

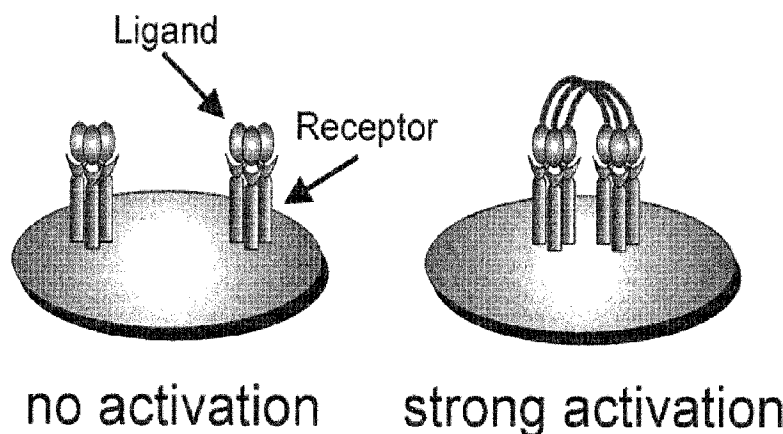


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(54) **Titre :** COMPOSITION COMPOSEE D'UN ANTIGENE LIE A UN LIGAND DE LA SUPERFAMILLE DES TNF
(54) **Title:** COMPOSITION COMPRISED OF ANTIGEN LINKED TO A TNF SUPERFAMILY LIGAND

TNFSF activity often requires ≥ 2 trimers
Receptor clustering is needed for activation



(57) **Abrégé/Abstract:**

The invention provides fusion proteins comprising antigens of infectious disease agents and cancer cells linked to multiple-trimer forms of TNF SuperFamily (TNFSF) ligands. The TNFSFs serve as vaccine adjuvants for increasing the immune response to the antigens. In particular, a fusion polypeptide strand that self-assembles inside cells into a multiple-trimer form of CD40 ligand (CD40L, TNFSF5) is provided. Other similar fusion proteins are also disclosed. The fusion proteins can be delivered to a host as isolated proteins, as nucleic acids used directly in DNA vaccination or carried and expressed by a viral vector such as adenovirus. In addition to use as a vaccine to prevent or ameliorate disease caused by an infectious agent, compositions of the invention may be used for the treatment of ongoing infection or for cancer immunotherapy.



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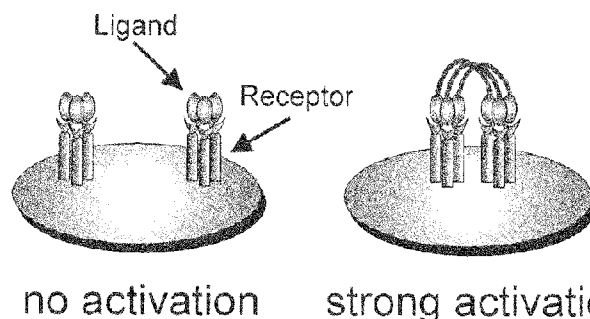


FIG. 1

(57) **Abstract**: The invention provides fusion proteins comprising antigens of infectious disease agents and cancer cells linked to multiple-trimer forms of TNF SuperFamily (TNFSF) ligands. The TNFSFs serve as vaccine adjuvants for increasing the immune response to the antigens. In particular, a fusion polypeptide strand that self-assembles inside cells into a multiple-trimer form of CD40 ligand (CD40L, TNFSF5) is provided. Other similar fusion proteins are also disclosed. The fusion proteins can be delivered to a host as isolated proteins, as nucleic acids used directly in DNA vaccination or carried and expressed by a viral vector such as adenovirus. In addition to use as a vaccine to prevent or ameliorate disease caused by an infectious agent, compositions of the invention may be used for the treatment of ongoing infection or for cancer immunotherapy.

COMPOSITION COMPRISED OF ANTIGEN LINKED TO A TNF SUPERFAMILY LIGAND

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[001] This invention was made with government support under Grant No. AI068489 awarded by the National Institutes of Health (NIH) of the United States of America. The government has certain rights in this invention.

FIELD OF THE INVENTION

[002] The present invention generally relates to compositions useful for generating or enhancing an immune response against an antigen, to methods for using the compositions, and to modified immune cells useful in such methods.

BACKGROUND OF THE INVENTION

[003] Industrial applications of vaccines: Vaccines are considered to be among the most cost-effective and health-preserving medical inventions ever developed. The rationale for vaccination is that pre-exposure of the host to a vaccine against a given infectious agent can ameliorate or prevent disease should the vaccinated individual become exposed to that agent at a later time. The gap in time between vaccination and possible exposure requires “memory” on the part of the immune system. This memory is embodied in the persistence of immune cells for years or even decades after vaccination. Creating vaccines that induce strong and lasting protection is a difficult task, given our incomplete knowledge of the immune system. Nevertheless, continuing advances in our understanding make possible new approaches to vaccine design.

[004] Vaccines against infectious agents: For microbial agents, many vaccines in use are comprised of live attenuated or non-virulent strains of the disease-causing microorganisms. Other vaccines are comprised of killed or otherwise inactivated microorganisms. Yet other vaccines utilize purified components of pathogen lysates, such as surface carbohydrates or recombinant pathogen-derived proteins. Vaccines that utilize live attenuated or inactivated pathogens typically yield a vigorous

immune response, but their use has limitations. For example, live vaccine strains can sometimes mutate back into disease-causing variants, especially when administered to immunocompromised recipients. Moreover, many pathogens, particularly viruses, undergo continuous rapid mutations in their genome, which allow them to escape immune responses to antigenically distinct vaccine strains.

[005] Vaccines for the prevention or treatment of cancer: As the understanding of immunity has developed, it became clear that the immune system also controls or attempts to control the development of malignancies (Dunn *et al.*, 2002;3(11):991-8). As a result, immunotherapy is now being used to eradicate or control certain human cancers. Some of the technology and concepts of vaccines against infectious agents also apply to using the immune system to fight cancers, both solid tumors and blood cancers such as leukemia. Patients at risk for cancer, such as those infected by cancer-associated viruses like human papilloma virus (HPV), can be protected from developing the particular cancer in question as exemplified by Gardasil® vaccination against human papilloma virus (HPV), which causes cervical cancer. Patients who already have cancer, such as prostate cancer, can also be helped by vaccination, as exemplified by the Provenge® vaccine which is an immunotherapy for prostate cancer.

[006] CD8+ T cells can recognize conserved antigens in many infectious agents and prevent disease: While these have been successful vaccines, there have been major problems constructing vaccines against antigens from rapidly mutating infectious agents such as influenza, HIV, and Plasmodium falciparum (a cause of malaria). In these cases and others, the infectious agent has surface protein(s) that can rapidly mutate to evade otherwise protective antibodies. Nevertheless, these agents also have relatively conserved and unchanging internal components as exemplified by nucleoprotein (NP) of influenza, Gag and Pol for HIV, and circumsporozoite surface protein (CSP) for Plasmodium falciparum. In these cases, antibodies (which can only bind to the surface of pathogens) are unable to bind to these more conserved and internal antigens. Instead, there is a well-established role for CD8+ T cells in controlling or clearing such infectious agents – provided that a strong enough CD8+ T cell response can be generated. To cite just three examples: (1) Protection from disease caused by influenza can be achieved by high levels of CD8+ T cells against the conserved nucleoprotein (NP) viral protein (Webster *et al.*, Eur J Immunol. 1980;10(5):396-401; Slutter *et al.*, J Immunol. 2013;190(8):3854-8. PMID: 3622175.). (2) Strong CD8+ T cell responses against the Gag and Pol proteins of simian immunodeficiency virus (SIV, a non-human primate

model for HIV infection) can protect macaques from developing AIDS after challenge with SIV (Hansen *et al.*, Nature. 2011;473(7348):523-7. PMID: 3102768). (3) CD8+ T cells against Plasmodium falciparum antigens can protect humans from malaria (Epstein *et al.*, Science. 2011;334(6055):475-80). Thus, there is an urgent and largely unmet need to develop better ways of eliciting strong CD8+ T cells to protect against infection.

[007] CD8+ T cells can recognize cancer antigens and cure malignancy: Similar to the situation with infectious agents, CD8+ T cells can also be generated against tumor cell antigens. As exemplified by Tumor-Infiltrating Lymphocytes (TILs), the passive administration of anti-tumor CD8+ T cells can be sufficient to cure patients of advanced cancers in a small percentage of cases (Restifo *et al.*, Nature Reviews Immunology. 2012;12(4):269-81). These CD8+ T cells recognize peptides termed “tumor antigens” where the tumor contains antigens either not found in normal tissue or present at much lower levels. As noted above, some tumor antigens are derived from tumorigenic viruses such as the E6 and E7 antigens in HPV-related cervical cancer. Other tumor antigens are derived from mutations in germline proteins such as the V600E mutation in the BRAF protein. Yet other tumor antigens are normal proteins such as HER-2/neu which is overexpressed in breast cancer, where the breast is a non-essential “disposable” tissue that can be sacrificed by an immune attack on breast-derived tissues. Here again, there is an urgent and largely unmet need to develop better ways of eliciting strong CD8+ T cell responses to protect against cancer or treat patients with already established malignant disease.

[008] Numerous licensed vaccines are live, attenuated viruses (LAV): As noted above, there is a major problem in the art which is that it has been difficult to develop industrial applicable vaccines that are able to generate antigen-specific CD8+ T cells. For viral infections, one of the best ways is to generate anti-viral CD8+ T cells is to vaccinate with a live, attenuated virus (LAV) vaccine. Familiar examples of LAV vaccines are the Measles/Mumps/Rubella (MMR) vaccine, Sabin poliovirus vaccine, FluMist® influenza vaccine, Yellow Fever Virus 17D vaccine, and Vaccinia smallpox vaccine. But it has been difficult to produce LAV vaccines against viral infections for a variety of reasons that include inefficient manufacturing process, a need for repeated vaccination with follow-up “booster” vaccination many years later, and the generally poor quality and low level of the CD8+ T cell response to many vaccine candidates.

[009] CD8+ T cells can cure cancer in humans but are difficult to generate: For cancers not associated with viruses, there is no possibility of developing an LAV type vaccine. Instead, tumor antigens must be identified or otherwise isolated or predicted and used for vaccination. To be curative for cancer, a substantial CD8+ T cell response is needed. This has been shown for regimens that isolate and expand tumor-infiltrating lymphocytes (TIL) which are CD8+ T cells grown ex vivo and then administered back to the patients. In these studies, a relatively high number of TIL CD8+ T cells is required to successfully eradicate and cure metastatic melanoma (Restifo *et al.*, Nature Reviews Immunology. 2012;12(4):269-81). Many seemingly auspicious cancer vaccines and immunotherapies turn out to be too weak to cure cancer when tested in vivo. For example, simply vaccinating with a tumor antigen peptide emulsified in **Montanide™ lipid as an immunostimulant fails** to cure cancer because the resulting CD8+ T cells do not enter the circulation and go to the tumors (Hailemichael *et al.*, Nat Med. 2013;19(4):465-72. PMID: 3618499).

[010] CD8+ T cells are stimulated by antigen peptides presented on MHC Class I (MHC-I): In order to understand the process for generating CD8+ T cells, it is helpful to review how they arise during a normal immune response. CD8+ T cells are named because they have the CD8 protein on their surface. CD8 works as a “co-receptor” along with the T cell receptor (TCR) to recognize peptide antigens (typically 7-11 amino acids in length) that are processed inside of cells by the cleavage of the intact proteins and then displayed on the surface of infected cells by major histocompatibility complex (MHC) Class I (MHC-I) molecules. These MHC-I molecules hold the peptide antigen in a “groove” and the CD8+ T cell then recognizes the peptide-MHC-I (pMHC-I) complex and becomes activated. CD8+ T cells that kill the infected cell are termed “cytotoxic” but they can also interfere with infectious agents by producing cytokines such as interferon-gamma (IFN-γ).

