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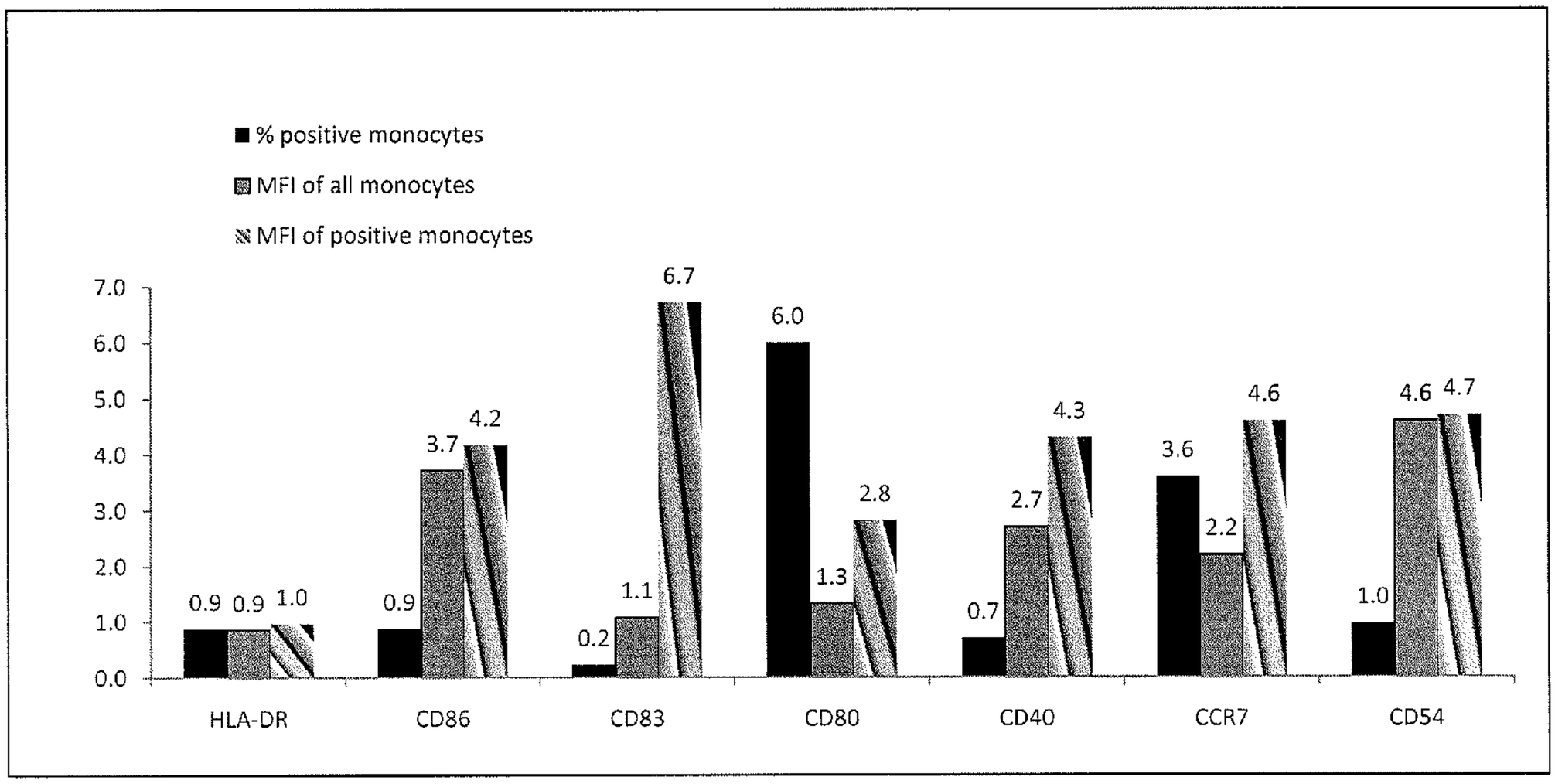
(54) Titre : COMPOSITION DE CELLULES IMMUNOSTIMULATRICES ACTIVEES POUR LE TRAITEMENT DE L'INFECTION

(54) Title: ACTIVATED IMMUNOSTIMULATORY CELL COMPOSITION FOR THERAPY OF INFECTION

Fig. 1



(57) Abrégé/Abstract:
Methods of making activated immunostimulatory cell compositions, activated immunostimulatory cell compositions, and methods of using those compositions to stimulate therapeutic immune responses to infectious organisms are described.

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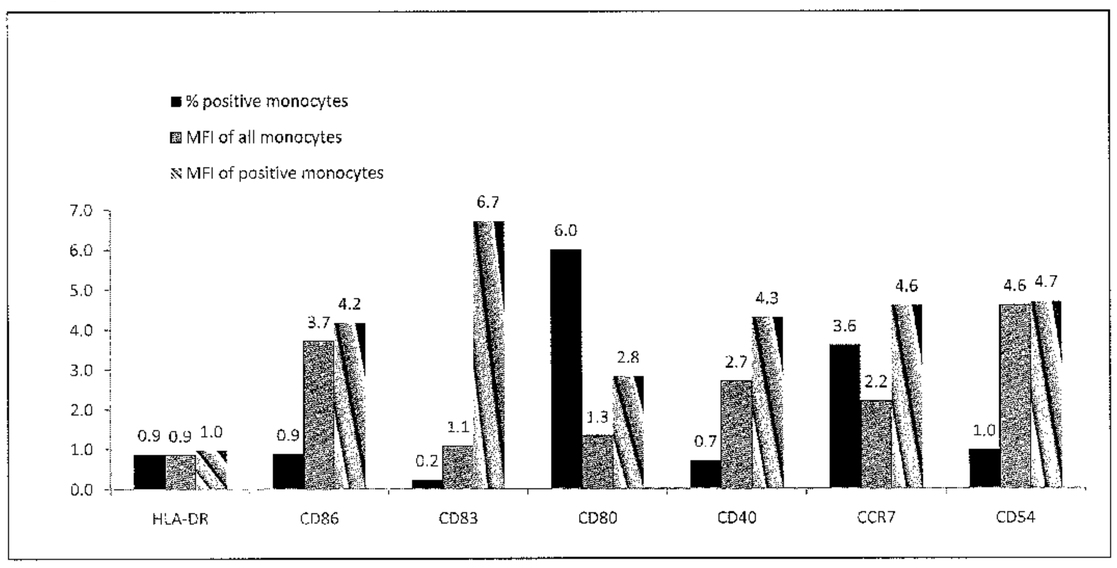
(54) Title: ACTIVATED IMMUNOSTIMULATORY CELL COMPOSITION FOR THERAPY OF INFECTION

Fig. 1



(57) Abstract: Methods of making activated immunostimulatory cell compositions, activated immunostimulatory cell compositions, and methods of using those compositions to stimulate therapeutic immune responses to infectious organisms are described.

WO 2013/136191 A1

ACTIVATED IMMUNOSTIMULATORY CELL COMPOSITION FOR THERAPY OF INFECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of U.S. Provisional Application No. 61/611,180, filed March 15, 2012, which is incorporated herein by reference.

FIELD AND BACKGROUND

[002] The present invention relates to activated immunostimulatory cell compositions, methods of preparing those compositions, and to uses of the compositions to treat conditions that may benefit from immunostimulation, such as infection.

[003] Viral or bacterial antigens are cleaved within infected cells into small peptides that are presented to immune cells as a complex with class I Major Histocompatibility molecules (MHC class I) also called Human Leukocyte Antigens (HLA class I, e.g. HLA A, B, or C). The MHC class I-peptide complex is recognized by the T Cell Receptor (TCR) complex of cytotoxic T lymphocytes (CTLs), which are CD8+ T cells. The interaction of TCR with MHC I-peptide complex causes release of substances from CTLs (perforin, granzymes, and granulysin) that destroy infected cells (Mullbacher et al. Proc. Natl. Acad. Sci. USA 1999; 96:13950).

[004] The initial CTL response is short-lived and requires amplification support from CD4+ T helper (Th) lymphocytes to continue. Differentiation and proliferation of Th cells occurs through their interaction with professional Antigen Presenting Cells (APCs), such as macrophages or interstitial dendritic cells (DCs) (Heath et al., Nat Immunol. 2009;10:1237–1244). DCs express several receptors for recognizing viruses such as the Toll-like receptors (e.g. TLR2, TLR3, TLR4, TLR7, TLR8) and C-type lectins (e.g. DEC-205, DCIR, CLEC9A) (Altfeld et al., 2011) (Altfeld et al., Nat Rev Immunol. 2011 Mar;11(3):176-86).

[005] APCs engulf viruses, bacteria, and apoptotic infected cells, process antigens from the engulfed material by cleaving their proteins into

small peptides, then present the peptides to naïve T cells. The process is similar to CTL stimulation by MHC class I + peptide, but CD4+ T cells recognize peptides presented in the context of MHC class II (e.g. HLA-DR). Upon recognition of MHC class II-peptide complexes by TCR, T cells acquire Th functions and produce IL-2, a cytokine that in turn amplifies the CTL response and induces development of T memory cells for sustained immunity (Keene JA, 1982;).

[006] T cell stimulation is thought to occur in at least two steps. (Marelli-Berg FM, 2007; Chambers CA 2001.) In the first step (signal 1), MHC/peptide complexes interact with the TCR. This step is not sufficient to fully stimulate the T cells. Instead, a second interaction between one of the APC-expressed co-stimulatory molecules (such as CD86, CD83, CD80 and CD40) and a corresponding ligand on T cells (signal 2) is required. T cells that receive signal 1 in the absence of signal 2 are unable to acquire helper functions (Chappert & Schwartz, 2010).

[007] The interaction of naïve T helper cells with APCs such as DCs polarizes the Th cells into Th1 and Th2 subsets, which differ by the patterns of cytokines that they produce. Th1 cells produce cytokines such as interferon gamma and IL-2 that activate proliferation of CTLs. Th2 cells produce the cytokines IL-4, IL-6, IL-10 and IL-13. Th1 cytokines are generally more beneficial in eliminating infections. For example INF-gamma is crucial for immunity against intracellular pathogens (Schoenborn & Wilson, 2007). (Schoenborn et al., Adv. Immunol. 2007, 96: 41–101.) Defects in proinflammatory Th1 responses, leading to biased Th2 cytokine profiles, have deleterious effects for the outcome of infections. (Netea et al, Antimicrob. Agents Chemother. 2005; 49(10):3991-96 (2005); Palucka & Banchereau, Curr Opin Immunol. 2002, 14(4):420-31.)

[008] Like Th cells, monocytes/macrophages phenotypes polarize in a process that is dependent on the balance between Th1/Th2 cytokines. The nomenclature for these polarized cells mirrors the Th1 and Th2 nomenclature and, like Th1 and Th2 T cells, M1 and M2 macrophages produce different patterns of cytokines. M1 macrophages predominantly produce IL-12, IL-23,

TNFα, IL-1, IL-6; whereas M2 macrophages produce high levels of IL-10 and IL-13 (Cassetta L, 2011; Benoit et al., J Immunol. 2008. 181(6):3733-9). M1 macrophages also express high levels of HLA-DR, while M2 macrophages express high levels of CD163 antigen. Fully polarized M1 and M2 macrophages are the extremes of a continuum of functional states.

[009] Another APC that is thought to also differentiate from peripheral blood monocytes is the myeloid dendritic cell (DC). Mature, activated DCs are extremely potent APCs. DC maturation is associated with up-regulation of MHC molecules, co-stimulatory molecules (CD86, CD83 CD80, CD40), adhesion molecules, such as CD11b, CD11c, and CD54, and the chemokine receptor CCR7 (Alvarez D, 2008; Banchereau et al., Annu Rev Immunol. 2000;18:767-811).

[010] The latter enables the DC to migrate from the peripheral tissue through the vessel walls to the T cell areas (Forster R, 2008). DC maturation not only ensures expression of molecules relevant for T cell stimulation, it also permits DC to reach the appropriate anatomical compartments in secondary lymphoid organs so that they can present antigens to naïve T cells. Cognate signals from T cells further activate DC (Cavanagh LL, 2002).

[011] Mature myeloid DCs are characterized by their ability to make IL-12 (Mariotti S, 2008). Because IL-12 promotes Th1 polarization, DCs that produce IL-12 are used in vaccine development (Trinchieri G: 2003).

[012] In addition to stimulating the adaptive, antigen-specific immune responses described above, DCs also play a role in stimulating innate (MHC-unrestricted) immunity. These cells include classical natural killer cells (NK cells), which do not express TCR (CD3-/CD56+ cells) and cytokine-induced killer T cells (CD3+/CD56+). NK cells can directly induce apoptosis of infected cells via the perforin-granzyme pathway or by expressing death-receptor ligands such as Fas ligand (Bryceson YT, 2011). IL-12-producing DCs can induce proliferation of NK cells (Walzer T, 2005). NK cells also cells release cytokines that promote differentiation of DCs (Ferlazzo G, 2009). Plasmacytoid DCs produce large amounts of type I interferons (IFN-α, IFN-β) in response to viral infection including HIV infection. Type I interferons

stimulate myeloid DCs and NK cells in a bystander fashion mediating killing of virus-infected cells (Trinchieri G, J Exp Med. 2010;207(10):2053-63).

[013] Thus, mature DC are an important cell type in generating both effective adaptive (T cell) and innate (NK, NKT cell) immune responses. Indeed, much research has focused on the application of DC-based vaccines as a therapy for infections, particularly chronic infections, and several clinical trials are underway for using dendritic cells in patients infected with human immunodeficiency virus (HIV) (Patham et al., Curr Med Chem. 2011.;18(26):3987-94). Accordingly, there is a great need for compositions comprising fully mature dendritic cells and other activated immune cells for treatment of conditions that may benefit from immunostimulation, such as therapy during infection.

SUMMARY OF CERTAIN EMBODIMENTS

[014] The present invention relates to activated immunostimulatory cell compositions, methods of preparing those compositions, and to uses of the compositions to treat conditions that may benefit from immunostimulation, such as infection.

[015] Accordingly, in one aspect the invention is directed to methods of treating a viral infection, comprising administering to an infected subject a composition mature dendritic cells, activated helper T cells, cytolytic T cells, and at least one other leukocyte cell type.

[016] In another aspect, the invention is directed to methods of treating a viral infection, the methods comprising: (a) incubating leukocytes under conditions of time and temperature to activate the leukocytes; (b) culturing the activated leukocytes in a supportive medium under conditions of time and temperature that induce maturation of dendritic cells in the composition; and (c) introducing the composition comprising dendritic cells into the subject to thereby treat the viral infection.

[017] In yet another aspect, the invention is directed to methods of treating a viral infection, the method comprising (a) incubating non-quiescent leukocytes in a supportive medium under conditions of time and temperature that induce maturation of dendritic cells in the composition, and (b) introducing

the composition comprising dendritic cells into the subject to thereby treat the viral infection.

[018] In still another aspect, the invention is directed to methods of treating a viral infection, the method comprising: (a) preparing a composition comprising mature dendritic cells by i) providing leukocytes, ii) allowing the leukocytes to transition from a quiescent to an active state by maintaining the leukocytes at room temperature for about 8 to 20 hours, iii) subjecting the leukocytes to hypo-osmotic shock, and iv) incubating the shocked leukocytes for 36 hours to 14 days in a supportive medium to thereby make a composition comprising mature DCs; and (b) introducing the composition comprising dendritic cells into the subject to thereby treat the viral infection.

[019] In similar aspects, the invention is directed to the use of any of the compositions of the invention in the preparation of a medicament for treating a viral infection.

[020] In other similar aspects, the invention is directed to any of the compositions of the invention for treating a viral infection.

[021] In some embodiments of these aspects of the invention, the composition comprising dendritic cells is exposed to at least one peptide epitope from the virus.

[022] In some embodiments of these aspects of the invention, the viral infection is a viral infection with human immunodeficiency virus (HIV), herpes simplex virus 1 (HSV1), herpes simplex virus 2(HSV2), hepatitis C virus (HCV), or human papilloma virus (HPV).

[023] In some embodiments of these aspects of the invention, the viral infection is an infection with HIV.

[024] In some embodiments of these aspects of the invention, the viral infection is chronic.

[025] In another aspect, the invention is directed to methods of treating an infection with an infectious organism, comprising administering to an infected subject a composition mature dendritic cells, activated helper T cells, cytolytic T cells, and at least one other leukocyte cell type.

[026] In another aspect, the invention is directed to methods of treating an infection with an infectious organism, the method comprising: (a) incubating leukocytes under conditions of time and temperature to activate the leukocytes; (b) culturing the activated leukocytes in a supportive medium under conditions of time and temperature that induce maturation of dendritic cells in the composition; and (d) introducing the composition comprising dendritic cells into the subject to thereby treat the viral infection.

[027] In still another aspect, the invention is directed to methods of treating an infection, the method comprising (a) incubating non-quiescent leukocytes in a supportive medium under conditions of time and temperature that induce maturation of dendritic cells in the composition, and (b) introducing the composition comprising dendritic cells into the subject to thereby treat the infection.

[028] In yet another aspect, the invention is directed to methods of treating an infection with an infectious organism, the method comprising: (a) preparing a composition comprising mature dendritic cells by: i) providing leukocytes, ii) allowing the leukocytes to transition from a quiescent to an active state by maintaining the leukocytes at room temperature for about 8 to 20 hours, iii) subjecting the leukocytes to hypo-osmotic shock, and iv) incubating the shocked leukocytes for 36 hours to 14 days in a supportive medium to thereby make a composition comprising mature DCs; and (b) introducing the composition comprising dendritic cells into the subject to thereby treat the infection.

[029] In similar aspects, the invention is directed to the use of any of the compositions of the invention in the preparation of a medicament for treating an infection with an infectious organism.

[030] In other similar aspects, the invention is directed to any of the compositions of the invention for treating an infection with an infectious organism.

[031] In some embodiments of these aspects of the invention, the composition comprising dendritic cells is exposed to at least one peptide epitope from the infectious organism.

[032] In some embodiments of these aspects of the invention, the infection is a bacterial infection.

[033] In some embodiments of these aspects of the invention, the infection is a fungal infection.

[034] In some embodiments of these aspects of the invention, the infection is a protozoal infection.

[035] In some embodiments of these aspects of the invention, the infection is with a prion protein.

[036] Additional objects and advantages of the embodiments in the application appear in part in the following description and in part will be obvious from the description, or they may be learned in practice. The objects and advantages of the embodiments will manifest themselves by means of the elements and combinations particularly pointed out in the appended claims.

[037] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

[038] The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion only. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

[039] **Figure 1** shows dendritic cell (DC) markers on monocytes expressed as fold increase after incubation at 37° C for 48 hours.

[040] **Figure 2** compares expression of DC markers before and after incubation at 37° C for 48h in adherent and non-adherent culture conditions.

[041] **Figures 3A-3B** compares expression of CD8 on monocytes before and after incubation at 37° C for 48 hours. **Fig. 3A** presents % CD8 positive cells. **Fig. 3B** presents the Mean Fluorescence Intensity (MFI) of CD8.

[042] **Figures 4A-4B** shows expression of the activation marker CD69 on lymphocyte subsets before and after incubation at 37° C for 48 hours in the presence or absence of the superantigen, *Staphylococcus aureus* Enterotoxin B. **Fig. 4A** shows the % positive cells among all lymphocytes, T cells, or CD3 negative cells. **Fig. 4B** presents the Mean Fluorescence Intensity (MFI) of CD69 on positive cells in each population.

[043] **Figure 5** shows expression of IL-2 receptor (CD25) on lymphocyte subsets before and after incubation at 37° C for 48 hours in the absence (48h) and presence (48 h SA) of superantigen, *Staphylococcus aureus* Enterotoxin B.

[044] **Figures 6A-6C** shows expression of CD40 on monocytes before and after incubation at 37° C for 48 hours in the absence and presence of superantigen, *Staphylococcus aureus* Enterotoxin B. **Fig. 6A** present the % CD40 positive cells. **Fig. 6B** presents the Mean Fluorescence Intensity. **Fig 6C** shows the fold increase after 48 hour incubation.

[045] **Figure 7A** shows IL-12 production before and after incubation at 37° C for 48 hours. **Figure 7B** shows IL-12 production in the absence and presence of superantigen, *Staphylococcus aureus* Enterotoxin B, before and after incubation at room temperature (RT) or 37° C for 48 hours.

[046] **Figure 8** shows IL-2 content in AICC and IL-2 production by washed cells of AICC resuspended in fresh serum after incubation at 37° C for 48 hours in the absence and presence of superantigen, *Staphylococcus aureus* Enterotoxin B.

