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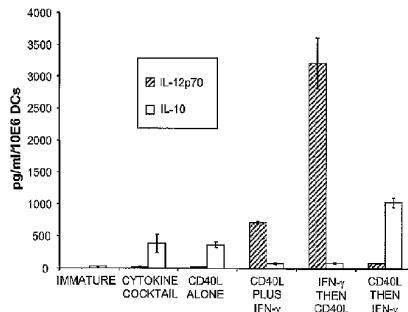
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(54) Title: MATURE DENDRITIC CELL COMPOSITIONS AND METHODS FOR CULTURING SAME



(57) **Abstract:** This invention provides methods to prepare and use immunostimulatory cells for enhancing an immune response. The invention provides a method for preparing mature dendritic cells (DCs), comprising the sequential steps of: (a) signaling isolated immature dendritic cells (iDCs) with a first signal comprising an interferon gamma receptor (IFN- $\gamma$ R) agonist and/or a tumor necrosis factor alpha receptor (TNF- $\alpha$ R) agonist to produce signaled dendritic cells; and (b) signaling said signaled dendritic cells with a second transient signal comprising an effective amount of a CD40 agonist to produce CCR7 $^{+}$  mature dendritic cells. Also provided by this invention are enriched populations of dendritic cells prepared by the methods of the invention. Such dendritic cells have enhanced immunostimulatory properties and increased IL-12 secretion and/or decreased IL-10 secretion. CD40 signaling can be initiated by one or more of polypeptide translated from an exogenous polynucleotide encoding CD40L (e.g., mRNA or DNA), an agonistic antibody to CD40 receptor or by CD40 ligand polypeptide. The enriched populations can be further modified by the administration of an immunogen to the DC. The DC will take up and process the immunogen on its cell surface.

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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**MATURE DENDRITIC CELL COMPOSITIONS AND METHODS FOR  
CULTURING SAME**

5 **CROSS REFERENCE TO RELATED APPLICATIONS**

This application claims priority to U.S. provisional application 60/522,512, filed 7 October 2004, the contents of which is specifically incorporated by reference.

**FIELD OF THE INVENTION**

10 The present invention relates to the generation of mature dendritic cells and their use in cell therapy and to educate immune effector cells. The mature dendritic cells can be generated from immature dendritic cells.

**BACKGROUND**

15 Cell therapy utilizes modified antigen presenting cells (APCs) or immune effector cells to initiate an immune response in a patient. Antigen presenting cells are central to cell therapy because they initiate the immune response. Indeed, they are the only cells capable of inducing a primary immune response from the T lymphocytes.

20 Dendritic cells (DC) are the most potent APCs involved in adaptive immunity. They coordinate the initiation of immune responses by naive T cells and B cells and induce antigen-specific cytotoxic T lymphocyte (CTL) responses. DCs are specialized in several ways to prime helper and killer T cells *in vivo*. For example, immature DCs that reside in peripheral tissues are equipped to capture antigens and to produce immunogenic MHC-peptide complexes. In response to maturation-inducing stimuli such as inflammatory cytokines, immature DCs develop into potent T cell stimulators by upregulating adhesion and costimulatory molecules. At the same time, they migrate into secondary lymphoid organs to select and stimulate rare antigen-specific T cells. However, potent stimulation of T cells occurs only after DC maturation, a process that increases the availability of MHC/peptide complexes on the cell surface, in addition to co-stimulatory molecules, that direct the effector 25 function of the responding T-cells. Indeed, immature DCs may be harmful in anti-tumor and other immunotherapies because they can induce immunotolerance rather than immunostimulation.

Co-stimulation is typically necessary for a T cell to produce sufficient cytokine levels that induce clonal expansion. One characteristic of dendritic cells which makes them potent antigen presenting cells is that they are rich in co-stimulatory molecules of the immune response, such as the molecules CD80 and CD86, which activate the molecule CD28, on T lymphocytes. In return, T-helper cells express CD40L, which ligates CD40 on DCs. These mutual interactions between DCs and T-cells leads to 'maturation' of the former, and the development of effector function in the latter. The expression of adhesion molecules, like the molecule CD54 or the molecule CD11a/CD18, facilitate the co-operation between the dendritic cells and the T-cells. Another special characteristic of dendritic cells is to deploy different functions depending on their stage of differentiation. Thus, the capture of the antigen and its transformation are the two principal functions of the immature dendritic cell, whereas its capacities to present the antigen in order to stimulate the T cells increases as the dendritic cells migrate into the tissues and the lymphatic ganglia. This change of functionality corresponds to a maturation of the dendritic cell. Thus, the passage of the immature dendritic cell to the mature dendritic cell represents a fundamental step in the initiation of the immune response. Traditionally, this maturation was followed by monitoring the change of the surface markers on the DCs during this process. Some of the more important cell surface markers characteristic of the different stages of maturation of the dendritic cells are summarized in Table I, below. However, the surface markers can vary depending upon the maturation process.

Table I

Cell type	Surface markers
Hematopoietic stem cell	CD34+
Monocytes	CD14++, DR+, CD86+, CD16+/-, CD54+, CD40+
Immature dendritic cell	CD14+/-, CD16-, CD80+/-, CD83-, CD86+, CD1a+, CD54+, DQ+, DR++
Mature dendritic cell	CD14-, CD83++, CD86++, CD80++, DR+++, DQ++, CD40++, CD54++, CD1a +/-

Mature DCs are currently preferred to immature DCs for immunotherapy. Only fully mature DC progeny lack GM-CSF Receptor (GM-CSF-R) and remain stably mature upon removal/in the absence of GM-CSF. Also, mature DCs have been shown to be superior in inducing T cell responses *in vitro* and *in vivo*. In contrast, immature DCs are reported to 5 induce tolerance *in vitro* (Jonuleit *et al.* (2000) *Exp. Med.* **192**:1213) as well as *in vivo* (Dhopakar *et al.* (2001) *Exp. Med.* **193**:233) by inducing regulatory T cells. Mature dendritic cells also are useful to take up and present antigen to T-lymphocytes *in vitro* or *in vivo*. The modified, antigen presenting DCs and/or T cells educated from these modified DCs have many applications, including diagnostic, therapy, vaccination, research, screening and 10 gene delivery.

It is difficult to isolate mature dendritic cells from peripheral blood because less than 1% of the white blood cells belongs to this category. Mature DCs are also difficult to extract from tissues. This difficulty, in combination with the potential therapeutic benefit of DCs in cell therapy, has driven research and development toward new methods to generate mature 15 dendritic cells using alternative sources. Several methods are reported to produce mature DCs from immature dendritic cells.

For example, Jonuleit *et al.* (*Eur J Immunol* (1997) **12**:3135-3142) disclose maturation of immature human DCs by culture in medium containing a cytokine cocktail (IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and PGE<sub>2</sub>).

20 WO 95/28479 discloses a process for preparing dendritic cells by isolating peripheral blood cells and enriching for CD34 $^{+}$  blood precursor cells, followed by expansion with a combination of hematopoietic growth factors and cytokines.

European Patent Publication EP-A-0 922 758 discloses the production of mature 25 dendritic cells from immature dendritic cells derived from pluripotential cells having the potential of expressing either macrophage or dendritic cell characteristics. The method requires contacting the immature dendritic cells with a dendritic cell maturation factor containing IFN- $\gamma$ .

European Patent Publication EP-B-0 633930 teaches the production of human 30 dendritic cells by first culturing human CD34 $^{+}$  hematopoietic cells (i) with GM-CSF, (ii) with TNF- $\alpha$  and IL-3, or (iii) with GM-CSF and TNF- $\alpha$  to induce the formation of CD1a $^{+}$  hematopoietic cells.

Patent Publication No. 2004/0152191 discloses the maturation of dendritic cells by contacting them with RU 41740.

U.S. Patent Publication No. 2004/0146492 teaches a process for producing recombinant dendritic cells by transforming hematopoietic stem cells followed by 5 differentiation of the stem cells into dendritic cells by culture in medium containing GM-CSF.

U.S. Patent Publication No. 2004/0038398 discloses methods for the preparation of substantially purified populations of DCs and monocytes from the peripheral blood of mammals. Myeloid cells are isolated from the mammal and DCs are separated from this 10 population to yield an isolated subpopulation of monocytes. DCs are then enriched by negative selection with anti-CD2 antibodies to remove T cells.

Although mature DCs are functionally competent and are therefore useful to induce antigen-specific T cells, not all mature DCs are optimized to induce these responses. It has been shown that some mature DCs may also stimulate T helper cells by secreting IL-12.

15 Macatonia *et al.* (1995) *Immunol.* **154**:507 1; Ahuja *et al.* (1998) *Immunol.* **161**:868 and Unintford *et al.* (1999) *Immunol.* **97**:588. IL-12 also has been shown to enhance antigen-specific CD8+ T cell response to antigen in an animal model. Schmidt *et al.* (1999) *Immunol.* **163**:2561.

20 Mosca *et al.* (2000) *Blood* **96**:3499, disclose that culture of DC in AIM V medium containing both soluble CD40L trimer and IFN $\gamma$  1b results in increased IL-12 expression in comparison to culture in medium containing only soluble CD40L trimer.

25 Koya *et al.* (2003) *J. Immunother.* **26**(5):451 report that IL-12 expression can be enhanced by transducing immature DCs, in the presence of IFN $\gamma$ , with a lentiviral vector encoding CD40 Ligand. Greater than 90% of the CD40L transduced DCs expressed CD83 on their cell surface. Unfortunately, lentiviral transduced cells are not suitable for therapeutic purposes, and proviral integration into the genome of the transduced cell can result in leukemia. Furthermore, persistent expression of CD40L may have detrimental effects on APC function and viability.

30 This work supplemented the earlier work of Mackey, *et al.* (1998) *J. Immunol.* **161**:2094 who reported that *in vivo*, DCs require maturation via CD40 to generate anti-tumor immunity. Similarly, Kuniyoshi, J.S. *et al.* (1999) *Cell Immunol.* **193**:48 have shown that

DCs treated with soluble trimeric CD40 Ligand plus IFN- $\gamma$  stimulated potent T-cell proliferation and induced T cells with augmented antigen-specific lysis. Kalady, M.F. *et al.* (2004) *J. Surg. Res.* **116**:24, reported that human monocytederived DCs transfected with mRNA encoding melanoma antigen MART-1 or influenza M1 matrix protein exposed to 5 different maturation stimuli added either simultaneously or sequentially showed variability in antigen presentation, IL-12 secretion and immunogenicity of effector T cells raised in the presence of these DCs. Most importantly, this study showed that the application of a 'cytokine cocktail' consisting of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and PGE<sub>2</sub>, followed by extracellular soluble CD40L protein was superior to applying all the agents simultaneously. However, 10 these authors did not study the combination of IFN- $\gamma$  signaling with transient CD40L signalling in a sequential process. Moreover, despite the production of IL-12 when IFN- $\gamma$  and CD40L are concomitantly added to the culture medium, the recent prior art shows that the resulting DCs are actually immunosuppressive, rather than pro-inflammatory (Hwu *et al.* (2000) *J. Immunol.* **164**: 3596 ; Munn *et al.* (2002) **297**:1867 ; and Grohmann *et al.* (2003) 15 *Trends Immunol.* **24**:242) due to the induction of an enzyme that metabolized tryptophan resulting in the starvation of responder T-cells that then fail to proliferate. Thus, current literature suggests that the combination of IFN- $\gamma$  and CD40L should not increase immunopotency. The present invention addresses the long-felt need to provide improved methods for DC maturation and mature DCs with enhanced immunopotency.

20

#### SUMMARY OF THE INVENTION

Applicants have discovered that potent immunostimulation occurs when immature dendritic cells are sequentially signaled with a first signal comprising an interferon gamma receptor (IFN- $\gamma$ R) agonist followed by a second signal comprising a CD40 agonist. 25 Accordingly, this invention provides a method for preparing mature dendritic cells (DCs), comprising the sequential steps of: (a) signaling isolated immature dendritic cells (iDCs) with a first signal comprising an interferon gamma receptor (IFN- $\gamma$ R) agonist, and optionally a TNF- $\alpha$ R agonist, to produce signaled dendritic cells; and (b) signaling said signaled dendritic cells with a second transient signal comprising an effective amount of a CD40 30 agonist to produce CCR7<sup>+</sup> mature dendritic cells.

In preferred embodiments, the immature DCs are further contacted with PGE<sub>2</sub> and

optionally with TNF- $\alpha$ . In alternative embodiments the method further comprises contacting the immature DCs, signaled DCs and/or CCR7 $^+$  mature dendritic cells with a compound selected from the group consisting of: galactosylceramides, glycosylceramides, galactofuranosylceramides, arabinopyranosylceramides,  $\alpha$ -C-galactosylceramides and  $\alpha$ -S-5 galactosylceramides. Preferably the compound is a galactosylceramide. Most preferably, the galactosylceramide is (2S, 3S, 4R)-1-O-(alpha-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000).

In another embodiment of the invention, the IFN- $\gamma$ R agonist can be replaced by a tumor necrosis factor alpha receptor (TNF- $\alpha$ R) agonist. Thus, the invention provides a 10 method for preparing an enriched population of mature dendritic cells (DCs), comprising sequentially signaling immature dendritic cells with a first signal comprising a tumor necrosis factor alpha receptor (TNF- $\alpha$ R) agonist followed by a second signal comprising a CD40 agonist, thereby preparing an enriched population of mature dendritic cells, wherein said signaling is in the absence of an effective amount of IL-1 $\beta$  or IL-6. Preferably, the 15 immature DCs are further contacted with PGE<sub>2</sub>.

Preferred IFN- $\gamma$ R agonists are mammalian IFN- $\gamma$ , preferably human IFN- $\gamma$  and active fragments thereof. Preferred TNF- $\alpha$ R agonists are mammalian TNF- $\alpha$ , preferably human TNF- $\alpha$  and active fragments thereof. Preferred CD40 agonists are mammalian CD40 20 Ligands (CD40L), preferably human CD40L and active fragments and variants thereof, as well as agonistic antibodies to CD40 receptor. Signaling can be initiated by providing the signaling agonist in the culture medium, introduction of the agonist into the cell, and/or upon translation within the dendritic cell of an mRNA encoding an agonistic polypeptide. The method can be practiced *in vivo* or *ex vivo*. Dendritic cells matured *ex vivo* according to the 25 methods of the invention can then be administered to the subject to induce or enhance an immune response.

Each of the dendritic cells can be further modified by the administration of an immunogen to the DC. The DC will take up and process the immunogen, and display it on its cell surface. The immunogen can be delivered *in vivo* or *ex vivo*. The matured, cultured DCs can be administered to a subject to induce or enhance an immune response. In yet a 30 further embodiment, the antigen loaded mature DCs are used to educate naïve immune effector cells.

In another aspect, the invention provides a composition comprising in vitro matured dendritic cells, such as CD83<sup>+</sup> CCR7<sup>-</sup> mature DCs and CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs. Mature dendritic cells of the invention express increased levels of IL-12 in comparison to immature dendritic cells, and/or express less than 500 pg IL-10 per million dendritic cells. The 5 compositions of the invention are useful to raise an immune response in a subject by administering to the subject an effective amount of the population.

#### BRIEF DESCRIPTION OF THE SEQUENCE LISTING

**SEQ ID NO:1** is a human CD40L cDNA. Nucleotides 40 to 825 represent the coding 10 region, including the ATG translation start codon and the TGA translational stop codon.

**SEQ ID NO:2** is an amino acid sequence for full length human CD40L protein.

**SEQ ID NO:3** is a human CD40 cDNA. Nucleotides 67 to 522 represent the coding region, including the ATG translation start codon and the TAG translational stop codon.

**SEQ ID NO:4** is an amino acid sequence for human CD40 (the receptor for CD40L).

15 **SEQ ID NO:5** is a human IFN- $\gamma$  cDNA. Nucleotides 109 to 609 represent the coding region, including the ATG translation start codon and the translational stop codon.

**SEQ ID NO:6** is an amino acid sequence for human IFN- $\gamma$ .

**SEQ ID NO:7** is a human TNF- $\alpha$  cDNA. Nucleotides 170 to 971 represent the coding region, including the ATG translation start codon and the TGA translational stop 20 codon.

**SEQ ID NO:8** is an amino acid sequence for human TNF- $\alpha$ .

**SEQ ID NO:9** is a mouse CD40L cDNA. Nucleotids 13 to 795 represent the coding region, including the ATG translation start codon and the TGA translational stop codon.

**SEQ ID NO:10** is an amino acid sequence for full length mouse CD40L protein.

25 **SEQ ID NO:11** is a CD40L 5' primer.

**SEQ ID NO:12** is a CD40L 3' primer.

**SEQ ID NO:13** is the DNA sequence corresponding to an optimized human CD40L mRNA.

**SEQ ID NO:14** is the CD40 Receptor 3'UTR.

30 **SEQ ID NO:15** is the untranslated region of final exon of the human beta-actin 3' UTR.

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**SEQ ID NO:16** is the minimal functional element of the human beta-actin 3' UTR.

**SEQ ID NO:17** is the simian rotavirus Gene 6 3'UTR.

**SEQ ID NO:18** is the minimal functional element of the simian rotavirus Gene 6 3'UTR.

5       **SEQ ID NO:19** is the human Hsp70 5'UTR (HSPA1A).

**SEQ ID NO:20** is the mouse VEGF 5'UTR.

**SEQ ID NO:21** is the minimal functional element of the mouse VEGF 5'UTR.

**SEQ ID NO:22** is the Spleen Necrosis Virus LTR RU5 Region.

**SEQ ID NO:23** is the Tobacco Etch Virus 5' Leader sequence.

10       **SEQ ID NOS:24-25** are HLA-A201 restricted MART-APL peptide, native peptide and PSA-1 peptide, respectively.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows that sequential maturation of DCs with IFN- $\gamma$  then soluble CD40L results in optimal IL-12p70 secretion. DCs were matured with cytokine cocktail, soluble CD40L alone, or with soluble CD40L plus IFN- $\gamma$ . Pre-incubation of immature DCs with 1000 U/ml of IFN- $\gamma$  for 18 hrs, followed by addition of soluble CD40L for a further 18 hrs results in maximum IL-12p70 release. Applying soluble CD40L first, followed by IFN- $\gamma$  is perceived as a negative signal, with minimal IL-12p70 release, accompanied by IL-10.

20       Figure 2 shows that HELA cells transfected with mRNA encoding CD40L and having a polyA tail of >400 nucleotides express cell surface protein, as defined by FACS analysis with anti-CD40L (CD154) antibody.

25       Figure 3 shows that IL-12p70 secretion from CD40L mRNA transfected cells is proportional to the size of the transfection payload. DCs were transfected with a titration of CD40L mRNA followed immediately by the addition of 1000 U/ml IFN- $\gamma$ . At least 4 $\mu$ g per million DCs of CD40L mRNA is required to induce significant levels of IL-12p70 release.

30       Figure 4 shows that at least 100 U/ml of IFN- $\gamma$  is required to synergize with the CD40L mRNA payload to induce maximal IL-12p70 secretion. DCs were transfected with 4 $\mu$ g CD40L mRNA per million cells, and immediately incubated with a titration of IFN- $\gamma$ . IL-12p70 and IL-10 were measured in culture supernatants after 24hrs.

Figure 5A shows that IL-12p70 secretion induced by CD40L/IFN- $\gamma$  occurs approximately 24hrs after transfection of DCs and culture in the presence of IFN- $\gamma$ . DCs

were transfected with 4 $\mu$ g CD40L mRNA per million cells, and immediately cultured with 1000 U/ml IFN- $\gamma$ . Supernatants were collected from replica cultures at the designated times, and assayed for IL-12p70 and IL-10 content.

5 **Figure 5B** shows that addition of TNF- $\alpha$  to CD40L mRNA transfected DCs results in the generation of IL-12p70, but the level of expression is less than that achieved with IFN- $\gamma$  as the co-maturation agent.

**Figure 5C** shows that the use of TNF- $\alpha$  as the co-maturation factor also results in elevated levels of IL-10 compared to the use of IFN- $\gamma$ .

Figures 6A and 6B show that DCs transfected with mRNA encoding CD40L  
10 demonstrate cellular expression as defined by FACS analysis with anti-CD40L (CD154) antibody. In Figure 6A, DCs were transfected with 4 $\mu$ g CD40L mRNA per million cells and analyzed at various time points. The majority of CD40L is localized within an intracellular compartment as demonstrated by a 4 hour time point where surface expression is considerably lower. Figure 6B shows that significant intracellular expression is evident at 60  
15 minutes with 27% positive DCs and increasing to 79% by 3 hours.

**Figure 6C** shows transient expression of CD40L protein post transfection of DC with CD40L encoding mRNA.

**Figure 7** shows that DCs transfected with CD40L mRNA and cultured in the presence of IFN- $\gamma$  secrete IL-12p70 despite the presence of an excess of blocking anti-  
20 CD40L antibody, CD40/CD40L interactions operate within an “intracellular” compartment. DCs were transfected 4  $\mu$ g CD40L mRNA and immediately cultured with 1000 U/ml IFN- $\gamma$  in the presence of either 10 or 50  $\mu$ g/ml of blocking anti-CD40L antibody. IL-12 p70 release is reduced by only 50%, indicating that intracellular signaling, rather than cell to cell  
signaling is the primary pathway for the induction of IL-12p70.

25 **Figure 8** shows that DCs transfected with CD40L mRNA and co-cultured with IFN- $\gamma$  require the presence of PGE<sub>2</sub> to enable chemokine dependent migration. DCs were transfected with a titration of CD40L mRNA and immediately incubated with 1000 U/ml IFN- $\gamma$  and 1  $\mu$ g/ml PGE<sub>2</sub>. DCs transfected with eGFP and matured with a cytokine cocktail containing PGE<sub>2</sub> represent a positive control. After 18 hrs of maturation, DCs from each  
30 culture condition were tested in “transwell” migration assays against the lymph node homing chemokines, CCL19 and 21. DC migration was proportional to the size of the CD40L mRNA payload.

**Figure 9** shows that DCs matured via transfection with CD40L mRNA and cultured in the presence of IFN- $\gamma$  and PGE<sub>2</sub> invoke efficient T-cell “recall responses” when compared to DCs matured in the presence of the “cytokine cocktail”. DCs were co-transfected with 2  $\mu$ g flu M1 mRNA per million cells as antigen payload, and 4  $\mu$ g eGFP mRNA control, and 5 subsequently matured with cytokine cocktail. Alternatively, DCs were co-transfected with 2  $\mu$ g flu M1 mRNA per million cells as antigen payload, and 4  $\mu$ g CD40L mRNA as the maturation payload. These latter cells were immediately cultured in 1000 U/ml IFN- $\gamma$  and 1  $\mu$ g/ml PGE<sub>2</sub> to complete the maturation process. After 24hrs, each DC population was used in ELISpot assays to recruit an anti-flu M1 recall responses, as determined by the frequency 10 of responding T-cells secreting IFN- $\gamma$ . DCs matured by transfection with CD40L mRNA in the presence of IFN- $\gamma$  and PGE<sub>2</sub> invoked a more potent anti-flu response.

**Figure 10** shows that DCs matured via transfection with CD40L mRNA and cultured in the presence of IFN- $\gamma$  and PGE<sub>2</sub> invoke efficient “primary T-cell responses” when compared to DCs matured in the presence of the “cytokine cocktail”. DCs were transfected 15 with 2  $\mu$ g MART-APL mRNA per million cells as antigen payload, and subsequently matured with cytokine cocktail. Alternatively, DCs were co-transfected with 2  $\mu$ g MART-APL mRNA per million cells as antigen payload, and 4  $\mu$ g CD40L mRNA as the maturation payload. These latter cells were immediately cultured in 1000 U/ml IFN- $\gamma$  and 1  $\mu$ g/ml PGE<sub>2</sub> to complete the maturation process. After 24 hrs, each DC population was used to raise T- 20 cell responses to MART-APL peptide sequences, generated from the transfected MART-APL mRNA payload, by co-culture of autologous naive CD8+ T-cells for 7 days in the presence of 0.2 U/ml of IL-2. After this first round of stimulation, T-cells were harvested and established in IL-2 ELISpot assays, restimulated with the appropriately matured, antigen loaded DCs. DCs matured by transfection with CD40L mRNA in the presence of IFN- $\gamma$  and 25 PGE<sub>2</sub> invoked a more potent anti-MART-APL response as determined by the frequency of responder CD8+ Tcells secreting IL-2.

**Figure 11** shows the induction of cytotoxic T-cells by DCs expressing MART-APL mRNA. Figure 11a shows that maturation of DCs using co-transfection with MART-APL mRNA as source of antigen, and CD40L mRNA, with the addition of soluble interferon- $\gamma$ /PGE<sub>2</sub> invokes an effective CTL response, whereas Figure 11b shows that DCs transfected 30 with MART-APL mRNA, but matured with a ‘cytokine cocktail’, do not. T2-PSA: T2 cells

pulsed with an HLA-A2 restricted peptide from prostate-specific antigen (PSA) as a negative control target. MART-T2: T2 cells pulsed with the HLA-A2 restricted MART epitope in its native sequence. MART-APL-T2: T2 cells pulsed with the HLA-A2 restricted MART epitope as the preferred 'altered peptide ligand'.

5 **Figure 12** shows the migratory capacity of PME-CD40L matured DCs in transwell assays to the lymph node chemokines, CCL19 and 21. Four independent healthy donors were tested in parallel, with each DC preparation being transfected with 1ug amplified total RCC tumor RNA, along with 4 ug CD40L RNA per million DCs. Migration assays were set up 24 hrs post transfection with the mRNA payloads.

10 **Figure 13** shows the induction of CTL responses from a healthy donor to the melanoma-associated antigen, MART-1. DCs were prepared and loaded with MART-1 RNA and matured via the 'CD40L base process' or DCs were prepared using the PME-CD40L process. DCs and purified CD8 T-cells were co-cultured in a 1:10 ratio, undergoing three rounds of stimulation in the presence of IL-2. The data shows  $^{51}\text{Cr}$  release cytotoxic assays 15 using MART-1 peptide pulsed T2 target cells across a range of effector-target ratios.

15 **Figure 14** shows the induction of a fully autologous CTL response to DCs loaded with total amplified RCC tumor RNA, PME-CD40L matured DCs. DCs and purified CD8 T-cells were co-cultured in a 1:10 ratio, undergoing three rounds of stimulation in the presence of IL-2. 5 days after the last stimulation, CD8 T-cells were restimulated with DCs 20 transfected with: total amplified RCC RNA, hTERT RNA, Survivin RNA, G250 RNA or negative control DCs transfected with eGFP RNA. The data is derived from identifying responder T-cells by cell surface staining for the activation marker, CD69, and simultaneously detection of intracellular IFN- $\gamma$  and IL-2. Intracellular cytokine responses were subdivided to identify IFN- $\gamma$  single positive (effector cells) from IFN- $\gamma$ /IL-2 double 25 positive (memory cells).

25 **Figure 15** shows the expansion of NKT-cells (a) and MART-1 reactive CTLs (b) by MART-1 RNA transfected CD40L base process matured DCs pulsed with KRN7000 or vehicle. The data clearly shows that KRN7000 pulsed DC can expand NKT-cells as defined by CD1d/KRN7000 tetramer staining, and that the presence of an expanded population of 30 NKT-cells can increase the concomitant recruitment of primary CTLs to MART-1, as defined by tetramer staining with MART-1/HLA-A2 tetramers.

Figure 16 shows the alignment of the human (SEQ ID NO:1) and mouse (SEQ ID NO:9) CD40L cDNAs. Figures 16A, 16B and 16C represent 3 consecutive pages of the alignment of SEQ ID NO:1 and 2.

Figure 17 shows the alignment of the human (SEQ ID NO:2) and mouse (SEQ ID NO:10) CD40L proteins.

Figure 18 shows the level of IL-12 expression by DC transfected with mRNA transcribed from pCR2.1 CD40L WT Delta X-E plasmid in 100 ug scale (Delta X-E1) or 1 mg scale (Delta X-E2) transcription reactions using mMessage mMachine T7 Ultra kit (Ambion). Reference RNA was transcribed from plasmid pCR2.1 CD40L WT. The transcribed CD40L RNAs were modified by addition of polyA tail using polyA plus kit (Epicentre). RNAs were transfected into DCs. Approximately 20 hrs post transfection the amount of IL-12 was measured in the supernatant of the matured DCs using Elisa. Negative: IL-12 expression measure in supernatant of DCs electroporated without any CD40L RNA.