[011] Considering the foregoing, it is highly desirable to find an industrially applicable means for producing vaccines that are highly effective for eliciting strong CD8+ T cells, CD4+ T cells, and antibody responses against infectious agents and tumor antigens.

[012] Need for antigen-presenting cells (APC) to generate antigen-specific CD8+ T cells: With this as an introduction, it can be appreciated that a key event in the generation of CD8+ T cells is to develop a cell type called an “antigen-presenting cell” (APC) that can present pMHC-I to uneducated or naïve CD8+ T cell precursors to induce them to divide, expand in numbers, and persist for

prolonged periods as highly active “memory” CD8+ T cells. To be effective at generating CD8+ T cells, an APC must both express peptide antigen on MHC-I (pMHC-I) that is recognized by the TCR (called “Signal 1”) and also co-stimulate the responding cells through additional receptor (called “Signal 2”) and even other receptors (called “Signal 3”). TCR stimulation by pMHC-I provides Signal 1 and generally stimulation of the CD28 receptor on CD8+ T cells provides Signal 2. Signal 3 can be provided in a non-redundant fashion either by soluble proteins such as interferon-alpha (Type I interferon) and/or interleukin-12 (IL-12) and/or cell surface molecules such as CD27 ligand (CD27L, also called CD70 or TNFSF7), 4-1BBL (also called CD137L or TNFSF9), and/or OX40L (also called CD134L or TNFSF4) (Sanchez and Kedl, Vaccine. 2012;30(6):1154-61. PMID: 3269501). What is needed is a vaccine approach that can activate an APC to provide all of these signals. This requires a good dendritic cell stimulus, also called an “immune adjuvant” or “adjuvant.”

[013] APC cross-presentation of extracellular antigens: The first requirement for an APC is to express peptide antigen on MHC-I (pMHC-I). The prototypic APC is the dendritic cell which takes up protein antigens from its environment, degrades these proteins into peptides, loads the resulting peptides onto MHC-I, and then presents the pMHC-I on their surface to provide the TCR stimulus that is Signal 1. This process is very different from cells infected by a microbial pathogen or tumor cells. In those cases, the protein antigen is produced within the cell itself – not taken up from the extracellular space – and then protein degradation products (which are peptides) are loaded onto MHC-I and exported to the cell surface as pMHC-I to provide Signal 1. What makes dendritic cells and other APCs special is that they can form pMHC-I from proteins in their environment, a phenomenon termed “cross-presentation.” For dendritic cells to do this, they must take up the protein antigen from their environment using one of a few very specialized receptors, including DEC205, CD11c, BDCA1, BDCA3, and/or CD40. After taking up protein antigen from the extracellular space, these receptors direct the delivery of the protein antigen into membrane-limited intracellular compartments (“endosomes”) where the protein can be digested into peptides and then transferred into compartments where MHC-I is being assembled. Of special important to the instant invention is that the best receptor on dendritic cells for processing protein antigen into pMHC-I (*i.e.*, crosspresentation) is the CD40 receptor (Chatterjee *et al.*, Blood. 2012;120(10):2011-20; Cohn *et al.*, J Exp Med. 2013;210(5):1049-63. PMID: 3646496). Therefore, it is highly desirable for a vaccine to include a protein antigen that is targeted toward the CD40 receptor on dendritic cells.

[014] Activation of the APC stimulates crosspresentation: A second requirement for an APC to crosspresent an exogenous protein antigen is for the APC to be “activated.” For dendritic cells, such activation is ideally provided by an effective stimulus through the CD40 receptor, which promotes crosspresentation and the formation of the pMHC-I Signal 1 (Delamarre *et al.*, J Exp Med. 2003;198(1):111-22). Similarly, B cells, which are another type of APC, can be activated by a CD40 receptor stimulus to crosspresent soluble protein antigens (Ahmadi *et al.*, Immunology. 2008;124(1):129-40).

[015] Crosspresentation of antigen by dendritic cells in the absence of CD40 stimulation leads to CD8+ T cell tolerance: DEC-205 is a receptor on dendritic cells and B cells recognized on mouse cells by the NLDC-145 monoclonal antibody (Inaba *et al.*, Cellular immunology. 1995;163(1):148-56). Bonifaz *et al.* showed that the binding portion of an anti-DEC205 antibody can be genetically fused to a model antigen, chicken ovalbumin (OVA). The injection of anti-DEC205/OVA fusion protein directs the OVA antigen to dendritic cells and leads to crosspresentation of OVA peptide antigen on MHC-I. However, while this treatment induces anti-OVA CD8+ T cells to divide and proliferate, these cells soon die off and are deleted. This results in specific tolerance for OVA that cannot be overcome by subsequent vaccination with OVA plus Complete Freund’s Adjuvant (CFA), which is usually considered to be a gold standard for vaccination (although CFA is far too inflammatory to be used in humans). However, if anti-DEC205/OVA fusion protein is combined with a stimulus for the CD40 receptor, then very strong anti-OVA CD8+ T cell responses result (Bonifaz *et al.*, J Exp Med. 2002;196(12):1627-38. PMCID: 2196060). This indicates that simply targeting antigens to dendritic cells alone (e.g., using a fusion protein of anti-DEC205 and antigen) does not succeed in eliciting high levels of efficacious and persisting antigen-specific CD8+ T cells. In fact, it shows that allowing antigen to be taken up by unactivated dendritic cells should be avoided because it will work against the goal of creating strong antigen-specific CD8+ T cell responses.

[016] Generating CD8+ T cell responses is best when antigen is delivered to dendritic cells in conjunction with an adjuvant: Although they did not use a CD40 stimulus, Kamath *et al.* (J Immunol. 2012;188(10):4828-37) developed a vaccine system for delivering an antigen either directly attached to an antigen or co-delivered with a separate, unattached antigen. When antigen was delivered to DCs in the absence of adjuvant, antigen-specific T cells were induced to proliferate but did not subsequently differentiate into effector cells. Instead, effective immunity was only induced when the

test vaccine provided antigen and adjuvant to the same individual DCs within a short window of time. These parameters are fulfilled when the antigen and adjuvant are linked in time and space as parts of the very same molecule, as provided by the instant invention.

[017] To fulfill the need for a vaccine that induces a strong CD8+ T cell responses, the instant invention provides for a composition that contains, for example, CD40 ligand (CD40L, TNFSF5, which is an agonist of the CD40 receptor) physically linked to a multimerization domain that organizes it into a highly active many-trimer structure in addition to being physically linked to an antigen. In this way, antigen can be targeted to dendritic cells via binding to the CD40 receptor on their surface and activates the dendritic cell simultaneously. This arrangement can thus avoid delivery of antigen to dendritic cells that do not become activated and which instead would induce antigen-specific CD8+ T cell tolerance. As a result, the compositions of the instant invention provide for a unusually high level of activity in inducing strong CD8+ T cell responses, where the TCRs of elicited CD8+ T cells show an exceptionally high level of avidity for pMHC-I and where a vaccine of the invention confers surprisingly profound protection from challenge by an infectious agent (Vaccinia encoding HIV-1 Gag as a model antigen). Variations on these compositions are expected to elicit very strong CD4+ T cells and B cell antibody responses in a similar fashion.

SUMMARY OF THE INVENTION

[018] The invention provides fusion proteins comprising antigens of infectious disease agents and cancer cells linked to many-trimer forms of TNF SuperFamily (TNFSF) ligands. The TNFSFs serve as vaccine adjuvants for increasing the immune response to the antigens. In particular, a fusion polypeptide strand that self-assembles inside cells into a many-trimer form of CD40 ligand (CD40L, TNFSF5) was shown to elicit surprisingly strong responses against an infectious disease agent and a tumor antigen. Other similar fusion proteins are contemplated and their construction provided for in the application. The fusion proteins can be delivered to a host either as nucleic acids used directly in DNA vaccination or carried and expressed by a viral vector such as adenovirus. It is contemplated that isolated fusion proteins could be also be administered with good effect. In addition to use as a vaccine to prevent or ameliorate disease caused by an infectious agent, compositions of the invention may be used for the treatment of ongoing infection or for cancer immunotherapy.

[019] To create a vaccine that effectively elicits strong CD8⁺ T cell responses, highly active forms of TNF Superfamily ligands (TNFSFs) were constructed as fusion proteins with test antigens from infectious disease agents and tumors. Using CD40 ligand (CD4OL, also called TNFSF5) as an exemplary TNFSF, the resulting fusion proteins were given to mice in the form of a DNA vaccine (by injection of plasmid DNA into muscle) as a means to deliver antigen to dendritic cells and activate these cells through their CD40 receptor at the same time. This approach minimizes the separate delivery of antigen to dendritic cells that have not been activated by adjuvant, which would otherwise result in CD8⁺ T cell tolerance as shown by Bonifaz *et al.* *CI Exp Med.* 2002;196(12):162738. PMID: 2196060) and Kamath *et al.* (*J Immunol.* 2012;188(10):4828-37). In the exemplary case, this invention combines one of the best vaccine adjuvants for dendritic cell activation (*i.e.*, CD4OL) along with targeting the antigen to dendritic cells by virtue of the antigen being operatively linked to CD4OL (the ligand for the CD40 receptor) which binds to CD40 and delivers the antigen to dendritic cells for cross-presentation as pMHC-I. Previous attempts to link CD4OL with antigen were flawed by defective molecular design and did not result in such a powerful vaccine. Instead, the approach of the instant invention provides a combination in such a way as to provide a surprisingly strong CD8⁺ T cell response that is highly protective. By selecting the appropriate antigen(s) and TNFSFs and an appropriate delivery method, applications include vaccines against infectious agents and malignant cells. Using fusion proteins directly or as their encoding nucleic acid sequences delivered by a DNA or RNA vaccine or by a viral vector such as adenovirus, the invention has substantial industrial application.

[019a] There is provided a fusion protein comprising: (a) a multimerization scaffold, composed of two or more trimeric collectin-like arms consisting each of three individual polypeptide strands, joined at their N-terminus by a disulfide-linked hub region, wherein the multimerization scaffold is selected from the group consisting of SPD, Acrp30, C11, HIB27, SPA, conglutinin, collectin-43, MBL1, and MBL2, and (b) two or more complete Tumor Necrosis Factor SuperFamily (TNFSF) receptor binders, comprising three receptor binder protein chains, wherein the two or more complete TNFSF receptor binders bind a complete TNFSF receptor to activate a cell, and wherein the TNFSF receptor binders are selected from the group consisting of CD40L (TNFSF5), CD27L (TNFSF7), CD137L (TNFSF9), OX40L (TNFSF4), GITRL, 4-1BBL, RANKL, LIGHT, CD70, and BAFF, wherein each of said receptor binder protein chains is operatively linked by a peptide bond to the C-terminus of one of said individual polypeptide strands; and (c) one or more antigens recognized by the immune system, wherein the amino acid sequence of the one or more antigens is contained within each of the individual polypeptide strands within the multimerization scaffold.

BRIEF DESCRIPTION OF THE DRAWINGS

[020] FIG. 1: Schematic drawing illustrating the need to cluster a TNFSF receptor such as the CD40 receptor on dendritic cells and other APCs in order to provide a strong cell stimulus. This requirement for clustering affects the design of an effective form of TNFSF ligand or an anti-TNFSF receptor-binding antibody.