[047] **Figure 9** shows IFN-gamma production in the presence of superantigen, *Staphylococcus aureus* Enterotoxin B, after incubation at 37° C for 48 hours and IFN-gamma production by washed cells resuspended in fresh serum after incubation at 37° C for 48 hours in the presence of Enterotoxin B.

DETAILED DESCRIPTION OF EMBODIMENTS

[048] As described in more detail below, the present invention relates to activated immunostimulatory cell compositions (AICCs), methods of preparing AICCs, and methods of using AICCs.

[049] An AICC of the invention includes functionally active monocytes differentiated into mature DCs, as shown by their cell surface marker profiles, their ability to present antigens such as superantigens to T cells, and their release of IL-12, a key factor promoting preferential Th1 polarization. T cells in the AICC are also activated. The interaction of the mature DC with T cells in an AICC in the presence of antigen causes upregulation of IL-2 receptor on T cells and release of IL-2 and IFN-g. When DCs in an AICC are exposed to antigen, IL-12 production drastically increases. Accordingly, an AICC of the current invention is a powerful tool for immune stimulation. For example, when administered *in vivo*, an AICC can change the cytokine balance to favor Th1 cytokines (*e.g.*, interferons, IL-2), which activate proliferation of CTLs and facilitates clearance of infectious organisms such as viruses and bacteria and clearance of cells infected with those organisms.

[050] Without being bound by theory, an AICC of the invention polarizes monocytes/macrophages into an M1 phenotype. As demonstrated in the working examples, the majority of monocytes/macrophages in AICC express high levels of HLA-DR and produce IL-12 and other M1 cytokines. M1 cytokines are known to overcome inhibitory effects on cellular immunity in the context of tumor environments. Accordingly, the cytokines in an AICC are also useful in overcoming the inhibitor effects that may be exerted by various infectious organisms.

[051] The principles and operation of the present invention may be better understood with reference to the drawings and accompanying

descriptions. Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

[052] It is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting. Further, the term "about" as used in connection with any and all values (including lower and upper ends of numerical ranges) includes a range of deviation of +/- 0.5% to +/- 20% (and values therebetween, *e.g.*, ± 1%, ± 1.5%, ± 2%, ± 2.5%, ± 3%, ± 3.5%, ± 4%, ± 4.5%, ± 5%, ± 5.5%, ± 6%, ± 6.5%, ± 7%, ± 7.5%, ± 8%, ± 8.5%, ± 9%, ± 9.5%, ± 10%, ± 10.5 %, ± 11%, ± 11.5%, ± 12%, ± 12.5%, ± 13, ± 13.5%, ± 14%, ± 14.5%, ± 15%, ± 15.5%, ± 16%, ± 16.5%, ± 17%, ± 17.5%, ± 18%, ± 18.5%, ± 19%, ± 19.5%, and ± 20%).

I. Methods of Making an Activated Immunostimulatory Cell Composition

[053] Leukocytes require activation to mediate an immune response. As used here, an activated immunostimulatory cell composition refers to a composition comprising at least one type of activated leukocyte. In this context, “activated” means that a cell has acquired one or more functional or phenotypic characteristics of an activated cell. Examples of characteristics of an activated (or “matured”) dendritic cell include, but are not limited to, production of IL-12; absent or low level production of IL-10, expression of one or more of the costimulatory molecules CD80, CD86, CD83, CD40, or CD1c (BDCA1), of one or more adhesion molecules such as CD56, CD11b, CD11c, or IGSF4 (SynCam and Nectin-like-2), of one or more lectin receptors such as CLEC9A (DNGR-1), of one or more chemokine receptors such as CCR7, of one or more Toll receptors such as TLR2, TLR3, TLR4, or TLR7, or TLR8, of one or more endocomal protein such as DC-LAMP, or of one or more transcription factors such as Id2, IRF8, or ICSBP; ability to activate naïve T

cells via antigen presentation; and ability to induce B cell differentiation into antibody secreting (plasma) cells. Examples of characteristics of an activated T cell include, but are not limited to, production of one or more of IL-2, IFN-gamma, IFN-alpha, or IFN-beta; expression of IL-2R; upregulation of T cell activation markers such as one or more of CD69, CD71 (transferrin receptor 1), CD28, or CD40L; and proliferation following exposure to antigen, cytotoxic function, or helper function.

[054] In one embodiment, an AICC is prepared from peripheral blood. Peripheral blood generally contains not only red blood cells (RBC) and platelets, but also leukocytes. Leukocytes, also known as "white blood cells," include monocytes (a "precursor" cell that differentiates into macrophages of various tissues and dendritic cells), lymphocytes (which includes T cells, B cells, natural killer (NK) cells, and natural killer T cells (NKT cells)), and granulocytes (which includes neutrophils, basophils, and eosinophils).

[055] Although whole peripheral blood is a convenient source of leukocytes, in alternate embodiments, an AICC is prepared using leukocytes isolated from blood from a central line, umbilical cord blood, placental blood, lymph, bone marrow, or lymphoid tissue such as lymph node or spleen. Leukocytes may be prepared by leukopheresis. Accordingly, the source of the leukocytes is not believed to be critical.

[056] When whole blood is used, leukocytes can be partially separated from red blood cells and platelets by preparing a "buffy coat" using density gradient separation of the different cell types. Accordingly, in some embodiments, the amounts of platelets and red blood cells present in an AICC are lower than that in whole blood.

[057] The starting materials for producing an AICC may be obtained from autologous or allogeneic sources. In one embodiment, an AICC is prepared from the patient who will ultimately be treated with the AICC; that is, the source is autologous. In other embodiments, an AICC is prepared from an individual other than the intended AICC recipient. In this case, the source is allogeneic.

[058] In those embodiments, involving allogeneic starting materials, these may be conveniently obtained from a blood bank. The samples may be screened by the blood bank for blood type (ABO, Rh) or specific human leukocyte antigen alleles such as, but not limited to, A2, B12 and C3, irregular antibodies to red cell antigens, and transfusion-transmittable diseases. More specifically, screening can be conducted with antibodies using an Abbott PRISM instrument against: Hepatitis B, C, HIV 1/2, HTLV and Syphilis (-HCV; HbsAg; anti-HIV 1/2 O+; and anti-HTLV I/II). The samples can also be screened for HIV, HCV and HBV by molecular methods (NAT-Nucleic Acid Testing). Molecular screening can be accomplished using commercially available instrumentation, e.g., the TIGRIS system of Chiron or any other methods which may be suitable forms of testing for such diseases.

[059] In one embodiment involving allogeneic sources, the sample is obtained from donors with the same blood type as the intended AICC recipient. In one embodiment, the donor(s) and recipient patient can be matched based on one or more HLA allele type. Alternatively, plasma samples can be obtained from donors with AB+ blood and the leukocytes can be obtained from donors with O- blood. Donors with AB+ blood are universal donors for plasma and donors with O- blood are universal donors for leukocytes. The plasma can be fresh, stored (e.g., at 1-6°C for less than 24 hours), dried, or otherwise pre-treated (e.g., pathogen-reduced plasma and solvent/detergent (SD) treated plasma). Regardless of the source, all necessary processing of the sample(s) can be carried out without the need for highly specialized equipment.

[060] In some embodiments, activated immunostimulatory cell composition may be prepared from smaller volumes of blood samples, with commensurate decreases in volumes of all solutions and use of smaller bags or other incubation vessels. Furthermore, use of these different size incubation vessels yields AICC with similar compositions. Use of smaller volumes provides the clinician with the ability to perform blood collection autonomously, without using an external blood bank. This may be useful when treating patients with otherwise healthy immune system.

[061] In some embodiments, a method of preparing an AICC comprises a) activating human leukocytes; b) incubating the activated leukocytes in an incubation composition under conditions of time and temperature to induce differentiation and maturation of dendritic cells (DC), thus producing an AICC. In one embodiment, step (b) also induces activation of lymphocytes.

[062] In one embodiment, the method further comprises contacting the DC with antigen or an antigenic peptide. In one embodiment, the antigen or antigenic peptide is contacted with the DC as they differentiate and mature in the incubation composition. That is, antigen or antigenic peptide is added during a part or all of the incubation of step (b). In one embodiment, the antigen or antigenic peptide is contacted with the DC after the incubation in the incubation composition is concluded. That is, the method further comprises a step (c) in which antigen or antigenic peptide is added to the AICC for a period of time sufficient to load DC with antigenic peptide.

[063] In one embodiment, an Activated Leukocyte Composition produced using the methods of WO 2010/100570, is used in preparing the AICC. In this embodiment, the Activated Leukocyte Composition corresponds to step (a) of the above embodiment of the method.

[064] In some embodiments, a method of preparing an AICC comprises a) isolating human leukocytes; b) optionally subjecting the leukocytes to hypo-osmotic shock; and c) incubating the shocked leukocytes in an incubation composition under conditions of time and temperature to induce differentiation and maturation of dendritic cells (DC), thus producing an AICC. In one embodiment, step (c) also induces activation of lymphocytes.

[065] In one embodiment, the method further comprises contacting the DC with antigen or an antigenic peptide. In one embodiment, the antigen or antigenic peptide is contacted with the DC as they differentiate and mature in the incubation composition. That is, antigen or antigenic peptide is added during a part or all of the incubation of step (c). In one embodiment, the antigen or antigenic peptide is contacted with the DC after the incubation in the incubation composition is concluded. That is, the method further

comprises a step (d) in which antigen or antigenic peptide is added to the AICC for a period of time sufficient to load DC with antigenic peptide.

[066] In one embodiment, an Activated Leukocyte Composition produced using the methods of WO 2010/100570, is used in preparing the AICC. In this embodiment, the Activated Leukocyte Composition corresponds to steps (a) and (b) of the above embodiment of the method.

[067] In some embodiments, the method comprises a) incubating human leukocytes under conditions of time and temperature to activate the leukocytes; b) optionally subjecting the leukocytes to hypo-osmotic shock; c) adding to the leukocytes of step b a physiologically acceptable salt solution in an amount effective to restore isotonicity; d) mixing the leukocytes of step c with a medium to form a second incubation composition; and e) incubating the second incubation composition under conditions of time and temperature to induce differentiation and maturation of dendritic cells (DC), thus producing an AICC. In one embodiment, step (e) also induces further activation of lymphocytes.

[068] In one embodiment, the method further comprises contacting the dendritic cells (DC) of step (e) with antigen or an antigenic peptide. In one embodiment, the antigen or antigenic peptide is contacted with the DC as they differentiate and mature in the incubation composition. That is, antigen or antigenic peptide is added during part or all of the incubation of step (e). In one embodiment, the antigen or antigenic peptide is contacted with the DC after the incubation in the incubation composition is concluded. That is, the method further comprises a step (f) in which antigen or antigenic peptide is added to the AICC for a period of time sufficient to load DC with antigenic peptide.

[069] In one embodiment, an Activated Leukocyte Composition produced using the methods of WO 2010/100570, is used in preparing the AICC. In this embodiment, the Activated Leukocyte Composition corresponds to steps (a) through (d) of the above embodiment of the method.

[070] In some embodiments, the method comprises a) incubating human leukocytes at room temperature for up to about 20 hours to activate

the leukocytes; b) subjecting the leukocytes to hypo-osmotic shock; c) adding to the leukocytes of step b a physiologically acceptable salt solution in an amount effective to restore isotonicity; d) mixing the leukocytes of step c with a medium to form a second incubation composition; and e) incubating the second incubation composition under conditions of time and temperature to induce differentiation and maturation of dendritic cells (DC), thus producing an AICC. In one embodiment, step (e) also induces further activation of lymphocytes.

[071] In one embodiment, the method further comprises contacting the dendritic cells (DC) of step (e) with antigen or an antigenic peptide. In one embodiment, the antigen or antigenic peptide is contacted with the DC as they differentiate and mature in the incubation composition. That is, antigen or antigenic peptide is added during part or all of the incubation of step (e). In one embodiment, the antigen or antigenic peptide is contacted with the DC after the incubation in the incubation composition is concluded. That is, the method further comprises a step (f) in which antigen or antigenic peptide is added to the AICC for a period of time sufficient to load DC with antigenic peptide.

[072] In one embodiment, an Activated Leukocyte Composition produced using the methods of WO 2010/100570, is used in preparing the AICC. In this embodiment, the Activated Leukocyte Composition corresponds to steps (a) through (d) of the above embodiment of the method.

[073] In some embodiments, the method comprises a) activating human leukocytes; b) mixing the leukocytes of step a with a medium to form a second incubation composition; and c) incubating the second incubation composition under conditions of time and temperature to induce differentiation and maturation of dendritic cells (DC), thus producing an AICC. Activation of leukocytes is indicated by a change in expression levels or in the number of leukocytes expressing an activation marker of leukocytes, such as CD11b and/or CD62L. Accordingly, in one embodiment, activation of the leukocytes is indicated by increased expression of CD11b in the leukocyte population. Increased expression of CD11b can be detected, for example, by flow

cytometry. Increased expression of CD11b encompasses an increase in the mean fluorescence intensity for CD11b on leukocytes, for example, the mean fluorescence intensity may be increased by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90%. Increased expression of CD11b also encompasses an increase in the percentage of leukocytes expressing CD11b (e.g., after correcting for background staining using an isotype control). For example, the percentage of leukocytes expressing CD11b may increase at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90% relative to expression of CD11b expression on leukocytes in a buffy coat. In one embodiment, activation of the leukocytes is indicated by reduced expression of CD62L in the leukocyte population. Reduced expression of CD62L can be detected, for example, by flow cytometry. Reduced expression of CD62L encompasses a decrease in the mean fluorescence intensity for CD62L on leukocytes, for example, the mean fluorescence intensity may be reduced by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90%. Reduced expression of CD62L also encompasses a decrease in the percentage of leukocytes expressing CD62L (e.g., after correcting for background staining using an isotype control). For example, the percentage of leukocytes expressing CD62L may decrease at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90% relative to expression of CD62L expression on leukocytes in a buffy coat. In one embodiment, CD62L and/or CD11b is measured on leukocytes that are granulocytes. In one embodiment, CD62L and/or CD11b is measured on leukocytes that are monocytes. In one embodiment, step (c) also induces further activation of lymphocytes.

[074] In addition, in any of the other embodiments involving activation of leukocytes, changes in expression of CD11b and/or CD62L, as discussed above, may be used alone, together, or in combination with additional markers and assays, as discussed elsewhere in the application, as an indicator of leukocyte activation.

[075] In one embodiment, the method further comprises contacting the dendritic cells (DC) of step (c) with antigen or an antigenic peptide. In one embodiment, the antigen or antigenic peptide is contacted with the DC as they differentiate and mature in the incubation composition. That is, antigen or antigenic peptide is added during part or all of the incubation of step (c). In one embodiment, the antigen or antigenic peptide is contacted with the DC after the incubation in the incubation composition is concluded. That is, the method further comprises a step (d) in which antigen or antigenic peptide is added to the AICC for a period of time sufficient to load DC with antigenic peptide.

[076] In one embodiment, an Activated Leukocyte Composition produced using the methods of WO 2010/100570, is used in preparing the AICC. In this embodiment, the Activated Leukocyte Composition corresponds to steps (a) and (b) of the above embodiment of the method.

[077] In general, in any of the methods of preparing an AICC, any composition in which the leukocytes have transitioned from a quiescent to a functionally active state can be used for the hypo-osmotic shock step. For example, as described in WO 2010/100570, leukocytes can be transitioned from a quiescent to functionally active state by incubating them at room temperature for up to about 20 hours. In one embodiment, the transition occurs by incubating the leukocytes for about 90 minutes to about 20 hours at room temperature. In one embodiment, the transition occurs by incubating the leukocytes for about 8 hours to about 20 hours at room temperature. In one embodiment, the transition occurs by incubating the leukocytes overnight at room temperature. In other embodiments, the temperature may be about 37°C. As noted, the details of this "transitioning" step are not essential and leukocytes may be obtained by any method for use in preparing an AICC.

[078] Further, since hypo-osmotic shock is a type of stress, in those embodiments of the method that include a step of hypo-osmotic shock other methods may be employed to stress the cells. That is, since various types of stress elicit cellular responses through the same highly conserved signaling pathway consisting of protein kinase cascades that result in the activation of

mitogen-activated proteins kinases (MAPKs), other stressors may be used in place of hypo-osmotic shock in any of the embodiments that mention hypo-osmotic shock. For example, in some embodiments, the methods of preparing an AICC comprise an optional step (in place of, or in addition to, hypo-osmotic shock) of subjecting the leukocytes to a stressor chosen from heat shock, hypoxia, treatment with any one or more of chlorpromazine, caffeine, vanadate, zymolyse, Congo red, calcofluor, rapamycin, or a mating pheromone, or by induction of actin depolymerization. Leukocyte activation also causes an increase in intracellular calcium, and there are many agonists that mimic this response. Accordingly, in still other embodiments, the methods of preparing an AICC comprise an optional step of subjecting the leukocytes to a calcium ionophore such as FMLP or PMA in place of or in addition to hypo-osmotic shock.

[079] In any of the embodiments of the various methods of producing an AICC, an incubation under "conditions of time and temperature to induce differentiation and maturation of dendritic cells (DC)" (which may optionally also activate lymphocytes and other cells) generally is an incubation of from about 24 hours to about 14 days at from about room temperature to about 37°C.

[080] In some embodiments, the incubation to induce differentiation and maturation of dendritic cells (DC) is at a temperature of about room temperature, *i.e.*, in the range of about 12°C to about 28°C. In one embodiment, the incubation is at a temperature of from about 16°C to about 25°C. In one embodiment, the incubation is at a temperature of from about 18-25°C. In yet another embodiment, the incubation is at a temperature of from about 20-25°C.

[081] In some embodiments, the incubation to induce differentiation and maturation of dendritic cells (DC) is at a temperature of about 37°C. In one embodiment, the incubation is at about 35°C to about 38°C. In one embodiment, the incubation is at 37°C +/- 0.5 °C.

[082] In some embodiments, the time period of incubation to induce differentiation and maturation of dendritic cells (DC) is from about 24 hours to

14 days. Thus, in some embodiments the incubation is for about 24, 30, 36, 42, 48, 54, 60, 66, 72, 84, 96, 108, 120, 132, 144, 156, 168, 192, 216, 240, 264, 288, 312, or about 336 hours, or for about any range of hours in between these values.

[083] In some embodiments, the time period of incubation to induce differentiation and maturation of dendritic cells (DC) is from about 48 to about 72 hours. In one embodiment, the incubation is for about 48 to about 72 hours at about 37°C. In one embodiment, the incubation is for about 48 hours at about 37°C. In one embodiment, the incubation is for about 72 hours at about 37°C. In one embodiment the incubation is for about 24 to about 72 hours at room temperature.

[084] In some embodiments, the incubation is in a cell incubator in an atmosphere containing 5% CO2 and at 100% humidity. In some embodiments the incubation is in gas-permeable bags and the bags are placed in a cell incubator in an atmosphere containing 5% CO_2 and 100% humidity. In other embodiments, tissue culture flasks or dishes are used in the method. In still other embodiments, combinations of bags systems and culture dishes or flasks are used in the method. In those embodiments involving incubation in a bag system, the bag system may be one of those described in WO2010/100570, or it can be a bag made from a different material, such as but not limited to fluorinated ethylene propylene (FEP) or Ethyl Vinyl Acetate (EVA), or in a tissue culture vessel or in any vessel.

[085] Any vessel used for incubation may also be treated or otherwise modified so that it becomes adhesive for leukocytes, which could be beneficial for leukocyte activation, differentiation of monocytes and activation/priming of lymphocytes. In one embodiment, one or more of the culture vessels used in the methods of preparing an AICC are non-adherent for dendritic cells. In another embodiment, the culture vessels used in the methods of preparing an AICC are treated to reduce cell adherence. In still another embodiment, one or more culture vessels used in the methods of preparing an AICC are treated to increase the adherence of cells.

[086] Any vessel used for an incubation may also contain scaffolds. The scaffolds may be in different shapes and in particular could be microbeads, biodegradable or not biodegradable, e.g., made of collagen, or made of PLA, PGA (polylactic acid, polyglycolic acid) or similar synthetic polymers, hydrogel scaffolds made of gelatin, hyaluronic acid alginated, fibrin sealer. Scaffolds or bags could be coated with adhesion receptors, extracellular matrix proteins such as fibronectin or laminin or with active binding peptides from extracellular matrices, such as RGD. Scaffolds or microbeads could be also coated with activating stimuli or stimulating antibodies, such as but not limited to activating antibodies against CD3, CD28, or CD40. In at least one embodiment, however, the method does not comprise microbeads or scaffolds coated with one or more activating stimuli or with one or more antibodies against CD3, CD28, or CD40.