#### MODES FOR CARRYING OUT THE INVENTION

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Australia or any other country.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. These methods are described in the following publications. See, e.g., Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2<sup>nd</sup> edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Ausubel *et al.* eds. (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); PCR: A PRACTICAL APPROACH (M. MacPherson *et al.* IRL Press at Oxford University Press (1991)); PCR 2: A PRACTICAL APPROACH (MacPherson, Hames and Taylor eds. (1995));

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ANTIBODIES, A LABORATORY MANUAL (Harlow and Lane eds. (1988)); USING  
ANTIBODIES, A LABORATORY MANUAL (Harlow and Lane eds. (1999)); and ANIMAL  
CELL

CULTURE (Freshney ed. (1987)).

**Definitions**

As used in the specification and claims, the singular form "a," "an" and "the" include 5 plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any 10 essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the 15 compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

All numerical designations, e.g., pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied (+) or (-) by increments of 0.1. It is to be understood, although not always explicitly stated, that the reagents described 20 herein are merely exemplary and that equivalents of such are known in the art.

The term "antigen" is well understood in the art and includes substances which are immunogenic, i.e., immunogen. It will be appreciated that the use of any antigen is envisioned for use in the present invention and thus includes, but is not limited to a self-antigen (whether normal or disease-related), an infectious antigen (e.g., a microbial antigen, 25 viral antigen, etc.), or some other foreign antigen (e.g., a food component, pollen, etc.). The term "antigen" or alternatively, "immunogen" applies to collections of more than one immunogen, so that immune responses to multiple immunogens may be modulated simultaneously. Moreover, the term includes any of a variety of different formulations of immunogen or antigen.

30 A "native" or "natural" or "wild-type" antigen is a polypeptide, protein or a fragment which contains an epitope, which has been isolated from a natural biological source, and which can specifically bind to an antigen receptor, when presented as an MHC/peptide

complex, in particular a T cell antigen receptor (TCR), in a subject.

The term "tumor associated antigen" or "TAA" refers to an antigen that is associated with a tumor. Examples of well known TAAs include gp100, MART and MAGE.

The terms "major histocompatibility complex" or "MHC" refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. In humans, the MHC is also known as the "human leukocyte antigen" or "HLA" complex. The proteins encoded by the MHC are known as "MHC molecules" and are classified into Class I and Class II MHC molecules. Class I MHC molecules include membrane heterodimeric proteins made up of an  $\alpha$  chain encoded in the MHC noncovalently linked with the  $\beta_2$ -microglobulin. Class I MHC molecules are expressed by nearly all nucleated cells and have been shown to function in antigen presentation to CD8 $^+$  T cells. Class I molecules include HLA-A, B, and C in humans. Class II MHC molecules also include membrane heterodimeric proteins consisting of noncovalently associated  $\alpha$  and  $\beta$  chains. Class II MHC molecules are known to function in CD4 $^+$  T cells and, in humans, include HLA-DP, -DQ, and -DR.

The term "antigen presenting cells (APCs)" refers to a class of cells capable of presenting one or more antigens in the form of peptide-MHC complex recognizable by specific effector cells of the immune system, and thereby inducing an effective cellular immune response against the antigen or antigens being presented. APCs can be intact whole cells such as macrophages, B-cells, endothelial cells, activated T-cells, and dendritic cells; or other molecules, naturally occurring or synthetic, such as purified MHC Class I molecules complexed to  $\beta_2$ -microglobulin. While many types of cells may be capable of presenting antigens on their cell surface for T-cell recognition, only dendritic cells have the capacity to present antigens in an efficient amount to activate naive T-cells for cytotoxic T-lymphocyte (CTL) responses.

The term "dendritic cells (DCs)" refers to a diverse population of morphologically similar cell types found in a variety of lymphoid and non-lymphoid tissues, Steinman (1991) Ann. Rev. Immunol. 9:271-296. Dendritic cells constitute the most potent and preferred APCs in the organism. While the dendritic cells can be differentiated from monocytes, they possess distinct phenotypes. For example, a particular differentiating marker, CD14 antigen, is not found in dendritic cells but is possessed by monocytes. Also, mature dendritic cells are not phagocytic, whereas the monocytes are strongly phagocytosing cells. It has been shown

that mature DCs can provide all the signals necessary for T cell activation and proliferation.

The term "immune effector cells" refers to cells capable of binding an antigen and which mediate an immune response. These cells include, but are not limited to, T cells, B cells, monocytes, macrophages, NK cells and cytotoxic T lymphocytes (CTLs), for example

5 CTL lines, CTL clones, and CTLs from tumor, inflammatory, or other infiltrates.

A "naïve" immune effector cell is an immune effector cell that has never been exposed to an antigen capable of activating that cell. Activation of naïve immune effector cells requires both recognition of the peptide:MHC complex and the simultaneous delivery of a costimulatory signal by a professional APC in order to proliferate and differentiate into

10 antigen-specific armed effector T cells.

"Immune response" broadly refers to the antigen-specific responses of lymphocytes to foreign substances. Any substance that can elicit an immune response is said to be "immunogenic" and is referred to as an "immunogen". All immunogens are antigens, however, not all antigens are immunogenic. An immune response of this invention can be

15 humoral (via antibody activity) or cell-mediated (via T cell activation).

As used herein, the term "educated, antigen-specific immune effector cell", is an immune effector cell as defined above, which has previously encountered an antigen. In contrast to its naïve counterpart, activation of an educated, antigen specific immune effector cell does not require a costimulatory signal. Recognition of the peptide:MHC complex is

20 sufficient.

"Activated", when used in reference to a T cell, implies that the cell is no longer in  $G_0$  phase, and begins to produce one or more cytotoxins, cytokines and other related membrane-associated proteins characteristic of the cell type (e.g.,  $CD8^+$  or  $CD4^+$ ), and is capable of recognizing and binding any target cell that displays the particular peptide/MHC complex on its surface, and releasing its effector molecules.

25 As used herein, the term "inducing an immune response in a subject" is a term understood in the art and refers to an increase of at least about 2-fold, or alternatively at least about 5-fold, or alternatively at least about 10-fold, or alternatively at least about 100-fold, or alternatively at least about 500-fold, or alternatively at least about 1000-fold or more in an immune response to an antigen (or epitope) which can be detected or measured, after introducing the antigen (or epitope) into the subject, relative to the immune response (if any) before introduction of the antigen (or epitope) into the subject. An immune response to an

antigen (or epitope), includes but is not limited to, production of an antigen-specific (or epitope-specific) antibody, and production of an immune cell expressing on its surface a molecule which specifically binds to an antigen (or epitope). Methods of determining whether an immune response to a given antigen (or epitope) has been induced are well

5 known in the art. For example, antigen-specific antibody can be detected using any of a variety of immunoassays known in the art, including, but not limited to, ELISA, wherein, for example, binding of an antibody in a sample to an immobilized antigen (or epitope) is detected with a detectably-labeled second antibody (e.g., enzyme-labeled mouse anti-human Ig antibody).

10 "Co-stimulatory molecules" are involved in the interaction between receptor-ligand pairs expressed on the surface of antigen presenting cells and T cells. Research accumulated over the past several years has demonstrated convincingly that resting T cells require at least two signals for induction of cytokine gene expression and proliferation (Schwartz, R.H. (1990) *Science* **248**: 1349-1356 and Jenkins, M.K. (1992) *Immunol. Today* **13**:69-73). One

15 signal, the one that confers specificity, can be produced by interaction of the TCR/CD3 complex with an appropriate MHC/peptide complex. The second signal is not antigen specific and is termed the "co-stimulatory" signal. This signal was originally defined as an activity provided by bone-marrow-derived accessory cells such as macrophages and dendritic cells, the so called "professional" APCs. Several molecules have been shown to enhance co- 20 stimulatory activity. These are heat stable antigen (HSA) (Liu, Y. *et al.* (1992) *J. Exp. Med.* 175:437-445), chondroitin sulfate-modified MHC invariant chain (Ii-CS) (Naujokas, M.F. *et al.* (1993) *Cell* **74**:257-268), intracellular adhesion molecule 1 (ICAM-1) (Van Sechteren, G.A. (1990)]. *Immunol.* **144**:4579-4586), B7-1, and B7-2/B70 (Schwartz, R.H. (1992) *Cell* 71:1065-1068). These molecules each appear to assist co-stimulation by interacting with their

25 cognate ligands on the T cells. Co-stimulatory molecules mediate co-stimulatory signal(s), which are necessary, under normal physiological conditions, to achieve full activation of naïve T cells. One exemplary receptor-ligand pair is the B7 family of co-stimulatory molecule on the surface of APCs and its counterreceptor CD28 or CTLA-4 on T cells (Freeman, *et al.* (1993) *Science* **262**:909-911; Young, *et al.* (1992)]. *Clin. Invest.* **90**:229 and

30 Nabavi, *et al.* (1992) *Nature* **360**:266-268). Other important co-stimulatory molecules are CD40, and CD54. The term "costimulatory molecule" encompasses any single molecule or combination of molecules which, when acting together with a MHC/peptide complex bound

by a TCR on the surface of a T cell, provides a co-stimulatory effect which achieves activation of the T cell that binds the peptide. The term thus encompasses B7, or other co-stimulatory molecule(s) on an antigen-presenting matrix such as an APC, fragments thereof (alone, complexed with another molecule(s), or as part of a fusion protein) which, together with MHC complex, binds to a cognate ligand and results in activation of the T cell when the TCR on the surface of the T cell specifically binds the peptide. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified co-stimulatory molecules (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

5 As used herein, the term "cytokine" refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth or proliferation. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1 $\alpha$ ), interleukin-1L (IL-1L), MIP-11, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO) and flt3 ligand. One embodiment of the present invention includes culture conditions in which an effective amount of IL-1 $\beta$  and/or IL-6 is excluded from the medium. Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA),

10 Genentech (South San Francisco, CA), Amgen (Thousand Oaks, CA), R&D Systems (Minneapolis, MN) and Immunex (Seattle, WA). It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

15 The terms "polynucleotide", "nucleic acid" and "nucleic acid molecule" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides, and/or their analogs. Nucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes, for example, single-stranded,

20 double-stranded and triple helical molecules, a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched

25

30

polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. In addition to a native nucleic acid molecule, a nucleic acid molecule of the present invention may also comprise modified nucleic acid molecules. As used herein, mRNA refers to an RNA that can be translated in a dendritic cell.

5 Such mRNAs typically are capped and have a ribosome binding site (Kozak sequence) and a translational initiation codon.

The term "peptide" is used in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or peptidomimetics. The subunits may be linked by peptide bonds. In another embodiment, the subunit may be linked by other bonds, 10 e.g., ester, ether, etc. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D and L optical isomers, amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein.

15 The term "genetically modified" means containing and/or expressing a foreign gene or nucleic acid sequence which in turn, modifies the genotype or phenotype of the cell or its progeny. In other words, it refers to any addition, deletion or disruption to a cell's endogenous nucleotides.

20 As used herein, "expression" refers to the processes by which polynucleotides are transcribed into mRNA and mRNA is translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA of an appropriate eukaryotic host expression may include splicing of the mRNA. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as 25 the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook *et al.* (1989) *supra*). Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences 30 described in methods known in the art, for example, the methods herein below for constructing vectors in general.

"Under transcriptional control" is a term understood in the art and indicates that

transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operatively linked to an element which contributes to the initiation of, or promotes, transcription. "Operatively linked" refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

5 A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria, or viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, 10 cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

"Gene delivery," "gene transfer," "transfection" and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide into a host cell, irrespective of 15 the method used for the introduction. Transfection refers to delivery of any nucleic acid to the interior of a cell. Gene delivery refers to the delivery of a nucleic acid that may be integrated into the host cell's genome, or that may replicate independently of the host cell genome. Gene delivery or gene transfer does not refer to introduction of an mRNA into a cell. Transfection methods include a variety of techniques such as electroporation, protein-based, lipid-based and cationic ion based nucleic acid delivery complexes, viral vectors, "gene gun" delivery and various other techniques known to those of skill in the art. The introduced polynucleotide can be stably maintained in the host cell or may be transiently expressed. In preferred embodiments, an mRNA is introduced into a DC and is transiently expressed. Stable maintenance typically requires that the introduced polynucleotide either 20 contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

25 A "viral vector" is defined as a recombinantly produced virus or viral particle that 30 comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors, alphavirus vectors and the like. Alphavirus vectors, such as Semliki Forest

virus-based vectors and Sindbis virus-based vectors, have also been developed for use in gene therapy and immunotherapy. See, Schlesinger and Dubensky (1999) *Curr. Opin. Biotechnol.* 5:434-439 and Zaks *et al.* (1999) *Nat. Med.* 7:823-827. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide

- 5 comprising the retroviral genome or part thereof, and a therapeutic gene. As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or nucleic acid sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be
- 10 modified such that it binds to a different host cell surface receptor or ligand to enter the cell. As used herein, "retroviral vector" refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism.

Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form which integrates into

- 15 the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad), pseudo adenoviral or adeno-associated virus (MV), vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a transgene.

Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses,

- 20 including over 50 serotypes. (See, e.g., WO 95/27071). Ads are easy to grow and do not require integration into the host cell genome. Recombinant Ad-derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. (See, WO 95/00655 and WO 95/11984). Wild-type MV has high infectivity and specificity integrating into the host cell's genome. (See, Hermonat and
- 25 Muzyczka (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470 and Lebkowski *et al.* (1988) *Mol. Cell. Biol.* 8:3988-3996).

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are known in the art. Such vectors are capable of transcribing RNA in vitro or in vivo, and are commercially available from sources such as Stratagene (La Jolla,

- 30 CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or in vitro transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons

or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

Gene delivery vehicles also include several non-viral vectors, including

5 DNA/liposome complexes, and targeted viral protein-DNA complexes. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. To enhance delivery to a cell, nucleic acids or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens, e.g., TCR, CD3 or CD4.

10 "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single 15 self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

Stringent hybridization conditions are as follows: Prehybridization of filters containing a nucleic acid of interest is carried out for 8 hrs to overnight at 65°C in buffer 20 composed of 6xSSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 hrs at 65°C, the preferred hybridization temperature, in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20x10<sup>6</sup> cpm of <sup>32</sup>P-labeled probe. Subsequently, filter washes are performed at 37°C for 1 h in a solution containing 2xSSC, 0.01% Ficoll, and 25 0.01% BSA, followed by a wash in 0.1xSSC at 50°C. for 45 min. Following the wash steps, the hybridized probes are detectable by autoradiography. Such methods are well known in the art and cited in Sambrook *et al.*, 1989; and Ausubel *et al.*, 1989.

A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to 30 another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity is determined using the well known BLAST alignment program and the default

parameters. Alternative programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + 5 SwissProtein + SPupdate + PIR. Details of these programs can be found at the following world wide web address: [ncbi.nlm.nih.gov/cgi-bin/BLAST](http://ncbi.nlm.nih.gov/cgi-bin/BLAST).

The term "isolated" means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, are normally associated with in nature. For example, with respect to a polynucleotide, an isolated 10 polynucleotide is one that is separated from the 5' and 3' sequences with which it is normally associated in the chromosome. As is apparent to those of skill in the art, a non-naturally occurring polynucleotide, peptide, polypeptide, protein, antibody, or fragment(s) thereof, does not require "isolation" to distinguish it from its naturally occurring counterpart. In addition, a "concentrated", "separated" or "diluted" polynucleotide, peptide, polypeptide, 15 protein, antibody, or fragment(s) thereof, is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than "concentrated" or less than "separated" than that of its naturally occurring counterpart. A polynucleotide, peptide, polypeptide, protein, antibody, or fragment(s) thereof, which differs from the naturally occurring counterpart in its primary sequence or for example, by its 20 glycosylation pattern, need not be present in its isolated form since it is distinguishable from its naturally occurring counterpart by its primary sequence, or alternatively, by another characteristic such as its glycosylation pattern. Although not explicitly stated for each of the inventions disclosed herein, it is to be understood that all of the above embodiments for each of the compositions disclosed below and under the appropriate conditions, are provided by 25 this invention. Thus, a non-naturally occurring polynucleotide is provided as a separate embodiment from the isolated naturally occurring polynucleotide. A protein produced in a bacterial cell is provided as a separate embodiment from the naturally occurring protein isolated from a eukaryotic cell in which it is produced in nature. A mammalian cell, such as dendritic cell is isolated if it is removed from the anatomical site from which it is found in an 30 organism.

"Host cell," "target cell" or "recipient cell" are intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of

exogenous nucleic acid molecules, polynucleotides and/or proteins. It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. The cells may be prokaryotic or eukaryotic, and

5 include but are not limited to bacterial cells, yeast cells, animal cells, and mammalian cells, e.g., murine, rat, simian or human.

A "subject" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

10 A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative". For example, where the purpose of the experiment is to determine a correlation of an immune response with a particular culture condition, it is generally preferable to use a positive control and a negative control.

By "cancer" is meant the abnormal presence of cells which exhibit relatively

15 autonomous growth, so that a cancer cell exhibits an aberrant growth phenotype characterized by a significant loss of cell proliferation control. Cancerous cells can be benign or malignant. In various embodiments, the cancer affects cells of the bladder, blood, brain, breast, colon, digestive tract, lung, ovaries, pancreas, prostate gland, or skin. The definition of a cancer cell, as used herein, includes not only a primary cancer cell, but also any cell

20 derived from a cancer cell ancestor. This includes metastasized cancer cells, and *in vitro* cultures and cell lines derived from cancer cells. Cancer includes, but is not limited to, solid tumors, liquid tumors, hematologic malignancies, renal cell cancer, melanoma, breast cancer, prostate cancer, testicular cancer, bladder cancer, ovarian cancer, cervical cancer, stomach cancer, esophageal cancer, pancreatic cancer, lung cancer, neuroblastoma, glioblastoma,

25 retinoblastoma, leukemias, myelomas, lymphomas, hepatoma, adenomas, sarcomas, carcinomas, blastomas, etc. When referring to a type of cancer that normally manifests as a solid tumor, a "clinically detectable" tumor is one that is detectable on the basis of tumor mass; e.g., by such procedures as CAT scan, magnetic resonance imaging (MRI), X-ray, ultrasound or palpation. Biochemical or immunologic findings alone may be insufficient to

30 meet this definition.

The term "culturing" refers to the *in vitro* maintenance, differentiation, and/or propagation of cells or in suitable media. By "enriched" is meant a composition comprising

cells present in a greater percentage of total cells than is found in the tissues where they are present in an organism. For example, the enriched cultures and preparations of CD83<sup>+</sup> CCR7<sup>+</sup> DCs and CD83<sup>+</sup> CCR7<sup>+</sup> DCs made by the methods of the invention are present in a higher percentage of total cells as compared to their percentage in the tissues where they are

5 present in an organism (e.g., blood, skin, lymph nodes, etc.).

A “composition” is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

A “pharmaceutical composition” is intended to include the combination of an active 10 agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use in vitro, in vivo or ex vivo.

As used herein, the term “pharmaceutically acceptable carrier” encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents.

15 The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin REMINGTON'S PHARM. SCI., 18th Ed. (Mack Publ. Co., Easton (1990)).

An “effective amount” is an amount sufficient to effect beneficial or desired results, such as enhanced immune response, treatment, prevention or amelioration of a medical 20 condition (disease, infection, etc). An effective amount can be administered in one or more administrations, applications or dosages. Suitable dosages will vary depending on body weight, age, health, disease or condition to be treated and route of administration.

As used herein, “signaling” means contacting an immature or mature dendritic cell 25 with an IFN- $\gamma$  receptor agonist, a TNF- $\alpha$  receptor agonist, a CD40L polypeptide or other CD40 agonist. In one embodiment, such agonists are provided externally, (e.g., in the cell culture medium). In another embodiment, the polypeptide agonist is provided via transfection of an immature or mature dendritic cell with a nucleic acid encoding the polypeptide. Alternatively, a nucleic acid aptamer agonist could be provided in the medium or by transfection. In cases where the polypeptide(s) is provided by transfecting a dendritic cell 30 with a nucleic acid encoding the polypeptide, signaling is effected upon translation of an mRNA encoding the polypeptide, rather than upon transfection with the nucleic acid. In one

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aspect, this invention provides methods for preparing enriched populations of mature dendritic cells (DCs) that induce potent immunostimulatory responses *in vivo* and/or *in vitro*. As used herein, the term “mature dendritic cells” means dendritic cells that demonstrate elevated cell surface expression of co-stimulator molecule CD83, compared to immature DCs (iDCs). Mature DCs of the invention include both CD83<sup>+</sup> CCR7<sup>+</sup> DCs and CD83<sup>+</sup> CCR7<sup>+</sup> DCs. The second signal, a CD40 agonist, can be given to either immature CD83<sup>-</sup> CCR7<sup>-</sup> DCs, or to CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs.

The literature (Schaft 2005, Bonehill 2004) suggests that post maturation electroporation of DCs with antigen-encoding RNA resulted in DCs with greater potency to invoke immune responses. Therefore, methods were developed to alter the ‘CD40L base process’ (sequential IFN- $\gamma$  signaling and CD40L signaling of CD83<sup>-</sup> iDCs), by altering the timing of the CD40L signaling to CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs (post phenotypic maturation). In this embodiment, DCs were first phenotypically matured by adding ‘inflammatory mediators’, IFN- $\gamma$  and TNF- $\alpha$ , and optionally PGE<sub>2</sub>, to the culture medium, and then electroporating with CD40L mRNA, and optionally antigen-encoding mRNA approximately 12-30 hours (preferably about 18 hrs) later. This novel process was named ‘PME-CD40L’, for Post Maturation Electroporation with CD40L to produce CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs. Cells harvested 4 hrs post electroporation and formulated as a vaccine were shown to mediate maximum immunopotency in *in vitro* assays (see examples). As yet a further enhancement, DCs can be pulsed with an activation ligand for NKT-cells, namely  $\alpha$ -galactosylceramide, so as to recruit this population of effector cells to the immune response. NKT-cells display facets of both T-helper and T-cytotoxic cells: NKT-cells can secrete IFN- $\gamma$ , display CD40L, and can secrete granzyme B, the latter to induce apoptosis in target cells. Thus, NKT-cell recruitment can lead to enhanced DC function by virtue of additional NKT-cell CD40L/DC-CD40 interactions, or amplify cell mediated immune responses by secreting helper cytokines, and/or contributing to a direct lytic effect on target cells.

After sequential signaling with the first signal (an IFN- $\gamma$  receptor agonist and/or a TNF- $\alpha$  receptor agonist) to iDCs, and the second signal (a CD40 agonist) to either CD83<sup>-</sup> CCR7<sup>-</sup> iDCs, or to CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs, the resulting DCs demonstrate (i) elevated cell surface expression of co-stimulator molecules CD80, CD83, and CD86, (ii) are CCR7<sup>+</sup>, and (iii) secrete IL-12 p70 polypeptide or protein, and/or secrete significantly reduced levels

(0 to 500 pg/per million DCs) of IL-10. In preferred embodiments, the mature CD83<sup>+</sup> CCR7<sup>+</sup> DCs of the invention produce at least 1000 pg IL-12/10<sup>6</sup> DCs, preferably at least 2000, 3000, 4000, 5000, or 6000 pg IL-12/10<sup>6</sup> DCs, more preferably at least 7000, 8000, 9000 or 10,000 pg IL-12/10<sup>6</sup> DCs, and most preferably at least 12,000, 15,000, 17,000 or 20,000 pg IL-12/10<sup>6</sup> DCs.

5 IL-10 and IL-12 levels can be determined by ELISA of culture supernatants collected at up to 36 hrs post induction of DC maturation from immature DCs. Wierda et al. (2000) *Blood* **96**:2917. Ajdary et al. (2000) *Infection and Immunity* **68**:1760.

Immature DCs can be isolated or prepared from a suitable tissue source containing DC precursor cells and differentiated in vitro to produce immature DC. For example, a 10 suitable tissue source can be one or more of bone marrow cells, peripheral blood progenitor cells (PBPCs), peripheral blood stem cells (PBSCs), and cord blood cells. Preferably, the tissue source is a peripheral blood mononuclear cell (PBMC). The tissue source can be fresh or frozen. In another aspect, the cells or tissue source are pre-treated with an effective amount of a growth factor that promotes growth and differentiation of non-stem or progenitor 15 cells, which are then more easily separated from the cells of interest. These methods are known in the art and described briefly in Romani, *et al.* (1994) *Exp. Med.* **180**:83 and Caux, C. *et al.* (1996) *Exp. Med.* **184**:695. In one aspect, the immature DCs are isolated from peripheral blood mononuclear cells (PBMCs). In a preferred embodiment, the PBMCs are treated with an effective amount of granulocyte macrophage colony stimulating factor (GM- 20 CSF) in the presence or absence of interleukin 4 (IL-4) and/or IL-13, so that the PBMCs differentiate into immature DCs. Most preferably, PBMCs are cultured in the presence of GM-CSF and IL-4 for about 4-7 days, preferably about 5-6 days, to produce immature DCs. In preferred embodiments, the first signal is given at day 4, 5, 6, or 7, and most preferably at day 5 or 6. In addition, GM-CSF as well as IL-4 and/or IL-13 may be present in the medium 25 at the time of the first and/or second signaling.

To increase the number of dendritic precursor cells in animals, including humans, one can pre-treat subjects with substances which stimulate hematopoiesis. Such substances include, but are not limited to G-CSF, and GM-CSF. The amount of hematopoietic factor to be administered may be determined by one skilled in the art by monitoring the cell 30 differential of individuals to whom the factor is being administered. Typically, dosages of factors such as G-CSF and GM-CSF will be similar to the dosage used to treat individuals recovering from treatment with cytotoxic agents. As an example, GM-CSF or G-CSF can be

administered for 4 to 7 days at standard doses prior to removal of source tissue to increase the proportion of dendritic cell precursors. U.S. Patent No. 6,475,483 teaches that dosages of G-CSF of 300 micrograms daily for 5 to 13 days and dosages of GM-CSF of 400 micrograms daily for 4 to 19 days result in significant yields of dendritic cells.

5        The methods of the invention produce an enriched population of mature CD83<sup>+</sup> CCR7<sup>+</sup> dendritic cells that are potent immunostimulatory agents. Specifically, the invention provides a method for preparing mature dendritic cells (DCs), comprising the sequential steps of: (a) signaling isolated immature dendritic cells (iDCs) with a first signal comprising an interferon gamma receptor (IFN- $\gamma$ R) agonist, and optionally a TNF- $\alpha$ R agonist, to

10      produce IFN- $\gamma$ R agonist signaled dendritic cells; and (b) signaling said IFN- $\gamma$ R agonist signaled dendritic cells with a second transient signal comprising an effective amount of a CD40 agonist to produce CCR7<sup>+</sup> mature dendritic cells. The invention further provides CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs and CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs. In preferred embodiments, the CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs and/or the CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs of the invention transiently express CD40L polypeptide. Preferably, CD40L is predominantly localized intracellularly, rather than on the cell surface. Most preferably, at least 60%, at least 70%, at least 80% or at least 90% of CD40L polypeptide is localized intracellularly.

In an alternative embodiment, the immature dendritic cells are signaled with an effective amount of a TNF- $\alpha$  receptor agonist followed by signaling with a CD40 agonist.

20      Thus, the invention provides a method for preparing mature dendritic cells (DCs), comprising sequentially signaling isolated immature dendritic cells with a first signal comprising a tumor necrosis factor alpha receptor (TNF- $\alpha$ R) agonist followed by a second signal comprising a CD40 agonist, wherein said signaling is in the absence of an effective amount of IL-1 $\beta$  and/or IL-6.