[021] FIG. 2: Agonistic anti-CD40 antibodies can cluster CD40 receptors so long as they bind to and are "mounted" on a nearby cell that expresses receptors for the Fc tail of the antibody molecule. Abbreviations: Fc γ R — the receptor for the Fc portion of immunoglobulin G (IgG). Anti-CD40 MAb - a monoclonal antibody that binds to CD40.

[022] FIG. 3: Molecular design of fusion proteins that create many-trimer forms of soluble CD40L. On the left is a schematic for a 1-trimer form of CD40L that cannot cluster the CD40 receptor and as a result is inactive, as shown by Haswell *et al.* (Eur J Immunol. 2001;31(10):3094-100) and Holler *et al.* (Mol Cell Biol. 2003;23(4):1428-40) and described in EP 1246925 B1. As previously described (Stone *et al.*, J Virol. 2006;80(4):1762-72) and presented in US 7,300,774 B1 and US 7,332,298 B2, and also in EP 1246925 B1, the extracellular domain (ECD) of CD40L can be genetically fused to scaffold-forming proteins such as Acrp30 (middle) or surfactant protein D (SPD) (right). The 2-trimer Acrp30-CD40L protein is also called MegaCD40L™ or CD40L hexamer, whereas the 4-trimer SP-D-CD40L protein is also called UltraCD40L™. These many-trimer forms of CD40L can cluster the CD40 receptor and act as a vaccine adjuvant. This occurs in part by activating dendritic cells (Miconnet and Pantaleo, Vaccine. 2008;26(32):4006-14).

[023] FIG. 4: Two- and four-trimer CD40L fusion proteins are vaccine adjuvants for CD8+ T cell responses. Mice were vaccinated by injecting “naked” plasmid DNA into muscle in order to test different forms of CD40L as an adjuvant for the HIV-1 Gag antigen. In Panel A, CD8+ T cell responses were detected as killing of P815 target cells pulsed with Gag peptide. In Panel B, CD8+ T cell responses were detected by measuring the number of individual interferon-gamma secreting cells in response to Gag peptide antigen using an ELISPOT assay. There was a distinct improvement in CD8+ T cell responses using a 2-trimer form of CD40L (Acrp30-CD40L) and more preferably a 4-trimer form of CD40L (SPD-CD40L) (Stone *et al.*, J Virol. 2006;80(4):1762-72). To show the general applicability of this approach, a similar vaccine assay system was used to show that other TNFSF ligands could be multimerized as 4-trimer proteins and used as vaccine adjuvants, including GITRL, 4-1BBL, OX40L, RANKL, LIGHT, CD70, and BAFF (Kanagavelu *et al.*, Vaccine. 2012;30(4):691-702. PMCID: 3253891).

[024] FIG. 5: Molecular design of multimeric CD40L fusion proteins containing an in-frame insertion encoding HIV-1 Gag as a model antigen. Top: pSPD-Gag-CD40L is a plasmid containing an antigen inserted into the protein strand that results in a 4-trimer form of CD40L. At the nucleic acid level, the codons for a model antigen, HIV-1 Gag, were positioned into the coding sequence of the SPD-CD40L construct. In the resulting translated protein, the N-terminus is comprised of a secretion signal peptide from SPD followed by an N-terminal sequence of SPD termed the “hub” which contains 2 cysteines in each strand, thereby producing disulfide bonds that (a) covalently

couple three individual polypeptide strands together to form an “arm” and (b) covalently couple 4 trimeric arms into the final 12-chain, 4-arm structure shown in the bottom left of the figure (where the inserted Gag antigen is shown as a solid bulge in each arm of the protein). Note that the Gag antigen sequence was positioned between the 105 and 106 amino acids of murine SPD protein, while retaining the previously constructed CD40L domain at the C-terminal end. Like the parent SPD-CD40L molecule, this protein strand of SPD-Gag-CD40L spontaneously self-assembles inside cells into a multimeric, many-trimer form of CD40L that is then secreted into the extracellular space. 2nd from Top: pTrimer-Gag-CD40L (labeled pTr-Gag-CD40L) is a plasmid constructed by deleting codons for amino acids 24-105 of murine SPD. This removes the hub region containing the 2-cysteines. Also included is the t-PA signal peptide sequence for secretion. This results in the production of a single-trimer, 1 “arm” form of the Gag antigen-CD40L protein, as shown in the bottom right of the figure (where the Gag antigen is shown as a solid bulge in this 1-trimer form of CD40L). 3rd from Top: pGag is the plasmid encoding amino acids for the p55 Gag antigen preceded by the t-PA signal sequence for secretion, as described by Qiu *et al.* (J Virol. 1999;73(11):9145-52). This is a control antigen construct that has no CD40L adjuvant. 4th from Top: pSPD-CD40L is the plasmid encoding a 4-trimer form of CD40L previously described by Stone *et al.* (J Virol. 2006;80(4):1762-72) and in US 7,300,774 B1 and US 7,332,298 B2. This is an adjuvant-only protein that does not contain an antigen. It can, however, be co-administered with an antigen plasmid such as pGag, as shown in FIG. 4.

[025] FIG. 6: pSPD-Gag-CD40L encodes a secreted protein. Panel A shows a Western blot of a reducing SDS-PAGE gel analysis of the culture media of 293T cells were transiently transfected with DNA for the plasmids shown. An antibody for murine CD40L was used to reveal the protein bands. As shown, pSPD-Gag-CD40L encodes a single protein of the expected size of 105 kDa. A single 105 kDa band was also observed using antibody to the p24 portion of Gag (not shown). Panel B shows a similar analysis using non-denaturing PAGE in the absence of a reducing agent. Multiple bands were observed at >200 kDa molecular weight, demonstrating the formation of large multimeric complexes. As is commonly observed in such analyses of collagen-like proteins, partial denaturation during processing can result in an unwinding of some of the collagen triple helix, which could thus lead to a less compact protein that moves more slowly through the gel during the electrophoretic process.

[026] FIG. 7: Qualifying assay for the biological activity of SPD-Gag-CD40L in vitro. Panel A: In vitro activity using a CD40 receptor NF- κ B indicator cell line. To produce soluble protein, 293T cells were transiently transfected with plasmids for pcDNA3.1 (empty vector control), pSPD-CD40L, or pSPD-Gag-CD40L and the protein-containing supernatants were collected 48 hours later. To determine the activity of the CD40L in these proteins, the culture media was added to cultures of 293 reporter cells containing an NF- κ B-driven gene for secreted alkaline phosphatase (SEAP) and expressing the CD40 receptor (CD40-293-SEAP reporter cells). If the CD40 receptor is activated by CD40L, then NF- κ B-driven SEAP production results in the secretion of SEAP which can be measured by a colorimetric enzyme assay at OD650 (Maurais *et al.*, Virology. 2009;385(1):227-32). In this assay, a single trimer of CD40L (R&D Systems, Inc., Minneapolis, MN) was entirely inactive and did not induce SEAP production (not shown), indicating the strict requirement for a many-trimer form of CD40L for activity in this assay. In contrast, both the pSPD-CD40L adjuvant protein and the new SPD-Gag-CD40L protein of the instant invention were active as CD40 receptor activators. Panel B: Stimulating activity on mouse bone marrow-derived dendritic cells (BMDDC). As in Panel A, culture supernatants from 293T cells transfected with pcDNA3.1 or pSPD-Gag-CD40L were incubated with BMDDC for 18 hours. Cells were washed, stained with fluorochrome-conjugated antibodies, and assayed by flow cytometry for the expression of activation and maturation markers. The SPD-Gag-CD40L protein upregulated CD80 and especially CD86 and CCR7, indicating that this fusion protein was fully capable of activating normal dendritic cells. As expected, the CD40 receptor was downregulated by exposure to SPD-Gag-CD40L. A cytokine mix was used as a positive control ("Mimic," consisting of 10 ng/ml of rhTNF-alpha, 10 ng/ml of rhIL-1beta, 1000 U/ml of rhIL-6 and 1 μ g/ml of PGE2; Sato *et al.*, Cancer Sci. 2003;94(12):1091-8). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to pcDNA3.1 supernatant. Data represents independent wells in the same experiment.

[027] FIG. 8: DNA vaccination with pSPD-Gag-CD40L demonstrates a surprisingly high level of CD8+ T cell responses. Panel A: DNA vaccination schedule. Mice were vaccinated three times at two-week intervals with an intramuscular injection of 100 μ g of plasmid DNAs. Panels B and C: CD8+ ELISPOT assay. To measure the Gag-specific CD8+ T cell response, spleen cells were collected 14 days after the last vaccination and tested by ELISPOT assays. Panel B shows cells producing interferon-gamma and Panel C shows cells producing IL-2. The control vaccination is pGag + pcDNA where empty pcDNA3.1 (pcDNA) was used to keep the total amount of DNA

constant. The previously reported mix of antigen and 4-trimer CD40L adjuvant plasmid is pGag + pSPD-CD40L which consists of separate plasmids for antigen and adjuvant, i.e., not present in the same secreted molecule. Surprisingly, pSPD-Gag-CD40L, the subject of the instant invention, resulted in a massive antigen-specific CD8⁺ T cell response (note that a broken Y-axis is needed to keep the results visible in the graph). In contrast, pGag + pIL-12 gave more modest CD8⁺ T cell responses, even though a pIL-12 plasmid is currently being evaluated in human vaccine trials. Panel C shows the same analysis using IL-2 ELISPOT assay and showed the surprising strength of pSPD-Gag-CD40L, the subject of the instant invention.

[028] FIG. 9: DNA vaccination with pSPD-Gag-CD40L demonstrates a surprising improvement in CD8⁺ T cell quality. *Panel A*: T cell receptor avidity for peptide antigen/MHC-I measured by ELISPOT assay. Splenocytes were cultured with serial dilutions of CD8⁺ T cell specific peptide AMQMLKETI for 18 hours. Splenocytes from mice vaccinated with pSPD-Gag-CD40L induced a significant increase in IFN- γ ELISPOTs following stimulation with Gag peptide AMQMLKETI at a concentration of 1 ng/ml and 10 pg/ml whereas there was essentially no activity at these doses using splenocytes from mice vaccinated with pGag antigen alone or a mixture of separate plasmids for pGag and pSPD-CD40L adjuvant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to pGag alone or pGag + SPD-CD40L vaccination. *Panel B*: IgG antibody responses against Gag antigen. Total IgG specific for Gag was measured by ELISA assay from mouse serum collected on day 42. Consistent with a previous study (Stone *et al.*, J Virol. 2006;80(4):1762-72), CD40L adjuvant used in this format is not an adjuvant for antibody responses.

[029] FIG. 10: The multi-trimer structure of SPD-Gag-CD40L is necessary for the improved vaccine effect. In Panels A and B, pTrimer-Gag-CD40L was used as 1-trimer control for 4-trimer pSPD-Gag-CD40L. As shown, the many-trimer structure was necessary for the strong adjuvant effect.

[030] FIG. 11: Protective effects of pSPD-Gag-CD40L vaccination measured by vaccinia-Gag viral challenge. BALB/c female mice were immunized intramuscularly with the plasmids shown on days 0, 14, and 28. Two weeks following the final vaccination, the mice were challenged intraperitoneally with 10E7 plaque-forming units (PFU) of vaccinia-Gag. Mice were sacrificed 5 days after viral challenge and the ovaries were harvested and analyzed for PFU. *Panel A*: Intramuscular DNA vaccination with pSPD-Gag-CD40L resulted in significantly greater protection from viral challenge.

In contrast, DNA vaccination with a mixture of pGag antigen plus pSPD-CD40L adjuvant as separate plasmids only induced a modest reduction in viral loads that was not significantly reduced compared to pGag antigen alone. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *Panel B*: Evaluation of a single trimer pTrimer-Gag-CD40L construct. As shown before, the multi-trimer structure of SPD-Gag-CD40L is necessary for the improved vaccine effect.

