(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
06 June 2019 (06.06.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/106432 A2

(51) International Patent Classification:
Not classified

(21) International Application Number:
PCT/IB2018/001508

(22) International Filing Date:
29 November 2018 (29.11.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/592,027 29 November 2017 (29.11.2017) US
62/673,407 18 May 2018 (18.05.2018) US

(71) Applicant: **ARATINGA.BIO AIO** [FR/FR]; 1 mail du Professeur Georges Mathé, 94800 Villejuif (FR).

(72) Inventors: **BAUCHE, Cécile**; 14 rue Auguste Perret, 75013 Paris (FR). **VAILLANT, Renaud**; 49 rue des Champs Elysées, 94250 Gentilly (FR). **SARRY, Emeline**; 8 rue Hoche, 92240 Malakoff (FR). **MOURLANE, Frédéric**; 22 rue Albert, 06000 Nice (FR).

(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, JO, 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,

(54) Title: VIRAL VECTOR CONSTRUCTS FOR EXPRESSION OF GENETIC ADJUVANTS MIMICKING CD40 SIGNALING

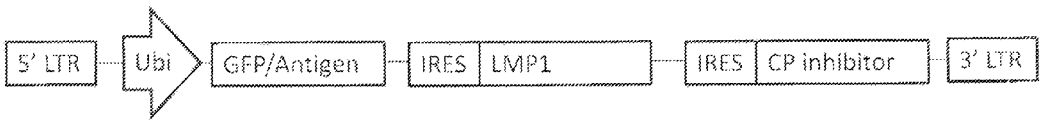


Fig. 2A

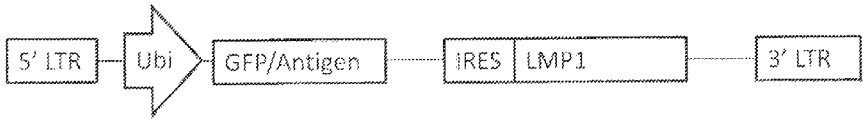


Fig. 2B

Fig. 2C

(57) Abstract: Viral vectors are provided for use as genetic immunotherapeutic agents, including preventive and therapeutic vaccines as well as compositions to enhance cellular immune responses. The vectors are particularly useful for treating or preventing cancer and infectious diseases. The vectors include lentiviral vectors that encode one or more antigens and an adjuvant, and optionally may encode one or more soluble checkpoint inhibitor molecules. The adjuvant is full-length latent membrane protein 1 (LMP1) from Epstein Barr virus which has been codon optimized for human expression.

WO 2019/106432 A2

[Continued on next page]

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

TITLE

Viral Vector Constructs for Expression of Genetic Adjuvants Mimicking CD40 Signaling

BACKGROUND

Canonical vaccine strategies based on the induction of antibody-based immune response have resulted in the eradication or near eradication of a number of previously fatal infectious diseases, such as smallpox, poliomyelitis and tetanus. Yet, these classical human vaccines have either been ineffective or unsafe for use in other infectious diseases, such as HIV and hepatitis, and for non-infectious illnesses such as cancer.

A new generation of immunotherapeutic products, aimed at inducing cellular immune responses, may overcome the limitations of traditional vaccines by recognizing and killing cancer cells and infected cells instead of the pathogen itself. Nucleic acid vaccines, and particularly viral vectors, have shown great potential to translate to the clinics.

Cancer cells and many infectious agents have ways of eluding the immune system, which makes creating effective vaccines difficult. Classical vaccines often require an adjuvant, e.g., aluminum salts, for optimal effectiveness, but conventional adjuvants are typically poor enhancers of cellular immune responses. Some strategies have been proposed to improve the quality and magnitude of the cellular immune response elicited by viral vectors. A new class of genetic adjuvants has been developed to improve cellular immune responses induced by vector-based immunotherapy. Genetic adjuvants consist of DNA sequences that encode immune regulatory molecules.

The cluster of differentiation 40 (CD40) is a membrane protein present on a variety of cells, most notably antigen-presenting cells such as dendritic cells (DC). CD40 is essential for the initiation and progression of cellular and humoral adaptive immunity, being involved in DC maturation, cytokine production, antibody isotype switching, memory B cell development, and germinal center formation, among other processes. The activation of CD40 requires that it become clustered in the membrane so that its cytoplasmic signaling domain forms a supramolecular signaling complex that subsequently activates different pro-inflammatory signaling pathways. The clustering of CD40 is initiated by either a multimeric form of its ligand (CD40 ligand or CD40L) or by anti-CD40 antibodies that must be arrayed on a nearby

cell via binding to Fc receptors. In its mRNA form, CD40 ligand has been used as an adjuvant in vaccines eliciting a cellular immune response (e.g., Argos Therapeutics AGS003, TriMix).

Stone et al. (WO 2013/0039942) discloses the use of a genetic adjuvant that induces a cellular immune response mimicking that of an activated CD40 receptor. In this approach a nucleic acid vaccine encodes latent membrane protein 1 (LMP1) of the Epstein Barr virus. Results have demonstrated that full length LMP1, when expressed in various forms (e.g., plasmids, mRNA, viruses, and vectors) spontaneously forms clusters, mimicking activated CD40L and its adjuvant effects. For example:

(i) macrophages infected by LMP1 expressing HIV-1 in vitro are stimulated to make immunostimulatory cytokines including IL-8, MIP-1beta, IL1-beta, IL-6, IL-12p70 and TNFalpha (without any production of IL-10, an immunosuppressive cytokine);

(ii) human dendritic cells are stimulated in vitro by infection with LMP1 expressing HIV-1 to produce stimulatory cytokines including IL-8, IL-1beta, TNF-alpha, IL-6, and IL-12p70;

(iii) human dendritic cells are stimulated by a single cycle SIV (scSIV) expressing LMP1 to produce stimulating cytokines including IL-8, IL1-beta, IL-6, IL-12p70 and TNFalpha;

(iv) in addition to being immunostimulatory, HIV-1-LMP1 and scSIV-LMP1 are also self-adjuvanting in vitro by enhancing the antigen presentation function of dendritic cells to induce the proliferation of HIV and SIV antigen-specific T-cells;

(v) HIV-LMPI stimulates DCs and macrophages in vitro to upregulate immunologically important cell surface costimulatory molecules like CD40, CD80, and CD83, and migration signals like CCR-7; and

(vi) mice intramuscularly injected three times every two weeks with a mix of plasmids encoding LMP1 and a melanoma specific antigen (gp100) are protected from tumor growth.

There is a need for self-adjuvanting vaccines that induce the intense cellular immune response required to break the immune tolerance observed in such indications as cancer, HIV, and other unmet medical needs.

SUMMARY

The present technology provides viral vectors encoding genetic adjuvants for improving immune responses, particularly cell-mediated immune responses, such as those directed against cancer or infections, and methods for using the viral vectors. The antigen and adjuvant constructs of the present technology have been optimized for use in human subjects.

One aspect of the technology is a viral vector encoding (i) a full length latent membrane protein 1 (LMP1) of the Epstein Barr virus that has been codon optimized for human expression; and (ii) one or more transgenes encoding one or more marker proteins, antigens, epitopes, or combinations thereof. In preferred embodiments, the viral vector is a lentiviral vector. In some embodiments, the vector includes a functional lentiviral integrase protein and can thereby integrate into the genome of the cells it is transducing.

The antigen may be a tumor antigen, viral antigen, or microbial antigen. Multiple antigens or selected epitopes of one or more antigens can be encoded by the vector. In certain embodiments, at least one antigen is selected from the group consisting of NY-ESO-1, mesothelin, PSA, MART-1, MART-2, Gp100, tyrosinase, p53, ras, MUC1, SAP-1, survivin, CEA, Ep-CAM, Her2, BRCA1/2, gag, reverse transcriptase, tat, circumsporozoite protein, HCV nonstructural proteins, hemaglutinins, and combinations thereof. In certain embodiments, the vector further encodes at least one immune checkpoint inhibitor molecule, such as an anti-CTLA-4 molecule, a PD1 blocker, a PDL1 blocker, or a combination thereof.

In certain embodiments, the viral vector includes more than one nucleic acid sequence. In some embodiments, the first nucleic acid sequence encodes one or more marker proteins, antigens, epitopes, or combinations thereof; the second nucleic acid sequence encodes a full length latent membrane protein 1 (LMP1) of the Epstein Barr virus that has been codon optimized for human expression; and optionally a third nucleic acid sequence encodes one or more immune checkpoint inhibitor molecules ("anti-checkpoints"). Preferably, the first and second, as well as the second and third, nucleic acid sequences are separated by a nucleic acid sequence encoding an internal ribosome entry site (IRES).

Another aspect of the technology is an immunotherapeutic formulation for preventing or treating a disease or condition in a subject including the viral vector. In preferred embodiments, the disease or condition is cancer or infection.

Another aspect of the technology is method of inducing an immune response against cancer or infection in a subject, the method including administering the viral vector or the immunotherapeutic formulation to a subject in need thereof. In some embodiments, administering the viral vector to the subject vaccinates the subject against cancer or infection.

In some embodiments, the cancer is selected from the group consisting of: melanoma, glioma, prostate cancer, breast cancer, cervical cancer, colorectal cancer, kidney cancer, lung cancer, lymphoma and pancreatic cancer. In some embodiments, the cancer harbors a tumor antigen listed above. In some embodiments, the cancer is sensitive to an anti-checkpoint. In some embodiments, the infectious disease is selected from the group consisting of: HIV/AIDS, hepatitis C, HPV, pneumonia, influenza, malaria, leishmaniosis, tuberculosis, Hansen's disease, rabies, dengue, Zika virus infection, Ebola virus infection, and schistosomiasis. In some embodiments, the infectious agent harbors a viral or microbial antigen listed above. In some embodiments, the infection disease is sensitive to an anti-checkpoint.

The technology also can be summarized with the following listing of embodiments.

1. A viral vector comprising a first nucleic acid sequence encoding an antigen or an antigenic epitope and a second nucleic acid sequence encoding a full length latent membrane protein 1 (LMP1) of the Epstein Barr virus that has been codon optimized for human expression.

2. The viral vector of embodiment 1, wherein the vector is a lentiviral vector.

3. The viral vector of embodiment 1 or 2, wherein the first nucleic acid sequence encodes a fusion protein comprising two or more antigens or two or more antigenic epitopes.

4. The viral vector of any of the preceding embodiments, wherein the second nucleic acid sequence comprises SEQ ID NO. 1.

5. The viral vector of any of the preceding embodiments, wherein the vector further comprises a third nucleic acid sequence encoding a soluble immune checkpoint inhibitor molecule.

6. The viral vector of embodiment 5, wherein the soluble immune checkpoint inhibitor molecule is selected from the group consisting of CTLA-4, PD-1, PDL-1, LAG-3, TIM 3, B7-H3, ICOS, IDO, 4-1BB, CD47 and combinations thereof.

7. The viral vector of any of the preceding embodiments, wherein the vector further comprises a fourth nucleic acid sequence encoding a functional lentiviral integrase protein, wherein the vector is self-inactivating.

8. The viral vector of any of the preceding embodiments, wherein the antigen is selected from the group consisting of NY-ESO-1, mesothelin, PSA, MART-1, MART-2, Gp100, tyrosinase, p53, ras, MUC1, SAP-1, survivin, CEA, Ep-CAM, Her2, BRCA1/2, gag, reverse transcriptase, tat, circumsporozoite protein, HCV nonstructural proteins, hemaglutinins, and combinations thereof.

9. An immunotherapeutic formulation for preventing or treating cancer or infection in a subject, the formulation comprising the viral vector of any of embodiments 1-8.

10. A method of inducing or enhancing an immune response against a cancer or an infectious disease in a subject, the method comprising administering the viral vector of any of embodiments 1-8 or the immunotherapeutic formulation of embodiment 9 to a subject in need thereof, whereby an immune response against said cancer or infectious disease is induced or enhanced in the subject.

11. The method of embodiment 10, whereby an immune response is induced or enhanced against a cancer, and the cancer is selected from the group consisting of: melanoma, glioma, prostate cancer, breast cancer, cervical cancer, colorectal cancer, kidney cancer, lung cancer, lymphoma and pancreatic cancer.

12. The method of embodiment 10, whereby an immune response is induced or enhanced against an infectious disease, and the infectious disease is selected from the group consisting of: HIV/AIDS, hepatitis C, HPV, pneumonia, influenza, malaria, leishmaniosis, tuberculosis, Hansen's disease, rabies, dengue, Zika, Ebola, and schistosomiasis.

13. The method of embodiment 10, wherein one or more cell surface markers selected from the group consisting of MHCI, MHCII, CCR7, CD25, CD40, CD69, CD80/86, and CD83 are upregulated in dendritic cells or macrophages.

14. The method of embodiment 10, wherein secretion by dendritic cells or macrophages of one or more cytokines selected from the group consisting of IL-1b, IL-6, IL-8, IFN-α, IFN-β, IL-10, and TNF-α is enhanced.

15. The method of embodiment 8, wherein the antigen is NY-ESO-1.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B show schematic representations of the secondary structure of LMP1 protein. FIG. 1A shows full length LMP1, and FIG. 1B shows truncated LMP1 with the intracytoplasmic domain removed.

FIGS. 2A-2C show schematic representations of the molecular constructs. FIG. 2A shows a construct containing (a) a promoter (human ubiquitin, Ubi); (b) a reporter gene (e.g., green fluorescent protein) or, alternatively, one antigen gene; (c) the genetic adjuvant LMP1; and (d) a gene encoding a soluble immune checkpoint inhibitor. FIG. 2B shows a construct containing (a) a promoter (human ubiquitin, Ubi); (b) a reporter gene (e.g., green fluorescent protein) or, alternatively, one or more antigen genes (e.g., NY-ESO-1 tumor antigen); and (c)

the genetic adjuvant LMP1. FIG. 2C shows a construct containing (a) a promoter (human ubiquitin, Ubi); (b) a combination of two antigen genes; and (c) the genetic adjuvant LMP1.

