled to a carrier molecule that is defective in neonatal Fc receptor (FcRn) binding.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a macrophage stimulating agent, not present in Group II; the special technical features of Group II include an anti-tumor agent, not present in Group I.

Groups I and II share the technical features including: administering an agent coupled to a carrier molecule that is defective in neonatal Fc receptor (FcRn) binding; wherein the carrier is a variant human albumin protein relative to SEQ ID NO: 1.

However, these shared technical features are previously disclosed by US 2016/0075763 A1 to Novozymes Biopharm DK A/S (hereinafter 'Novozymes').

Novozymes discloses administering an agent (administering a therapeutic compound (an agent); paragraph [0236]) coupled to a carrier molecule (conjugated (coupled) to an albumin variant (a carrier molecule); paragraph [0236]) that is defective in neonatal Fc receptor (FcRn) binding (that has reduced affinity for FcRn (is defective in neonatal Fc receptor (FcRn)) binding; paragraphs [0021], [0042], [0085]); wherein the carrier is a variant human albumin protein (wherein the carrier is a variant human albumin protein; paragraph [0236]) relative to SEQ ID NO: 1 (relative to SEQ ID NO: 2 (SEQ ID NO: 1); paragraph [0023, SEQ ID NO: 2; wherein SEQ ID NO: 2 is 100% identical to Applicants' SEQ ID NO: 1).

Since none of the special technical features of the Groups I and II inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Novozymes reference, unity of invention is lacking.