[031] FIG. 12: Adenoviral vector delivery of SPD-Gag-CD40L is surprisingly protective against virus challenge. BALB/c female mice were immunized intramuscularly on days 0 and 14 with adenovirus 5 (Ad5) expressing the HIV-1 Gag antigen (Ad5-Gag) or the SPD-Gag-CD40L construct (Ad5-SPD-Gag-CD40L). Two weeks following the final vaccination, mice were challenged intraperitoneally with vaccinia-Gag virus (10^7 PFU). Mice were sacrificed 5 days later and ovaries were harvested for vaccinia PFU determinations. Surprisingly, Ad5-SPD-Gag-CD40L vaccination reduced viral load by ~ 7 logs following vaccinia-Gag challenge. No detectable virus could be found in the mice that had received this vaccine, indicating complete protection (sterilizing immunity).

[032] FIG. 13: Construction and Western blot of SPD-gp100-CD40L. *Panel A*: Model of SPD-gp100-CD40L fusion. Amino acids 25 to 596 (sequence KVPRNQD to EAGLGQV) of human gp100 was inserted between amino acids 105 and 106 of murine SPD within the SPD-CD40L fusion construct. *Panel B*: Schematic diagram of expected SPD-gp100-CD40L 4-trimer structure. *Panel C*: Western blot analysis. 293T cells were transfected with DNA plasmid encoding gp100 or the SPD-gp100-CD40L fusion protein. After 48-hour culture, supernatant was collected and run on an SDS-PAGE gel in the presence of reducing agent. Western blot was performed using a polyclonal antibody to gp100.

[033] FIG. 14: Biological activity of SPD-gp100-CD40L. *Panel A*: In vitro activity of SPD-CD40L and SPD-gp100-CD40L was determined using a cell-based CD40 NF- κ B enzymatic reporter system. An equivalent amount of 293T supernatant from pcDNA3.1, pSPD-CD40L or pSPD-gp100-CD40L transfected cells was incubated with 293-CD40-SEAP NF- κ B reporter cells. *Panel B*: In vitro activity of SPD-gp100-CD40L was evaluated on mouse bone marrow derived mouse DC and compared to empty vector or Mimic cytokine positive control. * $p < 0.05$, ** $p < 0.01$ by Student's t test compared to pcDNA3.1 supernatant.

[034] FIG. 15: Immunotherapy of established B16F10 melanoma tumors. *Panel A*: Immunization schedule for B16-F10 tumor challenge and DNA/GVAX therapeutic vaccination, as indicated by arrows. B16F10 cells (50,000) were injected i.d. into the left flank of C57BL/6 mice on day 0. Mice were then immunized by i.m. injection of PBS or pSPD-gp100-CD40L plasmid on day 3, 10, and 17. GVAX, B16F10 tumor cells expressing GM-CSF, were irradiated at 5,000 rad and 1×10^6 cells injected subcutaneously on day 3, 6, and 9. *Panel B*: Tumor growth analysis. Each point represents the mean tumor volume in each group (n=5). We did not observe a statistical difference in tumor sizes between no treatment (PBS) and SPD-gp100-CD40L vaccination groups. *Panel C*: Survival analysis based on the date of death or when tumor size reached >1500 cm². No statistical differences in survival were observed between groups.

[035] FIG. 16: Immunotherapy of established B16F10 melanoma tumors by DNA vaccination with a combination of pSPD-gp100-CD40L, pIL-12p70 and pGM-CSF. *Panel A*: Immunization schedule for B16F10 tumor challenge and DNA/GVAX vaccination, as indicated by arrows. B16F10 cells (50,000) were injected i.d. into the left flank of the mice on day 0. Mice were immunized i.m. with PBS, pSPD-gp100-CD40L + pIL-12, pSPD-gp100-CD40L + pGM-CSF, or pSPD-gp100-CD40L + pIL-12 + pGM-CSF on day 3, 10, and 17. For GVAX therapy B16-F10 tumor cells expressing GM-CSF (GVAX), were irradiated at 5,000 rad and 1×10^6 cells were injected subcutaneously on day 3, 6, and 9. *Panel B*: Tumor growth analysis. Each point represents the mean tumor volume of animals in each group (n=5). There was a significant reduction in tumor growth kinetics for SPD-gp100-CD40L + IL-12 + GM-CSF vaccinated mice compared to other groups. (** p<0.01; *** p<0.001 compared to PBS or SPD-gp100-CD40L + IL-12 or SPD-gp100-CD40L + GM-CSF vaccination groups). *Panel C*: Survival analysis of mice. We observed a significant increase in survival and tumor free survival (date of tumor appearance) for pSPD-gp100-CD40L + pIL-12 + pGM-CSF vaccinated mice as compared to other groups (** p<0.01; *** p<0.001 compared to PBS, pSPD-gp100-CD40L + pIL-12, or pSPD-gp100-CD40L + pGM-CSF vaccination groups). *Panel D*: Tumor growth kinetics of individual mice from each treatment group.

[036] FIG. 17: Separate expression of gp100 and SPD-CD40L proteins fails to induce anti-tumor activity. As a control for pSPD-gp100-CD40L, several other anti-tumor treatment approaches were tested and found to be inferior. *Panel A*: Immunization schedule for B16F10 tumor challenge and DNA vaccination, as indicated by arrows. B16-F10 cells (50,000) were injected into the left flank of

the mice on day 0. Mice were immunized i.m. with PBS, pgp100, pgp100 + pIL-12, pgp100 + pGM-CSF, pgp100 + pIL-12 + pGM-CSF, or pgp100-IRES-SPD-CD40L + pIL-12 + pGM-CSF on day 3, 10, and 17. *Panel B*: Tumor growth analysis. Each point represents the mean tumor volume of animals in each group (n=5). We did not observe any statistical difference in tumor size between vaccination groups. *Panel C*: Survival analysis. We did not observe any statistical difference in survival of mice between groups.

BRIEF DESCRIPTION OF THE SEQUENCES

[037] SEQ ID NO 1: DNA sequence for muSP-D-Gag-muSP-D-muCD40L. This is the DNA sequence of a fusion protein using the murine sequences for SPD and CD40L. Due to minor differences between species, it is preferable to use a murine sequence for administration to mice, a macaque sequence for administration in monkeys (Stone *et al.*, Clin Vaccine Immunol. 2006;13(11):1223-30), a human sequence for administration to humans, and so. This minimizes the possibility of antibodies forming against a xenogeneic protein, other than the antigen contained in the construct. In this example, what is shown is the nucleic acid sequence used for the experiments shown in FIGs. 6-12. (Note that surfactant protein D is variously abbreviated as either 'SPD' or 'SP-D'. The location of the Gag antigen insert is shown in non-italicized type face.

[038] SEQ ID NO 2: Protein sequence for muSP-D-Gag-muSP-D-muCD40L. This is the translation of SEQ ID NO 1.

[039] SEQ ID NO 3: DNA sequence for tpa-muACRP30-gp120-muACRP30-muBAFF. This is a DNA sequence of a fusion protein using the previously described 2-trimer form of Acrp30-BAFF into which has been inserted a DNA sequence of HIV-1 gp120 envelope as an antigen. It is contemplated that the 2-trimer fusion protein encoded by this nucleic acid sequence will activate the Env gp120-binding B cell receptor (BCR) on B cells and simultaneously engage receptors for BAFF on these B cells that synergize with BCR engagement to stimulate the B cell to produce anti-Env antibodies.

[040] SEQ ID NO 4: Protein sequence for tpa-muACRP30-gp120-muACRP30-muBAFF. This is the translation of SEQ ID NO 3.

[041] SEQ ID NO 5: DNA sequence for muSP-D-gp100-muSP-D-muCD40L. This is the DNA sequence of a fusion protein using the murine sequences for SPD and CD40L. The inserted antigen (non-italicized sequence) is encoded by the nucleotide sequence for human gp100, a xenogenic antigen that has been found to be useful in melanoma studies in mice (Gold *et al.*, J Immunol. 2003;170(10):5188-94).

[042] SEQ ID NO 6: Protein sequence for muSP-D-gp100-muSP-D-muCD40L. This is the translation of SEQ ID NO 5.

[043] SEQ ID NO 7: DNA sequence for tpa-huIgG1Fc-gp120-GCN4-huAPRIL. This is a DNA sequence encoding a human t-PA signal sequence for protein secretion joined in-frame with the human IgG1 Fc region joined in-frame with HIV-1 Env gp120 joined in-frame with the GCN4 trimerization motif joined in-frame with the extracellular domain of human APRIL. It is contemplated that the 2-trimer fusion protein encoded by this nucleic acid sequence will activate the Env gp120-binding B cell receptor (BCR) on B cells and simultaneously engage receptors for APRIL on these B cells that synergize with BCR engagement to stimulate the B cell to produce anti-Env antibodies.

[044] SEQ ID NO 8: Protein sequence for tpa-huIgG1Fc-gp120-GCN4-huAPRIL. This is the translation of SEQ ID NO 7.

[045] SEQ ID NO 9: DNA sequence for huSP-D-NP-huSP-D-huCD40L-NST. It was previously found that some embodiments of SPD-CD40L can be equally or more active when the extracellular "stalk" region of CD40L is deleted. This stalk links the CD40L trimeric extracellular domain (ECD) with the transmembrane region that holds CD40L in the membrane. The SPD-CD40L-NST construct is disclosed in US 2009/0081157 A1 (see especially FIG. 21, Examples 1, 11, and 13).

The instant sequence comprises an insertion of coding sequences for the nucleoprotein (NP) antigen from influenza A. It is contemplated that the 4-trimer fusion protein encoded by this nucleic acid sequence will elicit strong CD8+ T responses against this conserved influenza antigen.

[046] SEQ ID NO 10: Protein sequence for huSP-D-NP-huSP-D-huCD40L-NST. This is the translation of SEQ ID NO 9.

[047] SEQ ID NO 11: DNA sequence for tpa-muACRP30-CSP1-muACRP30-muCD40L. This is a DNA sequence encoding a human t-PA signal sequence for protein secretion joined in-frame with a portion of the murine Acrp30 sequence joined in-frame with codons for the circumsporozoite protein-1 (CSP-1) of *Plasmodium yoelii* joined in-frame with a portion of the murine Acrp30 sequence joined in-frame with the extracellular domain of murine CD40L. *Plasmodium yoelii* is used for malaria vaccine studies because it causes a malaria-like disease in mice. CD8+ T cells directed against the CSP-1 antigen of this agent can provide immunity to malaria (Sedegah et al., Proc Natl Acad Sci U S A. 1998;95(13):7648-53). It is contemplated that mice vaccinated with this construct will be resistant to disease caused by intravenous challenge with *Plasmodium yoelii*-infected red blood cells.

[048] SEQ ID NO 12: Protein sequence for tpa-muACRP30-CSP1-muACRP30-muCD40L. This is the translation of SEQ ID NO 11.

[049] SEQ ID NO 13: DNA sequence for muSP-D-Gag-muSP-D-muRANKL. This is a DNA sequence encoding a portion of the murine SPD sequence joined in-frame with codons for HIV-1 Gag antigen joined in-frame with a portion of the murine Acrp30 sequence joined in-frame with the extracellular domain of murine RANKL. Of special note is the difference of position in placing the antigen within the sequence of the SPD “arms,” in this case shifted toward the 5' end (or N-terminal end in the protein) the equivalent of 10 codons in the SPD sequence. It is contemplated that this construct used as a vaccine will elicit strong immune responses in mice.

[050] SEQ ID NO 14: Protein sequence for muSP-D-Gag-muSP-D-muRANKL. This is the translation of SEQ ID NO 13.

