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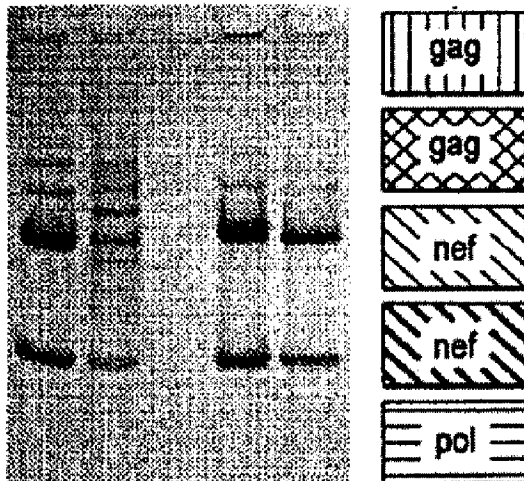
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(54) Title: ANTI-CD40 ANTIBODIES AND USES THEREOF



(57) Abrégé/Abstract:

The present description provides an antibody or an antigen binding fragment thereof that binds to CD40, comprising: (i) an antibody light chain variable region having 90% sequence identity to SEQ ID NO: 4 or 5 and an antibody heavy chain variable region having 90% sequence identity to SEQ ID NO: 3; an antibody light chain variable region having 90% sequence identity to SEQ ID NO: 7 and an antibody heavy chain variable region having 90% sequence identity to SEQ ID NO: 6; or the antibody light chain CDRs of SEQ ID NO: 41, 42 and 43 and the antibody heavy chain CDRs of SEQ ID NO: 44, 45 and 46.

## **ABSTRACT**

The present description provides an antibody or an antigen binding fragment thereof that binds to CD40, comprising: (i) an antibody light chain variable region having 90% sequence identity to SEQ ID NO: 4 or 5 and an antibody heavy chain variable region having 90% sequence identity to SEQ ID NO: 3; an antibody light chain variable region having 90% sequence identity to SEQ ID NO: 7 and an antibody heavy chain variable region having 90% sequence identity to SEQ ID NO: 6; or the antibody light chain CDRs of SEQ ID NO: 41, 42 and 43 and the antibody heavy chain CDRs of SEQ ID NO: 44, 45 and 46.

## ANTI-CD40 ANTIBODIES AND USES THEREOF

### Technical Field of the Invention

The present invention relates in general to the field of immunization, and more particularly, to novel anti-CD40 antibodies and anti-CD40 antibody-based vaccines.

### 5 Background Art

Without limiting the scope of the invention, its background is described in connection with antigen presentation. One example of vaccines and methods for antigen presentation is taught in United States Patent No. 7,118,751, issued to Ledbetter, et al., for DNA vaccines encoding an amino-terminus antigen linked to a carboxy-terminus domain that binds CD40. Briefly, vaccines are taught that target one or more antigens to a cell surface receptor to improve the antigen-specific humoral and cellular immune response. Antigen(s) linked to a domain that binds to a cell surface receptor are internalized, carrying antigen(s) into an intracellular compartment where the antigen(s) are digested into peptides and loaded onto MHC molecules. T cells specific for the peptide antigens are activated, leading to an enhanced immune response. The vaccine may comprise antigen(s) linked to a domain that binds at least one receptor or a DNA plasmid encoding antigen(s) linked to a domain that binds at least one receptor. A preferred embodiment of the invention targets HIV-1 env antigen to the CD40 receptor, resulting in delivery of antigen to CD40 positive cells, and selective activation of the CD40 receptor on cells presenting HIV-1 env antigens to T cells.

Another example is found in United States Patent Application No. 20080254026, filed by Li, et al., for antagonist anti-CD40 monoclonal antibodies and methods for their use. Briefly, compositions and methods are disclosed for use in therapy for treating diseases mediated by stimulation of CD40 signaling on CD40-expressing cells are provided. The methods comprise administering a therapeutically effective amount of an antagonist anti-CD40 antibody or antigen-binding fragment thereof to a patient in need thereof. The antagonist anti-CD40 antibody or antigen-binding fragment thereof is free of significant agonist activity, but exhibits antagonist activity when the antibody binds a CD40 antigen on a human CD40-expressing cell. Antagonist activity of the anti-CD40 antibody or antigen-binding fragment thereof beneficially inhibits proliferation and/or differentiation of human CD40-expressing cells, such as B cells.

Yet another example is taught in United States Patent Application No. 20080241139, filed by Delucia for an adjuvant combination comprising a microbial TLR agonist, a CD40 or 4-1BB agonist, and optionally an antigen and the use thereof for inducing a synergistic enhancement in cellular immunity. Briefly, this application is said to teach adjuvant combinations comprising at least one microbial TLR agonist such as a whole virus, bacterium or yeast or portion thereof such a membrane, spheroplast, cytoplasm, or ghost, a CD40

or 4-1BB agonist and optionally an antigen wherein all 3 moieties may be separate or comprise the same recombinant microorganism or virus are disclosed. The use of these immune adjuvants for treatment of various chronic diseases such as cancers and HIV infection is also provided.

United States Patent Application No. 20080199471, filed by Bernett, et al., is directed to optimized CD40 antibodies and methods of using the same. Briefly, this application is said to teach antibodies that target CD40, wherein the antibodies comprise at least one modification relative to a parent antibody, wherein the modification alters affinity to an FcγR or alters effector function as compared to the parent antibody. Also disclosed are methods of using the antibodies of the invention.

Finally, United States Patent Application No. 20080181915, file by Tripp, et al., is directed to a CD40 ligand adjuvant for respiratory syncytial virus. Briefly, this application is said to teach methods and adjuvants for enhancing an immune response to RSV in a host, wherein the methods and adjuvants comprise a source of a CD40 binding protein. Preferably, the CD40 binding protein is CD40L and the source is a vector comprising a promoter operatively linked to a CD40L coding region. The enhanced immune response produced by the adjuvants and methods of the current invention includes both increased expression of Th1 cytokines and increased production of antibody.

#### Disclosure of the Invention

In one embodiment, the present invention is a recombinant antibody or an antigen binding fragment thereof, both of which bind to CD40, comprising: at least one antibody light chain variable region of SEQ ID NOS: 2, 4, 5 or 7; and at least one antibody heavy chain variable region of SEQ ID NOS: 1, 3 or 6. In one aspect, the antibody further comprises a heavy chain constant region, wherein the heavy chain constant region comprises a gamma-1, gamma-2, gamma-3, or gamma-4 human heavy chain constant region or a variant of the human heavy chain constant region. In one aspect, the antibody further comprises a light chain constant region, wherein the light chain constant region comprises a lambda or a kappa human light chain constant region. In another aspect, the binding fragment is selected from group consisting of Fab, Fab', Fab'-SH, Fv, scFv, F(ab')<sub>2</sub>, and a diabody. In another aspect, the antibody comprises the polypeptide sequence of SEQ ID NOS: 1, 3 or 6, and/or the antibody comprises the polypeptide sequence of SEQ ID NOS: 2, 4, 5, or 7. In another aspect, the antibody is produced by a hybridoma anti-CD40\_12E12.3F3 (ATCC Accession No. PTA-9854 filed with the ATCC on 26/02/2009), anti-CD40\_12B4.2C10 (Deposit Submission No. HS446, ATCC Accession No. PTA-10652 filed with the ATCC on 17/02/2010), and anti-CD40\_11B6.1C3 (Deposit Submission No. HS440, ATCC Accession No. PTA-10653 filed with the ATCC on 17/02/2010). In another aspect, the antibody alone is capable of causing dendritic cells to secrete at least one of IL-6, MIP-1a, IL-12p40 or TNFalpha without prior activation of the dendritic cells. In one aspect, the antibody is capable of causing dendritic cells activated with GM-CSF and Interferon alpha to secrete at least one of IL-6, MIP-1 a, IP-10, IL-

10 or IL-12p40. In another aspect, the recombinant antibody comprises at least 90, 95, 99 or 100% sequence identity with at least one antibody light chain variable region of SEQ ID NOS: 2, 4, 5 or 7; and at least one antibody heavy chain variable region of SEQ ID NOS: 1, 3 or 6. In another aspect, the antibody is humanized.

- 5 Another embodiment of the present invention is a composition comprising an antibody or an antigen binding fragment thereof, in combination with a pharmaceutically acceptable carrier or diluent, wherein the antibody is the antibody of claim 1.

- Another embodiment of the present invention is a humanized recombinant antibody or an antigen binding fragment thereof, both of which bind to CD40, comprising: a) at least one antibody light chain variable region of SEQ ID NOS.: 2, 4, 5 or 7; and b) at least one antibody heavy chain variable region of SEQ ID NOS.: 1, 3 or 6. In one aspect, the antibody further comprises a heavy chain constant region, wherein the heavy chain constant region comprises a gamma-1, gamma-2, gamma-3, or gamma-4 human heavy chain constant region or a variant of the human heavy chain constant region. In one aspect, the antibody further comprises a light chain constant region, wherein the light chain constant region comprises a lambda or a kappa human light chain constant region. In another aspect, the binding fragment is selected from group consisting of Fab, Fab', Fab'-SH, Fv, scFv, F(ab')<sub>2</sub>, and a diabody. In another aspect, the antibody, or antigen binding fragment thereof, comprises the polypeptide sequence of SEQ ID NOS.: 1, 3 or 6, and/or the polypeptide sequence of SEQ ID NOS.: 2, 4, 5, or 7. In one aspect, the antibody comprises at least the variable region of anti-CD40\_12E12.3F3 (ATCC Accession No. PTA-9854), anti-CD40\_12B4.2C10 (Deposit Submission No. HS446, ATCC Accession No. PTA-10653), and anti-CD40\_11B6.1C3 (Deposit Submission No. HS440, ATCC Accession No. PTA-10652). In another aspect, the humanized antibody comprises the complementarity determining regions of: a) at least one antibody light chain variable region of SEQ ID NOS: 2, 4, 5 or 7; and b) at least one antibody heavy chain variable region of SEQ ID NOS: 1, 3 or 6 on a human antibody framework.
- 25 Another embodiment of the present invention is a composition comprising an antibody or an antigen binding fragment thereof, in combination with a pharmaceutically acceptable carrier or diluent, wherein the antibody is the antibody of claim a recombinant antibody or an antigen binding fragment thereof, both of which bind to CD40, comprising: at least one antibody light chain variable region of SEQ ID NO.: 2, 4, 5 or 7; and at least one antibody heavy chain variable region of SEQ ID NO.: 1, 3 or 6. In another aspect, the antibody comprises at least the variable region of the antibody anti-CD40\_12E12.3F3 (ATCC Accession No. PTA-9854), anti-CD40\_12B4.2C10 (ATCC Submission No. HS446, Accession No. PTA-10653), and anti-CD40\_11B6.1C3 (ATCC Submission No. HS440, Accession No. PTA-10652). In another aspect, the antibody

comprises at least one variable domain having 90, 95 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7.

Another embodiment of the present invention is an isolated nucleic acid encoding the polypeptide of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7. In one aspect, the nucleic acids further comprise nucleic acid sequences from human antibodies that humanize the antibody. In another aspect, the antibody comprises at least one variable domain having 90, 95 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7.

Another embodiment of the present invention is an expression vector comprising the isolated nucleic acid encoding the polypeptide of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS: 2, 4, 5, or 7, operably linked to control sequences recognized by a host cell transfected with the vector. In another aspect, the antibody comprises at least one variable domain having 90, 95 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7.

Another embodiment of the present invention is a host cell comprising the vector that encodes the isolated nucleic acid encoding the polypeptide of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS: 2, 4, 5, or 7. In another aspect, the antibody comprises at least one variable domain having 90, 95 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7.

Another embodiment of the present is a method of producing a polypeptide, comprising culturing the host cell comprising isolated nucleic acid encoding the polypeptide of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS: 2, 4, 5, or 7, under conditions wherein the nucleic acid sequence is expressed, thereby producing the polypeptide, and recovering the polypeptide from the host cell. In another aspect, the antibody comprises at least one variable domain having 90, 95 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7.

Another embodiment of the present invention is an expression vector comprising the isolated nucleic acid encoding the polypeptide of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS: 2, 4, 5, or 7, operably linked to control sequences recognized by a host cell transfected with the vector. In another aspect, the antibody comprises at least one variable domain having 90, 95 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7.

Another embodiment of the present invention is a method of producing a polypeptide, comprising culturing the host cell comprising a vector that comprises isolated nucleic acid encoding the polypeptide of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS: 2, 4, 5, or 7, under conditions wherein the nucleic acid sequence is expressed, thereby producing the polypeptide, and recovering the polypeptide from the host cell.

Another embodiment of the present invention is an isolated nucleic acid sequence encoding an antibody specific for CD40 comprising a light chain having the nucleic acid sequence of SEQ ID NO: 9, 11, 12 or 15

and a heavy chain having the nucleic acid sequence of SEQ ID NO: 8, 10 or 14. In one aspect, the binding fragment is an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH, Fv, scFv, F(ab')<sub>2</sub>, and a diabody. In another aspect, the antibody comprises at least one variable domain having 90, 95 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7.

Another embodiment of the present invention is a method to identify an acceptor germline sequence for a humanized antibody, which method comprises the steps of: a) identifying a non-human antibody that has the desired biological activity selected from at least one antibody light chain variable region of SEQ ID NO: 2, 4, 5 or 7; and at least one antibody heavy chain variable region of SEQ ID NO: 1, 3 or 6; b) determining the amino acid sequence of a non-human antibody VH and VL domains; and c) comparing the nonhuman antibody sequence to a group of human germline sequences, wherein the comparison comprises the substeps of: 1) assigning the sequence of non-human VH and VL domain sequences residue numbers; 2) delineating the CDR and FR regions in the sequence; 3) assigning a predetermined numerical score at each residue position for which the non-human and human germline sequences are identical; and 4) totaling all of the residue scores to generate a total score for each human germline sequence; and d) identifying the human germline sequence with the highest total residue score as the acceptor germline sequence. In one aspect, the non-human antibody is specific for CD40. In another aspect, the antibody comprises at least one variable domain having 90, 95 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7.

Another embodiment of the present invention is an antibody generated by the method comprising a) identifying a non-human antibody that has the desired biological activity selected from at least one antibody light chain variable region of SEQ ID NO: 2, 4, 5 or 7; and at least one antibody heavy chain variable region of SEQ ID NO: 1, 3 or 6; b) determining the amino acid sequence of a non-human antibody VH and VL domains; and c) comparing the nonhuman antibody sequence to a group of human germline sequences, wherein the comparison comprises the substeps of: 1) assigning the sequence of non-human VH and VL domain sequences residue numbers; 2) delineating the CDR and FR regions in the sequence; 3) assigning a predetermined numerical score at each residue position for which the non-human and human germline sequences are identical; and 4) totaling all of the residue scores to generate a total score for each human germline sequence; and d) identifying the human germline sequence with the highest total residue score as the acceptor germline sequence. In one aspect, the non-human antibody is specific for CD40. In another aspect, the antibody comprises at least one variable domain having 90, 95 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS.: 2, 4, 5, or 7.

Another embodiment of the present invention is a method of making an antibody comprising expressing in a host cell a recombinant antibody or an antigen binding fragment thereof, both of which bind to CD40, comprising: at least one antibody light chain variable region of SEQ ID NO: 2, 4, 5 or 7; and at least one antibody heavy chain variable region of SEQ ID NO: 1, 3 or 6. In one aspect, the host cell is a bacterial, fungal, insect, or mammalian cell. In another aspect, the antibody is a humanized antibody. In another aspect, the antibody comprises at least one variable domain having 90, 95, 99 or 100% sequence identity with a heavy chain variable domain of SEQ ID NOS: 1, 3 or 6, and/or SEQ ID NOS: 2, 4, 5, or 7.

Another embodiment of the present invention is a recombinant antibody or an antigen binding fragment thereof that binds to CD40, wherein the antibody alone is capable of causing dendritic cells to secrete at least one of IL-6, MIP-1a, IL-12p40 or TNFalpha without prior activation of the dendritic cells. In one aspect, the antibody comprises at least one variable domain having 90% sequence identity with at least one antibody light chain variable region of SEQ ID NOS: 2, 4, 5 or 7; and at least one variable domain having 90% sequence identity with one antibody heavy chain variable region of SEQ ID NOS: 1, 3 or 6. In another aspect, the antibody comprises the polypeptide sequence of SEQ ID NOS: 1, 3 or 6, the polypeptide sequence of SEQ ID NOS: 2, 4, 5, or 7, or both. In another aspect, the antibody is produced by a hybridoma selected from anti-CD40\_12E12.3F3 (ATCC Accession No. PTA-9854), anti-CD40\_12B4.2C10 (ATCC Submission No. HS446, Accession No. PTA-10653), and anti-CD40\_11B6.1C3 (ATCC Submission No. HS440, Accession No. PTA-10652). In another aspect, the antibody is humanized. In another aspect, the antibody is capable of causing dendritic cells activated with GM-CSF and Interferon alpha to secrete at least one of IL-6, MIP-1a, IP-10, IL-10 or IL-12p40. In another aspect, the antibody alone is capable of causing B cell proliferation of at least 10%, 20%, 25%, 28%, 30% or 35%.

Another embodiment of the present invention is a recombinant antibody or an antigen binding fragment thereof that binds to CD40, wherein the antibody alone is capable of causing B cell proliferation of at least 10% of the B cells. In one aspect, the percentage of B cells that proliferate is at least 15%, 20%, 25%, 28%, 30% or 35%. In one aspect, the antibody comprises at least one variable domain having 90% sequence identity with at least one antibody light chain variable region of SEQ ID NOS: 2, 4, 5 or 7; and at least one variable domain having 90% sequence identity with one antibody heavy chain variable region of SEQ ID NOS: 1, 3 or 6. In another aspect, the antibody comprises the polypeptide sequence of SEQ ID NOS: 1, 3 or 6, the polypeptide sequence of SEQ ID NOS: 2, 4, 5, or 7, or both. In another aspect, the antibody is produced by a hybridoma selected from anti-CD40\_12E12.3F3 (ATCC Accession No. PTA-9854), anti-CD40\_12B4.2C10 (ATCC Submission No. HS446, Accession No. PTA-10653), and anti-CD40\_11B6.1C3 (ATCC Submission No. HS440, Accession No. PTA-10652). In another aspect, the antibody is humanized. In another aspect, antibody alone is capable of causing dendritic cells to secrete at least one of IL-6, MIP-1a, IL-



12p40 or TNFalpha without prior activation of the dendritic cells. In another aspect, the antibody is capable of causing dendritic cells activated with GM-CSF and Interferon alpha to secrete at least one of IL-6, MIP-1a, IP-10, IL-10 or IL-12p40.

### Description of the Drawings

- 5 For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:  
Fig. 1 shows protein A affinity recombinant antibodies fused to various HIV peptides (lanes 1 to 5) secreted from transfected 293F cells, analyzed by reducing SDS-PAGE and Coomassie Brilliant Blue staining.  
Fig. 2 shows protein A affinity purified recombinant antibodies fused to various HIV peptides (Lanes 1 and  
10 2) secreted from transfected 293F cells, then analyzed by reducing SDS-PAGE and Coomassie Brilliant Blue staining.  
Fig. 3 shows protein A affinity purified recombinant antibodies fused to various HIV peptide strings (Lanes 1 to 5) secreted from transfected 293F cells, then analyzed by reducing SDS.PAGE and Coomassie Brilliant Blue staining.  
15 Fig. 4 shows protein A affinity purified recombinant antibodies fused to various HIV peptide strings (Lanes 1 to 6) secreted from transfected 293F cells, then analyzed by reducing SDS.PAGE and Coomassie Brilliant Blue staining.  
Fig. 5 describes the protocol used in vitro to assay the potency of  $\alpha$ CD40.LIPO5 HIV peptide fusion recombinant antibody ( $\alpha$ CD40.LIPO5 rAb) to elicit the expansion of antigen-specific T cells in the context of  
20 a PBMC culture.  
Fig. 6A-C shows HIV peptide-specific IFN $\gamma$  production in PBMCs from HIV patients incubated with various concentrations of anti-CD40.LIPO5 peptide string vaccine. C is the control group, which received no vaccine, and defines the baseline response of the culture to each peptide.  
Fig. 7 is a summary of  $\alpha$ CD40.LIPO5 peptide vaccine responses against the 5 peptide regions from 8 HIV  
25 patients.  
Fig. 8A-C shows that the  $\alpha$ CD40.LIPO5 HIV peptide vaccine elicits expansion of HIV peptide-specific T cells capable of secreting multiple cytokines – a desirable feature in a vaccine. Fig. 8A-C also shows that the  $\alpha$ CD40.LIPO5 HIV peptide vaccine elicits gag253, nef66, nef116 and pol325 peptide-specific responses characterized by production of multiple cytokines (patient A5).  
30 Fig. 9 shows the protocol for testing  $\alpha$ CD40.LIPO5 HIV peptide vaccine for its ability to direct the expansion of antigen-specific T cells resulting from targeted uptake by DCs and presentation of peptide epitopes on their surface MHC complex.

Fig. 10A-B shows the cytokine secretion in response to HIV peptides from DC-T cell co-cultures treated with various doses of  $\alpha$ CD40.LIPO5 HIV peptide vaccine (patient A10).

Fig. 11A-B shows PBMCs from patient A4 treated with the  $\alpha$ CD40.LIPO5 HIV peptide vaccine elicit expansion of antigen-specific T cells with specificity to the gag253 region, but not to the flexible linker sequences.

Fig. 12A is the  $\alpha$ CD40.LIPO5 HIV peptide vaccine heavy chain sequence showing flexible linker regions in bold, joining sequences underlined and HIV peptide regions shaded in grey. Fig. 12A shows PBMCs from patient A3 treated with the  $\alpha$ CD40.LIPO5 HIV peptide vaccine elicit expansion of antigen-specific T cells with specificities to the gag253, nef66, and nef116 regions, but not to the flexible linker sequences. Fig.

12B-1 and B-2 shows HIV antigen-specific T cell responses evoked from HIV patient A17 PBMCs incubated with 30 nM of three different HIV5 peptide DC targeting vaccines. Fig. 12C-1 and C-2 is a similar study to that show in Fig. 12B-1 and B-2, except that the PBMCs are from a different HIV patient (A2). Fig. 12D shows 15 different HIV peptide responses [5 peptide regions sampled in 3 patients], it was found that the anti-CD40.HIV5pep vaccine was superior to anti-DCIR.HIV5pep, anti-LOX-1.HIV5pep and non-LIPO5 mix for eliciting a broad range of HIV peptide-specific CD8+ and CD4+ T responses.

Fig. 13 shows the internalization of anti-CD40 mAb:IL-4DC. IL-4DCs were treated with 500 ng/ml of anti-CD40-Alexa 568.

Fig. 14 shows CD4 and CD8 T cell proliferation by DCs targeted with anti-CD40-HA1.  $5 \times 10^3$  IFNDCs loaded with 2  $\mu$ g/ml of anti-CD40-HA or control Ig-HA1 were co-cultured with CFSE-labeled autologous CD4+ or CD8+ T cells ( $2 \times 10^5$ ) for 7 days. Cells were then then stained with anti-CD4 or anti-CD8 antibodies. Cell proliferation was tested by measuring CFSE-dilution.

Fig. 15 shows a titration of HA1 fusion protein on CD4+ T proliferation. IFNDCs (5K) loaded with fusion proteins were co-cultured with CFSE-labeled CD4+ T cells (200K) for 7 days.

Fig. 16 shows IFNDCs targeted with anti-CD40-HA1 activate HA1-specific CD4+ T cells. CD4+ T cells were re-stimulated with DCs loaded with 5  $\mu$ M of indicated peptides, and then intracellular IFN $\gamma$  was stained.

Fig. 17 shows IFNDCs targeted with anti-CD40-HA1 activate HA1-specific CD4+ T cells. CD4+ T cells were re-stimulated with DCs loaded with indicated peptides for 36h, and then culture supernatant was analyzed for measuring IFN $\gamma$ .

Fig. 18 shows that targeting CD40 results in enhanced cross-priming of MART-1 specific CD8+ T cells. IFNDCs (5K/well) loaded with fusion proteins were co-cultured with purified CD8+ T cells for 10 days. Cells were stained with anti-CD8 and tetramer. Cells are from healthy donors (HLA-A\*0201+).

Fig. 19 shows targeting CD40 results in enhanced cross-priming of MART-1 specific CD8+ T cells (Summary of 8-repeated experiments using cells from different healthy donors).

Fig. 20 shows CD8<sup>+</sup> CTL induced with IFNDCs targeted with anti-CD40-MART-1 are functional. CD8<sup>+</sup> T cells co-cultured with IFNDCs targeted with fusion proteins were mixed with T2 cells loaded with 10  $\mu$ M peptide epitope.

Fig. 21 shows CD8<sup>+</sup> CTL induced with IFNDCs targeted with anti-CD40-Flu M1 are functional. CD8<sup>+</sup> T cells co-cultured with IFNDCs targeted with fusion proteins were mixed with T2 cells loaded with 1.0 nM peptide epitope.

Fig. 22 shows an outline of protocol to test the ability a vaccine composed of anti-CD4012E12 linked to PSA (prostate specific antigen) to elicit the expansion from a naïve T cell population. PSA-specific CD4<sup>+</sup> T cells corresponding to a broad array of PSA epitopes. Briefly, DCs derived by culture with IFN $\alpha$  and GM-CSF of monocytes from a healthy donor are incubated with the vaccine. The next day, cells are placed in fresh medium and pure CD4<sup>+</sup> T cells from the same donor are added. Several days later, PSA peptides are added and, after four hours, secreted gamma-IFN levels in the culture supernatants are determined.

Fig. 23 shows that many PSA peptides elicit potent gamma-IFN-production responses indicating that anti-CD4012E12 and similar anti-CD40 agents can efficiently deliver antigen to DCs, resulting in the priming of immune responses against multiple epitopes of the antigen.

Fig. 24 shows DCs targeted with anti-CD40-PSA induce PSA-specific CD8<sup>+</sup> T cell responses. IFNDCs were targeted with 1  $\mu$ g mAb fusion protein with PSA. Purified autologous CD8<sup>+</sup> T cells were co-cultured for 10 days. Cells were stained with anti-CD8 and PSA (KLQCVDLHV)-tetramer. Cells are from a HLA-A\*0201 positive healthy donor. The results demonstrate that anti-CD40 effectively deliver PSA to the DCs, which in turn elicit the expansion of PSA-specific CD8<sup>+</sup> T cells.

Fig. 25 a scheme (left) and the IFN $\gamma$  production by T cells of the pools of peptides and control for Donor 2.  $5 \times 10^3$  IFNDCs loaded with 2  $\mu$ g/ml of anti-CD40-Cyclin D1 were co-cultured with purified autologous CD4<sup>+</sup> T cells ( $2 \times 10^5$ ) for 8 days. Cells were then re-stimulated with 5  $\mu$ M of individual peptides derived from CyclinD1 for 5h in the presence of Brefeldin A. Cells were stained for measuring intracellular IFN $\gamma$  expression.

Fig. 26 shows a peptide scan and IFN $\gamma$  production by T cells obtained from the pools of peptides shown in Fig. 25 and control for Donor 2.  $5 \times 10^3$  IFNDCs loaded with 2  $\mu$ g/ml of anti-CD40-Cyclin D1 were co-cultured with purified autologous CD4<sup>+</sup> T cells ( $2 \times 10^5$ ) for 8 days. Cells were then re-stimulated with 5  $\mu$ M of individual peptides derived from CyclinD1 for 5h in the presence of Brefeldin A. Cells were stained for measuring intracellular IFN $\gamma$  expression.

Fig. 27 shows the expression and construct design for anti-CD40-MART-1 peptide antibodies.

Fig. 28 is a summary of the CD4<sup>+</sup> and CD8<sup>+</sup> immunodominant epitopes for MART-1.

Fig. 29 shows the expression and construct design for anti-CD40-gp100 peptide antibodies.

Fig. 30 shows the design for additional anti-CD40-gp100 peptide antibodies.

Fig. 31 shows the expression and construct design for additional anti-CD40-gp100 peptide antibodies.

Fig. 32 is a summary of the CD4<sup>+</sup> and CD8<sup>+</sup> immunodominant epitopes for gp100.

Fig. 33 shows the expression and construct design for additional anti-CD40-gp100 peptide antibodies.

5 Fig. 34 shows the results obtained with the various antibodies using an assay that detects signaling via CD40 ligation - read out as cell death.

Fig. 35 shows the binding of various constructs when the antibody has been made into a fusion protein with doc and then captures.

10 Figs. 36 and 37 compare cytokine production with our without the addition of GM-CSF and IFN $\alpha$  (Fig. 36 A-D), and soluble antibodies alone (Fig. 37A-D) incubated with the DCs for 24 hours.

Figure 38A-B demonstrates the effect of various concentrations of anti-CD40 antibodies of the present invention on B cell proliferation.

### Description of the Invention

15 While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include 20 the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

The invention includes also variants and other modification of an antibody (or "Ab") or fragments thereof, 25 e.g., anti-CD40 fusion protein (antibody is used interchangeably with the term "immunoglobulin"). As used herein, the term "antibodies or fragments thereof," includes whole antibodies or fragments of an antibody, e.g., Fv, Fab, Fab', F(ab')<sub>2</sub>, Fc, and single chain Fv fragments (ScFv) or any biologically effective fragments of an immunoglobulins that binds specifically to, e.g., CD40. Antibodies from human origin or humanized antibodies have lowered or no immunogenicity in humans and have a lower number or no immunogenic 30 epitopes compared to non-human antibodies. Antibodies and their fragments will generally be selected to have a reduced level or no antigenicity in humans.

As used herein, the terms "Ag" or "antigen" refer to a substance capable of either binding to an antigen binding region of an immunoglobulin molecule or of eliciting an immune response, e.g., a T cell-mediated immune response by the presentation of the antigen on Major Histocompatibility Antigen (MHC) cellular proteins. As used herein, "antigen" includes, but is not limited to, antigenic determinants, haptens, and immunogens which may be peptides, small molecules, carbohydrates, lipids, nucleic acids or combinations thereof. The skilled immunologist will recognize that when discussing antigens that are processed for presentation to T cells, the term "antigen" refers to those portions of the antigen (e.g., a peptide fragment) that is a T cell epitope presented by MHC to the T cell receptor. When used in the context of a B cell mediated immune response in the form of an antibody that is specific for an "antigen", the portion of the antigen that binds to the complementarity determining regions of the variable domains of the antibody (light and heavy) the bound portion may be a linear or three-dimensional epitope. In the context of the present invention, the term antigen is used on both contexts, that is, the antibody is specific for a protein antigen (CD40), but also carries one or more peptide epitopes for presentation by MHC to T cells. In certain cases, the antigens delivered by the vaccine or fusion protein of the present invention are internalized and processed by antigen presenting cells prior to presentation, e.g., by cleavage of one or more portions of the antibody or fusion protein.

As used herein, the term "antigenic peptide" refers to that portion of a polypeptide antigen that is specifically recognized by either B-cells or T-cells. B-cells respond to foreign antigenic determinants via antibody production, whereas T-lymphocytes are the mediate cellular immunity. Thus, antigenic peptides are those parts of an antigen that are recognized by antibodies, or in the context of an MHC, by T-cell receptors.

As used herein, the term "epitope" refers to any protein determinant capable of specific binding to an immunoglobulin or of being presented by a Major Histocompatibility Complex (MHC) protein (e.g., Class I or Class II) to a T-cell receptor. Epitopic determinants are generally short peptides 5-30 amino acids long that fit within the groove of the MHC molecule that presents certain amino acid side groups toward the T cell receptor and has certain other residues in the groove, e.g., due to specific charge characteristics of the groove, the peptide side groups and the T cell receptor. Generally, an antibody specifically binds to an antigen when the dissociation constant is 1 mM, 100 nM or even 10 nM.

As used herein, the term "vector" is used in two different contexts. When using the term "vector" with reference to a vaccine, a vector is used to describe a non-antigenic portion that is used to direct or deliver the antigenic portion of the vaccine. For example, an antibody or fragments thereof may be bound to or form a fusion protein with the antigen that elicits the immune response. For cellular vaccines, the vector for delivery and/or presentation of the antigen is the antigen presenting cell, which is delivered by the cell that is loaded with antigen. In certain cases, the cellular vector itself may also process and present the antigen(s) to T cells

and activate an antigen-specific immune response. When used in the context of nucleic acids, a "vector" refers a construct, which is capable of delivering, and preferably expressing, one or more genes or polynucleotide sequences of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

- As used herein, the terms "stable" and "unstable" when referring to proteins is used to describe a peptide or protein that maintains its three-dimensional structure and/or activity (stable) or that loses immediately or over time its three-dimensional structure and/or activity (unstable). As used herein, the term "insoluble" refers to those proteins that when produced in a cell (e.g., a recombinant protein expressed in a eukaryotic or prokaryotic cell or in vitro) are not soluble in solution absent the use of denaturing conditions or agents (e.g., heat or chemical denaturants, respectively). The antibody or fragment thereof and the linkers taught herein have been found to convert antibody fusion proteins with the peptides from insoluble and/or unstable into proteins that are stable and/or soluble. Another example of stability versus instability is when the domain of the protein with a stable conformation has a higher melting temperature ( $T_m$ ) than the unstable domain of the protein when measured in the same solution. A domain is stable compared to another domain when the difference in the  $T_m$  is at least about 2° C, more preferably about 4° C, still more preferably about 7° C, yet more preferably about 10° C, even more preferably about 15° C, still more preferably about 20° C, even still more preferably about 25° C, and most preferably about 30° C, when measured in the same solution.
- As used herein, "polynucleotide" or "nucleic acid" refers to a strand of deoxyribonucleotides or ribonucleotides in either a single- or a double-stranded form (including known analogs of natural nucleotides). A double-stranded nucleic acid sequence will include the complementary sequence. The polynucleotide sequence may encode variable and/or constant region domains of immunoglobulin that are formed into a fusion protein with one or more linkers. For use with the present invention, multiple cloning sites (MCS) may be engineered into the locations at the carboxy-terminal end of the heavy and/or light chains of the antibodies to allow for in-frame insertion of peptide for expression between the linkers. As used herein, the term "isolated polynucleotide" refers to a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof. By virtue of its origin the "isolated polynucleotide" (1) is not associated with all or a portion of a polynucleotide in which the "isolated polynucleotides" are found in nature, (2) is operably linked to a polynucleotide which it is not linked to in nature, or (3) does not occur in nature as part of a larger sequence. The skilled artisan will recognize that to design and implement a vector can be manipulated at the nucleic acid level by using techniques known in the art, such as those taught in Current Protocols in Molecular Biology, 2007 by John Wiley and Sons.

Briefly, the encoding nucleic acid sequences can be inserted using polymerase chain reaction, enzymatic insertion of oligonucleotides or polymerase chain reaction fragments in a vector, which may be an expression vector. To facilitate the insertion of inserts at the carboxy terminus of the antibody light chain, the heavy chain, or both, a multiple cloning site (MCS) may be engineered in sequence with the antibody sequences.

As used herein, the term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post expression modifications of the polypeptide, for example, glycosylations, acrylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. The term "domain," or "polypeptide domain" refers to that sequence of a polypeptide that folds into a single globular region in its native conformation, and that may exhibit discrete binding or functional properties.

A polypeptide or amino acid sequence "derived from" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 3-5 amino acids, preferably at least 4-7 amino acids, more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence.

As used herein, "pharmaceutically acceptable carrier" refers to any material that when combined with an immunoglobulin (Ig) fusion protein of the present invention allows the Ig to retain biological activity and is generally non-reactive with the subject's immune system. Examples include, but are not limited to, standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as an oil/water emulsion, and various types of wetting agents. Certain diluents may be used with the present invention, e.g., for aerosol or parenteral administration, that may be phosphate buffered saline or normal (0.85%) saline.

An antibody for use with the present invention comprises at least the variable region of anti-CD40\_12E12.3F3 (ATCC Accession No. PTA-9854), anti-CD40\_12B4.2C10 (Deposit No. HS446, ATCC Accession No. PTA-10653), and anti-CD40\_11B6.1C3 (Deposit No. HS440, ATCC Accession No. PTA-10652).

The invention provides an CD40 binding molecule comprising at least one immunoglobulin light chain variable domain (VL) which comprises in sequence hypervariable regions CDR1L, CDR2L and CDR3L, the CDR1L having the amino acid sequence SASQGISNYLN (SEQ ID NO.:41) the CDR2L having the amino acid sequence YTSILHS (SEQ ID NO.:42) and the CDR3L having the amino acid sequence QQFNKLPPPT

(SEQ ID NO.:43) the amino acid sequences of which are shown in SEQ ID NO. 37; and direct equivalents thereof for the anti-CD40\_11B6.1C3, or the anti-CD40\_12B4.2C10 antibodies.

Accordingly the invention provides an CD40 binding molecule which comprises an antigen binding site comprising at least one immunoglobulin heavy chain variable domain (VH) which comprises in sequence  
 5 hypervariable regions CDR1H, CDR2H and CDR3H, the CDR1H having the amino acid sequence GFTFSDYYMY (SEQ ID NO.:44), the CDR2H having the amino acid sequence YINSGGGSTYYPDTVKG (SEQ ID NO.:45), and the CDR3H having the amino acid sequence RGLPFHAMDY (SEQ ID NO.:46), the amino acid sequences of which are shown in SEQ ID NO. 38; and direct equivalents thereof the anti-CD40\_11B6.1C3, or the anti-CD40\_12B4.2C10 antibodies.

10 In one aspect the invention provides a single domain CD40 binding molecule comprising an isolated immunoglobulin light chain comprising a heavy chain variable domain (VL) as defined above. In another aspect the invention provides a single domain CD40 binding molecule comprising an isolated immunoglobulin heavy chain comprising a heavy chain variable domain (VH) as defined above.

In another aspect the invention also provides an CD40 binding molecule comprising both heavy (VH) and  
 15 light chain (VL) variable domains in which the CD40 binding molecule comprises at least one antigen binding site comprising: a) an immunoglobulin heavy chain variable domain (VL) which comprises in sequence hypervariable regions CDR1L, CDR2L and CDR3L, the CDR1L having the amino acid sequence SASQGISNYLN (SEQ ID NO.:41), the CDR2L having the amino acid sequence YTSILHS (SEQ ID NO.:42), and the CDR3L having the amino acid sequence QQFNKLPPT (SEQ ID NO.:43), the amino acid  
 20 sequences of which are shown in SEQ ID. NO. 1, and b) an immunoglobulin light chain variable domain (VH) which comprises in sequence hypervariable regions CDR1H, CDR2H and CDR3H, the CDR1H having the amino acid sequence GFTFSDYYMY (SEQ ID NO.:44), the CDR2' having the amino acid sequence YINSGGGSTYYPDTVKG (SEQ ID NO.:45), and the CDR3H having the amino acid sequence RGLPFHAMDY (SEQ ID NO.:46), the amino acid sequences of which are shown in SEQ ID NO. 38; and  
 25 direct equivalents thereof the anti-CD40\_11B6.1C3, or the anti-CD40\_12B4.2C10 antibodies..

Unless otherwise indicated, any polypeptide chain is herein described as having an amino acid sequence starting at the N-terminal end and ending at the C-terminal end. When the antigen binding site comprises both the VH and VL domains, these may be located on the same polypeptide molecule or, preferably, each domain may be on a different chain, the VH domain being part of an immunoglobulin heavy chain or  
 30 fragment thereof and the VL being part of an immunoglobulin light chain or fragment thereof.

As used herein, the term "CD40 binding molecule" refers to any molecule capable of binding to the CD40 antigen either alone or associated with other molecules having one or more the  $V_L$  and  $V_H$  CDRs taught herein, in some cases 2, 3, 4, 5, or all 6 CDRs. The binding reaction may be shown by standard methods



(qualitative assays) including, for example, a bioassay for determining by blocking the binding of other molecules to CD40 or any kind of binding or activity assays (e.g., activation, reduction or modulation of an immune response), with reference to a negative control test in which an antibody of unrelated specificity but of the same isotype, e.g., an anti-CD25 or anti-CD80 antibody, is used.

5 The present invention may also be made into a single chain antibody having the variable domains of the heavy and light chains of an antibody covalently bound by a peptide linker usually including from 10 to 30 amino acids, preferably from 15 to 25 amino acids. Therefore, such a structure does not include the constant part of the heavy and light chains and it is believed that the small peptide spacer should be less antigenic than a whole constant part.

10 As used herein, the term "chimeric antibody" refers to an antibody in which the constant regions of heavy or light chains or both are of human origin while the variable domains of both heavy and light chains are of non-human (e.g., mouse, hamster or rat) origin or of human origin but derived from a different human antibody.

As used herein, the term "CDR-grafted antibody" refers to an antibody in which the hypervariable complementarity determining regions (CDRs) are derived from a donor antibody, such as a non-human (e.g., mouse) antibody or a different human antibody, while all or substantially all the other parts of the immunoglobulin (e.g., the conserved regions of the variable domains, i.e., framework regions), are derived from an acceptor antibody (in the case of a humanized antibody -an antibody of human origin). A CDR-grafted antibody may include a few amino acids of the donor sequence in the framework regions, for instance

20 in the parts of the framework regions adjacent to the hypervariable regions.

As used herein, the term "human antibody" refers to an antibody in which the constant and variable regions of both the heavy and light chains are all of human origin, or substantially identical to sequences of human origin, not necessarily from the same antibody and includes antibodies produced by mice in which the mouse, hamster or rat immunoglobulin variable and constant part genes have been replaced by their human counterparts, e.g. as described in general terms in EP 0546073 B1, U.S. Pat. No. 5,545,806, U.S. Pat. No. 5,569,825, U.S. Pat. No. 5,625,126, U.S. Pat. No. 5,633,425, U.S. Pat. No. 5,661,016, U.S. Pat. No. 5,770,429, EP 0 438474 B1 and EP 0 463151 B1.