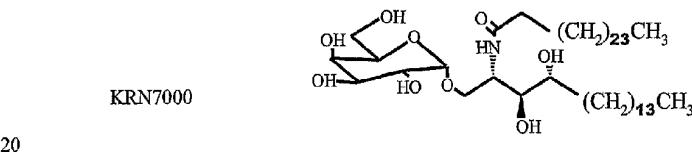
25      For either embodiment (IFN- $\gamma$ R agonist or TNF- $\alpha$ R agonist as a first signal), the second CD40 agonist signal can be given to either CD83<sup>+</sup> CCR7<sup>+</sup> iDCs, or to CD83<sup>+</sup> CCR7<sup>+</sup> mature DCs. In a preferred embodiment, the immature DCs and/or mature DCs are contacted with PGE<sub>2</sub>. Preferably the cells are contacted with PGE<sub>2</sub> at about the same time that they receive the first signal (an IFN- $\gamma$ R agonist or TNF- $\alpha$ R agonist). In preferred embodiments, 30      GM-CSF and at least one of IL-4 or IL-13 is present in the medium at the time the dendritic cells receive the first and second signals. In further embodiments, the method further

comprises contacting the immature dendritic cells, signaled dendritic cells, and/or CCR7<sup>+</sup> dendritic cells with a NKT cell ligand that can activate CD1d-restricted NKT cells and consequently potentiate innate and adoptive immunity. In preferred embodiments, the NKT cell ligand is a compound selected from the group consisting of:  $\alpha$ -galactosylceramides,

5  $\alpha$ -glucosylceramides,  $\alpha$ -6-deoxygalactosylceramides,  
 $\alpha$ -6-deoxygalactofuranosylceramides,  
 $\beta$ -6-deoxygalactofuranosylceramides,  $\alpha$ -arabinosylceramides,  $\alpha$ -C-galactosylceramides and  $\alpha$ -S-galactosylceramides. A preferred compound is the  $\alpha$ -galactosylceramide known as KRN7000 ((2S, 3S, 4R)-1-O-(alpha-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol).

Agelasphins, disclosed in JP patent 3068910, are a class of compounds originally discovered in a marine sponge which have an  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) structure and immunostimulating and anti-tumor activity. KRN7000 is a potent synthetic analog of agelasphins, disclosed in U.S. 5,767,092, the contents of which is incorporated by reference.

15 Additional useful analogs of agelasphins are disclosed U.S. 5,936,076, the contents of which is incorporated by reference. The structure of KRN7000 is shown below:

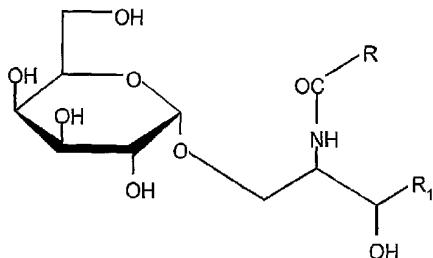


Glycosylceramide analogs of KRN7000 (e.g.,  $\alpha$ -galactosylceramides,  $\alpha$ -glucosylceramides,  $\alpha$ -6-deoxygalactosylceramides,  $\alpha$ -6-deoxygalactofuranosylceramides,  $\beta$ -6-deoxygalactofuranosylceramides,  $\beta$ -arabinosylceramides) are disclosed in U.S. 25 5,849,716, the contents of which is incorporated by reference. U.S. 5,780,441, the contents of which is incorporated by reference, discloses oligosaccharide (di-, tri-, tetra-, penta-) derivatives of KRN7000. Methods for using KRN7000 and related analogs to produce KRN7000 antigen loaded DCs, and to activate human NKT cells are disclosed in U.S. Ser No. 09/721,768 and U.S. 6,531,453, the contents of each are specifically incorporated by

reference.

U.S. 5,936,076, the contents of which is incorporated by reference, discloses  $\alpha$ -galactosylceramide compounds represented by the following formula:

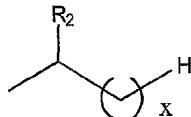
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10

wherein the fatty acid chain, R represents:

15



where  $R_2$  represents H or OH and  $X$  denotes an integer of 0-26 or R represents  $-(CH_2)_7CH=CH(CH_2)_7CH_3$  and

$R_1$  represents any one of the substituents defined by the following (a)-(e)

20

- (a)  $-CH_2(CH_2)_YCH_3$
- (b)  $-CH(OH)(CH_2)_YCH_3$
- (c)  $-CH(OH)(CH_2)_YCH(CH_3)_2$
- (d)  $-CH=CH(CH_2)_YCH$
- (e)  $-CH(OH)(CH_2)_YCH(CH_3)CH_2CH_3$

25

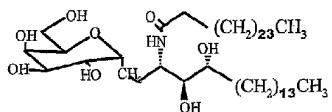
Wherein Y denotes an integer 5-17.

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WO 03/105769, U.S. 2004/0127429, the contents of which are incorporated by reference, and Shimieg J. *et al.*, (2003) *J. Exp. Med.* **198**:1631-1641 disclose the structure of  $\alpha$ -C-glycolipids, where the oxygen atom on glycoside bond of  $\alpha$ -glycosylceramides such as  $\alpha$ -galactosylceramide and  $\alpha$ -glucosylceramides is replaced by carbon atom. The structure of 5 a representative compound is shown below.

## C-GalCer

10



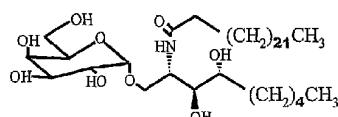
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WO 03/016326, the contents of which is incorporated, disclose KRN7000 analogs with truncated ceramide such as "C4" or "OCH" having the following structure:

## C4 (OCH)

20

25



30

U.S. 6,635,622, the contents of which is incorporated by reference, discloses  $\alpha$ -C-, N, or S-Glycolipids, wherein the oxygen atom on glycoside bond of a galactosylceramide is replaced by  $-(CH_2)_a-CH=CH-(CH_2)_{a'-}$ ,  $-(CH_2)_a-S(O)_{0-2}-CH_2-$ , or  $-NHCH_2-$ , wherein a and a' each denote an integer of 0-5 and a + a' is 5 or less.

35

In preferred embodiments, the IFN- $\gamma$ R agonist is IFN $\gamma$  or a biologically active fragment thereof. Preferably, the IFN $\gamma$  is a mammalian IFN $\gamma$ , most preferably a human IFN $\gamma$ . The cDNA and amino acid sequence of human IFN $\gamma$  are shown in SEQ ID NOs: 5 and 6, respectively. Preferably, the IFN $\gamma$  has the sequence shown in SEQ ID NO:6, or a fragment thereof. In one embodiment, the IFN- $\gamma$ R comprises a polypeptide having at least 80% sequence identity with SEQ ID NO:6. Preferably, the IFN- $\gamma$ R agonist has at least 85%.

90%, 95%, 97%, 98% or 99% sequence identity with SEQ ID NO:6. Methods for testing the activity of IFN- $\gamma$ R agonists are known to those of skill in the art, and some of these methods are described below. Immature DCs can be signaled by adding an IFN- $\gamma$ R agonist to the culture medium, or by expressing the IFN- $\gamma$ R agonist in the dendritic cell. In one embodiment, the

5 DC is transfected with an mRNA encoding an IFN- $\gamma$ R agonist, such as SEQ ID NO:6, or a biologically active fragment thereof. Signaling would then occur upon translation of the mRNA within the dendritic cell. Most preferably, the IFN- $\gamma$ R agonist is added to the culture medium containing immature DCs. In a preferred embodiment, the culture medium further comprises PGE<sub>2</sub> and/or GM-CSF plus IL-4 or IL-13.

10 The receptor for IFN- $\gamma$  has two subunits: IFN- $\gamma$ R1, the ligand-binding chain (also known as the  $\alpha$  chain) and IFN- $\gamma$ R2, the signal-transducing chain (also known as the  $\beta$  chain or accessory factor 1). These proteins are encoded by separate genes (*IFNGR1* and *IFNGR2*, respectively) that are located on different chromosomes. As the ligand-binding (or  $\alpha$ ) chains interact with IFN- $\gamma$  they dimerize and become associated with two signal-transducing (or  $\beta$ ) chains. Receptor assembly leads to activation of the Janus kinases JAK1 and JAK2 and phosphorylation of a tyrosine residue on the intracellular domain of IFN- $\gamma$ R1. This leads to the recruitment and phosphorylation of STAT1 (for 'signal transducers and activators of transcription'), which forms homodimers and translocates to the nucleus to activate a wide range of IFN- $\gamma$ -responsive genes. After signaling, the ligand-binding chains are internalized

15 and dissociate. The chains are then recycled to the cell surface. Bach *et al.* (1997) *Ann. Rev. Immunol.* 15, 563-591; and Lammes, Casanova and Kumararatne (2000) *Clin Exp Immunol* 121, 417-425. The crystal structure of the complex of human IFN- $\gamma$  with the soluble, glycosylated extracellular part of IFN- $\gamma$ R $\alpha$  (sIFN- $\gamma$ R $\alpha$ ) has been determined at 2.9 Å resolution using multiwavelength anomalous diffraction methods. Thiel *et al.* *Structure*

20 8:927-936 (2000).

25 In one assay, INF- $\gamma$  receptor agonists, such as IFN- $\gamma$  decrease Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in a time- and concentration-dependent manner in human intestinal epithelial Caco-2 cells. Na<sup>+</sup>-K<sup>+</sup>-ATPase activity can be determined as the difference between total and ouabain-sensitive ATPase. Treatment with IFN- $\gamma$  markedly increases the expression of total

30 and phospho-STAT1, this being accompanied by activation of p38 MAPK. p38 MAP kinase activity can be analyzed by Western blotting using the p38 MAP kinase assay kit. Total and

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phosphorylated STAT1 protein levels were detected using the PhosphoPlus® Stati. The transduction mechanisms set into motion by IFN- $\gamma$  involve the activation of PKC downstream STAT1 phosphorylation and Raf-1, MEK, ERK2 and p38 MAPK pathways. See Magro *et al.*, *Br J Pharmacol* advance online publication, July 26, 2004;

5 doi:10.1038/sj.bjp.0705895, the contents of which is incorporated by reference.

For the purpose of illustration, signaling with IFN- $\gamma$  receptor agonists, TNF- $\alpha$  receptor agonists and/or CD40 agonists can be provided by contacting a cell directly with IFN- $\gamma$  polypeptides and/or proteins and/or TNF- $\alpha$  polypeptides or proteins and/or CD40 agonists, respectively. Alternatively, signaling of a cell with IFN- $\gamma$ R agonists, TNF- $\alpha$ R agonists or CD40 agonists can occur upon translation of mRNA encoding such polypeptides or proteins within the dendritic cell. Thus, signaling occurs upon expression of IFN- $\gamma$ R agonist, TNF- $\alpha$ R agonist and CD40 agonist polypeptides and/or proteins.

10 The second signal used in the methods of the invention is a transient signal with a CD40 agonist. Persistent expression of a CD40 agonist polypeptide, such as constitutive expression of CD40L from a lentiviral vector as described by Koya *et al.*, *supra*, is not considered transient expression. The signal can be considered transient if the medium containing a CD40 agonist is removed from the DCs, or if the DCs are loaded with an mRNA encoding a CD40 agonist. The CD40 agonist signal can also be considered transient if the DCs are loaded/transfected with or with an expression vector encoding a CD40 agonist,

15 20 provided that either: 1) the promoter driving CD40 agonist expression is not constitutive in DCs, or 2) the expression vector does not integrate into the DC genome or otherwise replicate in DCs.

In preferred embodiments, the CD40 agonist is a CD40L polypeptide or a CD40 agonistic antibody. In general, ligands that bind CD40 may act as a CD40 agonist.

25 Applicants have demonstrated that administration of a second signal comprising CD40L to the cells by transfection of immature or mature DCs with CD40L mRNA produces subsequently modified DCs that induce immunostimulatory responses rather than immunosuppressive. In one embodiment, CD40L mRNA transfected dendritic cells are cultured in medium containing IFN $\gamma$  (and preferentially PGE<sub>2</sub> as well) immediately after

30 transfection and prior to translation of the CD40L mRNA to produce an effective amount of a CD40L signal. In this embodiment, although IFN $\gamma$  is added after transfection with CD40L

mRNA, the dendritic cells receive the IFN $\gamma$  signal prior to the signal resulting upon translation of the CD40L mRNA. Thus, the order in which the agents are delivered to the cells is important only in that CD40L signaling must occur after IFN- $\gamma$  signaling. As described in more detail below, the signaling of the DCs can occur *in vivo* or *ex vivo*, or 5 alternatively one or more set may occur *ex vivo* and the remaining steps of the method can occur *in vivo*.

In one embodiment, the CD40 agonist is an aptamer that binds CD40. Similarly, IFN- $\gamma$  and TNF- $\alpha$  could be replaced by aptamers, antibodies, and the like, that have a similar biological activity. Most preferably, the CD40 agonist is delivered as mRNA encoding 10 CD40L.

As used herein, "CD40 Ligand" (CD40L) shall encompass any polypeptide or protein that specifically recognizes and activates the CD40 receptor and activates its biological activity. The term includes transmembrane and soluble forms of CD40L. In preferred embodiments, the CD40 agonist is a mammalian CD40L, preferably a human CD40L. 15 Alignments of the human and mouse cDNAs and proteins are shown in Figures 16 and 17, respectively. A human CD40L cDNA and the corresponding amino acid sequence are shown in SEQ ID NOS:1 and 2, respectively. The open reading frame for CD40L is represented by nucleotides 40 to 822 of SEQ ID NO:1, while the TGA stop codon is at position 823 to 825. Also useful in the methods of the invention are truncated CD40L (residues 47 to 261 of SEQ 20 ID NO:2, encoded by nucleotide residues 178 to 825 of SEQ ID NO:1) and CD40L fragments encoded by nucleotides 43 to 825 of SEQ ID NO:1, 181 to 825 of SEQ ID NO:1, 193 to 825 of SEQ ID NO:1, 376 to 825 of SEQ ID NO:1, 379 to 825 of SEQ ID NO:1 and 400 to 825 of SEQ ID NO:1. In preferred embodiments, the CD40L polypeptide is selected from the group consisting of: a) a polypeptide comprising SEQ ID NO:2; b) a polypeptide 25 comprising amino acid residues 47 through 261 of SEQ ID NO:2; c) a polypeptide comprising amino acid residues 51 through 261 of SEQ ID NO:2; d) a polypeptide comprising amino acid residues 120 through 261 of SEQ ID NO:2; e) a polypeptide comprising amino acid residues 113 through 261 of SEQ ID NO:2; f) a polypeptide comprising amino acid residues 112 through 261 of SEQ ID NO:2; g) a polypeptide 30 comprising SEQ ID NO:10; h) a polypeptide comprising amino acid residues 35 through 261 of SEQ ID NO:2; i) a polypeptide comprising amino acid residues 34 through 225 of SEQ ID

NO:2; j) a polypeptide comprising amino acid residues 113 through 225 of SEQ ID NO:2; k) a polypeptide comprising amino acid residues 120 through 225 of SEQ ID NO:2; and l) a fragment of the polypeptide of any of (a) through (k), wherein said fragment binds CD40.

Preferably, the CD40L polypeptide is encoded by an mRNA comprising a

5 polynucleotide selected from the group consisting of: a) a polynucleotide of SEQ ID NO:1; b) a polynucleotide comprising nucleotides 40 to 822 of SEQ ID NO:1; c) a polynucleotide comprising nucleotides 178 to 822 of SEQ ID NO:1; d) a polynucleotide comprising nucleotides 190 to 822 of SEQ ID NO:1; e) a polynucleotide comprising nucleotides 397 to 822 of SEQ ID NO:1; f) a polynucleotide comprising nucleotides 376 to 822 of SEQ ID

10 NO:1; g) a polynucleotide of SEQ ID NO:9; h) a polynucleotide of SEQ ID NO:13; i) a polynucleotide having at least 80% sequence identity with any polynucleotide of (a) through (h); j) a polynucleotide hybridizing under stringent conditions to any polynucleotide of (a) through (h); and k) a polynucleotide of (a) through (j), further comprising a 3' untranslated sequence selected from the group consisting of the nucleic acids of SEQ ID NO:14, 15, 16,

15 17 or 18, and/or a 5' untranslated sequence selected from the group consisting of the nucleic acids of SEQ ID NO:19, 20, 21, 22, or 23.

Alternatively, the CD40L polypeptide is a polypeptide having at least 77% sequence identity to a polypeptide selected from the group consisting of: a) a polypeptide comprising SEQ ID NO:2; b) a polypeptide comprising amino acid residues 47 through 261 of SEQ ID

20 NO:2; c) a polypeptide comprising amino acid residues 51 through 261 of SEQ ID NO:2; d) a polypeptide comprising amino acid residues 120 through 261 of SEQ ID NO:2; e) a polypeptide comprising amino acid residues 113 through 261 of SEQ ID NO:2; f) a polypeptide comprising amino acid residues 112 through 261 of SEQ ID NO:2; g) a polypeptide comprising SEQ ID NO:10; h) a polypeptide comprising amino acid residues 35

25 through 261 of SEQ ID NO:2; i) a polypeptide comprising amino acid residues 34 through 225 of SEQ ID NO:2; j) a polypeptide comprising amino acid residues 113 through 225 of SEQ ID NO:2; k) a polypeptide comprising amino acid residues 120 through 225 of SEQ ID NO:2; and l) a fragment of the polypeptide of any of (a) through (k), wherein said fragment binds CD40.

30 CD40 was first characterized as a receptor expressed on B lymphocytes. Schonbeck and Libby (2001) *Cell Mol. Life Sci.* **58**:4. It was later discovered that engagement of B-cell

CD40 with CD40L expressed on activated T-cells is essential for T-cell dependent B-cell activation (i.e. proliferation, immunoglobulin secretion, and class switching). It was subsequently revealed that functional CD40 is expressed on a variety of cell types other than B-cells, including hematopoietic progenitor cells, T lymphocytes, basophils, eosinophils, 5 monocytes/macrophages, dendritic cells, epithelial cells, endothelial cells, smooth muscle cells, keratinocytes, fibroblasts and carcinomas. Schonbeck and Libby (2001) *supra*.

The CD40 Ligand was cloned in 1993 and reported by Gauchat, *et al.* (1993) FEBS Lett. **315**:259. Graf *et al.* mapped it to chromosome Xq26.3-q27.1 (Graf, *et al.* (1992) Eur. J. Immunol. **22**: 3191-3194). Shorter soluble forms of the cell-associated full-length 39 kDa 10 form of CD40 Ligand have been described with molecular weights of 33, and 18 kDa. Graf, *et al.* (1995) Eur. J. Immunol. **25**: 1749; Ludewig, *et al.* (1996) Eur. J. Immunol. **26**: 3137; Wykes, *et al.* (1998) Eur. J. Immunol. **28**:548. The 18 kDa soluble form generated via 15 intracellular proteolytic cleavage, which lacks the cytoplasmic tail, the transmembrane region and parts of the extracellular domain, but conserves the CD40 binding domain retains the ability to bind to CD40 receptor and therefore is an example of a CD40 receptor signaling agent. Graf, *et al.* (1995) *supra*.

U.S. Patent No. 5,981,724 discloses DNA sequences encoding human CD40 Ligand (CD40L) as well as vectors, and transformed host cells for the purpose of producing CD40L polypeptides. U.S. Patent No. 5,962,406 discloses DNA sequences encoding soluble forms of 20 human CD40L.

Exemplary sequences of mammalian homologs to CD40L have the following Genbank accession numbers: NM\_204733 (*Gallus gallus* (chicken)); DQ054533 (*Ovis aries* (sheep)); Z48469 (*Bos taurus* (cow)); AY333790 (*Canis familiaris* (dog)); *Macaca nemestrina* (pig-tailed macaque); AF344844 (*Callithrix jacchus* (white-tufted-ear 25 marmoset)); AF34481 (*Cercicebus torquatus atys* (sooty mangabey)); AF344860 (*Aotus trivirgatus* (douroucouli)); AF344859 (*Macaca mulatta* (rhesus monkey)); AF116582 (*Rattus norvegicus* (Norway rat)); and AF079105 (*Felus catus* (cat)).

The CD40 receptor can also be activated by use of CD40 agonist antibodies, antibody fragments, derivatives and variants thereof. CD40 agonist antibodies can be purchased from 30 commercial vendors such as Mabtech (Nacka, Sweden). Examples and methods to generate these agents are also provided infra. The literature also provides examples of CD40 agonist antibodies and antibody fragments. See, e.g., Osada, *et al.* (2002) **25**(2):176 and Ledbetter,

J.A. *et al.* (1997) Crit. Reviews in Immunol. 17:427.

As noted above, the agent having the biological activity of CD40L can be a polypeptide translated from an exogenous polynucleotide (mRNA or DNA) encoding CD40L. For example, the CD40L mRNA has the sequence of SEQ ID NO.: 1 or SEQ ID NO.:3. Alternatively, the cells are signaled with an effective amount of CD40L protein and/or polypeptide, for example, those having the sequence of SEQ ID NO.: 2 or SEQ ID NO.:4. Modified CD40L can also be used in the methods of this invention. For example, CD40L includes those molecules that have been altered through addition, subtraction, or substitution, either conservatively or non-conservatively, of any number of amino acids, provided that the resulting protein binds CD40 on the surface of DC. A "conservative alteration" is one that results in an alternative amino acid of similar charge density, hydrophilicity or hydrophobicity, size, and/or configuration (e.g., Val for Ile). In comparison, a "nonconservative alteration" is one that results in an alternative amino acid of differing charge density, hydrophilicity or hydrophobicity, size and/or configuration (e.g., Val for Phe). The means of making such modifications are well-known in the art and also can be accomplished by means of commercially available kits and vectors (for example, those available from New England Biolabs, Inc., Beverly, Mass.; Clontech, Palo Alto, Calif.).

When the agents are delivered as polynucleotides or genes encoding the agents, an effective amount of the polynucleotide can be replicated by any method known in the art. PCR technology is one means to replicate DNA and is the subject matter of United States Patent Nos. 4,683,195; 4,800,159; 4,754,065; and 4,683,202 and described in PCR: THE POLYMERASE CHAIN REACTION (Mullis *et al.* eds, Birkhauser Press, Boston (1994)) and references cited therein. Additional methods to generate polynucleotides are provided infra.

In embodiments of the invention, wherein immature dendritic cells are stimulated with an agonist of TNF- $\alpha$  receptor, followed by stimulation with a CD40 agonist, the method is performed in the absence of an effective amount of interleukin 1-beta (IL-1 $\beta$ ) and or interleukin 6 (IL-6). Methods for detecting the presence of proteins such as IL-1 $\beta$  and IL-6 are known in the art.

One of skill in the art can determine when the object of the method has been met by sampling a cell or small population of DCs from the population for the presence of mature

DCs expressing CD40L mRNA and/or CD40L polypeptide. In a further aspect, the mature CD83<sup>+</sup> CCR7<sup>+</sup> DCs of the invention express interleukin 12 (IL-12) p35 protein. In a further aspect, mature CD83<sup>+</sup> CCR7<sup>+</sup> DCs express IL-12 p70 protein, and/or express limited IL-10 (not more than 500 pg/ml/10<sup>6</sup> DCs).

5        The steps of the method can be practiced *in vivo* or *ex vivo*. When practiced *ex vivo*, the method can be practiced in an open or closed system. Methods and systems for culturing and enriching cell populations are known in the art. See, examples 1 and 2 of U.S. Patent Publication No. 2004/0072347. See also U.S. Patent Publication No. 2003/0235908, which describes closed systems for cell expansion.

10      In a further aspect, of this invention, the above method is modified by the addition of delivering to the immature or mature DCs an effective amount of an antigen which will be then be processed and presented by the mature DCs. Thus, the methods of the invention further comprise introducing into iDCs, signaled DCs or CCR7<sup>+</sup> mature DCs one or more antigens or a polynucleotide(s) encoding one or more antigens to produce an antigen-loaded

15      CCR7<sup>+</sup> mature DCs. The antigen or antigen-encoding polynucleotide can be introduced prior to said first signal. Alternatively, the antigen or antigen-encoding polynucleotide is delivered subsequent to said first signal and prior to said second signal. In another embodiment, the antigen or polynucleotide is delivered subsequent to said second signal or substantially concurrent with said second signal.

20      For example, antigens include, but are not limited to, pathogens, pathogen lysates, pathogen extracts, pathogen polypeptides, viral particles, bacteria, proteins, polypeptides, cancer cells, cancer cell lysates, cancer cell extracts, cancer cell specific polypeptides. Antigens can be naturally occurring or recombinantly produced. The immunogens can be delivered to the cells as polypeptides, proteins or as nucleic acids using methods known in

25      the art which are briefly described infra. Preferably, one or more polynucleotides encoding one or more antigens are introduced into the iDCs, signaled DCs or CCR7<sup>+</sup> mature DCs. The polynucleotide can be introduced into the DCs by methods known to those of skill in the art. In a preferred embodiment, the polynucleotide is introduced by electroporation. Most preferably, the polynucleotide is an mRNA. In preferred embodiments, the antigen or

30      antigen encoding mRNA is introduced together with an mRNA encoding a CD40 agonist or substantially concurrent with CD40 agonist signaling.

The methods can be further modified by contacting the cell with an effective amount of a cytokine or co-stimulatory molecule, e.g., GM-CSF, IL-4 and PGE<sub>2</sub>. In embodiments where the immature DCs are signaled with a TNF $\alpha$ R agonist followed by signaling with CD40 agonist, effective amounts of IL-1 $\beta$  and/or IL-6 are specifically excluded from the culture.

5 The antigen may be delivered in its "natural" form in that no human intervention was involved in preparing the antigen or inducing it to enter the environment in which it encounters the APC. Alternatively or additionally, the antigen may comprise a crude preparation, for example of the type that is commonly administered in a conventional allergy shot or in a tumor lysate. The antigen may alternatively be substantially purified, e.g., at least about 90% pure.

10 Where the antigen is a peptide, it may be generated, for example, by proteolytic cleavage of isolated proteins. Any of a variety of cleavage agents may be utilized including, but not limited to, pepsin, cyanogen bromide, trypsin, chymotrypsin, etc. Alternatively, 15 peptides may be chemically synthesized, preferably on an automated synthesizer such as is available in the art. Also, recombinant techniques may be employed to create a nucleic acid encoding the peptide of interest, and to express that peptide under desired conditions.

15 The antigen can alternatively have a structure that is distinct from any naturally-occurring compound. In certain embodiments of the invention, the antigen is a "modified antigen" in that the antigen has a structure that is substantially identical to that of a naturally-occurring antigen but that includes one or more deviations from the precise structure of the naturally-occurring compound. For instance, where the naturally-occurring antigen is a protein or polypeptide antigen, a modified antigen as compared with that protein or polypeptide antigen would have an amino acid sequence that differs from that of the 20 naturally-occurring antigen in the addition, substitution, or deletion of one or more amino acids, and/or would include one or more amino acids that differ from the corresponding amino acid in the naturally-occurring antigen by the addition, substitution, or deletion of one or more chemical moieties covalently linked to the amino acid. In one aspect, the naturally-occurring and modified antigens share at least one region of at least 5 amino acids that are at 25 least approximately 75% identical. Those of ordinary skill in the art will appreciate that, in comparing two amino acid sequences to determine the extent of their identity, the spacing

between stretches (i.e., regions of at least two) of identical amino acids need not always be precisely preserved. Naturally-occurring and modified protein or polypeptide antigens can show at least approximately 80% identity, more alternatively 85%, 90%, 95%, or greater than 99% identity in amino acid sequence for at least one region of at least 5 amino acids. Often,

5 it may be useful for a much longer region (e.g., 10, 20, 50, or 100 or more amino acids) of amino acid sequence to show the designated degree of identity.

In preferred embodiments, the antigen is delivered as a polynucleotide or gene encoding the antigen, so that expression of the gene results in antigen production either in the individual being treated (when delivered *in vivo*) or the cell culture system (when delivered 10 *in vitro*). Techniques for generating nucleic acids including an expressible gene, and for introducing such nucleic acids into an expression system in which any protein encoded by the expressible gene will be produced are known in the art and briefly described infra.

Preferrably, an mRNA encoding the antigen is introduced into the DC.

In one embodiment, the immunogen is delivered prior to said first signal, wherein the 15 first signal is an IFN $\gamma$ R agonist or TNF- $\alpha$ R. Alternatively, the immunogen is delivered subsequent to said first signal and prior to said second signal, or the immunogen is delivered subsequent to said second signal. In another embodiment, the immunogen is delivered substantially concurrent with said second signal.

The amount of antigen to be employed in any particular composition or application 20 will depend on the nature of the particular antigen and of the application for which it is being used, as will readily be appreciated by those of skill in the art.