[087] In some embodiments, the medium used for the incubation to produce an AICC is plasma or serum. In those embodiments utilizing serum, the serum may be obtained from a sample of plasma, which may be obtained from the same or a different whole blood sample (*i.e.*, from the same or a different human) as the leukocytes, that has been contacted with a coagulating agent at about 37°C. In some embodiments, the serum or plasma is obtained from a commercial or non-profit supplier and may be either fresh or in a storage-compatible form, such as frozen.

[088] In addition, although serum (particularly human serum) is often used in the incubation composition as the supportive medium, other supportive media may be used as well so long as it is a physiologic medium that supports release of cytokines, growth factors, and/or other soluble components from the activated leukocytes. For example, plasma may be used instead of serum. Other incubation medium that may also be used as supportive medium include culture medium, saline, or buffered saline solutions with optional addition of sugars and other components essential for cell viability and function such as amino acids (e.g. Lactated Ringer's solution, Acetated Ringer's solution, Hank's balanced salt solution (HBSS), Earle's balanced salt solution (EBSS), Standard saline citrate (SSC), HEPES-

buffered saline (HBS), Gey's balanced salt solution (GBSS)). Saline solutions and culture medium may also be supplemented with human serum or clinical grade animal serum, or serum substitutes. The incubation composition may alternatively, or in addition, contain serum proteins such as human or bovine albumin, gamma-globulin, transferrin or other proteins from different tissues, plant proteins, or plant extracts.

[089] In certain embodiments, leukocyte agonists such as complement proteins, chemokines, interferon-alpha, interferon-gamma, cytokines such as interleukin-4, granulocyte-macrophage–colony stimulating factor (GM-CSF), or interleukin-12, are added to the incubation. Monocyte differentiation to DCs *in vitro* can be induced using well-defined cytokine cocktails (Jensen SS, Gad M. 2010). Accordingly, in one embodiment, an incubation may be performed in the presence of cytokines such as interleukin-4 or GM-CSF. In other embodiments, an incubation may be performed with other substances that increase differentiation and maturation of dendritic cells and activation of lymphocytes and NK cells. For example, the CD40 co-stimulatory receptor on monocytes may be ligated by antibodies to CD40 or by a CD40 ligand (CD54) in the absence of cytokines (Brossart P, 1998). A CD40 independent activation of DC maturation can be induced by interaction with activated CD8 positive T cells (Ruedl C., 1999; Wirths, 2002). Accordingly, in one embodiment, exogenous, activated CD8 positive T cells may be added to the incubation. DC differentiation from monocytes *in vitro* can also be induced by DC interaction with NKT cells. DC differentiation results from NKT cell secretion of GM-CSF and IL-13, cytokines that were produced by the NKT cells upon activation by monocytes (Hegde, 2007). Accordingly, in one embodiment, exogenous, activated NKT cells may be added to the incubation. In other embodiments, DC differentiation and maturation may be promoted by the addition of one or more of GM-CSF, IL-4, IFN-gamma, IL-2, IFN-alpha, and TNF-alpha; and/or by addition of one or more bacterial products that interact with Toll receptors on DCs, such as but not limited to lipopolysaccharide (LPS), peptidoglycan (murein), double-

stranded RNA or its synthetic analog polyinosinic:polycytidylic acid (poly I:C), Resiquimod (R-848), and Picibanil (OK-432).

[090] It is also expressly contemplated that, in one or more embodiments of the methods, incubation occurs in the absence of one or more of the exogenously added factor(s) described above as involved in DC maturation. In one embodiment, all of the components needed for DC maturation are provided endogenously and no additional stimuli are added to the incubation composition. Nevertheless, various cytokines may be present in the incubation composition because they are released upon leukocyte activation during incubation. For example. CD40 ligand may be found in serum and on platelets that are part of the incubation composition. Similarly, activated $CD8^+$ T cells and NKT cells that are endogenously present in the incubation composition can interact with monocytes to support dendritic cell differentiation and maturation.

[091] Accordingly, in one embodiment, the incubation composition for producing an AICC does not include exogenous GM-CSF, exogenous IL-4, exogenous TNF, or an exogenous interferon (although one or more of GM-CSF, IL-4, TNF, or an interferon may be produced endogenously during the incubation). Thus, in one embodiment a method of preparing an AICC excludes the addition of one or more exogenous cytokine or interferon, the addition of reagent(s) that crosslink CD3 and/or CD28, the addition of reagent(s) that crosslink CD40, and/or the addition of other exogenous agents that promote dendritic cell maturation during the production of the AICC. Examples of exogenously added cytokines and exogenously added interferons that may be excluded from the practice of the method include any one or more of GM-CSF, IL-4, IFN-gamma, IL-2, IFN-alpha, or IL-2. Examples of exogenously added bacterial products that may be excluded from the practice of the methods include those known to interact with Toll receptors on DCs, such as but not limited to lipopolysaccharide (LPS), peptidoglycan (murein), double-stranded RNA or its synthetic analog polyinosinic:polycytidylic acid (poly I:C), Resiquimod (R-848), and Picibanil (OK-432).

[092] In some embodiments inhibitors of angiogenesis targeting VEGF signaling are added to the incubation composition. These include but are not limited to anti-VEGF antibodies (e.g. bevacizumab, ranibizumab), antibodies against VEGF receptors (e.g. Brivanib, targets VEGFR-2 and FGFR), inhibitors of the tyrosine kinase activity of the VEGF receptors (e.g., Sorafenib, Cediranib, Sunitinib), soluble receptor-decoys (e.g, VEGF Trap, also called aflibercept), or vascular-disrupting agents (e.g., ZD6126).

[093] In some embodiments adjuvants are added to the incubation composition. Examples of adjuvants include but are not limited to aluminium hydroxide, aluminium phosphate and calcium phosphate, adjuvants based on oil emulsions (Freund's emulsified oil adjuvants (complete and incomplete), Arlacel A, Mineral oil, emulsified peanut oil adjuvant (adjuvant 65), products from bacteria (their synthetic derivatives as well as liposomes) or gram-negative bacteria, endotoxins, cholesterol, fatty acids, aliphatic amines, paraffinic and vegetable oils, monophosphoryl lipid A, ISCOMs with Quil-A, and Syntex adjuvant formulations (SAFs).

[094] As discussed elsewhere, in some embodiments, any of the methods may further comprise a contacting step wherein one or more antigen or antigenic peptide is introduced. Examples of antigens include viral, bacterial, protazoal, and fungal antigens, as well as superantigens (*e.g.*, staphylococcal enterotoxins) and prion antigens. Generally speaking, antigens or antigenic peptides will enhance differentiation of monocytes into dendritic cells and prime lymphocytes specific for that antigen. Further, since antigen presentation on the cellular level involves an antigenic peptide presented in the context of a class I or class II molecule, the terms "antigen," "antigen peptide," and "antigenic peptide" should not be construed as requiring contact with an intact antigen or a particular peptide. Instead, the terms are used broadly to indicate that an antigen presenting cell is contacted with antigenic material that it may then either directly, or after further processing, present in the context of class I or class II molecules.

[095] Antigens and antigenic peptides, whether prepared from cell lysates or by recombinant expression of a protein or peptide, are incubated

with an AICC or during the production of an AICC at various concentrations for about 1 hour to about 24 hours at room temperature to about 37 °C. Examples of antigens/peptides include those listed below and any antigen/peptide used in the Examples section.

[096] In some embodiments, the intact antigen is used. Examples of intact antigens include proteins from viruses, bacteria, fungi, and protozoa that infect humans, as well as prion proteins.

[097] Exemplary viruses that may be used to provide viral antigens include, but are not limited to, human immunodeficiency virus (HIV-1, HIV-2); human papilloma virus; influenza virus; Dengue Fever; Japanese Encephalitis; yellow fever; hepatitis viruses (Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), non-A, non-B hepatitis); RSV (respiratory syncytial virus); HPIV 1; HPIV 3; adenovirus; and rhinovirus; EBV; CMV (cytomegalovirus); Herpes simplex viruses HSV-1 and HSV-2; BK; HHV6; Parainfluenza; Bocavirus; Coronavirus; LCMV; Mumps; Measles; Metapneumovirus; Parvovirus B; Rotavirus; West Nile Virus; and Ebola hemorrhagic fever virus.

[098] Exemplary bacterial antigens include, but are not limited to, bacterial antigens such as pertussis toxin, filamentous hemagglutinin, pertactin, FIM2, FIM3, adenylate cyclase, diptheria toxin, tetanus toxin, streptococcal M protein, lipopolysaccharides, mycolic acid, heat shock protein 65 (HSP65), the 30 kDa major secreted protein, antigen 85A 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 and other anthrax bacterial antigen components; rickettsiae bacterial antigens such as rompA and other rickettsiae bacterial antigen component. Also included with the bacterial antigens described herein are any other bacterial, mycobacterial, mycoplasmal, rickettsial, or chlamydial antigens.

[099] Exemplary fungal antigens include, but are not limited to, e.g., candida fungal antigen components; histoplasma fungal antigens such as heat shock protein 60 (HSP60) and other histoplasma fungal antigen components; cryptococcal fungal antigens such as capsular polysaccharides and other cryptococcal fungal antigen components; coccidiodes fungal antigens such as spherule antigens and other coccidiodes fungal antigen components; and tinea fungal antigens such as trichophytin and other coccidiodes fungal antigen components.

[0100] In other embodiments, a peptide epitope of the antigen (prepared either by proteolytic digestion or recombinantly) is used.

[0101] In some embodiments, the peptide antigen is a peptide antigen of HIV. Exemplary peptides include one, a combination, or all of a Tat peptide, a Nef peptide, a Rev peptide, a Gag peptide, an Env peptide, a Pol peptide, a Vpu peptide, a Vif peptide, and Padre. Examples of Gag peptides include Gag150, Gag433, Gag298, or a combination thereof. Examples of Env peptides include Env67. Examples of Pol peptides include Pol606. Examples of Vpu peptides include Vpu66. Examples of Vif peptides include Vif23 and Vif101.

[0102] In some embodiments, the peptide antigen is a peptide antigen of Hepatitis C Virus (HCV).

[0103] In some embodiments, the antigen is an antigen associated with a chronic viral infection. In one embodiment, the chronic viral infection is HIV, herpes simplex virus 1 (HSV1), herpes simplex virus 2 (HSV2), hepatitis C virus (HCV), or human papilloma virus (HPV).

[0104] In some embodiments, dendritic cells in an AICC are transfected with mRNA for one or more antigen from an infectious organism through electroporation, for example, using exponential decay wave or square-wave electroporators or other RNA pulsing apparatuses. In another embodiment, one or more antigen or antigen peptide is introduce into antigen presenting cells, such as dendritic cells in an AICC, using microparticle-based transfection, for example, as described in U.S. Patent No. 8,097,243. In still other embodiments, one or more antigen or antigen peptide is introduced

using adenovirus-based transduction, for example, as described in U.S. Patent No. 8,012,468 and in Butterfield et al., J. Immunotherapy 2008; 31:294-309; or using a retroviral vector as described in U.S. Patent No. 8,003,773.

[0105] In any of the embodiments, the method may result in one or more of differentiation of monocytes into maturate DC's, activation of lymphocytes, activation and/or proliferation of NK cells, or activation and/or proliferation of NKT cells.

[0106] In one embodiment, an AICC comprises "mature" DCs if the AICC includes cells that can stimulate activation (priming) of naïve T cells (as shown by expression of one or more of the T cell activation markers CD69, IL-2R, CD28, CD71, CD49d, CD40L, and/or by production of IL-2, IFN-alpha, IFN-beta, or IFN-gamma) and differentiation and proliferation of T helper and cytotoxic T cells in the presence of antigen. In another embodiment, the AICC comprises mature DC if there is an increase in production of IL-12. In another embodiment, the AICC comprises mature DC if there is an increase in the expression of one or more of the markers CD80, CD86, CD83, CD40, CD1c, CD56, CD11b, CD11c, IGSF4, CLEC9A, CCR7, TLR1, TLR3, TLR6, DC-LAMP, Id2, IRF8, or ICSBP on monocytes in the AICC. In one embodiment, an increase in expression is an increase in the number of one or more of molecules on the surface of a DC in the AICC (e.g., an increase in the mean fluorescence intensity (MFI) as determined by flow cytometry). An increase in the number of molecules is determined by comparing the MFI for that particular marker on a DC in the AICC that is suspected of being mature. In one embodiment, an AICC comprises "mature" DCs if there is an increase in the percentage of monocytes expressing one or more of the markers. In one embodiment, a monocyte is identified based on characteristic side scatter [SSC] and positive staining for the pan-leukocyte marker CD45 by flow cytometry, or by monocyte-specific CD14 staining. In one embodiment, the method results in an AICC in which both the MFI and the percentage of monocytes expressing at least one of the cell surface markers HLA-DR, CD54, CD86, CD83, CD80, CD40, and CCR7 increases. In one embodiment,

the method results in an AICC in which both the MFI and the percentage of monocytes expressing any combination or all of the cell surface markers HLA-DR, CD54, CD86, CD83, CD80, CD40, and CCR7 increases. In one embodiment, an increase is assessed relative to the starting leukocyte composition used to produce the AICC (*e.g.*, leukocytes in a buffy coat). In one embodiment, an increase is assessed relative to the cell composition used to being the incubation under conditions of time and temperature to induce differentiation and maturation of DC.

[0107] In one embodiment, an AICC will present antigens to naive T cells, causing the naïve T cells to differentiate into CD4 positive and CD8 positive cells, proliferate, produce IL-2, express IL-2 receptor, and produce interferons and other Th1 cytokines.

[0108] In another aspect, the methods may further comprise enriching an AICC of the invention for one or more cell populations. Compositions enriched for dendritic cells, T cells, NK cells, NKT cells, or other cell types can be prepared by cell sorting, panning, MACS, etc., using either positive or negative marker selection according to known methods.

[0109] In an additional embodiments, the methods may further comprise separating the cellular portion of the AICC from the liquid portion. In one embodiment, both the cellular and liquid (supernatant) portions are recovered. This may be accomplished, for example, by centrifuging the AICC and transferring the supernatant to a separate vessel. As described in the Examples, the supernatant is useful for therapy even in the absence of cells because of the cytokines and other soluble factors it contains. The cells in the pellet that forms following centrifugation may then be resuspended in any desired carrier. In other embodiments, the cells are removed from the liquid portion without recover of the cells, for example, by filtration. In still other embodiments, the cells are recovered without recovery of the supernatant, for example, by pelleting the cells and aspirating the supernatant.

[0110] In any of the embodiments, once an AICC is produced, the cells in the AICC may be isolated, either with or without additional concentration, and suspended in a carrier such as serum (which may be autologous or

allogeneic with respect to recipient) or some other physiologically acceptable isotonically normal liquid suitable for storing and administering cells. Examples of such solutions are described below, and include solutions used to restore isotonicity, cell culture medium, buffered saline, or any other biocompatible fluid or specially formulated clinically acceptable cell storage or cell cryopreservation medium.

II. Activated Immunostimulatory Cell Compositions

[0111] In another aspect, the invention relates to an Activated Immunostimulatory Cell Composition (AICC), which refers to any of the compositions produced by the methods of making an AICC described above. Accordingly, while "AICC" often refers to a cellular composition in the same carrier used in the incubation, as described above, an AICC also encompasses the cellular component in any carrier or excipient, as well as the liquid portion separated from the cellular portion.

[0112] Leukocytes in an AICC have certain characteristics that may be used to distinguish the individual leukocyte cell types or the composition as a whole. For example, monocytes in an AICC may express higher levels of CD54, HLA-DR and/or CD86 compared to freshly isolated monocytes. Further, monocytes in the AICC may express additional activation markers, such as one or more of CD8-alpha, CD83, CD80, CCR7, and/or CD40 compared to freshly isolated monocytes. An AICC composition may comprise a higher percentage or number of monocytes that have differentiated and matured into DCs than in a freshly prepared sample of, for example, peripheral blood leukocytes. Likewise, an AICC may contain a greater number or percentage of cells that are capable of activating/priming naïve lymphocytes than in a freshly prepared sample of, for example, peripheral blood leukocytes. Lymphocytes in the AICC may express higher surface levels of additional markers compared to freshly isolated lymphocytes, such as one or more of CD69, CD25, CD28, CD154, CD107a, and/or CD42d. In addition, leukocytes in the AICC may exhibit an increased ability to produce

cytokines, such as one or more of IL-2, IFN gamma, IFN alpha, IFN beta, TNF alpha, TNF beta, and/or IL-12, compared to freshly isolated leukocytes.

[0113] As noted, in some embodiments of the method, an Activated Leukocyte Composition (ALC) produced using a method of WO 2010/100570 is used in the method of preparing the Activated Immunostimulatory Cell Composition (AICC). Although leukocytes in the ALC may be at least partially activated, as described in the Examples the leukocytes in the AICC may achieve higher levels of activation than in the ALC. The higher level of activation of leukocytes in an AICC compared to leukocytes in an ALC may be shown by any one or more of the characteristics noted above for an AICC using the ALC as the comparator.

[0114] As shown in the working examples, an AICC may also be characterized and distinguished from known compositions in terms of minimum activation level of DCs, *e.g.*, as indicated by surface expression of HLA-DR, CD86, CD83, CD80, CD40, CD54, and CCR7 on monocytes; minimum activation level of lymphocytes, *e.g.*, as indicated by surface expression of CD69, CD25 (IL-2R), CD28, CD154/CD40L, and CD49d; and minimum activation levels of NK and NKT cells, *e.g.*, as indicated by surface expression of CD56, CD57, and CD107a. Surface expression of markers may be evaluated either as the percentage of cells expressing the marker or as the level of marker per cell.

[0115] In some embodiments, the final content of Activated Immunostimulatory Cell Composition (AICC) includes, in terms of the populations of leukocytes present, granulocytes, monocytes and lymphocytes. Specific amounts and relative percentages of the cells may differ based on the analysis techniques employed and on sample-to-sample variation. For example, when analysis is performed using a Cell Dyn Analyzer, the AICC generally contains about 45% to about 72% granulocytes (including neutrophils, eosinophils and basophils), about 3% to about 10% monocytes, and about 25% to about 50% lymphocytes. When analysis is performed using FACS (*e.g.*, using a side-scatter versus a forward-scatter dot plot analysis or versus CD45 and/or CD14 fluorescence), the AICC generally contains about

50% to about 70% granulocytes; about 5% to about 15% monocytes, and about 15% to about 35% lymphocytes.

[0116] Granulocytes include neutrophils, eosinophils and basophils. In some embodiments, an AICC contains about 25% to about 85% neutrophils, about 0 to about 9% eosinophils; about 1.5 to about 4% basophils, about 2% to about 40% monocytes (including dendritic cells), and about 4% to about 70% lymphocytes, based on the total number of leukocytes in the AICC.

[0117] In any of the embodiments, an AICC may further contain residual levels of red blood cells, generally in the amount of about 0.05 to about 0.2 million per microliter, and/or residual levels of platelets, generally in the amount about 1 to about 100 thousand per microliter.

[0118] In some embodiments, the subpopulations of lymphocytes in the AICC are in the general ranges as follows: about 20% to about 80% T cells (CD3+); about 5% to about 40% B cells (CD19+); about 5% to about 35% NK cells (CD3-/CD56+), and/or about 0.1% to about 35% of NKT cells (CD3+/CD56+). In some embodiments, among T cells there are about 5% to about 65% T helper cells (CD4+/CD3+) and about 5% to about 75% cytotoxic T lymphocytes (CTLs, CD8+/CD3+).

[0119] In other embodiments, there about 40% to about 60% T cells (CD3+); about 15% to about 30% B cells (CD19+); about 15% to about 30% NK cells (CD3-/CD56+), about 2% to about 20% of NKT cells (CD3+/CD56+). In some embodiments, among T cells there are about 15% to about 40% of T helper cells (CD4+/CD3+) and about 25% to about 50% of CTL (CD8+/CD3+).

[0120] The ratio between Th cells and CTLs is usually about 0.5 to 1.5.

[0121] In any of the embodiments, the levels of DC, lymphocyte, NK, and NKT cell markers, as well as percentages of cells expression those markers, may be determined as described in the methods of preparing an AICC, or as described in the Examples.

[0122] In one embodiment, an AICC comprises DCs, wherein at least about 5%, 10%, 15%, 20%, 25%, or 30% of the DC express CD8, as detected by flow cytometry compared to an isotype control.

[0123] In one embodiment, at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, or 60% of the monocytes in the AICC are positive for the marker CCR7, as detected by flow cytometry compared to an isotype control.