FIGS. 3A-3C show the expression levels of GFP transgene at 96 h post-transduction in human dendritic cells (3A), M1 macrophages (3B), and M2 macrophages (3C) after transduction with the indicated lentiviral vectors.

FIGS. 4A-4F show the activation and maturation of human dendritic cells and macrophages (M1 & M2) induced in vitro by the indicated lentiviral vectors. FIG. 4A shows the expression of a panel of upregulated markers in human GFP-positive dendritic cells at 96 h post-transduction with the indicated lentiviral constructs (expression normalized to GFP). "LMP1_RK" refers to a vector expressing wild-type LMP1. FIG. 4B shows the expression of a panel of upregulated cytokines in human dendritic cells at 96h post-transduction with the indicated lentiviral constructs (expression normalized to GFP). FIG. 4C shows the panel of upregulated markers in GFP-positive human M1 macrophages at 96 h post-transduction with the lentiviral constructs (expression normalized to GFP). FIG. 4D shows the panel of upregulated cytokines in human M1 macrophages at 96 h post-transduction with the lentiviral constructs (expression normalized to GFP). FIG. 4E shows the panel of upregulated markers in GFP-positive human M2 macrophages 96 h post-transduction with the lentiviral constructs (expression normalized to GFP). FIG. 4F shows the panel of upregulated cytokines in human M2 macrophages 96 h post-transduction with the lentiviral constructs (expression normalized to GFP).

FIGS. 5A-5C show a panel of cytokines secreted by human dendritic cells (FIG. 5A), M1 differentiated macrophages (FIG. 5B), and M2 differentiated macrophages (FIG. 5C) induced *in vitro* with the indicated lentiviral vectors at 48 h post-transduction.

DETAILED DESCRIPTION

The present technology provides viral vector constructs for the expression of genetic adjuvants for use in immunotherapeutic products and methods of using the vectors. The vector constructs can improve the quality and intensity of an immune response, such as those directed against cancer or infections, being especially suited to induce and/or enhance cell-mediated immune responses.

The present technology comprises one or more nucleic acid sequences that encode a full length LMP1 EBV protein that has been codon optimized for human expression and one or more antigens. In a typical embodiment, the technology provides activation of immune responses via aggregation of two or more LMP1 proteins in the cell membrane, which activates a CD40-like signal transduction pathway. After direct injection, introduction of the nucleic

acid sequences and consequent protein expression can occur in any type of cell, but preferably occur in immune cells. This technology can be used for traditional prophylactic or therapeutic vaccines against cancer and infectious diseases. In the experiments described herein, the viral vectors are expected to markedly enhance immune responses and protection from or treatment of infection and cancer.

"Vector" refers to a molecule containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, nucleic acid molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, such as in the production of antisense molecules, ribozymes or aptamers. Vectors can contain a variety of control sequences, which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operatively linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well.

A "construct" can be any type of engineered nucleic acid coding for gene products in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript generally is translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding genes of interest.

As used herein, "vaccine" includes all prophylactic and therapeutic vaccines.

An "adjuvant" can be any molecule or composition that activates or enhances an immune response to an antigen. An adjuvant may enhance the efficacy of a vaccine by helping to modify the immune response to particular types of immune system cells. An adjuvant may be an immunostimulant that triggers activation of antigen-presenting cells such as dendritic cells, macrophages, and B cells. Adjuvants are also understood to provide a "danger" signal indicating that the immune system should go into a state of alert. Adjuvants may act by facilitating antigen presentation by antigen-presenting cells, by activating macrophages and lymphocytes and/or by supporting the production of cytokines. Without an adjuvant, immune responses may either fail to progress or may be diverted into ineffective immunity or tolerance. Adjuvants are often needed for effective preventative or therapeutic vaccines, or for inducing an anti-tumor immune response. A "genetic adjuvant" is an adjuvant that is provided in the form of a nucleic acid, which is expressed by target cells to produce a molecule that functions as an adjuvant.

An antigen-presenting cell (APC) is any of a variety of cells capable of displaying, acquiring, or presenting at least one antigen or antigenic fragment on (or at) its cell surface. In general, the term "antigen-presenting cell" can refer to any cell that accomplishes the goal of the technology by aiding the enhancement of an immune response (i.e., from the T-cell or B-cell arms of the immune system) against an antigen or antigenic composition. Such cells can be defined by those of skill in the art, using methods disclosed herein and in the art. As is understood by one of ordinary skill in the art, and used herein certain embodiments, a cell that displays or presents an antigen normally or preferentially with a class II major histocompatibility molecule or complex to an immune cell is an "antigen-presenting cell." In certain aspects, a cell (e.g., an APC) may be fused with another cell, such as a recombinant cell or a tumor cell that expresses the desired antigen. Methods for preparing a fission of two or more cells are well known in the art. In some cases, the immune cell to which an antigen-presenting cell displays or presents an antigen is a CD4+ T or a CD8+ T cell. Additional molecules expressed on the APC or other immune cells may aid or improve the enhancement of an immune response. Secreted or soluble molecules, such as for example, cytokines and adjuvants, may also aid or enhance the immune response against an antigen. A dendritic cell (DC) is an antigen-presenting cell existing *in vivo*, *in vitro, ex vivo,* or in a host or subject, or which can be derived from a hematopoietic stem cell or a monocyte. Dendritic cells and their precursors can be isolated from a variety of lymphoid organs, e.g., spleen, lymph nodes, as well as from bone marrow and peripheral blood. The DC has a characteristic morphology with thin sheets (*lamellipodia*) extending in multiple directions away from the dendritic cell body. Typically, dendritic cells express high levels of major histocompatibility complex (MHC) and costimulatory (e.g., B7-1 and B7-2) molecules. Dendritic cells can induce antigen specific differentiation of T cells in vitro, and are able to initiate primary T cell responses in vitro and in vivo.

By the phrase "immune response" is meant induction of antibody and/or immune cell-mediated responses specific against an antigen or antigens or allergen(s) or drug or biologic. The induction of an immune response depends on many factors, including the immunogenic constitution of the challenged organism, the chemical composition and configuration of the antigen or allergen or drug or biologic, and the manner and period of administration of the antigen or allergen or drug or biologic. An immune response has many facets, some of which are exhibited by the cells of the immune system (e.g., B-lymphocytes, T-lymphocytes, macrophages, and plasma cells). Immune system cells may participate in the immune response through interaction with an antigen or allergen or other cells of the immune system, the release

of cytokines and reactivity to those cytokines. Immune responses are generally divided into two main categories—humoral and cell-mediated. The humoral component of the immune response includes production of antibodies specific for an antigen or allergen or drug or biologic. The cell-mediated component includes the generation of delayed-type hypersensitivity and cytotoxic effector cells against the antigen or allergen.

Activation or stimulation of the immune system may be mediated by the activation of immune effector cells, such as lymphocytes, macrophages, dendritic cells, natural killer cells (NK cells) and cytotoxic T lymphocytes (CTL). It can be mediated by activation and maturation of antigen presenting cells, such as dendritic cells. It can be mediated by the blockade of inhibitory pathways, such as by inhibiting immune checkpoint inhibitors.

By the term "LMP1 gene," is meant a native Epstein Barr virus LMPl-encoding nucleic acid sequence, e.g., the native Epstein Barr virus LMP1 gene; a nucleic acid having sequences from which a LMP1 cDNA can be transcribed; and/or allelic variants and homologs of the foregoing. An exemplary nucleic acid sequence of LMP1 is GenBank Accession No. M58153.1. The term encompasses double-stranded DNA, single-stranded DNA, and RNA.

By the term "LMP1 protein," is meant an expression product of a LMP1 gene or a protein that shares at least 65% (but preferably 75, 80, 85, 90, 95, 96, 97, 98, or 99%) amino acid sequence identity with the foregoing and displays a functional activity of a native LMP1 protein. A "functional activity" of a protein is any activity associated with the physiological function of the protein. LMP1 consists of an N-terminal transmembrane region linked to a C-terminal cell signaling region that is analogous to the CD40 receptor on immune cells. In addition to anchoring LMP1 into the membrane, the N-terminus of LMP1 self-aggregates and leads to clustering of LMP1 or any protein linked to the LMP1 N-terminal domain. The transmembrane (aggregation) domain of LMP1 protein is amino acids 1-190 of the amino acid sequence set forth in GenBank Accession No. AAA66330.1.

Latent membrane protein-1 (LMP1) is a gene in the Epstein-Barr Virus (EBV). Its N-terminus is composed of 6 contiguous transmembrane domains that anchor the protein into the membrane. FIG. 1 shows the structure of LMP1 protein showing a transmembrane domain 101 and an intracytoplasmic signaling domain 102. The intracytoplasmic domain of LMP1 is analogous to the signaling domain of the CD40 receptor.

As described supra, the cluster of differentiation 40 (CD40) receptor is a membrane protein present on a variety of cells, most notably antigen-presenting cells, such as dendritic cells (DC). CD40 is essential for the initiation and progression of cellular and humoral adaptive immunity, The activation of CD40 requires that it become clustered in the membrane so that

its cytoplasmic signaling domain forms a supramolecular signaling complex in concert with adaptor molecules in the TNF Receptor Activating Factor (TRAF) family (e.g., TRAF2, TRAF3, TRAF6), which subsequently activates different signaling pathways (e. g., nuclear factor κB (NFκB)-signaling pathways, mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), phospholipase Cγ (PLCγ) pathway). The clustering of CD40 is initiated by either a multimeric form of its ligand (CD40 ligand or CD40L) or by anti-CD40 antibodies that must be arrayed on a nearby cell via binding to Fc receptors. CD40 ligand (CD40L) is already used as adjuvant in vaccines eliciting a cellular immune response (e.g., Argos Therapeutics' AGS003, TriMix).

LMP1, however, needs no ligand or antibody to initiate signaling through its cytoplasmic domain since its N-terminal transmembrane domain spontaneously forms clusters in the cell membrane and thereby clusters the intracytoplasmic domain(s) that are connected to it via peptide bonds as a single polypeptide chain. In this sense, LMP1 is said to be "constitutively activated." Likewise, fusion proteins that link the N-terminal transmembrane domain to signaling domain(s) that require clustering in order to function can also be said to be "constitutively activated" and no longer need the ligand from the receptor from which they are taken.

Viral vectors of the present technology encode one or more nucleic acids sequences capable of activating or enhancing an immune response in a subject. The nucleic acids encode a full length latent membrane protein 1 (LMP1) of the Epstein Barr virus that has been codon optimized for human expression and in a typical embodiment provide activation of immune responses by a combination (i.e., multimerization) of two or more LMP1 proteins.

The viral vector can be any type of suitable vector, such as an expression vector or a plasmid. In preferred embodiments, the vector is a lentiviral vector. Lentiviral vectors are modified lentiviruses, derived, for example, from human immunodeficiency virus (HIV-1 or HIV-2), simian immunodeficiency virus (SIV), equine infectious encephalitis virus (EIAV), caprine arthritis encephalitis virus (CAEV), bovine immunodeficiency virus (BIV) and feline immunodeficiency virus (FIV). The modified lentiviral vectors have reduced pathogenicity. The vectors may also be modified to introduce beneficial therapeutic effects. Lentiviral vectors themselves are not toxic and, unlike other retroviruses, lentiviruses are capable of transducing non-dividing cells, in particular dendritic cells, allowing antigen presentation through the endogenous pathway.

In some embodiments, the lentiviral vector can be a recombinant DNA molecule, such as a plasmid. In some embodiments, the lentiviral vector further comprises a lentiviral vector

particle and associated proteins. A lentiviral vector particle (or lentiviral particle vector) comprises a lentiviral vector in association with viral proteins. Lentiviral vector particles may comprise single or double stranded nucleic acid molecules.

In preferred embodiments, the lentiviral vectors have the capacity for integration into the genome of the cells being transduced. In preferred embodiments, they contain a functional integrase protein. Non-integrating vector particles display genetic mutations that hinder the lentiviral vector particles capacity for integrating into the host genome. The term "transfection" and "transduction" refer to the process by which an exogenous DNA sequence is introduced into a eukaryotic host cell. Transfection is the non-viral delivery of nucleic acids (either DNA or RNA) and can be achieved by any one of a number of means including electroporation, microinjection, gene gun delivery, retroviral infection, lipofection, polymer-mediated delivery, and the like. Transduction refers to the delivery of nucleic acids by a virus or viral vector where the nucleic acids are typical DNA for a DNA virus and RNA for an RNA virus.

In some embodiments, the lentiviral vector is self-inactivating and does not contain an enhancer. Self-inactivating lentiviral vectors have modifications in the U3 (ΔU3) region of the 3′ LTR that render the vectors unable to replicate in the host cell. The U3 region encodes binding sites that are essential for basal promoter activity and viral replication, and elimination of these binding sites results in virtually complete inactivation of viral replication.

A myriad of factors can influence the efficacy of viral vectors, even after successful transduction and, optionally, integration into the host genome: gene expression and translation, protein folding, transport and turnover, and cell-to-cell interactions, to name a few. These factors depend, among other things, on the nucleic acid sequences encoded by the vector. Preferred DNA sequences for conducting the present technology include modifications of native sequences aimed at increasing viral vector efficacy and efficiency. These modifications include codon optimization for human use. The modifications may impact the rates of transcription and/or translation, as well as impact protein location in the cell and protein activity.