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The CD40 binding molecule of the invention can be a humanized antibody that comprises the CDRs obtained from the anti-CD40\_12E12.3F3, the anti-CD40\_11B6.1C3, or the anti-CD40\_12B4.2C10 antibodies. One

30 example of a chimeric antibody includes the variable domains of both heavy and light chains are of human origin, for instance those variable domains of the anti-CD40\_12E12.3F3 antibody that are part of SEQ ID NO.: 1 and SEQ ID NO.: 2, anti-CD40\_12B4.2C10 in SEQ ID NO.: 3 and SEQ ID NO.: 4 or SEQ ID NO.: 5; and/or anti-CD40\_11B6.1C3, SEQ ID NO.: 6 and SEQ ID NO.: 7, or combination thereof. The constant

region domains preferably also comprise suitable human constant region domains, for instance as described in "Sequences of Proteins of Immunological Interest", Kabat E. A. et al, US Department of Health and Human Services, Public Health Service, National Institute of Health. The nucleic acid sequences can be found in, e.g., SEQ ID NOS.: 8 and 9.

- 5 Hypervariable regions may be associated with any kind of framework regions, e.g., of human origin. Suitable framework regions were described Kabat E. A. One heavy chain framework is a heavy chain framework, for instance that of anti-CD40\_12E12.3F3 antibody that are part of SEQ ID NO.: 2; anti-CD40\_12B4.2C10 - SEQ ID NO.: 4 or SEQ ID NO.: 5, and/or anti-CD40\_11B6.1C3 - SEQ ID NO.: 7, or combination thereof, e.g., FR1<sub>L</sub>, FR2<sub>L</sub>, FR3<sub>L</sub> and FR4<sub>L</sub> regions. In a similar manner, SEQ ID NO. 1 shows  
10 the anti-CD40\_12E12.3F3 (or the equivalents for anti-CD40\_12B4.2C10 and anti-CD40\_11B6.1C3, SEQ ID NOS.: 3 and 6, respectively) heavy chain framework that includes the sequence of FR1<sub>H</sub>, FR2<sub>H</sub>, FR3<sub>H</sub> and FR4<sub>H</sub> regions. The CDRs may be added to a human antibody framework, such as those described in 7,456,260, issued to Rybak, et al., which teach new human variable chain framework regions and humanized antibodies comprising the framework regions, relevant portions and framework sequences.

- 15 To accomplish the engraftment at a genetic level, the present invention also includes the underlying nucleic acid sequences for the V<sub>L</sub> AND V<sub>H</sub> regions as well as the complete antibodies and the humanized versions thereof. The nucleic acid sequences of the present invention include SEQ ID NOS.: 8 and 9, which are the anti-CD40 antibody light and the heavy chains, respectively, as well as those nucleic acid sequences that include variable codon usage for the same amino acid sequences and conservative  
20 variations thereof having 85, 90, 95 or 100 % sequence identity at the nucleic or amino acid level. Likewise, the CDRs may have 85, 90, 95 or 100 % sequence identity at the nucleic or amino acid level, individually, in groups or 2, 3, 4 or 5 or all together.

- Monoclonal antibodies raised against a protein naturally found in all humans are typically developed in a non-human system e.g. in mice, and as such are typically non-human proteins. As a direct consequence of  
25 this, a xenogenic antibody as produced by a hybridoma, when administered to humans, elicits an undesirable immune response that is predominantly mediated by the constant part of the xenogenic immunoglobulin. Xenogenic antibodies tend to elicit a host immune response, thereby limiting the use of such antibodies as they cannot be administered over a prolonged period of time. Therefore, it is particularly useful to use single chain, single domain, chimeric, CDR-grafted, or especially human antibodies that are not likely to elicit a  
30 substantial allogenic response when administered to humans. The present invention includes antibodies with minor changes in an amino acid sequence such as deletion, addition or substitution of one, a few or even several amino acids which are merely allelic forms of the original protein having substantially identical properties.

The inhibition of the binding of CD40 to its receptor may be conveniently tested in various assays including such assays are described hereinafter in the text. By the term "to the same extent" is meant that the reference and the equivalent molecules exhibit, on a statistical basis, essentially identical CD40 binding inhibition curves in one of the assays referred to above. For example, the assay used may be an assay of competitive inhibition of binding of CD40 by the binding molecules of the invention.

5 Generally, the human anti-CD40 antibody comprises at least: (a) one light chain which comprises a variable domain having an amino acid sequence substantially identical to that shown in SEQ ID NO.: 1 starting with the amino acid at position 1 and ending with the amino acid at position 107 and the constant part of a human light chain; and (b) one heavy chain which comprises a variable domain having an amino acid sequence  
10 substantially identical to that shown in SEQ ID NO. 2 and the constant part of a human heavy chain. The constant part of a human heavy chain may be of the  $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$ ,  $\gamma 4$ ,  $\mu$ ,  $\beta 2$ , or  $\delta$  or  $\epsilon$  type, preferably of the  $\gamma$ -type, whereas the constant part of a human light chain may be of the  $\kappa$  or  $\lambda$  type (which includes the  $\lambda_1$ ,  $\lambda_2$  and  $\lambda_3$  subtypes) but is preferably of the  $\kappa$  type. The amino acid sequences of the general locations of the variable and constant domains are well known in the art and generally follow the Kabat nomenclature.

15 A CD40 binding molecule of the invention may be produced by recombinant DNA techniques. In view of this, one or more DNA molecules encoding the binding molecule must be constructed, placed under appropriate control sequences and transferred into a suitable host organism for expression.

In a very general manner, there are accordingly provided: (i) DNA molecules encoding a single domain CD40 binding molecule of the invention, a single chain CD40 binding molecule of the invention, a heavy or  
20 light chain or fragments thereof of a CD40 binding molecule of the invention; and (ii) the use of the DNA molecules of the invention for the production of a CD40 binding molecule of the invention by recombinant methods.

The present state of the art is such that the skilled worker in the art can synthesize the DNA molecules of the invention given the information provided herein, i.e., the amino acid sequences of the hypervariable regions  
25 and the DNA sequences coding for them. A method for constructing a variable domain gene is for example described in EPA 239 400.

Briefly, a gene encoding a variable domain of a MAb is cloned. The DNA segments encoding the framework and hypervariable regions are determined and the DNA segments encoding the hypervariable regions are removed so that the DNA segments encoding the framework regions are fused together with suitable restriction sites at the junctions.  
30 The restriction sites may be generated at the appropriate positions by mutagenesis of the DNA molecule by standard procedures. Double stranded synthetic CDR cassettes are prepared by DNA synthesis according to the sequences given in SEQ ID NO.: 1 and 3 or 2 and 4 (amino acid and nucleic acid sequences,

respectively). These cassettes are often provided with sticky ends so that they can be ligated at the junctions of the framework.

It is not necessary to have access to the mRNA from a producing hybridoma cell line in order to obtain a DNA construct coding for the CD40 binding molecules of the invention. For example, PCT application WO 90/07861 gives full instructions for the production of an antibody by recombinant DNA techniques given only written information as to the nucleotide sequence of the gene.

Briefly, the method comprises the synthesis of a number of oligonucleotides, their amplification by the PCR method, and their splicing to give the desired DNA sequence.

Expression vectors comprising a suitable promoter or genes encoding heavy and light chain constant parts are publicly available. Thus, once a DNA molecule of the invention is prepared it may be conveniently transferred in an appropriate expression vector. DNA molecules encoding single chain antibodies may also be prepared by standard methods, for example, as described in WO 88/1649. In view of the foregoing, no hybridoma or cell line deposit is necessary to comply with the criteria of sufficiency of description. For example, first and second DNA constructs are made that bind specifically to CD40. Briefly, a first DNA construct encodes a light chain or fragment thereof and comprises a) a first part which encodes a variable domain comprising alternatively framework and hypervariable regions, the hypervariable regions being in sequence CDR1<sub>L</sub>, CDR2<sub>L</sub> and CDR3<sub>L</sub>, the amino acid sequences of which are shown in SEQ ID NO.: 1; this first part starting with a codon encoding the first amino acid of the variable domain and ending with a codon encoding the last amino acid of the variable domain, and b) a second part encoding a light chain constant part or fragment thereof which starts with a codon encoding the first amino acid of the constant part of the heavy chain and ends with a codon encoding the last amino acid of the constant part or fragment thereof, followed by a stop codon.

The first part encodes a variable domain having an amino acid sequence substantially identical to the amino acid sequence as shown in SEQ ID NO.: 1, 2, 3, 4, 5, 6 or 7. A second part encodes the constant part of a human heavy chain, more preferably the constant part of the human  $\gamma$ 1 chain. This second part may be a DNA fragment of genomic origin (comprising introns) or a cDNA fragment (without introns).

The second DNA construct encodes a heavy chain or fragment thereof and comprises a) a first part which encodes a variable domain comprising alternatively framework and hypervariable regions; the hypervariable regions being CDR1<sub>H</sub> and optionally CDR2<sub>H</sub> and CDR3<sub>H</sub>, the amino acid sequences of which are shown in SEQ ID NO. 2; this first part starting with a codon encoding the first amino acid of the variable domain and ending with a codon encoding the last amino acid of the variable domain, and b) a second part encoding a heavy chain constant part or fragment thereof which starts with a codon encoding the first amino acid of the

constant part of the light chain and ends with a codon encoding the last amino acid of the constant part or fragment thereof followed by a stop codon.

The first part encodes a variable domain having an amino acid sequence substantially identical to the amino acid sequence as shown in SEQ ID NO. 2. The first part has the nucleotide sequence as shown in SEQ ID NO. 2 starting with the nucleotide at position 1 and ending with the nucleotide at position 321. Also preferably the second part encodes the constant part of a human light chain, more preferably the constant part of the human  $\kappa$  chain.

The invention also includes CD40 binding molecules in which one or more of the residues of CDR1<sub>L</sub>, CDR2<sub>L</sub>, CDR3<sub>L</sub>, CDR1<sub>H</sub>, CDR2<sub>H</sub> or CDR3<sub>H</sub> or the frameworks, typically only a few (e.g. FR1-4<sub>L</sub> or <sub>H</sub>), are changed from the residues shown in SEQ ID NO. 37 and SEQ ID NO. 38; by, e.g., site directed mutagenesis of the corresponding DNA sequences. The invention includes the DNA sequences coding for such changed CD40 binding molecules. In particular the invention includes a CD40 binding molecules in which one or more residues of CDR1<sub>L</sub>, CDR2<sub>L</sub> and/or CDR3<sub>L</sub> have been changed from the residues shown in SEQ ID NO. 37 and one or more residues of CDR1<sub>H</sub>, CDR2<sub>H</sub> and/or CDR3<sub>H</sub> have been changed from the residues shown in SEQ ID NO. 38, or the equivalents from SEQ ID NOS.: 1, 3 and 6.

Each of the DNA constructs are placed under the control of suitable control sequences, in particular under the control of a suitable promoter. Any kind of promoter may be used, provided that it is adapted to the host organism in which the DNA constructs will be transferred for expression. However, if expression is to take place in a mammalian cell, an immunoglobulin gene promoter may be used in B cells. The first and second parts may be separated by an intron, and, an enhancer may be conveniently located in the intron between the first and second parts. The presence of such an enhancer that is transcribed but not translated, may assist in efficient transcription. In particular embodiments the first and second DNA constructs comprise the enhancer of, e.g., a heavy chain human gene.

The desired antibody may be produced in a cell culture or in a transgenic animal. A suitable transgenic animal may be obtained according to standard methods that include micro injecting into eggs the first and second DNA constructs placed under suitable control sequences transferring the so prepared eggs into appropriate pseudo-pregnant females and selecting a descendant expressing the desired antibody.

The invention also provides an expression vector able to replicate in a prokaryotic or eukaryotic cell line, which comprises at least one of the DNA constructs above described. Each expression vector containing a DNA construct is then transferred into a suitable host organism. When the DNA constructs are separately inserted on two expression vectors, they may be transferred separately, i.e. one type of vector per cell, or co-transferred, this latter possibility being preferred. A suitable host organism may be a bacterium, a yeast or a

mammalian cell line, this latter being preferred. More preferably, the mammalian cell line is of lymphoid origin, e.g., a myeloma, hybridoma or a normal immortalized B-cell, which conveniently does not express any endogenous antibody heavy or light chain.

When the antibody chains are produced in a cell culture, the DNA constructs must first be inserted into either  
 5 a single expression vector or into two separate but compatible expression vectors, the latter possibility being preferred. For expression in mammalian cells it is preferred that the coding sequence of the CD40 binding molecule is integrated into the host cell DNA within a locus which permits or favors high level expression of the CD40 binding molecule.

In a further aspect of the invention there is provided a process for the product of a CD40 binding molecule  
 10 that comprises: (i) culturing an organism which is transformed with an expression vector as defined above; and (ii) recovering the CD40 binding molecule from the culture.

In accordance with the present invention it has been found that the anti-CD40<sub>12E12.3F3</sub>, anti-CD40<sub>12B4.2C10</sub> and/or anti-CD40<sub>11B6.1C3</sub> antibody appears to have binding specificity for human CD40. It is therefore most surprising that antibodies to this epitope, e.g. the anti-CD40<sub>12E12.3F3</sub>, anti-  
 15 CD40<sub>12B4.2C10</sub> and/or anti-CD40<sub>11B6.1C3</sub> antibody, are capable of delivering antigen efficiently into dendritic cells (DCs). Antibodies, in particular chimeric and CDR-grafted antibodies and especially human antibodies, which have binding specificity for the antigenic epitope of mature human CD40; and use of such antibodies for DC antigen loading are novel and are included within the scope of the present invention.

To use the anti-CD40 antibody of the present invention for treatment indications, the appropriate dosage will,  
 20 of course, vary depending upon, for example, the antibody disclosed herein to be employed, the host, the mode of administration and the nature and severity of the condition being treated. However, in prophylactic use, satisfactory results are generally found at dosages from about 0.05 mg to about 10 mg per kilogram body weight more usually from about 0.1 mg to about 5 mg per kilogram body weight. The frequency of dosing for prophylactic uses will normally be in the range from about once per week up to about once every 3  
 25 months, more usually in the range from about once every 2 weeks up to about once every 10 weeks, e.g., once every 4 to 8 weeks. The anti-CD40 antibody of the present can be administered parenterally, intravenously, e.g., into the antecubital or other peripheral vein, intramuscularly, or subcutaneously.

Pharmaceutical compositions of the invention may be manufactured in conventional manner, e.g., in a lyophilized form. For immediate administration it is dissolved in a suitable aqueous carrier, for example  
 30 sterile water for injection or sterile buffered physiological saline. If it is considered desirable to make up a solution of larger volume for administration by infusion rather as a bolus injection, it is advantageous to incorporate human serum albumin or the patient's own heparinized blood into the saline at the time of formulation. The presence of an excess of such physiologically inert protein prevents loss of antibody by

adsorption onto the walls of the container and tubing used with the infusion solution. If albumin is used, a suitable concentration is from 0.5 to 4.5% by weight of the saline solution.

One embodiment of the present invention provides an immunoconjugate comprising a humanized antibody of the invention, e.g., a humanized anti-CD40 antibody, linked to one or more effector molecules, antigen(s) and/or a detectable label(s). Preferably, the effector molecule is a therapeutic molecule such as, for example, one or more peptides that comprise one or more T cell epitopes, a toxin, a small molecule, a cytokine or a chemokine, an enzyme, or a radiolabel.

Exemplary toxins include, but are not limited to, *Pseudomonas* exotoxin or diphtheria toxin. Examples of small molecules include, but are not limited to, chemotherapeutic compounds such as taxol, doxorubicin, etoposide, and bleomycin. Exemplary cytokines include, but are not limited to, IL-1, IL-2, IL-4, IL-5, IL-6, and IL-12, IL-17, and IL-25. Exemplary enzymes include, but are not limited to, RNases, DNases, proteases, kinases, and caspases. Exemplary radioisotopes include, but are not limited to,  $^{32}\text{P}$  and  $^{125}\text{I}$ .

As used herein, the term "epitope" refers to a molecule or substance capable of stimulating an immune response. In one example, epitopes include but are not limited to a polypeptide and a nucleic acid encoding a polypeptide, wherein expression of the nucleic acid into a polypeptide is capable of stimulating an immune response when the polypeptide is processed and presented on a Major Histocompatibility Complex (MHC) molecule. Generally, epitopes include peptides presented on the surface of cells non-covalently bound to the binding groove of Class I or Class II MHC, such that they can interact with T cell receptors and the respective T cell accessory molecules.

**Proteolytic Processing of Antigens.** Epitopes that are displayed by MHC on antigen presenting cells are cleavage peptides or products of larger peptide or protein antigen precursors. For MHC I epitopes, protein antigens are often digested by proteasomes resident in the cell. Intracellular proteasomal digestion produces peptide fragments of about 3 to 23 amino acids in length that are then loaded onto the MHC protein. Additional proteolytic activities within the cell, or in the extracellular milieu, can trim and process these fragments further. Processing of MHC Class II epitopes generally occurs via intracellular proteases from the lysosomal/endosomal compartment. The present invention includes, in one embodiment, pre-processed peptides that are attached to the anti-CD40 antibody (or fragment thereof) that directs the peptides against which an enhanced immune response is sought directly to antigen presenting cells.

To identify epitopes potentially effective as immunogenic compounds, predictions of MHC binding alone are useful but often insufficient. The present invention includes methods for specifically identifying the epitopes within antigens most likely to lead to the immune response sought for the specific sources of antigen presenting cells and responder T cells.

The present invention allows for a rapid and easy assay for the identification of those epitopes that are most likely to produce the desired immune response using the patient's own antigen presenting cells and T cell repertoire. The compositions and methods of the present invention are applicable to any protein sequence, allowing the user to identify the epitopes that are capable of binding to MHC and are properly presented to T cells that will respond to the antigen. Accordingly, the invention is not limited to any particular target or medical condition, but instead encompasses and MHC epitope(s) from any useful source.

As used herein, the term "veneered" refers to a humanized antibody framework onto which antigen-binding sites or CDRs obtained from non-human antibodies (e.g., mouse, rat or hamster), are placed into human heavy and light chain conserved structural framework regions (FRs), for example, in a light chain or heavy chain polynucleotide to "graft" the specificity of the non-human antibody into a human framework. The polynucleotide expression vector or vectors that express the veneered antibodies can be transfected into mammalian cells for the expression of recombinant human antibodies which exhibit the antigen specificity of the non-human antibody and will undergo posttranslational modifications that will enhance their expression, stability, solubility, or combinations thereof.

#### 15 Antigens.

Examples of viral antigens for use with the present invention include, but are not limited to, e.g., HIV, HCV, CMV, adenoviruses, retroviruses, picornaviruses, etc. Non-limiting example of retroviral antigens such as retroviral antigens from the human immunodeficiency virus (HIV) antigens such as gene products of the gag, pol, and env genes, the Nef protein, reverse transcriptase, and other HIV components; hepatitis viral antigens such as the S, M, and L proteins of hepatitis B virus, the pre-S antigen of hepatitis B virus, and other hepatitis, e.g., hepatitis A, B, and C, viral components such as hepatitis C viral RNA; influenza viral antigens such as hemagglutinin and neuraminidase and other influenza viral components; measles viral antigens such as the measles virus fusion protein and other measles virus components; rubella viral antigens such as proteins E1 and E2 and other rubella virus components; rotaviral antigens such as VP7sc and other rotaviral components; cytomegaloviral antigens such as envelope glycoprotein B and other cytomegaloviral antigen components; respiratory syncytial viral antigens such as the RSV fusion protein, the M2 protein and other respiratory syncytial viral antigen components; herpes simplex viral antigens such as immediate early proteins, glycoprotein D, and other herpes simplex viral antigen components; varicella zoster viral antigens such as gpI, gpII, and other varicella zoster viral antigen components; Japanese encephalitis viral antigens such as proteins E, M-E, M-E-NS1, NS1, NS1-NS2A, 80% E, and other Japanese encephalitis viral antigen components; rabies viral antigens such as rabies glycoprotein, rabies nucleoprotein and other rabies viral antigen components. See *Fundamental Virology*, Second Edition, eds. Fields, B. N. and Knipe, D. M. (Raven Press, New York, 1991) for additional examples of viral antigens. The at least one viral antigen may



be peptides from an adenovirus, retrovirus, picornavirus, herpesvirus, rotaviruses, hantaviruses, coronavirus, togavirus, flavivirus, rhabdovirus, paramyxovirus, orthomyxovirus, bunyavirus, arenavirus, reovirus, papillomavirus, parvovirus, poxvirus, hepadnavirus, or spongiform virus. In certain specific, non-limiting examples, the at least one viral antigen are peptides obtained from at least one of HIV, CMV, hepatitis A, B, and C, influenza, measles, polio, smallpox, rubella; respiratory syncytial, herpes simplex, varicella zoster, Epstein-Barr, Japanese encephalitis, rabies, flu, and/or cold viruses.

In one aspect, the one or more of the antigenic peptides are selected from at least one of: Nef (66-97): VGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGL (SEQ ID NO.: 148); Nef (116-145): HTQGYFPDWQNYTPGQVRYPLTFGWLYKL (SEQ ID NO.: 149); Gag p17 (17-35): EKIRLRPGGKKKYKLKHIV (SEQ ID NO.: 150); Gag p17-p24 (253-284): NPPPIVGEIYKRWILGLNKIVRMYSPTSILD (SEQ ID NO.: 151); or Pol 325-355 (RT 158-188) is: AIFQSSMTKLEPFRKQNPDIYQYMDLY (SEQ ID NO.: 152). In one aspect, the fusion protein peptides are separated by one or more linkers selected from: SSVSPTTSVHPTPTSVFPPTPTKSSP (SEQ ID NO.: 11); PTSTPADSSTITPTATPTATPTIKG (SEQ ID NO.: 12); TVTPTATATPSAIVTTITPTATTKP (SEQ ID NO.: 13); or TNGSITVAATAPTVTPTVNAATPSAA (SEQ ID NO.: 14).

Antigenic targets that may be delivered using the anti-CD40-antigen vaccines of the present invention include genes encoding antigens such as viral antigens, bacterial antigens, fungal antigens or parasitic antigens. Pathogens include trypanosomes, tapeworms, roundworms, helminthes, malaria. Tumor markers, such as fetal antigen or prostate specific antigen, may be targeted in this manner. Other examples include: HIV env proteins and hepatitis B surface antigen. Administration of a vector according to the present invention for vaccination purposes would require that the vector-associated antigens be sufficiently non-immunogenic to enable long-term expression of the transgene, for which a strong immune response would be desired. In some cases, vaccination of an individual may only be required infrequently, such as yearly or biennially, and provide long-term immunologic protection against the infectious agent. Specific examples of organisms, allergens and nucleic and amino sequences for use in vectors and ultimately as antigens with the present invention may be found in U.S. Patent No. 6,541,011,

in particular, the tables that match organisms and specific sequences that may be used with the present invention.

Bacterial antigens for use with the anti-CD40-antigen vaccines disclosed herein include, but are not limited to, e.g., bacterial antigens such as pertussis toxin, filamentous hemagglutinin, pertactin, FIM2, FIM3, adenylate cyclase and other pertussis bacterial antigen components; diphtheria bacterial antigens such as diphtheria toxin or toxoid and other diphtheria bacterial antigen components; tetanus bacterial antigens such as tetanus toxin or toxoid and other tetanus bacterial antigen components; streptococcal bacterial antigens such

- as M proteins and other streptococcal bacterial antigen components; gram-negative bacilli bacterial antigens such as lipopolysaccharides and other gram-negative bacterial antigen components, *Mycobacterium tuberculosis* bacterial antigens such as mycolic acid, heat shock protein 65 (HSP65), the 30 kDa major secreted protein, antigen 85A and other mycobacterial antigen components; *Helicobacter pylori* bacterial antigen components; pneumococcal bacterial antigens such as pneumolysin, pneumococcal capsular polysaccharides and other pneumococcal bacterial antigen components; *Haemophilus influenza* bacterial antigens such as capsular polysaccharides and other *Haemophilus influenza* bacterial antigen components; anthrax bacterial antigens such as anthrax protective antigen and other anthrax bacterial antigen components; rickettsiae bacterial antigens such as *ompA* and other rickettsiae bacterial antigen component. Also included with the bacterial antigens described herein are any other bacterial, mycobacterial, mycoplasmal, rickettsial, or chlamydial antigens. Partial or whole pathogens may also be: *Haemophilus influenza*; *Plasmodium falciparum*; *Neisseria meningitidis*; *Streptococcus pneumoniae*; *Neisseria gonorrhoeae*; *Salmonella* serotype typhi; shigella; *Vibrio cholerae*; Dengue Fever; Encephalitis; Japanese Encephalitis; Lyme disease; *Yersinia pestis*; West Nile virus; yellow fever; tularemia; hepatitis (viral; bacterial); RSV (respiratory syncytial virus); HPIV 1 and HPIV 3; adenovirus; small pox; allergies and cancers.
- Fungal antigens for use with compositions and methods of the invention include, but are not limited to, e.g., candida fungal antigen components; histoplasma fungal antigens such as heat shock protein 60 (HSP60) and other histoplasma fungal antigen components; cryptococcal fungal antigens such as capsular polysaccharides and other cryptococcal fungal antigen components; coccidioides fungal antigens such as spherule antigens and other coccidioides fungal antigen components; and tinea fungal antigens such as trichophytin and other coccidioides fungal antigen components.
- Examples of protozoal and other parasitic antigens include, but are not limited to, e.g., *Plasmodium falciparum* antigens such as merozoite surface antigens, sporozoite surface antigens, circumsporozoite antigens, gametocyte/gamete surface antigens, blood-stage antigen pf 155/RESA and other plasmodial antigen components; toxoplasma antigens such as SAG-1, p30 and other toxoplasma antigen components; schistosoma antigens such as glutathione-S-transferase, paramyosin, and other schistosoma antigen components; *Leishmania major* and other leishmaniae antigens such as gp63, lipophosphoglycan and its associated protein and other leishmaniae antigen components; and *Trypanosoma cruzi* antigens such as the 75-77 kDa antigen, the 56 kDa antigen and other trypanosoma antigen components.
- Antigen that can be targeted using the anti-CD40-antigen vaccines of the present invention will generally be selected based on a number of factors, including: likelihood of internalization, level of immune cell specificity, type of immune cell targeted, level of immune cell maturity and/or activation and the like. In this embodiment, the antibodies may be mono- or bi-specific antibodies that include one anti-CD40 binding

domain and one binding domain against a second antigen, e.g., cell surface markers for dendritic cells such as, MHC class I, MHC Class II, B7-2, CD18, CD29, CD31, CD43, CD44, CD45, CD54, CD58, CD83, CD86, CMRF-44, CMRF-56, DCIR and/or Dectin-1 and the like; while in some cases also having the absence of CD2, CD3, CD4, CD8, CD14, CD15, CD16, CD 19, CD20, CD56, and/or CD57. Examples of

5 cell surface markers for antigen presenting cells include, but are not limited to, MHC class I, MHC Class II, CD45, B7-1, B7-2, IFN- $\gamma$  receptor and IL-2 receptor, ICAM-1 and/or Fc $\gamma$  receptor. Examples of cell surface markers for T cells include, but are not limited to, CD3, CD4, CD8, CD 14, CD20, CD11b, CD16, CD45 and HLA-DR.

Target antigens on cell surfaces for delivery include those characteristic of tumor antigens typically derived

10 from the cell surface, cytoplasm, nucleus, organelles and the like of cells of tumor tissue. Examples of tumor targets for the antibody portion of the present invention include, without limitation, hematological cancers such as leukemias and lymphomas, neurological tumors such as astrocytomas or glioblastomas, melanoma, breast cancer, lung cancer, head and neck cancer, gastrointestinal tumors such as gastric or colon cancer, liver cancer, pancreatic cancer, genitourinary tumors such cervix, uterus, ovarian cancer, vaginal cancer,

15 testicular cancer, prostate cancer or penile cancer, bone tumors, vascular tumors, or cancers of the lip, nasopharynx, pharynx and oral cavity, esophagus, rectum, gall bladder, biliary tree, larynx, lung and bronchus, bladder, kidney, brain and other parts of the nervous system, thyroid, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma and leukemia.

Examples of antigens that may be delivered alone or in combination to immune cells for antigen presentation

20 using the present invention includes tumor proteins, e.g., mutated oncogenes; viral proteins associated with tumors; and tumor mucins and glycolipids. The antigens may be viral proteins associated with tumors would be those from the classes of viruses noted above. Certain antigens may be characteristic of tumors (one subset being proteins not usually expressed by a tumor precursor cell), or may be a protein which is normally expressed in a tumor precursor cell, but having a mutation characteristic of a tumor. Other antigens include

25 mutant variant(s) of the normal protein having an altered activity or subcellular distribution, e.g., mutations of genes giving rise to tumor antigens.

Specific non-limiting examples of tumor antigens for use in an anti-CD40-fusion protein vaccine include, e.g., CEA, prostate specific antigen (PSA), HER-2/neu, BAGE, GAGE, MAGE 1-4, 6 and 12, MUC (Mucin) (e.g., MUC-1, MUC-2, etc.), GM2 and GD2 gangliosides, ras, myc, tyrosinase, MART (melanoma antigen), Pmel 17(gp100), GnT-V intron V sequence (N-acetylglucosaminyltransferase V intron V sequence),

30 Prostate Ca psm, PRAME (melanoma antigen),  $\beta$ -catenin, MUM-1-B (melanoma ubiquitous mutated gene product), GAGE (melanoma antigen) 1, MAGE, BAGE (melanoma antigen) 2-10, c-ERB2 (Her2/neu),

DAGE, EBNA (Epstein-Barr Virus nuclear antigen) 1-6, gp75, human papilloma virus (HPV) E6 and E7, p53, lung resistance protein (LRP), Bcl-2, Ki-67, Cyclin B1, gp100, Survivin, and NYESO-1

- In addition, the immunogenic molecule can be an autoantigen involved in the initiation and/or propagation of an autoimmune disease, the pathology of which is largely due to the activity of antibodies specific for a molecule expressed by the relevant target organ, tissue, or cells, e.g., SLE or MG. In such diseases, it can be desirable to direct an ongoing antibody-mediated (i.e., a Th2-type) immune response to the relevant autoantigen towards a cellular (i.e., a Th1-type) immune response. Alternatively, it can be desirable to prevent onset of or decrease the level of a Th2 response to the autoantigen in a subject not having, but who is suspected of being susceptible to, the relevant autoimmune disease by prophylactically inducing a Th1 response to the appropriate autoantigen. Autoantigens of interest include, without limitation: (a) with respect to SLE, the Smith protein, RNP ribonucleoprotein, and the SS-A and SS-B proteins; and (b) with respect to MG, the acetylcholine receptor. Examples of other miscellaneous antigens involved in one or more types of autoimmune response include, e.g., endogenous hormones such as luteinizing hormone, follicular stimulating hormone, testosterone, growth hormone, prolactin, and other hormones.
- Antigens involved in autoimmune diseases, allergy, and graft rejection can be used in the compositions and methods of the invention. For example, an antigen involved in any one or more of the following autoimmune diseases or disorders can be used in the present invention: diabetes, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjogren's Syndrome, including keratoconjunctivitis sicca secondary to Sjogren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis. Examples of antigens involved in autoimmune disease include glutamic acid decarboxylase 65 (GAD 65), native DNA, myelin basic protein, myelin proteolipid protein, acetylcholine receptor components, thyroglobulin, and the thyroid stimulating hormone (TSH) receptor.
- Examples of antigens involved in allergy include pollen antigens such as Japanese cedar pollen antigens, ragweed pollen antigens, rye grass pollen antigens, animal derived antigens such as dust mite antigens and

feline antigens, histocompatibility antigens, and penicillin and other therapeutic drugs. Examples of antigens involved in graft rejection include antigenic components of the graft to be transplanted into the graft recipient such as heart, lung, liver, pancreas, kidney, and neural graft components. The antigen may be an altered peptide ligand useful in treating an autoimmune disease.

- 5 It will be appreciated by those of skill in the art that the sequence of any protein effector molecule may be altered in a manner that does not substantially affect the functional advantages of the effector protein. For example, glycine and alanine are typically considered to be interchangeable as are aspartic acid and glutamic acid and asparagine and glutamine. One of skill in the art will recognize that many different variations of effector sequences will encode effectors with roughly the same activity as the native effector. The effector
- 10 molecule and the antibody may be conjugated by chemical or by recombinant means as described above. Chemical modifications include, for example, derivitization for the purpose of linking the effector molecule and the antibody to each other, either directly or through a linking compound, by methods that are well known in the art of protein chemistry. Both covalent and noncovalent attachment means may be used with the humanized antibodies of the present invention.
- 15 The procedure for attaching an effector molecule to an antibody will vary according to the chemical structure of the moiety to be attached to the antibody. Polypeptides typically contain a variety of functional groups; e.g., carboxylic acid (COOH), free amine ( $-NH_2$ ) or sulfhydryl ( $-SH$ ) groups, which are available for reaction with a suitable functional group on an antibody to result in the binding of the effector molecule. Alternatively, the antibody can be derivatized to expose or to attach additional reactive functional groups,
- 20 e.g., by attachment of any of a number of linker molecules such as those available from Pierce Chemical Company, Rockford Ill.
- The linker is capable of forming covalent bonds to both the antibody and to the effector molecule. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. Where the antibody and the effector
- 25 molecule are polypeptides, the linkers may be joined to the constituent amino acids through their side groups (e.g., through a disulfide linkage to cysteine). However, in a preferred embodiment, the linkers will be joined to the alpha carbon amino and carboxyl groups of the terminal amino acids.
- In some circumstances, it is desirable to free the effector molecule from the antibody when the immunoconjugate has reached its target site. Therefore, in these circumstances, immunoconjugates will
- 30 comprise linkages that are cleavable in the vicinity of the target site. Cleavage of the linker to release the effector molecule from the antibody may be prompted by enzymatic activity or conditions to which the immunoconjugate is subjected either inside the target cell or in the vicinity of the target site. When the target

site is a tumor, a linker that is cleavable under conditions present at the tumor site (e.g. when exposed to tumor-associated enzymes or acidic pH) may be used.

Exemplary chemical modifications of the effector molecule and the antibody of the present invention also include derivitization with polyethylene glycol (PEG) to extend time of residence in the circulatory system and reduce immunogenicity, according to well known methods (See for example, Lisi, et al., *Applied Biochem.* 4:19 (1982); Beauchamp, et al., *Anal Biochem.* 131:25 (1982); and Goodson, et al., *Bio/Technology* 8:343 (1990)).

The present invention contemplates vaccines for use in both active and passive immunization embodiments. Immunogenic compositions, proposed to be suitable for use as a vaccine, may be prepared most readily directly from immunogenic T-cell stimulating peptides prepared in a manner disclosed herein. The final vaccination material is dialyzed extensively to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle. In certain embodiment of the present invention, the compositions and methods of the present invention are used to manufacture a cellular vaccine, e.g., the antigen-delivering anti-CD40 binding portion of the antibody is used to direct the antigen(s) to an antigen presenting cell, which then "loads" the antigen onto MHC proteins for presentation. The cellular vaccine is, therefore, the antigen presenting cell that has been loaded using the compositions of the present invention to generate antigen-loaded antigen presenting cells.

When the vaccine is the anti-CD40 binding protein itself, e.g., a complete antibody or fragments thereof, then these "active ingredients" can be made into vaccines using methods understood in the art, e.g., U.S. Patent Nos. 4,608,251; 4,601,903; 4,599,231; 4,599,230; and 4,578,770.

Typically, such vaccines are prepared as injectables, e.g., as liquid solutions or suspensions or solid forms suitable for re-suspension in liquid prior to injection. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients, which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants, which enhance the effectiveness of the vaccines.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to generate an immune response. Precise amounts of cells or active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of a few thousand cells (to millions of cells) for cellular vaccines. For standard epitope or epitope delivery vaccines then the vaccine may be several

hundred micrograms active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may vary widely, however, certain embodiments herein will most likely be delivered intravenously or at the site of a tumor or infection directly. Regardless, any of the conventional methods for administration of a vaccine are applicable. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

In many instances, it will be desirable to have multiple administrations of the vaccine, e.g., four to six vaccinations provided weekly or every other week. A normal vaccination regimen will often occur in two to twelve week intervals or from three to six week intervals. Periodic boosters at intervals of 1-5 years, usually three years, may be desirable to maintain protective levels of the immune response or upon a likelihood of a remission or re-infection. The course of the immunization may be followed by assays for, e.g., T cell activation, cytokine secretion or even antibody production, most commonly conducted *in vitro*. These immune response assays are well known and may be found in a wide variety of patents and as taught herein.

A vaccine of the present invention may be provided in one or more "unit doses" depending on whether the nucleic acid vectors are used, the final purified proteins, or the final vaccine form is used. Unit dose is defined as containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses in association with its administration, i.e., the appropriate route and treatment regimen. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. The subject to be treated may also be evaluated, in particular, the state of the subject's immune system and the protection desired. A unit dose need not be administered as a single injection but may include continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of DNA/kg (or protein/Kg) body weight, with ranges between about 0.05, 0.10, 0.15, 0.20, 0.25, 0.5, 1, 10, 50, 100, 1,000 or more mg/DNA or protein/kg body weight are administered.

Likewise, the amount of anti-CD40-antigen vaccine delivered can vary from about 0.2 to about 8.0 mg/kg body weight. Thus, in particular embodiments, 0.4 mg, 0.5 mg, 0.8 mg, 1.0 mg, 1.5 mg, 2.0 mg, 2.5 mg, 3.0 mg, 4.0 mg, 5.0 mg, 5.5 mg, 6.0 mg, 6.5 mg, 7.0 mg and 7.5 mg of the vaccine may be delivered to an individual *in vivo*. The dosage of vaccine to be administered depends to a great extent on the weight and physical condition of the subject being treated as well as the route of administration and the frequency of treatment. A pharmaceutical composition that includes a naked polynucleotide prebound to a liposomal or viral delivery vector may be administered in amounts ranging from 1  $\mu$ g to 1 mg polynucleotide to 1  $\mu$ g to 100 mg protein. Thus, particular compositions may include between about 1  $\mu$ g, 5  $\mu$ g, 10  $\mu$ g, 20  $\mu$ g, 30  $\mu$ g, 40  $\mu$ g, 50  $\mu$ g, 60  $\mu$ g, 70  $\mu$ g, 80  $\mu$ g, 100  $\mu$ g, 150  $\mu$ g, 200  $\mu$ g, 250  $\mu$ g, 500  $\mu$ g, 600  $\mu$ g, 700  $\mu$ g, 800  $\mu$ g, 900  $\mu$ g

or 1,000 µg polynucleotide or protein that is bound independently to 1 µg, 5 µg, 10 µg, 20 µg, 3.0 µg, 40 µg, 50 µg, 60 µg, 70 µg, 80 µg, 100 µg, 150 µg, 200 µg, 250 µg, 500 µg, 600 µg, 700 µg, 800 µg, 900 µg, 1 mg, 1.5 mg, 5 mg, 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg, 70 mg, 80 mg, 90 mg or 100 mg vector.

Antibodies of the present invention may optionally be covalently or non-covalently linked to a detectable label. Detectable labels suitable for such use include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical methods. Useful labels in the present invention include magnetic beads (e.g. DYNABEADS<sup>TM</sup>), fluorescent dyes (e.g., fluorescein isothiocyanate, Texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g., <sup>3</sup>H, <sup>125</sup>I, <sup>35</sup>S, <sup>14</sup>C, or <sup>32</sup>P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (e.g. polystyrene, polypropylene, latex, etc.) beads.

Methods of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted illumination. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label.

The antibody and/or immunoconjugate compositions of this invention are particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity. The compositions for administration will commonly comprise a solution of the antibody and/or immunoconjugate dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well-known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of fusion protein in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Thus, a typical pharmaceutical immunoconjugate composition of the present invention for intravenous administration would be about 0.1 to 10 mg per patient per day. Dosages from 0.1 up to about 100 mg per patient per day may be used. Actual methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as REMINGTON'S PHARMACEUTICAL SCIENCE, 19TH ED., Mack Publishing Company, Easton, Pa. (1995).



The compositions of the present invention can be administered for therapeutic treatments. In therapeutic applications, compositions are administered to a patient suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health. An effective amount of the compound is that which provides either subjective relief of a symptom(s) or an objectively identifiable improvement as noted by the clinician or other qualified observer.

Single or multiple administrations of the compositions are administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the proteins of this invention to effectively treat the patient. Preferably, the dosage is administered once but may be applied periodically until either a therapeutic result is achieved or until side effects warrant discontinuation of therapy. Generally, the dose is sufficient to treat or ameliorate symptoms or signs of disease without producing unacceptable toxicity to the patient.

Controlled release parenteral formulations of the immunoconjugate compositions of the present invention can be made as implants, oily injections, or as particulate systems. For a broad overview of protein delivery systems see, Banga, A. J., THERAPEUTIC PEPTIDES AND PROTEINS: FORMULATION, PROCESSING, AND DELIVERY SYSTEMS, Technomic Publishing Company, Inc., Lancaster, Pa., (1995).

Particulate systems include microspheres, microparticles, microcapsules, nanocapsules, nanospheres, and nanoparticles. Microcapsules contain the therapeutic protein as a central core. In microspheres the therapeutic is dispersed throughout the particle. Particles, microspheres, and microcapsules smaller than about 1  $\mu\text{m}$  are generally referred to as nanoparticles, nanospheres, and nanocapsules, respectively. Capillaries have a diameter of approximately 5  $\mu\text{m}$  so that only nanoparticles are administered intravenously. Microparticles are typically around 100  $\mu\text{m}$  in diameter and are administered subcutaneously or intramuscularly.

Polymers can be used for ion-controlled release of immunoconjugate compositions of the present invention. Various degradable and non-degradable polymeric matrices for use in controlled drug delivery are known in the art (Langer, R., Accounts Chem. Res. 26:537-542 (1993)). For example, the block copolymer, poloxamer 407® exists as a viscous yet mobile liquid at low temperatures but forms a semisolid gel at body temperature, hydroxyapatite has been used as a microcarrier for controlled release of proteins, and/or liposomes may be used for controlled release as well as drug targeting of the lipid-capsulated drug. Numerous additional systems for controlled delivery of therapeutic proteins are known. See, e.g., U.S. Pat. Nos. 5,055,303, 5,188,837, 4,235,871, 4,501,728, 4,837,028 4,957,735 and 5,019,369, 5,055,303; 5,514,670; 5,413,797;

5,268,164; 5,004,697; 4,902,505; 5,506,206, 5,271,961; 5,254,342 and 5,534,496.