[0124] In one embodiment, at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, or 60% of the monocytes in the AICC are positive for the marker CD40, as detected by flow cytometry compared to an isotype control.

[0125] In one embodiment, at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, or 60% of the monocytes in the AICC are positive for the marker CD80, as detected by flow cytometry compared to an isotype control.

[0126] In one embodiment, at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, or 60% of the monocytes in the AICC are positive for the marker CD83, as detected by flow cytometry compared to an isotype control.

[0127] In one embodiment, the mean fluorescence intensity (MFI) of total monocytes for the marker CD86 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher fresh than on peripheral blood monocytes. In one embodiment, the mean fluorescence intensity (MFI) of monocytes for the marker CD86 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher than on monocytes in an ALC prepared according to WO 2010/100570. In these embodiments, total monocytes are determined by SSC and staining for a pan-leukocyte marker.

[0128] In one embodiment, the mean fluorescence intensity (MFI) of total monocytes for the marker CD83 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher fresh than on peripheral blood monocytes. In one embodiment, the mean fluorescence intensity (MFI) of monocytes for the marker CD83 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher than on monocytes in an ALC prepared

according to WO 2010/100570. In these embodiments, total monocytes are determined by SSC and staining for a pan-leukocyte marker.

[0129] In one embodiment, the mean fluorescence intensity (MFI) of total monocytes for the marker CD80 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher fresh than on peripheral blood monocytes. In one embodiment, the mean fluorescence intensity (MFI) of monocytes for the marker CD80 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher than on monocytes in an ALC prepared according to WO 2010/100570. In these embodiments, total monocytes are determined by SSC and staining for a pan-leukocyte marker.

[0130] In one embodiment, the mean fluorescence intensity (MFI) of total monocytes for the marker CD40 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher fresh than on peripheral blood monocytes. In one embodiment, the mean fluorescence intensity (MFI) of monocytes for the marker CD40 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher than on monocytes in an ALC prepared according to WO 2010/100570. In these embodiments, total monocytes are determined by SSC and staining for a pan-leukocyte marker.

[0131] In one embodiment, the mean fluorescence intensity (MFI) of total monocytes for the marker CCR7 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher fresh than on peripheral blood monocytes. In one embodiment, the mean fluorescence intensity (MFI) of monocytes for the marker CCR7 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher than on monocytes in an ALC prepared according to WO 2010/100570. In these embodiments, total monocytes are determined by SSC and staining for a pan-leukocyte marker.

[0132] In one embodiment, the mean fluorescence intensity (MFI) of total monocytes for the marker CD54 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher

fresh than on peripheral blood monocytes. In one embodiment, the mean fluorescence intensity (MFI) of monocytes for the marker CD54 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher than on monocytes in an ALC prepared according to WO 2010/100570. In these embodiments, total monocytes are determined by SSC and staining for a pan-leukocyte marker.

[0133] In one embodiment, the mean fluorescence intensity (MFI) of total monocytes for the marker CD8 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher fresh than on peripheral blood monocytes. In one embodiment, the mean fluorescence intensity (MFI) of monocytes for the marker CD8 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 fold higher than on monocytes in an ALC prepared according to WO 2010/100570. In these embodiments, total monocytes are determined by SSC and staining for a pan-leukocyte marker.

[0134] In one embodiment, at least about 5%, 10%, 15%, 20%, 25%, 30%, or 35% of the CD3 positive lymphocytes in an AICC are positive for the marker CD69, as detected by flow cytometry compared to an isotype control, when the AICC is prepared in the presence of a superantigen. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0135] In one embodiment, the mean fluorescence intensity (MFI) of CD3 positive lymphocytes for the marker CD69 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 fold higher when the AICC is prepared in the presence of a superantigen that mediates T cell-APC interaction. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0136] In one embodiment, at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, or 45% of the CD3 negative lymphocytes in an AICC are positive for the marker CD69, as detected by flow cytometry compared to an isotype control, when the AICC is prepared in the presence of a superantigen.

In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0137] In one embodiment, the mean fluorescence intensity (MFI) of CD3 negative lymphocytes for the marker CD69 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 fold higher when the AICC is prepared in the presence of a superantigen that mediates T cell-APC interaction. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0138] In one embodiment, at least about 5%, 10%, 15%, 20%, 25%, 30%, or 35% of the CD3 positive lymphocytes in an AICC are positive for the marker CD25, as detected by flow cytometry compared to an isotype control, when the AICC is prepared in the presence of a superantigen. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0139] In one embodiment, the mean fluorescence intensity (MFI) of CD3 positive lymphocytes for the marker CD25 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or 6.0 fold higher when the AICC is prepared in the presence of a superantigen that mediates T cell-APC interaction than in the preincubation composition or in AICC incubated without superantigen. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0140] In one embodiment, at least about 5%, 10%, 15%, 20%, 25%, 30%, or 35% of the CD3 negative lymphocytes are positive for the marker CD25, as detected by flow cytometry compared to an isotype control, when the AICC is prepared in the presence of a superantigen. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0141] In one embodiment, the mean fluorescence intensity (MFI) of CD3 negative lymphocytes for the marker CD25 is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 fold higher when the AICC is prepared in the presence of a superantigen than in the preincubation composition or when it is prepared in the absence of a superantigen. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0142] In one embodiment, at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, or 80% of the monocytes in an AICC incubated with a superantigen are positive for the marker CD40, as detected by flow cytometry compared to an isotype control, when the AICC is prepared in the presence of a superantigen. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0143] In one embodiment, the mean fluorescence intensity (MFI) of monocytes for the marker CD40 is at least about 1.0, 2.0, 3.0, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, or 10 fold higher than in the preincubation composition or when the AICC is prepared in the absence of a superantigen. In one embodiment, the T cell-APC superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0144] In one embodiment, the concentration of IL-12 in an AICC is at least about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 picograms per milliliter, as determined by ELISA. In one embodiment, the concentration of IL-12 in an AICC incubated with a superantigen is at least about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 fold higher when the AICC is prepared in the presence of a superantigen that mediates T cell-APC interaction than when it is prepared in the absence of a superantigen. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0145] In one embodiment, the concentration of IL-2 in an AICC is at least about 100, 500, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900, 4000 or more picograms per milliliter, as determined by ELISA. In one embodiment, the concentration of IL-2 is determined in an AICC incubated with a superantigen that mediates T cell-APC interaction. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL. In one embodiment, the superantigen is present during a 48 hour incubation at 37°C used to produce an AICC.

[0146] In one embodiment, the concentration of IFN-gamma (IFN-g) in an AICC is at least about 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, or 300 picograms per milliliter, as determined by ELISA. In one embodiment, the concentration of IFN-g in an AICC incubated with a superantigen is at least about 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0 fold higher when the AICC is prepared in the presence of a superantigen that mediates T cell-APC interaction than when it is prepared in the absence of a superantigen. In one embodiment, the superantigen is Staphylococcal Enterotoxin B (SEB) at 100 ng/mL.

[0147] In one embodiment, an AICC supports antigen presentation to naïve CD4 T cells, as shown by proliferation of the T cells when co-cultured with an AICC in the presence of antigen. In one embodiment, an AICC supports generation of T cells with a Th1 phenotype, as shown by the secretion of IL-2 and IFN-gamma by the T cells following co-culture with AICC.

[0148] In some embodiments, an AICC comprises T cell subsets in a ratio that is altered compared to the ratio of those same T cell subsets in peripheral blood. In one embodiment, an AICC comprises a ratio of CD4 T cells to CD8 T cells (CD4/CD8 ratio) that is less than about 1:1.

[0149] In one embodiment, an AICC has the characteristics of an AICC as described in the Examples with respect to one or more cell surface markers and/or cytokines. In one embodiment, an AICC has one or more of the characteristics of an "average" AICC presented in the Tables (that is, the characteristic reflects the mean +/- any standard deviation given in the Tables). In one embodiment, an AICC has one or more characteristics of a representative AICC as shown in the Figures, although any numbers in the Figures should be construed as "about" that number. In one embodiment, an AICC has a characteristic as described in the Examples following stimulation of the AICC with a superantigen.

[0150] In some embodiments, an AICC of the invention may be enriched for one or more cell populations. In one embodiment, an AICC is enriched for dendritic cells by negative selection of lymphocytes, for example,

using anti-CD3 and anti-CD19 antibodies. In one embodiment, an AICC is enriched for lymphocytes by negative selection of monocytes and dendritic cells, for example using cell adherence or using anti-HLA-DR or anti-CD40, or anti-CD14 antibodies, or combinations thereof. In one embodiment, an AICC is enriched for T cells by positive selection, for example, using anti-CD3 antibody on coated beads in MACS or anti-CD2 antibody in FACS.

[0151] Any of the AICC may be used therapeutically in the inventive methods. But as described in detail elsewhere, in some embodiments an AICC is incubated with antigens isolated for an infectious organism or produced by recombinant methods or any other means of deriving or isolating antigens or antigenic peptides from an infectious organism, for example by eluting peptides from infected cells. In some embodiments, the AICC, or the DCs within the AICC, is incubated with one or more viral, bacterial, fungal, or protozoal antigens. In some embodiments, an AICC or DC within the AICC, is incubated with superantigens such as bacterial products. The addition of antigen/peptides will result in further maturation of DC from monocytes and more specific activation/priming of T cells present in the AICC.

[0152] An AICC may be separated into cellular and liquid components. Accordingly, in one embodiment, an AICC comprises the cellular and liquid portions resulting directly from any of the methods. In one embodiment, an AICC comprises the cellular portion separated from the liquid portion, although the cellular component may be (re)-formulated in one or more carriers or excipients. In one embodiment, an AICC comprises the liquid portion separated from the cellular portion. It is believed that both the cellular components and the cell-free liquid portion possess therapeutically beneficial properties. For example, the cellular component comprises matured DC and other cell types and the (cell-free) liquid portion/component comprises various cytokines, such as IL-2 and IL-12.

[0153] In some embodiments adjuvants are added to an AICC. In one embodiment, the vaccine further comprises at least one antigen/peptide from one or more infectious organisms. Examples of adjuvants include but are not limited to aluminium hydroxide, aluminium phosphate, calcium phosphate,

Freund's complete adjuvant, Freund's incomplete adjuvant, Arlacel A, Mineral oil, emulsified peanut oil adjuvant (adjuvant 65), lipopolysaccharide (LPS), liposomes, endotoxins, cholesterol, fatty acids, aliphatic amines, paraffinic and vegetable oils, monophosphoryl lipid A, ISCOMs with Quil-A, and Syntex adjuvant formulations (SAFs).

[0154] AICC compositions may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. It may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, reflecting approval by the agency of the form of the composition or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

[0155] In an additional aspect, the invention provides an AICC formulated as a vaccine for treating an infection. In one embodiment the vaccine further comprises at least one adjuvant as described above. In one embodiment, the vaccine comprises an AICC formulated with at least one of the antigens described above. In one embodiment, the vaccine comprises an AICC that comprises matured dendritic cells, activated lymphocytes, at least 5 pg/mL IL-12, at least 1500 pg/mL IL-2, and at least 100 pg/mL IFN-gamma.

[0156] In another aspect, the invention provides an AICC for stimulating an immune response. The embodiments of this aspect include those described with respect to an AICC per se and those described with respect to the therapeutic uses of an AICC. For example, this aspect also relates to an AICC for treating an infection or for stimulating an immune response to an infectious organism or a cell infected with an infectious organism.

[0157] In an additional aspect, the invention provides for the use of any AICC in the preparation of a medicament for stimulating an immune response. The embodiments of this aspect include those described with respect to an

AICC per se and those described with respect to the therapeutic uses of an AICC. For example, this aspect also relates to the use of an AICC for preparing a medicament for treating an infection or for stimulating an immune response to an infectious organism or a cell infected with an infectious organism.

III. Therapeutic Uses

[0158] In another aspect, the invention provides methods in which an AICC is used as an immunostimulatory composition. According, the invention encompasses methods of stimulating an immune response to at least one antigen from an infectious organism, comprising administering an AICC of the invention to a subject. Subject includes both human and veterinary subjects.

[0159] An AICC may be administered to treat any type of infection. Examples of intact antigens include proteins from viruses, bacteria, fungi, and protozoa that infect humans, as well as prion protein antigens.

[0160] Any infectious virus may serve as a source of viral antigens. Exemplary viruses that may be used to provide viral antigens include, but are not limited to, human immunodeficiency virus (HIV-1, HIV-2); human papilloma virus (HPV); influenza virus; Dengue Fever; Japanese Encephalitis; yellow fever; hepatitis viruses (Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), non-A, non-B hepatitis); RSV (respiratory syncytial virus); HPIV 1; HPIV 3; adenovirus; and rhinovirus; EBV; CMV (cytomegalovirus); Herpes simplex viruses HSV-1 and HSV-2; BK; HHV6; Parainfluenza; Bocavirus; Coronavirus; LCMV; Mumps; Measles; Metapneumovirus; Parvovirus B; Rotavirus; West Nile Virus; and Ebola hemorrhagic fever virus.

[0161] Any species of bacteria may serve as a source of bacterial antigens. Exemplary bacterial antigens include, but are not limited to, bacterial antigens such as pertussis toxin, filamentous hemagglutinin, pertactin, FIM2, FIM3, adenylate cyclase, diptheria toxin, tetanus toxin, streptococcal M protein, lipopolysaccharides, mycolic acid, heat shock protein 65 (HSP65), the 30 kDa major secreted protein, antigen 85A 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 and other anthrax bacterial antigen components; rickettsiae bacterial antigens such as rompA and other rickettsiae bacterial antigen component. Also included with the bacterial antigens are any other bacterial, mycobacterial, mycoplasmal, rickettsial, or chlamydial antigens.

[0162] Any species of fungus may serve as a source of fungal antigens. Exemplary fungal antigens include, but are not limited to, e.g., candida fungal antigen components; histoplasma fungal antigens such as heat shock protein 60 (HSP60) and other histoplasma fungal antigen components; cryptococcal fungal antigens such as capsular polysaccharides and other cryptococcal fungal antigen components; coccidiodes fungal antigens such as spherule antigens and other coccidiodes fungal antigen components; and tinea fungal antigens such as trichophytin and other coccidiodes fungal antigen components.

[0163] In other embodiments, a peptide epitope of the antigen (prepared either by proteolytic digestion or recombinantly) is used.

[0164] In some embodiments, the peptide antigen is a peptide antigen of HIV. Exemplary peptides include one, a combination, or all of a Tat peptide, a Nef peptide, a Rev peptide, a Gag peptide, an Env peptide, a Pol peptide, a Vpu peptide, a Vif peptide, and Padre. Examples of Gag peptides include Gag150, Gag433, Gag298, or a combination thereof. Examples of Env peptides include Env67. Examples of Pol peptides include Pol606. Examples of Vpu peptides include Vpu66. Examples of Vif peptides include Vif23 and Vif101.

[0165] In some embodiments, the peptide antigen is a peptide antigen of Hepatitis C Virus (HCV).

[0166] In some embodiments, the antigen is an antigen associated with a chronic viral infection. In one embodiment, the chronic viral infection is

human immunodeficiency virus (HIV), herpes simplex virus 1 (HSV1), herpes simplex virus 2 (HSV2), hepatitis C virus (HCV), or human papilloma virus (HPV).

[0167] In some embodiments, an AICC is used directly in a method of stimulating an immune response to an infectious organism without further manipulation. In other embodiments, prior to administration an AICC is incubated (pulsed) with one or more antigens from the infectious organism. The antigens may be recombinantly prepared, partially purified from the infectious organism, prepared from cells infected with the organism, or a combination of techniques may be used to provide one or more antigens. In still other embodiments, an AICC is incubated with one or more "superantigen," such as bacterial products, prior to administration. Contacting an AICC with an antigen or superantigen prior to administration will result in further and more specific activation/priming of T cells present in the AICC.

[0168] Additional examples and details regarding addition of antigen and antigen peptides are presented in the description of methods of preparing an AICC.

[0169] In some embodiments adjuvants are added to the AICC prior to administration. Examples of adjuvants include but are not limited to aluminium hydroxide, aluminium phosphate, calcium phosphate, Freund's complete adjuvant, Freund's incomplete adjuvant, Arlacel A, Mineral oil, emulsified peanut oil adjuvant (adjuvant 65), lipopolysaccharide (LPS), liposomes, endotoxins, cholesterol, fatty acids, aliphatic amines, paraffinic and vegetable oils, monophosphoryl lipid A, ISCOMs with Quil-A, and Syntex adjuvant formulations (SAFs).

[0170] In general, application of the activated immunostimulatory cells composition is accomplished by one or more administrations of an AICC. In one embodiment, an AICC is administered systemically. Examples of systemic administration include intravenous, intramuscular, intraperitoneal, subcutaneous, and intradermal injection. In one embodiment, an AICC is administered locally, for example, topically, intradermally, or subcutaneously around a site of infection. In one embodiment, an AICC is administered

intranodally; that is, an AICC is injected into one or more lymph nodes associated with (for example, draining) a site of infection.

[0171] In some embodiment, an AICC is administered at a single site. In other embodiments, an AICC is administered at multiple sites.

[0172] In some embodiments, an AICC is administered by injection using a suitable syringe and needle (e.g., a 2 ml syringe fitted with an 18G or 25G needle). In those embodiments in which an AICC is injected directly into regional draining lymph nodes, administration of the AICC may be through a catheter or endoscopic device under surveillance of ultrasound, X-ray and other similar technologies. In some embodiments, injection occurs about every one centimeter to about every three centimeters throughout a site of local infection. In another embodiment, the injection is into tissue associated with lymph nodes draining a site of infection. In one embodiment, injection occurs about every one centimeter to about every three centimeters in the area of healthy tissues associated with lymph nodes draining a site of infection.

[0173] For injection, an AICC may be used directly. In this embodiment the incubation composition, which may contain cytokines that may help stimulate direct organism killing or killing of cells infected with the infectious organism, is administered along with the cellular portion of the AICC. In alternate embodiments, the liquid portion of the AICC may be removed, for example by centrifugation, and the cellular portion of an AICC then formulated in an aqueous solution (optionally more concentrated than the AICC), for example, in physiologically compatible buffers such as Hank's solution, Ringer's solution, or other physiological salt buffer, or in serum or plasma, including serum or plasma from the patient.

[0174] In another embodiment, an AICC is absorbed onto a physiologically inert and/or resorbable matrix or scaffold (e.g., collagen) and inserted by means of a press fit, into the lesion. This allows for a sustained delivery of the AICC into the site which benefits the patient in that the cells have a longer period in situ.

[0175] Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks, or until diminution of the disease state is achieved. Accordingly, in one embodiment an AICC is administered only once. In another embodiment, an AICC is administered at least two, three, four, five, or up to ten times or more. When the administration comprises at least two administrations, each administration may be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 days apart. Alternatively, each administration may be about one, two, three, four, five, or six months apart.

[0176] An AICC may be administered more than once if a clinician determines another application is necessary.

[0177] In the case of multiple administrations, the individual administrations may all be via the same route, or different routes of administration may be utilized for different administrations during the course of therapy.

[0178] The amount of an AICC to be administered will, of course, be dependent on the individual being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc. The dosage and timing of administration will be responsive to a careful and continuous monitoring of the individual's changing condition. Further, treatment algorithms should not be limited by the severity or type of infection, since an AICC may be more efficacious in patients presenting with disseminated or chronic infections.

[0179] In one embodiment, an AICC is used as a single treatment modality, where it may be administered once or more than once. In other embodiments, however, the AICC is administered as part of a combined treatment approach. In those embodiments involving combination therapy, an AICC is administered before, after, or in combination with other treatment modalities. An AICC may be used alone or in conjunction with any other conventional treatment for the specific type of infection.

[0180] Stimulation of an immune response against an infectious organism by an AICC can be demonstrated in various ways. For example, in one embodiment, administration of an AICC to a subject causes a reduction in fever or other systemic symptoms of infection. In still other embodiments, an AICC stimulates an immune response to an antigen of the infectious organism.

[0181] In any of the embodiments, responsiveness to the therapy can be measured by decreased serum concentrations of markers of infection, such as antibodies to the infectious organism. In certain embodiments, responsiveness to the therapy is measured by one or more of an increased overall survival time.

[0182] Irrespective of the nature of the treatment, an AICC may be useful in improving disease outcomes in patients. In addition, an AICC may also provide an analgesic effect.

EXAMPLES

[0183] Reference is now made to the following examples, which together with the above descriptions illustrate the invention in a non-limiting fashion.

[0184] Unless otherwise noted, the nomenclature used and the laboratory procedures utilized include standard techniques. See, for example, "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); "Animal Cell Culture" Freshney, R. L, ed. (1986); "Methods in Enzymology" Vol. 1-317, Academic Press; and Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996).