[051] SEQ ID NO 15: DNA sequence of huSP-D-WT1-huSP-D-huCD40L. This is a DNA sequence encoding a portion of the human SPD sequence joined in-frame with codons for the human WT1 protein joined in-frame with a portion of the human SPD sequence joined in-frame with the extracellular domain of human CD40L. WT1 is a tumor antigen present in many types of human cancer (Chaise *et al.*, Blood. 2008;112(7):2956-64). It is contemplated that this construct used as a vaccine will elicit strong immune responses in humans against cancer cells expressing the WT1 tumor antigen.

[052] SEQ ID NO 16: Protein sequence for huSP-D-WT1-huSP-D-huCD40L. This is the translation of SEQ ID NO 15.

[053] SEQ ID NO 17: DNA sequence of muSP-D-MAGE-A3-muSP-D-muBAFF. This is a contemplated DNA sequence encoding a portion of the murine SPD sequence joined in-frame with codons for the human MAGE-A3 tumor antigen (Groeper *et al.*, Int J Cancer. 2007; 120(2):337-43) joined in-frame with a portion of the murine SPD sequence joined in-frame with the extracellular domain of murine BAFF. Of note is that codons for 20 amino acids (PPGLPGIPGPMGARASVLSG) in the N-terminal half of the SPD arm have been deleted. This exemplifies how the SPD “arms” can be shortened N-terminal to the insertion site of the antigen sequence. Similar deletions in the C-terminal half of the SPD arm are also contemplated, as are deletions in both sides of the SPD arms that flank the antigen sequence insertion site.

[054] SEQ ID NO 18: Protein sequence of muSP-D-MAGE-A3-muSP-D-muBAFF. This is the translation of SEQ ID NO 17.

DEFINITIONS

[055] This disclosure uses art-recognized concepts and methods. The skilled artisan will be familiar with resources including the following: “Janeway’s Immunology” by Kenneth Murphy, Garland Science Press, 2011; “Fundamental Immunology” by William E. Paul, Lippincott Williams & Wilkins, 2008; “Cellular and Molecular Immunology, 7th Edition” by Abul K. Abbas, Andrew H. H. Lichtman, and Shiv Pillai, Elsevier Press, 2011; “Current Protocols in Immunology,” Wiley Press, 2012; and “Current Protocols in Molecular Biology,” Wiley Press, 2012. In addition, the following patents and applications are referred to: US 7,300,774B1; US 7,332,298 B2; US 2009/0081157 A1.

[056] Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Definitions of common terms in molecular biology can be found in Benjamin Lewin, Genes V, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCR Publishers, Inc., 1995 (ISBN 1-56081-569-8).

[057] In order to facilitate review of the various embodiments of this disclosure, the following explanations of specific terms are provided:

[058] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term "comprises" means "includes." The abbreviation, "e.g." is derived from the Latin *exempli gratia*, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[059] "C1q family protein" refers to a member of the C1q family. Exemplary C1q family proteins include, but are not limited to, C11, Acrp30, and HIB27. Preference is given to Acrp30. Like the collectins, C1q family members have 2 or more trimeric, collagen-like "arms" that provide the multivalent structures of these molecules. The instant invention utilized C1q family proteins as a multimerization scaffold by replacing their normal C-terminal "C1q" domains with a TNFSF receptor binding such as the ECD of a TNFSF ligand.

[060] "Collectin" refers to a member of the collectin family.

They include pulmonary surfactant A, pulmonary surfactant D, conglutinin, collectin-43, mannose-binding protein MBL1 or MBL2, and others. Preference is given to surfactant protein D (abbreviated alternatively as SP-D or SPD). All collectins have two or more trimeric collagen-like "arms" joined in the center at a "hub" and radiating outward to display their C-terminal ends. Each collectin has a C-terminal domain that typically binds to carbohydrate. When used as a multimerization scaffold in the instant invention, each collectin is made without the natural C-terminal end and a TNFSF ECD receptor binding domain is placed there instead. Preference is given to surfactant protein D which has four trimeric arms ending C-terminally.

[061] "Complete TNFSF receptor" is a term used herein in marked distinction to a single polypeptide chain often referred to as a TNFSF receptor protein

The nucleotide and peptide sequences of single TNFSF receptor polypeptide chains are listed in GenBank, SwissProt, and other databases. However, in actuality, single TNFSF receptor polypeptide chains are not found in isolation on the surface of cells. Instead, two or more TNFSF receptor chains are co-localized or linked. As an example, the Fas receptor (CD95) for Fas ligand (FasL) is held together in the absence of FasL by their N-terminal “pre-ligand association domains” or PLAD (Siegel *et al.*, Science. 2000;288(5475):2351-4). Similarly, there is a domain in the extracellular region of CD40 that holds this receptor together as 2 or more chains (Smulski *et al.*, J Biol Chem. 2013). Consequently, stimulation of TNFSF receptors generally does not involve simple bringing together of 2 or more receptor chains. When the ligand does bind to the receptor, computer modeling suggests that a ligand trimer engages three receptor chains (Bajorath *et al.*, Biochemistry. 1995;34(31):9884-92). Thus, this application uses the term “complete TNFSF receptor” to indicate that binding to a TNFSF receptor involves binding to 2 or preferably 3 receptor protein chains.

[062] “Immune system” refers to T cells, B cells, NK cells, dendritic cells, monocytes, and macrophages and the specialized tissues that contain them. The lymph nodes, lymphatics, and spleen are physical structures that housing many of the cells of the immune system. In addition, other immune system cells are found in non-lymphoid tissues and in blood. A characteristic of the immune system is that it responds to a first exposure to an antigen (primary response) in a set fashion but then responds more strongly and more quickly to a second exposure of an antigen (secondary response), which is a manifestation of immunological memory. The immune system responds to infectious agents and cancer by producing cells and effector molecules that kill the offending infectious agent or cancer cells. Among the cells that kill the attackers are T cells including CD4+ and CD8+ T cells. B cells make antibodies that can neutralize the infectivity of many infectious agents. T cells, monocytes, macrophages, and dendritic cells can make interferons that interfere with the replication of certain viruses.

[063] “Multimerization scaffold” refers to a molecular structure that confers upon the molecule into which it is incorporated an overall structure that is operatively linked to two or more TNFSF receptor binding domains, such that contact with the multimerized molecule leads to clustering of the complete TNFSF receptor in the membrane of a responding cell and thereby activates some or

all of the functional potential of the responding cell. A key concept of the instant invention is that a many-trimer form a TNFSF ligand is needed to stimulate a receptor-bearing responding cell. For example, structural studies of the GITRL/GITR interaction indicate that two closely localized trimers of GITRL are needed to bring together or “cluster” two complete GITR receptor (3 chains of GITR each) (Zhou *et al.*, Proc Natl Acad Sci U S A. 2008;105(14):5465-70). A multimerization scaffold is a molecular structure that provides for this close localization of 2 or more TNFSF receptor binding, typically 2 or more TNFSF ligand extracellular domains (ECD). In the instant invention, portions of collectins such as SPD or portions of C1q family members such as Acrp30 are used to make single polypeptide chains that self-assemble into multimerization scaffolds. Preference is shown for multimerization scaffolds that have “arms” capable of being operatively linked to TNFSF ECD trimers. Alternative embodiments are contemplated, such as multimerization scaffold that is operatively linked to single-chain antibodies that bind to a TNFSF receptor.

[064] “Operatively linked” refers to a method for joining two molecules. For polypeptides, this is preferably by a peptide bond, typically achieved by constructing a DNA or RNA template encoding the operatively linked fusion protein and then expressing the DNA or RNA in a cell or by an in vitro method. In some case, chemical crosslinkers can be used to construct multimeric forms of TNFSF receptor binding agents as described in US 6,482,411 B1 .

[065] “TNFSF” refers to a ligand in the Tumor Necrosis Factor (TNF) SuperFamily.

The TNFSFs are produced as trimeric Type II membrane molecules meaning that their N-terminus points inside the cell and their C-terminal end is extracellular, which is the reverse of most cell surface proteins. This makes these proteins very challenging to engineer using traditional fusion protein strategies.

[066] “TNFSF receptor binder” refers to a molecular fragment that binds to a TNFSF receptor. Exemplary TNFSF receptor binders (or binding domains) include the extracellular domain (ECD) of a TNFSF trimeric molecule or the receptor-binding portion of an antibody recognizing a TNFSF receptor. For a receptor-binding portion of an antibody, preference is give to single-chain antibody constructs (Ahmad *et al.*, Clin Dev Immunol. 2012;2012:980250. PMCID: 3312285). Exemplary TNFSF members whose extracellular domains can be used as TNFSF receptor binders include

CD40L (TNFSF5), CD27L (TNFSF7), CD137L (TNFSF9), OX40L (TNFSF4), GITRL, 4-1BBL, RANKL, LIGHT, CD70, and BAFF.

[067] “Tumor antigens” refers to proteins, carbohydrates, or lipids found on tumor cells against which the immune system can launch an attack. For a discussion of tumor antigens, see Kvistborg *et al.* (Curr. Opin Immunol. 25:284-290, 2013) and Cheever *et al.* (Clin Cancer Res 15, 5323-5337, 2009). Also contemplated as tumor antigens are antigenic peptides deduced from next-generation sequencing from the RNA or DNA of tumors, including exome sequencing (Segal *et al.*, Cancer Res. 2008;68(3):889-92; Castle *et al.*, Cancer Res. 2012;72(5):1081-91).

DETAILED DESCRIPTION OF THE INVENTION

[068] This invention describes, *inter alia*, molecules comprising fusion proteins and the nucleic acids that encode them in which the following protein coding domains are operably linked in the following order: a scaffold comprised of a portion of a collectin or C1q family protein or combinations of dimerizing/trimerizing motifs, an antigen (either following the scaffold or contained within the scaffold), and the extracellular domain of a TNF superfamily ligand. An exemplary fusion protein or nucleic acid that encodes it comprises the antigen, surfactant protein D (SPD) without its carbohydrate receptor domain, and the extracellular domain of CD40 ligand. Alternatives to surfactant protein D can also be used, including using immunoglobulin (Ig), Acrp30, a GCN4 multimerization motif, or similar proteins as scaffolds for CD40 ligand, other members of the TNF superfamily ligands, or other ligands or receptors, including gp96 or MHC molecules. In one embodiment, the molecules, compositions and/or fusion proteins of the invention do not contain portions of avidin or streptavidin.

[069] These fusion proteins are designed to allow the targeting of dendritic cells, macrophages, B cells or other antigen presenting cells with the antigen as well as providing necessary activation signals to induce maturation of the targeted dendritic cell, macrophage, B cell or other antigen presenting cell. This results in the optimal presentation of the antigen to the immune system, and a potent immune response in the treated individual, either T cell mediated or antibody mediated.

[070] In more detail, the instant invention provides a solution for the problem of vaccinating against infectious agents and for cancer immunotherapy. It provides a way to link an adjuvant in the

TNF SuperFamily (TNFSF) to an antigen such that the TNFSF adjuvant and antigen arrive at the same cell at the same time. In the case of CD8+ T cell responses, it is important to provide antigen to dendritic cells (DCs) and other antigen-presenting cells such that the protein antigen is processed by cleavage into peptides and loaded onto MHC-I for cross-presentation on the cell surface as pMHC-I complexes which in turn stimulates the T cell receptor (Signal 1). It is preferable to target the antigen to the CD40 receptor on DCs since this results in superior cross-presentation by a larger number of DC subtypes (Chatterjee *et al.*, Blood. 2012;120(10):2011-20). In addition, it is important to activate the DC that is presenting antigen in order that the DCs present the antigen-specific T cell with accessory signals (Signal 2 and Signal 3). If the DCs display only pMHC-I and are not activated to present other signals, then the resulting antigen-specific CD8+ T cell becomes tolerant and lacks protective effective functions (Bonifaz *et al.*, J Exp Med. 2002;196(12):1627-38. PMCID: 2196060). Stimulation of the CD40 receptor on DCs activates the DCs to provide these other signals and leads to profound CD8+ T cell responses (Bonifaz *et al.*, J Exp Med. 2004;199(6):815-24). Thus, the instant invention provides a strong vaccine for CD8+ T cells by fusing antigen to previously described multimeric forms of CD40L comprised of the extracellular domain (ECD) of CD40L fused to multimerization scaffolds employing portions of surfactant protein D (SPD) or Acrp30.