The antigen-loaded dendritic cells are useful for raising an immune response to the antigen(s). Thus, in one aspect, the invention provides a method of raising an immune response in a subject comprising administering to the subject an effective amount of the 25 immunogen loaded CCR7 $^+$  mature DCs. The loaded DCs may be allogeneic or autologous to the subject.

The invention further provides a method of stimulating immune effector cells, comprising culturing said cells in the presence of an antigen loaded CCR7 $^+$  mature DCs produced by the methods of invention to produce stimulated immune effector cells. In 30 another embodiment, the invention provides a method of enhancing immunity in a subject

comprising administering to the subject an effective amount of such stimulated immune effector cells.

In a further aspect of this invention, an effective amount of a cytokine and/or co-stimulatory molecule is delivered to the cells or patient, *in vitro* or *in vivo*. These agents can

- 5 be delivered as polypeptides, proteins or alternatively, as the polynucleotides or genes encoding them. Cytokines, co-stimulatory molecules and chemokines can be provided as impure preparations (e.g., isolates of cells expressing a cytokine gene, either endogenous or exogenous to the cell) or in a "purified" form. Purified preparations are preferably at least about 90% pure, or alternatively, at least about 95% pure, or yet further, at least about 99% pure. Alternatively, genes encoding the cytokines or inducing agents may be provided, so that gene expression results in cytokine or inducing agent production either in the individual being treated or in another expression system (e.g., an *in vitro* transcription/translation system or a host cell) from which expressed cytokine or inducing agent can be obtained for administration to the individual.

15 Where both cytokine and antigen are to be delivered to an individual, they may be provided together or separately. When they are delivered as polypeptides or proteins, they can be delivered in a common encapsulation device or by means of physical association such as covalent linkage, hydrogen bonding, hydrophobic interaction, van der Waals interaction, etc. In an alternative embodiment, the compounds are provided together, genes encoding

20 both are provided. For example, genes for both may be provided as part of the same nucleic acid molecule. In some embodiments, this nucleic acid molecule may be prepared so that both factors are expressed from a single contiguous polynucleotide, as a fusion protein in which the cytokine and the antigen are covalently linked to one another via a peptide bond. Alternatively or additionally, the genes may be linked to the same or equivalent control

25 sequences, so that both genes become expressed within the individual in response to the same stimuli. A wide variety of different control sequences, active in different host cells under different conditions are known in the art. These control sequences, including constitutive control sequences, inducible control sequences, and repressible control sequences, can be used in accordance with the present invention, though inducible or repressible sequences are 30 particularly preferred for applications in which additional control over the timing of gene expression is desired.

It is appreciated by those of skill in the art that administration of cytokine and/or antigen may optionally be combined with the administration of any other desired immune system modulatory factor such as, for example, an adjuvant or other immunomodulatory compound.

5 Antigens can also be delivered in the form of polynucleotides or genes encoding the antigens. The antigens can also be modified by linking a portion of sequence from a first polypeptide (e.g., a first antigen) to a portion of sequence from a second polypeptide (e.g., a second antigen, a signal sequence, a transmembrane domain, a purification handle, etc.) by means of a peptide bond. Those of ordinary skill in the art will appreciate the diversity of  
10 such fusion proteins for use in accordance with the present invention. Recombinant techniques further allow for the ready modification of the amino acid sequence of polypeptide or protein antigens, by substitution, deletion, addition, or inversion of amino acid sequences.

Where the immunogen is a fragment of an antigen, it may be generated, for example, 15 by proteolytic cleavage of isolated proteins. Any of a variety of cleavage agents may be utilized including, but not limited to, pepsin, cyanogen bromide, trypsin, chymotrypsin, etc. Alternatively, peptides may be chemically synthesized, preferably on an automated synthesizer such as is available in the art (see, for example, Stewart *et al.*, *Solid Phase Peptide Synthesis*, 2d. Ed., Pierce Chemical Co., 1984. Also, recombinant techniques may 20 be employed to create a nucleic acid encoding the peptide of interest, and to express that peptide under desired conditions (e.g., in a host cell or an in vitro expression system from which it can readily be purified).

In preferred embodiments, the antigen is from a cancer cell or a pathogen. 25 Preferably, the neoplastic cell is a renal cancer cell, a multiple myeloma cell or a melanoma cell. Preferred pathogens are HIV and HCV. In preferred embodiments, the antigen is delivered to the antigen presenting cell in the form of RNA isolated or derived from a neoplastic cell or a pathogen. Methods for RT-PCR of RNA extracted from any cell (e.g., a neoplastic cell or pathogen cell), and in vitro transcription are disclosed in copending PCT/US05/32710 and U.S. provisional patent application No. 60/525,076, the contents of 30 which are incorporated by reference.

The antigen employed in accordance with the present invention may be a naturally-occurring compound or may alternatively have a structure that is distinct from any naturally-occurring compound. In certain embodiments of the invention, the antigen is a "modified antigen" in that the antigen has a structure that is substantially identical to that of a naturally-occurring antigen but that includes one or more deviations from the precise structure of the naturally-occurring compound.

Also provided by this invention are the enriched populations of mature DCs prepared by any of the methods described herein. Mature DCs prepared by the methods of the invention have enhanced immunostimulatory characteristics. In another aspect, the invention 10 provides a method for storing an enriched population of mature DCs, comprising contacting an enriched dendritic cell population of the invention with a suitable cryopreservative under suitable conditions.

The compositions described herein are useful to raise an immune response in a subject by administering to the subject an effective amount of the enriched population of 15 cells, e.g., DCs, modified DCs, or educated immune effector cells. The cells can be allogeneic or autologous. They can be administered to a subject to raise or induce an immune response in a subject comprising administering to the subject an effective amount of the enriched populations as described above. The cells can be allogeneic or autologous to the subject. They can also be used to educate immune effector cells such as T cells by culturing 20 the immune effector cell in the presence and at the expense of a mature DC of this invention. The educated effector cells can also be used to enhance immunity in a subject by delivering to the subject an effective amount of these cells.

#### Methods for Generating and Delivering Polynucleotides

25 Certain embodiments of this invention require the use of polynucleotides. These can be generated and replicated using any method known in the art, e.g., one of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to replicate the DNA. Alternatively, they can be obtained by providing the linear sequence of the polynucleotide, appropriate primer molecules, chemicals such as enzymes and instructions for their 30 replication and chemically replicating or linking the nucleotides in the proper orientation to obtain the polynucleotides. In a separate embodiment, these polynucleotides are further

isolated. Still further, one of skill in the art can insert the polynucleotide into a suitable replication vector and insert the vector into a suitable host cell (prokaryotic or eukaryotic) for replication and amplification. The DNA so amplified can be isolated from the cell by methods well known to those of skill in the art. A process for obtaining polynucleotides by

- 5 this method is further provided herein as well as the polynucleotides so obtained.

In one embodiment, the agent (e.g., CD40L) is delivered as mRNA. RNA can be obtained by first inserting a DNA polynucleotide into a suitable host cell or preferably, by *in vitro* transcription. The DNA can be inserted by any appropriate method, e.g., by the use of an appropriate gene delivery vehicle (e.g., liposome, plasmid or vector) or by electroporation.

- 10 When the cell replicates and the DNA is transcribed into RNA; the RNA can then be isolated using methods well known to those of skill in the art, for example, as set forth in Sambrook *et al.* (1989) *supra*. For instance, mRNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook, *et al.* (1989) *supra* or extracted by nucleic-acid-binding resins following the accompanying instructions provided
- 15 by manufacturers.

In preferred embodiments the CD40L expression cassette contains a promoter suitable for *in vitro* transcription, such as the T7 promoter or SP6 promoter. Preferably, the *in vitro* transcribed CD40L or CD40 agonist mRNA is optimized for stability and efficiency of translation. For example, SEQ ID NO:13 represents an optimized CD40L mRNA,

- 20 wherein ATG codons in the 5' untranslated region have been altered to avoid incorrect initiation of translation.

mRNA stability and/or translational efficiency can also be increased by including 3'UTRs and or 5'UTRs in the mRNA. Preferred examples of 3'UTRs include those from human CD40,  $\beta$ -actin and rotavirus gene 6. Preferred examples of 5'UTRs include CD40L, 25 and the translational enhancers in the 5'UTRs of Hsp70, VEGF, spleen necrosis virus RU5, and tobacco etch virus.

For example, CD40L expression is normally regulated in part by 3'UTR-mediated mRNA instability, and therefore a large portion of the CD40L 3'UTR is not included in the current CD40L mRNA. CD40L is not normally expressed in DCs. In contrast, the CD40

- 30 Receptor is expressed in DCs and there is no evidence in the literature to indicate that its expression is regulated post-transcriptionally, particularly at the level of mRNA stability.

Including the CD40 Receptor 3'UTR (SEQ ID NO:14, or an active fragment thereof) at the 3' end or region of the CD40L mRNA would give the RNA 3' untranslated sequence similar to naturally occurring CD40 messages without imparting any unwanted regulatory activity.

Beta-Actin is an abundantly expressed gene in human non-muscle cells. The human 5 beta-actin promoter has been widely used to drive gene expression in mammalian cell lines and transgenic mice. Inclusion of the beta-actin 3'UTR plus flanking region has been demonstrated to further increase the level of mRNA accumulation from gene expression constructs containing the beta-actin promoter.

Qin and Gunning (1997) Journal of Biochemical and Biophysical Methods 36 pp. 63-72.

10 SEQ ID NO:15 represents the untranslated region of the final exon of the human beta-actin 3' UTR. SEQ ID NO:16 shows the minimal region of this 3'UTR.

The 3'UTR of the simian rotavirus gene 6 (SEQ ID NO:17) mRNA functions as an enhancer of translation in its capped, non-polyadenylated viral transcript. The 3'UTR has also been shown to enhance translation of a heterologous reporter mRNA in Rabbit

15 reticulocyte lysates. Yang et. al., 2004 Archives of Virology 149:303-321. The minimal functional element of this 3'UTR is shown in SEQ ID NO:18

The 5' UTR of the human hsp70 gene (SEQ ID NO:19) has been shown to increase translation of reporter mRNAs in the absence of stress induction and without dramatically influencing the message stability. Enhancer function has been demonstrated in a number of 20 human cell lines. Vivinus, et al., 2001 European Journal of Biochemistry 268:1908-1917.

The mouse VEGF 5' UTR (SEQ ID NO:20) enhances translation of a monocistronic reporter RNA and also has IRES (Internal Ribosome Entry Site) activity. Its enhancer activity has been demonstrated in rat, hamster and human cell lines. The full length 5'UTR is 1014 nucleotides, but a 163 nucleotide mutant version (SEQ ID NO:21) was shown to be 25 more active. Stein et al., 1998 Molecular and Cellular Biology 18:3112-3119.

The Spleen Necrosis Virus (SNV) is an avian retrovirus. The RU5 region of the viral 5' LTR (SEQ ID NO:22) stimulates translation efficiency of a non-viral reporter RNA in human 293 cells. Roberts and Boris-Lawrie 2000 Journal of Virology 74:8111-8118.

The 143 nucleotide 5' leader of the tobacco etch virus RNA (SEQ ID NO:23) 30 promotes cap-independent translation of reporter mRNAs in plant and animal cell lines. Although the leader sequence does not further enhance the translation of capped transcripts, the cap-independent CD40L expression in dendritic cells is a very attractive alternative to in

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vitro capping. Gallie *et al.* (1995) Gene 165:233-238. Niepel and Gallie (1999) Journal of Virology 73:9080-9088. Gallie, Journal of Virology (2001) 75:12141-12152.

Dendritic cells can be transfected with nucleic acids by methods known in the art, which include, but are not limited to calcium phosphate precipitation, microinjection or electroporation. They can be added alone or in combination with a suitable carrier, *e.g.*, a pharmaceutically acceptable carrier such as phosphate buffered saline. Alternatively or additionally, the nucleic acid can be incorporated into an expression or insertion vector for incorporation into the cells. Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression. Examples of vectors are viruses, such as baculovirus and retrovirus, bacteriophage, adenovirus, adeno-associated virus, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

Among these are several non-viral vectors, including DNA/liposome complexes, and targeted viral protein DNA complexes. To enhance delivery to a cell, the nucleic acid or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. This invention also provides the targeting complexes for use in the methods disclosed herein.

Polynucleotides are inserted into vector genomes using methods known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of restricted polynucleotide. These synthetic linkers contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA.

Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfecants in mammalian cells; enhancer/promoter sequences from the

5 immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColEl for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are known and available in the art.

10

#### **Preparation and Isolation of Proteins and Polypeptides**

Polypeptides and proteins are necessary components of various methods of this invention. The proteins and polypeptides can be obtained by chemical synthesis using a commercially available automated peptide synthesizer such as those manufactured by Perkin

15 Elmer/Applied Biosystems, Inc., Model 430A or 431A, Foster City, CA, USA. The synthesized protein or polypeptide can be precipitated and further purified, for example by high performance liquid chromatography (HPLC). Alternatively, the proteins and polypeptides can be obtained by known recombinant methods as described herein using the host cell and vector systems described below.

20 It is well known to those skilled in the art that modifications can be made to any peptide to provide it with altered properties. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D and L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein. Peptides for use in this invention can be modified to include unnatural amino acids. Thus, the peptides may comprise D-amino acids, a combination of D- and L-amino acids, and various "designer" amino acids (e.g.,  $\beta$ -methyl amino acids, C- $\alpha$ -methyl amino acids, and N- $\alpha$ -methyl amino acids, etc.) to convey special properties to peptides. Additionally, by assigning specific 25 amino acids at specific coupling steps, peptides with  $\alpha$ -helices  $\beta$  turns,  $\beta$  sheets,  $\gamma$ -turns, and 30 cyclic peptides can be generated. In a further embodiment, subunits of peptides that confer useful chemical and structural properties will be chosen. For example, peptides comprising

D-amino acids may be resistant to L-amino acid-specific proteases *in vivo*. Modified compounds with D-amino acids may be synthesized with the amino acids aligned in reverse order to produce the peptides of the invention as retro-inverso peptides. In addition, the present invention envisions preparing peptides that have better defined structural properties, 5 and the use of peptidomimetics, and peptidomimetic bonds, such as ester bonds, to prepare peptides with novel properties. In another embodiment, a peptide may be generated that incorporates a reduced peptide bond, *i.e.*, R<sub>1</sub>-CH<sub>2</sub>NH-R<sub>2</sub>, where R<sub>1</sub> and R<sub>2</sub> are amino acid residues or sequences. A reduced peptide bond may be introduced as a dipeptide subunit. Such a molecule would be resistant to peptide bond hydrolysis, *e.g.*, protease activity. Such 10 molecules would provide peptides with unique function and activity, such as extended half-lives *in vivo* due to resistance to metabolic breakdown, or protease activity. Furthermore, it is well known that in certain systems constrained peptides show enhanced functional activity (Hruby (1982) Life Sciences 31:189-199 and Hruby *et al.* (1990) Biochem 3, 268:249-262); the present invention provides a method to produce a constrained peptide that incorporates 15 random sequences at all other positions.

#### Methods for Isolating Stem Cells

Many methods are known in the art for the isolation and expansion of CD34<sup>+</sup> stem cells for *in vitro* expansion and differentiation into dendritic cells. See for example, U.S. 20 5,199,942, the contents of which is incorporated by reference. The following descriptions are for the purpose of illustration only and in no way are intended to limit the scope of the invention.

CD34<sup>+</sup> stem cells can be isolated from bone marrow cells or by panning the bone marrow cells or other sources with antibodies which bind unwanted cells, such as CD4<sup>+</sup> and 25 CD8<sup>+</sup> (T cells), CD45<sup>+</sup> (panB cells) and GR-1. For a detailed description of this protocol see, Inaba, *et al.* (1992) 3. Exp. Med. 176:1693-1702. Human CD34<sup>+</sup> cells can be obtained from a variety of sources, including cord blood, bone marrow explants, and mobilized peripheral blood. Purification of CD34<sup>+</sup> cells can be accomplished by antibody affinity procedures. See, for example, Paczesny *et al.* (2004) 3 Exp Med. 199: 1503-11; Ho, *et al.* (1995) Stem Cells 30 13 (suppl. 3):100-105; Brenner (1993) Journal of Hematology 2:7-17; and Yu, *et al.* (1995) PNAS 92:699-703.

**Differentiating Stem Cells into Immature Dendritic Cells**

CD34<sup>+</sup> stem cells can be differentiated into dendritic cells by incubating the cells with the appropriate cytokines. Inaba *et al.* (1994) *supra*, described the *in vitro* differentiation of murine stem cells into dendritic cells by incubating the stem cells with murine GM-CSF. In brief, isolated stem cells are incubated with between 1 and 200 ng/ml murine GM-CSF, and preferably about 20 ng/ml GM-CSF in standard RPMI growth medium. The media is changed with fresh media about once every other day. After approximately 5-7 days in culture, a large percentage of cells are dendritic, as assessed by expression of surface markers and morphology. Dendritic cells are isolated by fluorescence activated cell sorting (FACS) or by other standard methods.

Murine CD34<sup>+</sup> stem cells can be differentiated into dendritic cells by culturing the cells with murine GM-CSF. Typically, the concentration of GM-CSF in culture is at least about 0.2 ng/ml, and preferably at least about 1 ng/ml. Often the range will be between about 20 ng/ml and 200 ng/ml. In many preferred embodiments, the dose will be about 100 ng/ml.

IL-4 is optionally added in similar ranges for making murine DCs.

Human CD34<sup>+</sup> hematopoietic stem cells are preferably differentiated *in vitro* by culturing the cells with human GM-CSF and TNF- $\alpha$ . See for example, Szabolcs, *et al.* (1995) 154:5851-5861. Human GM-CSF is used in similar ranges, and TNF- $\alpha$  can also be added to facilitate differentiation. TNF- $\alpha$  is also typically added in about the same ranges. Optionally, SCF or other proliferation ligand (e.g., Flt3) is added in similar dose ranges to differentiate human DCs.

As is apparent to those of skill in the art, dose ranges for differentiating stem cells and monocytes into dendritic cells are approximate. Different suppliers and different lots of cytokine from the same supplier vary in the activity of the cytokine. One of skill can easily titrate each cytokine which is used to determine the optimal dose for any particular cytokine.

**Differentiation of Monocytes into Dendritic Cells**

DCs can be generated from frequent, but non-proliferating CD14<sup>+</sup> precursors (monocytes) in peripheral blood by culture in medium containing GM-CSF and IL-4 or GM-CSF and IL-13 (see, e.g., WO 97/29182). This method is described in Sallusto and Lanzavecchia (1994) *J. Exp. Med.* 179:1109 and Romani *et al.* (1994) *J. Exp. Med.* 180:83. Briefly, CD14<sup>+</sup> precursors are abundant so that pretreatment of patients with cytokines such

as G-CSF (used to increase CD34<sup>+</sup> cells and more committed precursors in peripheral blood) is reported to be unnecessary in most cases (Romani *et al.* (1996) *J. Immunol. Methods* 196:137). Others have reported that DCs generated by this approach appear rather homogenous and can be produced in an immature state or fully differentiated or mature. It 5 was shown that it is possible to avoid non-human proteins such as FCS (fetal calf serum), and to obtain fully and irreversibly mature and stable DCs by using autologous monocyte conditioned medium as maturation stimulus (Romani *et al.* (1996) *Immunol. Methods* 196:137; Bender *et al.* (1996) *J. Immunol. Methods* 196:121). However, in contrast to the instant invention, these studies did not result in mature DC having increased levels of IL-12 10 and/or decreased levels of IL-10.

#### Antigen Loading

Methods of loading dendritic cells with antigens are known to those of skill in the art. In one embodiment, the dendritic cells are cultured in medium containing the antigen. The 15 DCs then take up and process the antigen on the cell surface in association with MHC molecules. Preferably, the DCs are loaded with antigen by transfection with a nucleic acid encoding the antigen. Methods of transfecting DCs are known to those of skill in the art.

#### Isolation of and Expansion of T Cells

20 In some methods of this invention, T cells are isolated from mammals so that they can be educated (or activated) by the mature, modified DC *in vitro*. In one method, Ficoll-Hypaque density gradient centrifugation is used to separate PBMC from red blood cells and neutrophils according to established procedures. Cells are washed with modified AIM-V (which consists of AIM-V (GIBCO) with 2 mM glutamine, 10 µg/ml gentamicin sulfate, 50 25 µg/ml streptomycin) supplemented with 1% fetal bovine serum (FBS). T cells are enriched by negative or positive selection with appropriate monoclonal antibodies coupled to columns or magnetic beads according to standard techniques. An aliquot of cells is analyzed for cell surface phenotype including CD4, CD8, CD3 and CD14. For the purpose of illustration only, cells are washed and resuspended at a concentration of about 5 X 10<sup>5</sup> cells per ml of 30 AIM-V modified as above and containing 5% FBS and 100 U/ml recombinant IL-2 (rIL-2) (supplemented AIM-V). Where the cells are isolated from an HIV<sup>+</sup> patient, 25 nM CD4-PE40 (a recombinant protein consisting of the HIV-1-binding CD4 domain linked to the

translocation and ADP-ribosylation domains of *Pseudomonas aeruginosa* exotoxin A), or other similar recombinant cytotoxic molecule which selectively hybridizes to HIV is added to the cell cultures for the remainder of the cell expansion to selectively remove HIV infected cells from the culture. CD4-PE40 has been shown to inhibit p24 production in HIV-infected

5 cell cultures and to selectively kill HIV-1-infected cells.

To stimulate proliferation, OKT3 monoclonal antibody (Ortho Diagnostics) can be added to a concentration of 10 ng/ml and the cells are plated in 24 well plates with 0.5 ml per well. The cells are cultured at a temperature of about 37°C in a humidified incubator with 5% CO<sub>2</sub> for 48 hours. Media is aspirated from the cells and 1 ml of vector-containing supernatant 10 (described below) supplemented with 5 µl/ml of protamine sulfate, 100 U/ml rIL-2, 100 U/ml penicillin, 0.25 µg/ml amphotericin B/ml and an additional 100 µg/ml streptomycin (25 nM CD4-PE40 can be added).

#### Cell Isolation and Characterization

15 In another aspect, cell surface markers can be used to isolate the cells necessary to practice the method of this invention. For example, human stem cells typically express CD34 antigen while DCs express MHC molecules and costimulatory molecules (e.g., B7-1 and B7-2), a lack of markers specific for granulocytes, NK cells, B cells, and T cells. The expression of surface markers facilitates identification and purification of these cells. These methods of 20 identification and isolation include FACS, column chromatography, panning with magnetic beads, western blots, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, 25 radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like. For a review of immunological and immunoassay procedures in general, see Stites and Terr (eds.) 1991 *Basic and Clinical Immunology* (7th ed.) and Paul *supra*. For a discussion of how to make antibodies to selected antigens see Harlow and Lane (1989) *supra*.

30 Cell isolation or immunoassays for detection of cells during cell purification can be performed in any of several configurations, e.g., those reviewed in Maggio (ed.) (1980) *Enzyme Immunoassay* CRC Press, Boca Raton, Fla.; Tijan (1985) "Practice and Theory of

Enzyme Immunoassays," Laboratory Techniques in Biochemistry and Molecular Biology,

Elsevier Science Publishers B.V., Amsterdam; Harlow and Lane, *supra*; Chan (ed.) (1987)

Immunoassay: A Practical Guide Academic Press, Orlando, Fla.; Price and Newman (eds.)

(1991) Principles and Practice of Immunoassays Stockton Press, NY; and Ngo (ed.) (1988)

5 Non-isotopic Immunoassays Plenum Press, NY.

Cells can be isolated and characterized by flow cytometry methods such a FACS analysis. A wide variety of flow-cytometry methods are known. For a general overview of fluorescence activated flow cytometry see, for example, Abbas *et al.* (1991) Cellular and Molecular immunology W.B. Saunders Company, particularly chapter 3, and Kuby (1992)

10 Immunology W.H. Freeman and Company, particularly chapter 6. FACS machines are available, e.g., from Becton Dickinson.

Labeling agents which can be used to label cell antigen include, but are not limited to monoclonal antibodies, polyclonal antibodies, proteins, or other polymers such as affinity matrices, carbohydrates or lipids. Detection proceeds by any known method, such as

15 immunoblotting, western blot analysis, tracking of radioactive or bioluminescent markers, capillary electrophoresis, or other methods which track a molecule based upon size, charge or affinity.

#### Antibodies

20 Certain aspects of this method require the use of antibodies. Such antibodies can be monoclonal or polyclonal. They can be antibody derivatives or antibody variants. They can be chimeric, humanized, or totally human. Using a protein or a polypeptide one of skill in the art can generate additionally antibodies which specifically bind to the receptor. A functional fragment or derivative of an antibody also can be used including Fab, Fab', Fab2, Fab'2, and 25 single chain variable regions. Antibodies can be produced in cell culture, in phage, or in various animals, including but not limited to cows, rabbits, goats, mice, rats, hamsters, guinea pigs, sheep, dogs, cats, monkeys, chimpanzees, apes, etc. So long as the fragment or derivative retains specificity of binding for the protein or fragment thereof it can be used. Antibodies can be tested for specificity of binding by comparing binding to appropriate 30 antigen to binding to irrelevant antigen or antigen mixture under a given set of conditions. If the antibody binds to the appropriate antigen at least 2, 5, 7, and preferably 10 times more than to irrelevant antigen or antigen mixture then it is considered to be specific.

Techniques for making such partially to fully human antibodies are known in the art and any such techniques can be used. According to one embodiment, fully human antibody sequences are made in a transgenic mouse which has been engineered to express human heavy and light chain antibody genes. Multiple strains of such transgenic mice have been

5 made which can produce different classes of antibodies. B cells from transgenic mice which are producing a desirable antibody can be fused to make hybridoma cell lines for continuous production of the desired antibody. See for example, Russel *et al.* (2000) *Infection and Immunity* April 2000: 1820-1826; Gallo *et al.* (2000) *European J. of Immun.* 30:534-540; Green (1999) *J. of Immun. Methods* 231:11-23; Yang *et al.* (1999A) *J. of Leukocyte Biology* 66:401-410; Yang (1999B) *Cancer Research* 59(6):1236-1243; Jakobovits (1998) *Advanced Drug Delivery Reviews* 31:33-42; Green and Jakobovits (1998) *Exp. Med.* 188(3):483-495; Jakobovits (1998) *Exp. Opin. Invest. Drugs* 7(4):607-614; Tsuda *et al.* (1997) *Genomics* 42:413-421; Sherman-Gold (1997). *Genetic Engineering News* 17:14; Mendez *et al.* (1997) *Nature Genetics* 15:146-156; Jakobovits (1996) WEIR'S HANDBOOK OF

10 EXPERIMENTAL IMMUNOLOGY, THE INTEGRATED IMMUNE SYSTEM VOL. IV, 194.1-194.7; Jakobovits (1995) *Current Opinion in Biotechnology* 6:561-566; Mendez *et al.* (1995) *Genomics* 26:294-307; Jakobovits (1994) *Current Biology* 4:761-763; Arbones *et al.* (1994) *Immunity* 1:247-260; Jakobovits (1993) *Nature* 362:255-258; Jakobovits *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:2551-2555; Kucherlapati, *et al.* U.S. Patent No. 6,075,181.

15 Antibodies can also be made using phage display techniques. Such techniques can be used to isolate an initial antibody or to generate variants with altered specificity or avidity characteristics. Single chain Fv can also be used as is convenient. They can be made from vaccinated transgenic mice, if desired.

20 The antibodies of this invention also can be modified to create chimeric antibodies.

25 Chimeric antibodies are those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species. See, e.g., U.S. Patent No.: 4,816,567.

30 The term "antibody variant" also includes "diabodies" which are small antibody fragments with two antigen-binding sites, wherein fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH VL). See for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448. By using a linker that is too short to allow pairing

between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. *See also*, U.S. Patent No. 6,632,926 to Chen *et al.* which discloses antibody variants that have one or more amino acids inserted into a hypervariable region of the parent antibody and a binding affinity

5 for a target antigen which is at least about two fold stronger than the binding affinity of the parent antibody for the antigen. The term also includes post-translational modification to linear polypeptide sequence of the antibody or fragment. The term "antibody variant" further includes "linear antibodies". The procedure for making such variants is known in the art and described in Zapata *et al.* (1995) Protein Eng. 8(10):1057-1062. Briefly, these antibodies

10 comprise a pair of tandem Fd segments (VH ~CH1-VH ~CH1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

#### Methods to Detect Nucleic Acids

Various methods are known for quantifying the expression of a gene of interest (e.g.