EXAMPLE 1: Preparation of Exemplary Activated Immunostimulatory Cell Compositions (AICC)

[0185] Exemplary AICC were prepared as follows.

[0186] As an initial step, an activated leukocyte composition was prepared in accordance with the methods described in WO2010/100570. Briefly, buffy coat derived from a single unit of blood was incubated for 8-12 hours at room temperature. The cells were then subjected to hypo-osmotic shock ("HOS") by addition of water for 40-45 sec, and isotonicity restored by adding a NaCl solution. The cells were pelleted by centrifugation and the cell pellet was resuspended in 50 mL serum obtained from the plasma fraction derived from the same blood unit. The leukocyte suspension in serum was then incubated for 90 min at 37°C. The serum was then discarded and fresh serum added to the leukocytes to make a final concentration of 3-4 million/mL. This initial composition is referred to in the examples as the "Preincubation Composition" (PC).

[0187] Although the examples utilize a PC that was incubated in serum for 90 min at 37°C, it is expressly contemplated that leukocytes pelleted after hypo-osmotic shock may also be resuspended in, for example, serum, and directly incubated for a period of time, *e.g.*, 48 hours, to form the AICC.

[0188] In the current examples, the PC was concentrated by gentle centrifugation, removal of excess serum, and gentle resuspension of the leukocyte pellet in a volume of serum so that the leukocyte concentration was approximately 10 million/mL. The composition was then incubated for 48 or 72 hours (as indicated in the particular example) in a cell incubator at 37°C, 5% CO_2 and 100% humidity. The incubation was performed in gas-permeable FEP bags.

[0189] The current examples present results for AICC prepared using a concentrated PC so that the resulting AICC is also concentrated. A concentrated AICC may be better suited for clinical applications since the more concentrated the cells the smaller the injection volume needed to administer a sufficient amount of cells. But a comparison using non-concentrated PC showed that the leukocyte concentration did not affect the leukocyte composition in the AICC, either as assessed by percentage of cell types or by expression levels of specific markers. Accordingly, it is also

possible to use a non-concentrated PC and the examples should in no way be read as limited to an AICC prepared using a concentrated PC.

[0190] In the examples that follow, leukocytes in the Preincubated Composition (PC) were compared to leukocytes in the AICC prepared from the concentrated PC, unless otherwise clearly specified in the example.

EXAMPLE 2: Cell Population Analysis of an Activated Immunostimulatory Cell Composition (AICC)

[0191] The cell composition of the AICC was compared to that of the PC using two different cell counting methods: differential cell count on a Cell-Dyn Ruby Hematology Analyzer (Abbott Diagnostics) and flow cytometry analysis on a FACSCalibur™ (BD Biosciences). The Cell Dyn counts compare cell populations present in the PC ("before incubation") and in an AICC after incubation for 48 hours at 37°C. In the table, WBC denotes white blood cells, or leukocytes. Both the total WBC count and the percentage of leukocyte types in the WBC were determined. The numbers of red blood cells (RBC) and platelets in the sample were also determined. The results of the Cell Dyn counts are summarized in **Table 1.**

Table 1. Cell Dyn Hematology Analysis

Sample	WBC	% WBC					RBC	platelets
	$x10^6$/mL	granulocytes	lymphocytes	monocytes	eosinophils	basophils	$x10^6$/mL	$x10^3$/mL
before incubation	10.4±1.7	62.2±8.4	22.3±5.4	11.4±4.2	2.3±1.2	1.8±1.3	0.1±0.1	118±232
48h incubation	5.1±1.1	54.2±9.7	36.3±10.7	5.2±1.9	1.7±2.0	2.7±0.2	0.1±0.02	44±32

[0192] The data presented are mean±SD of 5 experiments each performed with a blood sample from a different blood donation. Each Cell Dyn Hematology Analyzer measurement was performed in triplicate and the average count was calculated.

[0193] The percentages of leukocyte subtypes present in the PC ("before incubation") and in an AICC after incubation for 48 hours at 37°C

were also determined by flow cytometry. Sampled cells were stained with pan-leukocyte antigen CD45 conjugated to peridinin chlorophyll protein (PerCP). CD45 staining permitted better resolution of leukocyte populations. Flow cytometry was performed using FACSCalibur™ (BD Biosciences) and population and marker analysis was done using FACSDiva™ software (BD Biosciences). Leukocyte populations were gated based on SSC and FL3 (CD45-PerCP) signals.

[0194] The percentage of granulocytes, lymphocytes, and monocytes in the leukocyte population was then determined. The results of the flow cytometry analysis of leukocyte populations are presented in **Table 2** as mean+SD of 5 experiments.

Table 2. Flow Cytometry Analysis of Leukocytes

sample	% WBC		
	Granulocytes	**Lymphocytes**	**Monocytes**
before incubation	62.2±12.9	21.5±5.5	9.7±4.3
48h incubation	59.0±11.0	25.7±6.8	10.6±4.8

[0195] The two counting methods produce slightly different results. Nevertheless, each method provides an acceptable measure of leukocyte subsets. The Cell-Dyn system is often used in clinical applications. But flow cytometry, as described below, can be advantageously used to determine the expression levels of individual molecules on the surface of an individual cell. In general, the AICC differed little from the PC in terms of percentages of the leukocyte subsets. The Cell-Dyn counts, however, indicate that the total number of leukocytes decreases during incubation to prepare the AICC.

[0196] The lymphocyte subset gated as described above based on SSC and CD45-PerCP fluorescence was further analyzed by flow cytometry. To separate T and B lymphocytes, samples were stained with an anti-CD3 antibody conjugated to fluorescein isothiocyanate (FITC) and with an anti-CD19 antibody conjugated to allophycocyanin (APC). Lymphocytes that were

$CD3^+/CD19^-$ were counted as T cells, while cells that were $CD3^-/CD19^+$ were counted as B cells. Samples were also triple-stained with anti-CD4-APC, anti-CD8-phycoerythrin (PE), and anti-CD3-FITC antibodies. $CD3^+$ lymphocytes were then separated into $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells (CTLs). NK and NKT cells were identified by double staining with anti-CD3-FITC and a mixture of anti-CD56 and anti-CD16 antibodies conjugated to PE ((CD56+CD16)-PE antibodies). NK cells were identified as $CD3^-/(CD56+CD16)^+$ cells and NKT cells were identified as $CD3^+/(CD56+CD16)^+$ cells. The results of 4-7 experiments performed with AICC produced from different blood donations are summarized in **Table 3** and presented as mean±SD.

Table 3. Lymphocyte Subset Analysis by Flow Cytometry.

conditions/ cell types	% T cells	% Th cells	% CTL	CD4/CD8 ratio	% NKT cells	% NK cells	% B cells
	$CD3^+$	$CD3^+/CD4^+$	$CD3^+/CD8^+$		$CD3^+/(CD56+CD16)^+$	$CD3^-/(CD56+CD16)^+$	$CD3^-/CD19^+$
before incubation	38.8±10.6	27.5±13.0	33.8±15.1	1.1±0.8	9.0±7.5	20.5±5.8	29.2±6.5
48h incubation	48.4±9.1	28.7±10.9	36.5±11.9	0.9±0.5	10.7±7.0	20.7±5.9	22.7±6.8

[0197] These results demonstrate a statistically significant increase from 38.8% to 48.4% (p=0.18) in the percentage of T-cells (CD3 positive) in the AICC compared to the percentage of T cells in the starting composition.

Example 3. Activation and Differentiation of Dendritic Cells (DCs)

Analysis of Dendritic Cell Markers on Monocytes

[0198] Differentiation of monocytes into dendritic cells (DC) was assessed by flow cytometry analysis of expression of the DC-specific markers HLA-DR, CD54, CD86, CD83, CD80, CD40 and CCR7 on monocytes. Monocytes were analyzed first in Preincubated Composition (PC) and then in AICC produced using 48 or 72 hour incubations in gas-permeable FEP bags. Sampled cells were double-stained with antibodies against each of the DC-specific markers and with an antibody to the pan-leukocyte antigen CD45 conjugated to peridinin chlorophyll protein (PerCP). The latter was used for

better resolution of leukocyte populations. Anti-HLA-DR and anti-CCR7 antibodies were conjugated to Allophycocyanin (APC). The rest of the DC-specific antibodies were conjugated to Phycoerythrin (PE).

[0199] Cells from each time point were washed with FACS staining solution (PBS, 2% Normal Mouse Serum; 0.02% Sodium Azide), aliquoted at $0.5x10^6$/tube and incubated with appropriate monoclonal antibodies for 30 min at 4°C in the dark. The second antibody (anti-CD45-PerCP) was added for 15 min at 4°C in the dark. After incubation, the cells were washed, resusupended in PBS, and analyzed on a FACSCalibur™ flow cytometer (BD Biosciences). Cells stained with irrelevant but isotype-matched antibodies under the same conditions were used as negative controls. The results were analyzed by FACSDiva™ software (BD Biosciences). Leukocyte populations were distinguished based on SSC and FL3 (CD45 positive, red fluorescence) signals.

[0200] The results of flow cytometry analysis of DC markers on monocytes are summarized in **Table 4** and a representative experiment is shown in **Figure 1**.

Table 4. Analysis of DC-Specific Markers on Monocytes

samples	HLA-DR	CD54	CD86	CD83	CD80	CD40	CCR7
before	46618 ±11003	6149 ±656	3150 ±721	5080 ±203	442 ±340	295 ±189	208 ±76
after 48h	31930 ±14702	33183 ±10424	17689 ±3492	1541 ±1623	944 ±432	1287 ±1151	741 ±315
fold increase	**0.6±0.3**	**5.6±2.4**	**6.0±1.8**	**2.9±2.5**	**3.9±3.9**	**4.7±3.6**	**3.7±1.8**
after 72h	47751 ±23925	28262 ±22334	14282 ±290	123 6±492	905 ±217	1910 ±646	990 ±57
fold increase	**1.1±0.4**	**5.0±4.4**	**2.4±3.1**	**0.8±1.0**	**1.6±1.8**	**5.4±4.8**	**8.1±6.5**

[0201] There are two parameters that can be used to characterize marker expression: 1) percentage of cells expressing the marker (% positive cells), and 2) the Mean Fluorescence Intensity (MFI) of the marker, which

depends on the number of marker molecules per cell. The data in **Table 4** present the MFI of all monocytes for each marker, presented as mean+SD of 7-8 experiments. Fold increase was calculated for each marker in each experiment, then mean+SD calculated. As shown by the standard deviation, there is variability among experiments. Nevertheless, the MFI is several fold higher in the AICC (whether incubated for 48 hours or 72 hours) compared to the PC for most of the markers related to DC differentiation and maturation, confirming monocyte differentiation into DC during a 48 hour incubation of PC at 37°C.

[0202] **Figure 1** presents the fold increase for AICC prepared using a 48 hour incubation compared to the PC starting composition for a representative experiment. Fold increase is shown for three parameters for each marker: 1) the number of cells expressing a certain marker (% positive cells, first bar), 2) MFI for all monocytes (middle bar) and 3) MFI of "positive" monocytes (third bar). MFI represents the fluorescence distribution in the monocyte population. A measure of MFI for positive monocytes is included so that any increase in MFI in a population of cells that is a small percentage of total monocytes can still be detected. HLA–DR expression was already high in the preincubation composition (PC) used to prepare the AICC. In the PC, 94+3% of monocytes were HLA-DR positive and the MFI was also high. HLA-DR expression did not increase further in the AICC. For markers CD86, CD83, CD40, and CD54, the "% positive cells" remained about the same or even decreased as in the case of CD83 upon incubation (fold increase ~0.2-1); however the MFI of all monocytes increased 2-5 fold mainly because the MFI of the positive cells increased many fold. For markers CD80 and CCR7 both % positive cells and MFI increased several fold.

[0203] These results show that AICC is enriched for mature DC compared to the starting composition.

[0204] The effect of incubating cells under conditions that promote cell adherence was also investigated. AICC was prepared using two different types of bags, one type with a regular surface and the other type with a surface treated to promote cell adhesion. Expression of DC-specific markers

was then compared for AICC prepared using the adherent and non-adherent bags. The results of this experiment are shown in **Figure 2**. Surprisingly, incubation in non-adherent bags resulted in higher MFI for DC markers compared to incubation in adherent bags. The effect was particularly notable for CD83, CD40, and CCR7.

Analysis of CD8-alpha Expression on Monocytes

[0205] While monocytes express low levels of CD8-alpha, a subpopulation of differentiated DCs express high levels of this marker (Merad M, 2000). CD8α(+) dendritic cells (DCs) are important *in vivo* for cross-presentation of antigens derived from intracellular pathogens and tumors because they secrete high levels of IL-12 (Mashayekhi M, 2011) and promote a Th1 phenotype of T helper cells (Maldonado-López R, 1999; Maldonado-López R, 2001).

[0206] CD8 expression on monocytes was measured by flow cytometry. Monocytes were analyzed in the Preincubated Composition (PC) and in AICC produced using 48 hour incubations in gas-permeable FEP bags. Sampled cells were double-stained with antibodies against CD8 conjugated to PE and with an antibody to the pan-leukocyte antigen CD45 conjugated to PerCP. Leukocyte populations were distinguished based on SSC and FL3 (CD45) signals so that CD8 expression in the monocyte population could be analyzed.

[0207] **Table 5** summarizes the results of 3 experiments. **Figure 3** presents a representative experiment.

Table 5. Expression of CD8 on Monocytes

conditions	% $CD8^+$ monocytes	MFI
before incubation	1.0±0.6	65283±9906
48h incubation	14.6±15.2	71244±23900

[0208] AICC prepared by incubation of the preincubation composition (PC) for 48 hours at 37°C had an increased percentage of monocytes expressing CD8 compared to the percentage of $CD8^+$ cells in the PC (**Table 5** and **Figure 3A**). The MFI of CD8 also increased (**Table 5** and **Figure 3B**).

Example 4. Effects of Superantigen Presentation by DC to T cells in AICC.

[0209] To confirm the functionality of DCs produced from monocytes during incubation of PC to produce AICC, Staphylococcal Enterotoxin B, a superantigen (SA), was added to the incubation mixture. Superantigens (SAs) resemble processed antigen peptides as they too engage MHC Class II molecules on antigen presenting cells and T cell receptor on T lymphocytes. SAs are advantageous, however, because unlike other antigens, they don't require intracellular processing. In addition, SAs stimulate about 20% of T cells bearing a certain family of T cell receptors; in contrast, most antigen peptides stimulate only around 0.001% of T cells because they only stimulate antigen-specific T cells. (Bhardwaj N, 1993). Thus, SAs can be used as a substitute for peptide antigen to test the ability of antigen presenting cells, such as DCs, to stimulate T cells.

Analysis of T Cell Activation Markers CD69 and CD25 (IL-2R)

[0210] Activation of lymphocytes in AICC in the presence of SA was assessed by flow cytometry analysis of the expression of a well known lymphocyte activation marker, CD69. CD69 expression increases rapidly on activated T cells, with peak expression occurring 18-48 hours after stimulation. (Simms & Ellis 1996.) Lymphocytes were analyzed first in Preincubated Composition (PC) and then in AICC following 48 or 72 hours incubation in serum in cell incubator (at 37°C, 5% CO2 and 100% humidity) in the absence or presence of different doses of the SA Staphylococcal Enterotoxin B (SEB) (Sigma Aldrich). The PC was produced and concentrated as described above in the Example 1. Phytohemagglutinin (PHA), which is a polyclonal T cell stimulator that does not require interaction with antigen presenting cells such as DC, was used as a control.

[0211] Lymphocytes were first double stained with anti-CD69-FITC antibody and anti-CD3-APC antibody (both from eBioscience), and then with anti-CD45-PerCP antibody. Cells were analyzed by flow cytometry. Mean+SD of 4 experiments is shown in **Table 6**. **Figure 4** presents a representative experiment.

Table 6. CD69 Expression on SA-Stimulated Lymphocytes

conditions	CD3pos CD69pos		CD3 neg CD69pos	
	% pos cells	MFI	% pos cells	MFI
before incubation	11.4±1.7	563±132	32.9±3.4	676±91
48h incubation	1.5±1.2	550±265	7.3±5.2	711±113
48h SA10	8.9±10.8	2157±301	16.0±14.0	1441±932
48h SA100	15.3±13.2	2954±30	26.7±13.9	1883±1056
48h PHA	7.1+1.5	1389+191	10.0+1.6	649+1

[0212] AICC at 48 hours contained a reduced number of CD69-positive T cells ($CD3^+$) and non-T cells ($CD3^-$) in the absence of SA. Addition of SA stimulated both subsets of lymphocytes, causing a dose-dependent increase in the percentage of CD69-positive cells (**Table 6** and **Figure 4A**) and in the Mean Fluorescence Intensity (MFI) of those cells (**Table 6** and **Figure 4B**). An increase in MFI reflects an increase in the number of CD69 molecules on each activated cell. The effect of SA was via a DC-dependent mechanism because phytohemagglutinin (PHA), an activator of lymphocytes that acts independent of antigen presentation by DCs, failed to produce similar effect, suggesting specific effect of SA via DC-dependent mechanism (**Table 6**).

[0213] The effect of SA on expression of IL-2 receptor (CD25) was also studied. Antigen presentation causes release of IL-2 from lymphocytes and upregulation of IL-2 receptor on their surface resulting in amplification of the immune response. The preincubation composition was incubated in serum for 48 hours at 37°C in the presence of 100 ng/mL SA. The resulting AICC was double stained with anti-CD25-PE antibody and anti-CD3-FITC antibody (both from eBioscience), and then with anti-CD45-PerCP antibody. The results of 4 experiments are summarized in **Table 7** and a representative experiment is shown in **Figure 5**.

Table 7. IL-2R Expression Following Superantigen Stimulation

conditions	CD25; all lymphocytes		CD25; T cells		CD25; CD3 neg cells	
	% positive	MFI	% positive	MFI	% positive	MFI
Before incubation	13.4±3.8	123±31	3.3±2.3	80±25	16.4±6.5	158±47
48 hour incubation	13.1±3.9	162±40	2.8±1.4	81±12	12.2±7.3	236±124
48 hour plus SA 100 ng/ml	22.9±11.5	550±319	5.6±4.0	529±359	18.8±14.3	606±334

[0214] Presentation of SA by DCs to lymphocytes increases the percentage of lymphocytes expressing IL-2R (**Table 7**, **Figure 5A**) and the number of IL-2R on each cell (MFI) (**Table 7**, **Figure 5B**). This effect was seen in both T cells and CD3-negative cells.

Analysis of Monocyte Differentiation Marker CD40

[0215] Surprisingly, addition of the superantigen (SA) during the 48 hour incubation used to make AICC not only promoted activation of lymphocytes but also stimulated further maturation of DCs. CD40 is a key co-stimulatory molecule on DCs that interacts with CD40L on T cells and induces production of IL-12 from DCs. Expression of CD40 therefore is an indication that DCs are functionally mature. To assess the maturation state of DC, levels of this marker were measured on monocytes in the preincubation composition and in AICC prepared either in the absence or presence of different amounts of superantigen. **Table 8** and **Figure 6** summarize the results of 3 independent experiments performed with AICC from different blood donations.

Table 8. CD40 Expression on Monocytes

Conditions	CD40 positive monocytes	
	% Positive	MFI
before incubation	24.7±33.0	683±175
48h incubation	14.2±14.6	3055±74
48h SA 10 ng/ml	51.8±22.2	6927±1508
48h SA 100 ng/ml	61.9±35.6	7358±2239
48H PHA 10μg/ml	13.3±12.7	3237±259

[0216] The percentage of CD40 positive cells in the AICC increased with increasing amounts of SA (**Table 8** and **Figure 6A**). Expression levels (MFI) of CD40 were also enhanced by addition of SA in dose-dependent manner (**Table 8** and **Figure 6B**). As shown by the MFI results, although levels of CD40 were high in AICC compared to the preincubation composition, the presence of SA resulted in even greater enhancement of CD40 levels on the cells (**Table 8**, "MFI" and **Figure 6C**). Phytohemagglutinin (PHA), an activator of lymphocytes that acts independently of antigen presentation by DCs, failed to produce this effect.

Analysis of IL-12 Content in AICC

[0217] When fully matured DCs interact with T cells, the DCs produce IL-12. Accordingly, the levels of IL-12 were also determined.