[071] Activation of DCs and other APCs is best performed by a many-trimer form of CD40L where 2 or more trimers are needed to cluster and thereby activate the CD40 receptors on DCs, as depicted in FIG. 1.

[072] The new understanding of agonistic anti-TNFSF receptor antibodies is shown in FIG. 2. In this case, the antibody is first bound to an adjacent cell via its Fc portion which binds to the Fc receptors on the adjacent cell type (Li and Ravetch, Science. 2011;333(6045):1030-4. PMCID: 3164589; Wilson *et al.*, Cancer Cell. 2011;19(1):101-13; White *et al.*, J Immunol. 2011;187(4):1754-63). This leads to two problems: (1) DCs and other APCs that are not adjacent to an FcR-bearing cell cannot be stimulated; and (2) if the antibody binds to certain FcRs, then it is possible that the adjacent cell will kill the DC by antibody-dependent cellular cytotoxicity (ADCC) or phagocytose the DC and eliminate it (Bulliard *et al.*, J Exp Med. 2013;210(9):1685-93. PMCID: 3754864). The later phenomenon may explain the severe depletion of CD40 B cells when an antibody against CD40 was tested in humans with cancer (Vonderheide *et al.*, J Clin Oncol. 2007;25(7):876-83). These

considerations set the stage for a new and better way to provide both antigen and CD40 stimulation to DCs and other APCs.

[073] Another approach was taken by Xiang *et al.* (J Immunol. 2001;167(8):4560-5) who made a fusion protein of tumor antigen (CEA) joined to the C-terminal end of CD40L (US 7,279,464 B2; US 6,923,958 B2). However, because the CD40L moiety is not located on the end of the protein, it could conceivably have impaired binding of the ligand to the CD40 receptor. No data were presented to rule out this concern, but the vaccine's effectiveness was modest.

[074] In a related approach, Zhang *et al.* (Proc Natl Acad Sci U S A. 2003;100(25):15101-6) fused a tumor antigen onto the N-terminus of the CD40L extracellular domain and delivered this construct using an adenovirus vector. In this case, the molecular design allowed for CD40L to bind unimpaired to its receptor. Even so, the effectiveness of this vaccine was relatively modest. This is expected when a 1-trimer form of CD40L is used rather than a receptor-clustering multi-trimer construct such as SPD-Gag-CD40L.

[075] Another approach was taken by Shirwan *et al.* who produced a fusion protein between the "core" region of bacterial streptavidin protein (CSA) and the extracellular domain of CD40L or 4-1BBL, as disclosed in US 8,017,582 B2 and in Schabowsky *et al.*, Exp Mol Pathol. 86:198-207, 2009. In this case, the N-terminal half of the fusion proteins consisted of CSA where streptavidin naturally assembles into a 4-chain molecule. This multimerism pulls together the covalently linked ECDs for CD40L or 4-1BBL. Since streptavidin binds to biotin and since proteins can be easily biotinylated, it was possible to biotinylate antigens such as chicken ovalbumin (OVA) or the tumor antigens E7 from HPV which allows them to bind non-covalently to CSA-CD40L or CSA-4-1BBL. However, in order to be active, CD40L must be used in a multi-trimer form that clusters together two or more CD40 receptors, as depicted in FIG. 1 of the instant application. The relative inactivity of a single trimer form of CD40L was demonstrated by Haswell *et al.* (Eur J Immunol. 31:3094-3100, 2001; see FIG. 3). In contrast, the CSA-CD40L forms a single trimer of CD40L, as depicted in FIG. 1B of Schabowsky *et al.*, which is not desirable from the perspective of efficient receptor stimulation. Furthermore, the biotin-streptavidin interaction in the design of Shirwan *et al.* is non-covalent. The antigen has been biotinylated which then allows it to bind to the streptavidin moiety in the CSA-CD40L complex. However, *in vivo*, there is free biotin present in biological fluids that can interfere with the formation of the CSA-CD40L/biotin-antigen complex or induce its dissociation. In

contrast, the instant invention utilizes antigen that has been covalently joined to CD40L by virtue of the peptide bonds that make up the SPD-antigen-CD40L fusion protein and thus the protein is not susceptible to dissociation in the presence of free biotin. Another important difference is that CSA is a xenogenic protein from bacteria that is highly antigenic in humans and other vertebrates (Meyer *et al.* Protein Science 2001;10(3):491-503; Yumura *et al.*, Protein Science 2013;22(2):213-21). In contrast, the fusion proteins of the instant invention can be constructed with primarily non-xenogenic proteins sequences such that the only major foreign protein component is the antigen selected for immunization. Therefore, in one embodiment of the present invention, the multimerization scaffold and the plurality of TNFSF receptor binder do not contain any xenogenic portions.

[076] Another system for producing many-trimer forms of OX40L was described by Weinberg *et al.* in US 7,959,925 B2.

In this system, fusion proteins are made by using an N-terminal immunoglobulin Fc domain which naturally dimerizes via interchain disulfide bonds. When this is joined to a trimerizing domain which is then joined to a TNFSF extracellular domain, it results in what is described as a hexamer or “dimer of trimers”. In the instant invention, SEQ ID NO:7 and SEQ ID NO:8 disclose a fusion protein that provides for a 2-trimer form of APRIL fused to the HIV-1 Env protein which is expected to elicit a strong antibody response to HIV-1. The skilled artisan will easily see how the extracellular domain of APRIL could be replaced by the extracellular domain of any other TNFSF ligand, and also how the HIV-1 Env antigen could be replaced by other antigens of interest. Such antigen-multimeric TNFSF fusion proteins are claimed by the instant invention. In addition, the skilled artisan could envision other dimerizing domains (such as that from CD4 or CD8) or other trimerizing domains (such as those from GCN4, TRAF2, thrombospondin 1, Matrilin-4, CMP (Matrilin-I), HSFI, or cubulin, as described in US 7,959,925 B2) or the trimerizing domain from the SPD “neck” region in US 6,190,886 .

[077] As described in the instant application, a surprisingly active vaccine can be made by incorporating an antigen with the arms of SPD in the 4-trimer SPD-CD40L construct that was previously developed by the inventors and shown in FIGs. 3 and 4. For demonstration purposes, the HIV-1 Gag antigen was inserted into the coding region for the SPD collagen-like arm as shown in SEQ ID NO:1 and SEQ ID NO:2 and depicted in FIG. 5. This fusion protein uses the natural SPD “arm” which has been shown to be 46 nm long in shadow electronmicroscopic studies. The

collagen-like triple helical structure and results from the class Gly-Xaa-Yaa collagen-like repeats in the protein which number 59 repeats in the arm. For the instant invention, the length of this arm can be varied in two ways: (1) Amino acid deletions can be introduced that truncate one Gly-Xaa-Yaa motif; and (2) the antigen can be inserted variably along the length of the arm. Considering the 177 amino acids in the 59 collagen-like repeats, the antigen domain can be positioned from 10 to 177 amino acids more C-terminal from the hub, or preferably from 20 to 140 amino acids more C-terminal from the hub, or more preferably from 40 to 120 amino acids more C-terminal from the hub. Likewise, the antigen domain can be placed closer or further from the TNFSF extracellular domain (ECD). For example, the antigen domain could be from 0 to 167 amino acids more N-terminal from the TNFSF ECD, or more preferably from 40 to 120 amino acids more N-terminal from the ECD. As non-limiting examples, SEQ ID 13 and SEQ ID 14 show a fusion protein where the antigen domain was shifted by 10 amino acid positions within the arm of SPD. Likewise, SEQ ID NO 17 and SEQ ID NO 18 show a fusion protein in which 20 amino acids have been removed from the SPD arm. With this as a guide, the skilled artisan will know that it is not critical exactly where in the SPD arm the antigen domain should be positioned.

[078] As previously described by the inventors, 2-trimer forms of TNFSF ligands can be made using Acrp30. FIGs. 3 and 4 show the design and vaccine adjuvant efficacy of an Acrp30-CD40L fusion protein. This molecule has two collagen-like arms. Accordingly, it is contemplated to place an antigen domain within the arms of Acrp30 as shown in SEQ ID 3 and SEQ ID 4 which place the HIV-1 Env antigen within the arms of an Acrp30-BAFF fusion protein. Analogous fusion proteins could be made from other collectin fusion proteins besides SPD-TNFSFs and from other C1q family molecules besides Acrp3-TNFSFs.

[079] A feature of these fusion proteins is that they can readily be made using the natural collectin or C1q family sequences and TNFSF sequences from a variety of organisms. It is preferable to use the murine coding sequences for studies in mice, the macaque coding sequences for studies in macaques, the human coding sequences for use in humans, etc. As non-limiting examples, the sequences shown provide fusion protein made using either murine or human sequences. Thus, animal vaccine uses are specifically contemplated as one use of the instant invention.

[080] In these cases, antigen was introduced into many-trimer forms of TNFSFs by standard genetic engineering methods familiar to the skilled artisan. Such fusion proteins can be made by

ligating together segments of genes or, more preferably, by ordering a custom synthesis from a commercial supplier (*e.g.* DNA2.0, Genset, Genewiz, and other suppliers). In other cases, it is possible to prepare antigenic peptides and TNFSF trimers separately and then link them together by chemical methods. The linking reagents and synthesis strategies that can be used are described in US 6,482,411 B1.

[081] There is a wide choice of antigens from infectious disease antigens, depending on the species in need of vaccination. Without limitation, these can be selected from the following list of disease-causing pathogens:

[082] Viruses such as influenza A and B, parainfluenza, poxviruses, ebola virus, hepadnavirus, filoform viruses such as marburg virus, dengue fever virus, influenza A and B, respiratory syncytial virus, measles (rubeola virus), human immunodeficiency virus (HIV), human papillomavirus (HPV), varicella-zoster, herpes simplex I and 2, cytomegalovirus, Epstein-Barr virus, JC virus, rhabdovirus, rotavirus, rhinovirus, adenovirus, orthomyxovirus, papillomavirus, parvovirus, picornavirus, poliovirus, mumps, rabies, reovirus, rubella, togavirus, retrovirus, coxsackieviruses, equine encephalitis, Japanese encephalitis, yellow fever, Rift Valley fever virus, hepatitis A, B, C, D, and E virus, hantavirus, coronavirus (including SARS and MERS), and the like;

[083] Microbial agents such as *Borrelia* species, *Bacillus anthracis*, *Borrelia burgdorferi*, *Bordetella pertussis*, *Camphylobacter jejuni*, *Chlamydia* species, *Chlamydial psittaci*, *Chlamydial trachomatis*, *Clostridium* species, *Clostridium tetani*, *Clostridium botulinum*, *Clostridium perfringens*, *Corynebacterium diphtheriae*, *Coxiella* species, an *Enterococcus* species, *Ehrlichia* species, *Escherichia coli*, *Francisella tularensis*, *Haemophilus* species, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Lactobacillus* species, a *Legionella* species, *Legionella pneumophila*, *Leptospirosis interrogans*, *Listeria* species, *Listeria monocytogenes*, *Mycobacterium* species, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Mycoplasma* species, *Mycoplasma pneumoniae*, *Neisseria* species, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Pneumococcus* species, *Pseudomonas* species, *Pseudomonas aeruginosa*, *Salmonella* species, *Salmonella typhi*, *Salmonella enterica*, *Rickettsia* species, *Rickettsia rickettsii*, *Rickettsia typhi*, *Shigella* species, *Staphylococcus* species, *Staphylococcus aureus*, *Streptococcus* species, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Treponema* species, *Treponema pallidum*, a *Vibrio* species, *Vibrio cholerae*, *Yersinia pestis*, and the like;

[084] Fungal, protozoan, and parasitic agents such as *Aspergillus* species, *Candida* species, *Candida albicans*, *Candida tropicalis*, *Cryptococcus* species, *Cryptococcus neoformans*, *Entamoeba histolytica*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Leishmania* species, *Nocardia asteroides*, *Plasmodium falciparum*, *Plasmodium vivax*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *Toxoplasma* species, *Trypanosoma brucei*, *Schistosoma mansoni*, *Pneumocystis jiroveci*, and the like.