Preferably, the DNA sequence encoding the full-length LMP1 with codon optimization for human use (LMP1 CO) includes SEQ ID NO:1 (below). The encoded amino acid sequence of full length LMP1 is shown below as SEQ ID NO:2.

ATGGATCTGGACCTGGAAAGAGGACCTCCTGGACCTAGACGGCCTCCTAGAGGACCACCTCTGAGCAGCTCTATTGGACTGGC
CCTGCTGCTGCTTCTGCTGGCTCTGCTGTTCTGGCTGTACATCATCATGAGCAACTGGACCGGCGGAGCACTGCTGGTGCTGT
ATGCCTTTGCTCTGATGCTGGTCATCATCATCCTGATCATCTTCATCTTCCGGCGGGACCTGCTGTGTCCTCTGGGAGCACTT
TGTCTGTTGCTGCTGATGATCACCCTCCTGCTGATCGCCCTGTGGAACCTGCATGGACAGGCCCTGTATCTGGGAATCGTGCT
GTTCATCTTCGGCTGCCTGCTGGTTCTCGGCCTGTGGATCTACCTGCTGGAAATCCTTTGGAGACTGGGCGCCACCATCTGGC

AGCTGCTGGCCTTTTTCCTGGCCTTCTTTCTGGATATCATCCTCCTCATCATTGCCCTGTACCTGCAGCAGAACTGGTGGACC
CTGCTGGTGGATCTGCTTTGGCTGCTGCTCTTTCTGGCCATCCTGATTTGGATGTACTACCACGGCCAGCGGCACAGCGGACGA
ACACCACCATGATGACAGCCTGCCTCATCCTCAGCAGGCCACCGACGATAGCAGCAACCAGAGCGACAGCAACAGCAACGAGG
GCAGACATCTGCTGCTGGTGTCTGGTGCTGGCGACGGACCTCCTCTGTGTTCTCAAAATCTTGGCGCCCCTGGCGGCGGACCA
AACAATGGACCTCAGGACCCCGACAACACCGACGACAATGGCCCTCAAGATCCTGATAATACCGATGACAACGGCCCACACGA
CCCTCTGCCTCAAGACCCAGATAACACAGACGATAACGGTCCACAAGATCCGGACAATACTGACGATAATGGACCCCACGATC
CACTGCCTCACAACCCTAGCGATAGCGCCGGAAATGATGGCGGACCTCCACAGCTGACCGACGAAGTGGAAAACAAAGGCGGA
GATCAGGGCCCTCCTCTGATGACCGATGGCGGAGGTGGACACTCTCACGATTCTGGCCACGACGGCATCGACCCTCATCTGCC
TACACTGCTGCTCGGCACATCTGGCTCTGGCGGCGACGATGATGATCCTCATGGACCTGTGCAGCTGAGCTACTACGAC
(SEQ ID NO:1)

MDLDLERGPPGPRRPPRGPPLSSSIGLALLLLLLALLFWLYIIMSNWTGGALLVLYAFALMLVIIILIIFIFRRDLLCPLGAL
CLLLLMITLLLIALWNLHGQALYLGIVLFIFGCLLVLGLWIYLLEILWRLGATIWQLLAFFLAFFLDIILLIIALYLQQNWWT
LLVDLLWLLLFLAILIWMYYHGQRHSDEHHHDDSLPHPQQATDDSSNQSDSNSNEGRHLLLVSGAGDGPPLCSQNLGAPGGGP
NNGPQDPDNTDDNGPQDPDNTDDNGPHDPLPQDPDNTDDNGPQDPDNTDDNGPHDPLPHNPSDSAGNDGGPPQLTEEVENKGG
DQGPPLMTDGGGGHSHDSGHDGIDPHLPTLLLGTSGSGGDDDDPHGPVQLSYYD (SEQ ID NO:2)

A useful control genetic adjuvant is provided by a truncated form of LMP1 (LMP1_CO delta IC) which has the intracytoplasmic signaling domain deleted. The DNA sequence of this form (codon optimized for expression in human cells) is shown below as SEQ ID NO:3, and the encoded amino acid sequence is shown as SEQ ID NO:4. The function of the signaling domain can be revealed by comparing the response to expression of SEQ ID NO:1 (including the signaling domain) to the response to expression of SEQ ID NO:3 (lacking the signaling domain).

ATGGATCTGGATCTCGAAAGAGGACCTCCTGGACCTAGACGGCCTCCTAGAGGACCACCTCTGAGCAGCTCTATTGGACTGGC
CCTGCTGCTGCTTCTGCTGGCTCTGCTGTTCTGGCTGTACATCATCATGAGCAACTGGACCGGCGGAGCACTGCTGGTGCTGT
ATGCCTTTGCTCTGATGCTGGTCATCATCATCCTGATCATCTTCATCTTCCGGCGGGACCTGCTGTGTCCTCTGGGAGCACTT
TGTCTGTTGCTGCTGATGATCACCCTCCTGCTGATCGCCCTGTGGAACCTGCATGGACAGGCCCTGTATCTGGGCATCGTGCT
GTTCATCTTCGGCTGCCTGCTGGTTCTCGGCCTGTGGATCTACCTGCTGGAAATCCTTTGGAGACTGGGCGCCACCATCTGGC
AGCTGCTGGCCTTTTTCCTGGCCTTCTTTCTGGATATCATCCTCCTCATCATTGCCCTGTACCTGCAGCAGAACTGGTGGACC
CTGCTGGTGGATCTGCTTTGGCTGCTGCTCTTTCTGGCCATCCTGATTTGGATGTACTACCACGGCCAGCGG (SEQ ID NO:3)

MDLDLERGPPGPRRPPRGPPLSSSIGLALLLLLLALLFWLYIIMSNWTGGALLVLYAFALMLVIIILIIFIFRRDLLCPLGAL
CLLLLMITLLLIALWNLHGQALYLGIVLFIFGCLLVLGLWIYLLEILWRLGATIWQLLAFFLAFFLDIILLIIALYLQQNWWT
LLVDLLWLLLFLAILIWMYYHGQR (SEQ ID NO:4)

The viral vectors of the present technology encode one or more antigens. The term "antigen" as used herein refers to a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. An antigen can be derived from organisms, subunits of proteins/antigens, killed or inactivated whole cells or lysates. Therefore, a skilled artisan realizes that any macromolecule, including virtually all proteins or peptides, can serve as antigens. Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan realizes that any DNA, which contains nucleotide sequences or partial nucleotide sequences of a pathogenic genome or a gene or a fragment of a gene for a protein that elicits an immune response results in synthesis of an antigen. Furthermore, one skilled in the art realizes that the present technology is not limited to the use of the entire nucleic acid sequence

of a gene or genome. The present technology includes, but is not limited to, the use of partial nucleic acid sequences of more than one gene or genome whose nucleic acid sequences are arranged in various combinations to elicit the desired immune response.

The antigen may be any antigen for which an enhanced immune response is desirable. Such antigens include, but are not limited to, antigens from pathogens that cause infectious disease for which a protective immune response may be elicited. For example, antigens from HIV include the proteins gag, env, pol, tat, rev, nef, reverse transcriptase, and other HIV components. The E6 and E7 proteins from human papilloma virus are also suitable antigens. Furthermore, the EBNA1 antigen from herpes simplex virus is suitable. Other viral antigens for use in the technology are hepatitis viral antigens such as the S, M, and L proteins of hepatitis B virus, the pre-S antigen of hepatitis B virus, and other hepatitis, e.g., hepatitis A, B, and C, viral components such as hepatitis C viral RNA; influenza viral antigens such as hemagglutinin, neuraminidase, nucleoprotein, M2, and other influenza viral components; measles viral antigens such as the measles virus fusion protein and other measles virus components; rubella viral antigens such as proteins E1 and E2 and other rubella virus components; rotaviral antigens such as VP7sc and other rotaviral components; cytomegaloviral antigens such as envelope glycoprotein B and other cytomegaloviral antigen components; respiratory syncytial viral antigens such as the RSV fusion protein, the M2 protein and other respiratory syncytial viral antigen components; herpes simplex viral antigens such as immediate early proteins, glycoprotein D, and other herpes simplex viral antigen components; varicella zoster viral antigens such as gpI, gpII, and other varicella zoster viral antigen components; Japanese encephalitis viral antigens such as proteins E, M-E, M-E-NS1, NS 1, NS 1-NS2A, 80% E, and other Japanese encephalitis viral antigen components; rabies viral antigens such as rabies glycoprotein, rabies nucleoprotein and other rabies viral antigen components; West Nile virus prM and E proteins; and Ebola envelope protein. See Fundamental Virology, Second Edition, eds. Knipe, D. M. and, Howley P. M. (Lippincott Williams & Wilkins, New York, 2001) for additional examples of viral antigens. In addition, bacterial antigens are also disclosed. Bacterial antigens which can be used in the compositions and methods of the technology include, but are not limited to, pertussis bacterial antigens such as pertussis toxin, filamentous hemagglutinin, pertactin, FIM2, FIM3, adenylate cyclase and other pertussis bacterial antigen components; diptheria bacterial antigens such as diptheria toxin or toxoid and other diptheria bacterial antigen components; tetanus bacterial antigens such as tetanus toxin or toxoid and other tetanus bacterial antigen components; streptococcal bacterial antigens such as M proteins and other streptococcal bacterial antigen components;

Staphylococcal bacterial antigens such as IsdA, IsdB, SdrD, and SdrE; gram-negative bacilli bacterial antigens such as lipopolysaccharides, flagellin, and other gram-negative bacterial antigen components; Mycobacterium tuberculosis bacterial antigens such as mycolic acid, heat shock protein 65 (HSP65), the 30 kDa major secreted protein, antigen 85A, ESAT-6, and other mycobacterial antigen components; Helicobacter pylori bacterial antigen components; pneumococcal bacterial antigens such as pneumolysin, pneumococcal capsular polysaccharides and other pneumococcal bacterial antigen components; haemophilus influenza bacterial antigens such as capsular polysaccharides and other haemophilus influenza bacterial antigen components; anthrax bacterial antigens such as anthrax protective antigen, anthrax lethal factor, and other anthrax bacterial antigen components; the F1 and V proteins from Yersinia pestis; rickettsiae bacterial antigens such as romps and other rickettsiae bacterial antigen components. Also included with the bacterial antigens described herein are any other bacterial, mycobacterial, mycoplasmal, rickettsial, or chlamydial antigens. Examples of protozoa and other parasitic antigens include, but are not limited to, plasmodium falciparum antigens such as merozoite surface antigens, sporozoite surface antigens, circumsporozoite antigens, gametocyte/gamete surface antigens, blood-stage antigen pf 1 55/RESA and other plasmodial antigen components; toxoplasma antigens such as SAG-1, p30 and other toxoplasma antigen components; schistosomae antigens such as glutathione-S-transferase, paramyosin, and other schistosomal antigen components; leishmania major and other leishmaniae antigens such as gp63, lipophosphoglycan and its associated protein and other leishmanial antigen components; and trypanosoma cruzi antigens such as the 75-77 kDa antigen, the 56 kDa antigen and other trypanosomal antigen components. Examples of fungal antigens include, but are not limited to, antigens from Candida species, Aspergillus species, Blastomyces species, Histoplasma species, Coccidiodomycosis species, Malassezia furfur and other species, Exophiala werneckii and other species, Piedraia hortai and other species, Trichosporum beigelii and other species, Microsporum species, Trichophyton species, Epidermophyton species, Sporothrix schenckii and other species, Fonsecaea pedrosoi and other species, Wangiella dermatitidis and other species, Pseudallescheria boydii and other species, Madurella grisea and other species, Rhizopus species, Absidia species, and Mucor species. Examples of prion disease antigens include PrP, beta-amyloid, and other prion-associated proteins.

In addition to the infectious and parasitic agents mentioned above, another area for desirable enhanced immunogenicity to a non-infectious agent is in the area of proliferative diseases, including but not limited to cancer, in which cells expressing cancer antigens are

desirably eliminated from the body. Tumor antigens which can be used in the compositions and methods of the technology include, but are not limited to, prostate specific antigen (PSA), breast, ovarian, testicular, melanoma, telomerase; multidrug resistance proteins such as P-glycoprotein; MAGE-1, alpha fetoprotein, carcinoembryonic antigen, mutant p53, papillomavirus antigens, gangliosides or other carbohydrate-containing components of melanoma or other tumor cells. It is contemplated by the technology that antigens from any type of tumor cell can be used in the compositions and methods described herein. The antigen may be a cancer cell, or immunogenic materials isolated from a cancer cell, such as membrane proteins. Included are survivin and telomerase universal antigens and the MAGE family of cancer testis antigens. Antigens which have been shown to be involved in autoimmunity and could be used in the methods of the present technology to induce tolerance include, but are not limited to, myelin basic protein, myelin oligodendrocyte glycoprotein and proteolipid protein of multiple sclerosis and CII collagen protein of rheumatoid arthritis.

In an embodiment, the DNA sequence encoding the full-length NY-ESO-1 tumor antigen includes SEQ ID NO:5 (below). The encoded amino acid sequence of full-length NY-ESO-1 is shown below as SEQ ID NO:6.