[0218] In the first experiment, the IL-12 concentration was measured in the PC and in AICC prepared using a 48 hour incubation at 37°C in FEP bags. Ten mL of concentrated PC was added to a bag and cells were incubated for 48 hours to prepare AICC. PC and AICC compositions were centrifuged at 3,500xg for 30 min at 15°C and IL-12 concentrations were measured in the supernatants with Diaclone™-ELISA for IL12p70 (active heterodimer) (Gen-Probe, Inc) according to the manufacturer's instructions. Serum without addition of the cells, either obtained on the day of PC production or incubated simultaneously with AICC for 48 hours at 37°C, was centrifuged the same way. The OD values of the serum were used as a

background and subtracted from the OD values of PC and AICC samples, respectively.

[0219] As shown in **Figure 7A**, the IL-12 concentration was increased in the AICC. Accordingly, the results provide additional evidence that monocytes differentiated into DCs that are capable of producing IL-12 in the AICC. Considering that monocytes comprise only about 10% of all the leukocytes in AICC, these results are quite significant.

[0220] In the second experiment (**Figure 7B**), PC was incubated at 37°C for 48 hours in the absence and in the presence of 100 ng/ml SA to produce AICC. Parallel cultures were incubated at room temperature (RT). Again the IL-12 content increased during 48 hour incubation at 37°C. In the presence of SA, however, IL-12 concentration increased more than 2 fold. These results suggest that further maturation of DC occurs in the presence of SA. When incubation was performed at RT, no SA effect was seen, confirming that IL-12 release was a biological process dependent on cell function (**Figure 7B**).

Analysis of IL-2 Production by AICC Lymphocytes

[0221] Naïve T cells activated by DCs via antigen presentationacquire a CD4 postive Th1 phenotype and produce large amounts of the T cell mitogen cytokine IL-2. Accordingly, if the AICC included activated T cells, those T cells should release IL-2 in the presence of antigen since the AICC contains mature DC.

[0222] To detect IL-2 release, IL-2 concentrations were measured in the preincubation composition (PC) and in the AICC at the end of incubation for 48 hours at 37°C in the presence and absence of 100 ng/ml SA. Samples were prepared as described for IL-12. After cell pelleting, IL-2 concentrations were measured in the supernatants using an IL-2 ELISA kit (eBioscience). As described above, pre-incubated and incubated serum samples were used as controls. In addition, the incubated cells (plus/minus SA) were pelleted, washed with culture medium, resuspended in fresh serum with no additives at $5x10^6$/mL, and placed in an incubator for 3 hours. The release of IL-2 into the

fresh serum was then measured. The results of 3 experiments using different batches of PC as the starting composition are summarized in **Table 9**.

Table 9. IL-2 Release Following Superantigen Stimulation

Conditions	**IL-2 concentration [pg/mL]**	**IL-2 release into fresh serum [pg/mL/3 hours]**
before incubation	below sensitivity	below sensitivity
48 hour incubation	below sensitivity	below sensitivity
48 hour incubation with SA	2562±1206	131±50

[0223] The data in **Table 9** show that DC in AICC are functionally active and present SA to T cells causing robust release of IL-2. The results in Table 9 also demonstrate that the activated lymphocytes continue IL-2 release after SA and other biologically active agents in AICC have been washed off. As shown in **Figure 8**, the concentrations of IL-2 in AICC produced in the presence of SA were high in all three samples derived from different subjects. There was a correlation between the concentration of IL-2 in incubated samples and the ability of lymphocytes to release IL-2 into fresh serum. Negligible amounts of IL-2 were produced when SA was not added to incubation medium (i.e., in the absence of antigen presentation).

Analysis of IFN-gamma Production by AICC Lymphocytes

[0224] CD4 T helper cells and CD8 cytotoxic T lymphocyte (CTL) effector T cells activated by DCs via antigen presentation produce large amounts of interferon gamma(IFN-g). IFN-g is a cytokine that is critical for innate and adaptive immunity and for tumor control. Accordingly, if the AICC included activated T cells, it should contain IFN-g.

[0225] IFN-g release during an incubation for 48 hours in the presence of SA was measured in the preincubation composition (PC) and in the AICC at the end of incubation for 48 hours at 37°C in the presence and absence of 100 ng/ml SA. Samples were prepared as described for IL-12. The concentrations of IFN-g was measured in supernatants after cell pelleting using IFN-g ELISA kit (eBioscience). Pre-incubated and incubated serum

samples were used as controls. The amount of IFN-g naturally present in the serum ranged from 1.5 to 5 pg/mL. These background serum concentrations were subtracted from the values obtained for experimental samples. In addition, the incubated cells (plus/minus SA) were pelleted, washed with culture medium and resuspended in fresh serum with no additives at $5x10^6$/mL. The cells were incubated for 3 hours and the release of IFN-g into the fresh serum was measured. The results of 4 experiments with different batches of PC are summarized in **Table 10**.

Table 10. IFN-g Release Following Superantigen Stimulation

Conditions	**IFN-g concentration [pg/mL]**	**IFN-g release into fresh serum [pg/mL/3 hours]**
before incubation	below sensitivity	below sensitivity
48 hour incubation	below sensitivity	below sensitivity
48 hour incubation with SA	190.2±86.6	39.5±15.2

[0226] The data in **Table 10** show that DC in AICC are functionally active and present SA to T cells causing robust release of IFN-g. The data in **Table 10** also demonstrate that the activated lymphocytes continue to release IFN-g after SA and other biologically active agents in the AICC have been washed off. As shown in **Figure 9**, there was a correlation between the concentrations of IFN-g in incubated samples and the ability of lymphocytes to release IFN-g into fresh serum. Negligible amounts of IFN-g are produced when SA is not added to incubation medium and no antigen presentation occurs.

Example 5. Expression of NK Cell Specific Activation Markers

[0227] Expression of two known NK cell activation markers, CD57 and CD107a, was also evaluated in the preincubation composition, and in AICC prepared by incubating for 48 hours at 37°C in the absence and presence of SA. Leukocytes were stained with antibodies against CD57 conjugated to APC or with antibodies against CD107a conjugated to PE. A second antibody against CD14 marker on monocytes conjugated to FITC or APC respectively

was added in order to achieve better resolution between leukocyte populations. Cells within a lymphocyte gate were analyzed. The results of two experiments are shown in **Table 11.**

Table 11. Expression of NK Markers on Lymphocytes

NK markers on Lymphocytes				
conditions	**CD57 lymph**		**CD107a lymph**	
	% positive	**MFI**	**% positive**	**MFI**
before incubation	43.8±1.3	14359±5353	47.5±2.6	420±94
48 hour incubation	49±1.0	16502±190	46.6±12.4	247±75
48 hour incubation with SA	42.6±8.4	12798±951	66.4±12.1	363±89

[0228] No significant differences were found in MFI for either marker. The percentage of CD107a positive cells increased (66.4% vs 47.5%) when incubation was performed in the presence of SA.

Example 6. Treatment of HIV Infection

[0229] The activated immunostimulatory cell composition is particularly useful for stimulating an immune response to HIV.

[0230] To stimulate an immune response to HIV, an AICC is prepared from an HIV infected individual. One µM zidovudine (Retrovir) can be added to AICC to avoid HIV infection. The AICC is stimulated (either during preparation or in a separate incubation afterward) with one or more antigens or antigenic peptides from HIV. Exemplary HIV antigens include one, a combination, or all of a Tat peptide, a Nef peptide, a Rev peptide, a Gag peptide, an Env peptide, a Pol peptide, a Vpu peptide, a Vif peptide, and Padre. Alternatively recombinant live vectors such as poxvirus vectors (*e.g.*, avipoxviruses or the attenuated poxvirus strain modified vaccinia virus Ankara (MVA)) constructed with viral protein expression cassettes are incubated with

AICC to transfect DCs with viral genes. The efficacy of transfection is confirmed by flow cytometry analysis of intracellular expression of viral proteins in CD86-positive cells in the composition (CD86 is used as a DC surface marker). Flow cytometry analysis of AICC cells stained with indicators of dead and apoptotic cells (7-AAD and Annexin V respectively) confirms antigen-loaded DC viability. The functional activity of DCs loaded with viral peptides is estimated *ex vivo* and in a clinical trial.

[0231] For *ex vivo* studies, induction of proliferation of $CD4^+$ and $CD8^+$ T cells from HIV-infected patients by DCs is tested. Patient's peripheral blood lymphocytes (mononuclear cells depleted of monocytes after adherence to plastic) are used as a source of T cells. Monocyte-depleted lymphocytes are labeled with CellTrace CFSE (Molecular Probes) and co-cultured with autologous AICC loaded with viral antigens for 6–7 days. CFSE labeled cells are analyzed by flow cytometry. Cells that proliferate after the co-culture have a lower intensity of CFSE in comparison with control. To identify CD4+ and CD8+ T cells, the cells are labeled with anti-CD3, anti-CD4, and anti CD8 antibodies (*e.g.*, NHP T Lymphocyte Cocktail, BD).

[0232] Activation of T cells in the AICC derived from HIV-infected patients and incubated with viral antigens is confirmed by expression of CD69 and IL-2R (CD25) on lymphocytes and by measuring production of IL-2 and INFg as described in Example 4.

[0233] For clinical studies, the AICC composition incubated with viral antigens is aspirated into a sterile syringe of any size, using an 18-gauge (18G) needle. Aspiration is performed slowly to minimize damage to the cells. While the size of the syringe and needle are by no means limiting, a large gauge needle is preferred for aspiration. This facilitates the transfer and reduces cell damage.

[0234] The AICC is administered by injecting the composition systemically, for example, by intravenous or subcutaneous administration. The AICC may also be injected into regional lymph nodes. The clinician may choose to administer repetitive doses of AICC if it is determined to be necessary based on clinical parameters.

[0235] The patient is monitored and the effect of the AICC on parameters such as plasma viral load and CD4 T cell counts are determined. Patient's peripheral blood lymphocytes (mononuclear cells depleted of monocytes after adherence to plastic) are collected and frozen at baseline and at various times after vaccination. They are used to evaluate specific immune responses directed against pooled HIV-1 peptides or the RNA sequences used for AICC loading. For example, in one test the number of interferon (IFN)–γ-producing T cell clones among patient's lymphocytes is estimated using ELISPOT. Other tests estimate the ability of T cells from the patient to proliferate in a co-culture with DCs from AICC pulsed with HIV-1 peptides as described above and/or to cause cytotoxic lysis of virally infected target cells pulsed with the corresponding peptide. Target cell apoptosis is analyzed by flow cytometry using Annexin V and caspase 3 staining.

[0236] All publications cited in the specification, including patent publications and non-patent publications, are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications are herein incorporated by reference to the same extent as if each individual publication were specifically and individually indicated as being incorporated by reference.

[0237] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

We claim:

1. A method of treating a viral infection, comprising administering to an infected subject a composition mature dendritic cells, activated helper T cells, cytolytic T cells, and at least one other leukocyte cell type.

2. A method of treating a viral infection, the method comprising: (a) incubating leukocytes under conditions of time and temperature to activate the leukocytes; (b) culturing the activated leukocytes in a supportive medium under conditions of time and temperature that induce maturation of dendritic cells in the composition; and (c) introducing the composition comprising dendritic cells into the subject to thereby treat the viral infection.

3. A method of treating a viral infection, the method comprising (a) incubating non-quiescent leukocytes in a supportive medium under conditions of time and temperature that induce maturation of dendritic cells in the composition, and (b) introducing the composition comprising dendritic cells into the subject to thereby treat the viral infection.

4. A method of treating a viral infection, the method comprising:
(a) preparing a composition comprising mature dendritic cells by

i) providing leukocytes,

ii) allowing the leukocytes to transition from a quiescent to an active state by maintaining the leukocytes at room temperature for about 8 to 20 hours,

iii) subjecting the leukocytes to hypo-osmotic shock, and

iv) incubating the shocked leukocytes for 36 hours to 14 days in a supportive medium to thereby make a composition comprising mature DCs; and

(b) introducing the composition comprising dendritic cells into the subject to thereby treat the viral infection.

5. The method of any one of the preceding claims, wherein the composition comprising dendritic cells is exposed to at least one peptide epitope from the virus.

6. The method of any one of the preceding claims, wherein the viral infection is a viral infection with human immunodeficiency virus (HIV), herpes simplex virus 1 (HSV1), herpes simplex virus 2(HSV2), hepatitis C virus (HCV), or papilloma virus (HPV).

7. The method of any one of the preceding claims, wherein the viral infection is an infection with HIV.

8. The method of any one of the preceding claims, wherein the viral infection is chronic.

9. A method of treating an infection with an infectious organism, comprising administering to an infected subject a composition mature dendritic cells, activated helper T cells, cytolytic T cells, and at least one other leukocyte cell type.

10. A method of treating an infection with an infectious organism, the method comprising: (a) incubating leukocytes under conditions of time and temperature to activate the leukocytes; (b) culturing the activated leukocytes in a supportive medium under conditions of time and temperature that induce maturation of dendritic cells in the composition; and (d) introducing the composition comprising dendritic cells into the subject to thereby treat the viral infection.

11. A method of treating an infection, the method comprising (a) incubating non-quiescent leukocytes in a supportive medium under conditions of time and temperature that induce maturation of dendritic cells in the composition,

and (b) introducing the composition comprising dendritic cells into the subject to thereby treat the infection.

12. A method of treating an infection with an infectious organism, the method comprising:

(a) preparing a composition comprising mature dendritic cells by:

i) providing leukocytes,

ii) allowing the leukocytes to transition from a quiescent to an active state by maintaining the leukocytes at room temperature for about 8 to 20 hours,

iii) subjecting the leukocytes to hypo-osmotic shock, and

iv) incubating the shocked leukocytes for 36 hours to 14 days in a supportive medium to thereby make a composition comprising mature DCs; and

(b) introducing the composition comprising dendritic cells into the subject to thereby treat the infection.

13. The method of any one claims 9-12, wherein the composition comprising dendritic cells is exposed to at least one peptide epitope from the infectious organism.

14. The method of any one of claims 9-13, wherein the infection is a bacterial infection.

15. The method of any one of claims 9-13, wherein the infection is a fungal infection.

16. The method of any one of claims 9-13, wherein the infection is a protozoal infection.

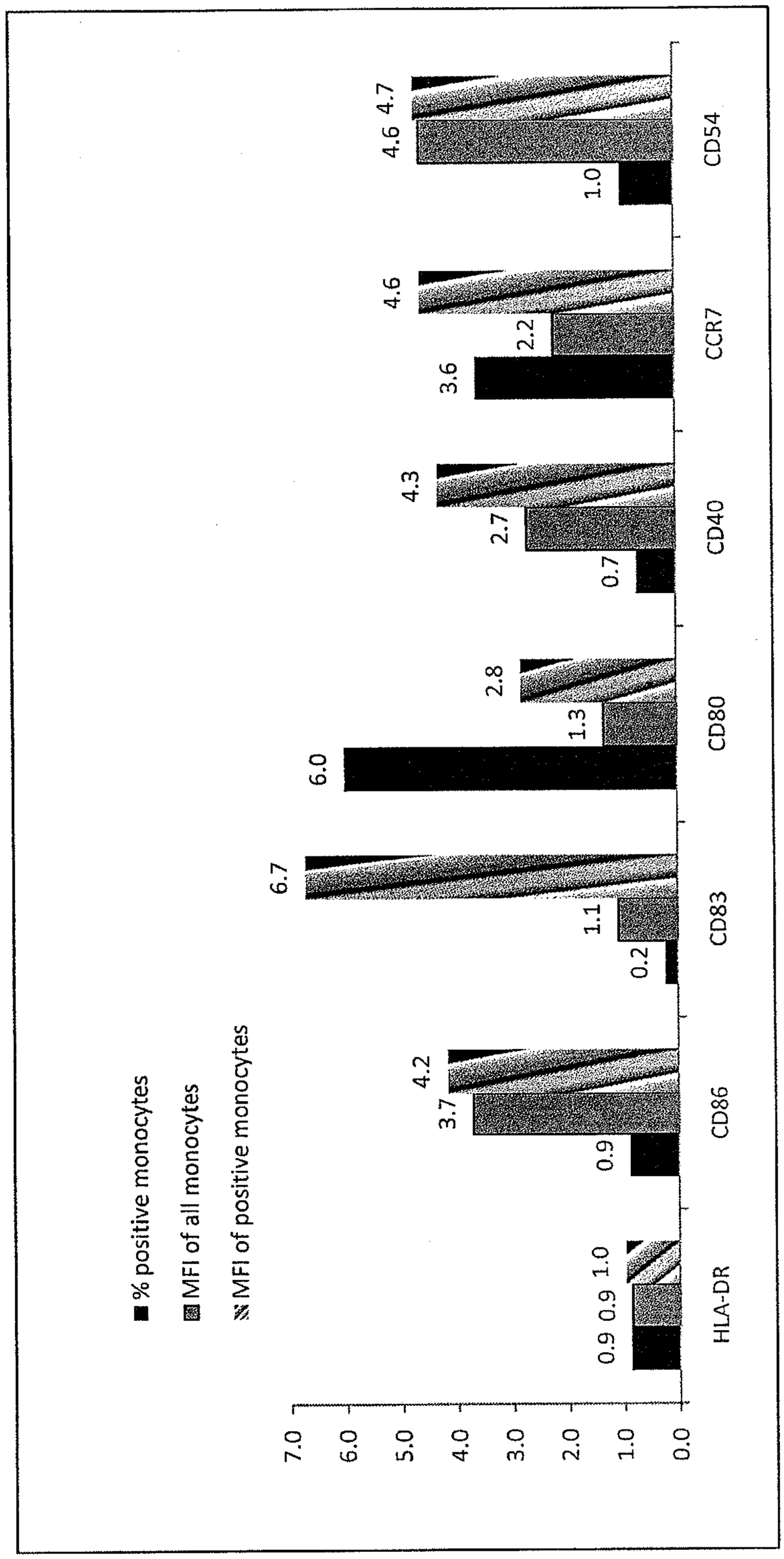

% positive monocytes
MFI of all monocytes
MFI of positive monocytes
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.9 0.9 1.0
HLA-DR
4.2
3.7
0.9
CD86
6.7
1.1
0.2
CD83
4.3
2.7
0.7
CD40
6.0
2.8
1.3
CD80
4.6
3.6
2.2
CCR7
4.6 4.7
1.0
CD54

Fig. 1

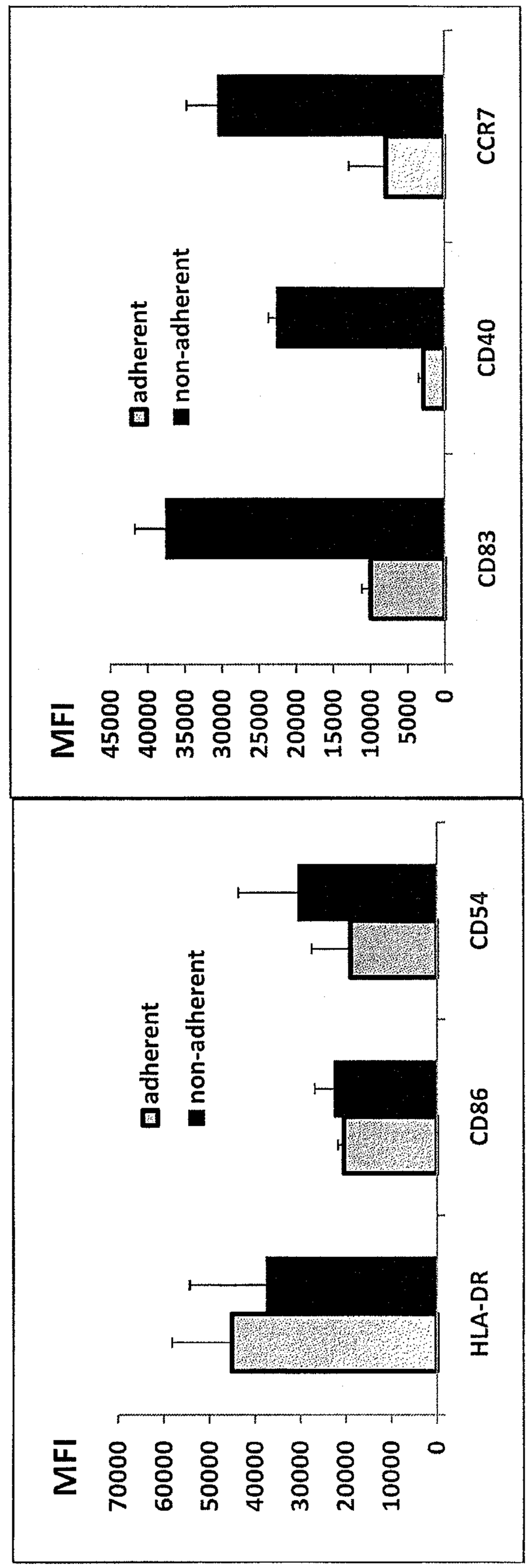

MFI
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
adherent
non-adherent
CD83
CD40
CCR7
MFI
70000
60000
50000
40000
30000
20000
10000
0
adherent
non-adherent
HLA-DR
CD86
CD54