[085] There is a wide choice of tumor antigens, depending on the species in need of cancer immunotherapy. Without limitation, these can be selected from the following list of cancer-associated antigens:

[086] gp100; WT1; Melan-A; tyrosinase; PSMA; HER-2/neu; MUC-I; PRAME; topoisomerase; BRAF V600E; bcr-Abl; sialyl-Tn; carcinoembryonic antigen; ErbB-3-binding protein-I; alpha-fetoprotein; and the cancer testis antigens MAGE-A1, MAGEA4, and NY-ESO-1; MART-1, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)-COI7 -1 AIGA 733, Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, ctv6, amll, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate specific membrane antigen (PSMA), MAGE-family of tumor antigens (e.g., MAGEA1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-AS, MAGE-A9, MAGE, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGEC5), GAGE-family of tumor antigens (e.g., GAGE-I, GAGEIn 2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGES, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, a-fetoprotein, E-cadherin, alpha-catenin, beta-catenin and gamma-catenin, p 120ctn, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL), EGFRviii, SSX-1, SSX-4, SSX-5, SCP-1, CT-7, cdc27, adenomatous polyposis coli protein (APC), fodrin, PI A, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papilloma virus proteins, Smad family of tumor antigens, LMP-1, LMP-2, EBV-encoded nuclear antigen (EBNA)-1, or c-erbB-2, and the like.

[087] There is a wide choice of delivery methods for the vaccines of the instant invention. Where the vaccine is comprised of a nucleic acid sequence, it can be delivered using a DNA or RNA vectors. Without limitation, these can be selected from the following list: Adenovirus (as shown in FIG. 12 for example), poxvirus including Modified Vaccinia Ankara, Herpesviruses, retroviruses,

lentiviruses, Newcastle Disease Virus, Mumps Virus, Measles Virus, Vesicular Stomatitis Virus, rhabdovirus, Para-influenza Virus, Sendai virus, Influenza Virus, Reovirus, and a Seneca Valley virus, alphavirus, Sindbis virus, Venezuelan Equine Encephalitis (VEE), Coxsackie virus, myxoma virus, viral organisms include those that are dsDNA viruses, ssDNA viruses, dsRNA viruses, (+) ssRNA viruses (-) ssRNA viruses, ssRNA-RT viruses, and dsDNA-RT viruses, and the like.

[088] Vaccines of the present invention can also be delivered as plasmid DNAs that include a promoter (e.g., CMV promoter) and a transcription termination and polyadenylation sequence. Such plasmids also include genes needed for growth in bacteria, but fragments of DNA can also be prepared by in vitro enzymatic synthesis. An exemplary plasmid used in the experiments in FIGs. 4 and 6-17 is pcDNA3.1 (Life Technologies, Inc., Carlsbad, CA) but other choices are available. The DNA can be delivered directly by injection into muscle (“naked” DNA vaccination) as shown in FIGs. 8-11 and 15-17. It can also be delivered by a number of means including electroporation, microinjection, gene gun delivery, lipofection, polymer-mediated delivery, and the like. The same methods can be used for RNA vaccination. In addition, for bacteria that enter cells such as Salmonella or Listeria, plasmid DNA can be introduced into these bacteria which then carry that DNA into the eukaryotic host cell, a process called “bactofection.”

[089] As another use of the instant invention, fusion proteins comprised of an antigen linked to a many-trimer TNFSF can be administered to APCs like dendritic cells ex vivo, as shown in FIGs. 7 and 14. Once the antigen has been delivered and the APCs activated, these DCs can then be delivered to a host as a cellular form of vaccination (Barth *et al.*, 2010;16(22):5548-56. PMID: 2994719).

[090] Without limitation, the following examples of invention are disclosed:

EXAMPLE 1: Vaccination to elicit CD8+ T Cells against HIV-1 Gag antigen

[091] CD40 ligand (CD40L, CD154) is a membrane protein that is important for the activation of dendritic cells (DCs) and DC-induced CD8+ T cell responses. To be active, CD40L must cluster CD40 receptors on responding cells. To produce a soluble form of CD40L that clusters CD40 receptors necessitates the use of a multi-trimer construct. With this in mind, a tripartite fusion protein was made from surfactant protein D (SPD), HIV-1 Gag as a test antigen, and CD40L, where SPD serves as a scaffold for the multi-trimer protein complex. This SPD-Gag-CD40L protein

activated CD40-bearing cells and bone marrow-derived DCs in vitro. Compared to a plasmid for Gag antigen alone (pGag), DNA vaccination of mice with pSPD-Gag-CD40L induced an increased number of Gag-specific CD8⁺ T cells with increased avidity for MHC-I-restricted Gag peptide and improved vaccine-induced protection from challenge by vaccinia-Gag virus. The importance of the multi-trimeric nature of the complex was shown using a plasmid lacking the N-terminus of SPD that produced a single trimer fusion protein. This plasmid, pTrimer-Gag-CD40L, was only weakly active on CD40-bearing cells and did not elicit strong CD8⁺ T cell responses or improve protection from vaccinia-Gag challenge. An adenovirus-5 (Ad5) vaccine incorporating SPD-Gag-CD40L was much stronger than Ad5 expressing Gag alone (Ad5-Gag) and induced complete protection (i.e., sterilizing immunity) from vaccinia-Gag challenge. Overall, these results show the potential of a new vaccine design in which antigen is introduced into a construct that expresses a multi-trimer soluble form of CD40L, leading to strongly protective CD8⁺ T cell responses.

[092] DNA vaccination induces both cellular and humoral responses against an encoded antigen, protecting animals against subsequent infection with a microbial pathogen. DNA vaccines are potent inducers of virus-specific T cell responses and studies have shown that prophylactic DNA vaccines, administered either alone or with recombinant viral vaccines as prime/boost vaccine, can provide protection against challenge with viral pathogens including SIV. The HIV-1 Gag antigen encoded within DNA or viral vector vaccines is known to induce measurable immune responses, providing a method to vaccinate against HIV-1. One strategy to enhance the effectiveness of DNA vaccines encoding weakly immunogenic antigens is by co-delivering genes encoding molecular adjuvants. TNF superfamily ligands (TNFSFL) including CD40L are costimulatory molecules involved in dendritic cell (DC) and T cell activation and have previously been tested as adjuvants to enhance immune responses in several vaccination studies.

[093] CD40L acts on DCs to induce or “license” CD8⁺ T cell responses. CD40L also works on DCs to diminish the immune suppression due to CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) and prevents the premature disappearance vaccine-generated CD8⁺ T cells. Consequently, we and others have examined the potential of CD40 stimulation as an adjuvant for vaccines designed to generate CD8⁺ T cell responses.

[094] CD40-mediated activation requires clustering of this receptor leading to the assembly of a supramolecular signaling complex inside cells. When CD40L is expressed on CD4⁺ T cells, the array

of membrane CD40L molecules ligates receptors on DCs and other cells to create a patch of clustered CD40 receptors that activates downstream events. For soluble ligands of CD40, some other way must be found to induce CD40 receptor clustering. Most reports on CD40 activation use agonistic anti-CD40 antibodies. It is now recognized that these antibodies only induce a CD40 signal if they are mounted onto Fc receptors (FcRs), thereby creating an array of anti-CD40 antibodies that can cluster the receptors on an adjacent CD40 receptor-bearing cell. This requirement restricts the effectiveness of anti-CD40 antibodies to tissue microenvironments that contain FcR-bearing cells. Other drawbacks of using anti-CD40 antibodies are their propensity to generate host antibodies against themselves, their toxicity for mice and humans, and their depleting effect on CD40-bearing B cells in the blood. These negative qualities argue against the routine use of agonistic anti-CD40 antibody as an adjuvant for vaccines given to otherwise healthy people in order to prevent infection by pathogens such as HIV-1.

[095] The use of CD40L presents an alternative to agonistic anti-CD40 antibodies as a vaccine adjuvant. CD40L is made as a Type II membrane protein but can be proteolytically cleaved from the cell surface and released as a soluble single trimer. By itself, a single trimer of CD40L is unable to provide clustering of CD40 receptors sufficient to generate a cell signal. Consequently, we devised fusion proteins in which the extracellular domain of CD40L is joined to a scaffold protein such as surfactant protein D (SPD). The resulting fusion protein, SPD-CD40L, is expected to form a plus sign-shaped 4-trimer molecule held together at its N-terminal “hub” by interchain cysteine bonds. Each “arm” of the SPD portion is a collagen-like triple helix that presents the CD40L trimers on the outside of the molecule for easy interaction with CD40 receptors. As expected, we previously found that SPD-CD40L activated DCs in vitro and was a strong vaccine adjuvant for CD8⁺ T cell responses against HIV-1 antigens.

[096] In the previous study, mice were vaccinated with plasmid DNAs for HIV antigens such as Gag (pGag) mixed in a single syringe with pSPD-CD40L. In the present study, we considered the effects of introducing the HIV-1 Gag antigen into the SPD-CD40L protein to create SPD-Gag-CD40L, a single chain peptide that retains the ability to form a multi-trimer structure capable of clustering and thereby activating the CD40 receptor. This molecular design resulted in a DNA vaccine that elicited much stronger Gag-specific CD8⁺ T cell responses capable of protecting mice from challenge by vaccinia virus engineered to express Gag (vaccinia-Gag). Since DNA vaccination

is relatively inefficient, viral delivery was also examined by introducing SPD-Gag-CD40L into an adenovirus-5 (Ad5) vaccine vector. The resulting Ad5-SPD-Gag-CD40L vaccine provided essentially total protection from vaccinia-Gag challenge, further attesting to the remarkable effectiveness of including the antigen inside the SPD-CD40L construct rather than administering SPD-CD40L as a separate adjuvant molecule.