Among various uses of the antibodies of the invention are included a variety of disease conditions caused by specific human cells. For example, for a humanized version of the mouse anti-CD40\_12E12.3F3 (ATCC  
 5 Accession No. PTA-9854), anti-CD40\_12B4.2C10 (Deposit No. HS446, ATCC Accession No. PTA-10453), and anti-CD40\_11B6.1C3 (Deposit No. HS440, ATCC Accession No. PTA-10453), antibodies disclosed herein, one application for antibodies is the treatment, contacting,, imaging, activation or deactivation of cells expressing CD40.

In another embodiment, this invention provides kits for the delivery of antigens, e.g., CD40 or an  
 10 immunoreactive fragment thereof, conjugated or in the form of a fusion protein with one or more T cell or B cell epitopes. A "biological sample" as used herein is a sample of biological tissue or fluid that contains the antigen. Such samples include, but are not limited to, tissue from biopsy, blood, and blood cells (e.g., white cells). Preferably, the cells are lymphocytes, e.g., dendritic cells. Biological samples also include sections of tissues, such as frozen sections taken for histological purposes. A biological sample is typically obtained  
 15 from a multicellular eukaryote, preferably a mammal such as rat, mouse, cow, dog, guinea pig, or rabbit, and more preferably a primate, such as a macaque, chimpanzee, or human. Most preferably, the sample is from a human. The antibodies of the invention may also be used in vivo, for example, as a diagnostic tool for in vivo imaging.

Kits will typically comprise a nucleic acid sequence that encodes an antibody of the present invention (or  
 20 fragment thereof) with one or more framework portions or multiple cloning sites at the carboxy-terminal end into which the coding sequences for one or more antigens may be inserted. In some embodiments, the antibody will be a humanized anti-CD40 Fv fragment, such as an scFv or dsFv fragment. In addition the kits will typically include instructional materials disclosing methods of use of an antibody of the present invention (e.g. for loading into dendritic cells prior to immunization with the dendritic cells, which can be  
 25 autologous dendritic cells). The kits may also include additional components to facilitate the particular application for which the kit is designed. Thus, for example, the kit may additionally contain methods of detecting the label (e.g. enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a sheep anti-mouse-HRP, or the like). The kits may additionally include buffers and other reagents routinely used for the practice of a particular method. Such kits and appropriate  
 30 contents are well known to those of skill in the art.

In another set of uses for the invention, antibodies targeted by antibodies of the invention can be used to purge targeted cells from a population of cells in a culture. For example, if a specific population of T cells is preferred, the antibodies of the present invention may be used to enrich a population of T cells having the

- opposite effect of the on-going immune response. Thus, for example, cells cultured from a patient having a cancer can be purged of cancer cells by providing the patient with dendritic cells that were antigen loaded using the antibodies of the invention as a targeting moiety for the antigens that will trigger an immune response against the cancer, virus or other pathogen. Likewise, the antibodies can be used to increase the population of regulatory T cells or drive the immune response toward or away from a cytotoxic T cell response or even drive a B cell response.

#### anti-CD40\_12E12.3F3

anti-CD40\_12E12.3F3\_H-V-hlgG4H-C – underlined region shows the Heavy chain V region amino acid sequence:

- 10 MNLGLSLIFLVLVLKGVQCEVKLVESGGGLVQPGGSLKLSCATSGFTFSDYYMYWVROTPEKRLE  
WVAYINSGGGSTYYPDTVKGRFTISRDNAKNTLYLOMSRLKSEDTAMYYCARRGLPFHAMDYWG  
QGTSVTVSSAKTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ  
SSGLYSLSSVVTVPSSSLGTKYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFEGGPSVFLFPPK  
PKDTLMISRTPEVTCVVDVVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQ  
15 DWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA  
VEWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLS  
LGKAS (SEQ ID NO.: 1)

anti-CD40\_12E12.3F3\_K-V-hlgGK-C – underlined region shows the Light chain V region amino acid sequence

- 20 MMSSAQFLGLLLLCFQGTRCDIQMTQTSSLSASLGDRVTISCSASQGISNYLNWYQOKPDGTVKLL  
IYYTSLHSGVPSRFSGSGSGTDYSLTIGNLEPEDATYYCOOFNKLPPTFGGGTKLEIKRTVAAPSVFI  
FPPSDEQLKSGTASVVCLLNFPYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKA  
DYEYHKVYACEVTHQGLSPVTKSFNRGEC- (SEQ ID NO.: 2)

#### anti-CD40\_12B4.2C10

- 25 anti-CD40\_12B4.2C10 Heavy Chain:

MEWSWIFLFLSGTAGVHSEVQLQQSGPELVKPGASVKMSCKASGYTFTDYVLHWVKQKPGQGLE  
WIGYINPYNDGTYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGYPAYSGYAMDYW  
GQGTSTVTVSSAKTTPPSVYPLAPGSAAQTNSMVTGLCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVL  
QKGEFV (SEQ ID No.: 3)

- 30 anti-CD40\_12B4.2C10 Light Chain:

MMSSAQFLGLLLLCFQGTRCDIQMTQTSSLSASLGDRVTISCRASQDISNYLNWYQOKPDGTVKLL  
IYYTSLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCHHGNTLPWTFGGGKLEIKRADAAPT  
SIFPPSSEQLTSGGASVVCFLNFPYKDNVKKIDGSRQNGVLNSWTDQDSKDSSTYSMSSTLTLT  
KDEYERHNSYTCEATHKTSTSPIVKSFNRECE (SEQ ID No.: 4)

- 35 anti-CD40\_12B4.2C10 Light Chain - alternative clone (17K6)

MDFQVQIFSLISASVIMSRGQIVLTQSPAILSASPGEKVTMTCSASSSVSYMYRYQQKPGSSPKPWI  
YGTSNLSAGVPAFSGSGSGTSYSLTISSMEAEADAATYYCQYHSYPLTFGAGTKLELKRADAAPT  
SIFPPSSEQLTSGGASVVCFLNFPYKDNVKKIDGSRQNGVLNSWTDQDSKDSSTYSMSSTLTLT  
KDEYERHNSYTCEATHKTSTSPIVKSFNRECE (SEQ ID No.: 5)

- 40 anti-CD40\_11B6.1C3

MGWSWIFLFLLSGTAGVLSEVQLQQSGPELVKPGASVKISCKASGYSFTGYMHVVKQSHVKSLE  
WIGRINPYNGATSYNQNFKDKASLTVDKSSSTAYMELHSLTSEDSAVYYCAREDYVYWGQGTTLT  
VSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQKGEFV  
(SEQ ID No.: 6)

**anti-CD40\_11B6.1C3 Light Chain:**

10 MKLPVRLVLVLMFWIPASSDVMVTQTPLSLPVSLGDAQSISRSSQSLVHSNGNTYLHWYLQKPGQ  
SPKLLIYKVSNRFSQVDFRSGSGSGTDFALKISRVEADLGVYFCSQSTHVPWTFGGGKLEIKRAD  
AAPTYSIFPSSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSRQNGVLNSWTDQDSKDYMSMSS  
TLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID No.: 7)

[anti-CD40\_12E12.3F3\_H-V-hlgG4H-C) – underlined region shows the Heavy chain V region sequence:

ATGAACCTTGGGGCTCAGCTTGATTTCCTTGTCCTTGTTTAAAGGTGTCCAGTGTGAAGTGAA  
GCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAACC  
TCTGGATTCACTTCAGTGACTATTACATGTATTGGGTTCGCCAGACTCCAGAGAAGAGGGCTGG  
AGTGGGTGCGATACATTAATTCTGGTGGTGGTAGCACCTATTATCCAGACACTGTAAAGGGCCG  
ATTCAACCATTCTCCAGAGACAATGCCAAGAACCCCTGTACCTGCAATGAGCCGGCTGAAGTCT  
GAGGACACAGCCATGTATTACTGTGCAAGACGGGGGTACCGTTCCATGCTATGGACTATTGGG  
GTCAAGGAACCTCAGTCAACCGTCTCCTCAGCCAAAACGAAGGGGCCATCCGTCTTCCCCCTGGC  
GCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCCGCCCTGGGCTGCCTGGTCAAGGACTACTTC  
CCCGAACCGGTGACGGTGTCTGTGAACTCAGGCGCCCTGACCAGCGGGCTGCACACCTTCCCCG  
CTGTCTACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCTCCAGCAGCTTG  
GGCAGGAAGACCTACACCTGCAACGTAGATCACAAGCCAGCAACACCAAGGTGGACAAGAGA  
GTTGAGTCCAAATATGGTCCCCATGCCACCCTGCCAGCACCTGAGTTCGAAGGGGGACCAT  
CAGTCTTCTGTTCCTCCCCCAAAACCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTCA  
GTGCGTGGTGGTGACGTGAGCCAGGAAGACCCGAGGTCCAGTTCAACTGGTACGTGGATGG  
CGTGGAGGTGCATAATGCCAAGACAAGCCGGGGAGGAGCAAGTTCAACAGCAGCATCCGTGT  
GGTCAGCGTCTCACCCTGTCACCAAGGACTGGCTGAAGCCGCAAGGAGTACAAGTGCAAGGT  
CTCCAACAAAGGCCTCCCGTCTCTCATCGAGAAAACCATCTCAAAGCCAAAGGGCAGCCCCG  
AGAGCCACAGGTGTACACCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTCAAGCT  
GACCTGCCTGGTCAAAGGCTTCTACCCACGCGACATCGCCGTGGAGTGGGAGCAAGTGGGCA  
GCCGGAGAACAACCTACAAGACCAACGCTCCCGTGTGAGTACCGACGGCTCTTCTCTCTAC  
AGCAGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGAATGTCTTCTCATGCTCCGTGATG  
CATGAGGCTTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAGGCTAGCT  
GA (SEQ ID NO.: 8)

[anti-CD40\_12E12.3F3\_K-V-hIgGK-C] – underlined region shows the Light chain V region sequence

40 ATGATGTCCTCTGCTCAGTTCCTTGGTCTCCTGTTGCTCTGTTTTCAAGGTACCAGATGTGATAT  
CCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTAGGAGACAGAGTCACCATCAGTTGC  
AGTGCAAGTCAGGGCATTAGCAATTATTTAAACTGGTATCAGCAGAAACCAGATGGAACCTGTTA  
AACTCCTGATCTATTACACATCAATTTTACACTCAGGAGTCCCCATCAAGGTTAGTGCGCAGTG  
GTCTGGGACAGATTATTCTCTCACCATCGGCAACCTGGAACCTGAAAGTATTGCCACTTACTATT  
GTCAGCAGTTTAATAAGCTTCTCTCCGACGTTCCGGTGAGGCCACCAAACCTCGAGATCAAA  
CGAAC  
CTGTGGCTGCCACATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAC  
TGCCT  
45 CTGTTGTGTGCCTGCTGAATAAATTCTATCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAA

CGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTA  
CAGCCTCAGCAGCACCCCTGACGCTGAGCAAAGCAGACTACGAGAAAACACAAAGTCTATGCCTG  
CGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTA  
G (SEQ ID NO.: 9)

5

**anti-CD40\_12B4.2C10\_H-V-hIgG4H-C heavy chain**

ATGGAATGGAGTTGGATATTTCTCTTTCTTCTGTACAGGAAGTGCAGGTGTCCACTCTGAGGTCCA  
GCTGCAGCAGTCTGGACCTGAGCTGGTAAAGCCTGGGGCTTCAGTGAAAGATGTCCTGCAAGGCT  
TCTGGATACACATTCAGTACTATGTTTTGCACTGGGTGAAACAGAAGCCTGGGCAGGGCCTTG  
10 AGTGGATTGGATATATTAATCCTTACAATGATGGTACTAAGTACAATGAGAAGTTCAAAGGCAA  
GGCCACACTGACTTCAGACAAAATCCTCCAGCACAGCCTACATGGAGCTCAGCAGCCTGACCTCT  
GAGGACTCTGCGGTCTATTACTGTGCAAGGGGCTATCCGGCCTACTCTGGGTATGCTATGGACT  
ACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAACGAAGGGGCCATCCGTCTTCCC  
CCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCCGCCCTGGGGTGCCTGGTCAAGGAC  
15 TACTTCCCCGAACCGGTGACGGTGTCTGGAACTCAGGCGCCCTGACCAAGCGCGTGCACACCT  
TCCCGGCTGTCTACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCTCCAGC  
AGCTTGGGCACGAAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGAC  
AAGAGAGTTGAGTCCAAATATGGTCCCCCATGCCACCCCTGCCAGCACCTGAGTTCGAAGGGG  
GACCATCAGTCTTCTGTCCCCCAAACCCAAGGACACTCTCATGATCTCCCGGACCCCTGA  
20 GGTCACGTGCGTGGTGGTGGACGTGAGCCAGGAAGACCCCGAGGTCCAGTTCAACTGGTACGT  
GGATGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGAGGAGCAGTTCAACAGCACGTA  
CCGTGTGGTCAGCGTCTCACCCTGTCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGC  
AAGGTCTCCAACAAAGGCCTCCCGTCTCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAG  
CCCCGAGAGCCACAGGTGTACACCCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTG  
25 AGCCTGACCTGCGCTGGTCAAAGGCTTCTACCCAGCGACATCGCCGTGGAGTGGGAGAGCAAT  
GGGCAGCCGGAGAACTACAAGACCACGCTCCCGTGTGGACTCCGACGGCTCCTTCTTCC  
TCTACAGCAGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCG  
TGATGCATGAGGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTGGGTAAAGC  
TAGCTGA (SEQ ID NO.: 10)

30

**anti-CD40\_12B4.2C10\_K-V-hIgGK-C (variant 1) light chain**

ATGGATTTTCAAGTGCAGATTTTCAGCTTCTGCTAATCAGTGCCTCAGTCATAATGTCCAGGG  
GACAAATTGTTCTACCCAGTCTCCAGCAATCCTGTCTGCATCTCCAGGGGAGAAAGGTACCCAT  
GACCTGCAGTGCCAGCTCAAGTGTAAGTTACATGTACAGGTACCAGCAGAAGCCAGGATCCTC  
35 ACCCAAACCCCTGGA'TTTATGGCACATCCAACCTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGC  
AGTGGATCTGGGACCTCTTATTCTCTACAATCAGCAGCATGGAGGCTGAAGATGCTGCCACTT  
ATTACTGCCAGCAATATCATAGTTACCCGCTCACGTTCCGTGCTGGGACCAAGTCTGAGTCAA  
ACGAAGTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGA  
ACTGCCTCTGTTGTGCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAAGG  
40 TGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGCAAGGACA  
GCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAGCAGACTACGAGAAAACACAAAGTCT  
ATGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAG  
AGTGTTAG (SEQ ID NO.: 11)

**45 anti-CD40\_12B4.2C10\_K-V-hIgGK-C (Variant 2) light chain**

ATGATGTCCTCTGCTCAGTTCCTTGGTCTCCTGTTGCTCTGTTTTCAAGGTACCAGATGTGATAT  
 CCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGC  
 AGGGCAAGTCAGGACATTAGCAATTATTTAACTGGTATCAGCAGAAACCAGATGGAAGTGT  
 AAATCCTGATCTACTACACATCAAGATTACACTCAGGAGTCCCATCAAGGTTCAAGTGGCAGTG  
 5 GGTCTGGAACAGATTATTCTCTCACCATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTT  
 TTGCCATCATGGTAATACGCTTCCGTGGACGTTCCGTGGAGGCACCAAGCTCGAGATCAAACGA  
 ACTGTGGCTGCACCATCTGTCTTCACTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAGTGC  
 CTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAAGGTGGAT  
 AACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACC  
 10 TACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTATGCC  
 TGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGT  
 TAG (SEQ ID NO.: 12)

anti-CD40\_11B6.1C3\_H-V-hlgG4H-C heavy chain

15 ATGGGATGGAGCTGGATCTTTCTTTCTCCTGTCAAGAACTGCAGGTGTCCTCTCTGAGGTCCA  
 GCTGCAACAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGCAAGGCT  
 TCTGGTTACTCATTCACTGGCTACTACATGCACTGGGTGAAGCAAAGCCATGTAAAGAGCCTTG  
 AGTGGATTGGACGTATTAATCCTTACAATGGTGCTACTAGCTACAACCAGAAATTTCAAGGACAA  
 GGCCAGCTTGACTGTAGATAAGTCTCCAGCACAGCCTACATGGAGCTCCACAGCCTGACATCT  
 20 GAGGACTCTGCAGTCTATTACTGTGCAAGAGAGGACTACGTCTACTGGGGCCAAGGCCACCTC  
 TCACAGTCTCCTCAGCCAAAACGAAGGGCCCATCCGTCTTCCCCCTGGCGCCCTGCTCCAGGAG  
 CACCTCCGAGAGCACAGCCGCCCTGGGCTGCTGTCAGGACTACTTCCCCGAACCGGTGACG  
 GTGTCGTGGAACTCAGGCGCCCTGACCAGCGCGTGCACACCTTCCCCGCTGTCTACAGTCCT  
 CAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACGAAGACCTA  
 25 CACCTGCAACGTAGATCAAAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGTCCAAATA  
 TGGTCCCCCATGCCCACCCTGCCCAGCACCTGAGTTCGAAGGGGGACCATCAGTCTTCTGTTT  
 CCCCCAAAACCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTACAGTGCCTGCTGGTGG  
 ACGTGAGCCAGGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGATGGCGTGGAGGTGCATA  
 ATGCCAAGACAAAGCCGCGGGAGGAGCAGTTCAAACAGCACGTACCGTGTGGTCAAGCCTCTCA  
 30 CCGTCTGCACCAAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCC  
 TCCCGTCTCCATCGAGAAAACCATCTCCAAGGCCAAAGGGCAGCCCCGAGAGCCACAGGTGT  
 ACACCTTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTACGCTGACCTGCCTGGTCA  
 AAGGCTTCTACCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACCT  
 ACAAGACCACGCCTCCCGTGTGACTCCGACGGCTCCTTCTTCTCTACAGCAGGCTAACCGT  
 35 GGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCA  
 CAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAAGCTAGCTGA (SEQ ID NO.: 14)

anti-CD40\_11B6.1C3\_K-V-hlgGK-C light chain

ATGAAGTTGCCTGTTAGGCTGTTGGTGGTGTGATGTTCTGGATTCTCTGCTTCCAGCAGTGATGTTGT  
 GATGACCCAAACTCCACTCTCCCTGCCTGTCACTTGGAGATCAAGCCTCCATCTCTTGCAGAT  
 40 CTAGTCAGAGCCTTGTACACAGTAATGGAAACACCTATTTACATTGGTACCTGCAGAAGCCAGG  
 CCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAACCGATTTTCTGGGGTCCAGACAGGTTT  
 AGTGGCAGTGGATCAGGGACAGATTTGCACTCAAGATCAGTAGAGTGGAGGCTGAGGATCTG  
 GGAGTTTATTTCTGCTCTCAAAGTACACATGTTCCGTGGACGTTCCGTGGAGGCCACCAAGCTCG  
 45 AGATCAAACGAAGTGTGGCTGCACCATCTGTCTTCACTTCCCGCCATCTGATGAGCAGTTGAA  
 ATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAG  
 TGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGC  
 AAGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAAACAC

AAAGTCTATGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACA  
GGGGAGAGTGTAG (SEQ ID NO:15)

Example 1: Anti-CD40 - HIV peptides vaccine

Five 19- to 32-amino-acid long sequences were selected from a multiplicity of cytotoxic T lymphocyte (CTL) epitopes identified in the HIV-1 Nef, Gag and Env proteins in the context of different MHC-class I molecules. It has been reported that CTL responses can be induced efficiently by lipopeptide vaccines in mice, in primates, and in humans. The five HIV peptides were then modified in C-terminal position by a (Palm)-NH<sub>2</sub> group and the five HIV peptide sequences have been well described in the scientific literature [e.g., Characterization of a multi-lipopeptides mixture used as an HIV-1 vaccine candidate (1999) Klinguer et al., Vaccine, Volume 18, 259-267] and in a patent application [Cytotoxic T lymphocyte-inducing lipopeptides and use as vaccines. Gras-Masse H. et al., Patent No. EP0491628 (1992-06-24); US 5871746 (1999-02-16)].

A very desirable HIV vaccine would be composed of recombinant anti-dendritic cell receptor antibody fused to the above HIV peptides. The present invention includes compositions and methods to efficiently produce proteins and HIV vaccines.

The sequences shown below are the amino-acid sequences of the five selected HIV peptides and the amino-acid positions within each HIV protein are in brackets.

Nef (66-97) is: VGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGL (SEQ ID NO.: 16)

Nef (116-145) is: HTQGYFPDWQNYTPGPGVRYPLTFGWLYKL (SEQ ID NO.: 17)

Gag p17 (17-35) is: EKIRLRPGGKKKYKLKHIV (SEQ ID NO.: 18)

Gag p17-p24 (253-284) is: NPPIPVGEIYKRWIILGLNKIVRMYSPTSILD (SEQ ID NO.: 19)

Pol 325-355 (RT 158-188) is: AIFQSSMTKILEPFRKQNPDIVIYQYMDDL (SEQ ID NO.: 20)

The present invention includes compositions and methods for assembling constructs encoding HIV peptides and Flexible linker sequences. The Heavy chain expression vectors typically have a Nhe I site [g|ctagc] appended to the Heavy chain C-terminal residue codon, or [for flex- v1 vectors] to the C-terminal codon of the flex-v1 sequence. Flexible linker sequences or HIV peptide sequences have an Spe I site [a|ctagt] preceding the N-terminal flexible linker or HIV peptide codon, a Nhe I site appended to the C-terminal flexible linker or HIV peptide codon, followed by a TGA stop codon, followed by a Eco RI site, followed by a Not I site. Such flexible linker or HIV peptide Spe I – Not I fragments are inserted into the Heavy chain vector prepared with Nhe I – Not I digestion. Nhe I and Spe I are compatible sites, but when ligated [g|ctagt] is no longer either a Nhe I or Spe I site. Thus additional Spe I – Not I flexible linker or HIV peptide fragments can be inserted into the new Nhe I – Not I interval distal to the initial flexible linker or HIV peptide. In this way, strings of HIV peptide and/or flexible linker coding regions can be appended to the expression vector Heavy chain coding region.

- Fig. 1 shows protein A affinity recombinant antibodies fused to various HIV peptides (lanes 1 to 5) secreted from transfected 293F cells, analyzed by reducing SDS-PAGE and Coomassie Brilliant Blue staining. Fig. 2 shows protein A affinity purified recombinant antibodies fused to various HIV peptides (Lanes 1 and 2) secreted from transfected 293F cells, then analyzed by reducing SDS-PAGE and Coomassie Brilliant Blue staining. Fig. 3 shows protein A affinity purified recombinant antibodies fused to various HIV peptide strings (Lanes 1 to 5) secreted from transfected 293F cells, then analyzed by reducing SDS-PAGE and Coomassie Brilliant Blue staining. Fig. 4 shows protein A affinity purified recombinant antibodies fused to various HIV peptide strings (Lanes 1 to 6) secreted from transfected 293F cells, then analyzed by reducing SDS-PAGE and Coomassie Brilliant Blue staining.
- 10 Example 2. HIV peptides vaccine – in vitro antigen-targeting biology
- Anti-CD40.LIPO5 HIV peptides vaccine tests on HIV patients in vitro. To study the ability of  $\alpha$ CD40.LIPO5 HIV peptide fusion recombinant antibody ( $\alpha$ CD40.LIPO5 rAb) to mediate antigen presentation, the fusion rAb was added to blood cells from HIV-infected individuals and measured cytokine production from peripheral blood mononuclear cells (PBMCs).
- 15 Fig. 5 describes the protocol used in vitro to assay the potency of  $\alpha$ CD40.LIPO5 HIV peptide fusion recombinant antibody ( $\alpha$ CD40.LIPO5 rAb) to elicit the expansion of antigen-specific T cells in the context of a PBMC culture. Briefly, PBMCs ( $2 \times 10^6$  cells/ml) from apheresis of HIV patients are incubated with a dose range of  $\alpha$ CD40.LIPO5 HIV peptide vaccine. On day 2, 100 U/ml IL-2 are added to the culture and then, the media is refreshed every 2 days with 100 U/ml IL-2. On day 10, the expanded cells are challenged for 48 h
- 20 with the individual long peptides corresponding to the 5 HIV peptide sequences incorporated in the  $\alpha$ CD40.LIPO5 HIV peptide fusion rAb. Then, culture supernatants are harvested and assessed for cytokine production (by the T cells with T cell receptor [TCR] specificities for peptide sequences) using multiplex beads assay (Luminex<sup>TM</sup>). Antigen-specific cytokine production detected in such an assay, if it depends on the presence of the anti-CD40.LIPO5 HIV peptide vaccine, reflects vaccine uptake by antigen presenting cells
- 25 [APC] in the culture, and processing [proteolytic degradation] and presentation of peptides on MHC. The antigen-MHC complexes are recognized by T cells with TCR that recognize only the particular HIV antigen-MHC complex. In a HIV patient, such cells are likely to be memory T cells that expanded in the patient in response to the HIV infection.
- Epitopes from all 5 HIV peptide regions of the vaccine can be presented by APCs. The scheme in Fig. 5 was
- 30 used to assay the in vitro expansion of HIV peptide-specific T cells in response to anti-CD40.LIPO5 peptide vaccine. Results from 7 individuals are shown in Fig. 6 and indicate that the  $\alpha$ CD40.LIPO5 HIV peptide fusion rAb elicited HIV peptide-specific IFN $\gamma$  responses in all of the patients studied. Thus, the  $\alpha$ -CD40.LIPO5 HIV peptide fusion rAb allows DCs to cross present at least 1 or 2 different peptides out of the



5 peptides within the vaccine to the T cells of each individual. However, the set of HIV peptides that stimulated IFN $\gamma$  production was different for each patient - most likely reflecting different pools of memory T cells for HIV specificity.

Fig. 6A-C shows the HIV peptide-specific IFN $\gamma$  production in PBMCs from HIV patients incubated with various concentrations of  $\alpha$ CD40.LIPO5 peptide string vaccine. C is the control group, which received no vaccine, and defines the baseline response of the culture to each peptide.

Fig. 7 is a summary of  $\alpha$ CD40.LIPO5 peptide vaccine responses against the 5 peptide regions from 8 HIV patients. The data are based on peptide-specific IFN $\gamma$  production. Fig. 7 shows that the antigen-specific responses observed in 8 HIV patients. The data demonstrate that all HIV peptide regions on the vaccine have the capacity to be processed and presented to T cells - assuming the likely situation that responses to these peptides will only be observed if the appropriate TCR-bearing cells are present. Thus, each patient has a characteristic spectrum of such cells.

The  $\alpha$ CD40.LIPO5 peptide vaccine can evoke the proliferation of antigen-specific T cells capable of secreting a wide spectrum of cytokines

Fig. 8A-C shows that  $\alpha$ CD40.LIPO5 HIV peptide vaccine elicits expansion of HIV peptide-specific T cells capable of secreting multiple cytokines - a desirable feature in a vaccine. In Fig. 8A-C  $\alpha$ CD40.LIPO5 HIV peptide vaccine elicits gag253, nef66, nef116 and pol325 peptide-specific responses characterized by production of multiple cytokines. This is patient A5.

Anti-CD40.LIPO5 HIV peptide vaccination of ex vivo DCs.

Fig. 9 shows the protocol for testing  $\alpha$ CD40.LIPO5 HIV peptide vaccine for its ability to direct the expansion of antigen-specific T cells resulting from targeted uptake by DCs and presentation of peptide epitopes on their surface MHC complex. Briefly, HIV patient monocytes are differentiated into DCs by culture for 2 days with IFN $\alpha$  and GM-CSF. Different doses  $\alpha$ CD40.LIPO5 HIV peptide vaccine or a mix of the 5 peptides are then added for 18 h. Autologous T cells were added to the co-culture (at a ratio of 1:20) on day 3. On day 5, 100 U/ml IL-2 are added to the culture and then, the media is refreshed every 2 days with 100 U/ml IL-2. On day 10, the expanded cells are rechallenged for 48 h with the individual long peptides corresponding to the 5 HIV peptide sequences incorporated in the  $\alpha$ CD40.LIPO5 HIV peptide fusion rAb. Then, culture supernatants are harvested and assessed for cytokine production using Luminex.

Fig. 10A-B shows the cytokine secretion in response to HIV peptides from DC-T cell co-cultures treated with various doses of  $\alpha$ CD40.LIPO5 HIV peptide vaccine. This is patient A10. The results in the patient A10 shown in Fig. 10 A-B demonstrate expansion of antigen-specific T cells corresponding to epitopes within the gag17, gag253, and pol325 HIV peptide regions. In most instances, there is concordance of responses between  $\alpha$ CD40.LIPO5 HIV peptide vaccine and non-LIPO5 vaccine [mixture of 5 non-lipidated HIV

peptides with sequences corresponding to those in the  $\alpha$ CD40.LIPO5 HIV peptide vaccine]. Thus, the  $\alpha$ CD40.LIPO5 HIV peptide vaccine functions well in this in vitro setting where cultured DCs effectively process and present the HIV antigens to T cells. This exemplifies use of the  $\alpha$ CD40.LIPO5 HIV peptide vaccine for ex vivo vaccination, whereby the 'vaccinated DCs' would be cryopreserved for future re-injection into the same patient.

$\alpha$ CD40.LIPO5 HIV peptide vaccine – possible immune effect of the flexible linker regions. It is possible that the flexible linker sequences interspersing the HIV peptide sequences within the  $\alpha$ CD40.LIPO5 HIV peptide vaccine themselves contain T cell epitopes. Fig. 11 A-B shows that patient A4 does not appear to have a significant pool of memory T cells with specificities to the five flexible linker sequences within  $\alpha$ CD40.LIPO5 HIV peptide vaccine. In Fig. 11 A-B, PBMCs from patient A4 treated with the  $\alpha$ CD40.LIPO5 HIV peptide vaccine elicit expansion of antigen-specific T cells with specificity to the gag253 region, but not to the flexible linker sequences. The protocol describe in Fig. 9 was used, with the flexible linker long peptides corresponding in sequence to the bold areas, the HIV peptides are in bold-italics, shown in the sequence below.

$\alpha$ CD40.LIPO5 HIV peptide vaccine heavy chain sequence showing flexible linker regions in bold, joining sequences underlined and HIV peptide regions shaded in bold italics.

QVTLKESGPGILQPSQTLSTCSFSGFSLSTSGMGLSWIRQPSGKGLEWLAHIYWDDDKRYNPSLKSR  
 LTISKDTSSNQVFLKITIVDTADAATYYCARSSHYGYGYGGYFDVWGAGTTVTVSSAKTKGPSVF  
 PLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLG  
 20 TKTYTCNVDHKPSNTKVDKRVESKYGPCCPPCAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVV  
 DVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGL  
 PSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP  
 VLDSGDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLGLGKASQTPTNTISVTPTN  
 NSTPTNNSNPKPNPASEKIRLRPGGKKKYKLKHIVASSVSPTTSVHPTPTSVPPTPTKSSPASNPI  
 25 PVGEIYKRWIHLGLNKIVMYSPTSILDA~~SPTSTPADSSTITPTATPTATPTTIKASHTQGYFPDWQN~~  
~~YTPGPGVRYPLTFGWLYKLASTVTPTATATPSAIVTTITPTATTKPASVGFVTPQVPLRPMYKAA~~  
~~VDLSHFLKEKGGLASTNGSITVAATPTVTPTVNATPSAAASAIQSSMTKILEPFRKQNPDIVIYQ~~  
 YMDDLYAS. (SEQ ID NO.:21).

In Fig. 12A, the PBMCs from patient A3 treated with the  $\alpha$ CD40.LIPO5 HIV peptide vaccine elicit expansion of antigen-specific T cells with specificities to the gag253, nef66, and nef16 regions, but not to the flexible linker sequences. The protocol described in Fig. 1 was used, with the flexible linker long peptides corresponding in sequence to the bold areas shown in Fig. 8.

- Fig. 12B-1 and B-2 shows HIV antigen-specific T cell responses evoked from HIV patient A17 PBMCs incubated with 30 nM of three different HIV5 peptide DC targeting vaccines. Cells were cultured for 10 days with IL-2 and then stimulated with individual long peptides corresponding to the 5 HIV peptide sequences encompassed within the DC-targeting vaccines. After 1 hr brefeldin A was added and incubation continued for a further 5 hrs before staining for FACS analysis. The FACS plots show IFN $\gamma$  and CD8 staining on CD3+ T cells. Circles indicate significant vaccine-evoked expansion of IFN $\gamma$ + cells compared to cells from PBMCs cultured without vaccine. CD8- cells are CD4+ T cells. The data show that that anti-CD40.HIV5pep vaccine evokes a strong expansion of nef66 (N66)-specific CD8+ T cells which is not seen with the other DC targeting vehicles.
- These are data based on the LIPO5 HIV peptide string. For example the anti-CD40 Heavy chain is anti-CD40\_12E12.3F3\_H-LV-hIgG4H-C-Flex-v1-Pep-gag17-f1-gag253-f2-nef116-f3-nef66-f4-pol158] with sequence:
- EVKLVESGGGLVQPGGSLKLSKATSGFTFSDYYMYWVRQTPEKRLEWVAYINSGGGSTYYPDTVK  
GRFTISRDNKNTLYLQMSRLKSEDTAMYCCARRGLPFHAMDYWGQGSVTVSSAKTKGSPVFP  
APCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLG  
15 TYTCNVDPKPSNTKVDKRVESKYGPPCPAPAFEGGSPVFLFPPKPKDTLMISRTPEVTCVVVDVS  
QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSI  
EKTISKAKGQPREPQVYITLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLD  
SDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLGLKASKQTPTNTISVTPTNNSTP  
20 TNNSNPKPNPASEKIRLRPGGKKKYKLKHIVASSVSPTTSVHPTPTSPPTPTKSSPASNPPIPVGEIY  
KRWILGLNKIVRMYSPTSILDASPTSTPADSSITPTATPTATPTIKGASHTQGYFPDWQNYTPGPGV  
RYPLTFGWLYKLASTVTPTATATPSAIVTITPTATTKPASVGFPVTPQVPLRPMTYKAAVDLSHFLK  
EKGGGLASTNGSITVAATAPTPTVPTVNATPSAAASAIFQSSMTKILEPFRKQNPDIVIYQYMDLDLYAS  
(SEQ ID NO.: 22).
- Fig. 12C-1 and C-2 is a similar study to that show in Fig. 12B, except that the PBMCs are from a different HIV patient (A2). The data show antigen-specific CD4+ and CD8+ T cell responses evoked by anti-CD40.HIV5pep but not the other DC-targeting vaccines, or by a mixture of the peptides themselves.
- Fig. 12D shows that, based on analysis of 15 different HIV peptide responses [5 peptide regions sampled in 3 patients], anti-CD40.HIV5pep vaccine is clearly superior to anti-DCIR.HIV5pep, anti-LOX-1.HIV5pep and non-LIPO5 mix for eliciting a broad range of HIV peptide-specific CD8+ and CD4+ T responses.
- The immunogenicity of the flexible linker sequences is of concern for the  $\alpha$ CD40.LIPO5 HIV peptide vaccine design. The limited datasets shown above, testing recall of T cells with specificities for epitopes within the flexible linker sequences, suggest that the human repertoire against these sequences is variable.

Also, the ability of these sequences to prime responses de novo is untested. Responses to the  $\alpha$ CD40.LIPO5 HIV peptide vaccine in monkeys can be tested using the present invention. If necessary, certain less desirable epitopes within these regions can be identified by a combination of predictive computational methods and peptide stimulation scans, and then eliminated by introducing mutational changes that abrogate the TCR interaction.

A humanized antibody includes the heavy chain variable region ( $V_H$ ) and a light chain variable region ( $V_L$ ), wherein the framework regions of the heavy chain and light chain variable regions are from a donor human antibody, and wherein the light chain complementarity determining regions (CDRs) have at least 80%, 90%, 95% or higher identity to CDR1<sub>L</sub> having the amino acid sequence SASQGISNYLN (SEQ ID NO.:41), the CDR2<sub>L</sub> having the amino acid sequence YTSILHS (SEQ ID NO.:23) and the CDR3<sub>L</sub> having the amino acid sequence QQFNKLPTT (SEQ ID NO.:23); and wherein the heavy chain complementarity determining regions comprise at least 80%, 90%, 95% or higher identity to the CDR1<sub>H</sub>, CDR2<sub>H</sub> and CDR3<sub>H</sub>, the CDR1<sub>H</sub> having the amino acid sequence GFTFSDYYMY (SEQ ID NO.:24), the CDR2<sub>H</sub> having the amino acid sequence YINSGGGSTYYPDTVKG (SEQ ID NO.:25), and the CDR3<sub>H</sub> having the amino acid sequence RGLPFHAMDY (SEQ ID NO.:26). For example, the humanized antibody may comprise a VL framework having at least 95% identity to the framework of SEQ ID NOS.: 2, 4, 5 or 7 and a VH framework that has at least 95% identity to the framework of SEQ ID NO.:1, 3 or 6. In another aspect, the donor CDR sequences are from anti-CD40\_12E12.3F3, anti-CD40\_12B4.2C10, anti-CD40\_11B6.1C3 or combinations of their heavy or light chains, and/or their variable regions and further, wherein the antibody or fragment thereof specifically binds to CD40.

Example 3. Prostate-specific antigen (PSA), Cyclin D1, MART-1, influenza viral nucleoprotein (NP) and HA1 subunit of influenza viral hemagglutinin (H1N1, PR8) and peptide screen.

Internalization of anti-CD40 mAb.  $1 \times 10^6$  IL-4DCs were incubated for 1 h in ice with 3 mg/ml human gamma globulin in PBS containing 3% BSA to block non-specific binding. Cells were pulsed for 30 minutes on ice with Alexa 568 labeled anti-CD40 mAb (all at 20 ng/ml final concentration in non-specific block). Cells were then washed and allowed to internalize surface bound antibodies for different times, between 0 and 90 minutes, at 37°C. Following internalization, cells were washed twice with ice-cold PBS containing 1% BSA and 0.05% sodium azide (PBA) and fixed in ice-cold 1% methanol-free formaldehyde (MFF) in PBS overnight at 4°C. Cells were permeabilized in PBS 3% BSA containing 0.5% saponin (PBAS) for 20 minutes at 4°C, and transferred to a 96-well round bottom polypropylene microtiter plate. After washing twice with ice-cold PBAS, cells were incubated for 1 h on ice with 3 mg/ml human gamma globulin in PBAS. BODIPY-phalloidin diluted in PBAS and incubated with cells for 1 hour in ice. Cells were further stained with TOPRO-II, as a nuclear counterstain. Slides were imaged on a Leica SP1 confocal microscope.

- Cells. Monoclonal antibodies for cell surface staining were purchased from BD Biosciences (CA). Monocytes ( $1 \times 10^6/\text{ml}$ ) from healthy donors were cultured in Cellgenics media (France) containing GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) or GM-CSF (100 ng/ml) and IFN $\alpha$  (500 Units/ml) (R&D, CA). For IFNDCs, cells were fed on day 1 with IFN $\alpha$  and GM-CSF. For IL-4DCs, the same amounts of cytokines were supplemented into the media on day one and day three. PBMCs were isolated from Buffy coats using Percoll<sup>TM</sup> gradients (GE Healthcare, Buckinghamshire, UK) by density gradient centrifugation. Total CD4+ and CD8+ T cells were purified by using StemCell kits (CA).
- Peptides. 15-mers (11 amino acid overlapping) for prostate-specific antigen (PSA), Cyclin D1, MART-1, influenza viral nucleoprotein (NP) and HA1 subunit of influenza viral hemagglutinin (H1N1, PR8), were synthesized (Mimotopes).
- DCs and T cell co-culture and cytokine expressions.  $5 \times 10^3$  DCs loaded with recombinant fusion proteins (anti-CD40-HA1, Control Ig-HA1, anti-CD40-PSA, anti-CD40-Cyclin D1, anti-CD40-MART-1, anti-MARCO-MART-1, and control Ig-MART-1) were co-cultured with  $2 \times 10^3$  CFSE-labeled CD4+ T cells for 8 days. Proliferation was tested by measuring CFSE dilution after staining cells with anti-CD4 antibody labeled with APC.
- For measuring the expression of intracellular IFN $\gamma$ , CD4+ T cells were restimulated with 1-5  $\mu\text{M}$  of indicated peptides for 5h in the presence of Brefeldin A. In separate experiments, CD4+ T cells were restimulated with peptides indicated for 36h, and then cytokines secreted by CD4+ T cells were measured by the Luminex.
- CD8+ T cells were co-cultured with DCs for 10 days in the presence of 20 units/ml IL-2 and 20 units/ml IL-7. On day 10 of the culture, CD8+ T cells were stained with anti-CD8 and tetramers indicated.
- CTL assay. On day 10 of the culture, a 5-h  $^{51}\text{Cr}$  release assay was performed. T2 cells pulsed with  $^{51}\text{Cr}$  first and then labeled with 10  $\mu\text{M}$  HLA-A2 epitope of MART-1 or 1 nM epitope of influenza viral M1. T2 cells without peptide were used as control. The mean of triplicate samples was calculated, and the percentage of specific lysis was determined using the following formula: percentage of specific lysis =  $100 \times (\text{experimental } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}) / (\text{maximum } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release})$ . The maximum release refers to counts from targets in 2.5% Triton X-100.
- Preparation of mAbs specific for human CD40. Receptor ectodomain.hlgG (human IgG1Fc) and AP (human placental alkaline phosphatase) fusion proteins were produced for immunizing mice and screening mAbs, respectively. A mammalian vector for human IgFc fusion proteins was engineered as described [*J. Immunol.* 163: 1973-1983 (1999)]. The mammalian expression vector for receptor ectodomain.AP proteins was generated using PCR to amplify cDNA for AP residues 133-1581 (gb|BC009647) while adding a proximal in-frame Xho I site and a distal 6C-terminal His residues followed by a TGA stop codon and Not I site. This Xho I – Not I fragment replaced the human IgG Fc coding sequence in the above ectodomain.IgG vector.