Fig. 2B

Fig. 2A

Fig. 3B

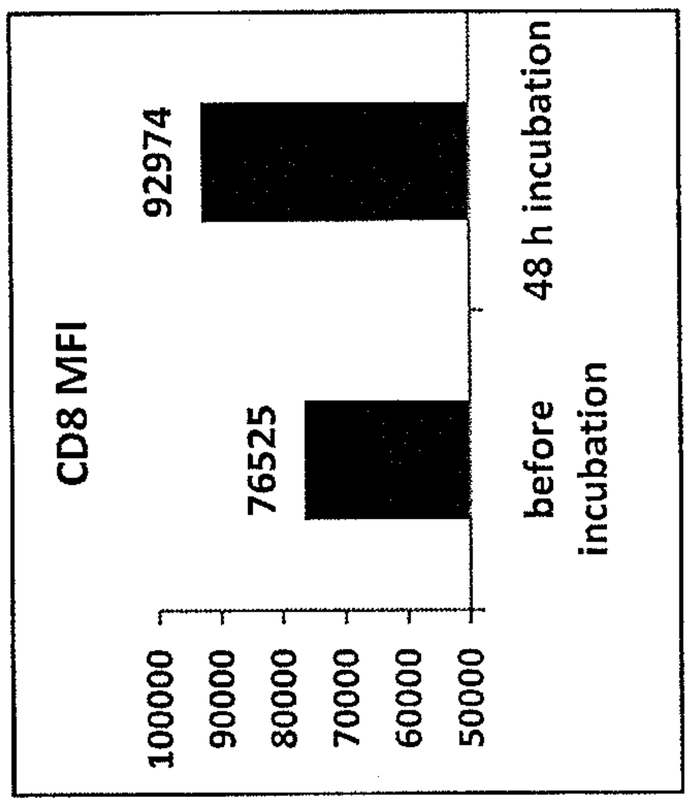
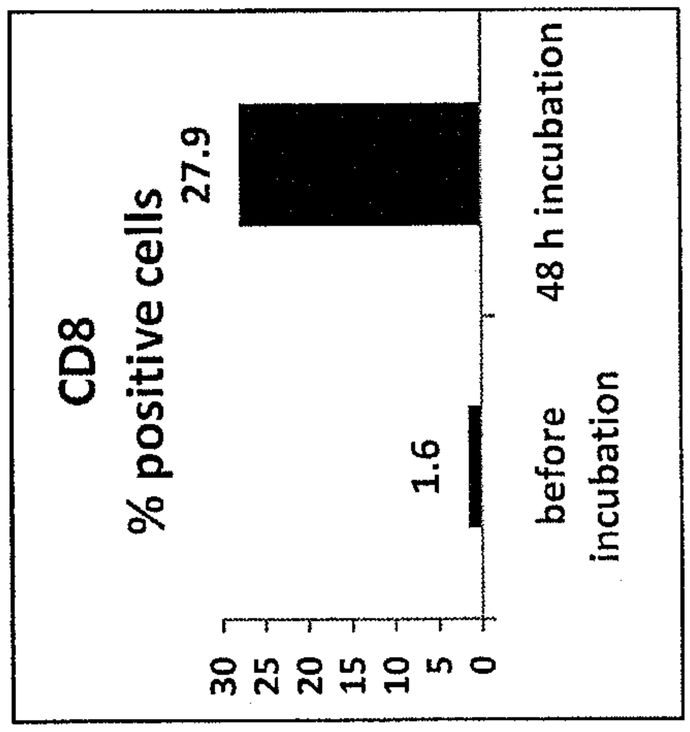

CD8 MFI
92974
76525
100000
90000
80000
70000
60000
50000
before incubation
48 h incubation

Fig. 3A


CD8
% positive cells
27.9
1.6
30
25
20
15
10
5
0
before incubation
48 h incubation

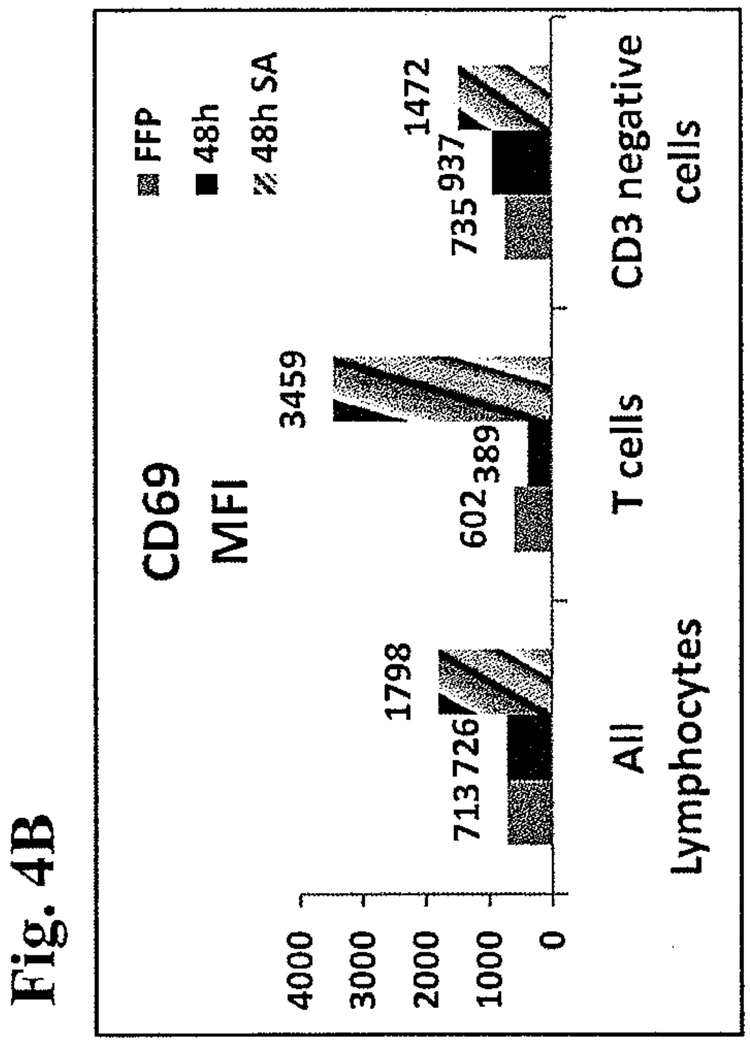

CD69
MFI
FFP
48h
48h SA
4000
3000
2000
1000
0
713
726
1798
602
389
3459
735
937
1472
All Lymphocytes
T cells
CD3 negative cells

Fig. 4B

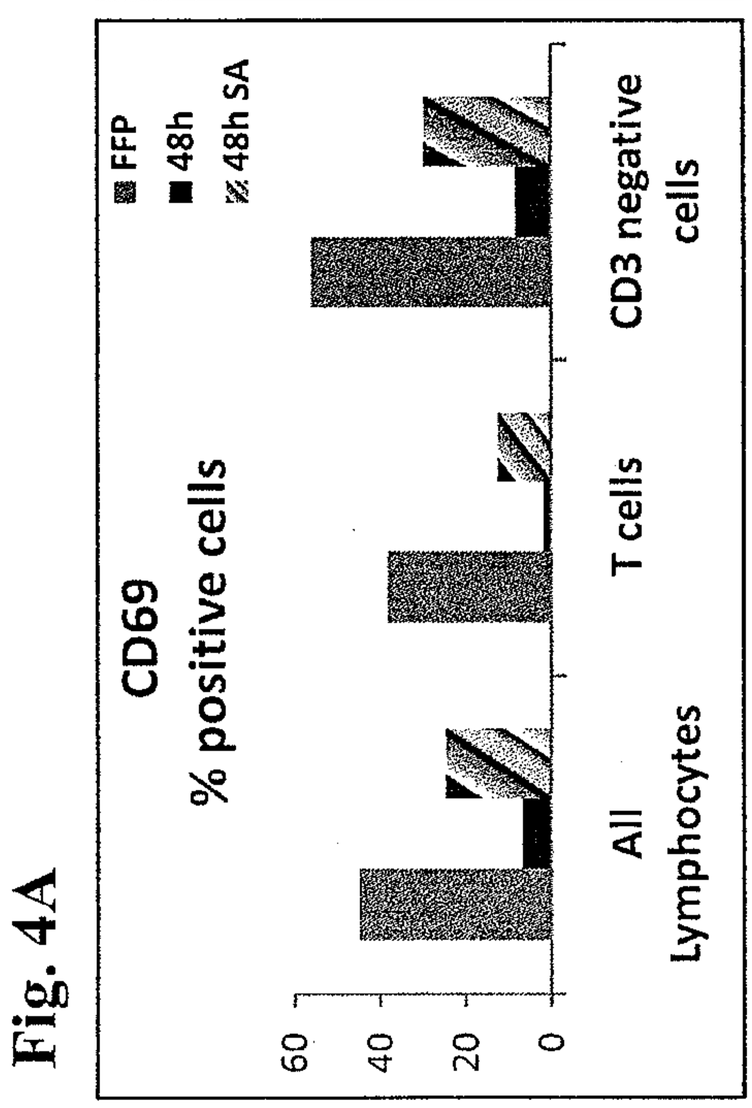

CD69
% positive cells
FFP
48h
48h SA
60
40
20
0
All Lymphocytes
T cells
CD3 negative cells

Fig. 4A

Fig. 5A

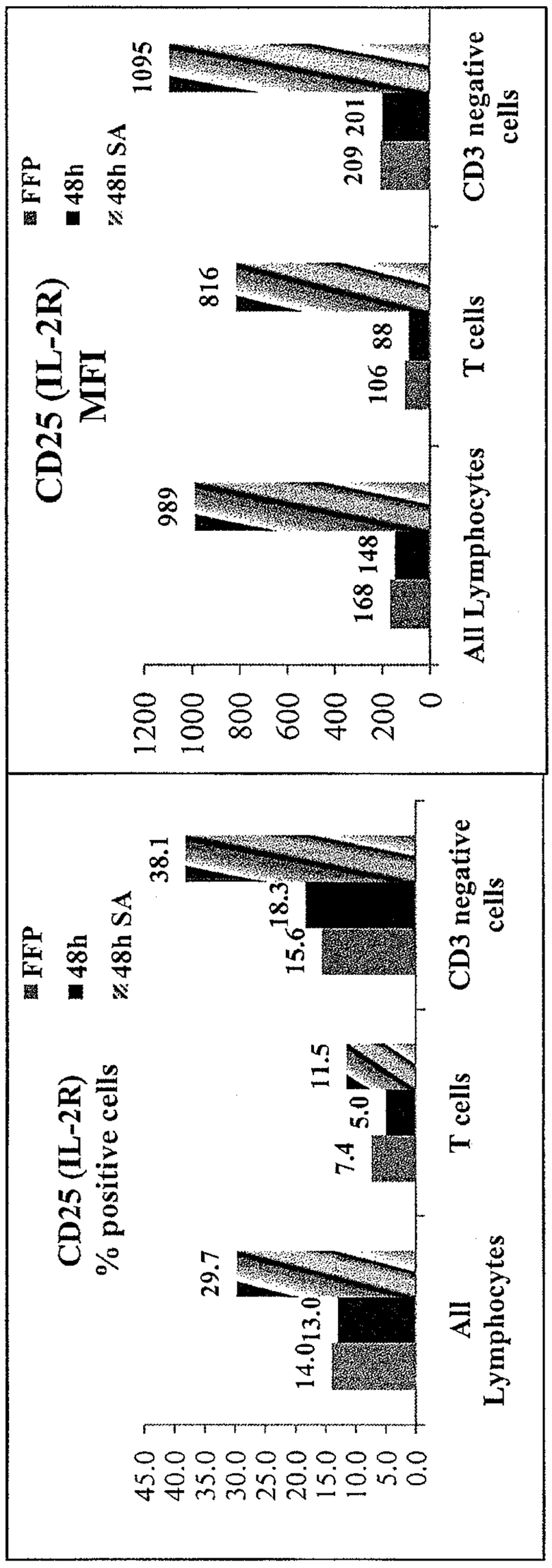

CD25 (IL-2R)
% positive cells
FFP
48h
48h SA
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
14.0
13.0
29.7
7.4
5.0
11.5
15.6
18.3
38.1
All Lymphocytes
T cells
CD3 negative cells
CD25 (IL-2R)
MFI
1200
1000
800
600
400
200
0
168
148
989
106
88
816
1095
209
201