15 CD40L and/or IL-12p35) and include but are not limited to hybridization assays (Northern blot analysis) and PCR based hybridization assays. In assaying for an alteration in mRNA level such as IL-12 p35 mRNA or CD40L mRNA, the nucleic acid contained in a sample can be first extracted. For instance, mRNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook *et al.* (1989), supra or

20 extracted by commercially available nucleic-acid-binding resins following the accompanying instructions provided by the manufacturers. The mRNA contained in the extracted nucleic acid sample can then detected by hybridization (e.g., Northern blot analysis) and/or amplification procedures using nucleic acid probes and/or primers, respectively, according to standard procedures.

25 Nucleic acid molecules having at least 10 nucleotides and exhibiting sequence complementarity or homology to the nucleic acid to be detected can be used as hybridization probes or primers in the diagnostic methods. It is known in the art that a "perfectly matched" probe is not needed for a specific hybridization. Minor changes in probe sequence achieved by substitution, deletion or insertion of a small number of bases do not affect the

30 hybridization specificity. In general, as much as 20% base-pair mismatch (when optimally aligned) can be tolerated. For example, a probe useful for detecting CD40L mRNA is at least about 80% identical to the homologous region of comparable size contained in a previously

identified sequence, e.g., see SEQ ID NOS: 1 or 3. Alternatively, the probe is at least 85% or even at least 90% identical to the corresponding gene sequence after alignment of the homologous region. The total size of fragment, as well as the size of the complementary stretches, will depend on the intended use or application of the particular nucleic acid

5 segment. Smaller fragments of the gene will generally find use in hybridization embodiments, wherein the length of the complementary region may be varied, such as between about 10 and about 100 nucleotides, or even full length according to the complementary sequences one wishes to detect.

Nucleotide probes having complementary sequences over stretches greater than about

10 10 nucleotides in length will increase stability and selectivity of the hybrid, and thereby improving the specificity of particular hybrid molecules obtained. One can design nucleic acid molecules having gene-complementary stretches of more than about 25 and even more preferably more than about 50 nucleotides in length, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by  
15 chemical means, by application of nucleic acid reproduction technology, such as the PCRTM technology with two priming oligonucleotides as described in U.S. Patent No. 4,603,102 or by introducing selected sequences into recombinant vectors for recombinant production.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for detecting  
20 hybridization and therefore complementary sequences. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. A fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents can also be used. In the case of  
25 enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

Hybridization reactions can be performed under conditions of different "stringency". Relevant conditions include temperature, ionic strength, time of incubation, the presence of  
30 additional solutes in the reaction mixture such as formamide, and the washing procedure. Higher stringency conditions are those conditions, such as higher temperature and lower sodium ion concentration, which require higher minimum complementarity between

hybridizing elements for a stable hybridization complex to form. Conditions that increase the stringency of a hybridization reaction are widely known and published in the art. See, Sambrook, *et al.* (1989) *supra*. One can also utilize detect and quantify mRNA level or its expression using quantitative PCR or high throughput analysis such as Serial Analysis of 5 Gene Expression (SAGE) as described in Verculescu *et al.* (1995) *Science* 270:484-487. Briefly, the method comprises isolating multiple mRNAs from cell or tissue samples suspected of containing the transcript. Optionally, the gene transcripts can be converted to cDNA. A sampling of the gene transcripts are subjected to sequence-specific analysis and quantified. These gene transcript sequence abundances are compared against reference 10 database sequence abundances including normal data sets for diseased and healthy patients. The patient has the disease(s) with which the patient's data set most closely correlates and for this application, includes the differential of the transcript.

In certain aspects, it may be necessary to use polynucleotides as nucleotide probes or 15 primers for the amplification and detection of genes or gene transcripts. A primer useful for detecting differentially expressed mRNA is at least about 80% identical to the homologous region of comparable size of a gene or polynucleotide. For the purpose of this invention, amplification means any method employing a primer-dependent polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA-polymerases such as T7 DNA polymerase, Klenow fragment of 20 *E. coli* DNA polymerase, and reverse transcriptase.

General procedures for PCR are taught in MacPherson *et al.*, PCR: A PRACTICAL APPROACH, (IRL Press at Oxford University Press (1991)). However, PCR conditions used for each application reaction are empirically determined. A number of parameters influence 25 the success of a reaction. Among them are annealing temperature and time, extension time,  $Mg^{2+}$  ATP concentration, pH, and the relative concentration of primers, templates, and deoxyribonucleotides.

After amplification, the resulting DNA fragments can be detected by agarose gel 30 electrophoresis followed by visualization with ethidium bromide staining and ultraviolet illumination. A specific amplification of differentially expressed genes of interest can be verified by demonstrating that the amplified DNA fragment has the predicted size, exhibits the predicated restriction digestion pattern, and/or hybridizes to the correct cloned DNA sequence. Other methods for detecting gene expression are known to those skilled in the art.

See, for example, International PCI Application No. WO 97/10365, U.S. Patent numbers 5,405,783, 5,412,087 and 5,445,934, 5,405,783; 5,412,087; 5,445,934; 5,578,832; 5,631,734; and LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY, Vol. 24: Hybridization with Nucleic Acid Probes, Tijssen, ed. Elscvier, N.Y. 5 (1993).

#### Methods for Detecting and Quantifying Protein or Polypeptides

A variety of techniques are available in the art for protein analysis and include, but are not limited to radioimmunoassays, ELISA (enzyme linked immunoradiometric assays), 10 "sandwich" immunoassays, immunoradiometric assays, *in situ* immunoassays (using e.g., colloidal gold, enzyme or radioisotope labels), western blot analysis, immunoprecipitation assays, immunofluorescent assays and PAGE-SDS.

#### Ex Vivo Therapy

15 As noted above, this invention also provides *ex vivo* therapeutic methods using the dendritic cells or educated T cells produced by the methods of this invention. For example, dendritic cells are transformed with an immunogen can be used to activate cytotoxic and helper T cells *in vitro*. Alternatively, the transformed dendritic cells are introduced into a mammal to activate the T cells *in vivo*. Yet further, T cells educated *in vitro* can be 20 introduced into a mammal where they are cytotoxic against target cells bearing antigenic peptides corresponding to those the T cells are activated to recognize on class I MHC molecules. These target cells are typically cancer cells, or infected cells which express unique antigenic peptides on their MHC class I surfaces.

Similarly, helper T-cells, which recognize antigenic peptides in the context of MHC 25 class II, can also be stimulated by the DCs of the invention, which comprise antigenic peptides both in the context of class I and class II MHC. Helper T-cells also stimulate an immune response against a target cell. As with cytotoxic Tcells, helper T-cells are stimulated with the recombinant DCs *in vitro* or *in vivo*.

The dendritic cells and T cells can be isolated from the mammal into which the DCs 30 and/or activated T cells are to administered. Alternatively, the cells can be allogeneic provided from a donor or stored in a cell bank (e.g., a blood bank).

**In Vivo Therapy**

T cells or dendritic cells produced by the methods of this invention can be administered directly to the subject to produce T cells active against a selected immunogen. Administration can be by methods known in the art to successfully deliver a cell into ultimate

5 contact with a subject's blood or tissue cells.

The cells are administered in any suitable manner, often with pharmaceutically acceptable carriers. Suitable methods of administering cells in the context of the present invention to a subject are available, and, although more than one route can be used to administer a particular cell composition, a particular route can often provide a more

10 immediate and more effective reaction than another route. Preferred routes of administration include, but are not limited to intradermal and intravenous administration.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical

15 compositions of the present invention. Most typically, quality controls (microbiology, clonogenic assays, viability tests), are performed and the cells are reinfused back to the subject, preceded by the administration of diphenhydramine and hydrocortisone. See, for example, Korbling *et al.* (1986) Blood 67:529-532 and Haas *et al.* (1990) Exp. Hematol. 18:94-98.

20 Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, intranodal and subcutaneous routes, and carriers include aqueous isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous

25 sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Intradermal and intravenous administration are the preferred method of administration for DCs or T cells of the invention.

The dose of cells (e.g., activated T cells, or dendritic cells) administered to a subject is in an effective amount, effective to achieve the desired beneficial therapeutic response in

30 the subject over time, or to inhibit growth of cancer cells, or to inhibit infection.

For the purpose of illustration only, the method can be practiced by obtaining and

saving blood samples from the subject prior to infusion for subsequent analysis and comparison. Generally at least about  $10^4$  to  $10^6$  and typically, between  $1 \times 10^8$  and  $1 \times 10^{10}$  cells are infused intravenously or intraperitoneally into a 70 kg patient over roughly 60-120 minutes. In one aspect, administration is by intravenous infusion. Vital signs and oxygen

5 saturation by pulse oximetry are closely monitored. Blood samples are obtained 5 minutes and 1 hour following infusion and saved for analysis. Cell re-infusions are repeated roughly every month for a total of 10-12 treatments in a one year period. After the first treatment, infusions can be performed on an outpatient basis at the discretion of the clinician. If the re-infusion is given as an outpatient, the participant is monitored for at least 4 hours following

10 the therapy.

For administration, cells of the present invention can be administered at a rate determined by the effective dose, the LD-50 (or other measure of toxicity) of the cell type, and the side-effects of the cell type at various concentrations, as applied to the mass and overall health of the subject. Administration can be accomplished via single or divided doses.

15 The cells of this invention can supplement other treatments for a condition by known conventional therapy, including cytotoxic agents, nucleotide analogues and biologic response modifiers. Similarly, biological response modifiers are optionally added for treatment by the DCs or activated T cells of the invention. For example, the cells are optionally administered with an adjuvant, or cytokine such as GM-CSF, IL-12 or IL-2.

20

#### **In Vitro Assays and Kits**

The present invention provides commercially valuable kits to practice the maturation methods of the invention. In one aspect, the kit comprises IFN $\gamma$  polypeptide, or an expression cassette for expressing IFN $\gamma$  mRNA in vivo or in vitro, and CD40L polypeptide, or an expression cassette for expressing CD40L mRNA in vivo or in vitro expression of CD40L. In another aspect, the kit comprises TNF $\alpha$  polypeptide, or an expression cassette for expressing TNF $\alpha$  mRNA in vivo or in vitro, and CD40L polypeptide, or an expression cassette for expressing CD40L mRNA in vivo or in vitro expression of CD40L. The kits may further comprise a RNA polymerase for in vitro transcription.

25

#### **Methods to Assess Immunogenicity**

The immunogenicity of the antigen presenting cells or educated T cells produced by

the methods of the invention can be determined by well known methodologies including, but not limited to the following:

5        <sup>51</sup>Cr-release lysis assay. Lysis of peptide-pulsed <sup>51</sup>Cr-labeled targets by antigen-specific T cells can be compared. "More active" compositions will show greater lysis of targets as a function of time. The kinetics of lysis as well as overall target lysis at a fixed timepoint (e.g., 4 hours) may be used to evaluate performance. Ware *et al.* (1983) 3. *Immunol.* 131:1312.

10      Cytokine-release assay. Analysis of the types and quantities of cytokines secreted by T cells upon contacting modified APCs can be a measure of functional activity. Cytokines can be measured by ELISA or ELISPOT assays to determine the rate and total amount of cytokine production. Fujihashi *et al.* (1993) *J. Immunol. Meth.* 160:181; Tanquay and Killion (1994) *Lymphokine Cytokine Res.* 13:259.

15      In vitro T cell education. The compositions of the invention can be assayed for the ability to elicit reactive T cell populations from normal donor or patient-derived PBMC. In this system, elicited T cells can be tested for lytic activity, cytokine-release, polyclonality, and cross-reactivity to the antigenic epitope. Parkhurst *et al.* (1996) *Immunol.* 157:2539.

20      Transgenic animal models. Immunogenicity can be assessed *in vivo* by vaccinating HLA transgenic mice with the compositions of the invention and determining the nature and magnitude of the induced immune response. Alternatively, the hu-PBL-SCID mouse model allows reconstitution of a human immune system in a mouse by adoptive transfer of human PBL. These animals may be vaccinated with the compositions and analyzed for immune response as previously mentioned in Shirai *et al.* (1995) *J. Immunol.* 154:2733; Mosier *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:2443.

25      Proliferation Assays. T cells will proliferate in response to reactive compositions. Proliferation can be monitored quantitatively by measuring, for example, <sup>3</sup>H-thymidine uptake. Caruso *et al.* (1997) *Cytometry* 27:71.

Primate models. A non-human primate (chimpanzee) model system can be utilized to

monitor *in vivo* immunogenicities of HLA-restricted ligands. It has been demonstrated that chimpanzees share overlapping MHC-ligand specificities with human MHC molecules thus allowing one to test HLA-restricted ligands for relative *in vivo* immunogenicity. Bertoni *et al.* (1998) *Immunol.* 161:4447.

5

Monitoring TCR Signal Transduction Events. Several intracellular signal transduction events (e.g., phosphorylation) are associated with successful TCR engagement by MHC-ligand complexes. The qualitative and quantitative analysis of these events have been correlated with the relative abilities of compositions to activate effector cells through TCR engagement.

10 Salazar *et al.* (2000) *Tnt. J. Cancer* 85:829; Isakov *et al.* (1995) *J. Exp. Med.* 181:375).

In accordance with the above description, the following examples are intended to illustrate, but not limit, the various aspects of this invention.

### 15 Experimental Examples

#### Reagents

Histopaque 1077 and Tween 20 were purchased from Sigma (St Louis, MO). PBS and X-VIVO 15 were purchased from Cambrex (East Rutherford, NJ). AIM-V medium, 20 Iscove's modified Dulbecco's medium and RPMI 1640 medium along with Trypan Blue and Fetal Bovine Serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Viaspan was purchased from Dupont Pharma Labs (Wilmington, DE). GM-CSF, IL-4, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IFN- $\gamma$  were all purchased from R&D Systems (Minneapolis, MN). PGE<sub>2</sub> was purchased from Cayman Chemicals (Ann Arbor, CA). Soluble CD40L was purchased from Alexis 25 Biochemicals (San Diego CA). Human AB serum was purchased from Valley Biochemical (Winchester, VA).

Chemokines CCL19 and CCL21 were purchased from Peprotech (Rocky Hill, NJ). Phenotyping antibodies (HLA-ABC, HLA-DR, CD80, CD86, CD83, CD14, and negative isotype controls), ELISpot antibody pairs (IFN- $\gamma$  and IL-2) ELISA sets (IL-12 and IL-10) and 30 streptavidin-HRP were all purchased from BD Pharmingen (San Diego, CA) along with BD Opt EIA reagent set B pH9.5. AEC peroxidase substrate was purchased from Vector labs

(Vector Labs, Burlingame, CA). Blocking anti-CD40L antibody was purchased from eBioscience. CD1d/α-galactosylceramide (KRN7000) tetramer and native KRN7000 were kind gifts from Kirin Brewery, Pharmaceuticals Division, Tokyo, Japan. MART-1/HLA-A201 tetramers were purchased from Beckman-Coulter (Miami, FL)

5

**DC generation**

Human PBMCs were isolated from Leukapheresis collections from healthy volunteers provided by Life Blood (Memphis, Tennessee). PBMCs were prepared by Ficoll-Histopaque density centrifugation and washed four times in PBS at room temperature. 2 x 10<sup>8</sup> PBMCs were re-suspended in 30 ml AIM-V medium and allowed to adhere to 150 cm<sup>3</sup> plastic flasks for 2 hours at 37°C. Non-adherent cells were removed and remaining cells cultured in X-VIVO 15 medium, supplemented with GM-CSF (1000 U/ml) and IL-4 (1000 U/ml), for 5-6 days at 37°C, 5% CO<sub>2</sub>.

15 **Cloning of CD40L**

T cells were stimulated with PMA in RPMI for 1 hr. Cells were harvested and washed with PBS once. Total RNA was extracted using QIAGEN RNeasy procedure. One microgram of total RNA from activated T cells was taken into one tube RT-PCR reaction using Gene Amp Gold kit (Applied Bioscience) using a high fidelity Advantage Polymerase (Clontech). Gene specific primers for CD40L sequence correspond to bases 47 and 859 of CD40L sequence CD40L 5' primer: 5'-GCATCATCGAACATACAACC-3' (SEQ ID NO. 11) and CD40 3' primer: 5'-GTATTATGAAGACTCCCAGCG-3' (SEQ ID NO. 12). The PCR fragment was purified and subcloned into pCR2.1 vector using T4 DNA ligase (Invitrogen). Sequence analysis of the CD40L open reading frame and alignment with a GenBank consensus sequence revealed presence of two mutations. One mutation was conservative and did not lead to amino acid change. Another substitution resulted in a functional amino acid change Asn-Ser. Site directed mutagenesis was performed to correct the non-conservative amino acid change back to asparagine. Briefly, 10-40 ng of CD40L PCR2.1 plasmid DNA was used in site directed mutagenesis using custom 5' phosphorylated HPLC purified primers (QIAGEN), PFU Ultra enzyme with accompanying 10X PCR buffer (Stratagene) and dNTPS (Clontech). Following the PCR reaction, Dpn I restriction

enzyme (Promega) was added and incubated for 1 hour at 37°C to digest away parental template. Five microliters of this reaction was then transformed into Oneshot MACH T1R competent cells (Invitrogen) and plated out on freshly made ampicillin containing LB plates. Six colonies were selected and grown as 3 mL cultures overnight in LB containing

- 5 ampicillin. DNA was isolated using plasmid miniprep (QIAGEN). An aliquot of purified DNA for each clone was submitted to the University of North Carolina (UNC) sequencing facility for sequence analysis of the CD40L open reading frame using M13F and M13R primers (Invitrogen). All the clones were then aligned to a consensus GenBank Sequence for CD40L using DNASTAR Seqman analysis software. Clone #2 (renamed CD40L WT PCR
- 10 2.1) was selected for containing the correct mutagenized bases.

#### **Generation of mRNAs for transfection of DCs**

CD40L WT PCR 2.1 plasmid was linearized using SphI restriction enzyme and purified by phenol/chloroform extraction followed by ethanol precipitation. The linear template was reconstituted in water and transcribed *in vitro* using mMessage mMachine T7 Ultra kits (Ambion) following the manufacturer's directions. An aliquot of RNA was saved for final analysis prior to proceeding to polyadenylation reaction. Polyadenylated RNA was purified using RNeasy column (QIAGEN) following protocol for RNA cleanup. RNA was eluted in water and stored in individual size aliquots below -150°C. PolyA tail length was determined by the comparative analysis of non-polyadenylated RNA and final product using RNA Bioanalyzer 2100.

#### **Electroporation of DCs**

Prior to electroporation, DCs were harvested and washed in PBS and then re-suspended in chilled Viaspan® (Barr Laboratories) at  $4 \times 10^7$ /ml in 0.5ml or  $2.5 \times 10^7$ /ml in 0.2 ml and placed on ice. DCs were mixed with mRNA (1 or  $2 \mu\text{g}/10^6$  for mRNA encoding antigen and  $4 \mu\text{g}/10^6$  for CD40L mRNA) and placed in a 4 mm gap electroporation cuvette and electroporated using Biorad apparatus. Immediately after electroporation, DCs were washed in X-VIVO 15 medium and finally re-suspended in X-VIVO 15 supplemented with GM-CSF (800 U/ml) and IL-4 (500 U/ml) at  $1 \times 10^6$ /ml and cultured for either 4 or 24 hours at 37°C in low adherence six well plates (BD Biosciences, Franklin Lakes, NJ). Additional maturation stimuli, described below, were also added at this point.

**DC maturation – CD40L base process.**

Following electroporation, DCs transfected with CD40L mRNA were treated with IFN- $\gamma$  (1000 U/ml) or TNF- $\alpha$  (10 ng/ml) or a combination of IFN- $\gamma$  and PGE<sub>2</sub> (1  $\mu$ g/ml). By 5 comparison, immature DCs were transfected with various antigen-encoding mRNAs and were then treated with a “cytokine cocktail” comprising of TNF- $\alpha$  (10 ng/ml), IL-1 $\beta$  (10 ng/ml), IL-6 (100 ng/ml) and PGE<sub>2</sub> (1  $\mu$ g/ml) or soluble CD40L (200 ng/ml) plus enhancer (1  $\mu$ g/ml) with either simultaneous or sequential addition of 1000 U/ml IFN- $\gamma$ .

**10 DC maturation – PME-CD40L process.**

Immature DCs were phenotypically matured on Day 5 of culture with TNF- $\alpha$  (10 ng/ml), IFN- $\gamma$  (1000 U/ml) and PGE<sub>2</sub> (1  $\mu$ g/ml). On day 6, DCs were harvested and electroporated with antigen and CD40L mRNA as described above, and cultured in X-VIVO 15 media containing 800 U/ml GM-CSF and 500 U/ml IL-4 for 4hrs prior to harvest, or 15 formulation for vaccine production.

**DC maturation with the CD40L base process, in combination with  $\alpha$ -galactosylceramide (KRN7000)**

100 ng/ml of KRN7000 was pulsed onto the CD40L base process DCs immediately 20 post electroporation in combination with 500 U/ml IFN- $\gamma$  and 1  $\mu$ g/ml PGE<sub>2</sub>, for 24 hrs of culture.

**Flow Cytometry analysis of DCs**

10<sup>6</sup> DCs were harvested and re-suspended in chilled PBS/ 1% FCS. Phycoerythrin 25 (PE) or FITC conjugated antibodies specific for MHC molecules (HLA-ABC, HLA-DR), co-stimulatory molecules (CD80, CD86), maturation markers (CD83) and monocyte markers (CD14) were mixed with 1x10<sup>5</sup> DCs per well in a 96 well plates (BD Biosciences) and incubated at 4°C for a minimum of 15 minutes. Isotype matched antibodies were used as controls. After thorough washing, fluorescence analysis was performed with a FACScalibur 30 flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences).

Intracellular expression of CD40L was determined as follows: 2 x 10<sup>5</sup> DCs or HeLa

## PATENT

cells were harvested at various time points post transfection with CD40L mRNA and re-suspended in 250  $\mu$ L of Cytofix/Cytoperm solution (BD Biosciences) for a minimum of 10 minutes up to 2 hours at 4°C. Cells were washed twice with 2 ml staining buffer (PBS, BSA, NaN<sub>3</sub>, and EDTA), re-suspended in 0.5 ml staining buffer and stored over night at 4°C. Cells 5 were re-suspended in 2.0 ml Perm/Wash solution (BD Biosciences) for 15 minutes, centrifuged and re-suspended in 100  $\mu$ L Perm/Wash solution. 20  $\mu$ L of mouse anti-human CD40L PE and anti-human CD40 APC (BD Biosciences) or mouse IgG1 PE and IgG1 APC (BD Biosciences) was added to each DC preparation collected and permeabilized at each time point, and incubated at 4°C for 30 minutes in the dark. Cells were washed twice with 1 10 ml Perm/Wash solution and re-suspended in staining buffer prior to flow cytometric analysis.

Intracellular cytokine staining (ICS) was performed as follows: 1x10<sup>6</sup>/ml primed CD8+ T cells, removed from co-culture on day 19 and re-stimulated in 200  $\mu$ l R10 media with PME DC targets (RCC, survivin, G250, hTERT or eGFP) at 37°C; 5% CO<sub>2</sub> for 1 hour prior to the addition of brefeldin A (BD GolgiPlug, Cat No. 555029) at 1  $\mu$ l/ml. Cells 15 incubated at 37°C for a further 16 hours. Cells were washed and resuspended in 150  $\mu$ l FACS buffer with 5  $\mu$ l CD8 per CP-cy5.5 (BD 341051) and incubated at 4°C. After 30 minutes cells were washed twice and resuspended in 2% paraformaldehyde (PFA). Cells were subsequently washed after 10 minutes, and then permeabilized in 0.1% saponin for 10 minutes at room temperature (RT), prior to incubation with 2  $\mu$ l of blocking antibody, Mouse 20 IgG1 pure (BD 349040). After 10 minutes incubation at RT, 0.5  $\mu$ l IFN- $\gamma$  – APC (BD 554681), 10  $\mu$ l IL-2 – FITC (BD 554702) and 10  $\mu$ l CD69-PE (BD 555531) antibodies were added to each sample tube. Samples were incubated for 30 minutes in the dark at RT. Cells were resuspended in 2% PFA following a final wash in 0.1% saponin. Analysis undertaken by FACS cytometry, collecting 100,000 events.

25

**CD40L functional analysis when expressed from mRNA in HeLa cells**

HeLa cells were grown in 10% FBS/DMEM and then harvested and electroporated in 4 mm cuvettes with GFP and CD40L RNA (20  $\mu$ g each/5x10<sup>6</sup> cells). Post-transfection recovery was ~70% and the cells were plated in 6 well dishes and allowed to grow overnight. 30 Following the overnight incubation, transfected HELA cells were harvested by scraping and stained with either mouse IgG1-PE or anti-human CD40L-PE (both from BD Biosciences,

San Diego, CA) to look for cell surface expression of CD40L.  $2 \times 10^5$  cells/tube were stained with 10  $\mu\text{g}/\text{ml}$  of antibody in 1% FBS/PBS for 30 minutes at 4°C. The cells were analyzed using a FACScaliber flow cytometer and Cellquest software (BD Biosciences). To analyze the function of the HeLa expressed CD40L,  $1 \times 10^6$  immature dendritic cells were co-5 cultured with  $1 \times 10^6$  HeLa cells in 5% huAB serum/RPMI supplemented with 1000 U/ml of IFN- $\gamma$  (R&D Systems, Minneapolis, MN) in 6 well dishes (2 mls total volume) overnight. A blocking CD40L monoclonal antibody (24-31 from eBioscience) was included at 10  $\mu\text{g}/\text{ml}$  in matched wells to confirm that cell surface expression of the protein was required for stimulating the dendritic cells. The culture supernatant was harvested after 18-24 hours and 10 expression of the cytokines IL-10 and IL-12 analyzed by ELISA (BD Biosciences).

**Migration Assay**

Chemotaxis of DCs was measured by migration through a 8  $\mu\text{m}$  pore size polycarbonate filter in 24 well transwell chambers (Corning Costar, Acton, MA). 5% human 15 AB serum in Iscoves modified Dulbecco's medium or AIM-V medium containing 3-300 ng/ml CCL19, 5-250 ng/ml CCL21, a combination of both or medium alone was added to the lower chamber.  $1-5 \times 10^5$  DCs in 0.1 ml were added to the upper chamber and incubated for 2-3 hours at 37°C. Lower chamber harvested into 5ml tubes (BD Biosciences) and re-suspended in 0.1 ml PBS and viable cell counts undertaken using trypan blue.

20

**ELISpot**

PVDF membrane ELISpot plates (Millipore, Billerica, MA) were coated with 5  $\mu\text{g}/\text{mL}$  monoclonal anti-IFN- $\gamma$  or anti-IL-2 capture antibody (BD Pharmingen, San Diego, CA) and incubated at 4°C for 24 hours. After incubation, plates were washed with 25 PBS/0.05% Tween 20, and blocked with 5% human AB serum/RPMI 1640 medium for 1 hour. PBMCs, T-cells, or CD8 enriched T cells, were plated at  $1 \times 10^5$  cells/well and mRNA transfected, antigen-loaded DC targets at  $1 \times 10^4$  cells/well for a 10:1 effector:target ratio, and incubated at 37°C, 5% CO<sub>2</sub> for a minimum of 16 hours.

Following incubation, plates were washed 6 times, and anti-IFN- $\gamma$  detection antibody 30 (BD Pharmingen) or anti-IL-2 detection antibody (BD Pharmingen) was added to the appropriate plates at 1  $\mu\text{g}/\text{ml}$  for 2 hours. After a further six washes, Streptavidin-HRP (BD Pharmingen) was added to each well for 1 hour. Finally, after another wash cycle, color

development was undertaken with AEC Peroxidase Substrate for 5-15 minutes and stopped with water. The plates were left to air dry prior to analysis on CTL Immunospot Plate Reader (CTL, Cleveland, OH).