ATGCAGGCTGAAGGCAGAGGAACAGGCGGATCTACAGGCGACGCTGATGGACCTGGCGGACCTGGAATACCAGATG
GCCCAGGTGGAAATGCAGGCGGACCAGGCGAAGCTGGTGCTACAGGTGGAAGAGGACCTAGAGGTGCTGGCGCCGCTAGAGCT
TCTGGTCCTGGTGGCGGAGCACCTAGAGGACCTCATGGCGGAGCTGCCTCTGGCCTGAATGGCTGTTGTAGATGTGGCGCCAG
AGGACCCGAAAGCCGGCTGCTGGAATTCTACCTGGCCATGCCTTTCGCCACACCTATGGAAGCCGAGCTGGCCAGAAGAAGCC
TGGCTCAAGACGCTCCTCCTCTTCCTGTTCCTGGCGTGCTGCTGAAAGAATTCACCGTGTCCGGCAACATCCTGACCATCAGA
CTGACAGCCGCCGACCACAGACAGCTCCAGCTGAGCATTAGCAGCTGCCTCCAGCAGCTGAGCCTGCTGATGTGGATCACCCA
GTGCTTTCTGCCCGTGTTTCTGGCCCAGCCTCCAAGCGGACAGAGAAGA (SEQ ID NO:5)

MQAEGRGTGGSTGDADGPGGPGIPDGPGGNAGGPGEAGATGGRGPRGAGAARASGPGGGAPRGPHGGAASGLNGCC
RCGARGPESRLLEFYLAMPFATPMEAELARRSLAQDAPPLPVPGVLLKEFTVSGNILTIRLTAADHRQLQLSISSCLQQLSLL
MWITQCFLPVFLAQPPSGQRR (SEQ ID NO:6)

The antigen may be a portion of an infectious agent such as HZV-1, EBV, HBV, influenza virus, SARS virus, poxviruses, malaria, or HSV, by way of non-limiting examples, for which vaccines that mobilize strong T-cell mediated immunity (via dendritic cells) are needed.

The term “cancer” as used herein is defined as a hyperproliferation of cells whose unique trait—loss of normal controls—results in unregulated growth, lack of differentiation, local tissue invasion, and metastasis. Examples include but are not limited to, melanoma, non-small cell lung, small-cell lung, lung, hepatocarcinoma, leukemia, retinoblastoma, astrocytoma, glioblastoma, gum, tongue, neuroblastoma, head, neck, breast, pancreatic, prostate, renal, bone, testicular, ovarian, mesothelioma, cervical, gastrointestinal, lymphoma, brain, colon, sarcoma or bladder.

The term "tumor" denotes at least one cell or cell mass in the form of a tissue neoformation, in particular in the form of a spontaneous, autonomous and irreversible excess growth, which is more or less disinhibited, of endogenous tissue, which growth is as a rule associated with the more or less pronounced loss of specific cell and tissue functions. This cell or cell mass is not effectively inhibited, in regard to its growth, by itself or by the regulatory mechanisms of the host organism, e.g. melanoma or carcinoma. Tumor antigens not only include antigens present in or on the malignant cells themselves, but also include antigens present on the stromal supporting tissue of tumors including endothelial cells and other blood vessel components. In a related aspect, “neoplastic” refers to abnormal new growth and thus means the same as tumor, which may be benign or malignant. Further, such neoplasia would include cell proliferation disorders.

In preferred embodiments, an immune checkpoint inhibitor molecule will be encoded within the viral vector, enhancing the immune response against a tumor. The immune checkpoint inhibitor molecule can be, but is not limited to, an anti-CTLA-4 molecule, a PD1 blocker, and a PDL1 blocker. The immune checkpoint inhibitor molecule can be a protein, such as an antibody, or a soluble protein encoded by the vector, or a nucleic acid sequence with anticheckpoint activity.

In certain embodiments, the viral vector may include more than one expression cassette. In some embodiments, the viral vector particles may include more than one nucleic acid molecule, such as two or three nucleic acid molecules, which may be delivered separately or operatively linked. In some embodiments, the second nucleic acid encodes an antigen and/or immune checkpoint inhibitor molecule. In some embodiments, the third nucleic acid encodes an antigen and/or immune checkpoint inhibitor molecule different from that encoded by the second nucleic acid molecule.

In one aspect, the technology is an immunotherapeutic formulation for preventing or treating a disease or condition in a subject. The vaccine includes a therapeutically effective amount of the viral vector. The disease may be any disease in which vaccination against an agent is desirable, such as cancer or an infection.

In another aspect the technology is a method for inducing or enhancing an immune response against cancer or infection in a subject. The method includes administering a therapeutically effective amount of the viral vector or immunotherapeutic formulation to a subject in need thereof.

EXAMPLES

Example 1. Molecular Constructs.

Vectors are constructed containing: (a) a promoter, preferably a human ubiquitin promoter; (b) a reporter gene (e.g., green fluorescent protein) or, alternatively, one or more antigens fused into a single transgene (e.g., NY-ESO-1 tumor antigen); (c) an IRES followed by an adjuvant gene (i.e., LMP1 or LMP1 CO); and (d) optionally, an IRES followed by soluble immune checkpoint inhibitor genes (FIGS. 2A-2C). Preferably, the sequences are in the aforementioned order, but the genes can be situated in the vector in any other suitable order. Control vectors are also constructed that have some but not all the above-mentioned regions.

Example 2. Production of Viral Vectors.

Lentiviral vectors were produced by transient calcium-phosphate transfection of HEK 293T cells as described in Nasri et al. (2014). HEK 293T cells were seeded at 1.6 x 10^8 cells in a two chamber Cell Stack (Corning) in 250 mL of complete culture medium and 20 maintained 24h in an incubator with a humidified atmosphere of 5% CO_2 at 37° C to adhere. For each vector produced, one cell stack was transfected as follows. The lentiviral backbone plasmid (235 µg), the envelope coding plasmid (47 µg), and the packaging plasmid (235 µg) were mixed with 8.6 mL of sterile distilled water and 3.0 mL of $CaCl_2$. The DNA mix was then added drop by drop to 12.1 mL of 37° C pre-warmed HBS 2X, pH 7.1, and the 24.2 mL of precipitate obtained was added to the culture medium of the cells after 30 minutes of incubation at room temperature. The transfected cells were incubated at 37°C, 5% CO_2. The medium was replaced 24 h after transfection by 210 mL of harvest medium without serum and phenol red, and the viral supernatant was harvested after an additional 24h, clarified by centrifugation for 5 min at 2500 rpm. The harvest clarified bulk (210 mL) was treated for 30 min with DNase I in the presence of $MgCl_2$ to cleave any residual DNA, and concentrated by centrifugation for 1 h at 22000 rpm, 4°C. Vector pellets were resuspended in 70 µl of Tris-trehalose (50 mM), pooled in a 1.5 mL microtube and divided into 50 µL aliquots, frozen and stored at ≤ -70°C.

Production yields were a bit less with adjuvanted vectors compared to GFP or NY-ESO-1vector due to the presence of a longer DNA cassette. However, for all adjuvanted constructs, titers were at least in the 10^9 TU/mL range and were consistent among different production runs. No issue that would impact industrial bioproduction was observed.

Example 3. *In Vitro* Effects of Adjuvanted Lentiviral Vectors Activating the CD40L Pathway.

Fresh human dendritic cells and macrophages were obtained from healthy human donors (leukocyte cones) over a density gradient. CD14-positive monocytes were purified from PBMC using a magnetic isolation kit (positive selection) and plated in 6-well plates in complete RPMI. Monocytes were differentiated into DCs with GM-CSF and IL-4 using published methods. A10 % medium change was made after 3 days to replenish cytokines, and cells were harvested after a total of 6 days of culture using non-enzymatic cell dissociation solution. DCs were then re-plated in complete RPMI + 4μg/ml of polybrene + lentiviral construct (at an MOI of 15) + GM-CSF and IL-4. After 2 hours, 700 μl of complete RPMI + GM-CSF/IL-4 was added, and cells were cultured for 96 hours in total. Additional control wells were stimulated with IFN-γ and LPS for 96 hours, to act as a positive control for activation marker expression.

CD14-positive monocytes were differentiated into M1 or M2 macrophages with GM-CSF (M1) or M-CSF (M2). A 10 % medium change was made after 3 days to replenish cytokines, and cells were harvested after a total of 6 days of culture using non-enzymatic cell dissociation solution. M1/M2 macrophages were then re-plated in 300 μl of complete RPMI + 4μg/ml of polybrene + lentiviral construct (at a MOI of 15) + M-CSF). After 2 hours, 700 μl of complete RPMI + M-CSF was added, and cells were cultured for 96 hours in total. Additional control wells were stimulated with IFN-gamma and LPS (M1) or IL-13 and IL-4 (M2) for 96 hours in total, to act as a positive control for activation marker expression.

Human DCs and macrophages were transduced with a MOI of 15 with lentiviral vectors containing expression cassettes as described below:

Construct 1: GFP

Construct 2: GFP-IRES-LMP1_RK (LMP1 gene not codon optimized)

Construct 3: GFP-IRES-LMP1_CO (LMP1 gene codon optimized)

Control Construct 4: LMP1(dIC)

See FIGS. 2A-2B for illustrations of the adjuvant constructs and FIG. 1 B for the control construct.

Dendritic cell and macrophage proliferation was quantified after 96 h of culture. Triplicate samples were pulsed with ^{3}H-TdR and cultured overnight before being harvested on a Skarton harvester and the incorporation of radioactive thymidine determined by standard scintillation counting on a Trilux liquid scintillation counter. Proliferation was slightly reduced with adjuvanted vectors compared to GFP vector, most likely due to the presence of a longer DNA cassette. As already mentioned, viability of the transduced cells was determined by staining with a fixable viability dye before analysis using a BD FACS Canto System flow

cytometer. While slight differences were observed between the adjuvanted vectors, no significant toxicity was found.

Expression of GFP was determined for cells transduced with each construct by measuring the fluorescence with BD FACS Canto System flow cytometer after 96 h of culture, and the results are shown in FIGS. 3A (dendritic cells), 3B (M1 macrophages) and 3C (M2 macrophages).

The percentages of viable and GFP-positive cells were determined by gating on debris excluded/viable/single cells. Three independent experiments were carried out with PBMCs isolated from different donors. Graphed data represent means of duplicates of a representative experiment. The results presented in FIGS. 3A (dendritic cells), 3B (M1 macrophages) and 3C (M2 macrophages) show that, for all cell types, differences were observed between the adjuvanted vectors, with control vectors containing the LMP1 with no codon optimization showing lower expression of GFP.

Activation and maturation of the dendritic cells and macrophages elicited by the lentiviral vectors were evaluated by measuring the expression of surface markers and assessing their cytokine and chemokine release profile. To determine levels of lentiviral integration and DC/macrophage activation, cells were harvested after 48 or 96 h culture, stained with a fixable viability dye and a panel of staining antibodies recognizing the following surface markers; CD25, CD40, CD69, CD80/86, CD83, CCR7, MHC I and MHC II, before analysis using a BD FACS Canto System flow cytometer. Cell frequencies and geometric mean (Gmean) marker expression values were determined by gating on debris excluded/viable/single cells. Only double positive cells (GFP-positive, marker-positive) were taken into consideration. For both dendritic cells and macrophages, activation of the CD40 pathway was assessed by measuring the production the immune-stimulatory cytokines IL-8, IL-1beta, TNF-alpha, IL-6, and IL-12p70 after 96 h of culture by Luminex analysis with a Bioplex 200 system with high throughput fluidics (BioRad). The production of immune-suppressive cytokine IL-10 was measured as control. Three independent experiments were carried out with PBMCs isolated from different healthy donors. Graphed data represent means of duplicates of a representative experiment. The results are presented in FIGS. 4A (dendritic cells, membrane markers), 4B (dendritic cells, cytokines), 4C (M1 macrophages, membrane markers), 4D (M1 macrophages, cytokines), 4E (M2 macrophages, membrane markers) and 4F (M2 macrophages, cytokines).

For transduced dendritic cells, the results for expression of surface markers by GFP positive cells showed that compared to non-adjuvanted construct or construct adjuvanted with the wild type LMP1, LMP1 codon optimized construct upregulated the expression of the

following immune activation markers: CCR7 (+40% increase); CD25 (2-fold increase) and MHC II (2.5-fold increase). Consistent with the upregulation of these activation markers, increases in cytokine expression were as follows: pro-inflammatory IL-6 (2-fold increase), pro-inflammatory IL-8 (2-fold increase) and pro-inflammatory IL-1-beta (2-fold increase). Dendritic cells transduced with the control vector lacking the LMP1 intracellular domain exhibited levels of marker expression and cytokine secretion similar to the GFP control. Similarly, in transduced M1 macrophages, the results for expression of markers by GFP-positive cells showed that the construct adjuvanted with the codon-optimized LMP1 upregulated the expression of immune activation markers: CCR7 (5-fold compared to control), CD25 (10-fold compared to control), CD69 (3.5-fold compared to control), and MHCII (9-fold factor compared to control). Consistent with the upregulation of these activation markers, increases in cytokine expression in cells transduced with codon optimized LMP1 were as follows: pro-inflammatory IL-8 (2.5-fold compared to control) and pro-inflammatory TNF-alpha (2-fold compared to control). As observed with dendritic cells, removal of the intracellular domain reduced the adjuvant properties of LMP1 construct. Finally, in transduced M2 macrophages, the results for expression of markers by GFP-positive cells showed that the construct adjuvanted with the codon-optimized LMP1 upregulated the expression of immune activation markers: CCR7 (6-fold compared to control), CD25 (3-fold compared to control), CD40 (+50% increase), CD69 (2-fold compared to control), and MHCII (9-fold compared to control). The upregulation of these activation markers had a limited impact on cytokine expression in cells transduced with codon optimized LMP1. Pro-inflammatory TNF-alpha (+25% increase) and IL-8 (+20% increase) were increased compared with transduced with the GFP control vector. Low pro-inflammatory cytokine secretions were consistently observed with the M2 control stimulus conditions (IL-4/IL-13), which suggests that this cell type was more difficult to activate under these experimental conditions.