Fig. 5B

Fig. 6A

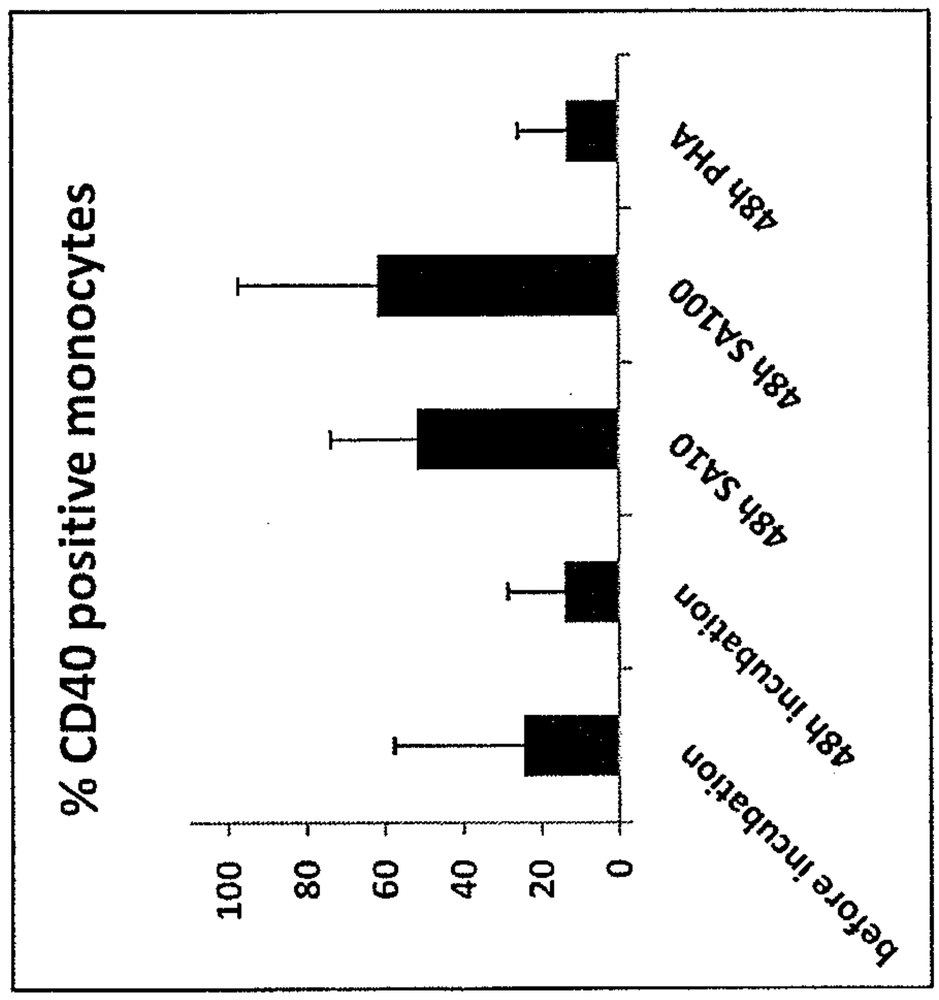


Fig. 6B

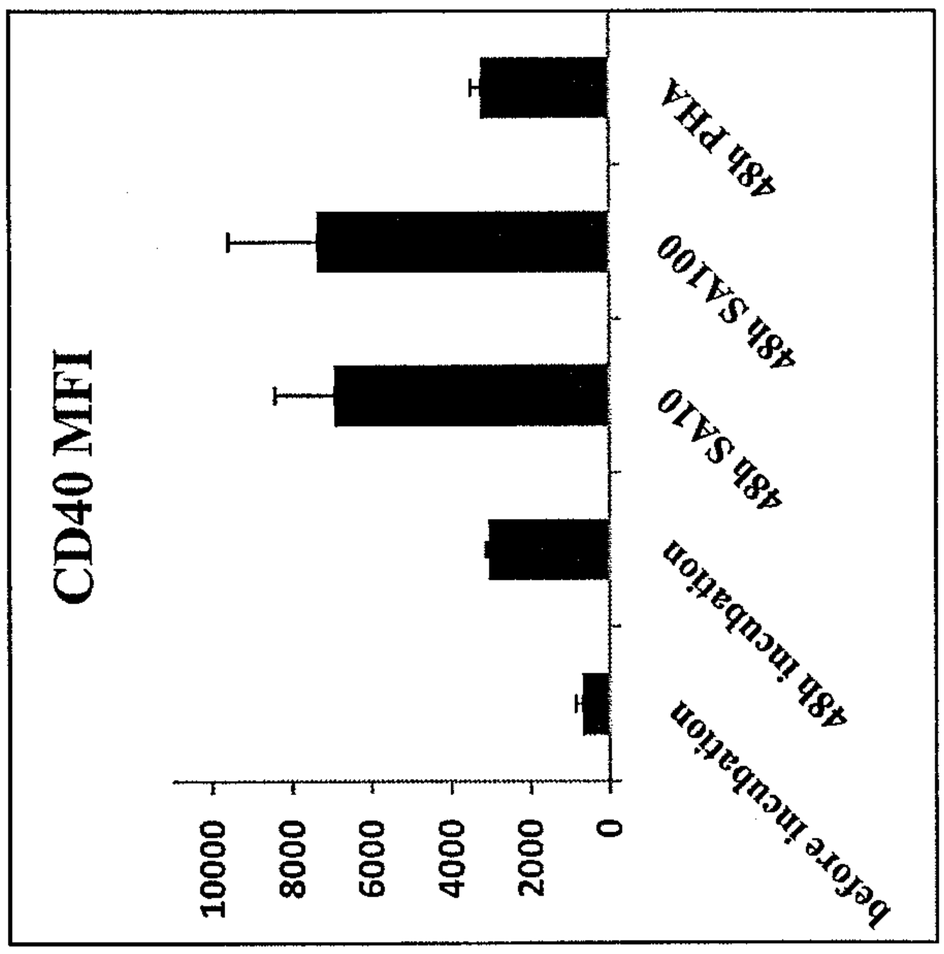


Fig. 6C

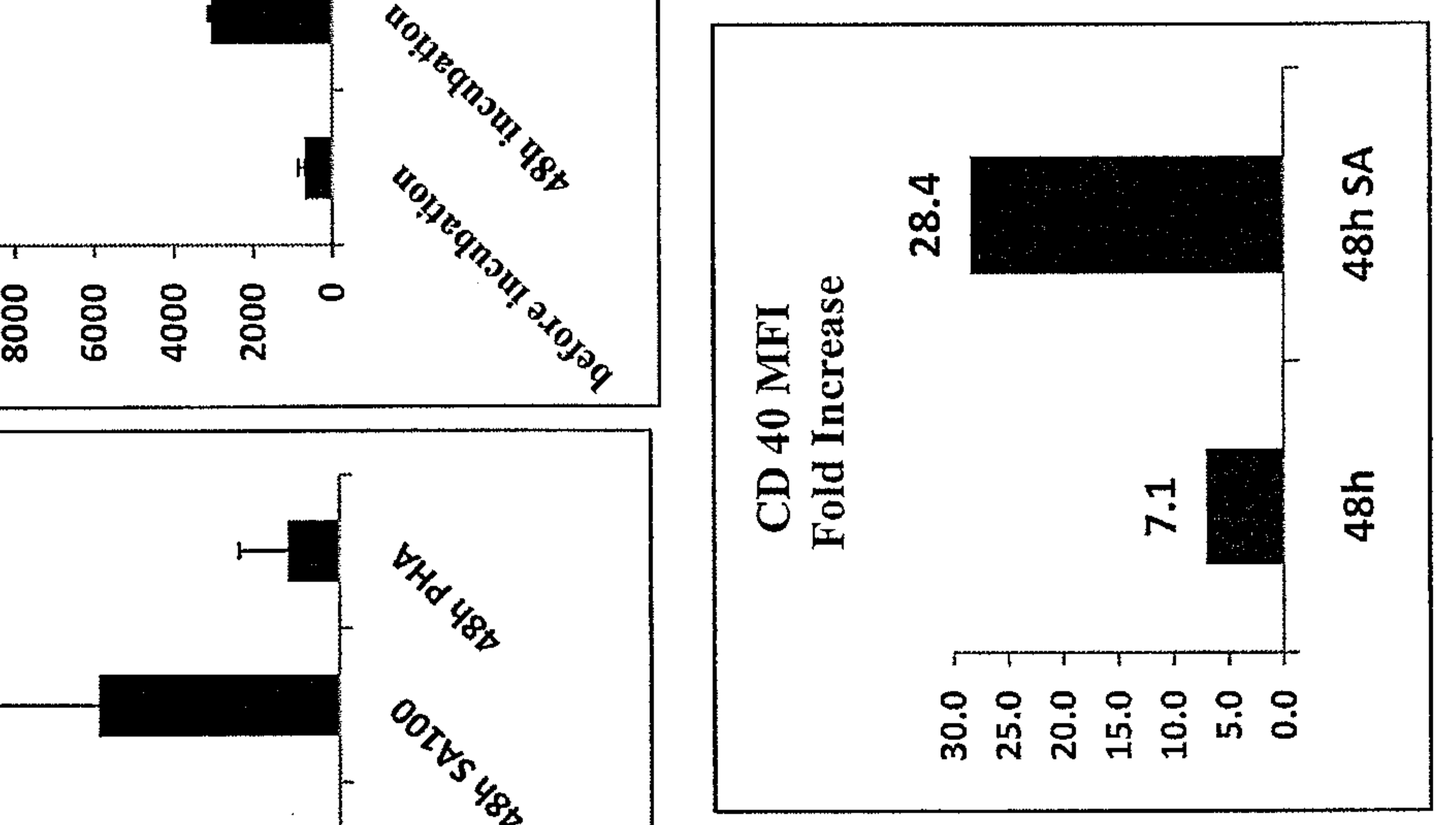


Fig. 7A

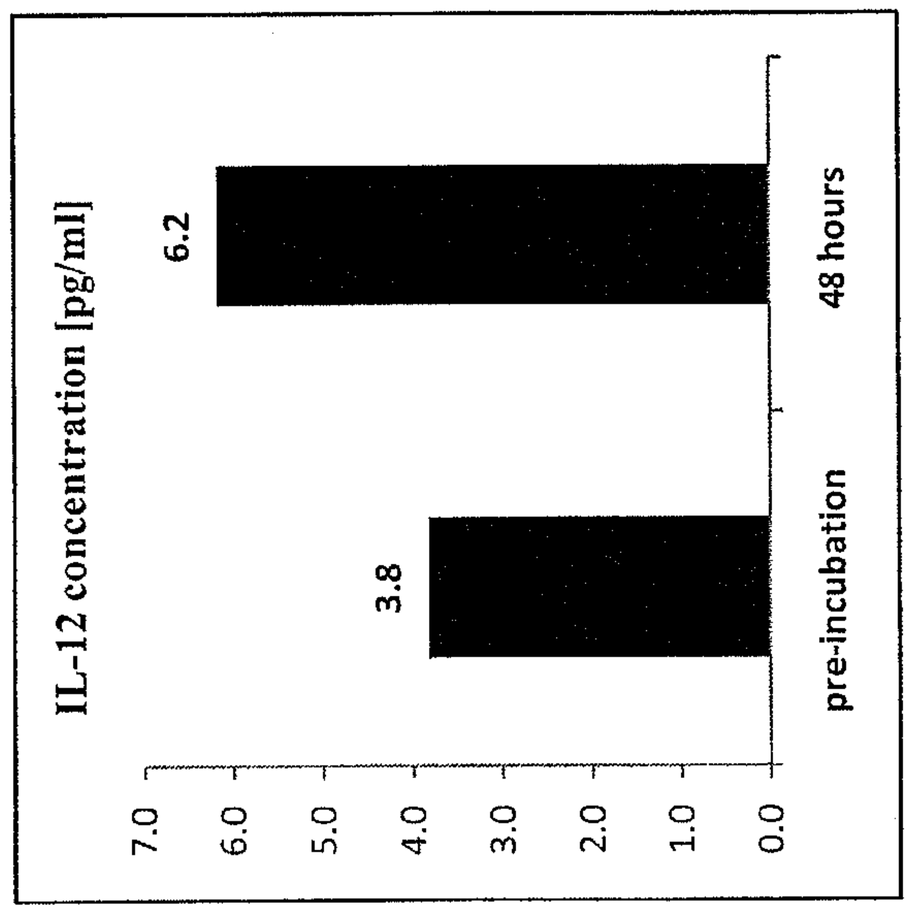
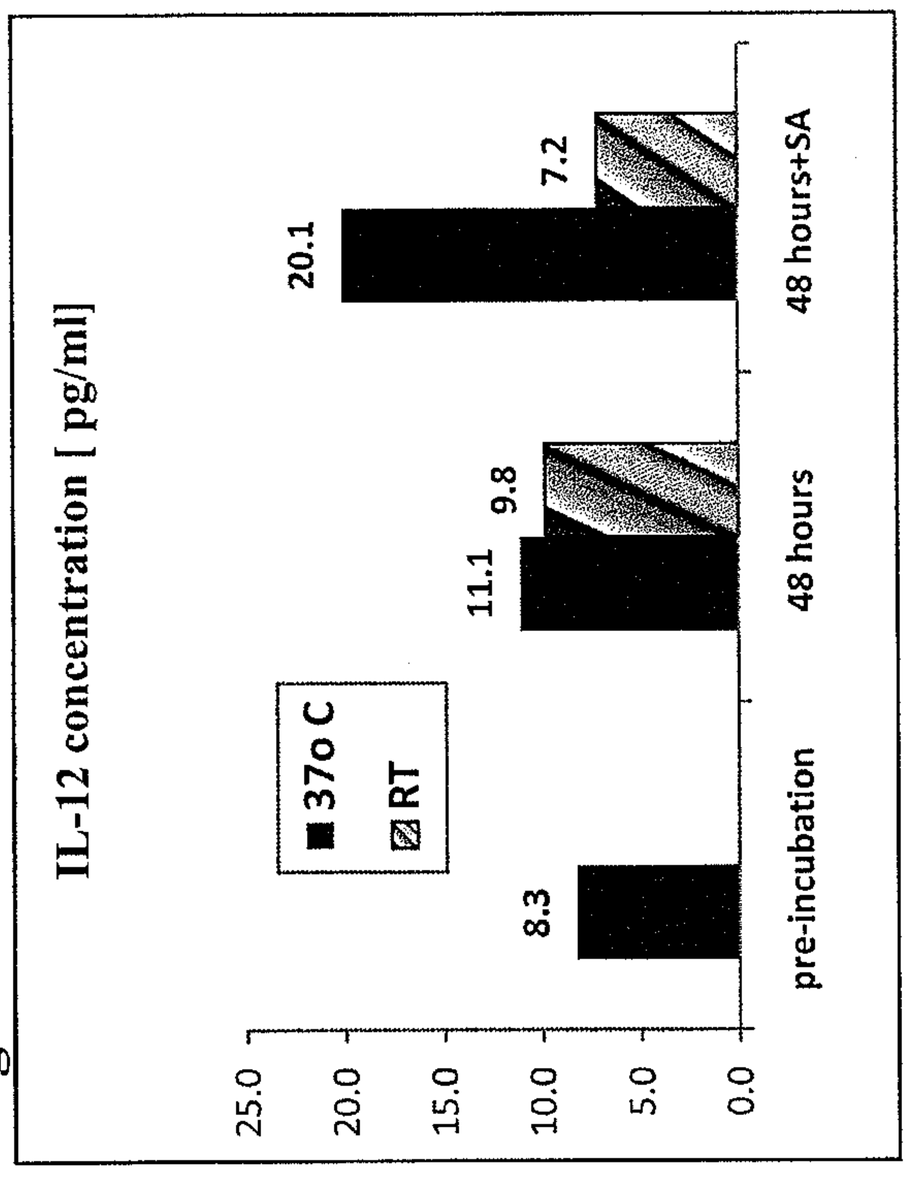

IL-12 concentration [pg/ml]
6.2
3.8
pre-incubation
48 hours
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0

Fig. 7B


IL-12 concentration [pg/ml]
37o C
RT
20.1
7.2
11.1
9.8
8.3
pre-incubation
48 hours
48 hours+SA
25.0
20.0
15.0
10.0
5.0
0.0

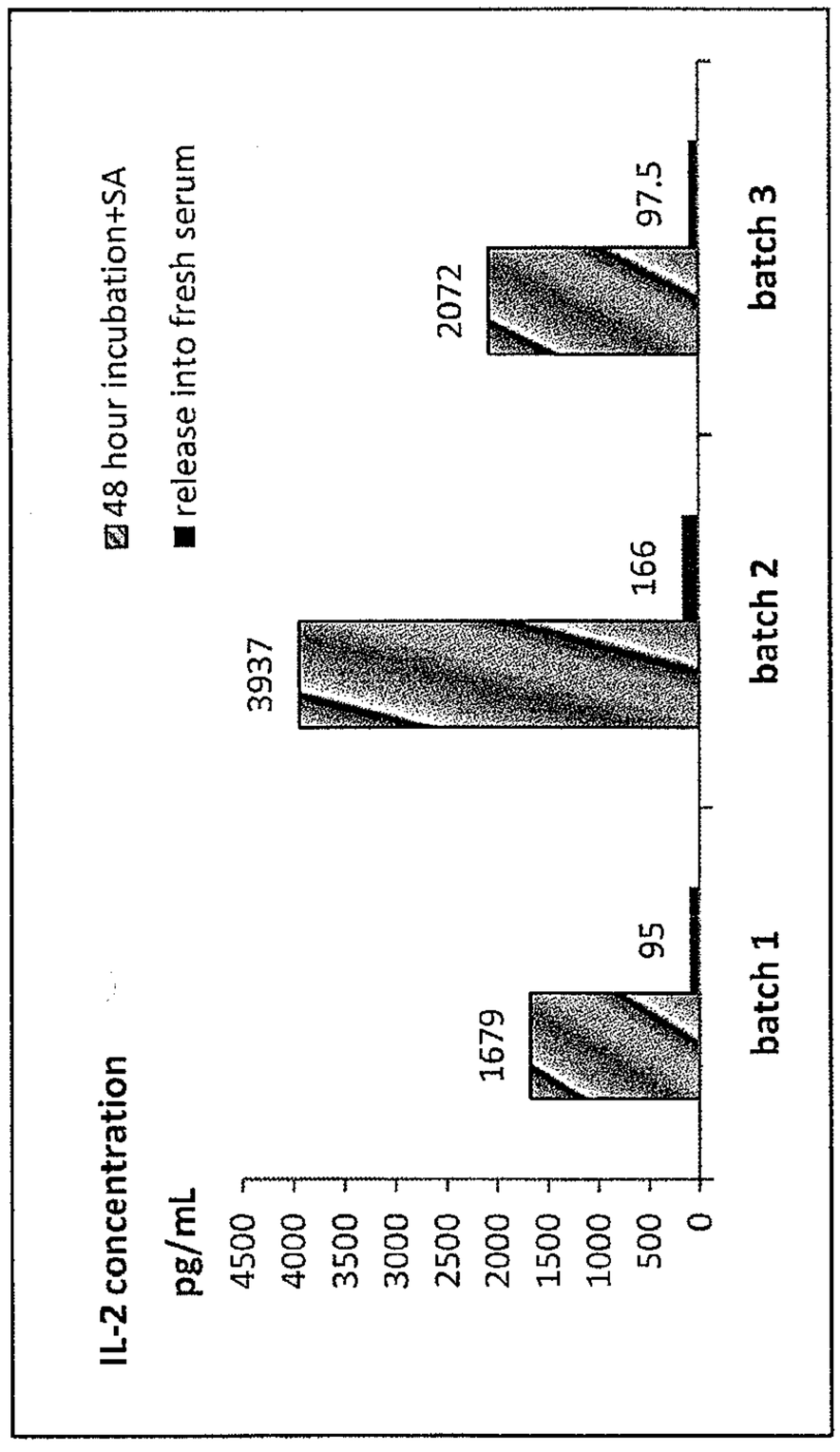

48 hour incubation+SA
release into fresh serum
IL-2 concentration
pg/mL
4500
4000
3500
3000
2500
2000
1500
1000
500
0
3937
166
2072
97.5
1679
95
batch 2
batch 3
batch 1

Fig. 8

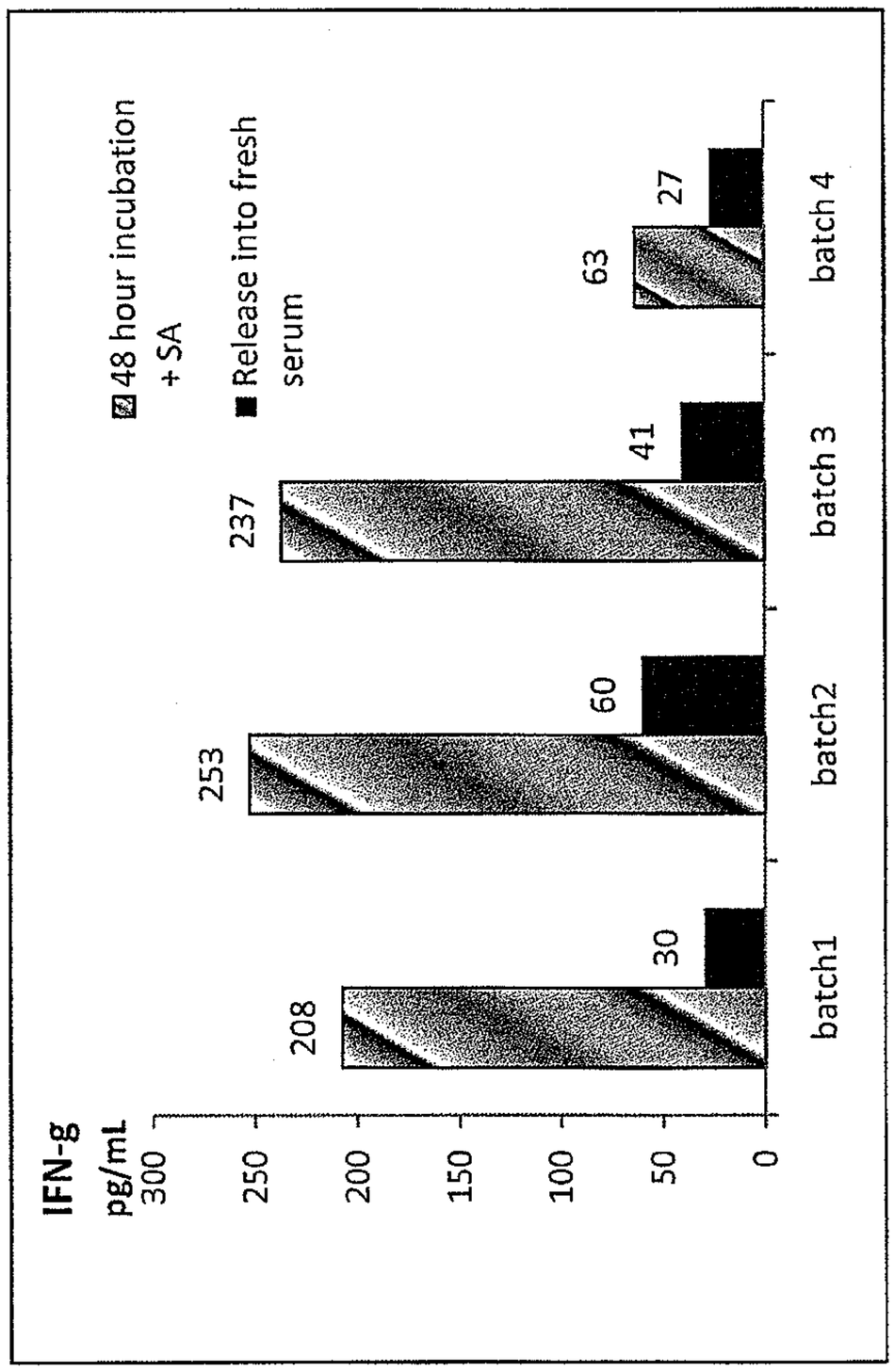
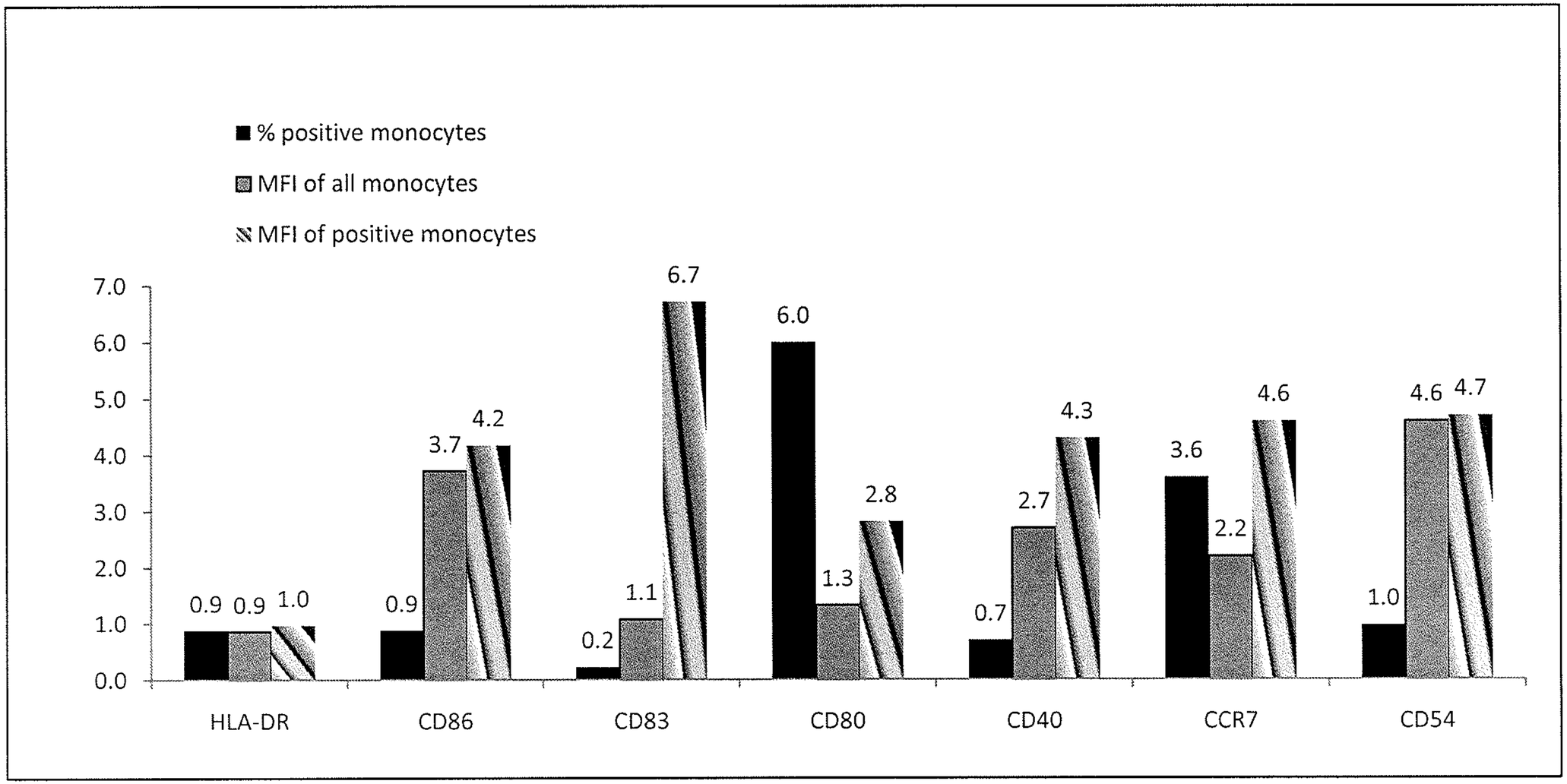

IFN-g pg/mL
300
250
200
150
100
50
0
208
30
253
60
batch1
batch2
237
41
63
27
batch 3
batch 4
48 hour incubation + SA
Release into fresh serum

Fig. 9

Fig. 1


% positive monocytes
MFI of all monocytes
MFI of positive monocytes
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.9 0.9 1.0
0.9 3.7 4.2
0.2 1.1 6.7
6.0 1.3 2.8
0.7 2.7 4.3
3.6 2.2 4.6
1.0 4.6 4.7
HLA-DR
CD86
CD83
CD80
CD40
CCR7
CD54