Fusion proteins were produced using the FreeStyle™ 293 Expression System (Invitrogen, CA) according to the manufacturer's protocol (1 mg total plasmid DNA with 1.3 ml 293Fectin reagent /L of transfection). Receptor ectodomain.hIgG was purified by 1 ml HiTrap protein A affinity chromatography (GE Healthcare, CA) eluted with 0.1 M glycine, pH 2.7. Fractions were neutralized with 2M Tris, and then dialyzed against

5 PBS.

Mouse mAbs were generated by conventional technology. Briefly, six-week-old BALB/c mice were immunized i.p. with 20 µg of receptor ectodomain.hIgGFc fusion protein with Ribi adjuvant, then boosted with 20 µg antigen ten days and fifteen days later. After three months, the mice were boosted again three days prior to taking the splens. Three to four days after a final boosting, draining lymph nodes (LN) were  
 10 harvested. B cells from spleen or LN cells were fused with SP2/O-Ag 14 cells (ATCC). Hybridoma supernatants were screened to analyze mAbs specific to the receptor ectodomain fusion protein compared to the fusion partner alone, or to the receptor ectodomain fused to alkaline phosphatase [*J. Immunol.* 163: 1973-1983 (1999)]. Positive wells were then screened in FACS using 293F cells transiently transfected with expression plasmids encoding full-length receptor cDNAs. Selected hybridomas were single cell cloned and  
 15 expanded in CELLline flasks (Integra, CA). Hybridoma supernatants were mixed with an equal volume of 1.5 M glycine, 3 M NaCl, 1× PBS, pH 7.8 (binding buffer) and tumbled with MabSelect resin (GE Healthcare, CA) (800 ml /5ml supernatant). The resin was washed with binding buffer and eluted with 0.1 M glycine, pH 2.7. Following neutralization with 2 M Tris, mAbs were dialyzed against PBS.

Expression and purification of recombinant mAbs. Total RNA was prepared from hybridoma cells using  
 20 RNeasy kit (Qiagen, CA) and used for cDNA synthesis and PCR (SMART RACE kit, BD Biosciences) using supplied 5' primers and gene specific 3' primers (mIgGκ, 5'ggatgggtgggaagatggatacagttgggtgcagcatc3' (SEQ ID NO.:48); mIgG2a, 5'ccaggcatcctagagtcaccgaggagccagt3') (SEQ ID NO.:49). PCR products were then cloned (pCR2.1 TA kit, Invitrogen) and characterized by DNA sequencing (MC Lab, CA). Using the derived sequences for the mouse heavy (H) and light (L) chain variable (V)-region cDNAs, specific primers were  
 25 used to PCR amplify the signal peptide and V-regions while incorporating flanking restriction sites for cloning into expression vectors encoding downstream human IgGκ or IgG4H regions. The vector for expression of chimeric mVk-hlgc was built by amplifying residues 401-731 (gi|63101937|) flanked by Xho I and Not I sites and inserting this into the Xho I – Not I interval of pIRES2-DsRed2 (BD Biosciences). PCR was used to amplify the mAb Vk region from the initiator codon, appending a Nhe I or Spe I site then CACC,  
 30 to the region encoding (e.g., residue 126 of gi|76779294|), appending a distal Xho I site. The PCR fragment was then cloned into the Nhe I – Not I interval of the above vector. The control human IgGκ sequence corresponds to gi|49257887| residues 26-85 and gi|21669402| residues 67-709. The control human IgG4H vector corresponds to residues 12-1473 of gi|19684072| with S229P and L236E substitutions, which stabilize

a disulphide bond and abrogate residual FcR interaction [*J. Immunol.* 164: 1925-1933 (2000)], inserted between the Bgl II and Not I sites of pIRES2-DsRed2 while adding the sequence 5'gctagctgattaataa 3' instead of the stop codon. PCR was used to amplify the mAb VH region from the initiator codon, appending CACC then a Bgl II site, to the region encoding residue 473 of gi|19684072|. The PCR fragment was then

5 cloned into the Bgl II – Apa I interval of the above vector.

Expression and purification of Flu HA1 fusion protein. The Flu HA1 antigen coding sequence is a CipA protein [*Clostridium. thermocellum*] gi|479126| residues 147-160 preceding hemagglutinin [Influenza A virus (A/Puerto Rico/8/34(H1N1))] gi|126599271| residues 18-331 with a P321L change and with 6 C-terminal His

10 residues was inserted between the Heavy chain vector Nhe I and Not I sites to encode recombinant antibody-HA1 fusion proteins (rAb.HA1). Similarly, recombinant antibody-PSA fusion proteins (rAb.PSA) were encoded by inserting gi|34784812| prostate specific antigen residues 101-832 with proximal sequence

CTAGCGC (SEQ ID NO.:27) (Nhe I site and CipA spacer) and a distal Not I site into the same Heavy chain

15 vector. Recombinant antibody proteins were expressed and purified as described above for hFc fusion proteins. In some cases the rAb. antigen coding region and the corresponding L chain coding region were transferred to separate cetHS-puro UCOE vectors (Millipore, CA). The use of UCOE vectors in combination with a preadapted serum free, suspension cell line allowed for rapid production of large quantities of protein

20 [*Cytotechnology* 38, 43-46 (2002).] CHO-S cells grown in CD-CHO with GlutaMAX and HT media supplement (Invitrogen) were seeded at  $5 \times 10^5$  ml 24h prior to transfection in 500 ml Corning Erlenmeyer flasks and incubated in 8% CO<sub>2</sub> at 125 rpm. On the day of transfection,  $1.2 \times 10^7$  cells with viability at least

95% were added to a final volume of 30 ml in a 125 ml flask in CD-CHO with GlutaMAX. 48 ml of FreeStyle Max reagent (Invitrogen) in 0.6 ml of OptiPRO SFM (Invitrogen) was added with gentle mixing to

25 24 mg of See 1-linearized light chain vector and 24 mg of See 1-linearized Heavy chain vector mixed and sterile filtered in 0.6 ml of OptiPRO SFM. After 20 min, the DNA-lipid complex was slowly added to the 125 ml CHO-S culture flask with swirling. Cells were incubated 24h before adding 30 ml of a combined

media solution of CD-CHO with CHO-M5 (Sigma, C0363 component of CHO Kit 1) containing 5 mg/ml of puromycin (A.G. Scientific, CA), 2xGlutaMAX and 0.25xPen/Strep (Invitrogen). At day 2, another 5 mg/ml of puromycin was added directly to the culture and selection was allowed to proceed ~10-14 days while

30 following cell viability from six days post transfection. The viable cell count dropped and when the viable density is  $\sim 2-3 \times 10^6$ /ml, the cells were transferred to fresh selection medium (CD CHO-S + CHO M5 with 2X GlutaMAX, 0.25xPen/Strep, 10 mg/ml Puromycin) at 1E6/ml. Frozen cell stocks were prepared when viability reached >90%. Cells were split in selection medium when cell density exceeded  $2 \times 10^6$ /ml until

sealed to 4×250 ml in 500 ml flasks. Supernatant was harvested when cell viability dropped below 80% with a maximum final cell density  $\sim 7 \times 10^6$ /ml. Endotoxin levels were less than 0.2 units/ml.

- Expression and purification of recombinant Flu M1 and MART-1 proteins. PCR was used to amplify the ORF of Influenza A/Puerto Rico/8/34/Mount Sinai (H1N1) M1 gene while incorporating an Nhe I site distal to the initiator codon and a Not I site distal to the stop codon. The digested fragment was cloned into pET-28b(+) (Novagen), placing the M1 ORF in-frame with a His6 tag, thus encoding His.Flu M1 protein. A pET28b (+) derivative encoding an N-terminal 169 residue cohesin domain from *C. thermocellum* (unpublished) inserted between the Nco I and Nhe I sites expressed Coh.His. For expression of Cohesin-Flex-hMART-1-PeptideA-His, the sequence
- 10 GACACCACCGAGGCCCGCCACCCCCACCCCCCGTGACCACCCCCACCACCACCGACCGGAAG  
GGCACCACCGCCGAGGAGCTGGCCGGCATCGGCATCCTGACCGTGATCCTGGGCGGCAAGCGG  
ACCAACAACAGCACCCCCACCAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGGCCG (SEQ ID NO.:28) (encoding DTTEARHPHPVTTPTTDRKGT***TAEE******LAGIGILT******VL***GGKRTNNSTPTKGFCRYP SHWRP (SEQ ID NO.:29) - the italicized residues are the immunodominant HLA-A2-restricted peptide and the underlined residues surrounding the peptide are from MART-1) was inserted between the Nhe I and Xho I sites of the above vector. The proteins were expressed in *E. coli* strain BL21 (DE3) (Novagen) or T7 Express (NEB), grown in LB at 37°C with selection for kanamycin resistance (40 µg/ml) and shaking at 200 rounds/min to mid log phase growth when 120 mg/L IPTG was added. After three hours, the cells were harvested by
- 20 centrifugation and stored at -80°C. *E. coli* cells from each 1 L fermentation were resuspended in 30 ml ice-cold 50 mM Tris, 1 mM EDTA pH 8.0 (buffer B) with 0.1 ml of protease inhibitor Cocktail II (Calbiochem, CA). The cells were sonicated on ice 2x 5 min at setting 18 (Fisher Sonic Dismembrator 60) with a 5 min rest period and then spun at 17,000 r.p.m. (Sorvall SA-600) for 20 min at 4°C. For His.Flu M1 purification the 50 ml cell lysate supernatant fraction was passed through 5 ml Q Sepharose beads and 6.25 ml 160 mM Tris, 40
- 25 mM imidazole, 4 M NaCl pH 7.9 was added to the Q Sepharose flow through. This was loaded at 4 ml/min onto a 5 ml HiTrap chelating HP column charged with Ni<sup>++</sup>. The column-bound protein was washed with 20 mM NaPO<sub>4</sub>, 300 mM NaCl pH 7.6 (buffer D) followed by another wash with 100 mM H<sub>3</sub>COONa pH 4.0. Bound protein was eluted with 100 mM H<sub>3</sub>COONa pH 4.0. The peak fractions were pooled and loaded at 4 ml/min onto a 5 ml HiTrap S column equilibrated with 100 mM H<sub>3</sub>COONa pH 5.5, and washed with the
- 30 equilibration buffer followed by elution with a gradient from 0 - 1 M NaCl in 50 mM NaPO<sub>4</sub> pH 5.5. Peak fractions eluting at about 500 mM NaCl were pooled. For Coh.Flu M1.His purification, cells from 2 L of culture were lysed as above. After centrifugation, 2.5 ml of Triton X114 was added to the supernatant with incubation on ice for 5 min. After further incubation at 25°C for 5 min, the supernatant was separated from



the Triton X114 following centrifugation at 25°C. The extraction was repeated and the supernatant was passed through 5 ml of Q Sepharose beads and 6.25 ml 160 mM Tris, 40 mM imidazole, 4 M NaCl pH 7.9 was added to the Q Sepharose<sup>TM</sup> flow through. The protein was then purified by Ni<sup>++</sup> chelating chromatography as described above and eluted with 0-500 mM imidazole in buffer D.

- 5 Fig. 13 shows the internalization of anti-CD40 mAb:IL-4DC. IL-4DCs were treated with 500 ng/ml of anti-CD40-Alexa 568. Fig. 14 shows CD4 and CD8 T cell proliferation by DCs targeted with anti-CD40-HA1. 5x10<sup>3</sup> IFNDCs loaded with 2 ug/ml of anti-CD40-HA or control Ig-HA1 were co-cultured with CFSE-labeled autologous CD4+ or CD8+ T cells (2x10<sup>5</sup>) for 7 days. Cells were then stained with anti-CD4 or anti-CD8 antibodies. Cell proliferation was tested by measuring CFSE-dilution. Fig. 15 shows a titration of
  - 10 HA1 fusion protein on CD4+ T proliferation. IFNDCs (5K) loaded with fusion proteins were co-cultured with CFSE-labeled CD4+ T cells (200K) for 7 days. Fig. 16 shows IFNDCs targeted with anti-CD40-HA1 activate HA1-specific CD4+ T cells. CD4+ T cells were restimulated with DCs loaded with 5 uM of indicated peptides, and then intracellular IFN $\gamma$  was stained. Fig. 17 shows IFNDCs targeted with anti-CD40-HA1 activate HA1-specific CD4+ T cells. CD4+ T cells were restimulated with DCs loaded with indicated
  - 15 peptides for 36h, and then culture supernatant was analyzed for measuring IFN $\gamma$ . Fig. 18 shows that targeting CD40 results in enhanced cross-priming of MART-1 specific CD8+ T cells. IFNDCs (5K/well) loaded with fusion proteins were co-cultured with purified CD8+ T cells for 10 days. Cells were stained with anti-CD8 and tetramer. Cells are from healthy donors (HLA-A\*0201+). Fig. 19 shows targeting CD40 results in enhanced cross-priming of MART-1 specific CD8+ T cells (Summary of 8-repeated experiments
  - 20 using cells from different healthy donors). Fig. 20 shows CD8+ CTL induced with IFNDCs targeted with anti-CD40-MART-1 are functional. CD8+ T cells co-cultured with IFNDCs targeted with fusion proteins were mixed with T2 cells loaded with 10 uM peptide epitope. Fig. 21 shows CD8+ CTL induced with IFNDCs targeted with anti-CD40-Flu M1 are functional. CD8+ T cells co-cultured with IFNDCs targeted
  - 25 with fusion proteins were mixed with T2 cells loaded with 1.0 nM peptide epitope. Fig. 22 shows an outline of protocol to test the ability a vaccine composed of anti-CD4012E12 linked to PSA (prostate specific antigen) to elicit the expansion from a naïve T cell population. PSA-specific CD4+ T cells corresponding to a broad array of PSA epitopes. Briefly, DCs derived by culture with IFN $\alpha$  and GM-CSF of monocytes from a healthy donor are incubated with the vaccine. The next day, cells are placed in fresh medium and pure CD4+ T cells from the same donor are added. Several days later, PSA peptides are added and, after four hours,
  - 30 secreted gamma-IFN levels in the culture supernatants are determined.
- Fig. 23 shows that many PSA peptides elicit potent gamma-IFN-production responses indicating that anti-CD4012E12 and similar anti-CD40 agents can efficiently deliver antigen to DCs, resulting in the priming of immune responses against multiple epitopes of the antigen. The peptide mapping of PSA antigens. 5x10<sup>3</sup>

IFNDCs loaded with 2 ug/ml of anti-CD40-PSA were co-cultured with purified autologous CD4+ T cells ( $2 \times 10^5$ ) for 8 days. Cells were then restimulated with 5 uM of individual peptides derived from PSA for 36h. The amount of IFN $\gamma$  was measured by Luminex. Cells are from healthy donors.

Fig. 24 shows DCs targeted with anti-CD40-PSA induce PSA-specific CD8+ T cell responses. IFNDCs were targeted with 1 ug mAb fusion protein with PSA. Purified autologous CD8+ T cells were co-cultured for 10 days. Cells were stained with anti-CD8 and PSA (KLQCVDLHV)-tetramer. Cells are from a HLA-A\*0201 positive healthy donor. The results demonstrate that anti-CD40 effectively deliver PSA to the DCs, which in turn elicit the expansion of PSA-specific CD8+ T cells. Briefly,  $5 \times 10^3$  IFNDCs loaded with 2 ug/ml of anti-CD40-PSA were co-cultured with purified autologous CD8+ T cells ( $2 \times 10^5$ ) for 10 days. Cells were then stained with tetramer. Cells are from HLA-0\*201 positive healthy donor.

Fig. 25 a scheme (left) and the IFN $\gamma$  production by T cells of the pools of peptides and control for Donor 2.  $5 \times 10^3$  IFNDCs loaded with 2 ug/ml of anti-CD40-Cyclin D1 were co-cultured with purified autologous CD4+ T cells ( $2 \times 10^5$ ) for 8 days. Cells were then restimulated with 5 uM of individual peptides derived from CyclinD1 for 5h in the presence of Brefeldin A. Cells were stained for measuring intracellular IFN $\gamma$  expression.

Fig. 26 shows a peptide scan and IFN $\gamma$  production by T cells obtained from the pools of peptides shown in Fig. 25 and control for Donor 2.  $5 \times 10^3$  IFNDCs loaded with 2 ug/ml of anti-CD40-Cyclin D1 were co-cultured with purified autologous CD4+ T cells ( $2 \times 10^5$ ) for 8 days. Cells were then restimulated with 5 uM of individual peptides derived from CyclinD1 for 5h in the presence of Brefeldin A. Cells were stained for measuring intracellular IFN $\gamma$  expression.

In conclusion, delivering antigens to DCs, the most potent antigen presenting cells, via CD40 is an efficient way to induce and activate antigen specific both CD4+ and CD8+ T cell-mediated immunity. Thus, vaccines made of anti-CD40 mAb will induce potent immunity against cancer and infections.

Peptide information:

HA1 sequences:

MKANLLVLLCALAAADADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLED SHNGKLCR (SEQ ID NO.:30)

LKGIAPLQLGKCNIAGWLLGNPECDPLLPVRSWSYIVETPNSENGICYPGDFIDYEELRE (SEQ ID NO.:31)

QLSSVSSFERFEIFPKESSWPNHNTNGVTAACSHEGKSSFYRNLLWLTEKEGSYPKLKNS (SEQ ID NO.:32)

YVNKKGKEVLVLWGIHPPNSKEQQNLYQENAYVSVVTSNYNRRFTPEIAERP KVRDQA (SEQ ID NO.:33)

GRMNYWTLTKPGDTIIFEANGNLIAPMYAFALSRGFGSGIITSNASMHECNTKCQTPLG (SEQ ID NO.:34)

AINSSLPYQNIHPVTIGECPKYVRSKLRMVTGLRNIPSI (SEQ ID NO.:35)

Sequences of peptides in Fig. 17

5 Peptide 22: SSFERFEIFPKESSWPN (SEQ ID NO.:36)

Peptide 45: GNLIAPWYAFALSRGFG (SEQ ID NO.:37)

Peptide 46: WYAFALSRGFGSGIITS (SEQ ID NO.:38)

NP sequences:

10 MASQGTKRSYEQMETDGERQNATEIRASVGKMIGGIGRFYIQMCTELKLSDYEGRLIQNS (SEQ ID NO.:39)

LTIERMVLSAFDERRNKYLEEHPSAGKDPKKTGGPIYRRVNGKWMRELILYDKEEIRRIW (SEQ ID NO.:30)

RQANNGDDATAGLTHMMIWHSNLNDATYQRTRALVRTGMDPRMCSLMQGSTLPRRSGAAG (SEQ ID NO.:41)

15 AAVKGVGTMMELVRMIKRGINDRNFWRGENGRKTRIAYERMCNILKGKFQTAAQKAMMD (SEQ ID NO.:42)

QVRESRNPNGNAEFEDLTFLARSALILRGSAVHKSLPACVYGPVAVASGYDFEREGYSLVG (SEQ ID NO.:43)

20 IDPFRLQLNSQVYSLIRPNENPAHKSQLVWMACHSAAFEDLRVLSFIKGTKVLPRGKLST (SEQ ID NO.:44)

RGVQIASNENMETMESSTLELRSRYWAIRTRSGGNTNQRASAGQISIQPTFSVQRNLFF (SEQ ID NO.:45)

DRTTIMAAFNNGTEGRTSDMRTEIIRMMESARPEDVSFQGRGVFELSDEKAASPIVPSFD (SEQ ID NO.:46)

25 MSNEGSYFFGDNAEEYDN (SEQ ID NO.:48)

Sequences of peptides in Fig. 23

Peptide 22: GKWVRELVLVDKEEIRR (SEQ ID NO.:49)

Peptide 33: RTGMDPRMCSLMQGSTL (SEQ ID NO.:50)

Peptide 46: MCNILKGKFQTAAQKAM (SEQ ID NO.:51)

30 Prostate specific antigen (PSA) sequence

MWVPVVFLLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVHPQWV (SEQ ID NO.:52)

- LTAAH CIRNKS VILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPGDDSSHD (SEQ ID NO.:53)
- LMLLR LSEPAELTDAVKVMDLPTQEPALGTTCTYASGWGSIEPEEFLTPKKLQCVDLHVIS (SEQ ID NO.:54)
- 5 NDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITSWGSEPCALPERP (SEQ ID NO.:55)
- SLYTKVVHYRKWIKDTIVANP (SEQ ID NO.:56)
- Sequences of peptides in Fig. 23
- Peptide 1: APLILSRIVGGWECE (SEQ ID NO.:57)
- 10 Peptide 4: ECEKHSQPWQVLVAS (SEQ ID NO.:58)
- Peptide 25: GDDSSHD LMLLR LSE (SEQ ID NO.:59)
- Peptide 26: SHDLMLLR LSEPAEL (SEQ ID NO.:60)
- Peptide 49: SGDSGGPLVCNGVLQ (SEQ ID NO.:61)
- Peptide 54: GSEPCALPERPSLYT (SEQ ID NO.:62)
- 15 Peptide 56: ERPSLYTKVVHYRKW (SEQ ID NO.:63)
- Peptide 58: VVHYRKWIKDTIVAN (SEQ ID NO.:64)
- Cyclin D1 sequence
- MRSYRFSDYLHMSVSFSNDMDLFCGEDSGVFSGESTVDFSSSEVD SWPGDSIACFIEDER (SEQ ID NO.:65)
- 20 HFVPGHDYLSRFQTRSLDASAREDSVAWILKVQAYYNFQPLTAYLAVNYMDRFLYARRLP (SEQ ID NO.:66)
- ETSGWPMQLLAVACLSLAAKMEEILVPSLDFQVAGVKYLFEAKTIKRMELLVLSVLDWR (SEQ ID NO.:67)
- LRSVTPFD FISFFAYKIDPSGTF LGFFISHATEIILSNIKEASFLEYWPSSIAAAAILCV (SEQ ID NO.:68)
- 25 ANELPSLSSVVP HESPETWCDGLSKEKIVRCYRLMKAMAIENNRLNTPKVIAKLRVSVR (SEQ ID NO.:69)
- ASSTLTRPSDESSFS SSPCKRRKLSGYSWVGDETSTN (SEQ ID NO.:70)
- Sequences of peptides in Fig. 26.
- Peptide 7: DRVLRAMLKAEETCA (SEQ ID NO.:71)
- 30 Peptide 8: RAMLKAEETCAPSVS (SEQ ID NO.:72)
- Peptide 10: TCAPSVSYFKCVQKE (SEQ ID NO.:73)

MART-1 Antigen. MART-1 is a tumor-associated melanocytic differentiation antigen. Vaccination with MART-1 antigen may stimulate a host cytotoxic T-cell response against tumor cells expressing the melanocytic differentiation antigen, resulting in tumor cell lysis.

Fig. 27 shows the expression and construct design for anti-CD40-MART-1 peptide antibodies. Fig. 28 is a summary of the CD4<sup>+</sup> and CD8<sup>+</sup> immunodominant epitopes for MART-1. Figs. 27 and 28 show the use of the flexible linker technology to permit the successful expression of recombinant anti-DC receptor targeting antibodies fused to significant (~2/3) parts of human MART-1. Recombinant antibody fused at the Heavy chain C-terminus to the entire MART-1 coding region is not at all secreted from production mammalian cells [not shown]. The Flex-v1-hMART-1-Pep-3-f4-Pep-1 adduct is particularly well expressed and is one preferred embodiment of a MART-1-targeting vaccine, as is the Flex-v1-hMART-1-Pep-3-f4-Pep-1-f3-Pep-2 adduct which bears a maximum load of MART-1 epitopes. Slide 2 of the MART-1 powerpoint presentation shows that these adducts can be successfully appended to multiple anti-DC receptor vehicles.

The sequence below is a Heavy chain – hMART-1 peptides string of pep3-pep1-pep2 fusion protein where each hMART1 peptide sequence [bold-italics] is separated by a inter-peptide spacer f [shown in bold]. In this case, a 27-amino-acid long linker flex-v1(v1) [italics] derived from cellulosomal anchoring scaffoldin B precursor [Bacteroides cellulosolvens- described in the gag-nef vaccine invention disclosure] was inserted between the Heavy chain C-terminus and the hMART1 peptides-flexible spaces string. The underlined AS residues are joining sequences.

[manti-CD40\_12E12.3F3\_H-LV-hIgG4H-C-Flex-v1-hMART-1-Pep-3-f4-Pep-1] C981 is:

EVKLVESGGGLVQPGGSLKLSCATSGFTFSDYYMYWVRQTPEKRLEWVAYINSGGGSTYYPDTVK  
GRFTISRDNKNTLYLQMSRLKSEDTAMYYCARRGLPFHAMDYWGQGTSTTVSSAKTKGPSVFPL  
APCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGK  
TYTCNVDPKPSNTKVDKRVESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS  
QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSI  
EKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLD  
SDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLKASKQTPTNTISVTPTNNSTPT  
NNSNPKPNPASGFDHRDSKVSLQEKNCEPVVPNAPPAYEKLSAEQSPPPYSPASTNGSITVAATAPT  
VTPTVNATPSAAASMPREDAHFIYGYPKKGHGHSYTTAEAAAGIGILTVILGAS (SEQ ID NO.:74)

[manti-CD40\_12E12.3F3\_H-LV-hIgG4H-C-Flex-v1-hMART-1-Pep-3-f4-Pep-1-f3-Pep-2] C978 is:

EVKLVESGGGLVQPGGSLKLSCATSGFTFSDYYMYWVRQTPEKRLEWVAYINSGGGSTYYPDTVK  
GRFTISRDNKNTLYLQMSRLKSEDTAMYYCARRGLPFHAMDYWGQGTSTTVSSAKTKGPSVFPL  
APCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGK  
TYTCNVDPKPSNTKVDKRVESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS

QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSI  
 EKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLD  
 SDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLGLKASQTPTNTISVTPTNNSTPT  
 NNSNPKPNPASGFDHRDSKVSLQEKNCEPVVPNAPPAYEKLSAEQSPPPYSPASTNGSITVAATAPT  
 5 VTPTVNATPSAAASMPREDAHFIYGYPKKGHGHSYTTAEAAAGIGILTVILGASTVTPTATATPSAI  
 VTTITPTATTKPASVLLIGCWYCRRRNGYRALMDKSLHVGTCALTRRCPEGAS (SEQ ID  
 NO.:75)

[mAnti-DCIR\_9E8\_H-LV-hlgG4H-C-Flex-v1-hMART-1-Pep-3-f4-Pep-1] C1012 is:

QVTLKESGPGILQPSQTLSTCSFSGFSLSTSGMGLSWIRQPSGKGLEWLAHIYWDDDKRYNPSLKSR  
 10 LTISKDTSSNQVFLKITIVDTADAATYYCARSSHYGYGYGGYFDVWGAGTTVTVSSAKTKGPSVF  
 PLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLG  
 TKTYTCNVDPKPSNTKVDKRVESKYGPPCPPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVV  
 DVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGL  
 PSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP  
 15 VLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLGLKASQTPTNTISVTPTNNS  
 TPTNNSNPKPNPASGFDHRDSKVSLQEKNCEPVVPNAPPAYEKLSAEQSPPPYSPASTNGSITVAATA  
 PTVTPTVNATPSAAASMPREDAHFIYGYPKKGHGHSYTTAEAAAGIGILTVILGAS (SEQ ID NO.:76)

[mAnti-DCIR\_9E8\_H-LV-hlgG4H-C-Flex-v1-hMART-1-Pep-3-f4-Pep-1-f3-Pep-2] C1013 is:

QVTLKESGPGILQPSQTLSTCSFSGFSLSTSGMGLSWIRQPSGKGLEWLAHIYWDDDKRYNPSLKSR  
 20 LTISKDTSSNQVFLKITIVDTADAATYYCARSSHYGYGYGGYFDVWGAGTTVTVSSAKTKGPSVF  
 PLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLG  
 TKTYTCNVDPKPSNTKVDKRVESKYGPPCPPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVV  
 DVSQPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPS  
 SIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL  
 25 DSDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLGLKASQTPTNTISVTPTNNSTP  
 TNNNSNPKPNPASGFDHRDSKVSLQEKNCEPVVPNAPPAYEKLSAEQSPPPYSPASTNGSITVAATAP  
 TVTPTVNATPSAAASMPREDAHFIYGYPKKGHGHSYTTAEAAAGIGILTVILGASTVTPTATATPSA  
 IVTTITPTATTKPASVLLIGCWYCRRRNGYRALMDKSLHVGTCALTRRCPEGAS (SEQ ID  
 NO.:77)

30 MART-1 DNA Sequence:

MART-1 constructs with 3 peptides, Start/stop sites are underlined, peptide 1 is bold, peptide 2 is bold-italics  
 and peptide 3 is bold-underlined:

- AACACCGACAACAACAGATGATCTGGATGCAGCTAGTGGGTTTGATCATCGGGACAGCAAAG  
TGTCTCTTCAAGAGAAAACTGTGAACCTGTGGTTCCCAATGCTCCACCTGCTTATGAGAA  
ACTCTCTGCAGAACAGTCACCACCACCTTATTCACCTGCTAGTACCAACGGCAGCATCACCG  
TGGCCGCCACCGCCCCACCGTGACCCCCACCGTGAACGCCACCCCCAGCGCCGCCGCTAGTAT  
5 GCCAAGAGAAGATGCTCACTTCATCTATGGTTACCCCAAGAAGGGGCACGGCCACTCTTACACCA  
CGGCTGAAGAGGGCCGCTGGGATCGGCATCTGACAGTGATCCTGGGAGCTAGTACCGTGACCCC  
CACCGCCACCGCCACCCCCAGCGCCATCGTGACCACCATCACCCCCACCGCCACCACCAAGCCC  
GCTAGTGTCTTACTGCTCATCGGCTGTTGGTATTGTAGAAGACGAAATGGATACAGAGCCT  
TGATGGATAAAAGTCTTCATGTTGGCACTCAATGTGCCTTAACAAGAAGATGCCACAAG  
10 AAGGGTgaGCGGCCGCATCGAAGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCA  
TGC (SEQ ID NO.:78)  
MART1-Peptide 3, the italicized portion is the CD4+ immunodominant epitope.  
GFDHRDSKVSLQEKNCPEVVPNAPPAYEKLAEQSPPPYSP (SEQ ID NO.:79)  
Flex-4  
15 ASTNGSITVAATAPTVTPTVNATPSAAAS (SEQ ID NO.:80)  
MART1-Peptide 1 the italicized portion is the CD4+ immunodominant epitope and the underlined-italicized  
portion is the CD8+ immunodominant epitope  
MPREDAHFIYGYPKKGHGHSYTTAEAAAGIGILTVILG (SEQ ID NO.:81)  
Flex-3: ASTVTPTATATPSAIVTTITPTATTKPAS (SEQ ID NO.:82)  
20 MART1 - Peptide 2 the italicized portion is the CD4+ immunodominant epitope.  
VLLIGCWYCRRRNGYRALMDKSLHVGTCALTRRC PQEG (SEQ ID NO.:83)  
MART1 constructs with two peptides:  
Peptide 3 is bold-italics-underlined, flex-4 is bold and Peptide 1 is bold-italics-underlined:  
**GFDHRDSKVSLQEKNCPEVVPNAPPAYEKLAEQSPPPYSP**ASTNGSITVAATAPTVTPTVNATPSA  
25 **AASMPREDAHFIYGYPKKGHGHSYTTAEAAAGIGILTVILG**AS (SEQ ID NO.:84)  
Protein Sequence: C978. rAB-cetHS-puro[manti-CD40\_12E12.3F3\_H-LV-hIgG4H-C-Flex-v1-hMART-1-  
Pep-3 (bold-italics-underlined)-f4 (bold)-Pep-1 (bold-italics)-f3 (italics)-Pep-2 (bold-underlined)]  
MNLGLSLIFLVVLKGVQCEVKLVESGGGLVQPGSLKLSCATSGFTFSDYYMYWVRQTPEKRLE  
WVAYINSGGGSTYYPDTVKGRFTISRDNANTLYLQMSRLKSEDTAMYYCARRGLPFHAMDYWG  
30 QGTSVTVSSAKTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ  
SSGLYSLSSVVTVPSSSLGTQKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFEGGPSVFLFPPK  
PKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQ  
DWLNGKEYKCKVSNKGLPSSIEKTIKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA

VEWESNGQPENNYKTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLS  
 LGKASQTPTNTISVTPTNNSPTNNSNPKNPAS**GFDHRDSKVSLOEKNC****EPVVPNAPPAYEKL****SAEQ**  
**SPPPYSPASTNGSITVAATAPTVTPTVNATPSAAASMPREDAHFIYGYPKKGHGHSYTTAEAA****GI**  
**GILTVILGASTVTPTATATPSAIVTTITPTATTKPASVLLIGCWYCRRRNGYRALMDKSLHVGTOCA**

5 **LTRRCPOEGAS** (SEQ ID NO.:85)

Protein Sequence: C981. rAB-cetHS-puro[manti-CD40\_12E12.3F3\_H-LV-hlgG4H-C-Flex-v1-hMART-1-  
 Pep-3 (bold-italics-underlined)-f4-(bold)-Pep-1](bold-underlined)

MNLGLSLIFLVLVLKGVQCEVKLVESGGGLVQPGGSLKLSCATSGFTFSDYYMYWVRQTPEKRLE  
 WVAYINSGGGSTYYPDVTVKGRFTISRDNAKNTLYLQMSRLKSEDTAMYYCARRGLPFHAMDYWG  
 10 QGTSVTVSSAKTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ  
 SSGLYSLSSVVTVPSSSLGKTYTCNVDHKPSNTKVDKRVESKYGPPCPPAPEFEGGPSVFLFPPK  
 PKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQ  
 DWLNGKEYCKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA  
 VEWESNGQPENNYKTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLS

15 LGKASQTPTNTISVTPTNNSPTNNSNPKNPAS**GFDHRDSKVSLOEKNC****EPVVPNAPPAYEKL****SAEQ**  
**SPPPYSPASTNGSITVAATAPTVTPTVNATPSAAASMPREDAHFIYGYPKKGHGHSYTTAEAA**  
**GIGILTVILGAS** (SEQ ID NO.:86)

GP100 Antigen. GP100 antigen is a melanoma-associated antigen. When administered in a vaccine  
 formulation, gp100 antigen may stimulate a cytotoxic T cell HLA-A2.1-restricted immune response against  
 20 tumors that express this antigen, which may result in a reduction in tumor size.

GP100 ectodomain coding region fused to recombinant antibody Heavy chain coding region is not at all  
 secreted by production mammalian cells [not shown]. The total sequence is shown below – italics residues  
 are the leader sequence and the transmembrane domain, the peptides are in bold-italics and the  
 transmembrane domain is italics-underlined.

25 **MDLVLRCLLHLAVIGALLAVGATK****VPRNQDWLGVSRLRTKAWNRQLYPEWTEAQR****LD****CWRGGQ**  
**VSLKVSNDGPTLIGANASFSIALNFP****GSQKVL****PDGQVIWVNNTIINGSQVWGGQPVYPQETDDACIFP**  
**DGGPCPSGSWSQKRSFVYVW****KTWGQYWQV****LG****GPVSGLSIGTGRAMLGTH****TMEVT****VYHRRGSR****SY**  
**VPLAHSSSAFTITDQVPFSVSV****SQLRALDGGNKHFLRNQPLTFALQLHDP****SGYLAEADLSYT****WDFG**  
**SSGTLISRALVVTHTYLEPGPVTAQVVLQAAIPLTSCGSSPVPGTTD****GHRPTAEAPNTTAGQVPTTEV**  
 30 **VGTT****PGQA****PTAEP****SGTTSVQVPTTEVISTAPVQMPTAESTGMTPEKVPVSEVMGTTLAEMSTPEATG**  
**MTPAEVSIVVLSOTTAQVTTTEWVETTARELPIEPEGPDASSIMSTESITGSLGPLLDGTATLRLVK**  
**RQVPLDCVLYRYGSFSVTLDIVQIESAEILQAVPSGEGDAFELTVSCQGGLPKEACMEISSPGCQPP**



AQRLCQPVLPSPACQLVLHQILKGGSGTYCLNVSLADTNSLAVVSTQLIMPGQEAGLGQVPLIVGILL  
VLMAVVLASLIYRRRLMKQDFSVPQLPHSSSHWLRLPRIFCSCPIGENSPLLSGQQV (SEQ ID NO.:87)

- Known HLA-A0201 restricted peptides sequences are: GP100 M: 209-217 (2M): IMDQVPFSV (SEQ ID NO.:88); 209-217 WT: ITDQVPFSV (SEQ ID NO.:89) GP100 M: 280-288 (9V): YLEPGPVTV (SEQ ID NO.:90) 280-288 WT: YLEPGPVTA (SEQ ID NO.:91) GP100 WT: 154-162: KTWGQYWQV (SEQ ID NO.:92)

Fig. 29-33 show the gp100 adducts which were successfully expressed as secreted anti-DC receptor targeting vaccines. These employed the use of the flexible linker sequences and fragmentation and shuffling of the gp100 ectodomain coding region. Preferred embodiments of gp100 vaccine adducts are described.

- Fig. 29 shows the expression and construct design for anti-CD40-gp100 peptide antibodies. Fig. 30 shows the design for additional anti-CD40-gp100 peptide antibodies. Fig. 31 shows the expression and construct design for additional anti-CD40-gp100 peptide antibodies. Fig. 32 is a summary of the CD4<sup>+</sup> and CD8<sup>+</sup> immunodominant epitopes for gp100. Fig. 33 shows the expression and construct design for additional anti-CD40-gp100 peptide antibodies.

- rAB-cetHS-puro[manti-CD40\_12E12.3F3\_H-LV-hIgG4H-C-Flex-hgp100-Pep-1-f4-Pep-3-f3-Pep-4-f4-Pep-5-f3-Pep-2] C1285, the peptides are bold-italics, flexible linkers are bold and the underlined AS residues are joining sequences:

EVKLVESGGGLVQPGGSLKLSCATSGFTFSYYMYWVRQTPEKRLEWVAYINSGGGSTYYPDTVK  
 GRFTISRDNKNTLYLQMSRLKSEDTAMYYCARRGLPFHAMDYWGQGTSTVTVSSAKTKGPSVFPL  
 20 APCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGKTK  
 TYTCNVDHKPSNTKVDKRVESKYGPCCPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVDVDS  
 QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSI  
 EKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLD  
 SDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLQKASDTTEPATPTTPVTPTT  
 25 TKVPRNQDWLGVSRLRTKAWNRQLYPEWTEAQRLD CWRGGQVSLKVSNDGPTLIGANASFSIAL  
NFPGSQKVLDPGQVIWVNNTHINGSQVWGGQPVYPQETDDACIFPDGGPCPSGWSQKRSFYVYWK  
TWGQYWQVLGGPVSGLSIGTGRAMLGTHTMEVTYHRRGSQSYVPLAHSSSAFTITDQVPFSVSVS  
QLRALDGGNKHFLRNQASTNGSITVAATAPTVTPVTPVATPSAAASGTTDGHRPTTEAPNTTAGQV  
 PTTEVVGTTTGGQAPTAEPSTGTSVQVPTTEVISTAPVQMPTAESTGMTPEKVPVSEVMGTTLAEMST  
 30 PEATGMTPAEVSIVVLSGTTAAASTVTPTATATPSAIVTTITPTATTKPASQVTTTEWVETTARELPI  
PEPEGPDASSIMSTESITGSLGPLLDGTATLRLVKRQVPLDCVLYRYGSFSVTLDIVQASTNGSITVA  
ATAPTYTPTVNATPSAAASGIESAEILQAVPSGEGDAFELTVSCQGGLPKEACMEISSPGCQPPAQR  
LCQPVLPSPACQLVLHQILKGGSGTYCLNVSLADTNSLAVVSTQLIVPGILLTGQEAGLGQASTVTPT

ATATPSAIVTTITPTATTKPASPLTFALQLHDPSGYLAEADLSYTWDFGDSSGTLISRALVVTHTYLE  
 PGPVTAQVVLQAAIPLTSCGSSPVPAS (SEQ ID NO.:93)

rAB-cetHS-puro[hlgG4H-C-Flex-hgp100-Pep-1-f4-Pep-3-f3-Pep-4-f4-Pep-5-f3-Pep-2] C1286:

- 5 RVTMSADMSSENSFYKLDSVTAADTAVYYCAAGHLVMGFQAHWGQKLVSVSPASTKGPSVFPL  
 APCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTK  
 TYTCNVDPKPSNTKVDKRVESKYGPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS  
 QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSI  
 EKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLD  
 10 SDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLGLKASDTEPATPTPTPTPTPT  
 TKVPRNQDWLGVSRLRTKAWNRQLYPEWTEAQRLDCWRGGQVSLKVSNDGPTLIGANASFSIAL  
 NFPGSQKVLDPGQVIWVNNIINGSQVWGGQPVYPQETDDACIFPDGGPCPSGSWSQKRSFVYVWK  
 TWGQYWQVLGGPVSGLSIGTGRAMLGTHMEVTVYHRRGSQSYVPLAHSSSAFTITDQVPFSVSVS  
 QLRALDGGNKHFLRNQASTNGSITVAATAPTPTPTVNATPSAAASGTTDGHRTTEAPNTTAGQV  
 15 PTTEVVGTTPGQAPTAEPSTTSVQVPTTEVISTAPVQMPTAESTGMTPEKVPVSEVMGTTLAEMST  
 PEATGMTPAEVSIVVLSGTTAAASTVTPTATATPSAIVTTITPTATTKPASQVTTTEWVETTARELP  
 PEPEGPDASSIMSTESITGSLPLLDGTATLRLVKRQVPLDCVLYRYGSFSVTLDIVQASTNGSITVA  
 ATAPTPTPTVNATPSAAASGIESAEHLQAVPSGEGDAFELTVSCQGGLPKEACMEISSPGCQPPAQR  
 LCQPVLPSPACQLVLHQILKGGSGTYCLNVSLADTNSLAVVSTQLIVPGILLTGQEAGLGQASTVTPT  
 20 ATATPSAIVTTITPTATTKPASPLTFALQLHDPSGYLAEADLSYTWDFGDSSGTLISRALVVTHTYLE  
 PGPVTAQVVLQAAIPLTSCGSSPVPAS (SEQ ID NO.:94)

gp100: - Nucleic Acid Sequence. Peptide 1-underlined, Peptide 2-italics, Peptide 3-bold, Peptide 4-bold-  
 underlined, Peptide 5 bold-italics.