In conclusion, GFP-expressing vectors adjuvanted with codon optimized LMP1 showed significant activation of DCs and macrophages compared with control vectors and vectors adjuvanted with wild type LMP1.

Example 4. In Vitro Effects of Adjuvanted Lentiviral Vectors Activating the CD40L Pathway and Coding for NY-ESO-1 Tumor Antigen.

Fresh human dendritic cells and macrophages were obtained from healthy human donors (leukocyte cones) over a density gradient. CD14-positive monocytes were purified from PBMC using a magnetic isolation kit (positive selection) and were plated in 6-well plates

in complete RPMI. Monocytes were differentiated into DCs with GM-CSF and IL-4 using published methods. A 10 % medium change was made after 3 days to replenish cytokines, and cells were harvested after a total of 6 days of culture using non-enzymatic cell dissociation solution. DCs were then re-plated in 600 µl of complete RPMI + 4 µg/ml of polybrene + lentiviral construct (at an MOI of 40) + GM-CSF and IL-4. After 2 hours, 1.4 ml of complete RPMI + GM-CSF/IL-4 was added, and cells were cultured for 48 hours in total. Additional control wells were stimulated with IFN-γ and LPS for 96 hours, to act as a positive control for activation marker expression.

CD14-positive monocytes were differentiated into M1 or M2 macrophages with GM-CSF (M1) or M-CSF (M2). A 10 % medium change was made after 3 days to replenish cytokines, and cells were harvested after a total of 6 days of culture using non-enzymatic cell dissociation solution. M1/M2 macrophages were then re-plated in 600 µl of complete RPMI + 4 µg/ml of polybrene + lentiviral construct (at an MOI of 40) + M-CSF). After 2 hours, 1.4 ml of complete RPMI + M-CSF was added, and cells were cultured for 48 hours in total. Additional control wells were stimulated with IFN-gamma and LPS (M1) or IL-13 and IL-4 (M2) for 96 hours in total, to act as a positive control for activation marker expression.

Human DCs and macrophages were transduced with a MOI of 40 with lentiviral vectors containing expression cassettes as described below:

Construct 1: NY-ESO-1

Construct 2: NY-ESO-1-IRES-LMP1_RK (LMP1 gene not codon optimized)

Construct 3: NY-ESO-1-IRES-LMP1_CO (LMP1 gene not codon optimized)

Control Construct 4: NY-ESO-1-IRES-LMP1(dIC)

See FIGS. 2A-2B for illustrations of the adjuvant constructs and FIG. 1 B for the control construct.

Dendritic cell and macrophage viability was quantified after 48 h of culture with a fixable viability dye before analysis using a BD FACS Canto System flow cytometer. No significant differences were observed between the adjuvanted vectors, no significant toxicity was found.

Expression of NY-ESO-1 was determined for cells transduced with each construct by intracellular staining with a specific antibody measuring the fluorescence with BD FACS Canto System flow cytometer after 48 h of culture, and the results are shown in FIGS. 5A (dendritic cells), 5B (M1 macrophages) and 5C (M2 macrophages). The percentages of viable and NY-ESO-1-positive cells were determined by gating on debris excluded/viable/single cells. Three independent experiments were carried out with PBMCs isolated from different donors.

Graphed data represent means of duplicates of a representative experiment. The results presented in FIGS. 5A (dendritic cells), 5B (M1 macrophages) and 5C (M2 macrophages) show that, for all cell types, reduced expression levels of NY-ESO-1 were observed with the adjuvanted vectors.

Activation of the CD40 pathway in dendritic cells and macrophages elicited by the lentiviral vectors was evaluated by measuring the production the immune-stimulatory cytokines IL-8, IL-1beta, TNF-alpha and IL-6 after 48 h of culture by Luminex analysis with a Bioplex 200 system with high throughput fluidics (BioRad). The production of immune-suppressive cytokine IL-10 was measured as control. Three independent experiments were carried out with PBMCs isolated from different healthy donors. Graphed data represent means of duplicates of a representative experiment. The results are presented in FIGS. 6A (Dendritic cells), 6B (M1 macrophages) and 6C (M2 macrophages).

For transduced dendritic cells, the results for cytokine expression by NY-ESO-1 positive cells showed that compared to non-adjuvanted construct adjuvanted with the wild type LMP1, LMP1 codon optimized construct upregulated the secretion of the following pro-inflammatory cytokines: IL-1-beta (10-fold increase compared to control), IL-6 (3-fold increase compared to control), TNF-alpha (4-fold increase compared to control) and IL-8 (4-fold increase compared to control). Dendritic cells transduced with the control vector lacking the LMP1 intracellular domain exhibited a cytokine pattern similar to the NY-ESO-1 control. Similarly, in transduced M1 macrophages, increases in pro-inflammatory cytokine expression in cells transduced with codon optimized LMP1 were as follows: IL-1-beta (2.5-fold increase compared to control), IL-6 (6-fold increase compared to control), TNF-alpha (7-fold increase compared to control) and IL-8 (5-fold increase compared to control). Likewise, in transduced M2 macrophages, LMP1 codon optimized construct upregulated the secretion of the following pro-inflammatory cytokines compared to non-adjuvanted construct: IL-1-beta (2.5-fold increase compared to control), IL-6 (5-fold increase compared to control), TNF-alpha (3-fold increase compared to control) and IL-8 (5-fold increase compared to control). As already observed with dendritic cells, the removal on the intracellular domain reduced the adjuvant properties of LMP1 construct observed in M1 and M2 differentiated macrophages.

In conclusion, tumor antigen-expressing vectors adjuvanted with codon optimized LMP1 showed significant activation of DCs and macrophages compared with control vectors and vectors adjuvanted with wild type LMP1.

Example 5. *In Vivo* Immunogenicity in Healthy Mice Treated with Single or Multiple Antigens Shows Superior immunogenicity of LMP1-Lentiviral Vectors.

Healthy mice are treated with different viral vectors containing expression cassettes encoding (a) human ubiquitin; (b) one tumor antigen; and (c) LMP1. Short- and long-term evaluation of *in vivo* immunogenicity is conducted by either by FACS analysis of mouse blood, or by ELISPOT INFgamma on spleen cells, both of which allow for the detection and quantification of specific immune cells such as CD4+ and CD8+ T cells targeting the antigen present in the vector. Treatment with lentiviral vector coding for an antigen(s) and LMP1 is expected to increase specific immunogenicity when compared to the same lentiviral vectors without the LMP1.

Example 6. *In Vivo* Immunogenicity in Mouse Models of Specific Tumors Shows Superior Effectiveness of Lentiviral Vectors Containing a Combination of Multiple Antigens and LMP1 as Adjuvant.

Mouse models of specific tumors are treated with lentiviral vectors containing expression cassettes encoding human ubiquitin as promoter and at least one of the following: an indication-specific antigen; LMP1. Mice are divided into different treatment groups according to vector type and construct, dose and number of injections. Experimental groups are administered vectors encoding: only the indication-specific antigen(s); only LMP1; or indication-specific antigens and LMP1. *In vivo* efficacy and immunogenicity are evaluated by tumor growth rates, survival, and detection of antigen specific T-cells (using IFN-gamma ELISpot or FACS analysis of the CD4+ and CD8+ T cells). Lentiviral vectors encoding indication specific antigens and LMP1 molecules are expected to induce the most potent and long-lasting immune response of all experimental groups, thus inducing a higher survival rate and lower tumor growth in the treated groups of mice.

Example 7. *In Vivo* Immunogenicity in Mouse Models of Specific Anti-Checkpoint Sensitive Tumors.

Mouse models of specific tumors are treated with lentiviral vectors containing expression cassettes encoding human ubiquitin promoter and at least one of the following: an indication-specific antigen; LMP1; and one or more anti-checkpoint inhibitor molecules. Mice are divided into different treatment groups according to vector constructs, dose, and number of injections. Experimental groups are administered vectors encoding: only the indication-specific antigen(s); only LMP1; only anti-checkpoint inhibitor molecules; indication-specific

antigens and LMP1; indication-specific antigens and anti-checkpoint inhibitor; or indication-specific antigens, LMP1, and anti-checkpoint molecules. *In vivo* efficacy and immunogenicity is evaluated by tumor growth rates, survival, and detection of antigen specific T-cells (IFN-gamma ELISpot or FACS analysis of the CD4+ and CD8+ T cells). Lentiviral vectors encoding indication-specific antigen, LMP1, and anti-checkpoint inhibitor molecules are expected to induce the most potent and long-lasting immune response of all experimental groups.

References

Barry, M. et al. Role of endogenous endonucleases and tissue site in transfection and CpG mediated immune activation after naked DNA injection. Hum Gene Ther, 10 (15) (1999), pp. 2461–2480.

McNamara, M. et al. RNA-Based Vaccines in Cancer Immunotherapy. J Immunol Res. 2015; 2015: 794528.

Nasri et al., Production, Purification and Titration of a Lentivirus-Based Vector for Gene Delivery Purposes, Cytotechnology 66,1031-8 (2014).

CLAIMS

What is claimed is:

1. A viral vector comprising a first nucleic acid sequence encoding an antigen or an antigenic epitope and a second nucleic acid sequence encoding a full length latent membrane protein 1 (LMP1) of the Epstein Barr virus that has been codon optimized for human expression.

2. The viral vector of claim 1, wherein the vector is a lentiviral vector.

3. The viral vector of claim 1 or claim 2, wherein the first nucleic acid sequence encodes a fusion protein comprising two or more antigens or two or more antigenic epitopes.

4. The viral vector of any of the preceding claims, wherein the second nucleic acid sequence comprises SEQ ID NO. 1.

5. The viral vector of any of the preceding claims, wherein the vector further comprises a third nucleic acid sequence encoding a soluble immune checkpoint inhibitor molecule.

6. The viral vector of claim 5, wherein the soluble immune checkpoint inhibitor molecule is selected from the group consisting of CTLA-4, PD-1, PDL-1, LAG-3, TIM 3, B7-H3, ICOS, IDO, 4-1BB, CD47, and combinations thereof.

7. The viral vector of any of the preceding claims, wherein the vector further comprises a fourth nucleic acid sequence encoding a functional lentiviral integrase protein, wherein the vector is self-inactivating.

8. The viral vector of any of the preceding claims, wherein the antigen is selected from the group consisting of NY-ESO-1, mesothelin, PSA, MART-1, MART-2, Gp100, tyrosinase, p53, ras, MUC1, SAP-1, survivin, CEA, Ep-CAM, Her2, BRCA1/2, gag, reverse transcriptase, tat, circumsporozoite protein, HCV nonstructural proteins, hemaglutinins, and combinations thereof.

9. An immunotherapeutic formulation for preventing or treating cancer or infection in a subject, the formulation comprising the viral vector of any of claims 1-8.

10. A method of inducing or enhancing an immune response against a cancer or an infectious disease in a subject, the method comprising administering the viral vector of any of claims 1-8 or the immunotherapeutic formulation of claim 9 to a subject in need thereof, whereby an immune response against said cancer or infectious disease is induced or enhanced in the subject.

11. The method of claim 10, whereby an immune response is induced or enhanced against a cancer, and the cancer is selected from the group consisting of: melanoma, glioma, prostate cancer, breast cancer, cervical cancer, colorectal cancer, kidney cancer, lung cancer, lymphoma and pancreatic cancer.

12. The method of claim 10, whereby an immune response is induced or enhanced against an infectious disease, and the infectious disease is selected from the group consisting of: HIV/AIDS, hepatitis C, HPV, pneumonia, influenza, malaria, leishmaniosis, tuberculosis, Hansen's disease, rabies, dengue, Zika, Ebola, and schistosomiasis.

13. The method of any of claims 10-12, wherein one or more cell surface markers selected from the group consisting of MHCI, MHCII, CCR7, CD25, CD40, CD69, CD80/86, and CD83 are upregulated in dendritic cells or macrophages.

14. The method of any of claims 10-13, wherein secretion by dendritic cells or macrophages of one or more cytokines selected from the group consisting of IL-1b, IL-6, IL-8, IFN-α, IFN-β, and TNF-α is enhanced.