5 **ELISA**

The method as laid out by BD Pharmingen for IL-12 and IL-10 ELISA sets (BD Pharmingen) using BD Opt EIA reagent set B pH 9.5. Briefly, ELISA plates (BD Biosciences) were coated with anti-IL-12p70 or anti-IL-10 ELISA capture antibody in coating buffer for 24 hours at 4°C. Plates underwent blocking with 200 µl per well 10% FCS/PBS for one hour prior to the addition of standards (BD Pharmingen) and supernatant samples, in duplicate, at 100 µl per well and incubated at room temperature for 2 hours. Plates were washed and anti-cytokine detection antibody added, incubated for one hour, the plates washed and solutions replaced with 100 µl of streptavidin-HRP and further incubated for one hour at room temperature. Again plates were washed and color development substrates applied for 10-20 minutes, followed by cessation of color development with stop solution. Plate analysis undertaken using Bio-Tek instruments ELx800 plate reader with KC junior software (Winooski, VT). The results show the number of picograms/ml/10<sup>6</sup> DCs. Because the assays were set up so that 1 ml corresponds to 10<sup>6</sup> DCs, the results can also be expressed as number of picograms/10<sup>6</sup> DCs. For example, 3000 pg/ml/10<sup>6</sup> DCs is equivalent to 3000 pg/10<sup>6</sup> DCs.

**CTL induction**

Mature dendritic cells transfected with mRNAs were co-cultured with CD8 purified T-cells. All co-cultures were performed in R-10 media (10% FBS, RPMI-1640 supplemented with 10 mM HEPES pH 7.4, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM sodium glutamate, 55 µM β-mercaptoethanol). All cell culture reagents were from Invitrogen (Carlsbad, CA). CD8<sup>+</sup> cells were purified using the CD8<sup>+</sup> T Cell Isolation kit II (Miltenyi Biotec, Auburn, CA) from non-adherent cells harvested from the monocyte adherence step. The CD8<sup>+</sup> cells are mixed with dendritic cells prepared as described above at 10:1 CD8<sup>+</sup>:DC. For the first seven days the cells were cultured in media supplemented with 0.2 U/ml IL-2 (R&D Systems, Minneapolis, MN) and then aliquoted into

24-well tissue culture dishes at 1 ml (1x10<sup>6</sup> CD8<sup>+</sup> cells)/well. Following this initial seven day incubation the CD8<sup>+</sup> cells were harvested, counted and re-cultured with fresh DC stimulators at 10:1 in media supplemented with 5 U/ml IL-2. Again the cells were cultured for one week and then restimulated with fresh DC and 20 U/ml IL-2. CTL assays were

5 performed 3 or 7 days following the third stimulation.

#### CTL assay

T2 cells were previously pulsed with 10 µg/ml of either the HLA-A201 restricted MART-APL peptide (LAGIGILTV; SEQ ID NO:24) or native peptide (AAGIGILTV; SEQ

10 ID NO:25) or PSA-1 peptide (FLTPKKLQCV; SEQ ID NO:26) by overnight incubation in FBS/RPMI media, and washed prior to use as CTL targets. Dendritic cell targets were transfected with either GFP mRNA, MART-1 APL mRNA, Flu-M1 mRNA, as described above and incubated overnight without maturation. Pulsed T2 cells were incubated with 100 µCi of Na<sup>51</sup>Cr (Perkin-Elmer Life and Analytical Sciences, Inc., Boston, MA) for 90 minutes  
15 at 37° C. Excess <sup>51</sup>Cr was washed away and 5000 labeled targets incubated with various E:T ratios of CD8<sup>+</sup> cells for 4 hours. Non-specific lysis was reduced by the addition of unpulsed T2 cells at 25,000 cells per well. Released <sup>51</sup>Cr was measured in the supernatant by scintillation counting. Total release was calculated by addition of 1% Triton X-100 to the targets while spontaneous release was calculated by addition of media alone. Percent lysis  
20 was calculated using the formula (sample cpm released-spontaneous cpm)/(total cpm released- spontaneous cpm released).

#### Induction of MART-1 specific CTLs employing KRN7000-pulsed CD40L base process matured DC

25 DCs were generated as described above, employing the 'CD40L base process', and loaded with mRNA encoding MART-1. Post electroporation, DCs were incubated with KRN7000, IFN-γ and PGE<sub>2</sub>. DCs and PBMCs were co-cultured at a 1:10 ratio in the presence of 20 U/ml IL-2. PBMCs were restimulated three times under the same conditions, and the frequency of CTL induction determined by staining with MART-1/A2 tetramers, and  
30 the expansion of NKT-cells enumerated using KRN7000/CD1d tetramers by FACS.

**Results of Experimental Examples****Sequential maturation with Interferon- $\gamma$  and CD40L optimizes IL-12p70 secretion**

Immature DCs were prepared by 6 day culture of adherent cells PBMCs in X-VIVO

5 15 media, inclusive of GM-CSF and IL-4. DCs were recovered on Day 6 and electroporated with 2  $\mu$ g of eGFP encoding mRNA per million DCs, and matured for 36 hrs with "cytokine cocktail". Alternatively, maturation was achieved by culturing the DCs in the presence of IFN- $\gamma$  and soluble CD40L, applied simultaneously, or sequentially. DCs were monitored for increased expression of co-stimulatory molecules, but most importantly for the secretion of  
10 IL-12p70 versus IL-10. Figure 1 shows that DCs matured with the cytokine cocktail secrete excess IL-10 in comparison to IL-12p70 into the culture supernatant over the 36 hr culture period. By contrast, DCs matured simultaneously with soluble CD40L plus IFN- $\gamma$  secrete excess IL-12p70. However, sequential application of IFN- $\gamma$  for 18 hrs, followed by the addition of soluble CD40L directly to the culture, and an additional 18hr culture period  
15 resulted in significantly enhanced levels of IL-12p70 secretion. Unexpectedly, the application of soluble CD40L, followed by IFN- $\gamma$ , prevented significant secretion of IL-12p70. In conclusion, the sequential delivery of an innate stimulus to "prime" DC maturation (IFN- $\gamma$ ), followed by a surrogate T-helper cell signal delivered by soluble CD40L, optimizes DC maturation for IL-12p70 secretion.

20

**Co-culture of HeLa cells, transfected with mRNA encoding CD40L, with immature DCs results in the induction of DC derived IL-12p70.**

Figure 2 shows that HeLa cells transfected with mRNA encoding CD40L results in significant cell surface expression of CD40L protein after 24 hrs of culture, as defined by an  
25 anti-CD40L antibody and flow cytometry. CD40L mRNA transfected HeLa cells were co-cultured with immature DCs, in the presence of 1000 U/ml IFN- $\gamma$ . Table II shows that HeLa cells transfected with an extended poly-A tail (> 400 'A's) are capable of inducing significant IL-12p70 secretion when cultured with immature DCs over the 18 hr culture period. Importantly, the inclusion of a blocking anti-CD40L antibody prevents IL-12p70 secretion,  
30 and confirms the identity and functional importance of protein encoded by the transfected mRNA sequence.

TABLE II

HeLa cells transfected with CD40L encoding mRNA, when co-cultured with immature DCs in the presence of IFN- $\gamma$ , results in the secretion of IL-12p70. Inclusion of 'blocking' anti-CD40L antibody in the culture prevents the induction of IL-12p70.

	Immature DCs IL-12p70 (pg/ml)	"Cocktail" matured and reactivated DCs IL-12p70 (pg/ml)
DC's alone	-	4.9
(a) HeLa/ > 400 polyA + IFN- $\gamma$	372	26.3
(b) HeLa/ > 400 polyA + IFN- $\gamma$ + 24-31	-	2.5

5

(a) HeLa cells were transfected with 4 $\mu$ g of CD40L mRNA bearing greater than 400 nucleotide poly-A tail, and incubated with DCs in the presence of IFN- $\gamma$ .

(b) As (a) but in the presence of blocking anti-CD40L antibody (24-31).

10 **Dendritic cells transfected with CD40L mRNA, and cultured in the presence of IFN- $\gamma$  secrete IL-12p70.**

Immature DCs were harvested after 6 days in culture with GM-CSF and IL-4, and transfected with a titration of CD40L mRNA (400-polyA), and immediately cultured in the 15 presence of 1000 U/ml IFN- $\gamma$ . Figure 3 shows that supernatants harvested after 18 hrs of culture contain excess IL-12p70 over IL-10, and that at least 4  $\mu$ g of CD40L mRNA per million DCs is required for optimal cytokine secretion. Increasing the CD40L mRNA payload above 4  $\mu$ g per million DCs results in a significant reduction in DC yield post 20 maturation (data not shown). In a parallel experiment, immature DCs were transfected with 4  $\mu$ g CD40L mRNA per million cells, and a titration of IFN- $\gamma$  immediately applied to the

cultures. Figure 4 shows that at least 100 U/ml of IFN- $\gamma$  is required to support optimal induction of IL-12p70. Figure 5a shows that IL-12p70 appears at detectable levels 6 to 8 hrs post transfection and coculture with IFN- $\gamma$ , with optimal accumulation in the culture supernatant being recorded between 20 and 24 hrs. By contrast, the substitution of 10 ng/ml TNF- $\alpha$  for IFN- $\gamma$  also supports IL-12 production, but at reduced levels (Figure 5b). Moreover, IFN- $\gamma$  results in concomitantly lower levels of IL-10 production than does TNF- $\alpha$  (Figure 5c)

**Induction of IL-12p70 by DCs transfected with CD40L mRNA is dependent on**

10 **“intracellular signaling” as opposed to cell-cell interactions.**

Figure 2 demonstrates that CD40L protein translated from mRNA can be expressed on the cell surface of the transfected cells, and that the protein retains the ability to appropriately signal DCs for IL-12p70 secretion as a consequence of its interaction with its counterpart on DCs, namely CD40. To determine the cellular distribution of CD40L in 15 transfected DCs, and to confirm its functional identity, DCs were harvested at various time points post transfection, the presence of CD40L on the cell surface, or intracellular compartments was determined. Figures 6a and 6b show that the majority of CD40L is localized within an intracellular compartment, and that significant protein expression (27% DCs CD40L positive) was not apparent until 60 minutes post transfection. Thus, although 20 IFN- $\gamma$  is applied immediately post transfection, the delivery of the maturation events is *sequential*, with the IFN- $\gamma$  signal preceding that of CD40L. As shown in Figure 1, sequential maturation of DCs with IFN- $\gamma$  and CD40L optimizes for IL-12p70 secretion. In addition, 25 Figure 7 shows that CD40L transfected and IFN- $\gamma$  treated DCs, when cultured in the presence of excess blocking anti-CD40L antibody for 18hrs post transfection, still secrete significant levels of IL-12p70. This data shows that CD40L/CD40 interactions, which are required for IL-12p70 production in this system, can take place within the intracellular compartment.

**Frequency of CD40L positive cells over time**

Immature DCs were transfected with 4  $\mu$ g CD40L mRNA per  $10^6$  DC, and co-matured with 1000 U/ml IFN- $\gamma$ . Alternatively, and by way of negative control for CD40L 30 staining, immature DCs were matured with ‘cytokine cocktail’. Maximum frequency of

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expression is achieved around 3 to 4 hrs post transfection with CD40L RNA (see Figure 6b), although 80% of DCs express CD40L when the cells are fixed and permeabilized, cell surface staining only detects approximately 15% of the DCs (Figure 6c). This data shows that the bulk of the CD40L protein is retained within the DC, and is not expressed at the cell surface. CD40L protein is transiently expressed, with the majority of DCs becoming CD40L negative 26 hrs post transfection. The expression of CD40, the cognate receptor molecule for CD40L, is not altered by transfection of DCs with mRNA encoding CD40L, when compared to DCs receiving 'cytokine cocktail' only.

10 **PGE<sub>2</sub> is required to induce DC migration on maturation with CD40L and IFN- $\gamma$**

In addition to the capacity to secrete IL-12p70 and exhibit a mature phenotype, typically defined as cells expressing elevated levels of co-stimulatory molecules such as CD80, CD83 and CD86 (see Table III), DCs must display the capacity to migrate, if they are going to be capable of homing to a lymph node *in vivo*. Several studies have shown that 15 PGE<sub>2</sub> primes mature DCs for migration (Luft et al. (2002) Blood 100: 1362, Scandella et al. (2002) Blood 100: 1354). Figure 8 shows that the inclusion of 1  $\mu$ g/ml PGE<sub>2</sub>, in addition to IFN- $\gamma$ , enables the maturing DCs to migrate, and that the acquisition of this migratory potential is proportional to the CD40L mRNA payload. Thus, CD40L contributes to not only the maturing DC phenotype, and dominant IL-12p70 profile (see Table II), but also to 20 priming for migration. By contrast, DCs matured by transfection with CD40L mRNA and cultured in the presence of IFN- $\gamma$ , but in the absence of PGE<sub>2</sub>, fail to migrate (data not shown), despite displaying significant cell surface expression of the chemokine receptor, CCR7.

TABLE III

Phenotypic analysis and secreted cytokine profile of DCs undergoing maturation induced by either 'Cytokine Cocktail', or CD40L plus IFN- $\gamma$  and PGE<sub>2</sub>

	Immature DC	(a) Flu/eGFP mRNA	(b) Mart-APL mRNA	(c) Flu/CD40L mRNA	(d) Mart-APL/CD40L mRNA
<b>DC markers</b>		<b>Cytokine Cocktail</b>	<b>Cytokine Cocktail</b>	<b>IFN-<math>\gamma</math>/PGE<sub>2</sub></b>	<b>IFN-<math>\gamma</math>/PGE<sub>2</sub></b>
HLA-ABC	99.7%	98.6%	99.5%	99.9%	99.9%
<b>HLA-DR</b>	<b>95.0%</b>	<b>99.6%</b>	<b>99.7%</b>	<b>99.8%</b>	<b>99.5%</b>
CD83	23.2%	98.3%	99.2%	99.6%	99.3%
CD14	0.3%	1.7%	2.9%	3.2%	4.9%
CD56	2.8%	3.3%	3.2%	2.8%	2.1%
CD19	1.8%	1.1%	2.1%	3.2%	3.2%
CD3	2.8%	2.4%	3.1%	2.8%	3.1%
CD86	59.3%	99.7%	100.0%	100.0%	100.0%
CD80	28.8%	99.0%	99.5%	99.2%	99.5%
CD1a	51.6%	49.1%	52.2%	48.6%	49.9%
CD209	95.8%	95.5%	96.1%	96.4%	95.9%
CCR7	3.2%	47.4%	35.5%	35.4%	36.2%
(e) IL-12 (pg/ml)	N/A	27.5	59.0	<b>1456.3</b>	<b>1350.0</b>
(f) IL-10 (pg/ml)	N/A	<b>948.8</b>	<b>810.0</b>	187.7	165.5

5 DCs were prepared from adherent monocytes and cultured for 6 days in GM-CSF/IL-4. On harvesting, DCs were transfected with various mRNA payloads and subjected to maturation for a further 24 hrs. DCs were again harvested, and the cells stained for various cell surface markers, particularly those associated with increased function, namely co-stimulation and migration. Supernatants from the maturation cultures were collected and 10 subjected to IL-12p70 and IL-10 cytokine analysis.

(a) DCs were transfected with 2  $\mu$ g per million cells with flu mRNA as antigen-encoding payload, in addition to 4  $\mu$ g per million cells eGFP mRNA. eGFP mRNA allows for

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confirmation of transfection by FACS, and to act as a substitute control for the 4ug per million cells CD40L mRNA maturation payload, in the alternate process. These flu/eGFP transfected DCs were matured in the presence of the “cytokine cocktail”.

(b) DCs were transfected with 2  $\mu$ g per million cells with MART-APL mRNA as antigen-encoding payload, and subjected to maturation with the “cytokine cocktail”.

(c) DCs were transfected with 2  $\mu$ g per million cells with flu mRNA as antigen-encoding payload, concomitant with 4  $\mu$ g per million cells CD40L mRNA as the maturation payload. These cells were immediately placed in culture with IFN- $\gamma$  and PGE<sub>2</sub> as described in materials and methods.

(d) DCs were transfected with 2  $\mu$ g per million cells with MART-APL as antigen-encoding payload, concomitant with 4  $\mu$ g per million cells CD40L mRNA as the maturation payload. These cells were immediately placed in culture with IFN- $\gamma$  and PGE<sub>2</sub> as described in materials and methods.

(e) IL-12p70 secretion from DCs undergoing maturation.

(f) IL-10 secretion from DCs undergoing maturation.

**DCs sequentially matured via transfection with CD40L mRNA and IFN- $\gamma$ /PGE<sub>2</sub> invoke potent T-cell recall responses.**

To determine the “immunopotency” of DCs matured via CD40L mRNA transfection and IFN- $\gamma$ /PGE<sub>2</sub>, DCs were co-transfected with 2  $\mu$ g mRNA encoding flu matrix protein per million DCs in addition to the CD40L mRNA and IFN- $\gamma$ /PGE<sub>2</sub> culture environment. 18hrs post transfection, DCs were harvested, washed, and co-cultured with autologous T-cells in IFN- $\gamma$  ELISpot assays. Figure 9 shows that DCs matured via CD40L/IFN- $\gamma$ /PGE<sub>2</sub> display increased immunopotency, compared to DCs transfected with flu mRNA and matured with ‘cytokine cocktail’, as defined by the frequency of flu-specific IFN- $\gamma$  spots in the assay.

**DCs sequentially matured via transfection with CD40L mRNA and IFN- $\gamma$ /PGE<sub>2</sub> invoke primary responses.**

Recall responses, such as that described in Figure 9, are less dependent on the presence of DCs expressing optimized co-stimulatory molecules and supporting cytokine environments. Therefore, DCs were tested for their ability to invoke primary immune responses to the melanoma associate antigen, MART-1, to which many healthy donors maintain a high naive T-cell precursor frequency. As HLA-A201 donors were preferentially used, DCs were transfected with an mRNA encoding MART-1 in which the A2 restricted determinant was optimized by mutation of the mRNA sequence by site directed mutagenesis, such that the alanine at position 27 was substituted by leucine, and here referred to as MART-APL (Valmori, D et al (1998) J. Immunol. 160:1750). DCs co-transfected with 2  $\mu$ g MART-APL mRNA with 4  $\mu$ g CD40L mRNA and immediately pulsed with IFN- $\gamma$ /PGE<sub>2</sub> for 18 hrs were compared to DCs loaded solely with MART-APL, and matured overnight with the "cytokine cocktail". Antigen-loaded and matured DCs were added to purified autologous CD8<sup>+</sup> T-cells, and cultured for 7 days in the presence of 0.2 U/ml human IL-2. After this period, T-cells were recovered and co-cultured with a second round of antigen-loaded DC stimulators as appropriate in an IL-2 ELISpot assay. Figure 10 shows that CD8<sup>+</sup> T-cells cultured in the presence of DCs matured via CD40L and IFN- $\gamma$ /PGE<sub>2</sub> results in a highly significant increase in T-cells capable of IL-2 secretion in a specific response to the optimized MART-APL epitope originally encoded within the MART-APL mRNA sequence. In conclusion, DCs exposed to sequential maturation via IFN- $\gamma$ /PGE<sub>2</sub> and CD40L are significantly more potent at raising primary immune responses than DCs matured with the currently accepted standard "cytokine cocktail". Moreover, Figure 11 shows that CTLs generated with MART-APL loaded DCs matured with the 'cytokine cocktail' fail to mediate CD4-independent CD8-mediated cytotoxicity against T2 cells pulsed with the appropriate HLA-A2 restricted MART-APL peptide (Figure 11b). By contrast, CTLs generated on CD40L/IFN- $\gamma$ /PGE<sub>2</sub> matured DCs are fully active, and kill the MART-APL peptide pulsed T2 targets (Figure 11a).

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*PATENT***Phenotypic analysis of immature DCs maturing under the PME-CD40L process**

DCs were matured on Day 5 with the PME-CD40L process described herein. Specifically monocytes were cultured in medium GM-CSF and IL-4 for 5 days to produce immature CD83<sup>-</sup> DCs. On day 5, the immature DCs were fed with medium containing 5 TNF $\alpha$ , IFN $\gamma$  and PGE<sub>2</sub> (TIP). On day 6, the post TIP phenotype was determined (see Table IV). As shown in Table IV, the majority of cells were positive for CD80, CD83, CD86 and CD209. These DCs were also CCR7 negative (data not shown). The low percentage of 10 CD14<sup>+</sup> cells represent monocytes that did not differentiate into dendritic cells. On day 6, the CD83<sup>+</sup> CCR7<sup>-</sup> DCs were co-transfected (via electroporation) with 1  $\mu$ g mRNA prepared from amplified renal cell carcinoma RNA and 4  $\mu$ g CD40L mRNA per million cells. CD40L expression was measured at 4 hours post transfection. The cells were cryopreserved in liquid nitrogen at 4 hrs post transfection. The post thaw recovery and viability were measured immediately after thawing, and at 24 hours post thawing. As can be seen, at 24 hours post thaw, the majority of DCs became CCR7+. The CCR7+ DCs were also positive for CD80, 15 CD83 and CD86. The results of 3 separate runs are shown in Table IV.

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TABLE IV

Run data	Run 1	Run 2	Run 3
Seeding density per flask	200 x 10 <sup>6</sup>	200 x 10 <sup>8</sup>	200 x 10 <sup>6</sup>
<b>Number of flasks seeded</b>	<b>18</b>	<b>20</b>	<b>20</b>
Post TIP Recovery (%)	8	24	15
Post TIP Viability (%)	97	95	93
<b>Number of cuvettes</b>	<b>14</b>	<b>15 (limited)</b>	<b>15 (limited)</b>
4hr post electroporation Recovery (%)	64	43	73
4hr post electroporation Viability (%)	91	89	85
Number of vaccine doses from Run	13	9	15
Post thaw Recovery (%)	86	94	85
Post thaw Viability (%)	88	88	78
<b>Predicted doses per 30 flasks</b>	<b>21</b>	<b>28</b>	<b>30</b>
<b>4hr CD40L expression</b>	<b>84</b>	<b>76</b>	<b>49</b>
<b>Post TIP DC phenotype</b>			
% CD14	0.8	0.5	12
% CD80	100	100	98
% CD83	99	92	82
% CD86	100	100	100
% CD209	98	99	100
<b>mDC phenotype (post thaw)</b>			
% CD14	3	0.3	1.4
% CD80	99	100	100
% CD83	100	100	98
% CD86	100	100	100
% CD209	98	100	100
% CCR7	53	12	32
<b>24hr post thaw % CCR7</b>	<b>93</b>	<b>93</b>	<b>95</b>
<b>24hr post thaw 'washout'</b>			
% viability	50	67	63
% recovery	36	46	73
<b>24hr post thaw transwell migration</b>			
% Migration - media control	1.1	0.78	1.2
% Migration - 100ng/ml CCL19 and 21	74	107	70

**DCs matured via the PME-CD40L process are highly migratory in response to lymph**5 **node homing chemokines, CCL19 and 21.**

PME-CD40L matured DCs were assayed for migration in response to chemokines, CCL19 and 21, twenty-four hours after co-transfection with total amplified RCC RNA and CD40L RNA. Figure 12 shows that using four independent donors, that PME-CD40L matured DCs are highly migratory, consistent with the very high level of CCR7 expression 10 achieved 24 hrs post electroporation with the PME-CD40L process (see Table IV).

**DCs matured via the PME-CD40L process show significantly enhanced immunopotency over DCs matured with the 'CD40L base process'.**

Despite the induction of primary immune responses by the 'CD40L base process', the 5 'post maturation electroporation-CD40L' process, whereby DCs are first matured with TNF- $\alpha$ , IFN- $\gamma$  and PGE<sub>2</sub>, prior to electroporation with CD40L plus antigen-encoding mRNA, results in a significant improvement in CTL activity using the MART antigen model system. (Figure 13). In addition, the PME-CD40L process was tested for the induction of IFN- $\gamma$  and IL-2 responses using fully autologous materials derived from a renal cell carcinoma patient: 10 patient DCs were prepared as described above for the PME-CD40L process, and electroporated with autologous total amplified RCC tumor RNA. The antigen loaded DCs were cultured with autologous patient CD8 T-cells, and the resulting responder CTL were studied by intracellular cytokine staining in response to the eliciting DC, and to individual DCs transfected with the tumor-associated antigens, hTERT, Survivin and the RCC specific 15 antigen, G250. DCs transfected with eGFP encoding mRNA were used as negative control stimulators. Figure 14 shows that patient T-cells responded to the total amplified RCC RNA loaded DCs, and also to the three tumor-associated antigens, with both IFN- $\gamma$  and IL-2 frequencies higher than that induced by the eGFP mRNA transfected negative control. (Response to eGFP subtracted from total response to each RCC associated DC target)

20

**DCs matured by the 'base CD40L process' and pulsed with KRN7000 can recruit NKT-cells which enhance the induction of primary CTLs.**

MART-1 mRNA-loaded, CD40L base process matured DC, pulsed with KRN7000, increase the frequency of NKT-cells in PBMC cultures versus the same mature RNA loaded 25 DCs pulsed with vehicle in place of KRN7000, as defined by CD1d/KRN7000-tetramer staining (Figure 15a). Using tetramer analysis for responder CTL (MART-1/HLA-A2), the presence of KRN7000 pulsed onto MART-1 mRNA transfected DC significantly increases the frequency of MART reactive T-cells (Figure 15b). Thus, the expansion of NKT-cells in the PBMC cultures provides an amplification loop, probably achieved by NKT-cell derived 30 'help', that can support primary CD8 CTL development.

**Optimization of CD40L mRNA**

The CD40L RNA used in the original DC experiments demonstrating a preferred way of maturation was transcribed from plasmid template pCR2.1 CD40L WT. The preferred CD40L RNA contains an ARCA cap analog and polyA tail. The plasmid pCR2.1 CD40L 5 WT was modified by removal of an XbaI – EcoRV fragment located 5' of the initiator ATG codon. The fragment encompassed 32 nucleotides of vector sequence and contained three cryptic potential initiator ATG codons. The rationale for this modification was that these additional ATG's might interfere with efficient initiation of CD40L translation by competing with the accurate CD40L translation initiation site. Coding sequence of the CD40L remained 10 unaffected by these modifications. CD40L RNA transcribed from the modified plasmid template performed better than the current CD40L reference standard in two independent DC transfection experiments as measured by induction of IL-12 expression. The modified plasmid is referred to as pCR2.1 CD40L WT Delta X-E.

In addition we wished to determine whether expression of the CD40L RNA can be 15 further optimized by placing the CD40L 5' untranslated region directly upstream of the CD40L initiator codon. The pCR2.1 CD40L WT Delta X-E plasmid was further modified by the insertion of 39 bp CD40L 5' untranslated sequence located immediately upstream of the CD40L translation start site. RNA transcribed from this plasmid did not perform as well as the RNA described from CD40L WT Delta X-E but rather, performed similarly to the current 20 CD40L transcribed from pCR2.1 CD40L WT (Figure 18). Therefore the pCR2.1 CD40L WT Delta X-E plasmid is the preferred plasmid. The DNA sequence corresponding to the CD40L RNA transcribed from the pCR2.1 CD40L WT Delta X-E plasmid is shown in SEQ ID NO:11. The ATG start codon begins at position 41.

The Claims Defining the Invention are as Follows:

1. A method for preparing mature dendritic cells (DCs), comprising the sequential steps of:
  - 5 (a) signaling isolated immature dendritic cells (iDCs) with a first signal comprising an interferon gamma receptor (IFN- $\gamma$ R) agonist in the presence of a TNF- $\alpha$ R agonist, to produce signaled CD83 $^{+}$  mature dendritic cells; and
  - (b) signaling said CD83 $^{+}$  mature dendritic cells with a second transient signal comprising an effective amount of a CD40 agonist to produce CD83 $^{+}$  CCR7 $^{+}$  mature dendritic cells, wherein said second signal is effected upon translation of an mRNA encoding said CD40 agonist, and further wherein said cells are transfected with said mRNA.
2. A method according to claim 1, further comprising contacting said iDCs or said 5 signaled DCs with PGE<sub>2</sub>.
3. A method according to claim 1 or 2, wherein steps (a) and (b) further comprise contacting said cells with GM-CSF and at least one of IL-4 or IL-13.
4. A method according to any one of claims 1 to 3, wherein the CD40 agonist is an 10 agonistic antibody to CD40.
5. A method according to any one of claims 1 to 3, wherein said CD40 agonist is a CD40L polypeptide.
- 25 6. A method according to claim 5, wherein said CD40L polypeptide is selected from the group consisting of:
  - (a) a polypeptide comprising SEQ ID NO:2;
  - (b) a polypeptide comprising SEQ ID NO:10; and
  - 30 (c) a fragment of the polypeptide of any of (a) or (b) wherein said fragment selectively binds CD40.
7. A method according to claim 5, wherein said CD40L polypeptide is a polypeptide having at least 77% sequence identity to a polypeptide selected from the group consisting of:
  - (a) a polypeptide comprising SEQ ID NO:2;
  - (b) a polypeptide comprising SEQ ID NO:10; and
  - 35 (c) a fragment of the polypeptide of any of (a) or (b) wherein said fragment selectively binds CD40.