- 25 GATACAACAGAACCTGCAACACCTACAACACCTGTAACAACACCGACAACAACAAAAGTACCC  
AGAAACCAGGACTGGCTTGGTGTCTCAAGGCAACTCAGAACCAAAGCCTGGAACAGGCAGCTG  
TATCCAGAGTGACAGAAAGCCAGAGACTTGACTGCTGGAGAGGTGGTCAAGTGCTCCCTCAAG  
GTCAGTAATGATGGGCCTACACTGATTGGTGCAAATGCCTCCTTCTCTATTGCCCTGAACTTCCC  
TGGAAGCCAAAAGGTATTGCCAGATGGGCAGGTTATCTGGGTCAACAATACCATCATCAATGG  
GAGCCAGGTGTGGGAGGACAGCCAGTGTATCCCCAGGAAACTGACGATGCCTGCATCTTCCCT  
 30 GATGGTGGACCTTGCCCATCTGGCTCTTGGTCTCAGAAGAGAAAGCTTTGTTTATGTCTGGAAGA  
CCTGGGGCCAATACTGGCAAGTTCTAAGGGGCCAGTGTCTGGGCTGAGCATTGGGACAGGCA  
GGGCAATGCTGGGCACACACACCATGGAAGTGACTGTCTACCATCGCCGGGGATCCCAGAGCT  
ATGTGCCTCTTGCTCATTCCAGCTCAGCCTTACCATTACTGACCAGGTGCCTTTCTCCGTGAGC

GTGTCCCAGTTGCGGGCCTTGGATGGAGGGAACAAGCACTTCCTGAGAAATCAGGCTAGTACC  
 AACGGCAGCATCACCGTGCCGCCACCGCCCCACCGTGACCCCCACCGTGAACGCCACCCCCA  
 GCGCCGCCGCTAGTGGCACCACAGATGGGCACAGGCCAACTGCAGAGGCCCTAACACCACAGCTG  
 GCCAAGTGCCCTACTACAGAAGTTGTGGGTACTACACCTGGTCAGGCGCCAACTGCAGAGCCCTCTGG  
 5 AACCACATCTGTGCAGGTGCCAACCACTGAAGTCATAAGCACTGCACCTGTGCAGATGCCAACTGCAG  
 AGAGCACAGGTATGACACCTGAGAAGGTGCCAGTTTCAGAGGTATGGGTACCACACTGGCAGAGAT  
 GTCAACTCCAGAGGCTACAGGTATGACACCTGCAGAGGTATCAATTGTGGTGCTTTCTGGAACCACAG  
 CTGCAGCTAGTACCGTGACCCCCACCGCCACCGCCACCCCCAGCGCCATCGTGACCACCATCAC  
 CCCCCCGCCACCACCAAGCCCCGCTAGTCAGGTAACAACCTACAGAGTGGGTGGAGACCACA  
 10 GCTAGAGAGCTACCTATCCCTGAGCCTGAAGGTCCAGATGCCAGCTCAATCATGTCTACG  
 GAAAGTATTACAGGTTCCCTGGGCCCCCTGCTGGATGGTACAGCCACCTTAAGGCTGGTG  
 AAGAGACAAGTCCCCCTGGATTGTGTTCTGTATCGATATGGTTCCTTTTCCGTACCCCTGG  
 ACATTGTCCAGGCTAGTACCAACGGCAGCATCACCGTGGCCGCCACCGCCCCACCGTGACCC  
 CCACCGTGAACGCCACCCCCAGCGCCGCCGCTAGTGGTATTGAAAGTGCCGAGATCCTGCAG  
 15 GCTGTGCCGTCCGGTGAGGGGGATGCATTTGAGCTGACTGTGTCCTGCCAAGGCGGGCT  
 GCCCAAGGAAGCCTGCATGGAGATCTCATCGCCAGGGTGCCAGCCCCCTGCCAGCGGGCT  
 GTGCCAGCCTGTGCTACCCAGCCCAGCCTGCCAGCTGGTTCTGCACCAGATACTGAAGGG  
 TGGCTCGGGGACATACTGCCTCAATGTGTCTCTGGCTGATACCAACAGCCTGGCAGTGGT  
 CAGCACCCAGCTTATCGTGCCTGGGATTCTTCTCACAGGTCAAGAAGCAGGCCTTGGGCA  
 20 GTAAAGCTAGTACCGTGACCCCCACCGCCACCGCCACCCCCAGCGCCATCGTGACCACCATCAC  
 CCCACCGCCACCACCAAGCCCCGCTAGTCCTCTGACCTTTGCCCTCCAGCTCCATGACCCTAGTGG  
 CTATCTGGCTGAAGCTGACCTCTCCTACACCTGGGACTTTGGAGACAGTAGTGGAACCCCTGATCT  
 CTCGGGCACYTGTGGTCACTCATACTTACCTGGAGCCTGGCCCAGTCACTGCCAGGTGGTCCCTG  
 CAGGCTGCCATTCTCTCACCTCCTGTGGCTCCTCCCCAGTTCCA GCTAGC TGA (SEQ ID  
 25 NO.:95)

GP100-Peptide 1 – Nucleic Acid Sequence.

GATACAACAGAACCTGCAACACCTACAACACCTGTAACAACACCGACAACAACAAAAGTACCC  
 AGAAACCAGGACTGGCTTGGTGTCTCAAGGCAACTCAGAACCAAGCCTGGAACAGGCAGCTG  
 TATCCAGAGTGGACAGAAAGCCAGAGACTTGACTGCTGGAGAGGTGGTCAAGTGTCCCTCAAG  
 30 GTCAGTAATGATGGGCCTACACTGATTGGTGAAATGCCTCCTTCTCTATTGCCCTTGAACCTCCC  
 TGGAAGCCAAAAGGTATTGCCAGATGGGCAGGTTATCTGGGTCAACAATACCATCATCAATGG  
 GAGCCAGGTGTGGGAGGACAGCCAGTGTATCCCCAGGAACTGACGATGCCTGCATCTTCCCT  
 GATGGTGGACCTTGCCCATCTGGCTCTTGGTCTCAGAAGAGAAGCTTTGTTTATGTCTGGAAGA

CCTGGGGCCAATACTGGCAAGTTCTAGGGGGCCCAGTGTCTGGGCTGAGCATTGGGACAGGCA  
 GGGCAATGCTGGGCACACACACCATGGAAGTGAAGTGTCTACCATCGCCGGGGATCCCAGAGCT  
 ATGTGCCTCTTGCTCATTCCAGCTCAGCCTTCACCATTACTGACCAGGTGCCTTTCTCCGTGAGC  
 GTGTCCCAGTTGCGGGCCTTGGATGGAGGGAACAAGCACTTCCTGAGAAATCAG (SEQ ID

5 NO.:96)

Protein Sequence:

DTTEPATPTTPVTTPTTTKVPRNQDWLGVSRLRTKAWNRQLYPEWTEAQRDCWRGGQVSLKVS  
 NDGPTLIGANASFSIALNFPQSQKVLDPGQVIWVNNTIINGSQVWGGQPVYPQETDDACIFPDGGPCP  
 SGSWSQKRSFVYVWKTWQYVQVLGGPVSGLSIGTGRAMLGTHTMEVTYHRRGSQSYVPLAHS

10 SSAFTITDQVPFSVSVSQLRALDGGNKHFLRNQ (SEQ ID NO.:97)

GP100-Peptide 3

GGCACACAGATGGGCACAGGCCAACTGCAGAGGCCCTAACACCACAGCTGGCCAAGTGCCT  
 ACTACAGAAAGTTGTGGTACTACACCTGGTCAGGCGCCAACTGCAGAGCCCTCTGGAACCAT  
 CTGTGCAGGTGCCAACCCTGAAGTCATAAGCACTGCACCTGTGCAGATGCCAACTGCAGAGA  
 15 GCACAGGTATGACACCTGAGAAGGTGCCAGTTTCAGAGGTGATGGGTACCACACTGGCAGAGA  
 TGTCAACTCCAGAGGCTACAGGTATGACACCTGCAGAGGTATCAATTGTGGTGCTTTCTGGAAC  
 CACAGCTGCA (SEQ ID NO.:98)

Protein Sequence:

GTTDGHRTAEAPNTTAGQVPTTEVVGTTPGQAPTAEPSGTTSVQVPTTEVISTAPVQMPTAESTGM

20 TPEKVPVSEVMGTTLAEMSTPEATGMTPAEVSIVVLSGTTAA (SEQ ID NO.:99)

GP100-Peptide 4:

CAGGTAACAACACAGAGTGGGTGGAGACCACAGCTAGAGAGCTACCTATCCCTGAGCCTGAA  
 GGTCCAGATGCCAGCTCAATCATGTCTACGGAAAGTATTACAGGTTCCCTGGGCCCCCTGCTGG  
 ATGGTACAGCCACCTTAAGGCTGGTGAAGAGACAAGTCCCCCTGGATTGTGTTCTGTATCGATA

25 TGGTTCCTTTTCCGTACCCCTGGACATTGTCCAG (SEQ ID NO.:100)

Protein Sequence:

QVTTTEWVETTARELPIPEPEGPDASSIMSTESITGSLGPLLDGTATLRLVKRQVPLDCVLYRYGSFSV  
 TLDIVQ (SEQ ID NO.:101)

GP100-Peptide 5

30 GGTATTGAAAGTGCCGAGATCCTGCAGGCTGTGCCGTCCGGTGAGGGGGATGCATTTGAGCTGA  
 CTGTGTCCTGCCAAGGCGGGCTGCCCAAGGAAGCCTGCATGGAGATCTCATCGCCAGGGTGCCA  
 GCCCCCTGCCAGCGGCTGTGCCAGCCTGTGCTACCCAGCCCAGCCTGCCAGCTGGTTCTGCAC  
 CAGATACTGAAGGGTGGCTCGGGGACATACTGCCTCAATGTGTCTCTGGCTGATACCAACAGCC

TGGCAGTGGTCAGCACCCAGCTTATCGTGCCTGGGATTCTTCTCACAGGTCAAGAAGCAGGCCT  
TGGGCAG (SEQ ID NO.:102)

Protein Sequence:

GIESAEILQAVPSGEGDAFELTVSCQGGLPKEACMEISSPGCQPPAQRLCQPVLPSPACQLVLHQILK  
5 GGSOTYCLNVSLADTNSLAVVSTQLIVPGILLTGQEAGLGQ (SEQ ID NO.:103)  
GP100-Peptide 2

CCTCTGACCTTTGCCCTCCAGCTCCATGACCCTAGTGGCTATCTGGCTGAAGCTGACCTCTCCTA  
CACCTGGGACTTTGGAGACAGTAGTGGAAACCCTGATCTCTCGGGCACYTGTGGTCACTCATACT  
TACCTGGAGCCTGGCCAGTCACTGCCAGGTGGTCCTGCAGGCTGCCATTCTCTCACCTCCTG  
10 TGGCTCCTCCCAGTTCAGCTAGC (SEQ ID NO.:104)

Protein Sequence:

PLTFALQLHDPGYLEADLSYTWDFGDSSGTLISRAXVVTHTYLEPGPVTAQVVLQAAIPLTSCGS  
SPVPAS (SEQ ID NO.:105)

Cyclin B1 Antigen. Cyclin B1, also known as CCNB1, is a human gene that encodes a regulatory protein  
15 involved in mitosis. Cyclin B1 complexes with p34(cdc2) to form the maturation-promoting factor (MPF).  
Two alternative transcripts are known that are the result of alternative transcription initiation sites. A first  
transcript encodes a constitutively expressed transcript. The second transcript is a cell cycle-regulated  
transcript expressed predominantly during G2/M phase.

The following amino acid sequence is human cyclin B1. Two peptide regions known to contain T cell  
20 epitopes are highlighted in bold-underlined and italics-underlined.

MALRVTRNSKINAENKAKINMAGAKRVPTAPAATSKPGLRPTALGDIGNKVSEQLQAKMPMKKE  
AKPSATGKVIDKKLPKPLEKVPMLVPVPVSEPVPEPEPEPEPEPVKEEKLSPILVDTASPSMETSG  
CAPAEEDLCQAFSDVILAVNDVDAEDGADPNLCSEYVKDIYAYLRQLEEEQAVRPKYLLGREVTGN  
MRAILIDWLQVQMKFRLLQETMYMTVSIIDRFMONNCVPKKMLQLVGVMTAMFIASKYEEMYP  
25 PEIGDFAFVTDNTYTKHQIRO**MEMKILRALNFGLRPLPLHFLRRASKIGEVDVEQHTLAKYLMET**  
**MLDY**DMVHFPPSQAAGAFCLALKILDNGEWTPTLQHYLSYTEESLLPVMQHLAKNVVMVNQGLT  
KHMTVKNKYATSKHAKISTLPQLNSALVQDLAKAVAKVHHHHHH (SEQ ID NO.:106)

Peptide-1 MEMKILRALNFGLRPLPLHFLRRASKIGEVDVEQHTLAKYLMELTMDY (SEQ ID  
NO.:107)

30 Peptide-2

DWLQVQMKFRLLQETMYMTVSIIDRFMONNCVPKK (SEQ ID NO.:108)

Fig. 35 shows a summary of relative expression levels of prototype Cyclin B1 vaccines secreted from  
transfected mammalian 293F cells. The flexible linker sequences facilitate secretion.

C1189 rAB-cetHS-puro[manti-CD40\_12E12.3F3\_H-LV-hlgG4H-C-Flex-v1 (bold)-hCyclinB1-Peptide-2(*italics*)-Peptide-1 (bold -*italics*)-f4 (bold)] [AS linkers -underlined]

EVKLVESGGGLVQPGGSLKLSCATSGFTFSDYYMYWVRQTPEKRLEWVAYINSGGGSTYYPDTVK  
 GRFTISRDNANTLYLQMSRLKSEDTAMYYCARRGLPFHAMDYWGQGTSTVSSAKTKGPSVFPL  
 5 APCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTPVSSSLGTK  
 TYTCNVDPKPSNTKVDKRVESKYGPCCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVDVS  
 QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYCKVSNKGLPSSI  
 EKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLD  
 SDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLSLOKASQTPTNTISVTPTNNT  
 10 PTNNSNPKNPASDWLVQVQMKFRLLQETMYMTVSIIDRFMQNNCVPKKAMEMKILRALNFGLRPL  
PLHFLRRASKIGEVDVEQHTLAKYLMELTMLDYASTNDSITVAATAPTPTVPTVNATPSAAAS (SEQ  
 ID NO.:109)

Above is the sequence of the mature secreted Heavy chain for one form of anti-CD4012E12-cyclin B1  
 vaccine. The AS residues are from joining restriction sites. The DNA coding sequence is shown below, and  
 15 this includes the signal peptide.

ATGAACTTGGGGCTCAGCTTGATTTTCCTTGTCCTTGTTTTAAAAGGTGTCCAGTGTGAAGTGAA  
 GCTGCTGGAGTCTGGGGGAGGCTTAGTGACGCCCCGAGGGTCCCTGAAACTCTCCTGTGCAACC  
 TCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCGCCAGACTCCAGAGAAGAGGCTGG  
 AGTGGGTGCGCATACATTAATTCTGGTGGTGGTAGCACCTATTATCCAGACACTGTAAAGGGCCG  
 20 ATTACCATCTCCAGAGACAATGCCAAGAACCCCTGTACCTGCAAATGAGCCGGCTGAAGTCT  
 GAGGACACAGCCATGTATTACTGTGCAAGACGGGGGTACCGTTCCATGCTATGGACTATTGGG  
 GTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAACGAAGGGCCCATCCGTCTCCCCCTGGC  
 GCCCTGCTCCAGGACACCTCCGAGAGCACAGCCGCCCTGGGCTGCCTGGTCAAGGACTACTTC  
 CCCGAACCGGTGACGGTGTCTGGAACTCAGGCGCCCTGACCAGCGCGTGCACACCTTCCCGG  
 25 CTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCTCCAGCAGCTTG  
 GGCACGAAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGACAAGAGA  
 GTTGAGTCCAAATATGGTCCCCCATGCCACCCCTGCCAGCACCTGAGTTCGAAGGGGGACCAT  
 CAGTCTTCCTGTTCCCCCAAAACCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTAC  
 GTGCGTGGTGGTGGACGTGAGCCAGGAAGACCCGAGGTCCAGTTCAACTGGTACGTGGATGG  
 30 CGTGGAGGTGCATAATGCCAAGACAAAACCGCGGGAGGAGCAGTTCAACAGCACGTACCGTGT  
 GGTCAGCGTCTCACCCTCCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGT  
 CTCCAACAAAGGCCTCCCGTCTCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCG  
 AGAGCCACAGGTGTACACCCTGCCCCCATCCAGGAGGAGATGACCAAGAACCAGGTACGCCT

GACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCA  
 GCCGGAGAACAACACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTAC  
 AGCAGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTGATG  
 CATGAGGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAAGCTAGTC  
 5 AGACCCCCACCAACACCATCAGCGTGACCCCCACCAACAACAGCACCCCCACCAACAACAGCA  
 ACCCCAAGCCCAACCCCGCTAGTGACTGGCTAGTACAGGTTCAAATGAAATTCAGGTTGTTGCA  
 GGAGACCATGTACATGACTGTCTCCATTATTGATCGGTTTCATGCAGAATAATTGTGTGCCCAAG  
 AAGGCTAGTATGGAAATGAAGATTCTAAGAGCTTTAACTTTGGTCTGGGTGGGCTCTACCTT  
 TGCACCTTCTTCGGAGAGCATCTAAGATTGGAGAGGTTGATGTGAGCAACATACTTTGGCCAA  
 10 ATACCTGATGGAACTAACTATGTTGGACTATGCTAGTACCAACGACAGCATACCCGTGGCCGCC  
 ACCGCCCCCACCCTGACCCCCACCCTGAACGCCACCCCCAGCGCCGCCGCTAGCTGA (SEQ ID  
 NO.:110)

C1143 rAB-cetHS-puro[manti-CD40\_12E12.3F3\_H-LV-hlgG4H-C-Flex-v1 (bold)-hCyclinB1-Peptide-  
 2(*italics*)-f3 (bold)] [AS linkers -underlined].

15 EVKLVESGGGLVQPGGSLKLSCATSGFTFSDYYMYWVRQTPEKRLEWVAYINSGGGSTYYPDTVK  
 GRFTISRDNANTLYLQMSRLKSEDTAMYYCARRGLPFHAMDYWGQTSVTVSSAKTKGPSVEPL  
 APCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTK  
 TYTCNVDHKPSNTKVDKRVESKYGPCCPPCAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS  
 QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSI  
 20 EKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLD  
 SDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLGLKASQTPTNTISVTPTNST  
 PTNNSNPKNPASDWLVQVQMKFRLLQETMYMTVSIIDRFMQNNCVPKKASTVTPATATPSAIVTTI  
 TPTATTKPAS (SEQ ID NO.:111)

25 Above is the sequence of the mature secreted Heavy chain for one form of anti-CD4012E12-cyclin B1  
 vaccine. The AS residues are from joining restriction sites. The DNA coding sequence is shown below, and  
 this includes the signal peptide.

ATGAACTTGGGGCTCAGCTTGAATTTTCTTGTCTTGTTTTAAAAGGTGTCCAGTGTGAAGTGAA  
 GCTGGTGGAGTCTGGGGGAGGCTTACTGCAGCCCGGAGGGTCCCTGAAACTCTCCTGTGCAACC  
 TCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCCGCCAGACTCCAGAGAAGAGGCTGG  
 30 AGTGGGTCGCATACATTAATTCTGGTGGTGGTAGCACCTATTATCCAGACACTGTAAAGGGCCG  
 ATTCACCATCTCCAGAGACAATGCCAAGAACACCTGTACCTGCAAATGAGCCGGCTGAAGTCT  
 GAGGACACAGCCATGTATTACTGTGCAAGACGGGGGTTACCGTTCCATGCTATGGACTATTGGG  
 GTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAACGAAGGGCCCATCCGCTTCTCCCCCTGGC

GCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCCGCCCTGGGCTGCCTGGTCAAGGACTACTTC  
 CCCGAACCGGTGACGGTGTCTGTGAACTCAGGCGCCCTGACCAGCGCGGTGCACACCTTCCCGG  
 CTGTCTTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTG  
 GGCACGAAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGACAAGAGA  
 5 GTTGAGTCCAAATATGGTCCCCCATGCCCACCCTGCCAGCACCTGAGTTCGAAGGGGGACCAT  
 CAGTCTTCCTGTTCCCCCAAAAACCAAGGACACTCTCATGATCTCCCGGACCCCTGAGOTCAC  
 GTGCGTGGTGGTGGACGTGAGCCAGGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGATGG  
 CGTGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTTCAACAGCAGTACCGTGT  
 GGTACGCGTCTCACCCTCCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGT  
 10 CTCCAACAAAGGCTCCCGTCTCCATCGAGAAAACCATCTCCAAGGCAAAGGGCAGCCCCG  
 AGAGCCACAGGTGTACACCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTACGCT  
 GACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCA  
 GCCGGAGAACAATAAGACACGCCTCCCGTGTGGACTCCGACGGCTCCTTCTTCTCTAC  
 AGCAGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTGATG  
 15 CATGAGGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAAGCTAGTC  
 AGACCCCCACCAACACCATCAGCGTGACCCCCACCAACAACAGCACCCCCACCAACAACAGCA  
 ACCCCAAGCCCAACCCCGCTAGTGACTGGCTAGTACAGGTTCAAATGAAATTCAGGTTGTTGCA  
 GGAGACCATGTACATGACTGTCTCATTATTGATCGGTTTCATGCAGAATAATTGTGTGCCAAG  
 AAGGCTAGTACCGTGACCCCCACCGCCACCGCCACCCCCAGCGCCATCGTGACCACCATCACCC  
 20 CCACCGCCACCACCAAGCCCGCTAGCTGA (SEQ ID NO.:112)  
 C911 rAB-cetHS-puro[manti-CD40\_12E12.3F3\_H-LV-hlgG4H-C-Flex-v1 (bold)-hCyclinB1-Peptide-1  
 (italics)-f4 (bold)]  
 EVKLVESGGGLVQPGGSLKLSCATSGFTFSYYMYWVRQTPEKRLEWVAYINSGGGSTYYPDVTK  
 GRFTISRDNKNTLYLQMSRLKSEDTAMYYCARRGLPFHAMDYWGQTSVTVSSAKTKGPSVFPL  
 25 APCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGK  
 TYTCNVDPKPSNTKVDKRVESKYGPPCPPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS  
 QEDPEVFQFNWYVDGVEVHNAKTKPREEQFNSTYRVSVLTVLHQDWLNGKEYKCKVSNKGLPSSI  
 EKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLD  
 SDGSFFLYSRLTVDKSRWQEGNVFSCVMHEALHNHYTQKSLSLGLKASQTPTNTISVTPTNNST  
 30 PTNNSNPKNPASMEMKILRALNFGRLPLHLRRASKIGEVDVEQHTLAKYLMELTMLDYASTNGS  
 ITVAATAPTPTPTVNATPSAAAS (SEQ ID NO.:114)  
 C911 rAB-cetHS-puro[manti-CD40\_12E12.3F3\_H-LV-hlgG4H-C-Flex-v1 (bold)-hCyclinB1-Peptide-1  
 (italics)-f4 (bold)] nucleic acid sequence.



ATGAACTTGGGGCTCAGCTTGATTTTCCTTGTCCTTGTTTTAAAAGGTGTCCAGTGTGAAGTGAA  
 GCTGGTGGAGTCTGGGGGAGGCTTAGTGAGCCCGGAGGGTCCCTGAAACTCTCCTGTGCAACC  
 TCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCCGCCAGACTCCAGAGAAGAGGCTGG  
 AGTGGGTCCGCATACATTAATTCTGGTGGTGGTAGCACCTATTATCCAGACACTGTAAAGGGCCG  
 5 ATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTACCTGCAAATGAGCCGGCTGAAGTCT  
 GAGGACACAGCCATGTATTACTGTGCAAGACGGGGGTACCGTTCCATGCTATGGACTATTGGG  
 GTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAACGAAGGGCCCATCCGTCTTCCCCCTGGC  
 GCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCCGCCCTGGGCTGCCTGGTCAAGGACTACTTC  
 CCCGAACCGGTGACGGTGTCTGTGGAACCTCAGGCGCCCTGACCAGCGCGTGCACACCTTCCCGG  
 10 CTGTCTACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTG  
 GGCACGAAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGACAAGAGA  
 GTTGAGTCCAAATATGGTCCCCCATGCCACCCCTGCCCAGCACCTGAGTTCGAAGGGGGACCAT  
 CAGTCTTCTGTTCCCCCAAAACCCAAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTAC  
 GTGCGTGGTGGTGGACGTGAGCCAGGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGATGG  
 15 CGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTTCAACAGCACGTACCGTGT  
 GGTCAGCGTCCTCACCCTCCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGT  
 CTCCAACAAAGGCCTCCCGTCTCCATCGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCG  
 AGAGCCACAGGTGTACACCCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTGAGCCT  
 GACCTGCCTGGTCAAAGGCTTCTACCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCA  
 20 GCCGGAGAACAACACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTAC  
 AGCAGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGTCCCGTGATG  
 CATGAGGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAAGCTAGTC  
 AGACCCCCACCAACACCATCAGCGTGACCCCCACCAACAACAGCACCCCCACCAACAACAGCA  
 ACCCCAAGCCCCAACCCCGCTAGTATGGAAATGAAGATTCTAAGAGCTTTAAACTTTGGTCTGGG  
 25 TCGGCCTCTACCTTTGCACTTCCTTCGGAGAGCATCTAAGATTGGAGAGGTTGATGTCCAGCAA  
 CATACTTTGGCCAAATACCTGATGGAATACTATGTTGGACTATGCTAGTACCAACGGCAGCA  
 TCACCGTGGCCGCCACCGCCCCACCGTGACCCCCACCGTGAACGCCACCCCCAGCGCCGCCG  
 TAGCTGA (SEQ ID NO.:115)

D-type Cyclin Antigen. D-type cyclins are predominantly expressed in the G1 phase of the cell cycle. The  
 30 expression pattern of cyclin D1 has been extensively studied in certain cancer types including lymphoma and  
 non-small cell lung cancer. Approximately 30 percent of breast carcinomas are Cyclin D1 positive. Over  
 expression of Cyclin D1 is now a well established criterion for the diagnosis of Mantle Cell Lymphoma, a  
 malignant, non-Hodgkin's lymphoma which is characterized by a unique chromosomal translocation t(11;14).

Cyclin D1 – Peptide 1-bold, Peptide 2-bold-underlined, Peptide-3 italics, Peptide 4-underlined.

- MEHQLLCCEVETIRRAYPDANLLNDRVLRAMLKAEETCAPSVSYFKCV**QKEVLP**SMRKIVAT  
**WMLEVCEEOKCEEEVF**PLAMNYLDRFLSLEPVKKSRLOLLGATCMFVASKMKETIPLTAEKL  
**CIYTDNSIRPEELLQ**MELLV**LNKLKWNLAAMTPHDFIEHFLSKMPEAEENKQIRKHAQT**FVALCATDV  
 5 **KFISNPPSMVAAGSVVAAVOGLNLRSPNNFLSYRYL**TRFLSRVIKCDPDCLRACQEOIEALLESSLRQ  
**AOQNMDPKAAEEEEEEEEVDLACTPTDVRD**VDI (SEQ ID NO.:116)

Pep-1

MEHQLLCCEVETIRRAYPDANLLNDRVLRAMLKAEETCAPSVSYFKCV (SEQ ID NO.:117)

Pep-2

- 10 QKEVLP**SMRKIVATWMLEVCEEOKCEEEVF**PLAMNYLDRFLSLEPVKKSRLOLLGATCMFVASKM  
 KETIPLTAEKL**CIYTDNSIRPEELLQ**MELL (SEQ ID NO.:118)

Pep-3

LVNKLKWNLAAMTPHDFIEHFLSKMPEAEENKQIRKHAQT**FVALCATDV**KFISNPPSMV (SEQ ID  
 NO.:119)

- 15 Pep-4

AAGSVVAAVQGLNLRSPNNFLSYRYLTRFLSRVIKCDPDCLRACQEQIEALLESSLRQAQQNMDPK  
 AEEEEEEEEVDLACTPTDVRD**VDI** (SEQ ID NO.:120)

Table 1. Clone-Antibody Correlation.

Name	Clone	Isotype
PAB176	AB13_22.11B6.2C6	IgG1k
PAB176	AB13.22.11B6.1C3 (HS440) - subclone	
PAB177	AB13_22.11C7.1D6	IgG2b k
PAB180	AB13_22.11H12.1G1	IgG1k
PAB188	AB13_22.12B4.2C10	IgG1k
PAB1574		
PAB187	AB13_22.12E12.3F3	IgG1k
PAB366		
PAB525		
PAB530		
PAB594		
PAB1400		
PAB1700		
PAB184	AB13_22.15C11.3G12	IgG1k
PAB181	AB13_22.19B5.4C11	IgG2a k
PAB183	AB13_22.24A3.3F1	IgG2b k
PAB178	AB13_22.24C9.2A6	IgG2b k
PAB189	AB13_22.2G2.1A5	IgG2b k
PAB194	AB13_22.3C7.1G5	IgG2a k
PAB1573		
PAB193	AB13_22.7G10.2D5	IgG2a k
PAB1572		

PAB182 PAB1435	AB13_22.8A4.3G10	IgG1k
PAB179	AB13_22.8F6.2C7	IgG2b k
PAB190	AB13_22.9A11.2A11	IgG1 lam

Fig. 34 shows the results obtained with the various antibodies using an assay that detects signaling via CD40 ligation - read out as cell death. CD40 itself can send such signals, but the intracellular domain of FAS is used for comparison when expressed in CHO cells (Fas CHO v. CHO). Briefly, CHS-S cells were transfected with expression vectors for either hCD40ectodomain<sup>TM</sup> fused to FAS intracellular domain, or hCD40. These cells proliferate normally, but signaling through CD40 ligation activated apoptotic signals. After 48 hours, MTT is added to the culture and reduction in dye is measured, which is directly proportional to the content of active mitochondria (i.e., live cells).

ELISA. The plates were coated with either CD40 ecto (human or NHP coh) then mAbs. anti-mIgG HRP or CBD doc/then CD40 ecto (coh = cohesin, NHP = non human primate, HRP = horseradish peroxidase) then mAbs and then anti-mIgG HRP or Capture is anti-mIgG then Mabs then biotinylated CD40 ecto (human or NHP coh). Cytokine production was measured as described in the examples above.

Figs. 35 shows the binding of various constructs when the antibody has been made into a fusion protein with doc and then captures. Figs. 36 and 37 compare cytokine production with our without the addition of GM-CSF and IFN $\alpha$  (Fig. 36 A-D), and soluble antibodies alone (Fig. 37A-D) incubated with the DCs for 24 hours. Figure 38A-B demonstrates the effect of various concentrations of anti-CD40 antibodies of the present invention on direct B cell proliferation.

B cell Proliferation. B cells from PBMC of healthy donors were enriched by B cell enrichment kit (from BD). CFSE-labeled  $5 \times 10^4$  B cells were cultured in RPMI medium containing 10 % FCS in the presence of 50 units/ml IL-2 for 6 days. B cell proliferation was tested by measuring CFSE dilution using flow cytometry. Surprisingly, it was found that antibodies were able to cause B cell proliferation at various dilutions, while an immunoglobulin control and an anti-CD40 antibody (data not shown) did not.

The various constructs shown herein demonstrate the that CD40 antibodies (e.g., 12E12) are capable of strong activation as variable domains when: (1) the antibody is reconfigured as a recombinant mouse  $\nu$  region human IgG4 C region chimera, and (2) the activity can be retained in the context of (1) with H-chain - C-terminal antigen added. These variable region-peptide fusion proteins and/or complexes enhance greatly vaccine efficacy.

It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, kit, reagent, or composition of the invention, and vice versa. Furthermore, compositions of the invention can be used to achieve methods of the invention.

It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains.

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The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

15

As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

20

The term "or combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, MB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

25

## CLAIMS

1. An antibody or an antigen binding fragment thereof that binds to CD40, comprising:
  - (i) a light chain variable region comprising an amino acid sequence of SEQ ID NO:7 and a heavy chain variable region comprising an amino acid sequence of SEQ ID NO:6; or
  - (ii) a humanized version of (i).
2. The antibody of claim 1, further comprising a heavy chain constant region, wherein the heavy chain constant region comprises a gamma-1, gamma-2, gamma-3, or gamma-4 human heavy chain constant region or a variant of the human heavy chain constant region.
3. The antibody of claim 1 or 2, further comprising a light chain constant region, wherein the light chain constant region comprises a lambda or a kappa human light chain constant region.
4. The antibody of any one of claims 1-3, wherein the binding fragment is selected from group consisting of Fab, Fab', Fab'-SH, Fv, scFv, F(ab')<sub>2</sub>, and a diabody.
5. The antibody of claim 1, wherein the antibody is produced by the hybridoma anti-CD40\_11B6.1C3 (ATCC Accession No. PTA-10652).
6. The antibody of any one of claims 1-5, wherein the antibody alone is capable of causing dendritic cells to secrete at least one of IL-6, MIP-1a, IL-12p40 or TNFalpha without prior activation of the dendritic cells.
7. The antibody of any one of claims 1-6, wherein the antibody is capable of causing dendritic cells activated with GM-CSF and Interferon alpha to secrete at least one of IL-6, MIP-1a, IP-10, IL-10 or IL-12p40.
8. The antibody of any one of claims 1-7, wherein the antibody is humanized.
9. A composition comprising an antibody or an antigen binding fragment thereof, in combination with a pharmaceutically acceptable carrier or diluent, wherein the antibody or antigen binding fragment thereof is as defined in any one of claims 1-7.
10. The composition of claim 9, wherein antibody is humanized.
11. A humanized recombinant antibody or an antigen binding fragment thereof, both of which bind to

CD40, wherein the antibody or antigen binding fragment comprises a humanized version of a light chain variable region consisting of an amino acid sequence of SEQ ID NO:7 and a humanized version of a heavy chain variable region consisting of an amino acid sequence of SEQ ID NO:6.

12. The antibody of claim 11, further comprising a heavy chain constant region, wherein the heavy chain constant region comprises a gamma-1, gamma-2, gamma-3, or gamma-4 human heavy chain constant region or a variant of the human heavy chain constant region.
13. The antibody of claim 11 or 12, wherein the humanized antibody comprises the complementarity determining regions of: a) the antibody light chain variable region consisting of an amino acid sequence of SEQ ID NO:7; and b) the antibody heavy chain variable region consisting of an amino acid sequence of SEQ ID NO:6 on a human antibody framework.
14. The antibody of any one of claims 11-13, further comprising a light chain constant region, wherein the light chain constant region comprises a lambda or a kappa human light chain constant region.
15. The antibody of any one of claims 11-14, wherein the binding fragment is selected from group consisting of Fab, Fab', Fab'-SH, Fv, scFv, F(ab')<sub>2</sub>, and a diabody.
16. The antibody of any one of claims 11-15, wherein the antibody alone is capable of causing dendritic cells to secrete at least one of IL-6, MIP-1a, IL-12p40 or TNFalpha without prior activation of the dendritic cells.
17. The antibody of any one of claims 11-16, wherein the antibody is capable of causing dendritic cells activated with GM-CSF and Interferon alpha to secrete at least one of IL-6, MIP-1a, IP-10, IL-10 or IL- 12p40.
18. A composition comprising an antibody or an antigen binding fragment thereof, in combination with a pharmaceutically acceptable carrier or diluent, wherein the antibody or antigen binding fragment thereof is as defined in any one of claims 11-17.
19. One or more nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO:15 encoding a light chain or a binding fragment thereof and comprising the nucleotide sequence of SEQ ID NO:14 encoding a heavy chain or a binding fragment thereof.
20. The one or more nucleic acids of claim 19, wherein the binding fragment encodes for an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH, Fv, scFv, F(ab')<sub>2</sub>, and a diabody.

21. A method of making an antibody comprising: expressing in a host cell a recombinant antibody or an antigen binding fragment thereof, both of which bind to CD40, wherein the recombinant antibody or antigen binding fragment comprises: (i) an antibody light chain variable region consisting of an amino acid sequence of SEQ ID NO:7 and an antibody heavy chain variable region consisting of an amino acid sequence of SEQ ID NO:6; or (ii) a humanized version of (i).
22. The method of claim 21, wherein the host cell is a bacterial, fungal, insect, or mammalian cell.
23. The method of claim 21 or 22, wherein the antibody is a humanized antibody.
24. The method of any one of claims 21-23, wherein the antibody alone is capable of causing dendritic cells to secrete at least one of IL-6, MIP-1a, IL-12p40 or TNFalpha without prior activation of the dendritic cells.
25. The method of any one of claims 21-24, wherein the antibody is capable of causing dendritic cells activated with GM-CSF and Interferon alpha to secrete at least one of IL-6, MIP-1a, IP-10, IL-10 or IL- 12p40.