15. The viral vector of claim 8, wherein the antigen is NY-ESO-1.

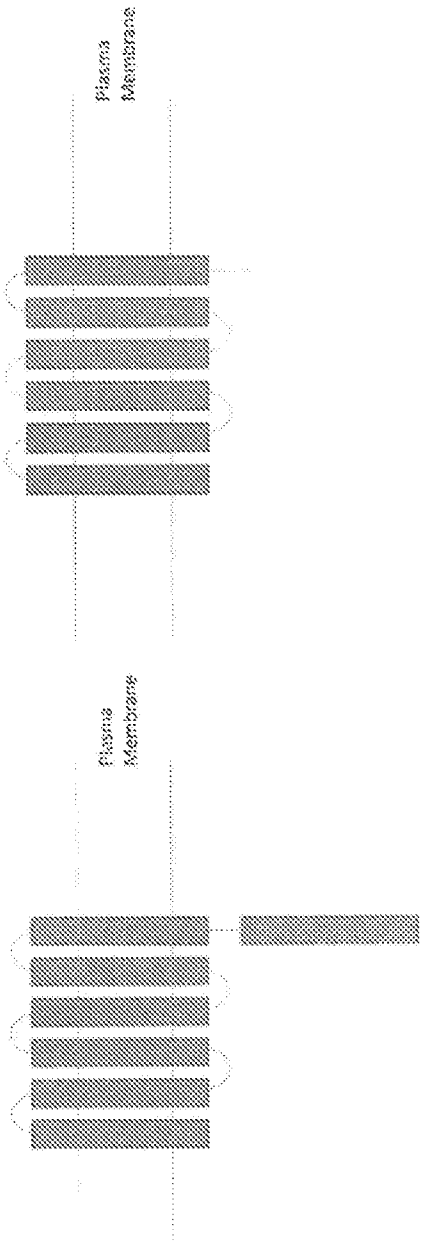

Plasma Membrane
Plasma Membrane
Truncated LMP1 (no intracytoplasmic region)
- Codon optimized form
- Used as control for effect of intracytoplasmic region
Full length LMP1
- Codon optimized
- Non-codon optimized

Fig. 1B

Fig. 1A

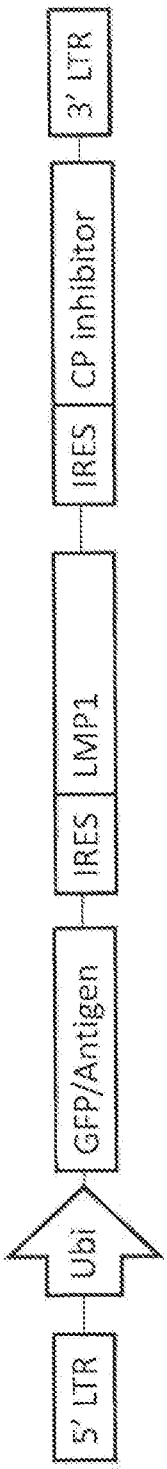


Fig. 2A

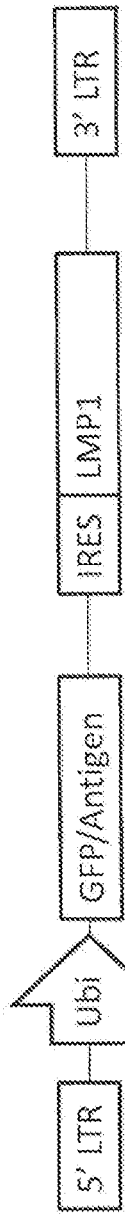


Fig. 2B

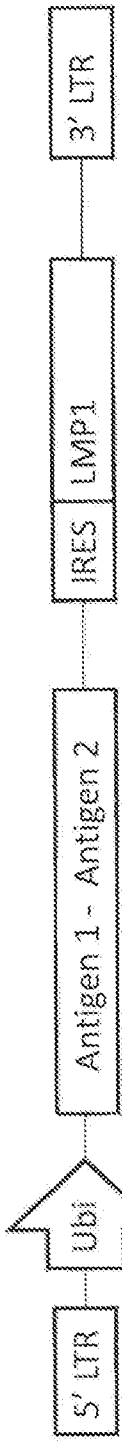


Fig. 2C

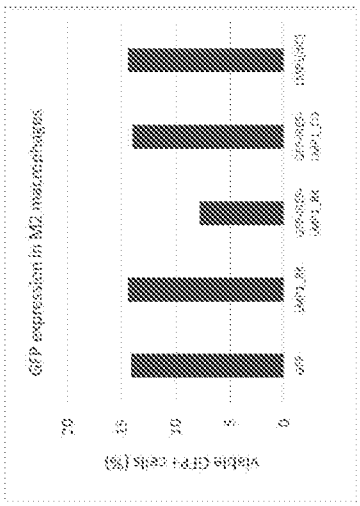


Fig 3C

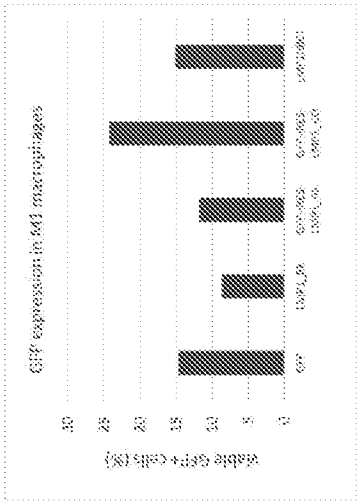
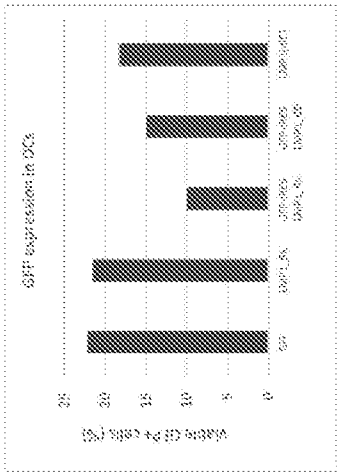


Fig 3B



3A

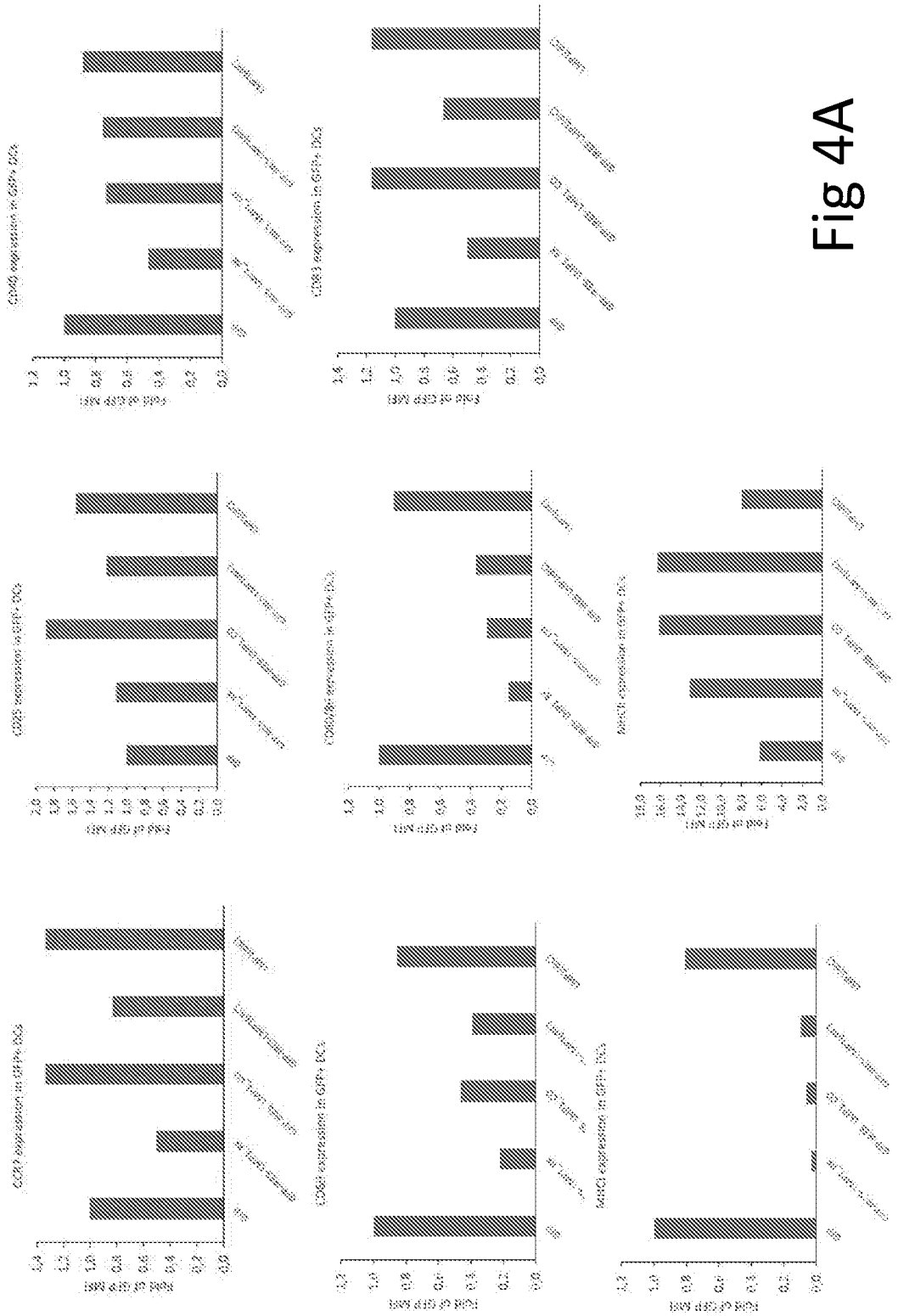


Fig 4A

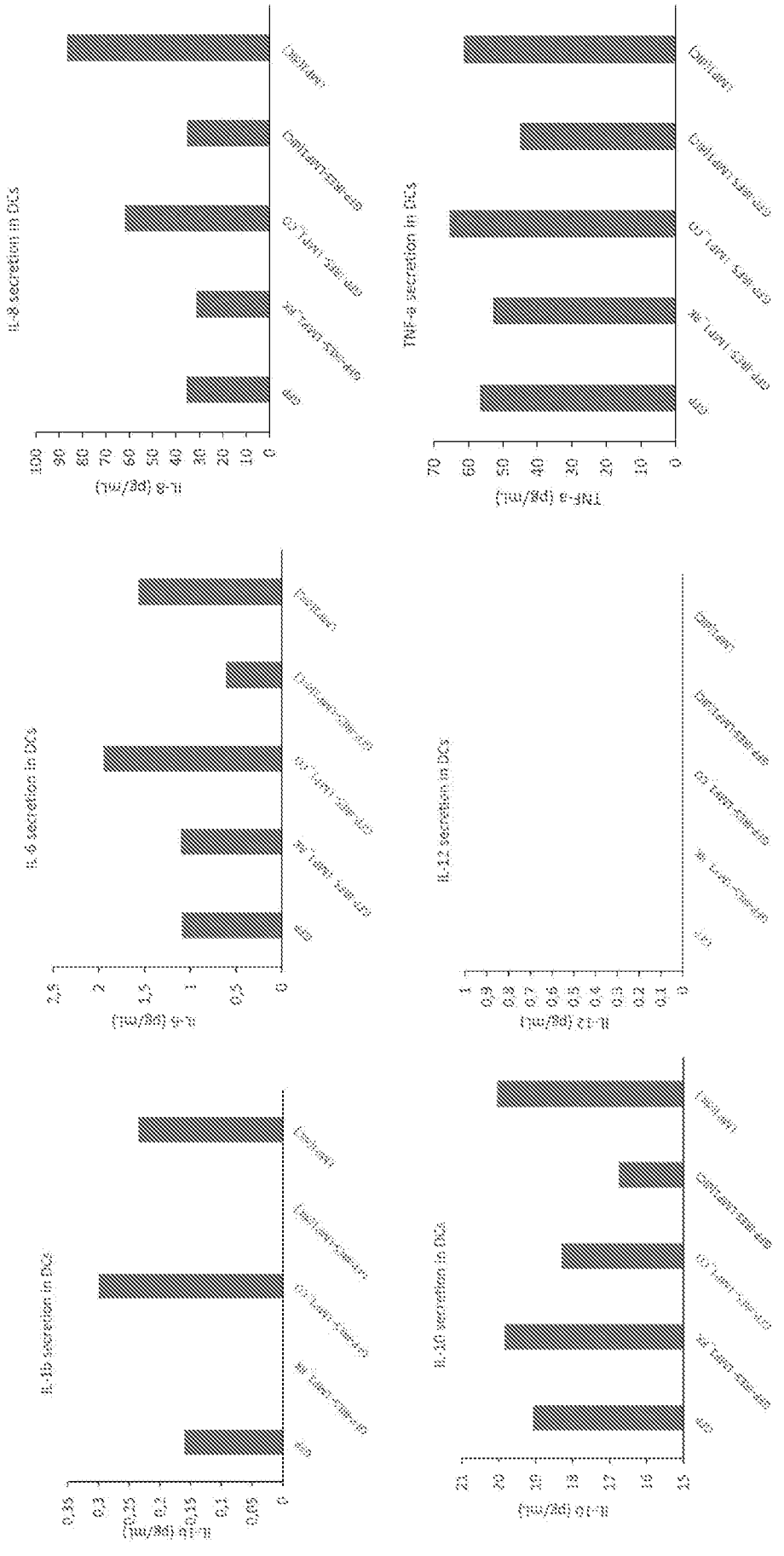

IL-8 secretion in DCs
IL-8 (pg/mL)
TNF-α secretion in DCs
TNF-α (pg/mL)
IL-6 secretion in DCs
IL-6 (pg/mL)
IL-12 secretion in DCs
IL-12 (pg/mL)
IL-1b secretion in DCs
IL-1b (pg/mL)
IL-10 secretion in DCs
IL-10 (pg/mL)