8. A method according to any one of claims 1 to 7, wherein said first signal is effected upon translation of an mRNA encoding a IFN- $\gamma$ R agonist.
- 5 9. A method according to any one of claims 1 to 7, wherein said first signal is effected upon contacting said iDCs with a IFN- $\gamma$ R agonist.
10. A method according to any one of claims 1 to 9, wherein said cells are transfected with said mRNA using electroporation.
- 0 11. A method according to any one of claims 1 to 10, wherein said mRNA encodes a CD40L polypeptide and comprises a polynucleotide selected from the group consisting of:
  - (a) a polynucleotide of SEQ ID NO:1;
  - (b) a polynucleotide comprising nucleotides 40 to 822 of SEQ ID NO:1;
  - 5 (c) a polynucleotide of SEQ ID NO:9;
  - (d) a polynucleotide of SEQ ID NO:13;
  - (e) a polynucleotide having at least 80% sequence identity with any polynucleotide of (a), (b), (c), or (d);
  - (f) a polynucleotide hybridizing under stringent conditions to any polynucleotide of (a) through (d); and
  - (g) a polynucleotide of (a) through (f), further comprising a 3' untranslated sequence selected from the group consisting of the nucleic acids of SEQ ID NO:14, 15, 16, 17 or 18, and/or a 5' untranslated sequence selected from the group consisting of the nucleic acids of SEQ ID NO:19, 20, 21, 22, or 23.
- 20 12. A method according to any one of claims 1 to 11, wherein the CCR7 $^+$  mature DCs secrete from 0 to 500 pg IL-10 per 10 $^6$  DCs.
- 25 13. A method according to any one of claims 1 to 12, wherein the CCR7 $^+$  mature DCs secrete IL-12 p70 protein.
14. A method according to any one of claims 1 to 13, wherein the immature dendritic cells are prepared from CD34 $^+$  hematopoietic stem or progenitor cells.
- 30 15. A method according to any one of claims 1 to 13, wherein the immature dendritic cells are prepared from peripheral blood mononuclear cells (PBMCs).

16. A method according to claim 15, wherein the immature dendritic cells are prepared by contacting said PBMCs with GM-CSF in the presence of IL-4 and/or IL-13.
17. A method according to any one of claims 1 to 16, wherein said IFN- $\gamma$ R agonist is IFN- $\gamma$  or a biologically active fragment thereof.
18. A method according to claim 17, wherein the IFN- $\gamma$  has the sequence of SEQ ID NO:6.
19. A method according to any one of claims 1 to 16, wherein said IFN- $\gamma$ R agonist comprises a polypeptide having at least 80% sequence identity with SEQ ID NO:6.
20. A method according to any one of claims 1 to 19, further comprising introducing into said iDCs, signaled DCs or CCR7<sup>+</sup> mature DCs one or more antigens or a polynucleotide(s) encoding one or more antigens to produce antigen-loaded CCR7<sup>+</sup> mature DCs.
21. A method according to claim 20, wherein the antigen is selected from the group consisting of pathogens, pathogen lysates, pathogen extracts, pathogen polypeptides, cancer cells, cancer cell lysates, cancer cell extracts, and cancer cell specific polypeptides.
22. A method according to claim 21, wherein a polynucleotide(s) encoding the antigen(s) is introduced into said iDCs, signaled DCs or CCR7<sup>+</sup> mature DCs.
23. A method according to claim 22, wherein said polynucleotide is introduced by electroporation.
24. A method according to claim 22 or 23, wherein the polynucleotide is an mRNA.
25. A method according to claim 24, wherein the antigen encoding mRNA is introduced together with an mRNA encoding a CD40 agonist.
26. A method according to any one of claims 20 to 25, wherein the antigen or polynucleotide is introduced prior to said first signal.
27. A method according to any one of claims 20 to 25, wherein the antigen or polynucleotide is delivered subsequent to said first signal and prior to said second signal.

28. A method according to any one of claims 20 to 25, wherein the antigen or polynucleotide is delivered subsequent to said second signal.
29. A method according to any one of claims 20 to 25, wherein the antigen or polynucleotide is delivered substantially concurrent with said second signal.
30. A method of raising an immune response in a subject comprising administering to the subject an effective amount of an antigen loaded CCR7<sup>+</sup> mature DCs produced by a method according to any one of claims 20 to 29.
31. A method according to claim 30, wherein the DCs are allogeneic to the subject.
32. A method according to claim 30, wherein the DCs are autologous to the subject.
33. A method of stimulating immune effector cells, comprising culturing said cells in the presence of an antigen loaded CCR7<sup>+</sup> mature DCs produced by a method according to any one of claims 20 to 29 to produce stimulated immune effector cells.
34. A method of enhancing immunity in a subject comprising administering to the subject an effective amount of stimulated immune effector cells produced by a method according to claim 33.
35. A method according to claim 34, wherein the TNF- $\alpha$ R agonist is TNF- $\alpha$ .
36. A method according to any one of claims 1 to 9, wherein said mRNA encoding a CD40 agonist is introduced into said mature CCR7<sup>+</sup> dendritic cells by transfection.
37. A method according to claim 36, wherein the mRNA is introduced by electroporation.
38. A method according to claim 34, wherein said mRNA encoding a CD40 agonist is cotransfected with mRNA encoding an antigen.
39. A method according to claim 37, further comprising contacting said immature dendritic cells with PGE<sub>2</sub>.
40. A method according to claim 1, further comprising contacting said mRNA loaded dendritic cells with PGE<sub>2</sub>.

41. A method according to claim 1, further comprising contacting said immature dendritic cells, said signaled dendritic cells, or said CCR7<sup>+</sup> dendritic cells with a NKT cell ligand.

5 42. A method according to claim 41, wherein said NKT cell ligand is a compound selected from the group consisting of:  $\alpha$ -galactosylceramides,  $\alpha$ -glucosylceramides,  $\alpha$ -6-deoxygalactosylceramides,  $\alpha$ -6-deoxygalactofuranosylceramides,  $\beta$ -6-deoxygalactofuranosylceramides,  $\beta$ -arabinosylceramides,  $\alpha$ -C-galactosylceramides and  $\alpha$ -S-galactosylceramides.

0 43. A method according to claim 42, wherein said compound is (2S, 3S, 4R)-1-O-(alpha-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000).

5 44. A method according to claim 41, wherein said CCR7<sup>+</sup> dendritic cells are contacted with said compound.

5 45. A method according to claim 41, wherein said immature DCs are contacted with said compound.

20 46. A method according to claim 41, wherein said signaled DCs are contacted with said compound.

47. A CCR7<sup>+</sup> mature dendritic cell (DC) prepared by a method according to claim 1.

48. A dendritic cell according to claim 47, wherein said cell is transfected using electroporation.

25 49. A dendritic cell according to claim 47 or 48, wherein the method further comprises contacting said iDCs or said signaled DCs with PGE2.

30 50. A dendritic cell according to any one of claims 47 to 49, wherein the method steps (a) and (b) further comprise contacting said cells with GM-CSF and at least one of IL-4 or IL-13.

51. A dendritic cell according to any one of claims 47 to 50, wherein the CD40 agonist is selected from an agonistic antibody to CD40, an aptamer that binds to CD40 and a CD40L polypeptide, wherein optionally said CD40 agonist is CD40L, wherein optionally said CD40L polypeptide is selected from the group consisting of:

35 (a) a polypeptide comprising SEQ ID NO:2;

(b) a fragment of the polypeptide of step (a) wherein said fragment binds CD40, or wherein CD40L polypeptide is a polypeptide having at least 77% sequence identity to a polypeptide selected from the group consisting of:

- (i) a polypeptide comprising SEQ ID NO:2;
- (ii) a fragment of the polypeptide of (a) wherein said fragment binds

CD40.

52. A dendritic cell according to any one of claims 47 to 51, wherein said mRNA encodes a CD40L polypeptide and comprises a polynucleotide selected from the group consisting of:

- (a) a polynucleotide of SEQ ID NO:1;
- (b) a polynucleotide comprising nucleotides 40 to 822 of SEQ ID NO:1;
- (c) a polynucleotide having at least 80% sequence identity with any polynucleotide of (a) or (b);
- (d) a polynucleotide hybridizing under stringent conditions to any polynucleotide of (a) through (c); and
- (e) a polynucleotide of (a) through (d), further comprising a 3' untranslated sequence selected from the group consisting of the nucleic acids of SEQ ID NO: 14, 15, 16, 17, or 18, and/or a 5' untranslated sequence selected from the group consisting of the nucleic acids of SEQ ID NO: 19, 20, 21, 22, or 23.

53. A dendritic cell according to any one of claims 47 to 52, wherein the CCR7<sup>+</sup> mature DCs secrete from 0 to 500 pg IL-10 per 10<sup>6</sup> DCs and/or secrete IL-12 p70 protein, and/or wherein the immature dendritic cells are prepared from one of the following:

- (a) CD34+ hematopoietic stem or progenitor cells;
- (b) peripheral blood mononuclear cells (PBMCs); or
- (c) by contacting said PBMCs with GM-CSF in the presence of IL-4 and/or IL-13.

54. A dendritic cell according to any one of claims 47 to 53, which is antigen loaded and the method further comprises introducing into said iDCs, signaled DCs or CCR7<sup>+</sup> mature DCs one or more antigens or a polynucleotide(s) encoding one or more antigens to produce [[an]] antigen-loaded CCR7<sup>+</sup> mature DCs.

55. A dendritic cell according to claim 54, wherein the antigen is selected from pathogens, pathogen lysates, pathogen extracts, pathogen polypeptides, cancer cells, cancer cell lysates, cancer cell extracts and cancer cell specific polypeptides.

56. A dendritic cell according to claim 54 or 55, wherein mRNA(s) encoding the antigen(s) is introduced into said DCs together with the mRNA encoding a CD40 agonist.

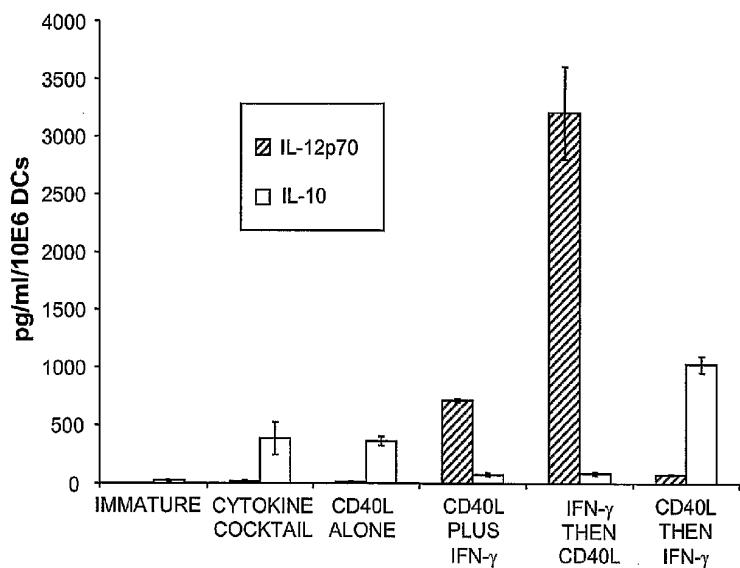
5 57. A dendritic cell according to any one of claims 47 to 56, wherein said TNF- $\alpha$ R agonist is TNF- $\alpha$ .

0 58. A dendritic cell according to any one of claims 47 to 57, wherein the IFN- $\gamma$ R agonist is IFN- $\gamma$  or a biologically active fragment thereof, wherein optionally the IFN- $\gamma$  has the sequence of SEQ ID NO:6 or said IFN- $\gamma$  agonist comprises a polypeptide having at least 80% sequence identity with SEQ ID NO:6.

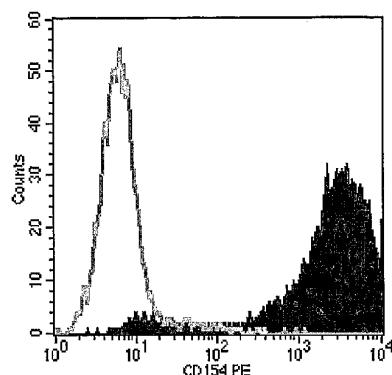
5 59. A dendritic cell according to any one of claims 47 to 58, further comprising contacting said immature dendritic cells, said signaled dendritic cells, or said CCR7 $^{+}$  dendritic cells with a NKT cell ligand, wherein optionally said NKT cell ligand is a compound selected from the group consisting of:  $\alpha$ -galactosylceramides,  $\alpha$ -glucosylceramides,  $\alpha$ -6-deoxygalactosylceramides,  $\alpha$ -6-deoxygalactofuranosylceramides,  $\beta$ -6- deoxygalactofuranosylceramides,  $\beta$ -arabinosylceramides,  $\alpha$ -C-galactosylceramides and  $\alpha$ -S-galactosylceramides, and further optionally wherein said compound is (2S, 3S, 4R)-1-O-(alpha-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000).

!0 60. An enriched population of mature dendrites according to any one of claims 47 to 59.

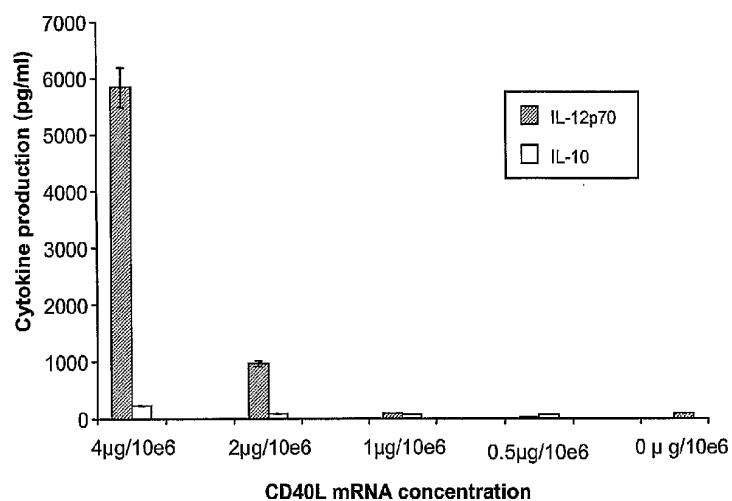
25 61. A cytotoxic T lymphocyte (CTL) obtained by a method comprising culturing said immune effector cells in the presence of an antigen loaded CCR7 $^{+}$  mature dendritic according to any one of claims 8 to 14 when used in enhancing immunity in a subject.

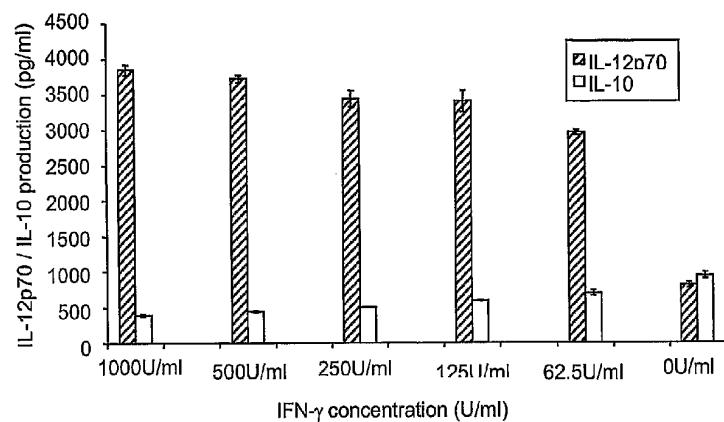
**FIG. 1**

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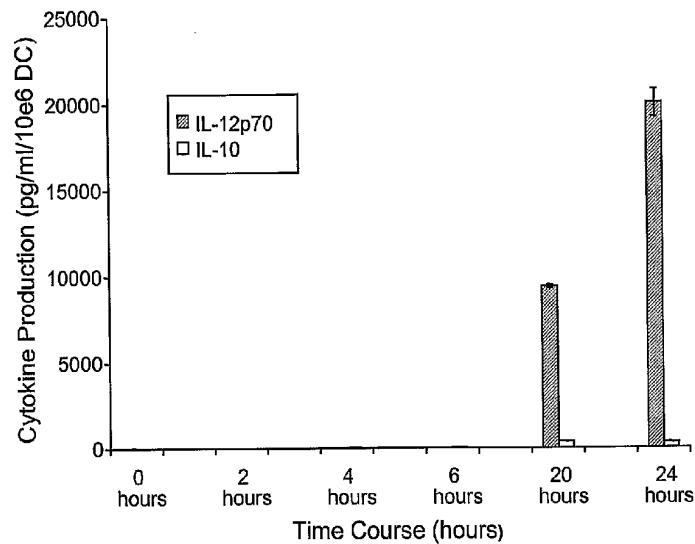
**FIG. 2**

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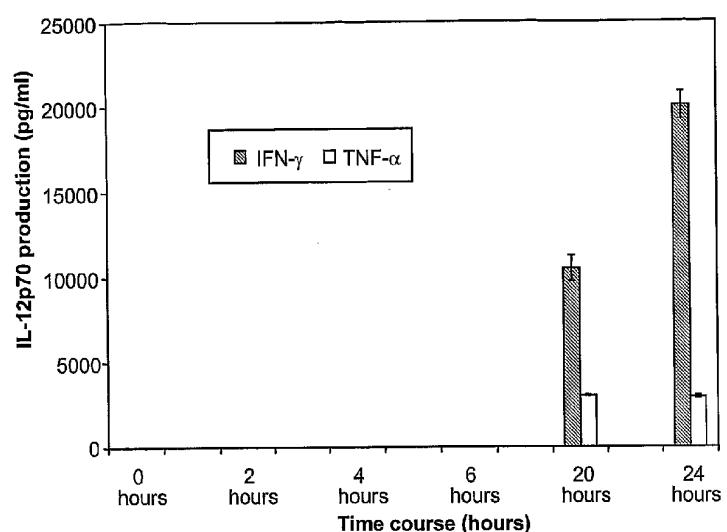
**FIG. 3**

**FIG. 4**

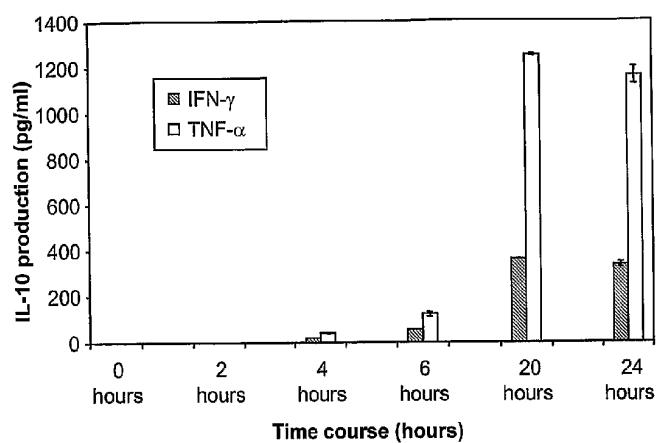
5/23

**FIG. 5a**

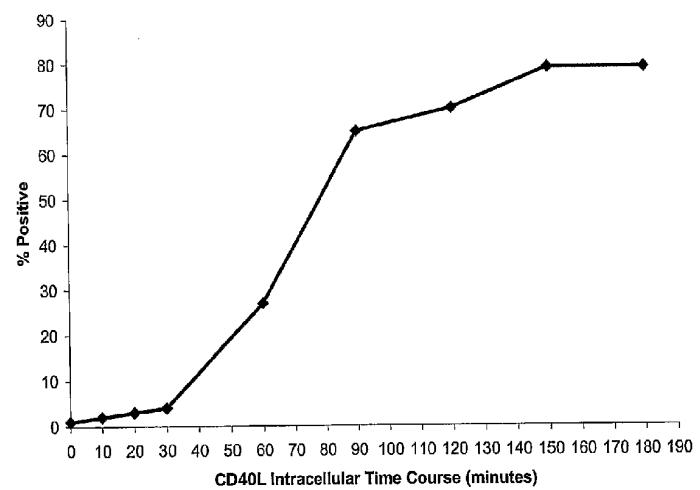
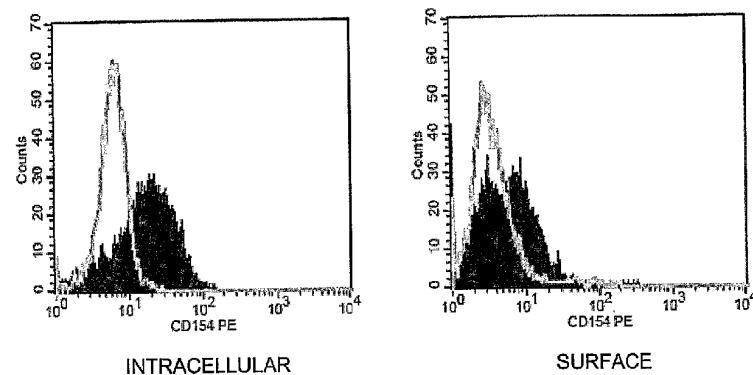
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**FIG. 5b**

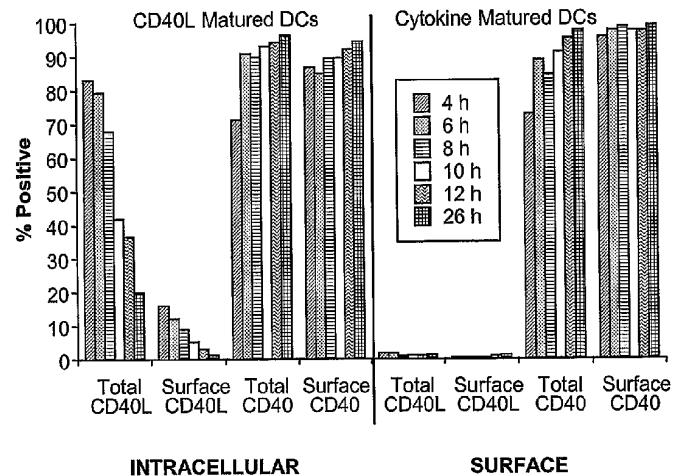
7/23

**FIG. 5c**

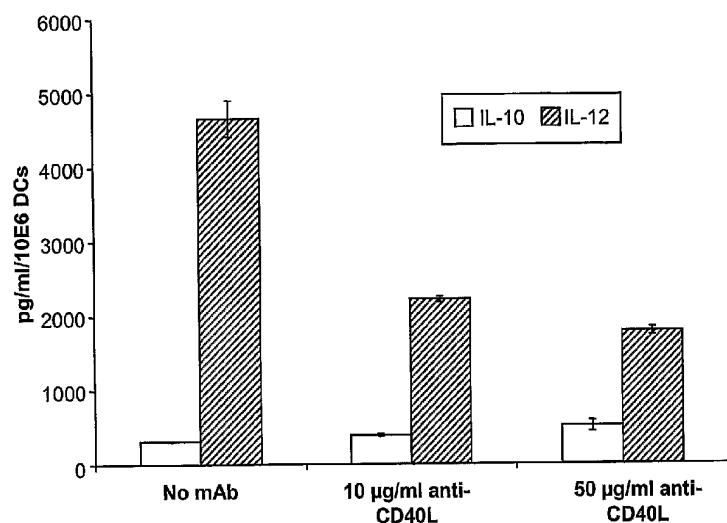
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**FIG. 6A****FIG. 6B**

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**FIG. 6C**

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**FIG. 7**

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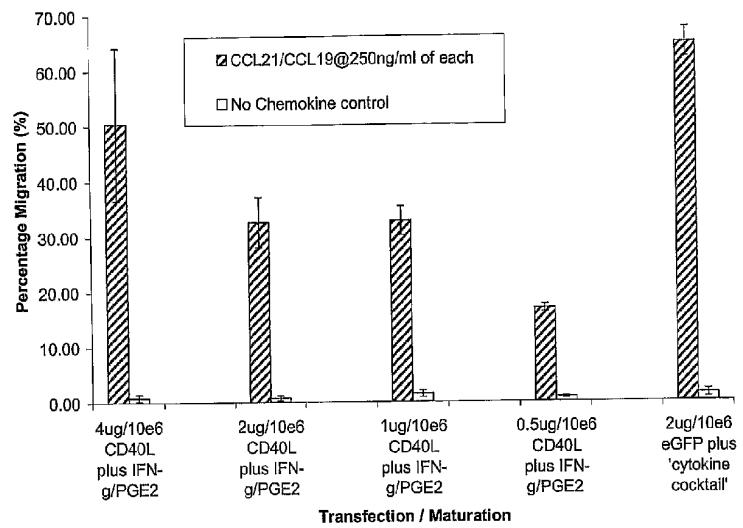
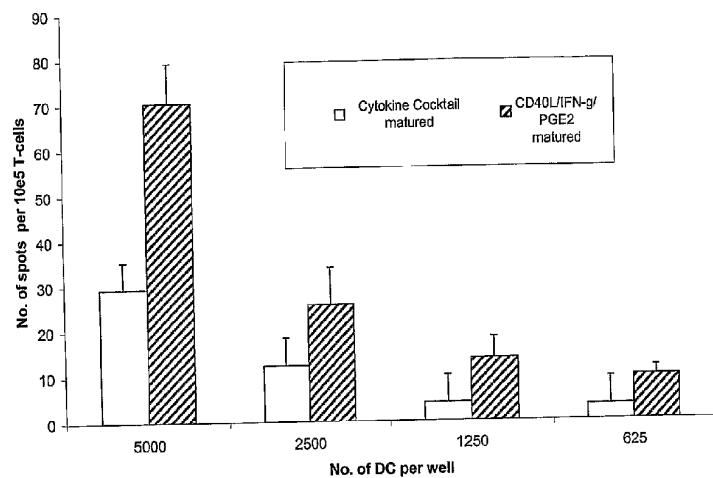
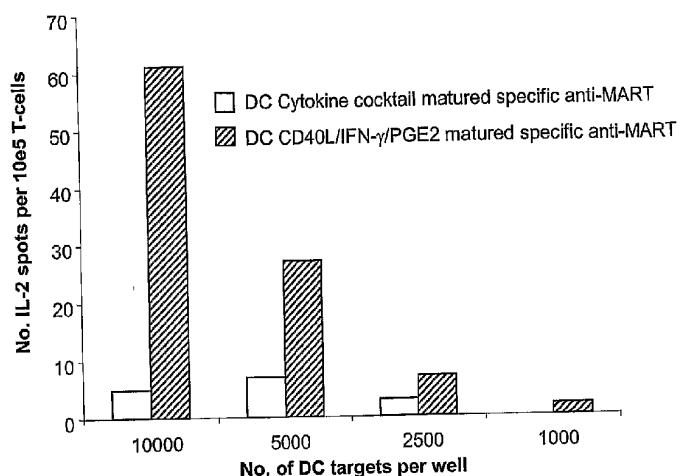