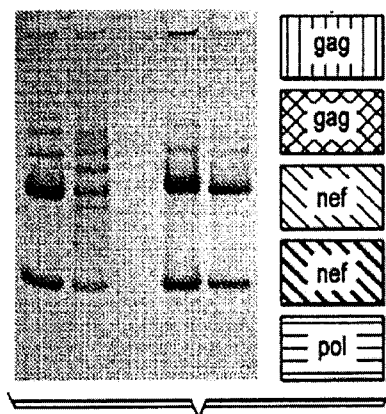


FIG. 1

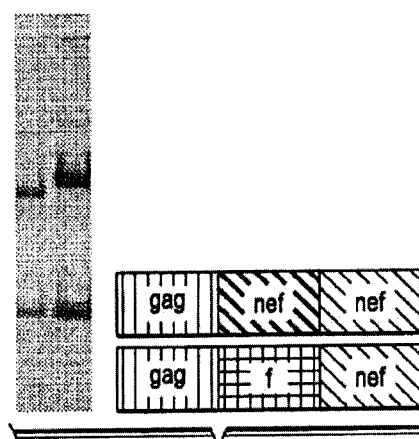


FIG. 2

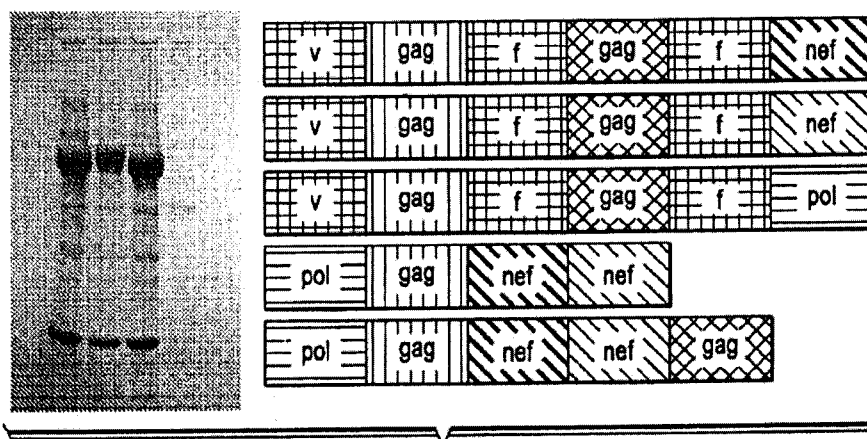


FIG. 3



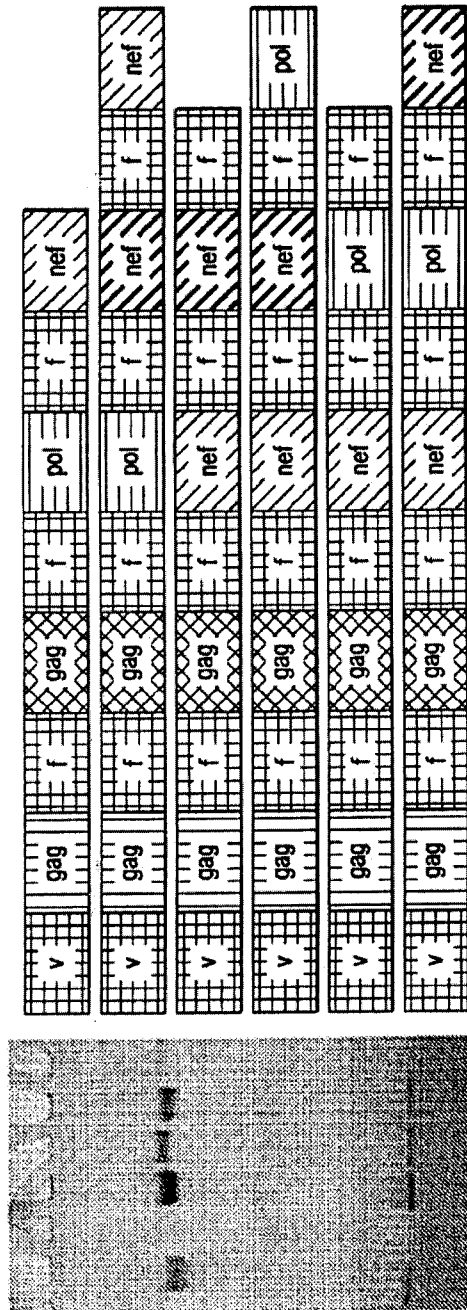


FIG. 4

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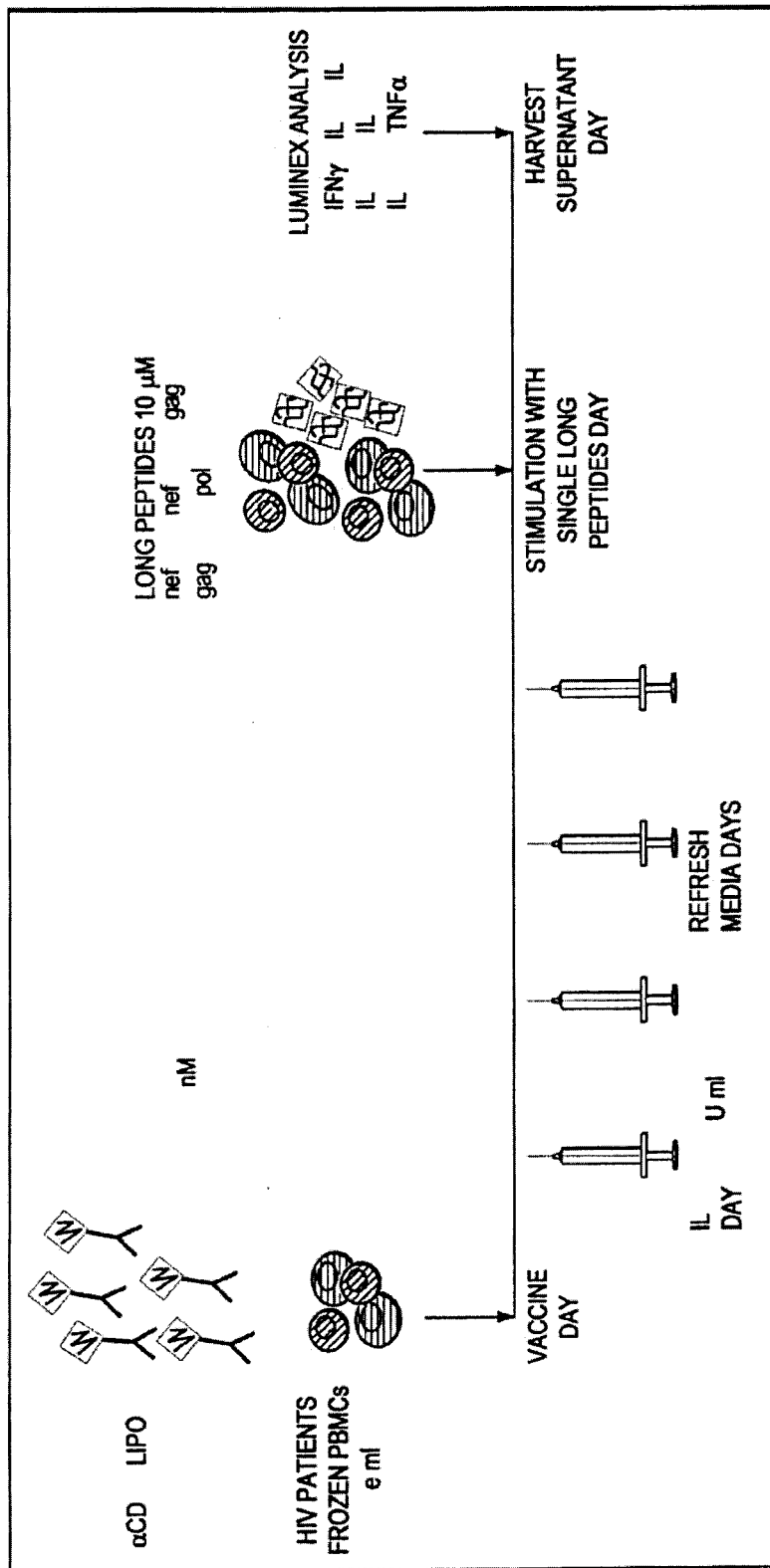
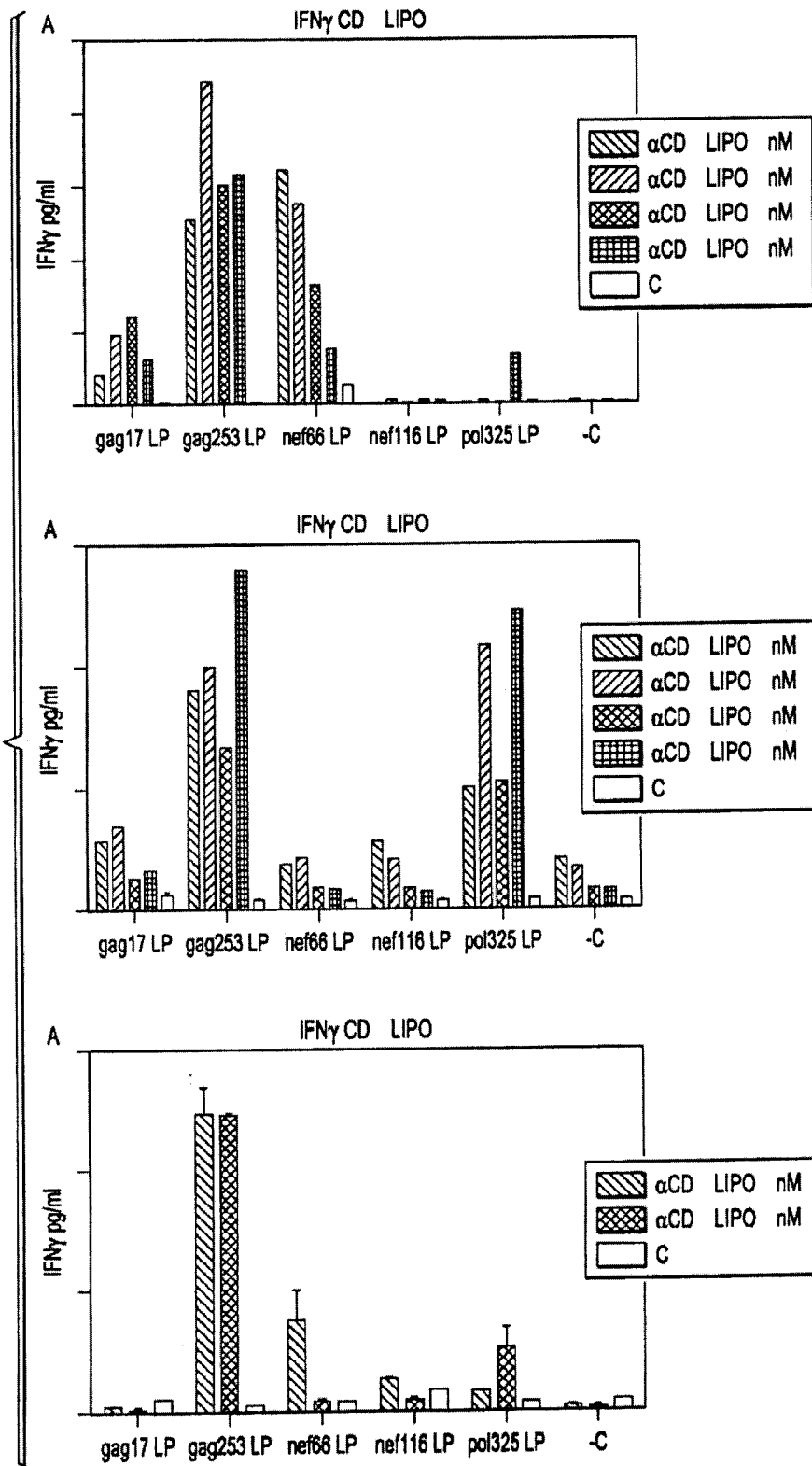


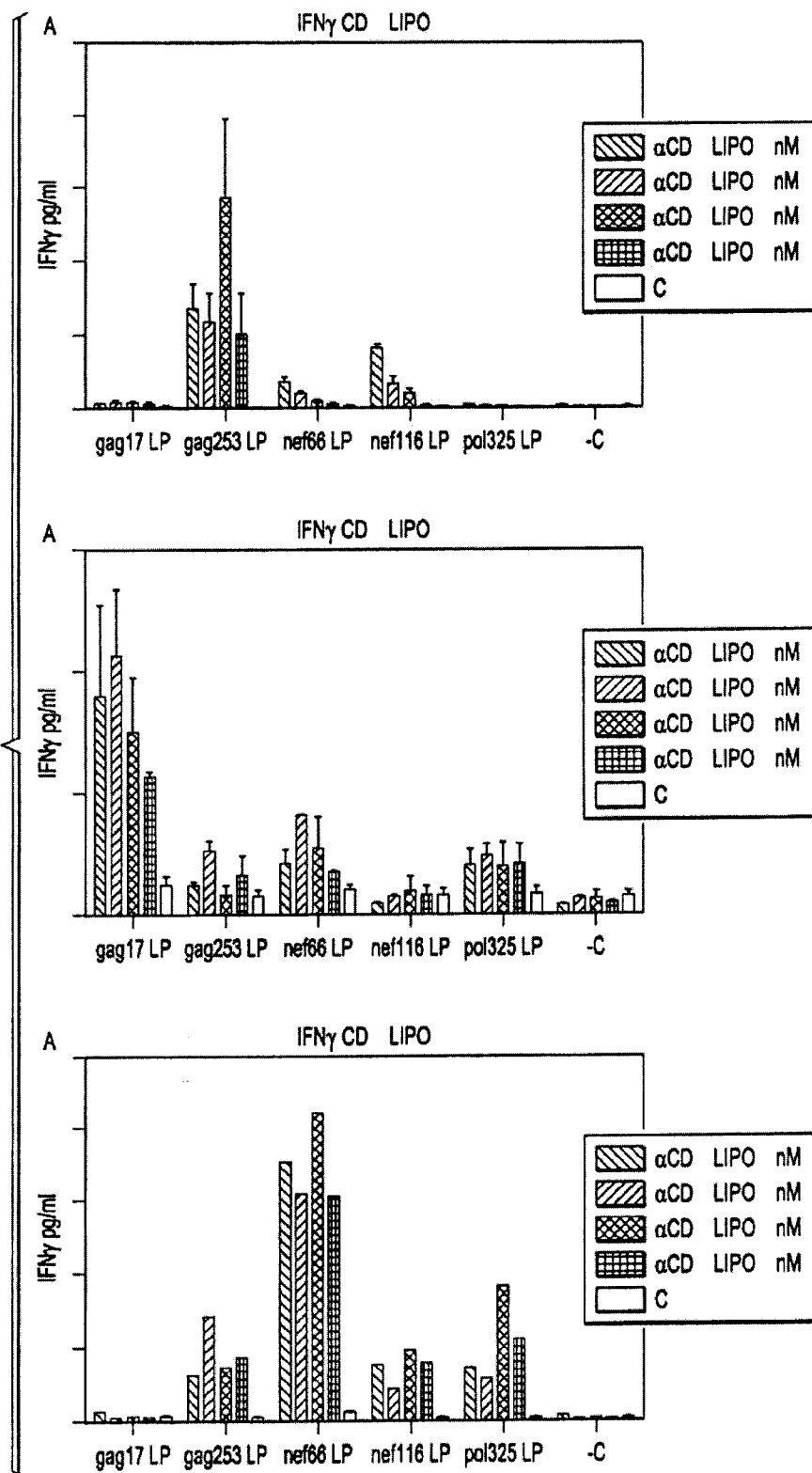
FIG. 5

FIG. 6A

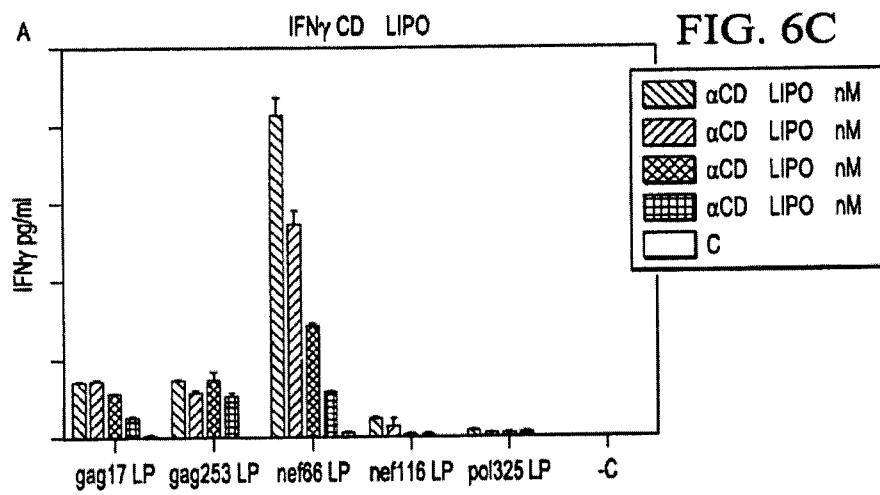


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FIG. 6B



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	Gag	Gag	Nef	Nef	Pol
A	WEAK RESPONSE	STRONG RESPONSE	STRONG RESPONSE	NO RESPONSE	NO RESPONSE
A	NO RESPONSE	STRONG RESPONSE	NO RESPONSE	NO RESPONSE	STRONG RESPONSE
A	NO RESPONSE	STRONG RESPONSE	NO RESPONSE	NO RESPONSE	WEAK RESPONSE
A	NO RESPONSE	STRONG RESPONSE	NO RESPONSE	NO RESPONSE	NO RESPONSE
A	WEAK RESPONSE	NO RESPONSE	WEAK RESPONSE	NO RESPONSE	WEAK RESPONSE
A	NO RESPONSE	WEAK RESPONSE	STRONG RESPONSE	WEAK RESPONSE	WEAK RESPONSE
A	NO RESPONSE	STRONG RESPONSE	NO RESPONSE	NO RESPONSE	NO RESPONSE
A	WEAK RESPONSE	WEAK RESPONSE	STRONG RESPONSE	NO RESPONSE	NO RESPONSE

STRONG RESPONSE

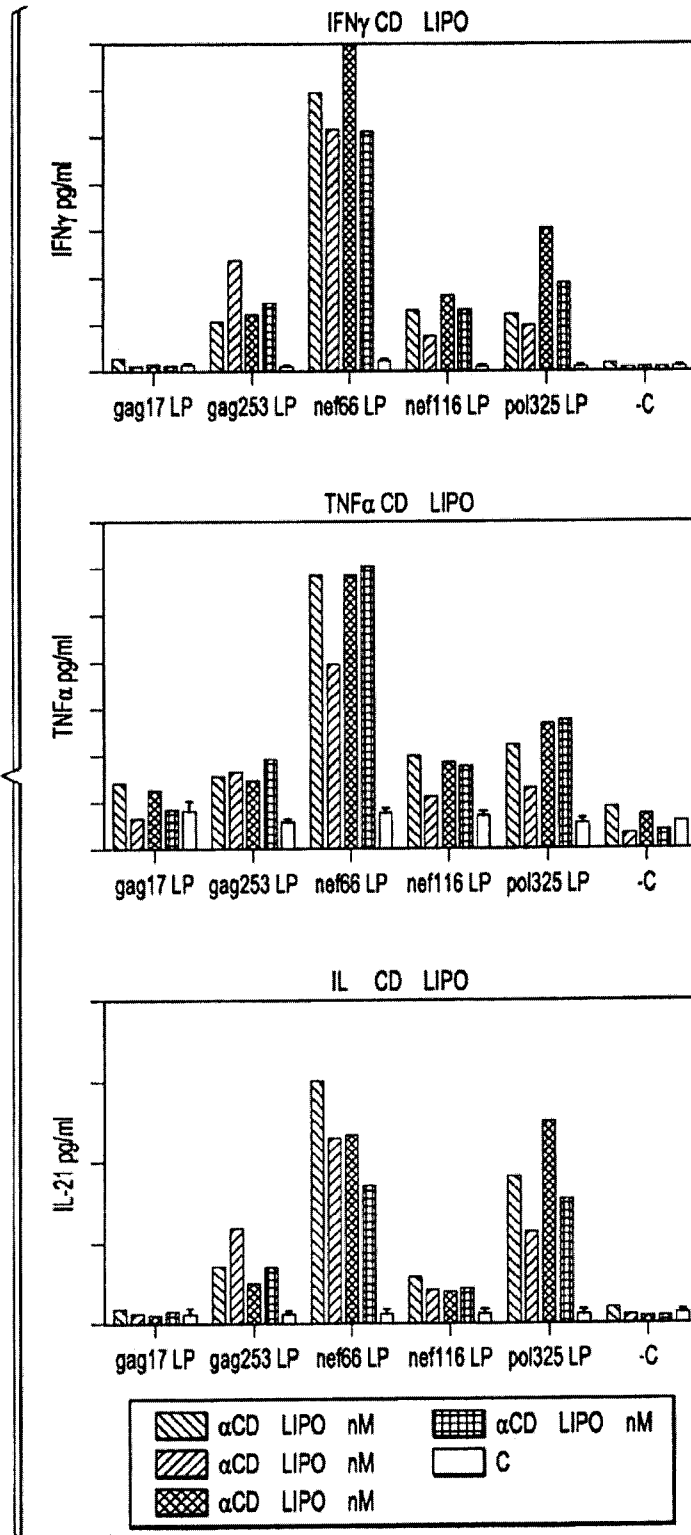
MEDIUM RESPONSE

WEAK RESPONSE

NO RESPONSE

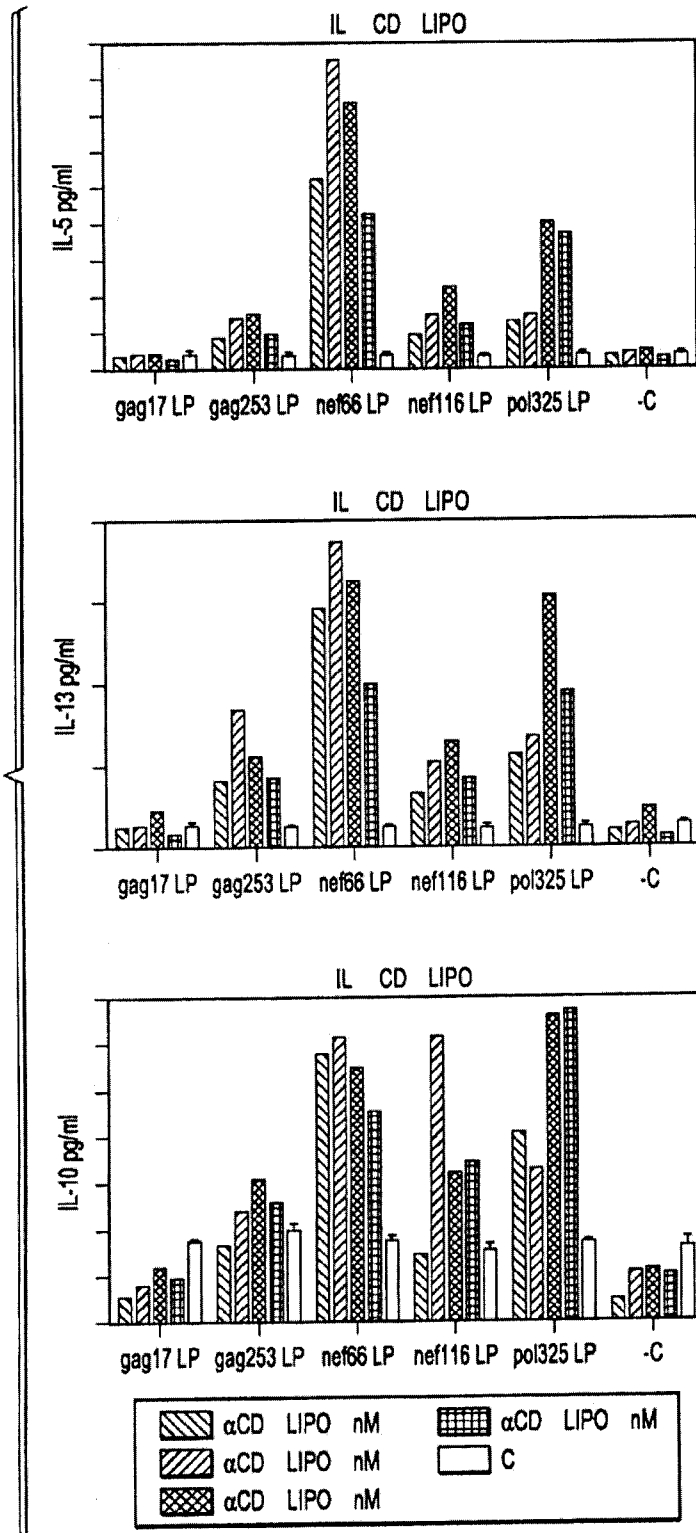
FIG. 7

FIG. 8A



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FIG. 8B





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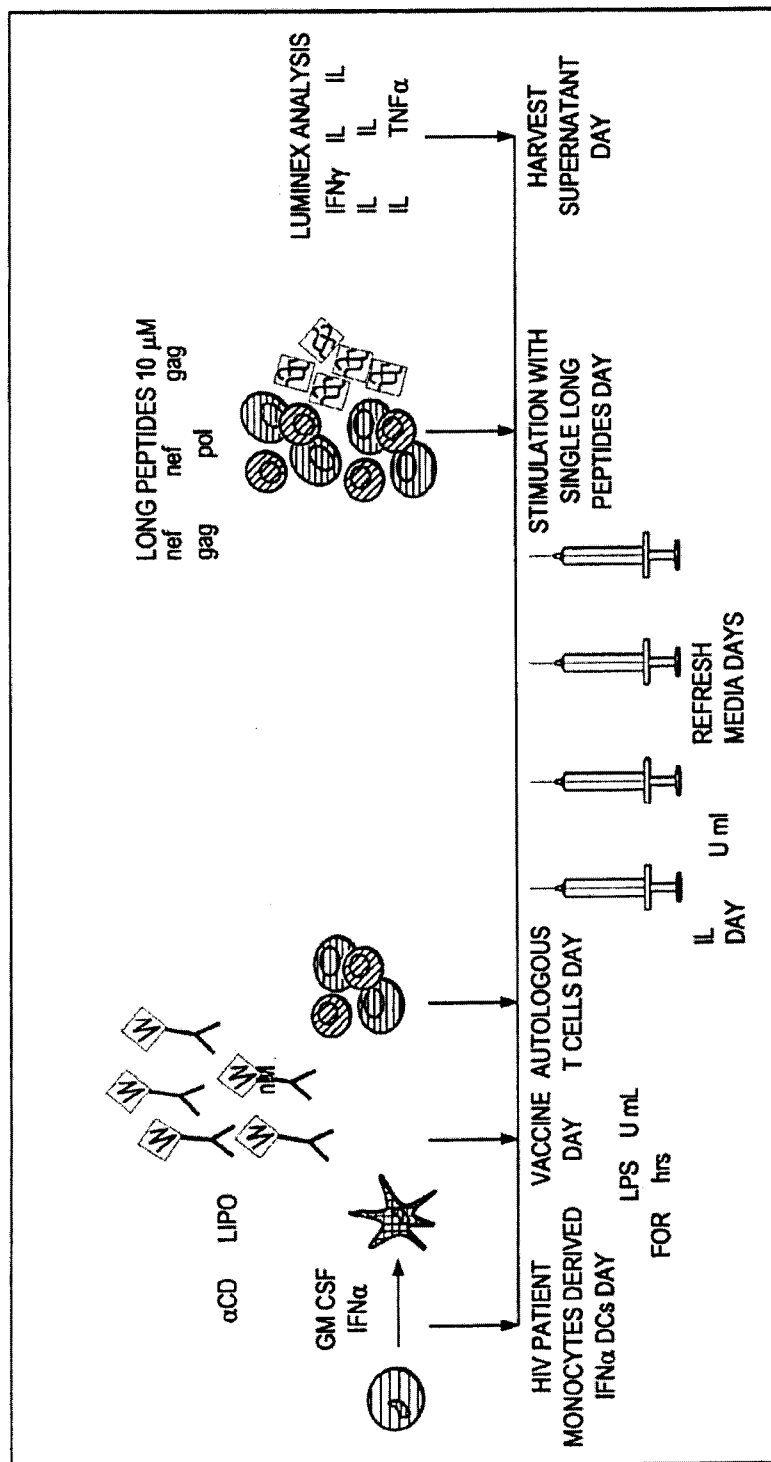
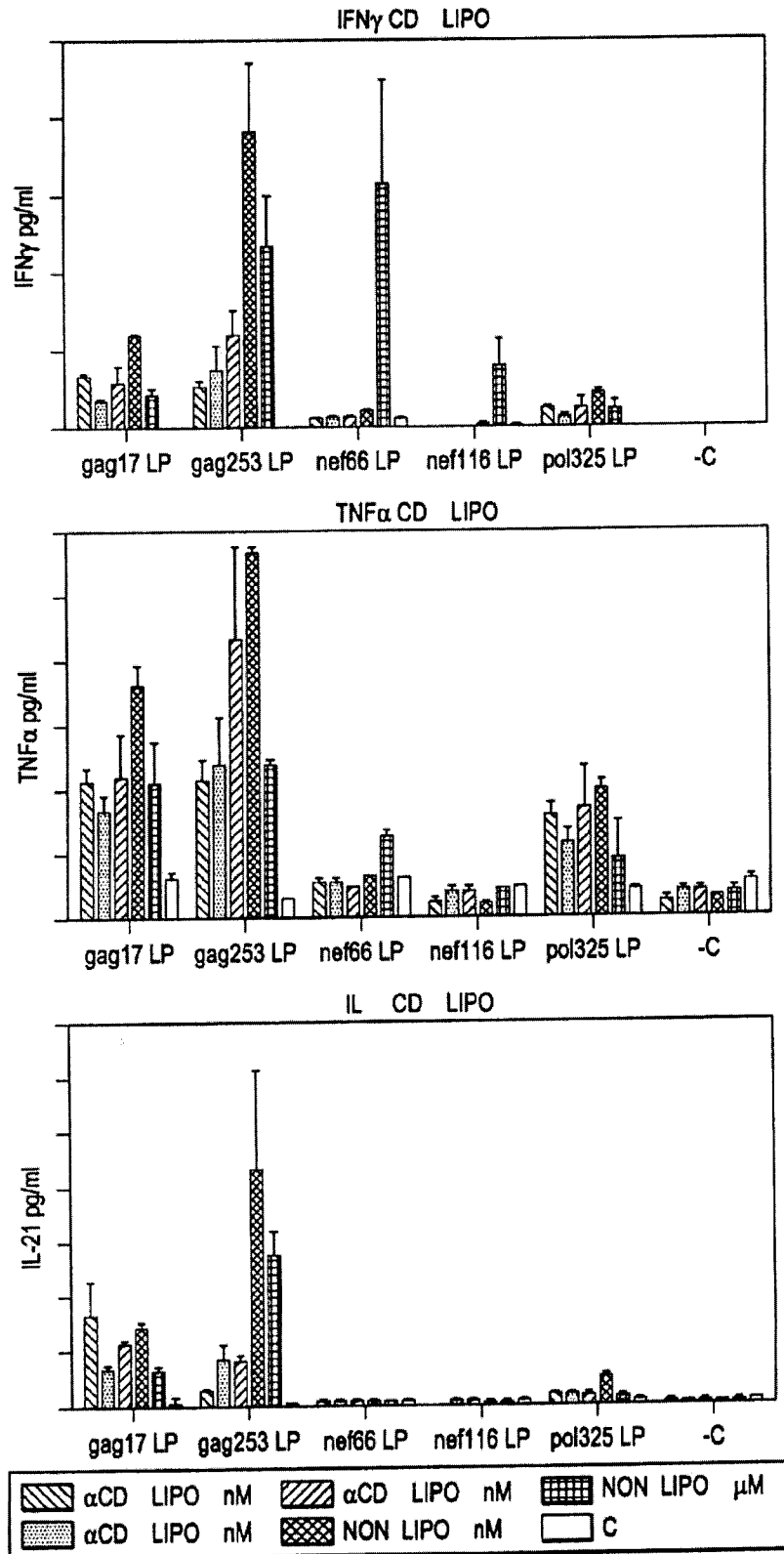


FIG. 9

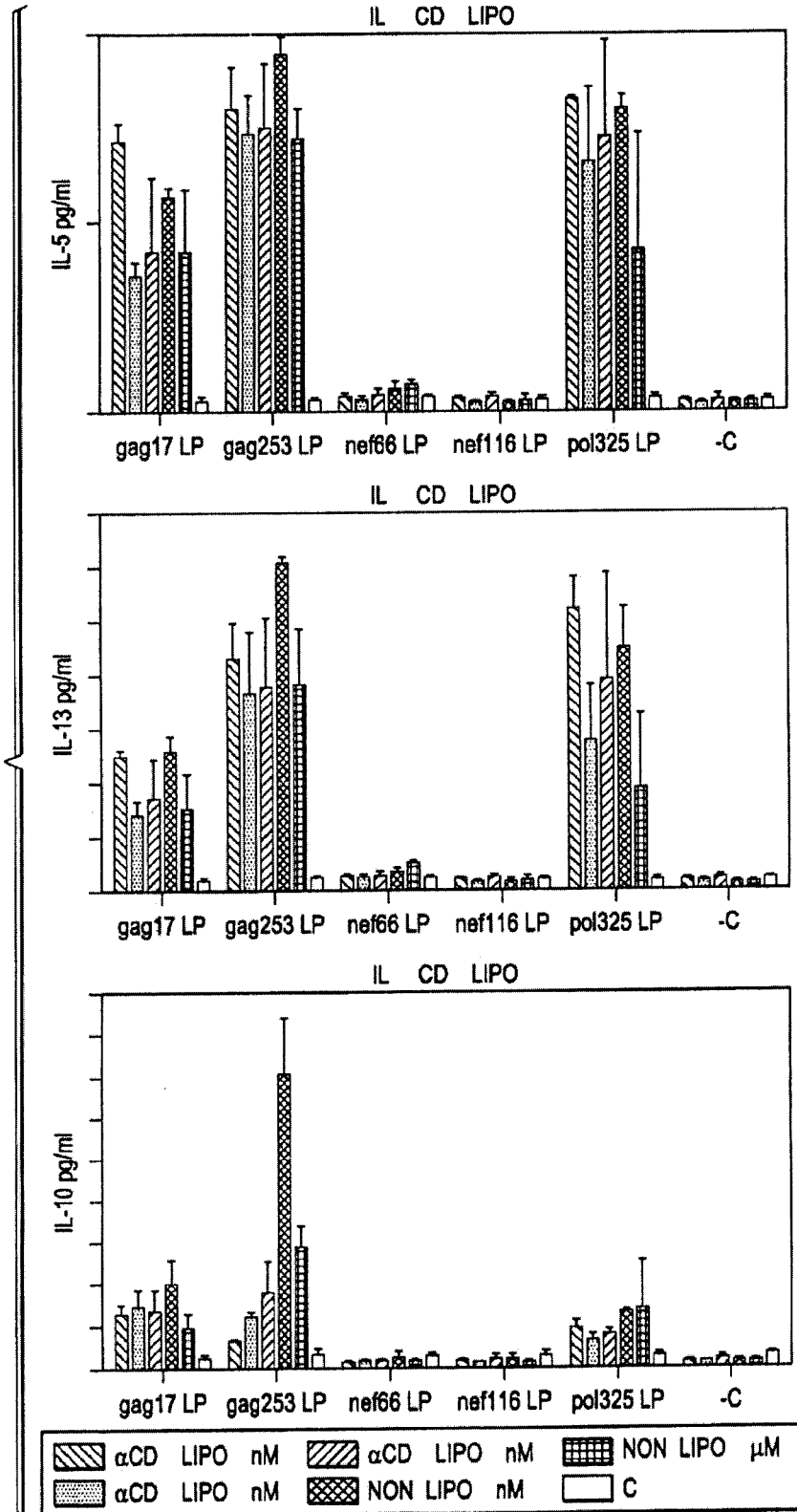
6/36

FIG. 10A



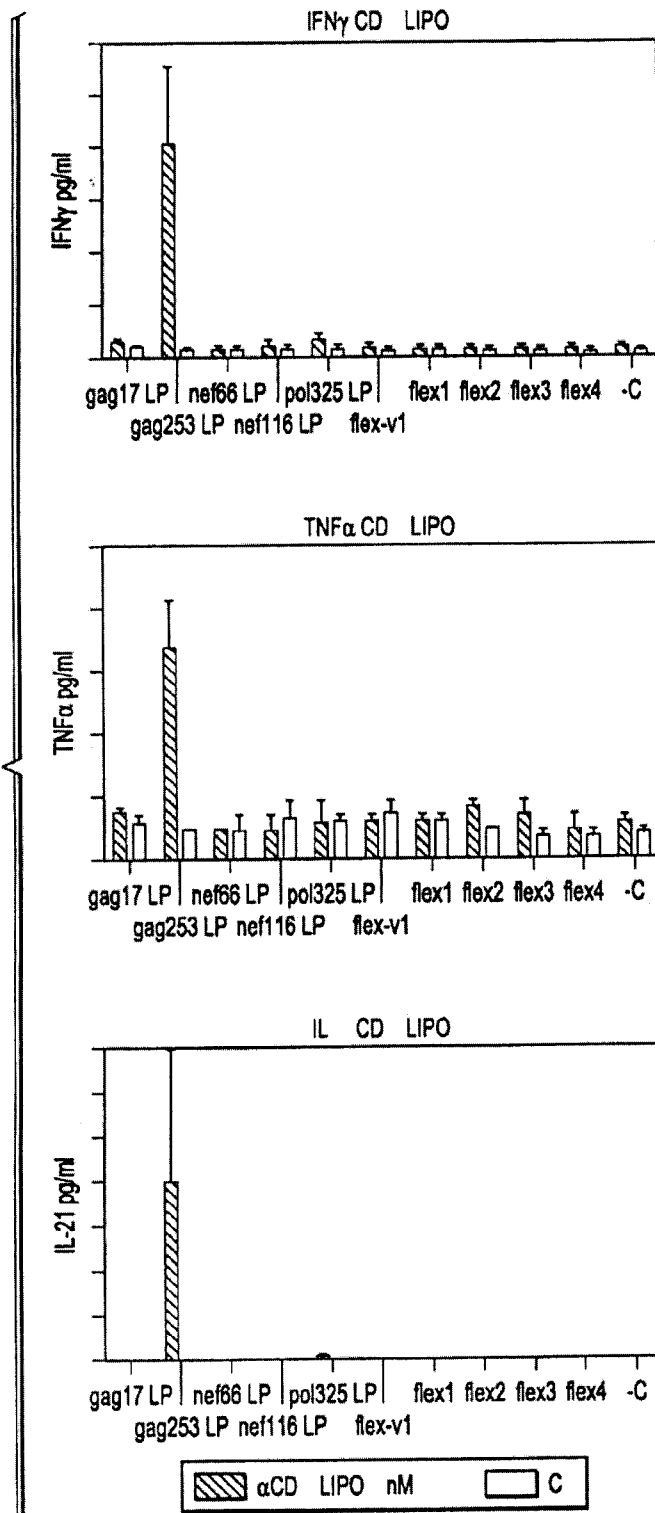
6/36/1

FIG. 10B



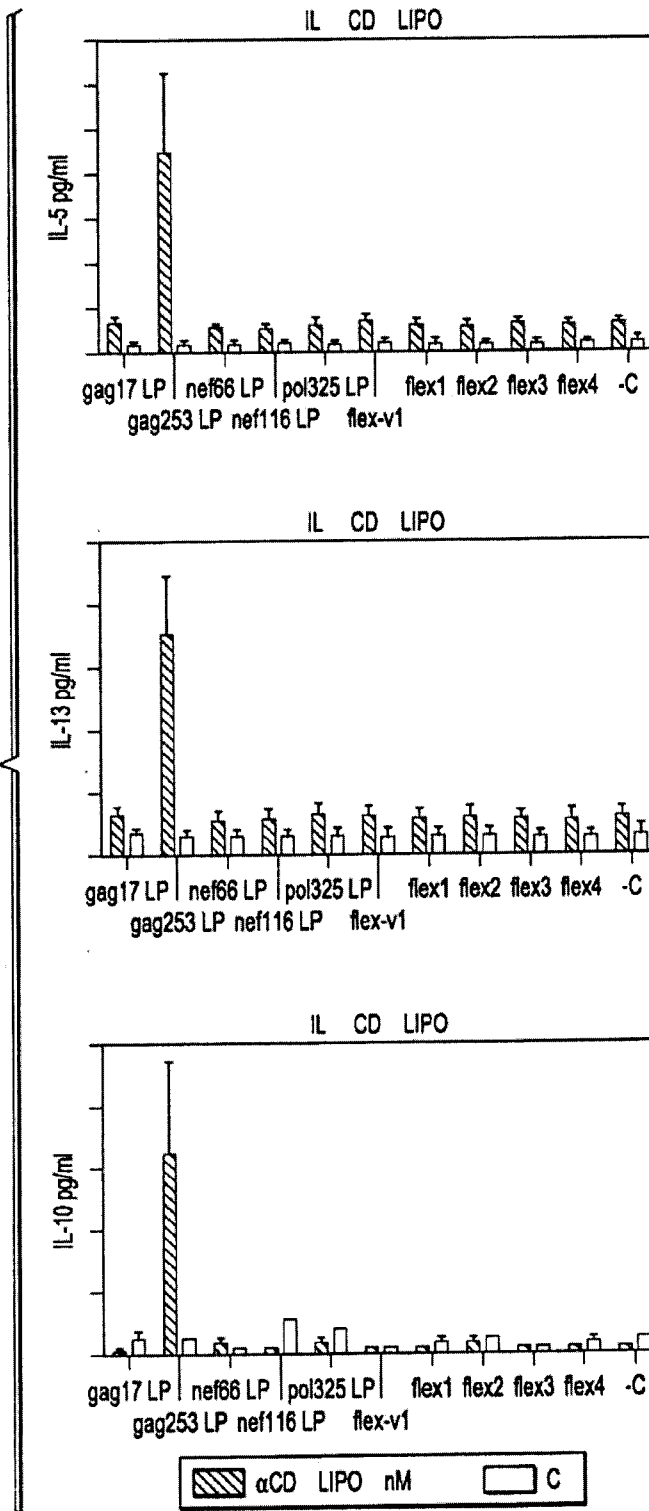
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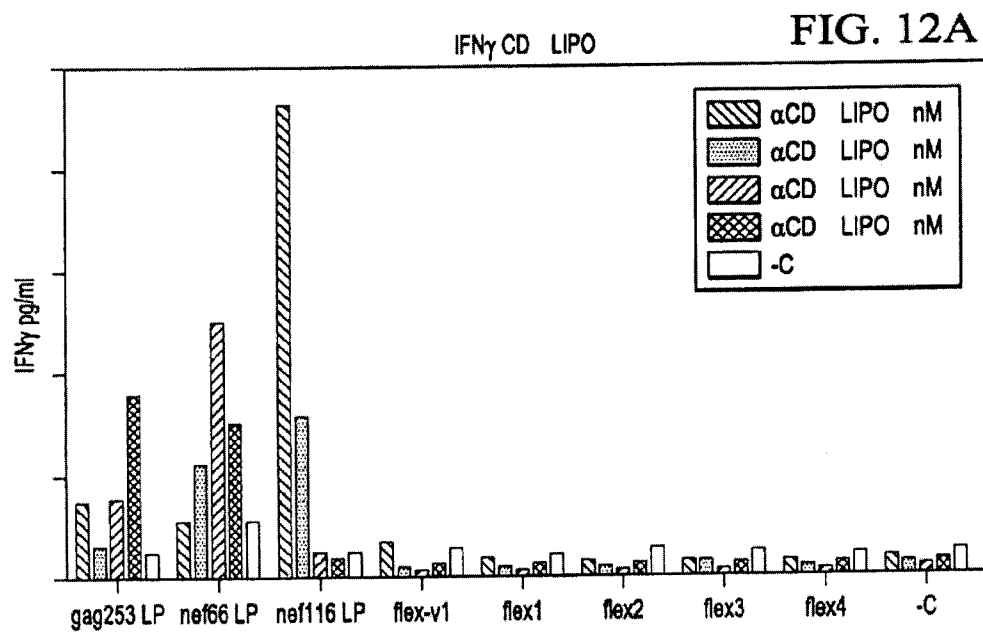
FIG. 11A