Fig 4B

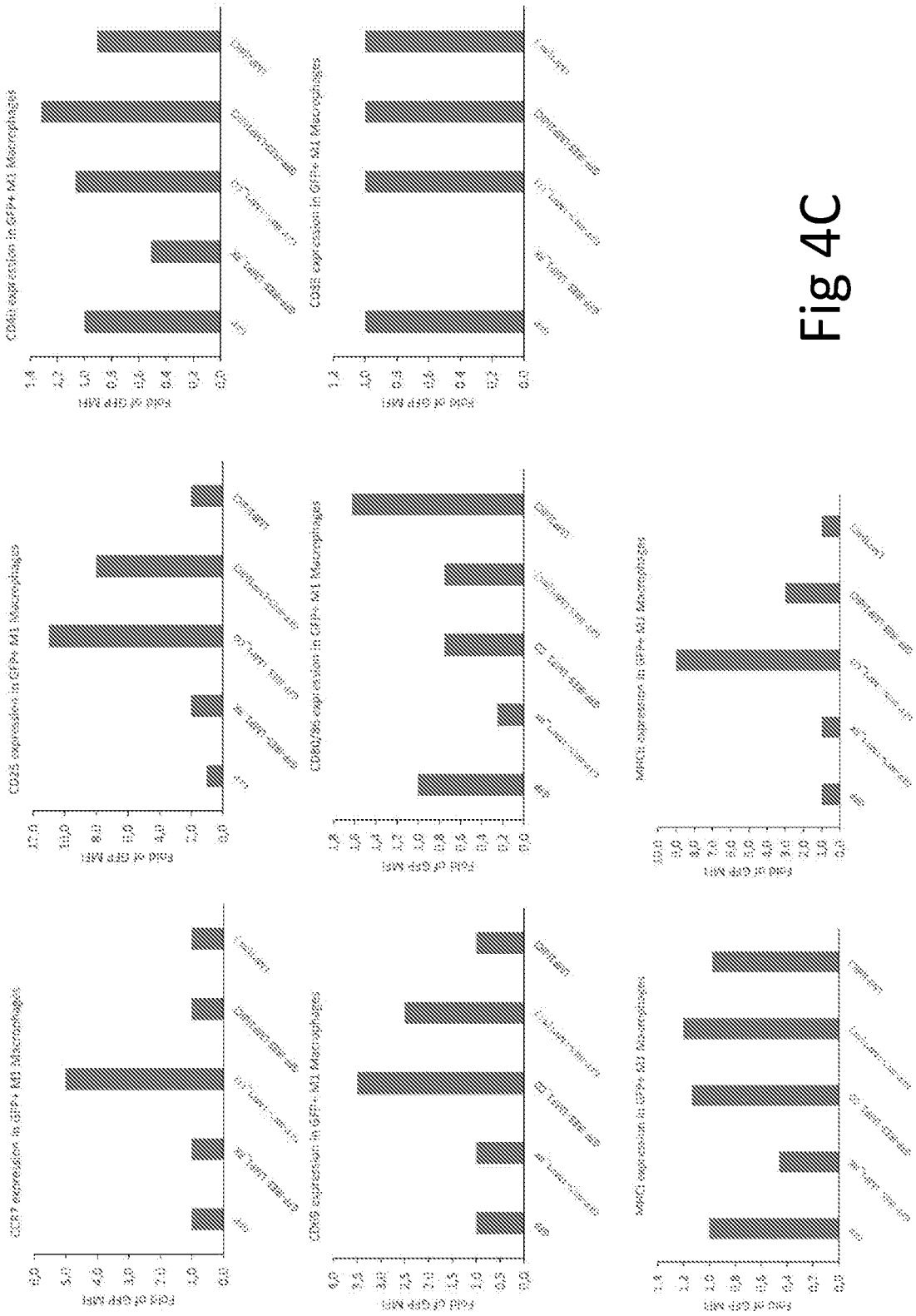

CD80 expression in GFP+ M1 Macrophages
CD86 expression in GFP+ M1 Macrophages
CD80/86 expression in GFP+ M1 Macrophages
CD25 expression in GFP+ M1 Macrophages
CD69 expression in GFP+ M1 Macrophages
MHCII expression in GFP+ M1 Macrophages
CD57 expression in GFP+ M1 Macrophages
Fold of GFP MFI
M1
M1 + Tumor Cells 24h
M1 + Tumor Cells 48h
M1 + Tumor Cells 72h
Tumor Cells

Fig 4C

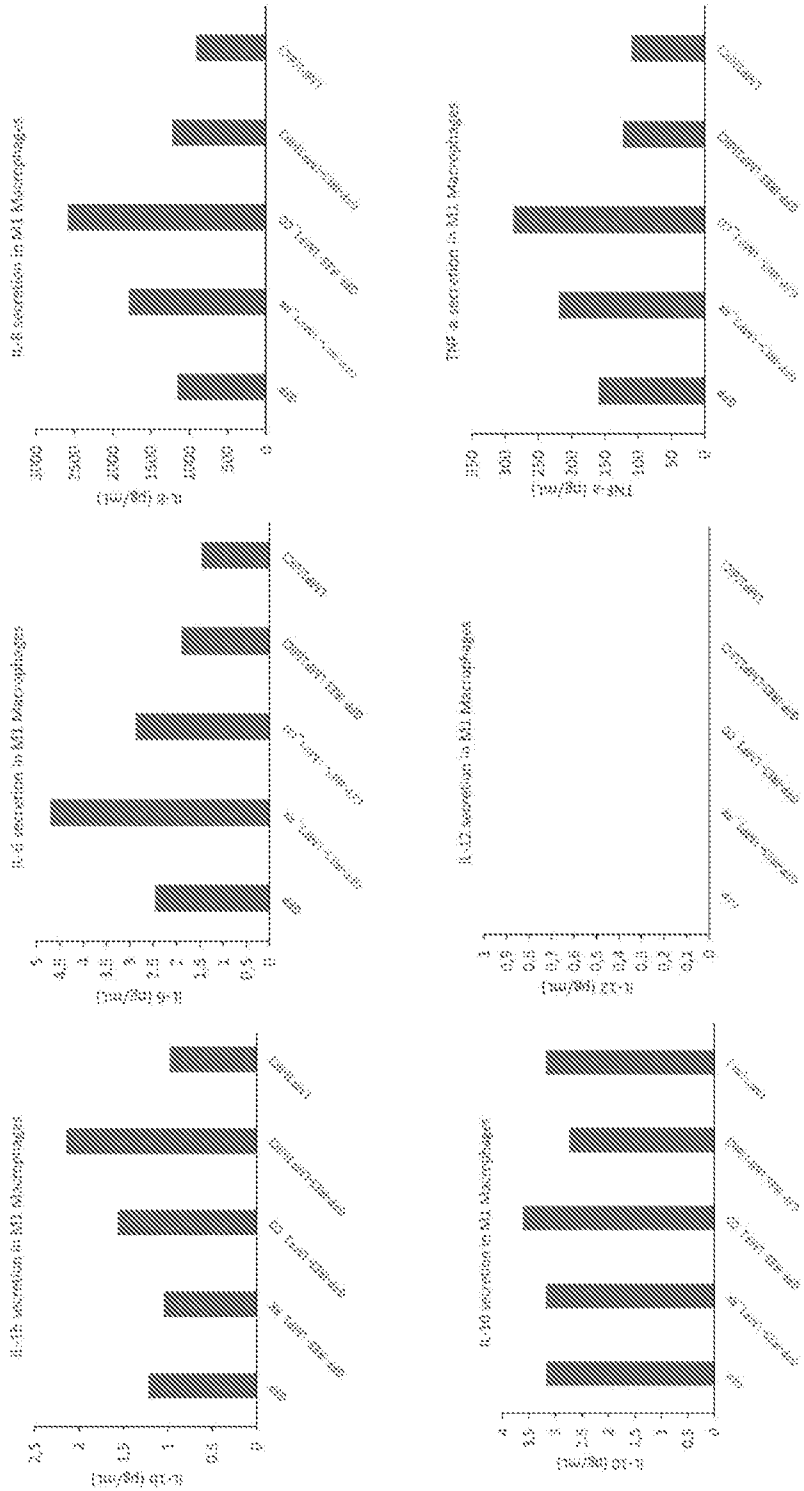


Fig 4D

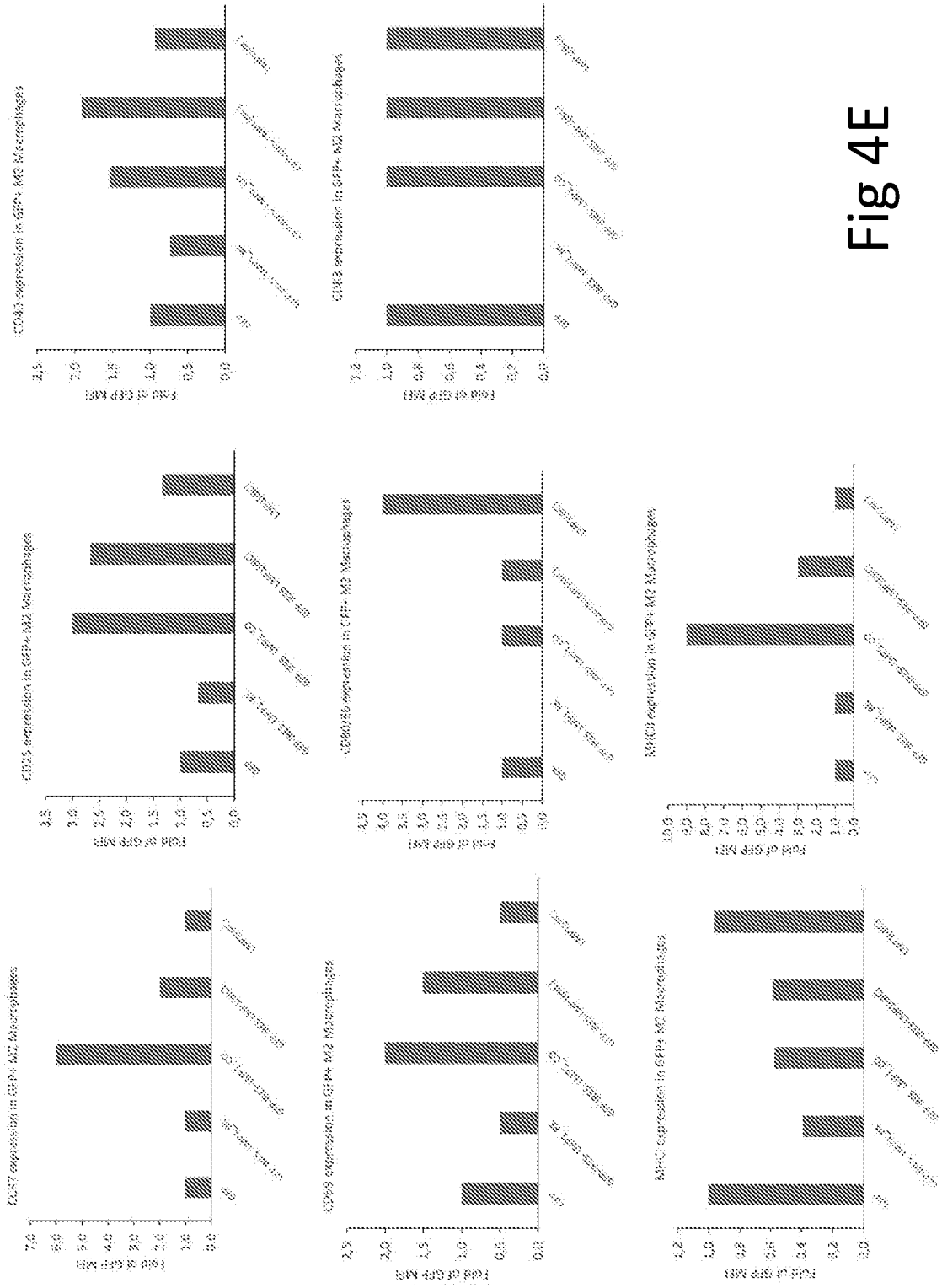


Fig 4E

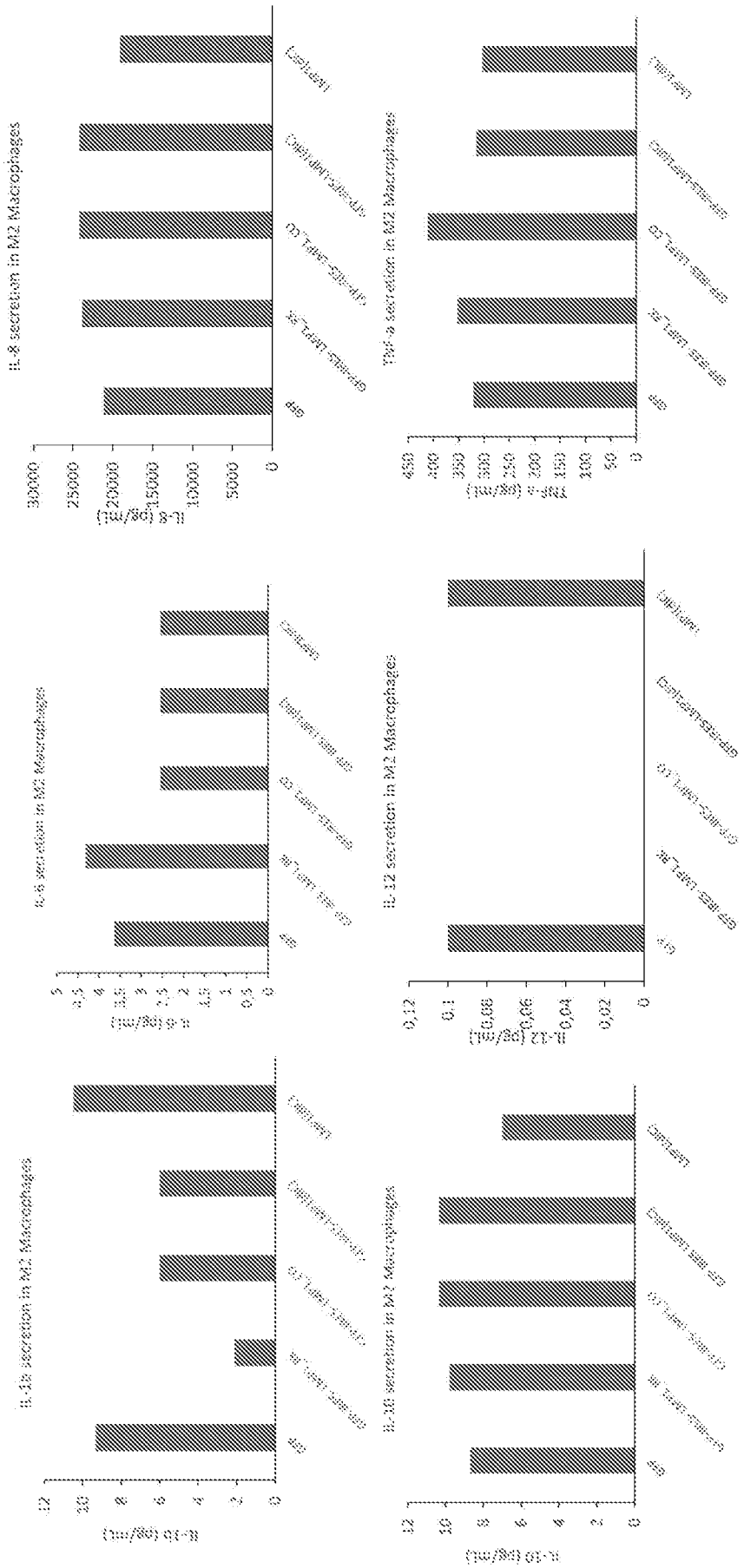

IL-8 secretion in M2 Macrophages
IL-8 (pg/mL)
30000
25000
20000
15000
10000
5000
0
IL-6 secretion in M2 Macrophages
IL-6 (pg/mL)
IL-10 secretion in M2 Macrophages
IL-10 (pg/mL)
TNF-a secretion in M2 Macrophages
TNF-a (pg/mL)
450
400
350
300
250
200
150
100
50
0
IL-12 secretion in M2 Macrophages
IL-12 (pg/mL)
0,12
0,1
0,08
0,06
0,04
0,02
0
IL-16 secretion in M2 Macrophages
IL-16 (pg/mL)