FIG. 8

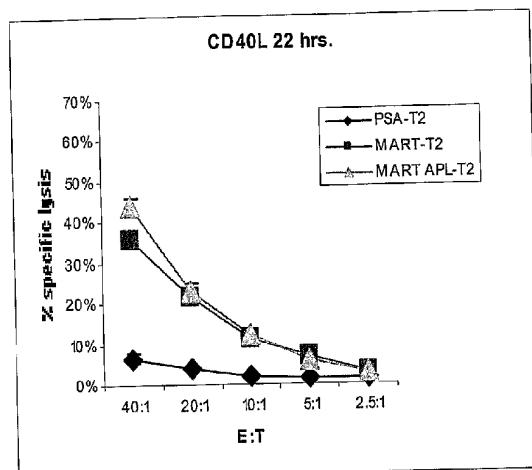
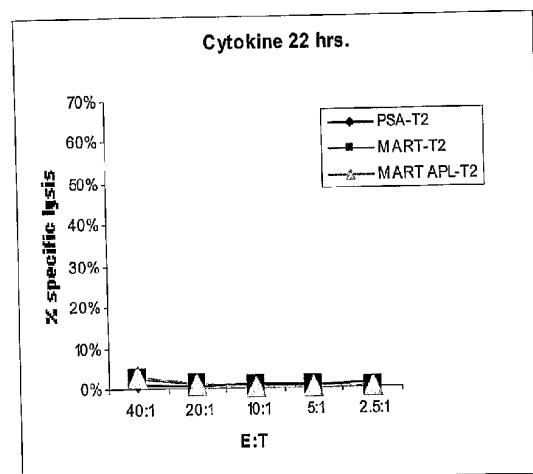
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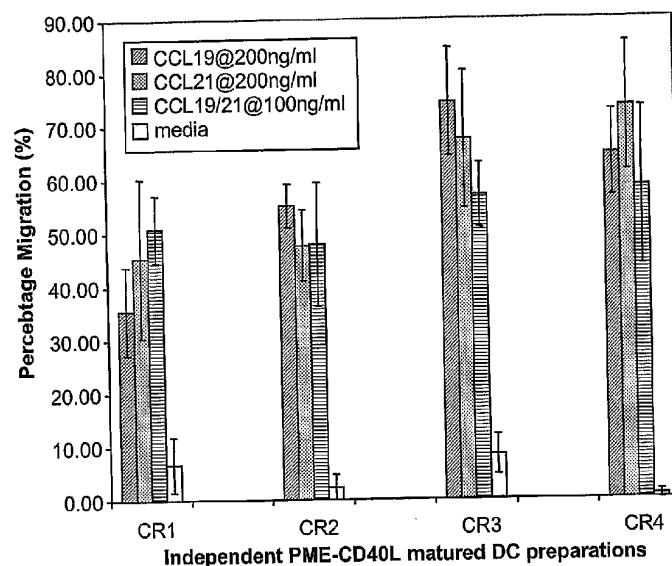
**FIG. 9**

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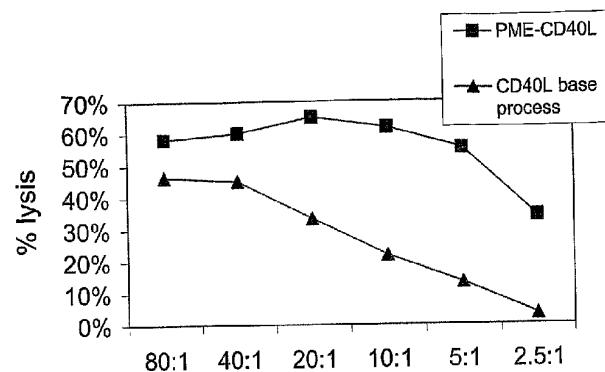
**FIG. 10**

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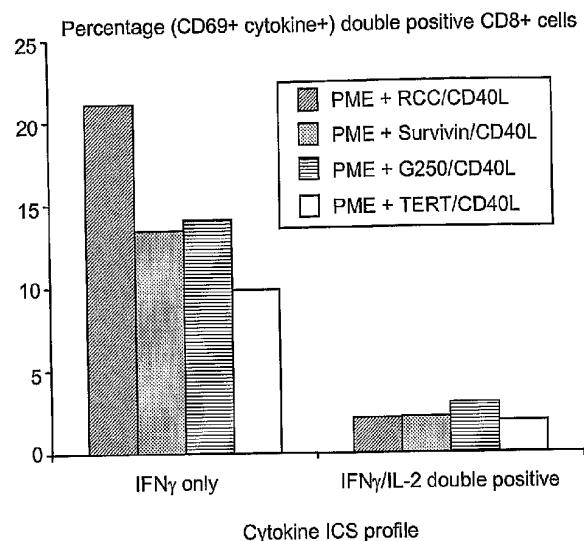
**FIG. 11a****FIG. 11b**

**FIG. 12**

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**FIG. 13**

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**FIG. 14**

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FIG. 15a

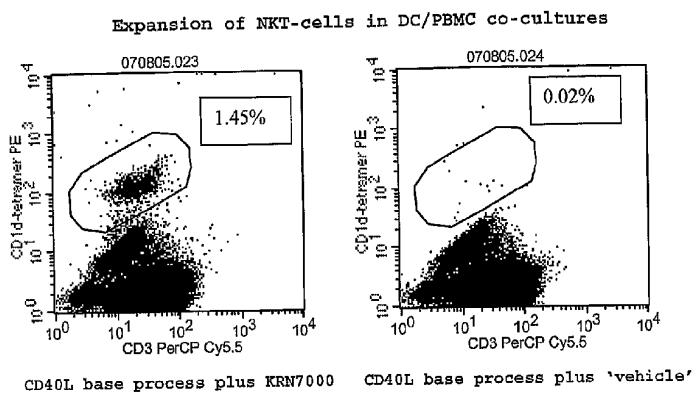
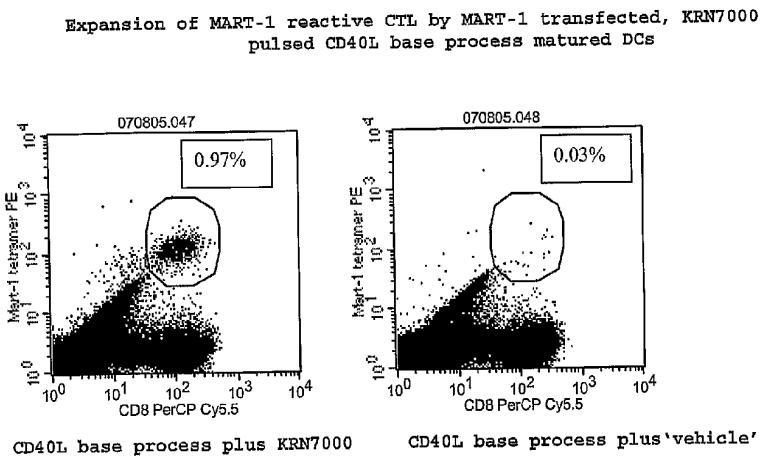


FIG. 15b



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FIG. 16a

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FIG. 16b

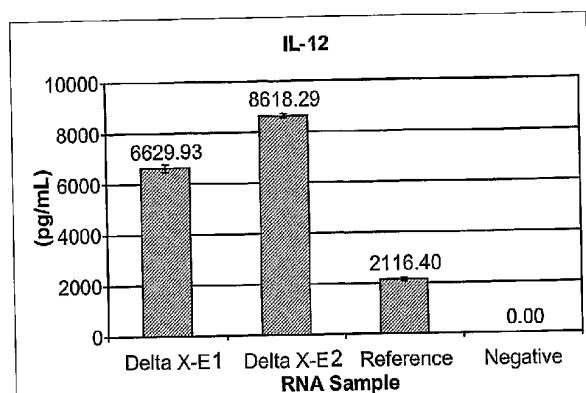
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**FIG. 16c**

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## FIGURE 17

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**Figure 18**

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SEQUENCE LISTING

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Kirin Beer Kabushiki Kaisha  
Healey, Don  
Tcherepanova, Irina  
Adams, Melissa  
Hinohara, Atsushi

<120> MATURE DENDRITIC CELL COMPOSITIONS AND METHODS FOR CULTURING SAME

<130> MER030WO

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10 15 20  
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25 30 35  
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Lys Glu Glu Thr Lys Lys Glu Asn Ser Phe Glu Met Gln Lys Gly Asp  
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Thr	Thr	Ser	Val	Leu	Gln	Trp	Ala	Glu	Lys	Gly	Tyr	Tyr	Thr	Met	Ser	
135																145
aac	aac	ttg	gtt	acc	ctg	gaa	aat	ggg	aaa	cag	ctg	acc	gtt	aaa	aga	534
Asn	Asn	Leu	Val	Thr	Leu	Glu	Asn	Gly	Lys	Gln	Leu	Thr	Val	Lys	Arg	
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caa	gga	ctc	tat	tat	atc	tat	gcc	caa	gtc	acc	tcc	tgt	tcc	aat	cgg	582
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Glu	Leu	Gln	Pro	Gly	Ala	Ser	Val	Phe	Val	Asn	Val	Thr	Asp	Pro	Ser	
230																245
caa	gtg	agc	cat	ggc	act	ggc	ttc	acg	tcc	ttt	ggc	tta	ctc	aaa	ctc	822
Gln	Val	Ser	His	Gly	Thr	Gly	Phe	Thr	Ser	Phe	Gly	Leu	Leu	Lys	Leu	
250																260
tga	acagtgtcac	tttgaggct	gtggggagc	tgacgctggg	agtcttcata											875
atacagcaca	gcggtaagc	ccacccctg	ttaactgcct	atttataacc	ctaggatcct											935
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caggtcacat	gaaacaaaaa	cggccctgc	tccataagag	cttatatac	tgaagcagca											1055
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gtctttccat	ggataatgc	tttgatttat	cagtgaagat	gcagaaggga	aatggggagc											1235
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 Arg Leu Asp Lys Ile Glu Asp Glu Arg Asn Leu His Glu Asp Phe Val  
 50 55 60  
 Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly Glu Arg Ser Leu Ser  
 65 70 75 80  
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 85 90 95  
 Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys Glu Asn Ser Phe Glu  
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 Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val Ile Ser  
 115 120 125  
 Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu Lys Gly  
 130 135 140  
 Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu Glu Asn Gly Lys Gln  
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 165 170 175  
 Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile Ala Ser  
 180 185 190  
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 195 200 205  
 Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly Gln Gln Ser Ile His  
 210 215 220  
 Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe Val Asn  
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## MER030WO2.ST25.txt

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Met Val Arg Leu Pro Leu Gln Cys Val Leu Trp Gly Cys Leu	
1 5 10	
ctg acc gct gtc cat cca gaa cca ccc act gca tgc aga gaa aaa cag	156
Leu Thr Ala Val His Pro Glu Pro Pro Thr Ala Cys Arg Glu Lys Gln	
15 20 25 30	
tac cta ata aac aat cag tgc tgg tct ttg tgc cag cca gga cag aaa	204
Tyr Leu Ile Asn Ser Gln Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys	
35 40 45	
ctg gtg agt gac tgc aca gag ttc act gaa acg gaa tgc ctt cct tgc	252
Leu Val Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys	
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ggt gaa agc gaa ttc cta gac acc tgg aac aga gag aca cac ttc cac	300
Gly Glu Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Phe His	
65 70 75	
cag cac aaa tac tgc gac ccc aac cta ggg ctt cgg gtc cag cag aag	348
Gln His Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys	
80 85 90	
ggc acc tca gaa aca gac acc atc tgc acc tgg aac gaa ggc tgg cac	396
Gly Thr Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His	
95 100 105 110	
tgt acg agt gag gcc tgg gag agc tgg gtc ctg cac cgc tca tgc tcg	444
Cys Thr Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser	
115 120 125	
ccc ggc ttt ggg gtc aag cag att gac atc tgc cag cca cat ttc ccc	492
Pro Gly Phe Gly Val Lys Gln Ile Asp Ile Cys Gln Pro His Phe Pro	
130 135 140	
aag gac cgc ggt ttg aac ctt ctg atg tag atgagctctg acatttggaaag	542
Lys Asp Arg Gly Leu Asn Leu Leu Met	
145 150	
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gcatgctgaa gtcctgattt ctccaggatcc ccaggatcgg ctgagagccc tggtggtat	662
ccccatcatc ttccggatcc tggccat cctcttgggtt ctggcttta tcaaaaaggt	722
ggccaagaag ccaaccaata aggcccccca ccccaagcag gaaccccagg agatcaattt	782
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MERU30WO2.ST25.txt

ccaacccggtc	acccaggagg	atggcaaaga	gagtgcac	tcagtgcagg	agagacagt	902
aggctgcacc	cacccaggag	tgtggccacg	tggcaaca	ggcagttggc	cagagagcct	962
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aagccatata	cacagatgcc	cattgcagca	ttgtttgtga	tagtgaacaa	ctgaaagctg	1322
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cttttaaaa	aagtacatgc	ttttatgtat	gtatattgcc	tatggatata	tgtataaata	1502
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aaaaaaaaaa						1570

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&lt;211&gt; 151

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 4

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Ala	Val	His	Pro	Glu	Pro	Pro	Thr	Ala	Cys	Arg	Glu	Lys	Gln	Tyr	Leu
						20			25			30			
Ile	Asn	Ser	Gln	Cys	Cys	Ser	Leu	Cys	Gln	Pro	Gly	Gln	Lys	Leu	Val
						35			40			45			
Ser	Asp	Cys	Thr	Glu	Phe	Thr	Glu	Thr	Glu	Cys	Leu	Pro	Cys	Gly	Glu
						50			55			60			
Ser	Glu	Phe	Leu	Asp	Thr	Trp	Asn	Arg	Glu	Thr	His	Phe	His	Gln	His
						65			70			75			80
Lys	Tyr	Cys	Asp	Pro	Asn	Leu	Gly	Leu	Arg	Val	Gln	Gln	Lys	Gly	Thr
						85			90			95			
Ser	Glu	Thr	Asp	Thr	Ile	Cys	Thr	Cys	Glu	Glu	Gly	Trp	His	Cys	Thr
						100			105			110			
Ser	Glu	Ala	Cys	Glu	Ser	Cys	Val	Leu	His	Arg	Ser	Cys	Ser	Pro	Gly
						115			120			125			
Phe	Gly	Val	Lys	Gln	Ile	Asp	Ile	Cys	Gln	Pro	His	Phe	Pro	Lys	Asp
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 Met Lys Tyr  
 1  
 aca agt tat atc ttg gct ttt cag ctc tgc atc gtt ttg ggt tct ctt 165  
 Thr Ser Tyr Ile Leu Ala Phe Gln Leu Cys Ile Val Leu Gly Ser Leu  
 5 10 15  
 ggc tgt tac tgc cag gac cca tat gta aaa gaa gca gaa aac ctt aag 213  
 Gly Cys Tyr Cys Gln Asp Pro Tyr Val Lys Glu Ala Glu Asn Leu Lys  
 20 25 30 35  
 aaa tat ttt aat gca ggt cat tca gat gta gcg gat aat gga act ctt 261  
 Lys Tyr Phe Asn Ala Gly His Ser Asp Val Ala Asp Asn Gly Thr Leu  
 40 45 50  
 ttc tta ggc att ttg aag aat tgg aaa gag gag agt gac aga aaa ata 309  
 Phe Leu Gly Ile Leu Lys Asn Trp Lys Glu Glu Ser Asp Arg Lys Ile  
 55 60 65  
 atg cag agc caa att gtc tcc ttt tac ttc aaa ctt ttt aaa aac ttt 357  
 Met Gln Ser Gln Ile Val Ser Phe Tyr Phe Lys Leu Phe Lys Asn Phe  
 70 75 80  
 aaa gat gac cag agc atc caa aag agt gtg gag acc atc aag gaa gac 405  
 Lys Asp Asp Gln Ser Ile Gln Lys Ser Val Glu Thr Ile Lys Glu Asp  
 85 90 95  
 atg aat gtc aag ttt ttc aat agc aac aaa aag aaa cga gat gac ttc 453  
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 Glu Lys Leu Thr Asn Tyr Ser Val Thr Asp Leu Asn Val Gln Arg Lys  
 120 125 130  
 gca ata cat gaa ctc atc caa gtg atg gct gaa ctg tcg cca gca gct 549  
 Ala Ile His Glu Leu Ile Gln Val Met Ala Glu Leu Ser Pro Ala Ala  
 135 140 145  
 aaa aca ggg aag cga aaa agg agt cag atg ctg ttt caa ggt cga aga 597  
 Lys Thr Gly Lys Arg Lys Arg Ser Gln Met Leu Phe Gln Gly Arg Arg  
 150 155 160  
 gca tcc cag taa tggttgtcct gcctgcata tttgaatttt aaatctaaat 649  
 Ala Ser Gln  
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 ctatttata atatcca ttatttatata gggaaataata ttttttagact catcaatcaa 709  
 ataagtttttataatataatagcaa cttttgtgtat atgaaaatgtat atatcttataatatgtat  
 Page 6 769

## MERU30WO2.ST25.txt

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aag	atccat	gggttgtgt	tttatttac	ttgatgatac	aatgaacact	tataagtgaa	949	
gtg	tactat	ccagttact	ccggtttga	aatatgcctg	caatctgagc	cagtgc	1009	
atg	gcatgtc	agacagaact	tgaatgtgtc	aggtgaccct	gatgaaaaca	tagcatctca	1069	
ggag	atttca	tgcctgg	tc	ttccaaat	at	tgtgactgta	cccaaatgga	1129
aag	taactca	tttgtt	aaa	tatcaat	ata	tgaataaagt	gtaagttcac	1189
aact								1193

&lt;210&gt; 6

&lt;211&gt; 166

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 6

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Gly	Ser	Leu	Gly	Cys	Tyr	Cys	Gln	Asp	Pro	Tyr	Val	Lys	Glu	Ala	Glu
							20			25		30			

Asn	Leu	Lys	Lys	Tyr	Phe	Asn	Ala	Gly	His	Ser	Asp	Val	Ala	Asp	Asn
						35		40		45					

Gly	Thr	Leu	Phe	Leu	Gly	Ile	Leu	Lys	Asn	Trp	Lys	Glu	Glu	Ser	Asp
						50		55		60					

Arg	Lys	Ile	Met	Gln	Ser	Gln	Ile	Val	Ser	Phe	Tyr	Phe	Lys	Leu	Phe
65						70			75			80			

Lys	Asn	Phe	Lys	Asp	Asp	Gln	Ser	Ile	Gln	Lys	Ser	Val	Glu	Thr	Ile
						85		90		95					

Lys	Glu	Asp	Met	Asn	Val	Lys	Phe	Phe	Asn	Ser	Asn	Lys	Lys	Lys	Arg
						100		105		110					

Asp	Asp	Phe	Glu	Lys	Leu	Thr	Asn	Tyr	Ser	Val	Thr	Asp	Leu	Asn	Val
115						120			125						

Gln	Arg	Lys	Ala	Ile	His	Glu	Leu	Ile	Gln	Val	Met	Ala	Glu	Leu	Ser
130						135			140						

Pro	Ala	Ala	Lys	Thr	Gly	Lys	Arg	Lys	Arg	Ser	Gln	Met	Leu	Phe	Gln
145						150			155		160				

Gly	Arg	Arg	Ala	Ser	Gln										
					165										

&lt;210&gt; 7

&lt;211&gt; 1669

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

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 Met Ser Thr  
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 Glu Ser Met Ile Arg Asp Val Glu Leu Ala Glu Glu Ala Leu Pro Lys  
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 aag aca ggg ggg ccc cag ggc tcc agg cgg tgc ttg ttc ctc agc ctc  
 Lys Thr Gly Gly Pro Gln Gly Ser Arg Arg Cys Leu Phe Leu Ser Leu  
 20 25 30 35  
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 Phe Ser Phe Leu Ile Val Ala Gly Ala Thr Thr Leu Phe Cys Leu Leu  
 40 45 50  
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 His Phe Gly Val Ile Gly Pro Gln Arg Glu Glu Phe Pro Arg Asp Leu  
 55 60 65  
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 Ser Leu Ile Ser Pro Leu Ala Gln Ala Val Arg Ser Ser Ser Arg Thr  
 70 75 80  
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 Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro Gln Ala Glu  
 85 90 95  
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 Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn  
 100 105 110 115  
 ggc gtg gag ctg aga gat aac cag ctg gtg gtg cca tca gag ggc ctg  
 Gly Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu  
 120 125 130  
 tac ctc atc tac tcc cag gtc ctc ttc aag ggc caa ggc tgc ccc tcc  
 Tyr Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser  
 135 140 145  
 acc cat gtg ctc ctc acc cac acc atc agc cgc atc gcc gtc tcc tac  
 Thr His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr  
 150 155 160  
 cag acc aag gtc aac ctc ctc tct gcc atc aag agc ccc tgc cag agg  
 Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg  
 165 170 175  
 gag acc cca gag ggg gct gag gcc aag ccc tgg tat gag ccc atc tat  
 Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr  
 180 185 190 195  
 ctg gga ggg gtc ttc cag ctg gag aag ggt gac cga ctc agc gct gag  
 Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu  
 200 205 210

## MERU30WO2.ST25.txt

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Cys Leu Leu His Phe Gly Val Ile Gly Pro Gln Arg Glu Glu Phe Pro 50 55 60	
Arg Asp Leu Ser Leu Ile Ser Pro Leu Ala Gln Ala Val Arg Ser Ser 65 70 75 80	
Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro 85 90 95	
Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu 100 105 110	

MER030WO2.ST25.txt

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 115 120 125

Glu Gly Leu Tyr Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly  
 130 135 140

Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala  
 145 150 155 160

Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro  
 165 170 175

Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu  
 180 185 190

Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu  
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Gln Val Tyr Phe Gly Ile Ile Ala Leu  
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 Ala Thr Gly Leu Pro Ala Ser Met Lys Ile Phe Met Tyr Leu Leu Thr  
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gtt ttc ctt atc acc caa atg att gga tct gtg ctt ttt gct gtg tat 147  
 Val Phe Leu Ile Thr Gln Met Ile Gly Ser Val Leu Phe Ala Val Tyr  
 30 35 40 45

ctt cat aga aga ttg gat aag gtc gaa gag gaa gta aac ctt cat gaa 195  
 Leu His Arg Arg Leu Asp Lys Val Glu Glu Val Asn Leu His Glu  
 50 55 60

gat ttt gta ttc ata aaa aag cta aag aga tgc aac aaa gga gaa gga 243  
 Asp Phe Val Ile Lys Lys Leu Lys Arg Cys Asn Lys Gly Glu Gly  
 65 70 75

tct tta tcc ttg ctg aac tgt gag gag atg aga agg caa ttt gaa gac 291  
 Ser Leu Ser Leu Leu Asn Cys Glu Glu Met Arg Arg Gln Phe Glu Asp  
 80 85 90

ctt gtc aag gat ata acg tta aac aaa gaa gag aaa aaa gaa aac agc 339  
 Leu Val Lys Asp Ile Thr Leu Asn Lys Glu Glu Lys Lys Glu Asn Ser  
 95 100 105

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MER030WO2.ST25.txt

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Val	Ser	Glu	Ala	Asn	Ser	Asn	Ala	Ala	Ser	Val	Leu	Gln	Trp	Ala	Lys	
																140
130																
aaa	gga	tat	tat	acc	atg	aaa	agc	aac	ttg	gta	atg	ctt	gaa	aat	ggg	483
Lys	Gly	Tyr	Tyr	Met	Lys	Ser	Asn	Leu	Val	Met	Leu	Glu	Asn	Gly		
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145																
aaa	cag	ctg	acg	gtt	aaa	aga	gaa	gca	ctc	tat	tat	gtc	tac	act	caa	531
Lys	Gln	Leu	Thr	Val	Lys	Arg	Glu	Gly	Leu	Tyr	Tyr	Val	Tyr	Thr	Gln	
																160
160																165
gtc	acc	tcc	tgc	tct	aat	cgg	gag	cct	tcg	agt	caa	cgc	cca	ttc	atc	579
Val	Thr	Phe	Cys	Ser	Asn	Arg	Glu	Pro	Ser	Ser	Gln	Arg	Pro	Phe	Ile	
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175																180
gtc	ggc	ctc	tgg	ctg	aag	ccc	agg	agt	gga	tct	gag	aga	atc	tta	ctc	627
Val	Gly	Leu	Trp	Leu	Lys	Pro	Ser	Ser	Gly	Ser	Glu	Arg	Ile	Leu	Leu	
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190																195
aag	gcg	gca	aat	acc	cac	agt	tcc	tcc	cag	ctt	tgc	gag	cag	cag	tct	675
Lys	Ala	Ala	Asn	Thr	His	Ser	Ser	Ser	Gln	Leu	Cys	Glu	Gln	Gln	Ser	
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210																215
gtt	cac	ttg	ggc	gga	gtg	ttt	gaa	tta	caa	gct	ggt	gct	tct	gtg	ttt	723
Val	His	Leu	Gly	Gly	Val	Phe	Glu	Leu	Gln	Ala	Gly	Ala	Ser	Val	Phe	
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225																230
gtc	aac	gtg	act	gaa	gca	agc	caa	gtg	atc	cac	aga	gtt	ggc	ttc	tca	771
Val	Asn	Val	Thr	Glu	Ala	Ser	Gln	Val	Ile	His	Arg	Val	Gly	Phe	Ser	
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240																245
tct	ttt	ggc	tta	ctc	aaa	ctc	tga	acagtgcgct	gtccttaggct	gcagcagggc						825
Ser	Phe	Gly	Leu	Leu	Lys	Leu										
																255
255																260
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<213>	Mus musculus															
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MER030WO2.ST25.txt

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Ile Thr Gln Met Ile Gly Ser Val Leu Phe Ala Val Tyr Leu His Arg			
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Arg Leu Asp Lys Val Glu Glu Val Asn Leu His Glu Asp Phe Val			
50 55 60			
Phe Ile Lys Lys Leu Lys Arg Cys Asn Lys Gly Glu Gly Ser Leu Ser			
65 70 75 80			
Leu Leu Asn Cys Glu Glu Met Arg Arg Gln Phe Glu Asp Leu Val Lys			
85 90 95			
Asp Ile Thr Leu Asn Lys Glu Glu Lys Lys Glu Asn Ser Phe Glu Met			
100 105 110			
Gln Arg Gly Asp Glu Asp Pro Gln Ile Ala Ala His Val Val Ser Glu			
115 120 125			
Ala Asn Ser Asn Ala Ala Ser Val Leu Gln Trp Ala Lys Lys Gly Tyr			
130 135 140			
Tyr Thr Met Lys Ser Asn Leu Val Met Leu Glu Asn Gly Lys Gln Leu			
145 150 155 160			
Thr Val Lys Arg Glu Gly Leu Tyr Tyr Val Tyr Thr Gln Val Thr Phe			
165 170 175			
Cys Ser Asn Arg Glu Pro Ser Ser Gln Arg Pro Phe Ile Val Gly Leu			
180 185 190			
Trp Leu Lys Pro Ser Ser Gly Ser Glu Arg Ile Leu Leu Lys Ala Ala			
195 200 205			
Asn Thr His Ser Ser Ser Gln Leu Cys Glu Gln Ser Val His Leu			
210 215 220			
Gly Gly Val Phe Glu Leu Gln Ala Gly Ala Ser Val Phe Val Asn Val			
225 230 235 240			
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Leu Leu Lys Leu			
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21

## MER030WO2.ST25.txt

<212> DNA  
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 <223> Optimized human CD40L  
  
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 acttactgtt ttctttatca cccagatgtat tgggtcagca ctttttgcgt tgtagtctca 180  
 tagaagggtt gacaagatag aagatgaaag gaatcttcat gaagatTTT tattcatgaa 240  
 aacgatacag agatgcaaca caggagaaag atccttattcc ttactgaact gtgaggagat 300  
 taaaagccag ttgtgaaggc ttgtgaagga tataatgtta aacaaagagg agacgaaagaa 360  
 agaaaacagc ttgtgaatgc aaaaagggtga tcagaatcct caaattgcgg cacatgtcat 420  
 aagttagggcc agcagtaaaa caacatctgt ttacagtgg gctgaaaaag gatactacac 480  
 catgagcaac aacttggtaa ccctggaaaa tggaaacag ctgaccgtta aaagacaagg 540  
 actcttattt atctatgccc aagtcaccc ttgttccaat cgggaaggctt cgagtcaagc 600  
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 <212> DNA  
 <213> Artificial Sequence  
  
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 <223> CD40 Receptor 3'UTR  
  
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MER030WO2.ST25.txt

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<212>	DNA					
<213>	Homo sapiens					
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aactctgtaa	gtaaggatgc	gtatacgcatt	tcgctacact	gagttatca	ctctgtatgtt	120
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## MER030WO2.ST25.txt

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## MER030WO2.ST25.txt

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<210> 22  
 <211> 180  
 <212> DNA  
 <213> Spleen necrosis virus

<400> 22  
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<210> 23  
 <211> 143  
 <212> DNA  
 <213> Tobacco etch virus

<400> 23  
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<210> 24  
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 <212> PRT  
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<220>  
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<210> 25  
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 <212> PRT  
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<220>  
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<210> 26

## MER030WO2.ST25.txt

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