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FIG. 11B





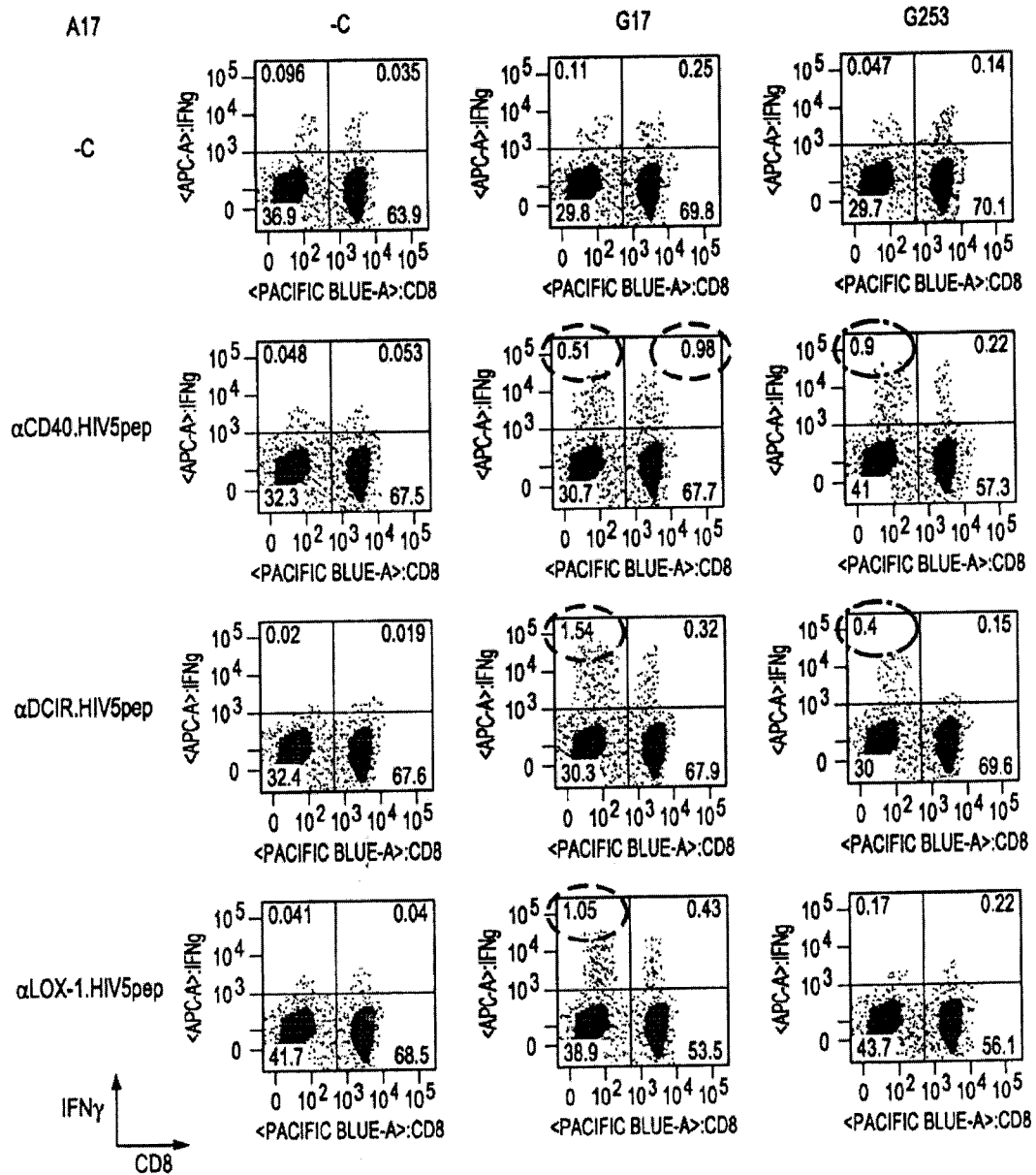


FIG. 12B-1

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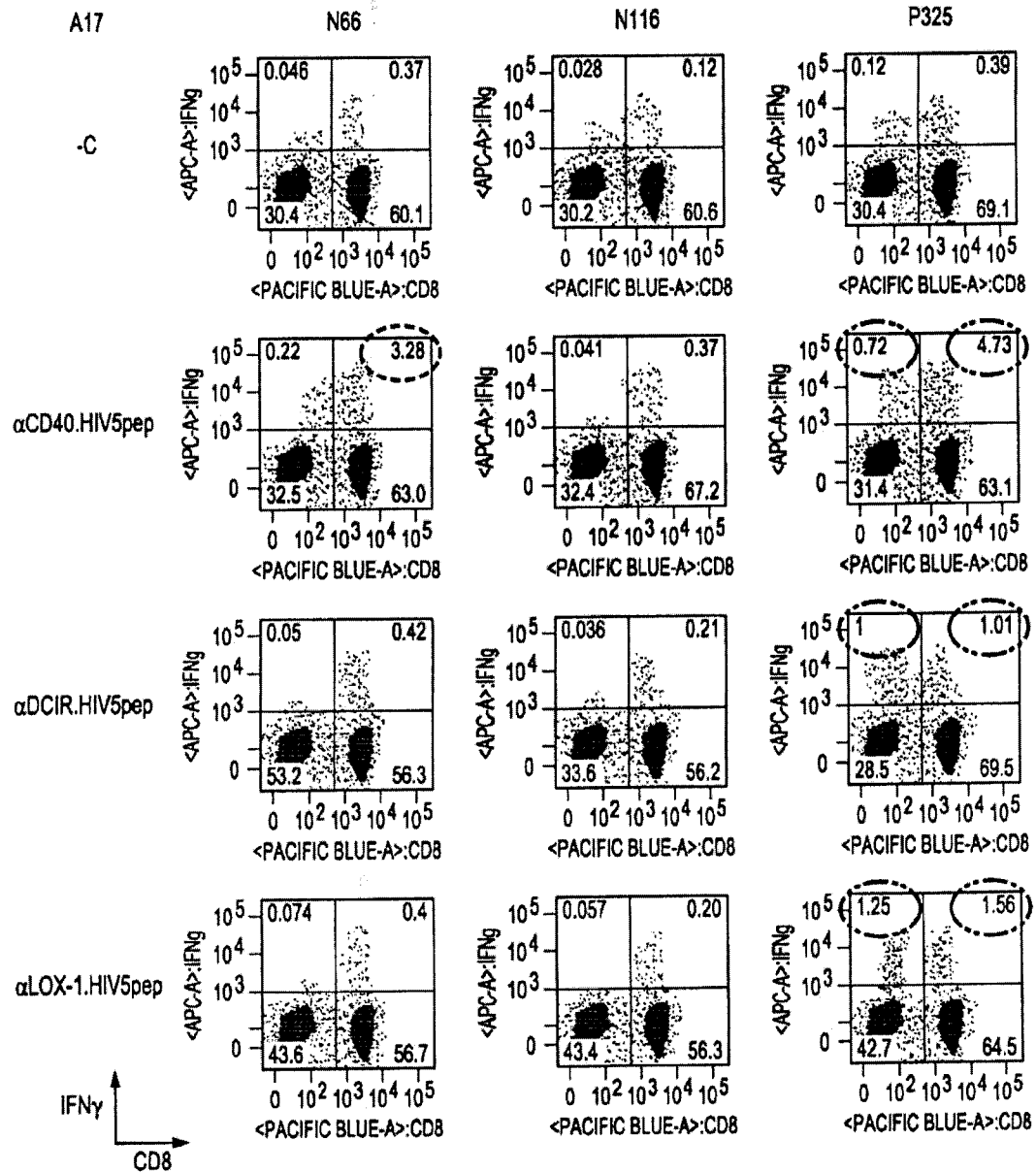


FIG. 12B-2



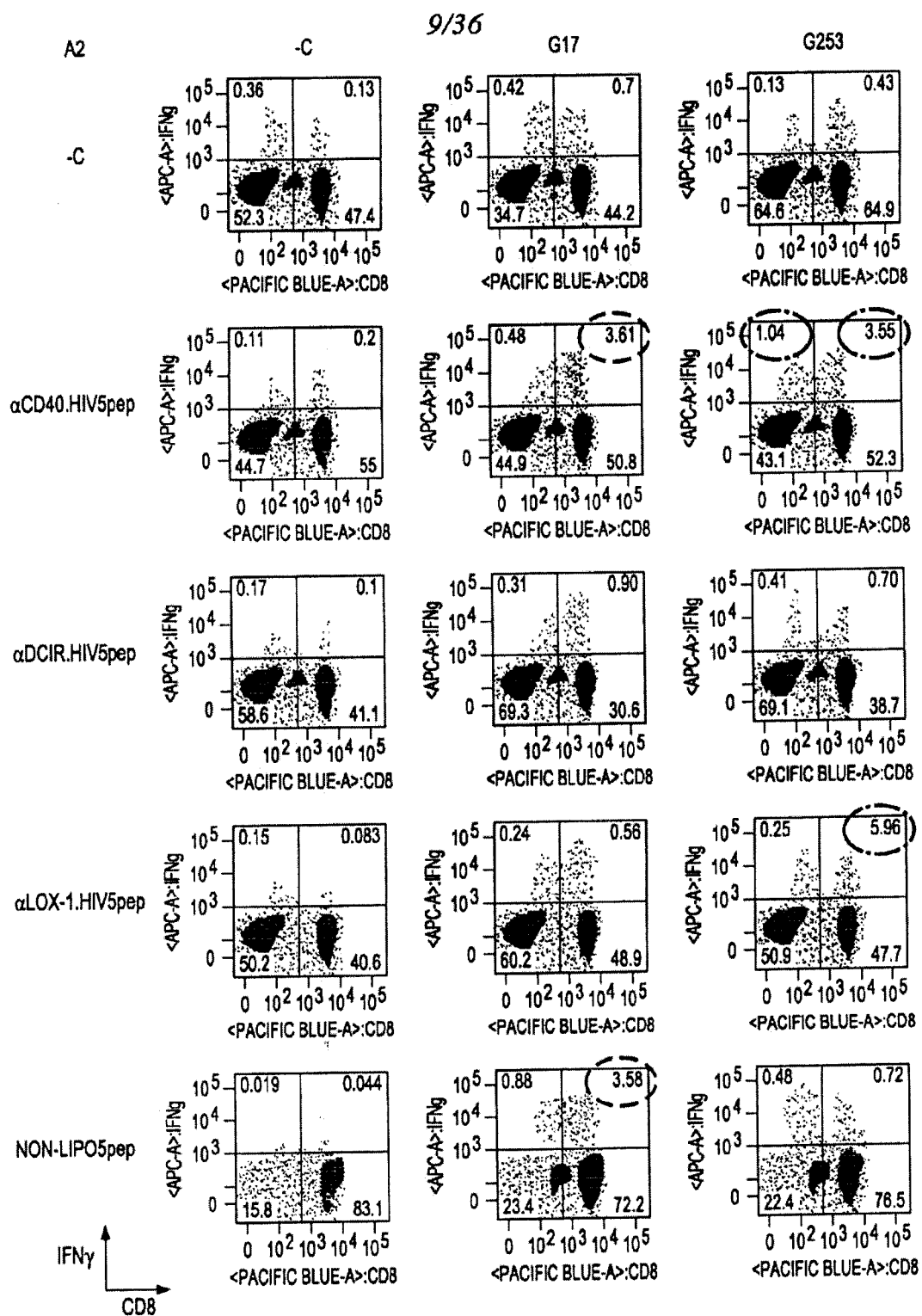


FIG. 12C-1

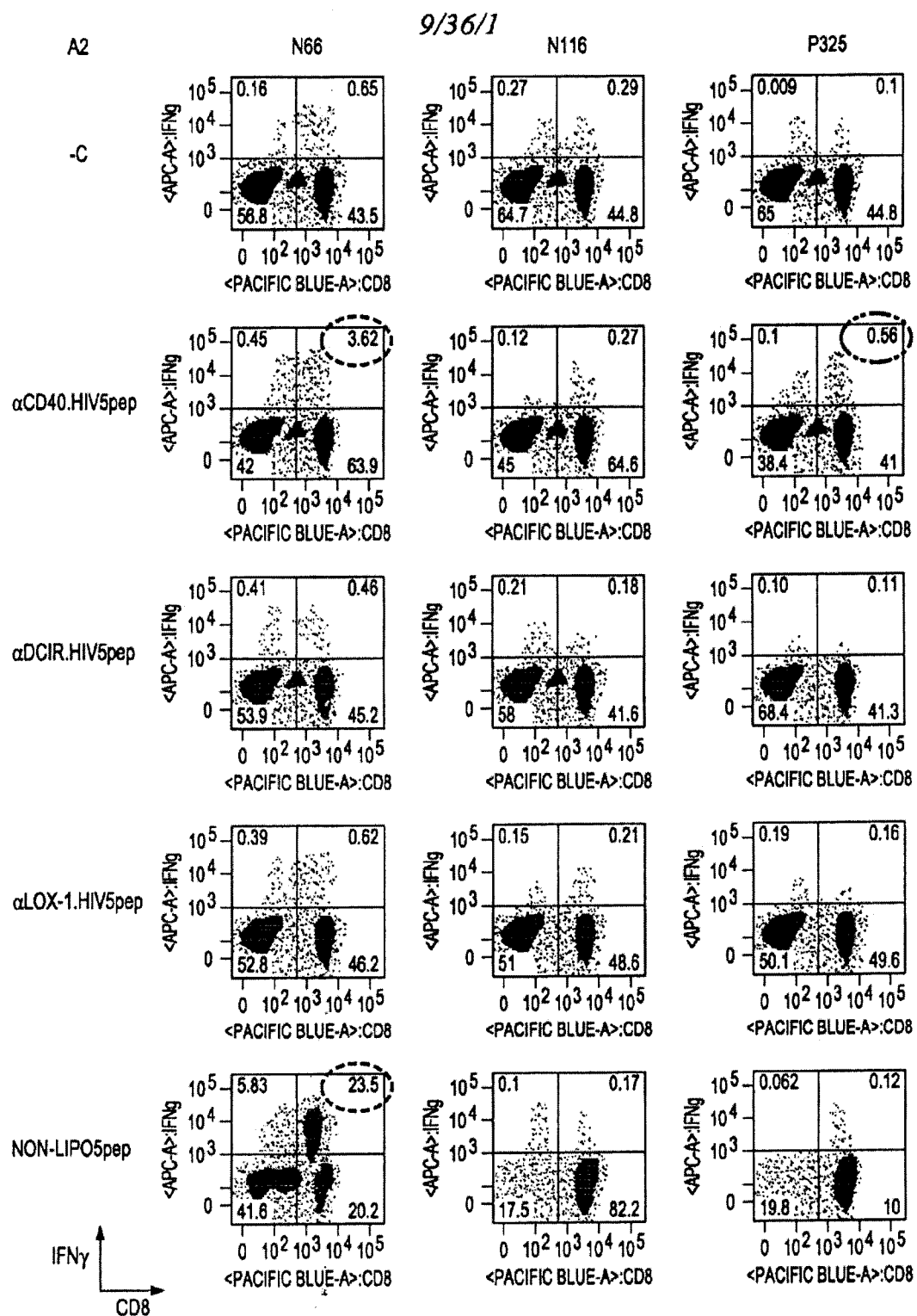


FIG. 12C-2

CD4<sup>+</sup> AND CD8<sup>+</sup> T CELLS RESPONSES AFTER TARGETING OF HIV  
PEPTIDE ANTIGENS THROUGH DIFFERENT ENDOCYTIC RECEPTORS

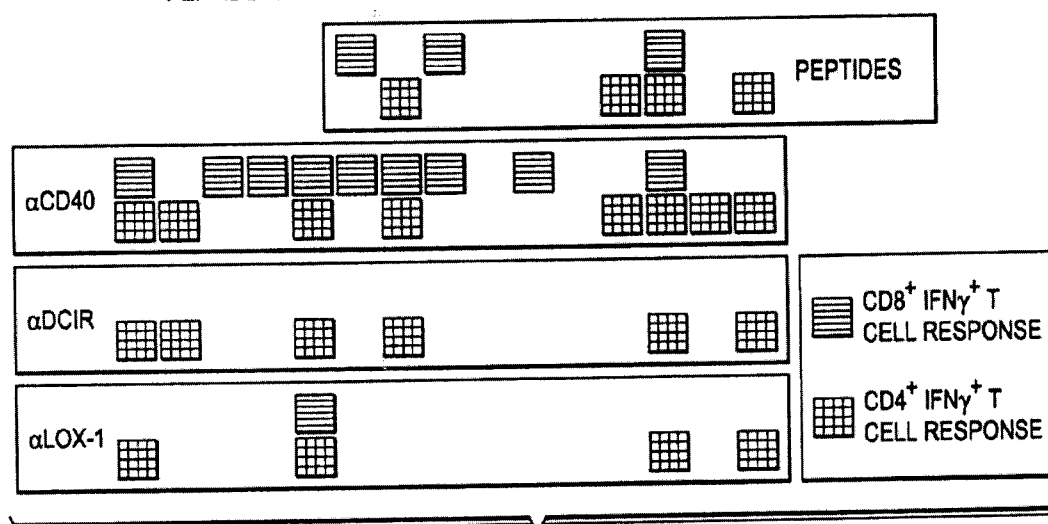


FIG. 12D

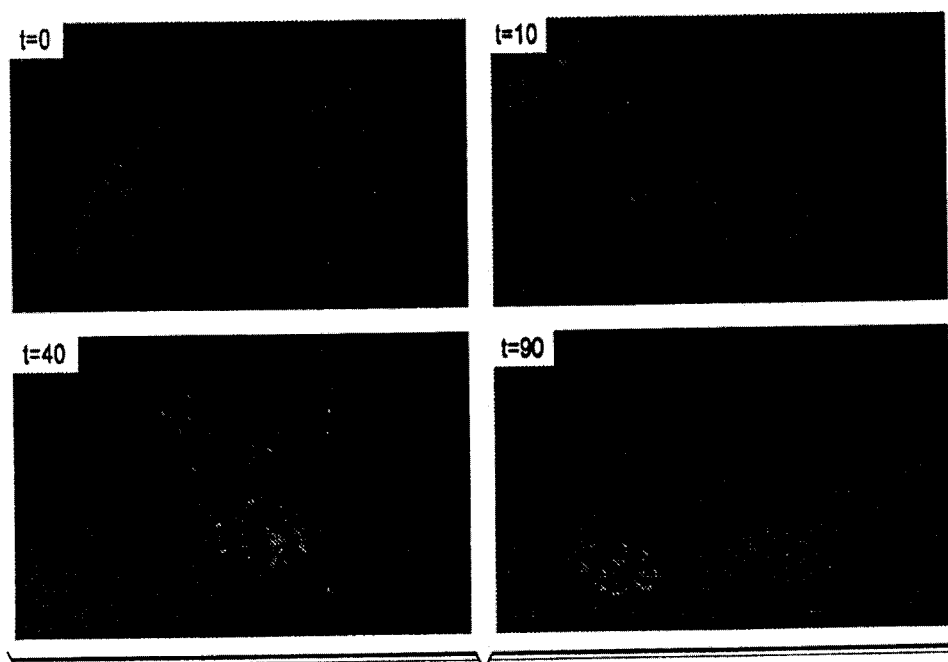
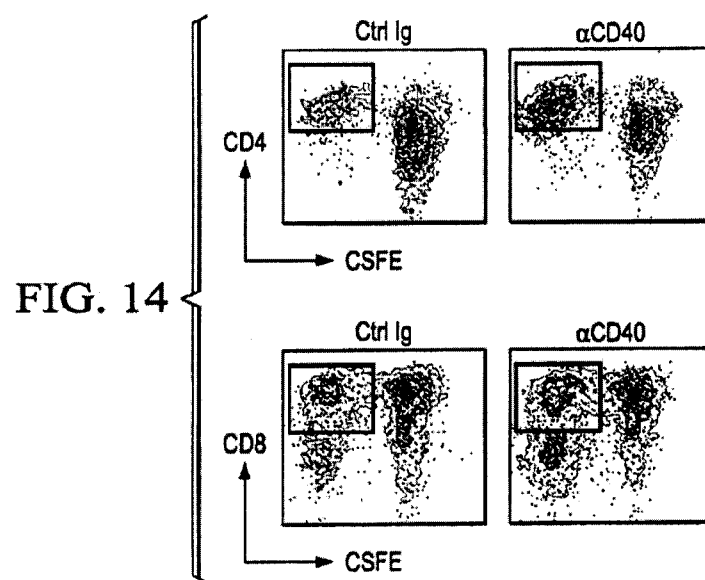
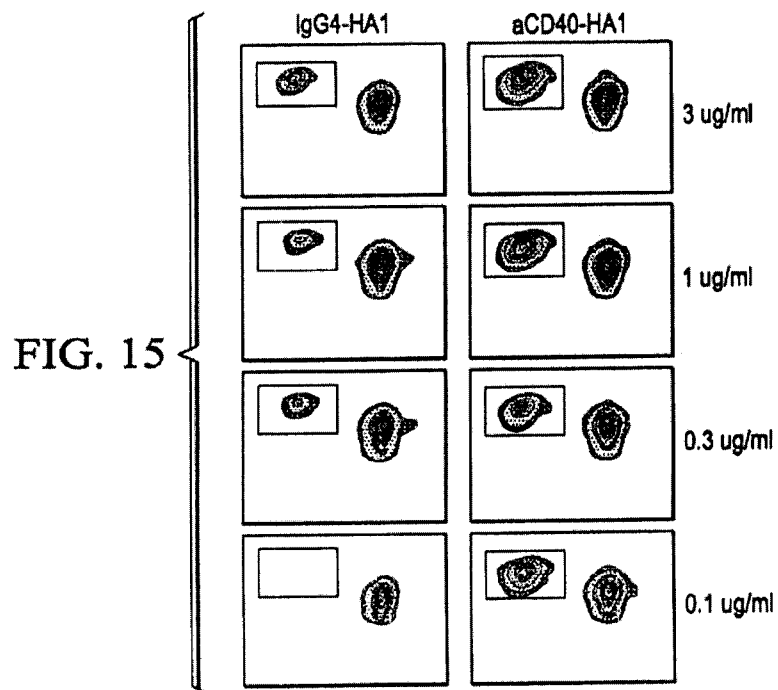


FIG. 13





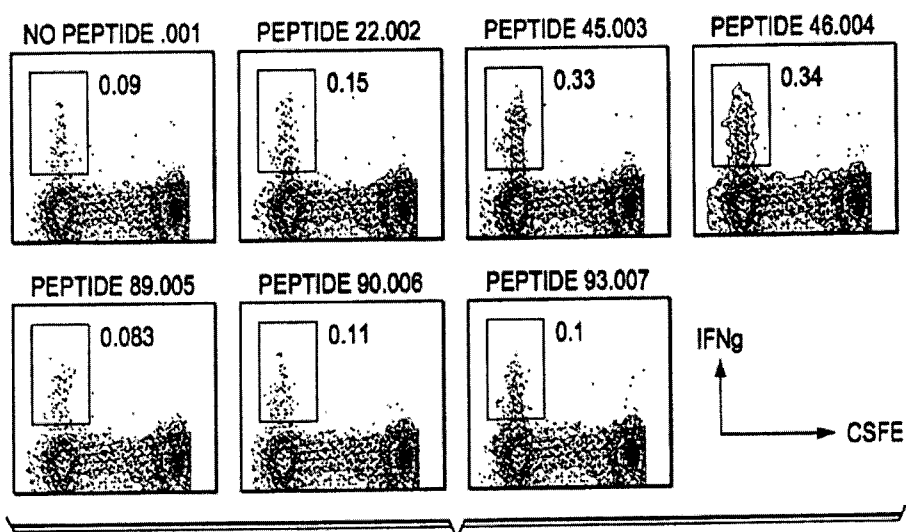
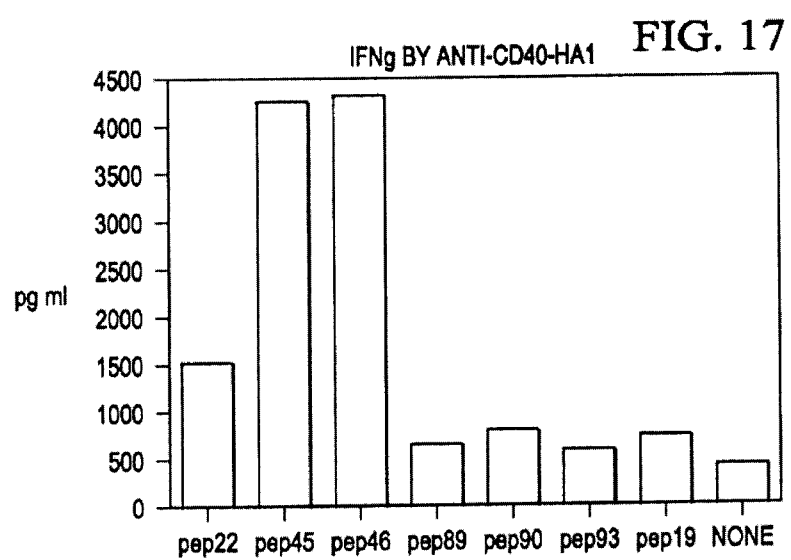


FIG. 16





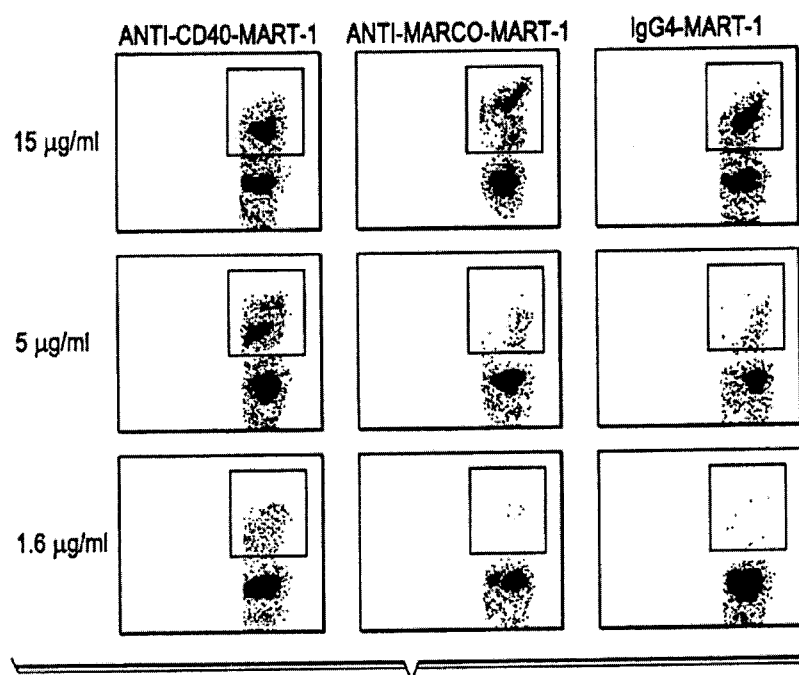


FIG. 18

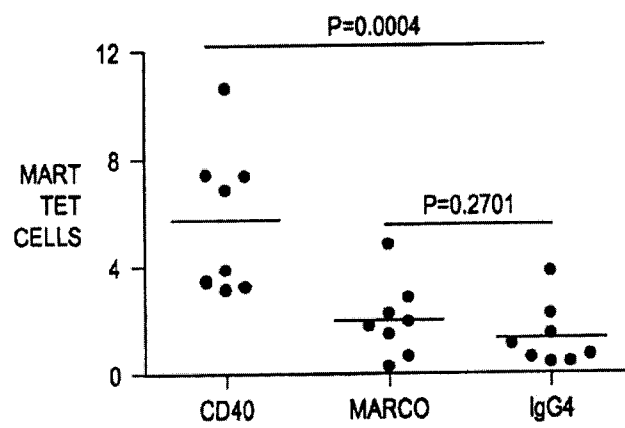


FIG. 19

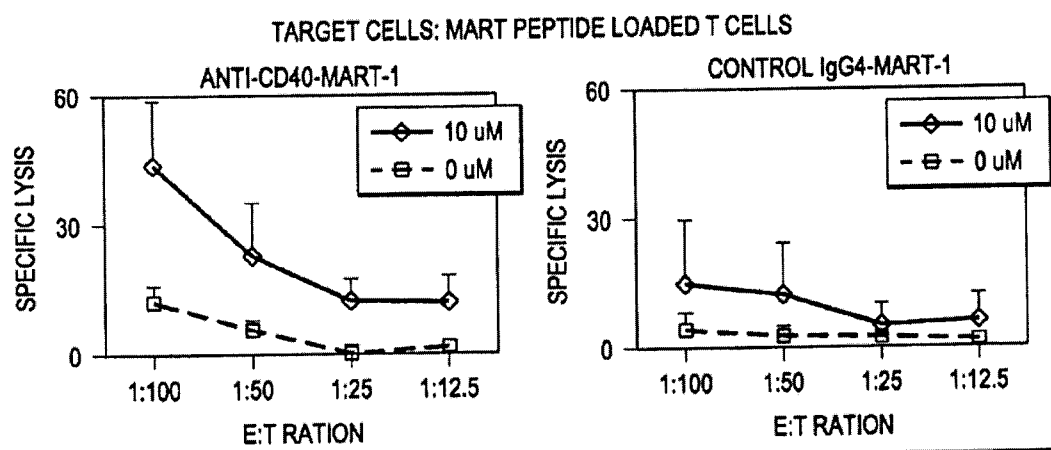


FIG. 20

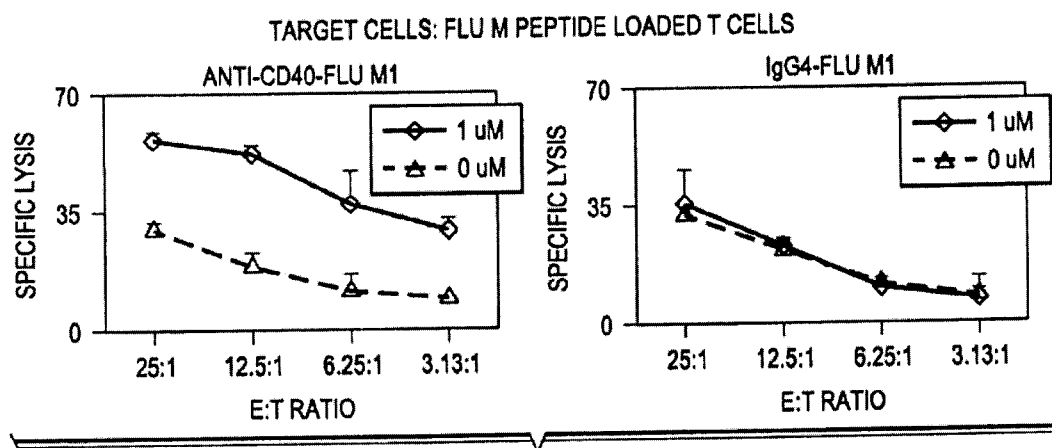


FIG. 21

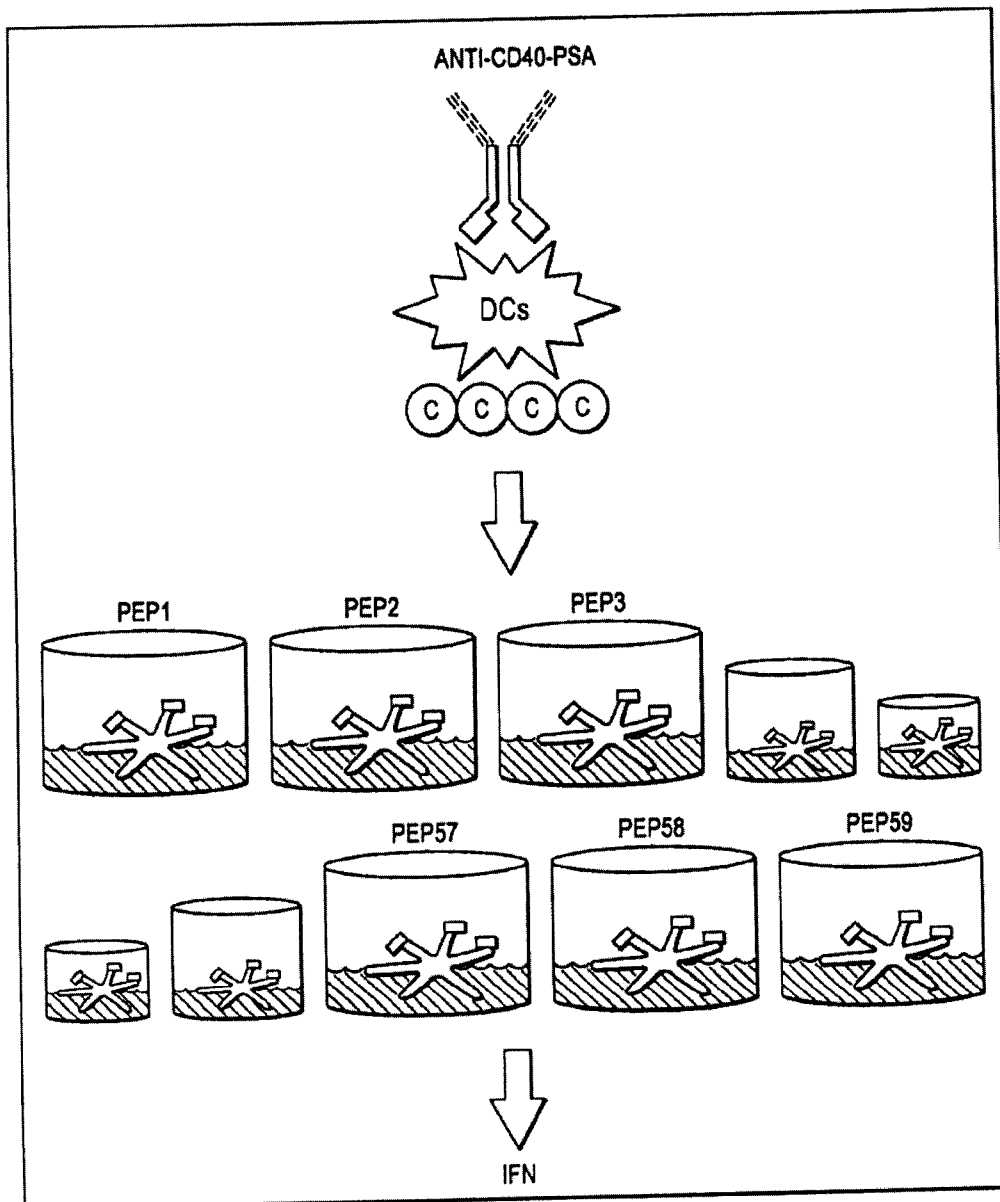
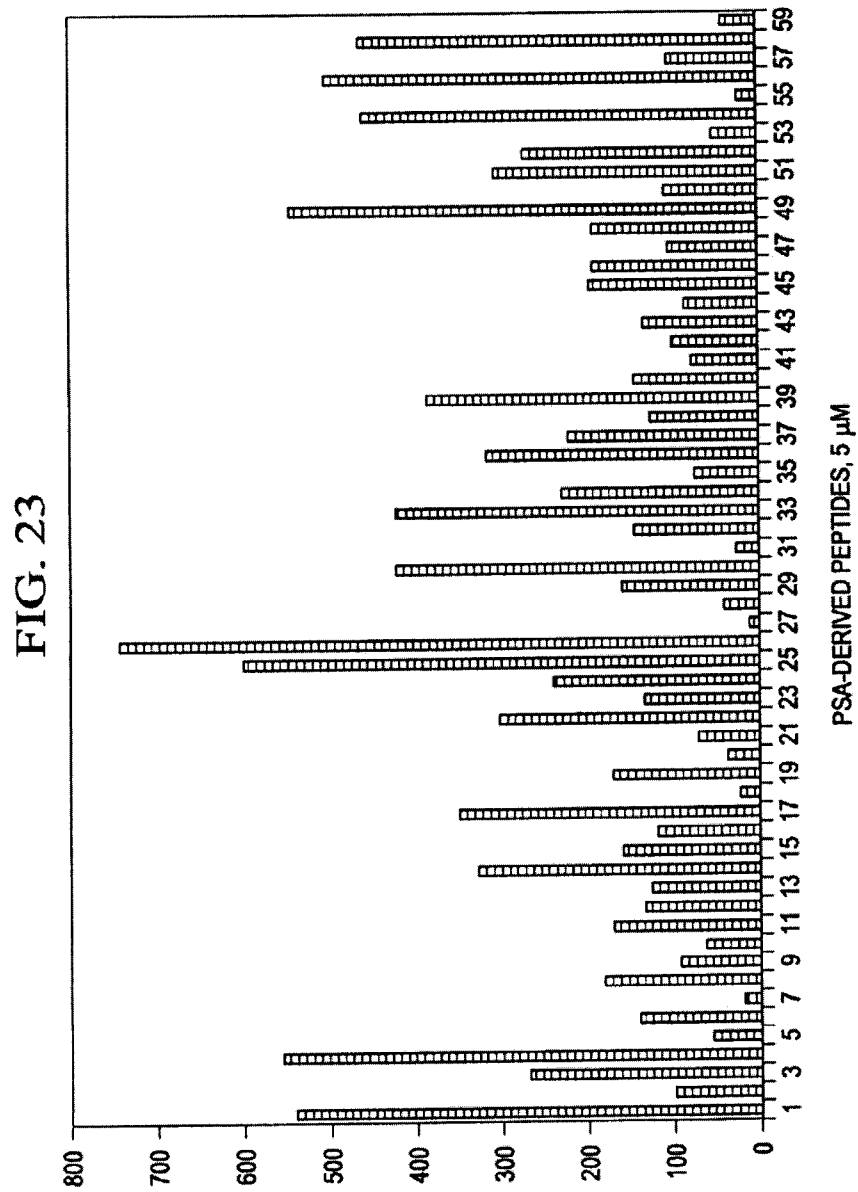


FIG. 22



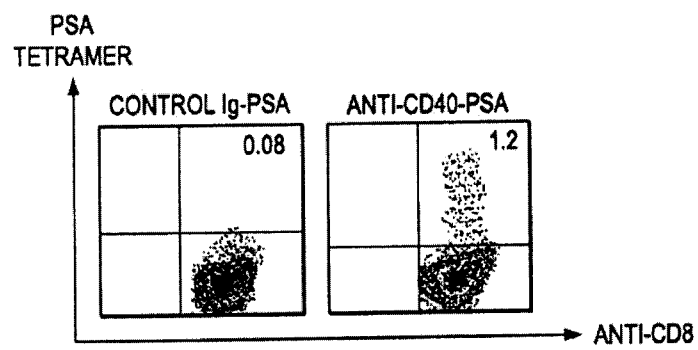
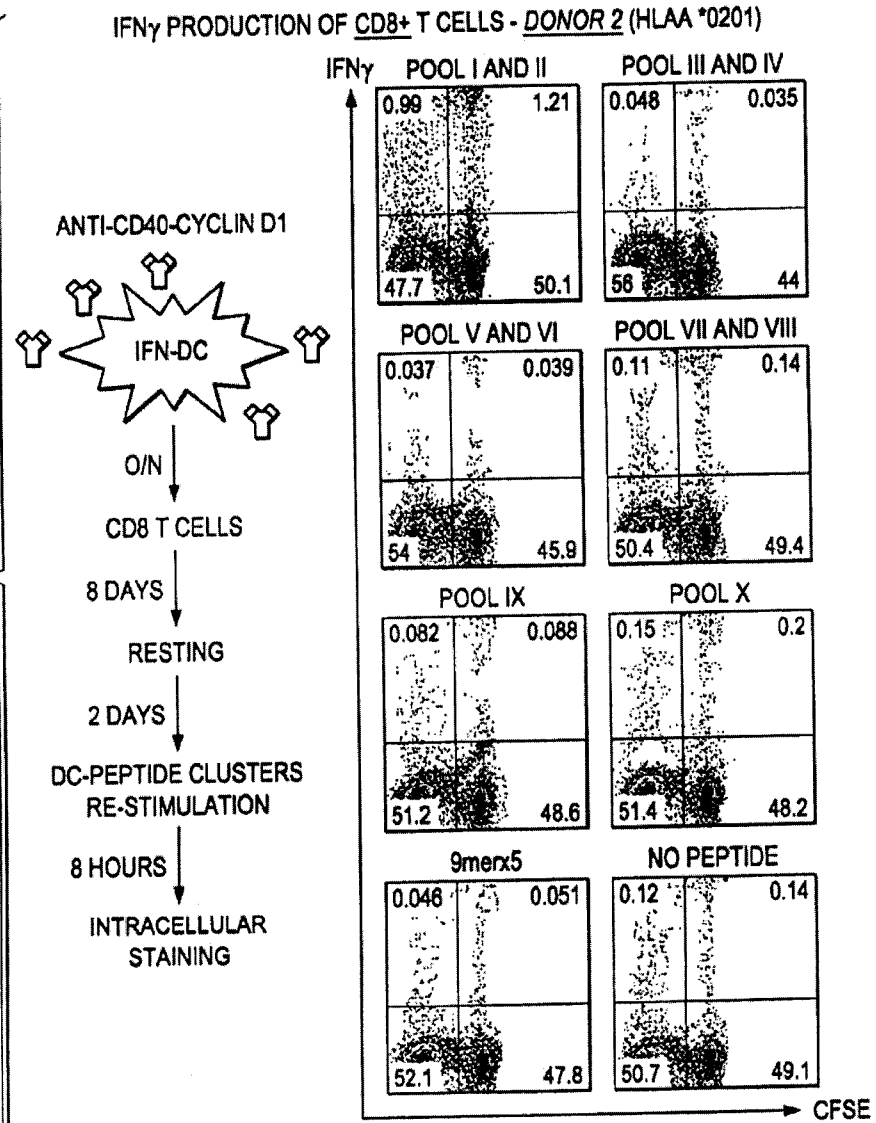