Fig 4F

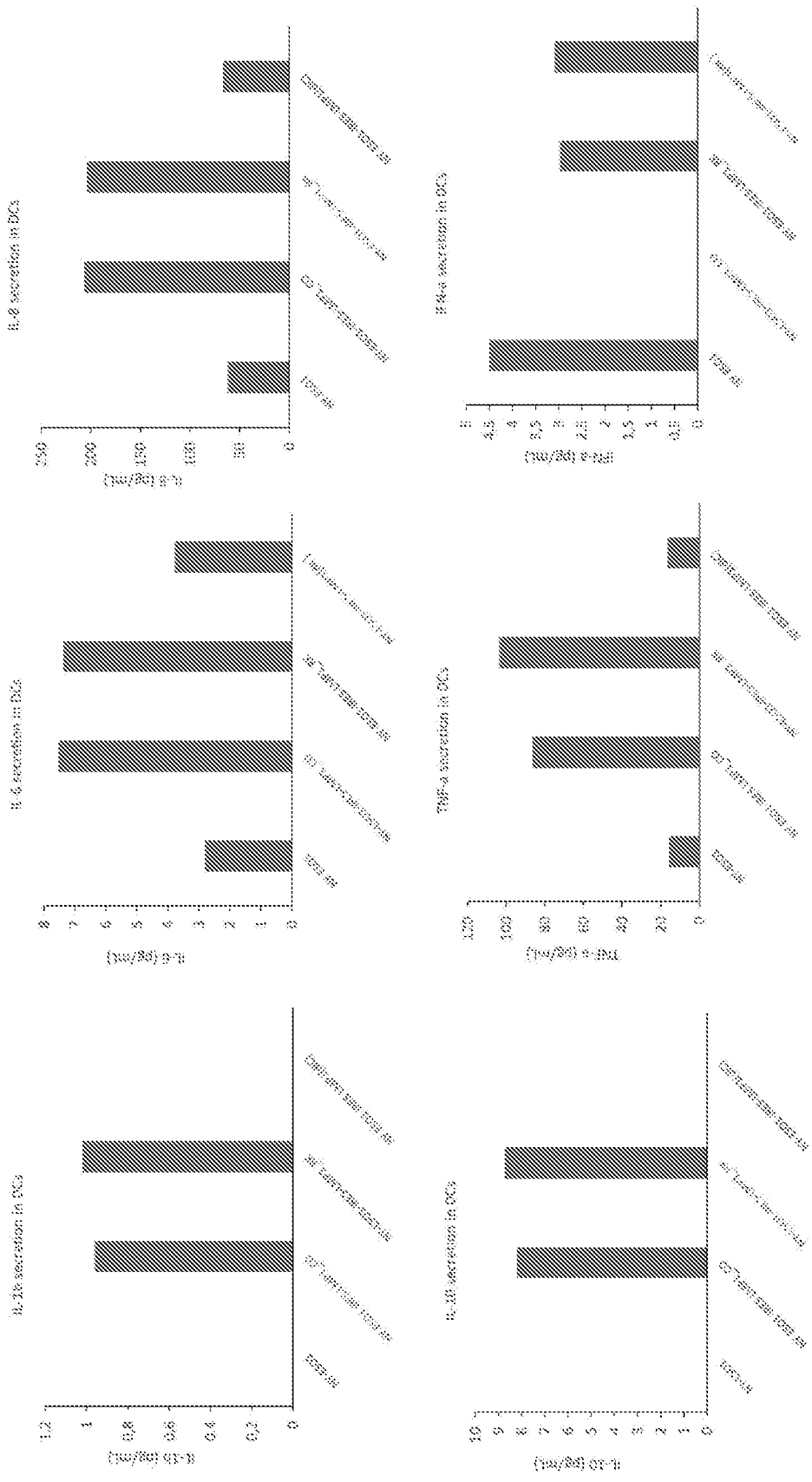

IL-6 secretion in DCs
IL-6 (pg/mL)
IL-10 secretion in DCs
IL-10 (pg/mL)
IL-12 secretion in DCs
IL-12 (pg/mL)
TNF-α secretion in DCs
TNF-α (pg/mL)
IFN-α secretion in DCs
IFN-α (pg/mL)
IL-8 secretion in DCs
IL-8 (pg/mL)

Fig 5A

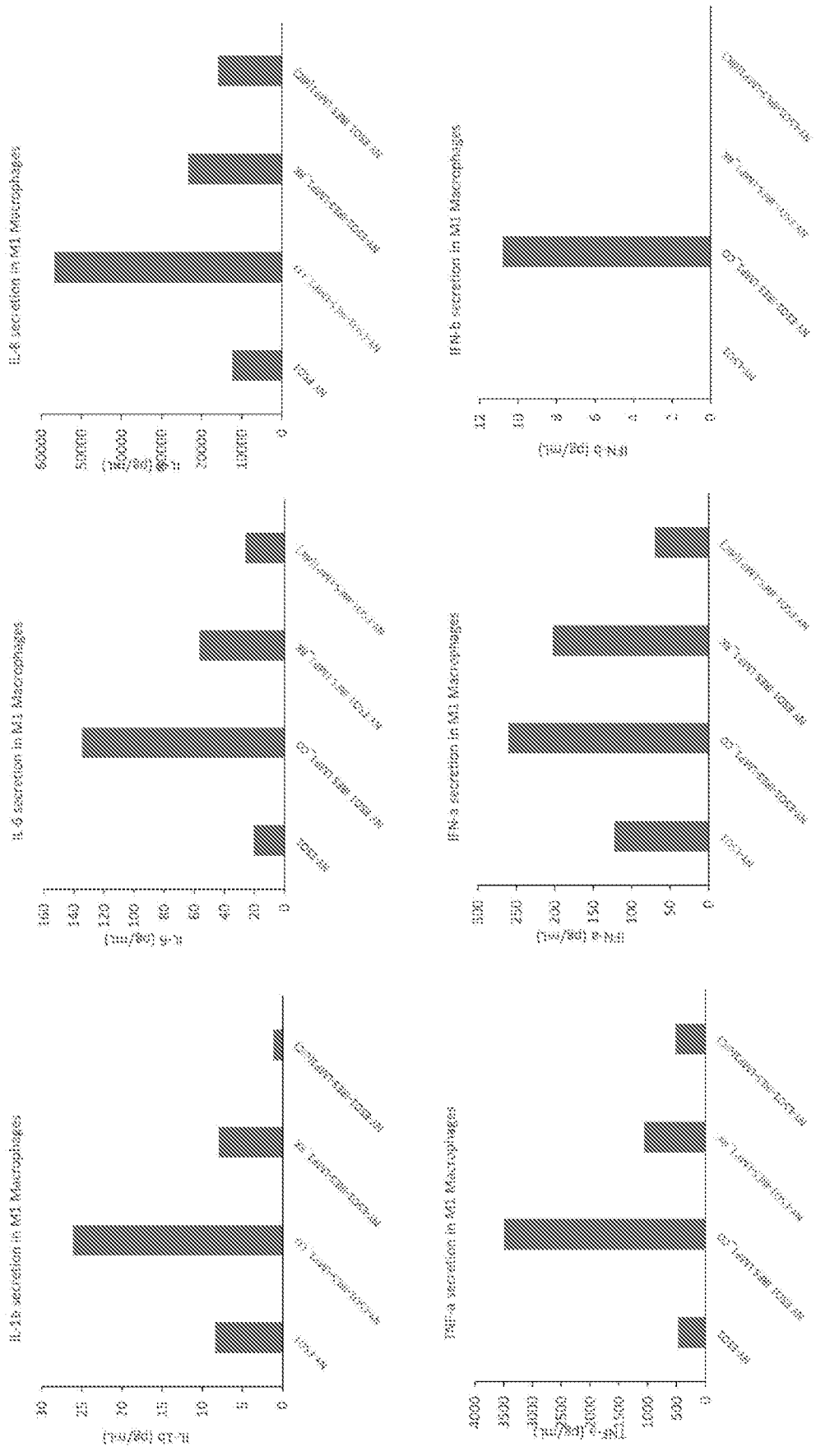

IL-12p secretion in M1 Macrophages
IL-6 secretion in M1 Macrophages
IL-8 secretion in M1 Macrophages
TNF-α secretion in M1 Macrophages
IFN-α secretion in M1 Macrophages
IFN-b secretion in M1 Macrophages

Fig 5B

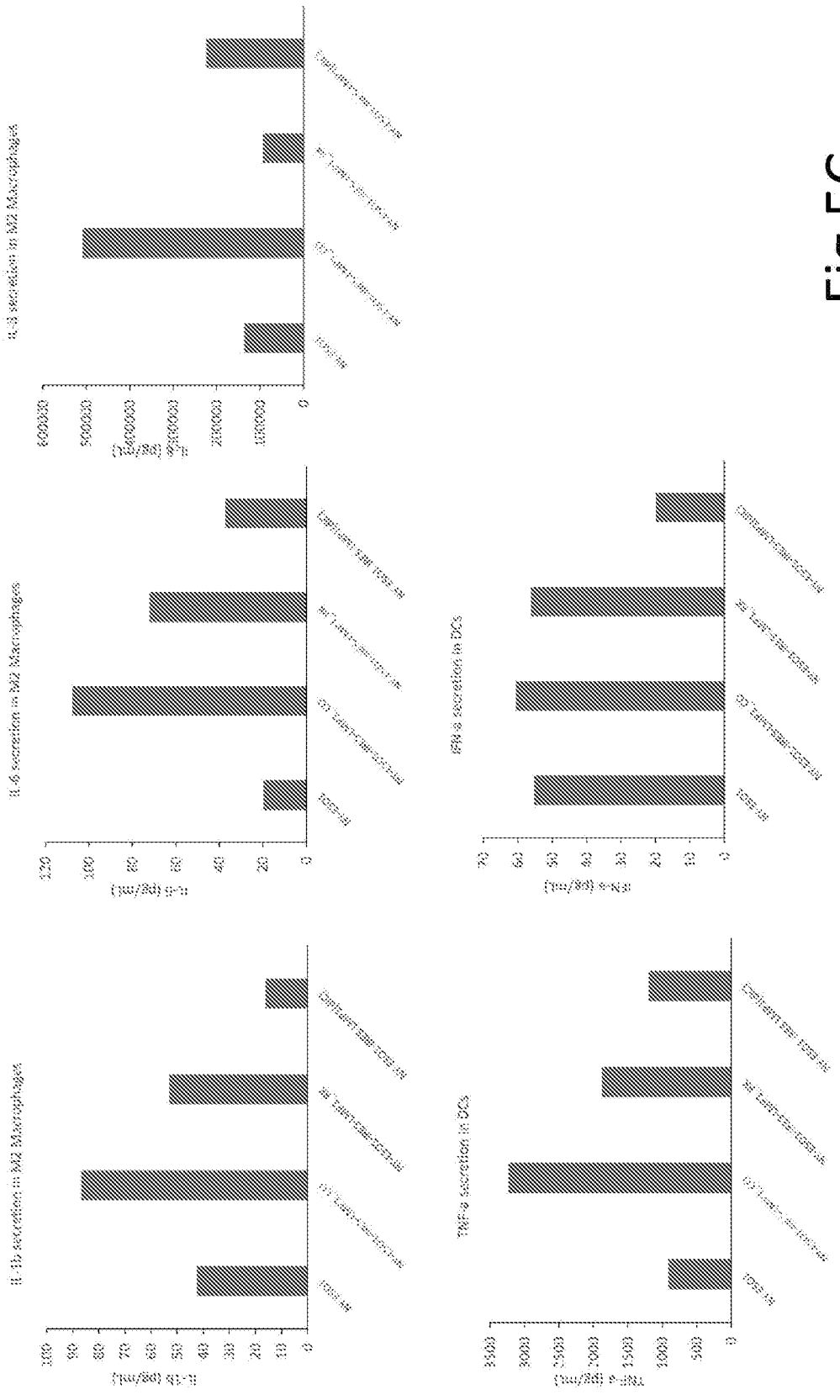

IL-1b secretion in M2 Macrophages
IL-6 secretion in M2 Macrophages
IL-8 secretion in M2 Macrophages
TNF-a secretion in DCs
IFN-a secretion in DCs

Fig 5C