FIG. 24

FIG. 25





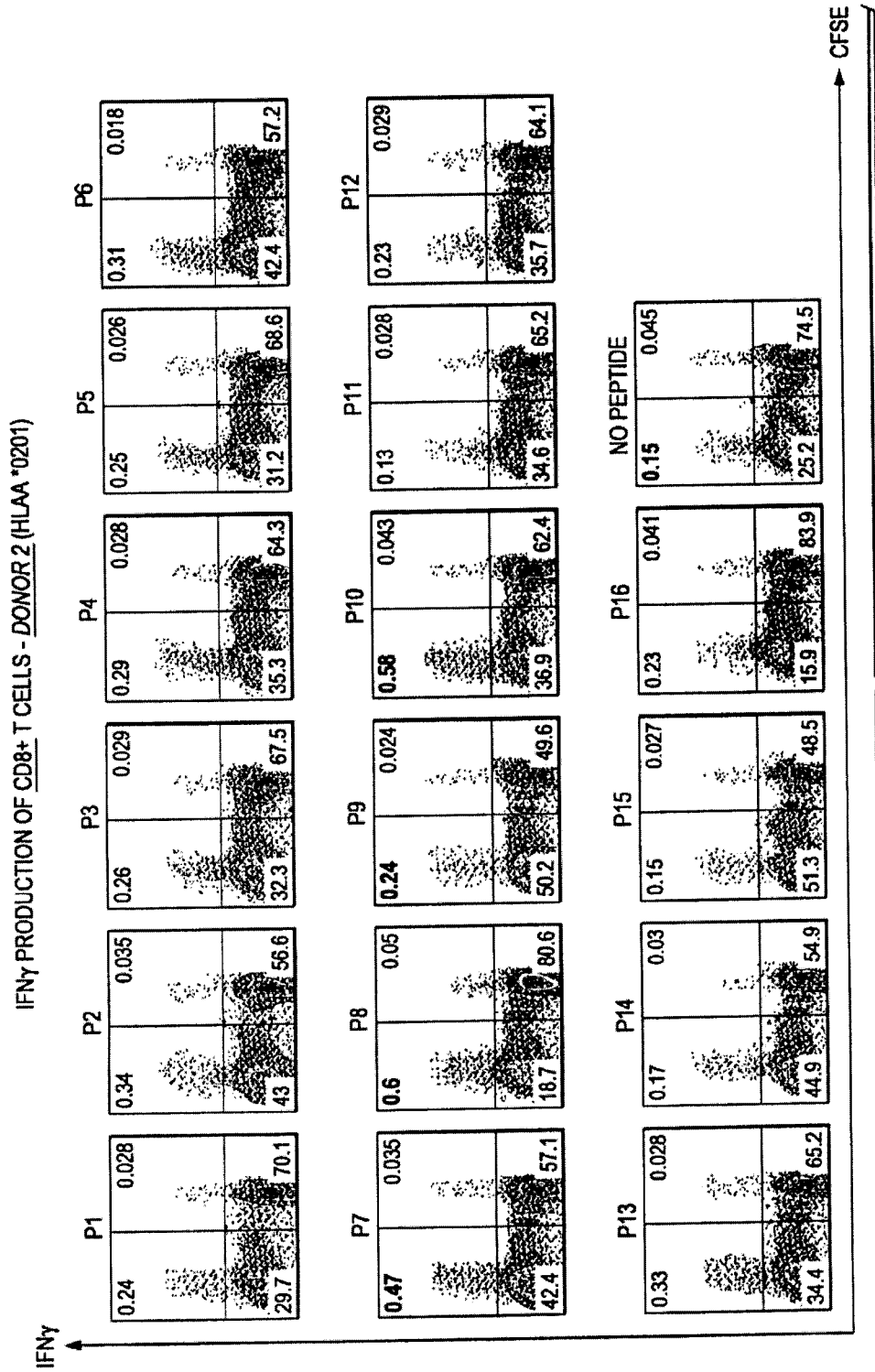


FIG. 26

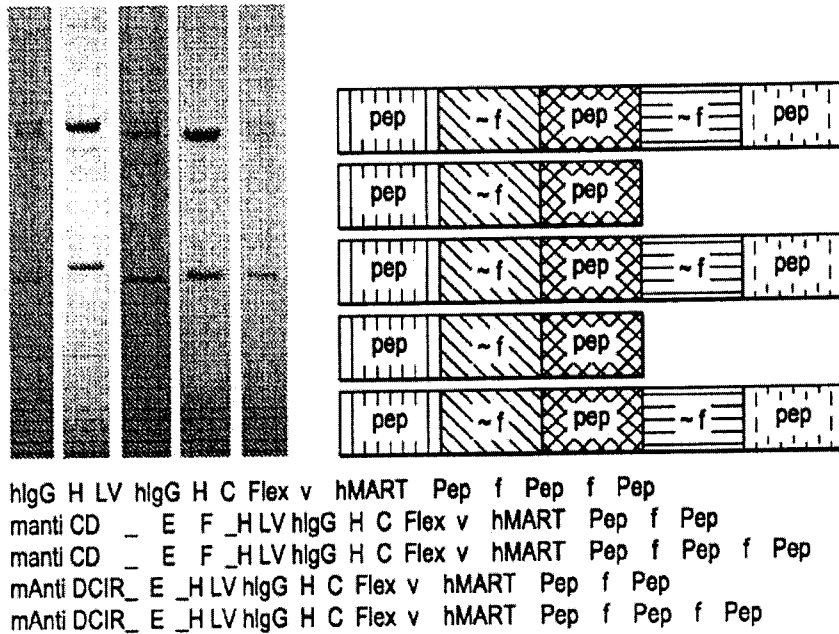


FIG. 27

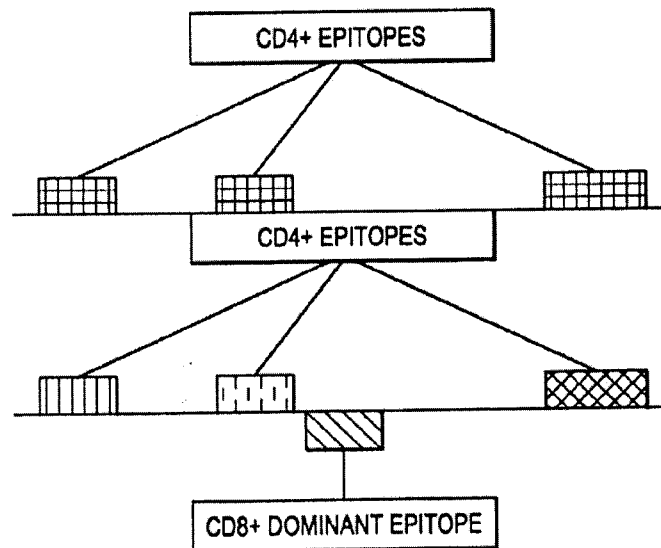


FIG. 28

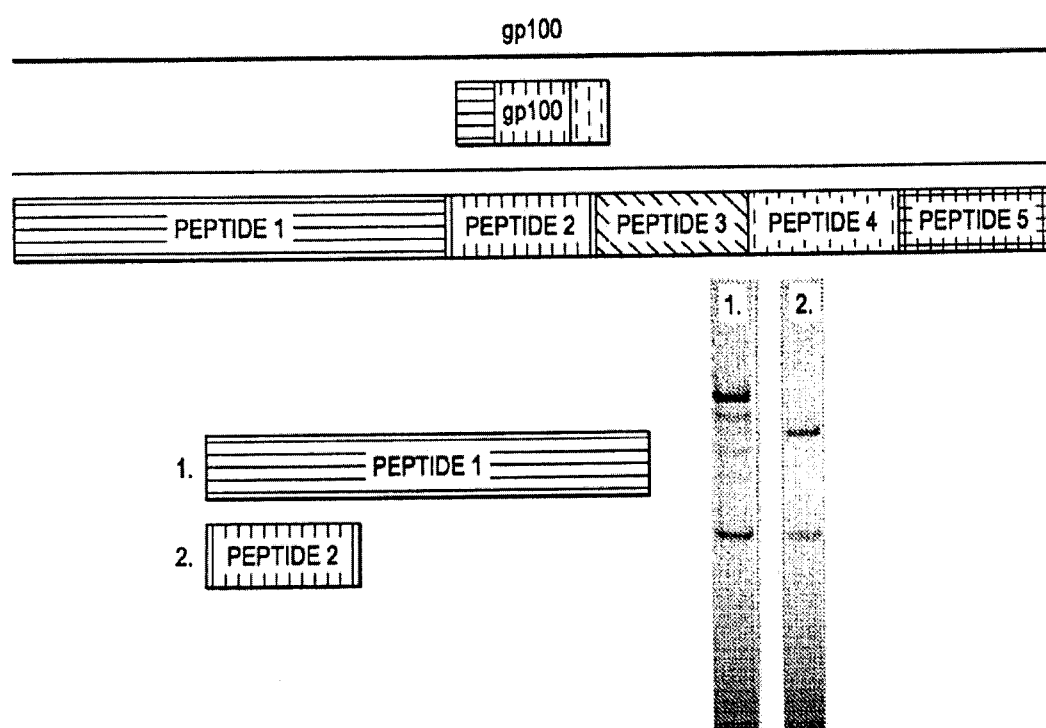


FIG. 29

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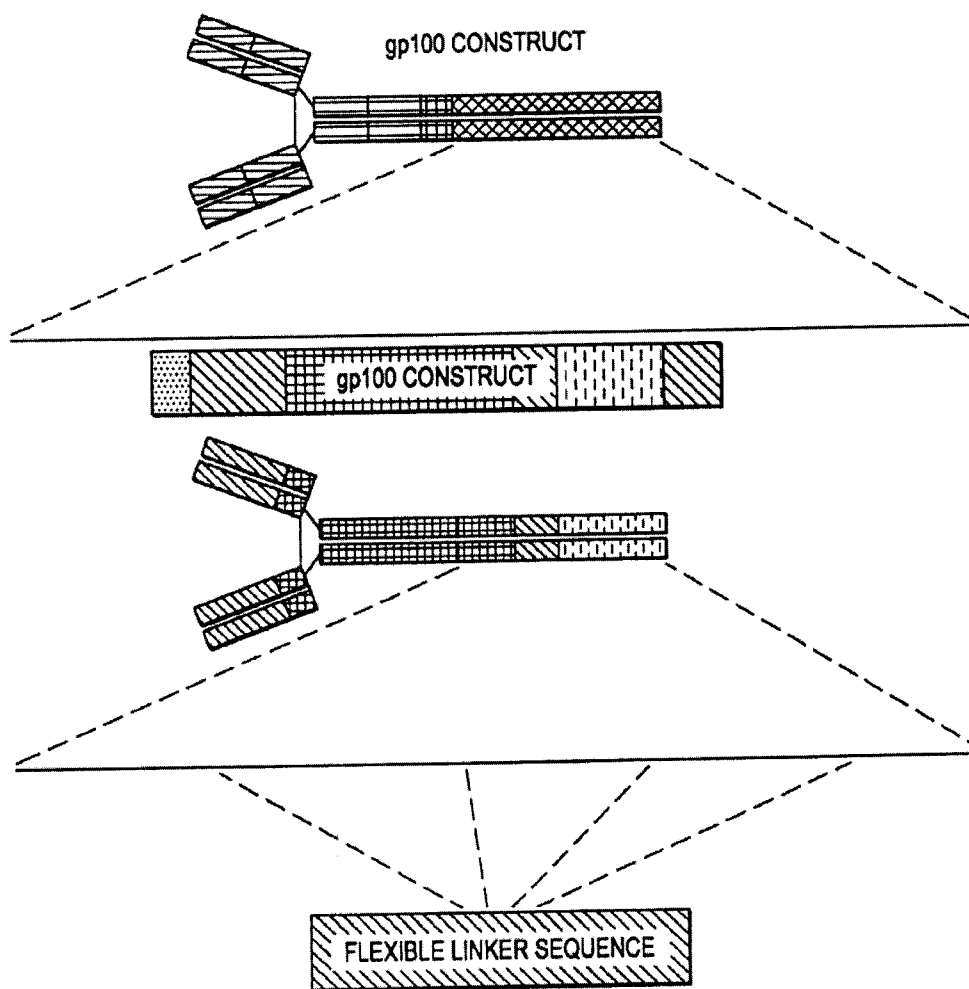


FIG. 30

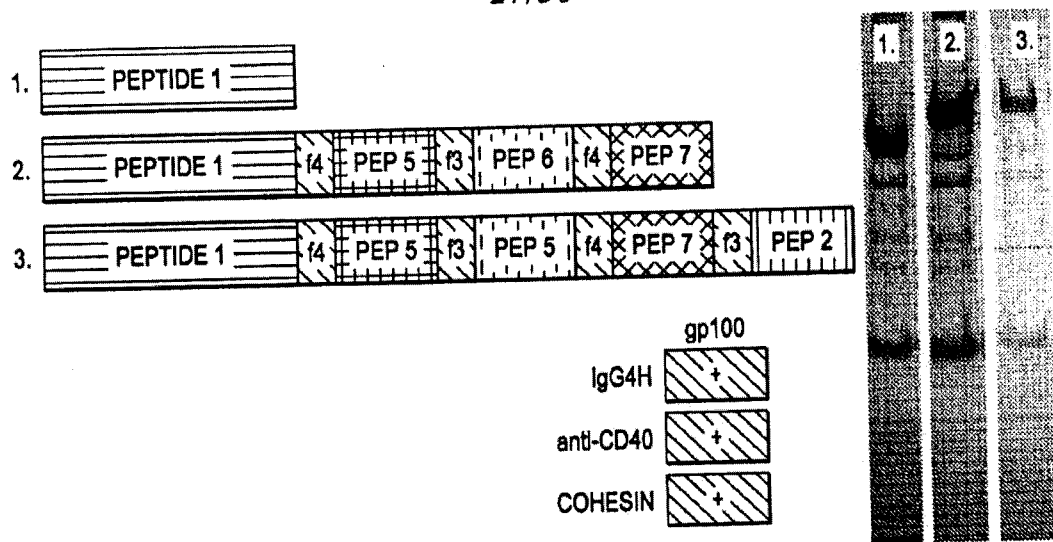


FIG. 31

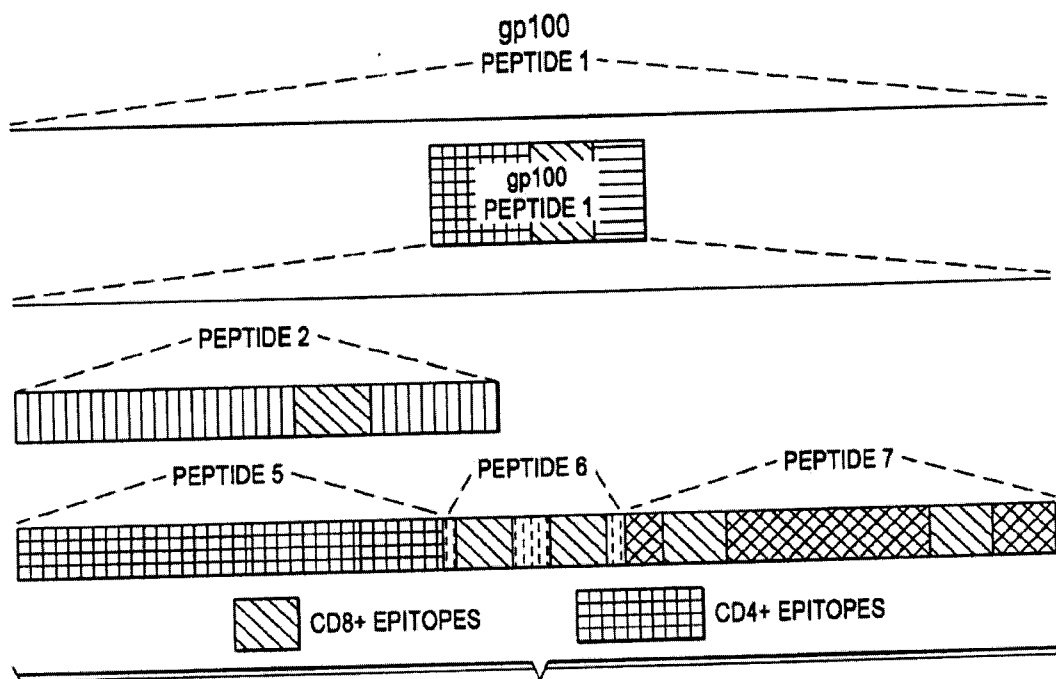


FIG. 32

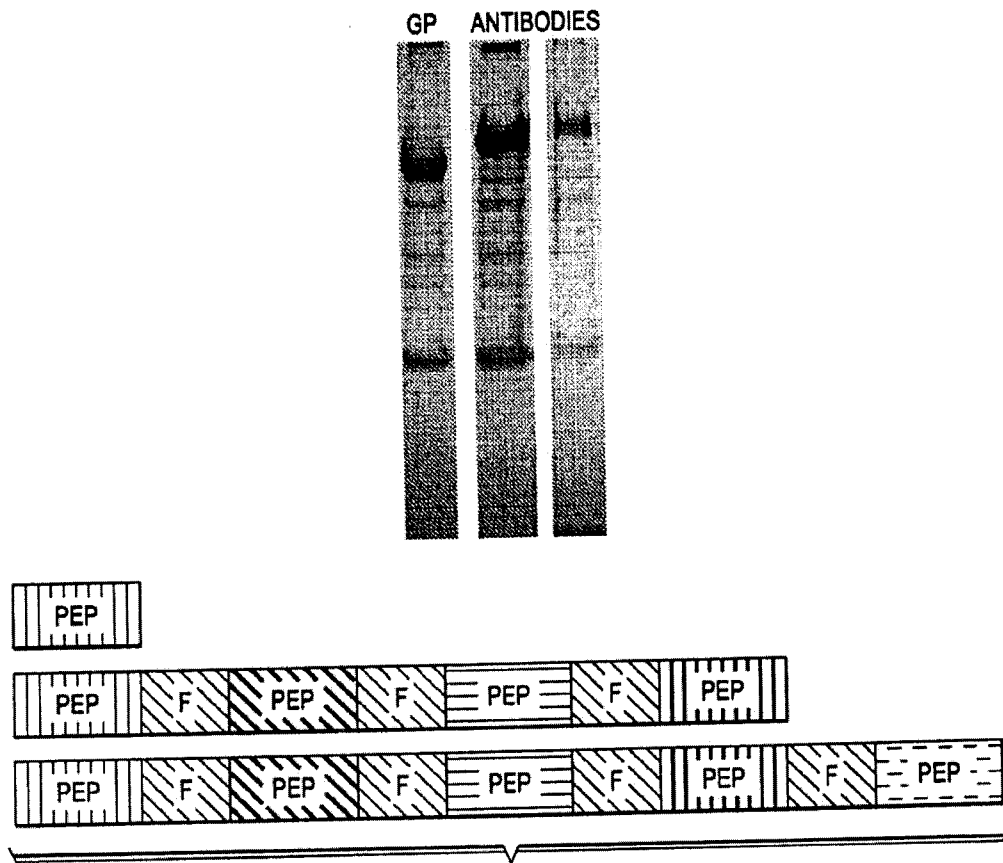


FIG. 33

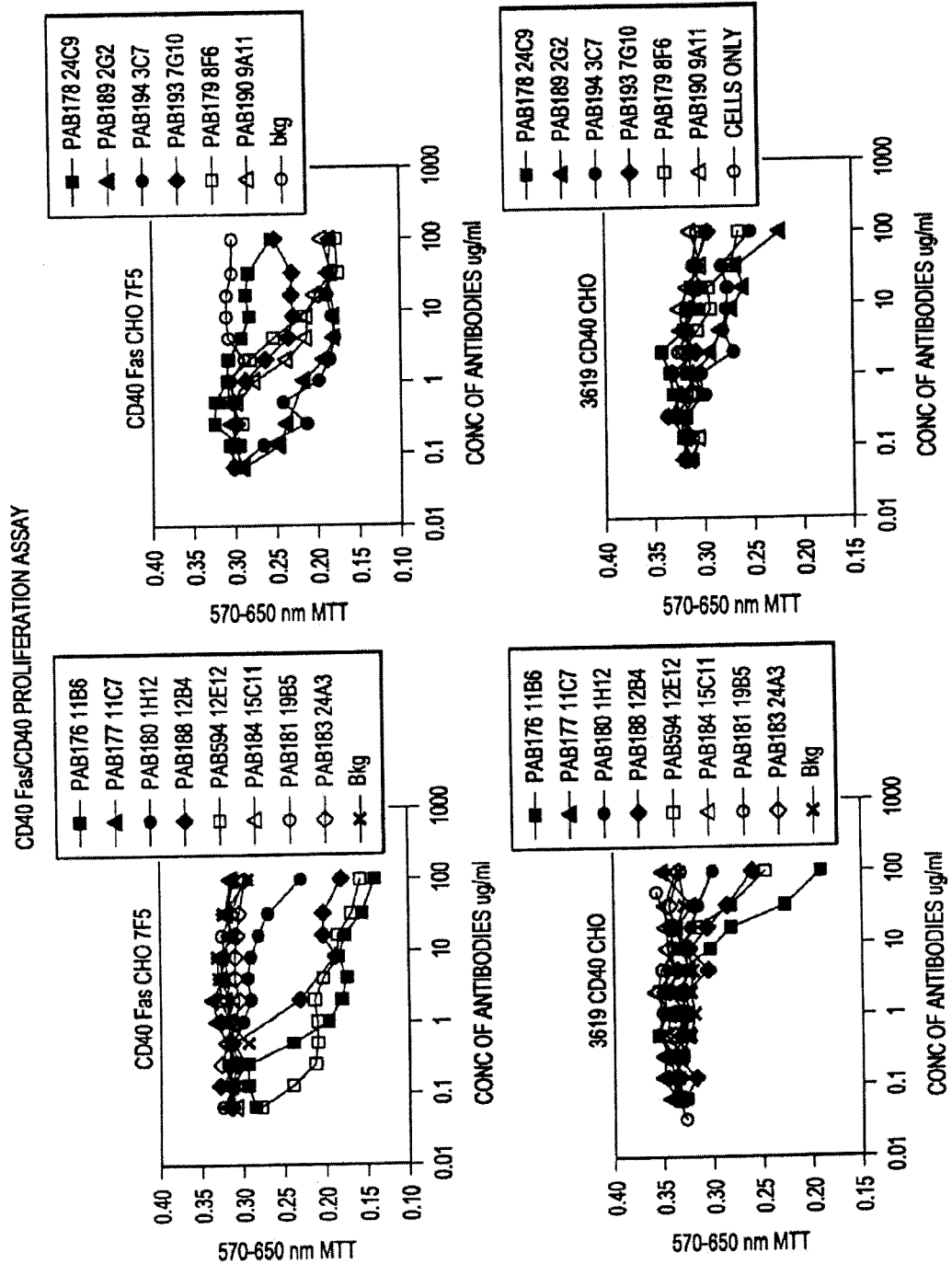


FIG. 34

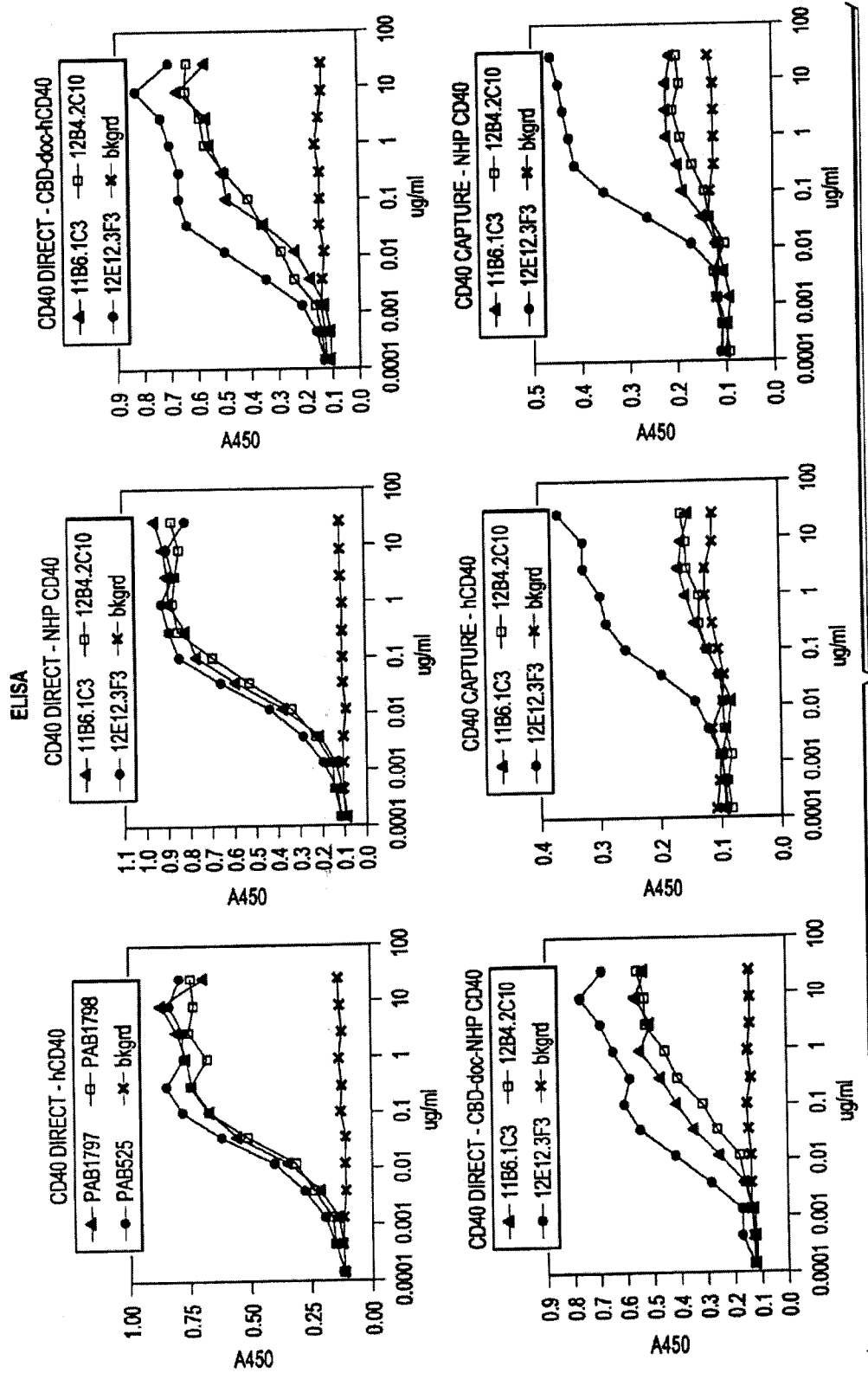


FIG. 35



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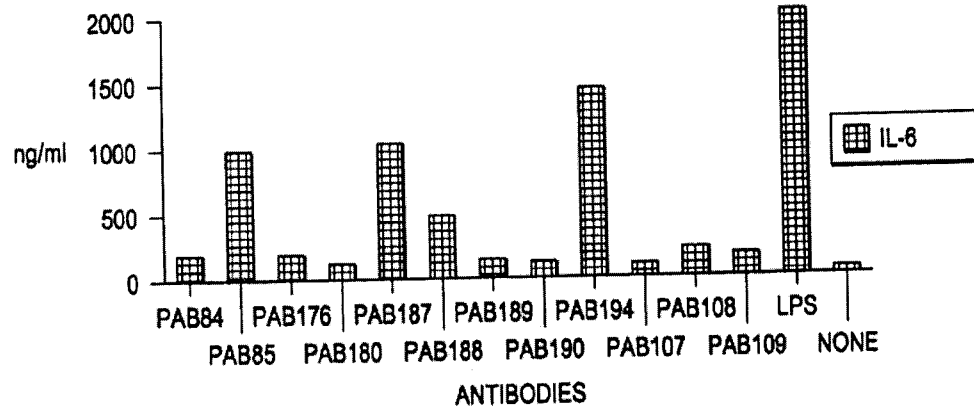


FIG. 36A

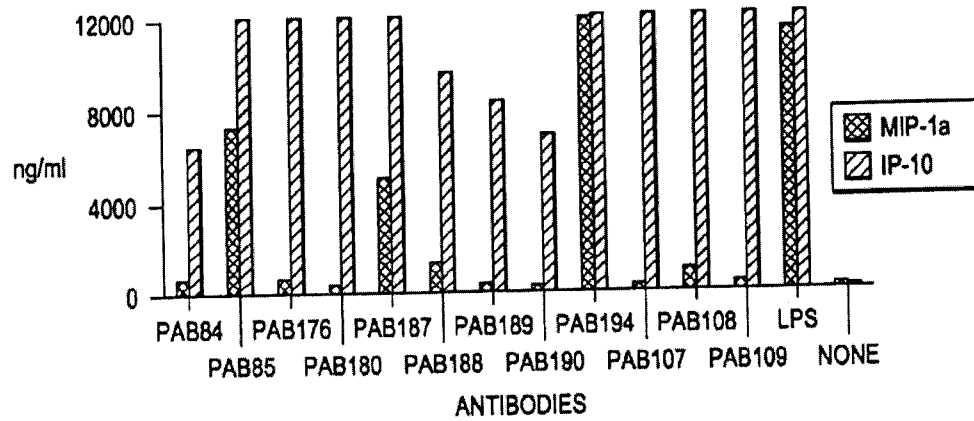


FIG. 36B

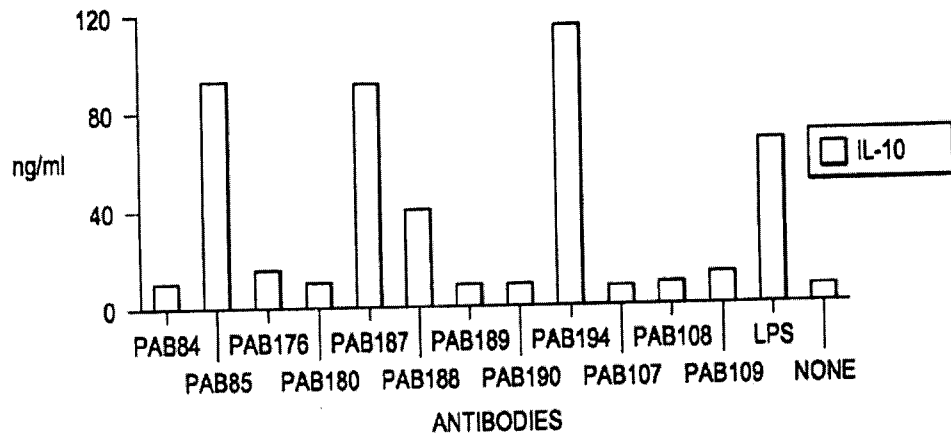


FIG. 36C

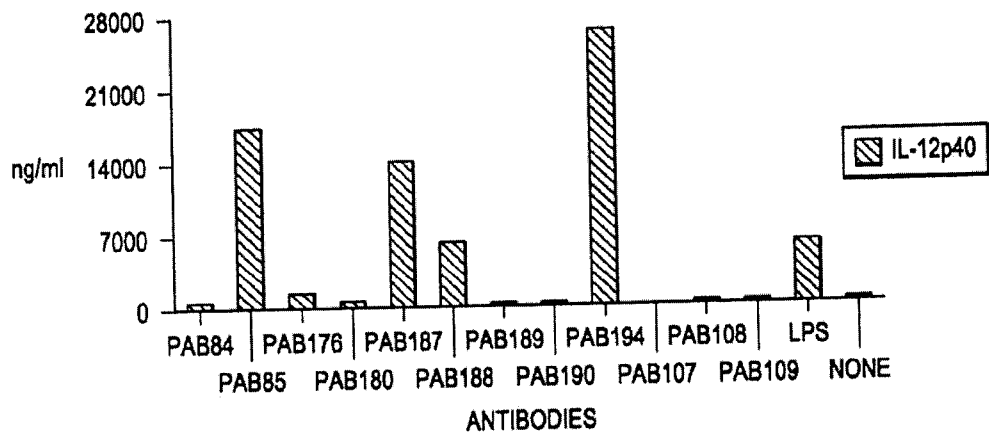


FIG. 36D

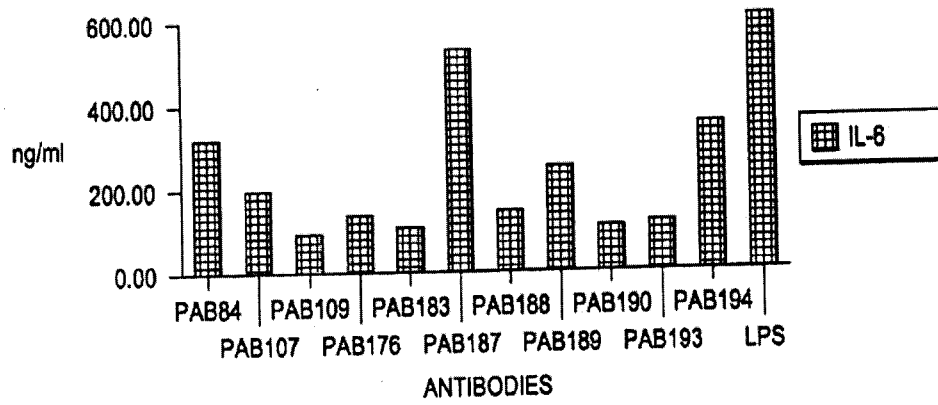


FIG. 37A

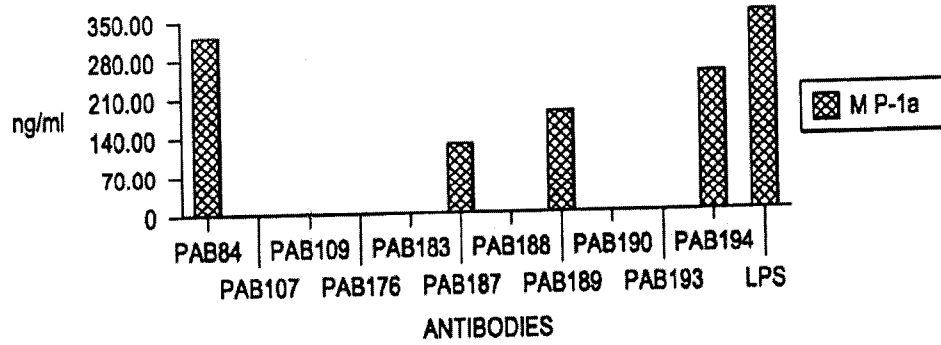


FIG. 37B

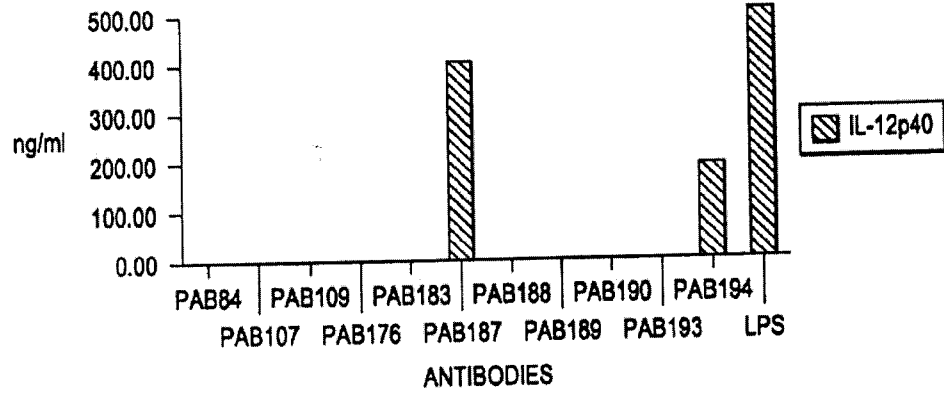


FIG. 37C

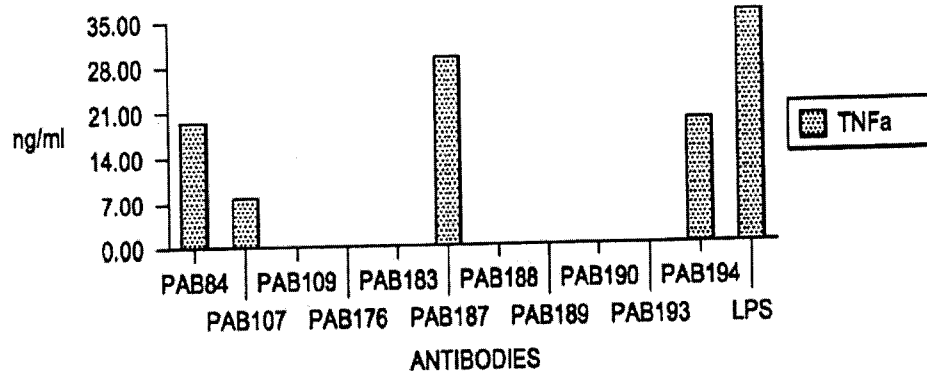


FIG. 37D

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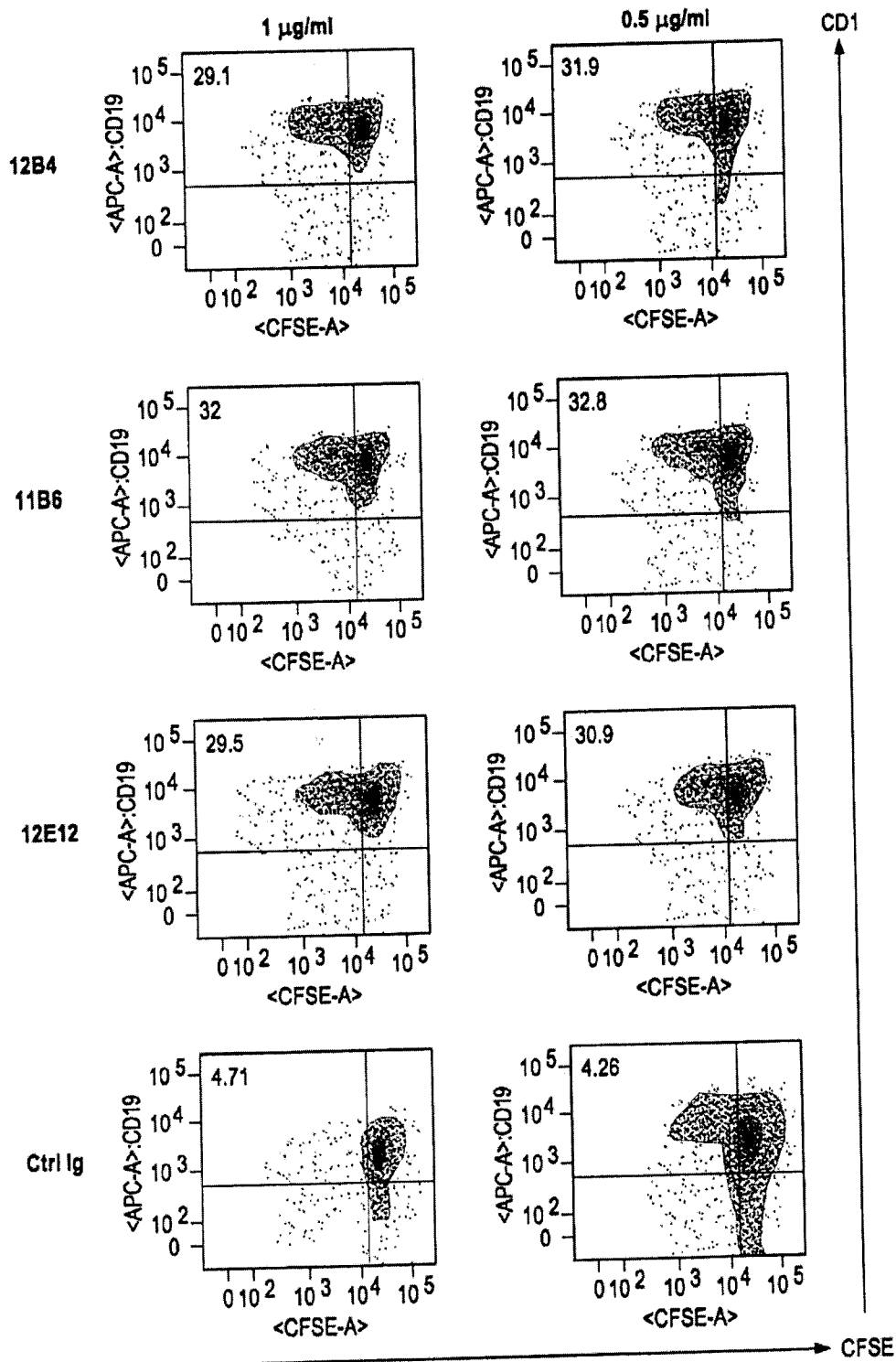


FIG. 38A

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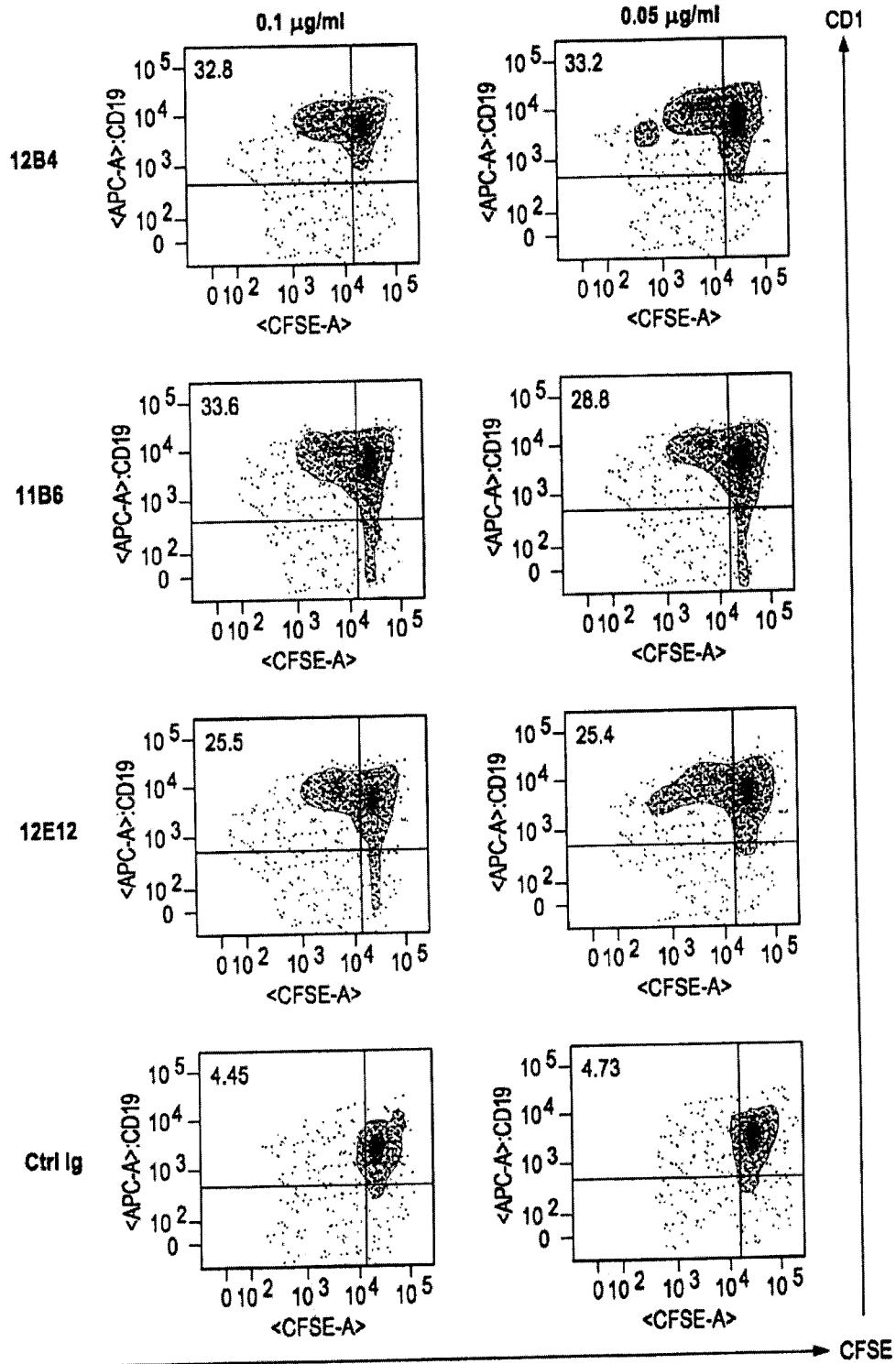
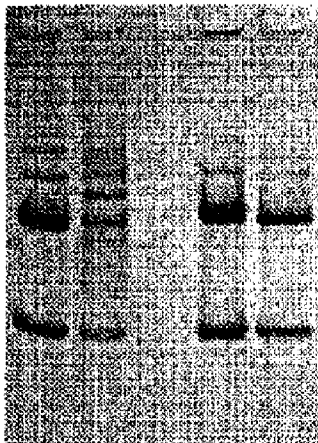


FIG. 38B

LAST SHEET ADDED  
TOTAL OF SHEETS - 48



gag

gag

nef

nef

pol