Materials and Methods

Construction and preparation of DNA plasmids

[097] To construct a HIV-1 Gag DNA vaccine (pGag), the gag coding sequence was fused with the first 21 amino acids of human tissue plasminogen activator (t-PA) as a signal peptide as described previously (Stone *et al.*, J Virol. 2006;80(4):1762-72). A DNA construct encoding murine SPD-CD40L was also previously described (Stone *et al.*, J Virol. 2006;80(4):1762-72). To construct SPD-Gag-CD40L, the p55 gag sequence from pGag was inserted into the “arm” portion of murine SPD between amino acids 105 and 106 within the construct SPD-CD40L (*i.e.* between peptide sequence GERGLSG and PPGLPGI of murine SPD) (see FIG. 5). To construct pTrimer-Gag-CD40L, the ScGag coding sequence was fused with amino acid 106 of mouse SPD within construct SPD-CD40L (*i.e.* fusing ScGag to a fragment of SPD-CD40L starting at peptide sequence PPGLPGI), thereby deleting the N-terminal portion of SPD that contains the dicystine-containing “hub” region needed for self-assembly into a 4-armed molecule. As a result, this construct is expected to form single trimers of Gag-SPD-CD40L (see FIG. 5). Plasmid pIL-12p70, encoding mouse single chain IL-12, was purchased from Invivogen Inc. All plasmids were propagated in *Escherichia coli* strain TOP10™. Endotoxin-free DNA plasmid preparations were prepared using an Endofree Giga™ plasmid kit (Qiagen™). Plasmids were further purified to remove residual endotoxins with additional Triton -X114™ extractions as previously described (Stone *et al.*, J Virol. 2006;80(4):1762-72). Plasmid endotoxin level was <0.2 EU/ml for all constructs as confirmed by LAL endotoxin assay (Lonza Inc.). Gag protein secretion for all Gag-containing constructs was confirmed by p24 ELISA assay on supernatants from transfected 293T cells.

Transient transfection and Western blotting of protein constructs

[098] 293T cells were transiently transfected with plasmid constructs using Genjet-plus™ Transfection Reagent (Signagen Laboratories, Iamsville, MD). A control transfection with GFP

plasmid was used to confirm transfection efficiency of each reaction. Forty-eight hours later, supernatants were centrifuged and filtered with a 0.45 μ m filter to remove debris. Filtered supernatant was reduced with 2-mercaptoethanol, loaded onto sodium-dodecyl sulfate–10% polyacrylamide gels (10% SDS-PAGE) (BioRad), electrophoresed, and blotted onto PVDF membranes (Pierce). The membranes were blocked using 5% (w/v) dry milk and then probed with goat anti-mouse CD40L antibody (R&D Systems), followed by incubation with anti-goat horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch). The protein bands were developed onto X-ray film using chemiluminescence. To further evaluate high molecular weight complexes, a non-denaturing PAGE was performed in the absence of SDS and reducing agent.

CD40 in vitro activity assay

[099] A CD40 receptor-bearing reporter cell line (CD40-293-SEAP) was used to monitor CD40L-mediated activation. This 293-derived cell line constitutively expresses human CD40 receptor along with the gene for secreted alkaline phosphatase (SEAP) gene under control of NF- κ B (Maurais *et al.*, Virology. 2009;385(1):227-32). Briefly, 80,000 CD40-293-SEAP reporter cells, grown in DMEM medium with 10% FBS, were plated in each well of a 96-well plate. A total of 100 μ l of SPD-Gag-CD40L, SPD-CD40L or pcDNA3.1 transfected 293T supernatant was added to the reporter cells for 24 h in triplicate at various dilutions. On the following day, 10 μ l/well of the supernatants was added to the wells of a 96-well assay plate together with 100 μ l/well of QUANTI-Blue™ Alkaline Phosphatase substrate (InvivoGen). The plates were incubated for 20 min at 20°C and OD was read at 650 nm.

DC activation and maturation assay

[100] Bone marrow-derived murine DCs were generated by standard methods (Inaba *et al.*, Cellular immunology. 1995;163(1):148-56) with the following modifications: Bone marrow cells were obtained from C57BL/6 mice and washed in RPMI 1640 media. The cells were then placed in tissue culture treated T75 flasks at a concentration of 1×10^6 cells per ml in 20 ml complete RPMI (RPMI 1640 with 10% FBS, 20 μ g/ml gentamycin sulfate, 50 μ M 2-mercaptoethanol), and 20 ng/ml murine recombinant GM-CSF and 10 ng/ml murine recombinant IL-4 (Peprotech, Rocky Hill, NJ)). Cells were cultured at 37°C, 5% CO₂ and on day 3, media was replaced with fresh complete RPMI containing cytokines. On day 5, cells were harvested and washed and resuspended in complete RPMI

at 5×10^5 cells/ml. A total of 2 ml was added to each well of 6-well tissue culture treated plates. Subsequently, 300 μ l of supernatant containing SPD-Gag-CD40L or DC activation cytokine mix (containing TNF, IL-1 β , IL-6, and PGE2) was added and cells were incubated for 36 hours. Cells were harvested and stained with hamster anti-mouse CD11c clone N418 PE-Cyanine7 conjugate (eBioscience, San Diego, CA) combined with one of the following antibodies: anti-mouse CD80 clone 16-10A1, anti-mouse CD86 clone GL1, anti-mouse CD40 clone 1C10, anti-mouse CD83 clone Michel-17, anti-mouse MHC Class II (I-A/I-E) clone M5-114.15.2, and anti-mouse CCR7 clone 4B12 (all from eBioscience). After flow cytometry analysis, the mean fluorescence intensity for each antibody was calculated for CD11c+ dendritic cells under each experimental condition. **FlowJo™ 7.6.4** flow cytometry analysis software (FlowJo™, Ashland, OR) was used for analysis. Three independent wells were analyzed for each condition.

Production of recombinant adenovirus containing Gag antigen or SPD-Gag-CD40L

[101] The construction of replication-deficient adenovirus (pAdEasy-1) containing codon-optimized Gag with a t-PA signal peptide or SPD-Gag-CD40L was performed as described by the manufacturer (AdEasy™ Adenoviral vector system, Agilent Technology, Inc.). Briefly, gene constructs were PCR amplified and cloned into the pAdenoVator-CMV5 (AdenoVator™) shuttle vector (Qbiogene). CMV5-shuttle vector clones were confirmed by sequencing and then electroporated into BJ5183 cells containing the pAdEasy-1 plasmid to induce homologous recombination. The recombined pAdEasy-1 vector was linearized and transfected into AD293 cells (Stratagene). Following propagation in AD293 cells, recombinant Ad5 viruses were purified and concentrated using the Adeno-X™ Mega purification kit (Clontech). The concentration of Ad5 viral particles (vp) was determined by measuring the absorbance at 260 nm and 280 nm, and calculated using the formula $\text{vp/ml} = \text{OD}_{260} \times \text{viral dilution} \times 1.1 \times 10^{12}$. To determine infectious units, viruses were titrated using the Adeno-x™ Rapid Titer kit (Clontech).

Mice and immunization schedule

[102] Female BALB/c mice (7–8 weeks old) were used in all vaccination experiments. Animals were housed at the University of Miami under the guidelines of the National Institutes of Health (NIH, Bethesda, MD). All animal experiments were performed in accordance with national and institutional guidance for animal care and were approved by the IACUC of the University of Miami.

Different groups of mice were immunized with plasmid DNA or Ad5 viruses for immunological and vaccinia challenge experiments.

[103] DNA Immunization Schedule: DNA was injected intramuscularly into the quadriceps muscle of both hind limbs. Vaccinations were given three times at two-week intervals with 100 µg of SPD-Gag-CD40L or 100µgGag plasmid mixed with either 20 µg of pcDNA3.1, pSPD-CD40L, or pIL-12p70 plasmids. Doses were administered in a total volume of 100 µl PBS (50 µl per limb). Control mice were injected with 100 µg of pcDNA3.1 empty vector.

[104] Splenocyte preparation: Two weeks following the final DNA immunization, mice were euthanized and spleens were removed. Single cell splenocyte preparations were obtained by passage through a 40 µm nylon cell strainer (BD Falcon). Erythrocytes were depleted with lysis buffer (Sigma) and splenocytes washed thoroughly using R10 media (RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 50 µM 2-mercaptomethanol, 100 U/ml of penicillin, 100 µg/ml streptomycin, and 10 mM HEPES).

[105] Adenovirus Immunization Schedule: Five mice per group were immunized by intramuscular injection with Ad5 constructs twice at a two-week interval. Viral vector was injected in a total volume of 100 µl PBS (50 µl per limb) in the quadriceps muscles of both hind limbs.

Enzyme linked immunospot (ELISPOT) assay

[106] IFN-γ and IL-2 ELISPOT assays were performed to determine antigen specific cytokine secretion from immunized mouse splenocytes. ELISPOT assays were carried out per manufacturer's protocol (R&D Systems) using 96-well MAIP plates (Millipore). Freshly prepared vaccinated mouse splenocytes (1×10^5 cells/well) were added to each well of the plate and stimulated for 18 h at 37 °C, 5% CO₂ in the presence of HIV-1 Gag peptide AMQMLKETI (10 µg/ml or as described). A c-myc peptide (negative control) and PMA/Ionomycin (positive control) were evaluated to calculate the minimum and maximum number of antigen-specific ELISPOTs respectively. After 18 h, spots were developed with AEC substrate kit (Vector Laboratories) according to manufacturer's instructions. The membrane was read by automated plate reader (CTL Immunospot) for quantitative analyses of the number of IFN-γ or IL-2 spots forming counts (SFC) per million cells plated, subtracting negative control values.

T Cell Receptor Avidity ELISPOT Assay

[107] ELISPOT was performed as described, stimulating the cells with 1 µg/ml, 10–3 µg/ml, or 10–5 µg/ml of Gag peptide (AMQMLKETI) to evaluate the number of T cells able to secrete IFN-γ at limiting peptide concentrations.

ELISA assay for anti-Gag IgG responses

[108] Anti-Gag antibody production was measured by ELISA assay. HIV-1 p55 Gag protein (10 µg/ml) was coated onto 96-well ELISA plates overnight at 4 °C. Mouse sera at varying dilutions (1:30, 1:120, 1:480 and 1:1,920) were added to Gag-coated wells and incubated at room temperature for 2 h with shaking. After the plates were washed, Gag antigen specific IgG antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Inc.). Signal was developed using BluePhos™ substrate (KPL, Inc.). Plates were analyzed using a 96-well plate absorbance reader at 650 nm. Endpoint titers were calculated as the highest dilution with more than twice the background absorbance of control wells.

Vaccinia-Gag virus challenge

[109] Two weeks following DNA or Ad5 immunization, mice were challenged i.p with 1 x 10⁷ vp vaccinia-gag virus vP1287 as described (Qiu *et al.*, J Virol. 1999;73(11):9145-52). Five days following challenge, mice were sacrificed and ovaries were removed and homogenized in 500 µl PBS. For measurement of virus titers, samples were sonicated and evaluated in triplicate by 10-fold serial dilution on Vero cells plated in 24 well plates. Following 48-hour incubation, the plates were stained with 0.1% (w/v) crystal violet in 20% ethanol. Plaques were counted and expressed as the plaque-forming units (PFU) of virus in total lysate volume (PFU/mouse).

Statistical analysis

[110] All error bars represent standard error from the mean. Graph pad Prism 6.0 software was used to calculate significance by one way ANOVA for multiple comparison or by two-tailed Student's t test, comparing mice vaccinated with SPD-Gag-CD40L, Gag, or Gag antigen + adjuvant (SPD-CD40L or IL-12p70). In all figures, p values are labeled by asterisks denoting p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***). Any unlabeled comparisons were not statistically significant between groups.

Results

Construction and expression of multi-trimer SPD-Gag-CD40L

[111] CD40L is naturally produced as a Type II membrane protein on the surface of activated CD4+ T cells and other cells. When an activated CD4+ T cell comes in contact with a DC, an immunological synapse forms that clusters CD40 receptors in the DC membrane, which in turn initiates downstream events in the DC. To mimic this situation using a soluble CD40L protein, a many trimer form of CD40L is needed since single trimers of CD40L do not provide an effective stimulus (reviewed in Kornbluth *et al.*, International Reviews of Immunology. 2012;31(4):279-88). Consequently, multi-trimer soluble forms of CD40L were developed by fusing SPD with the CD40L extracellular domain, where SPD provides a self-assembling scaffold for multimerization. SPD-CD40L mimics the multivalent nature of membrane CD40L and was previously shown to activate B-cells, macrophages and dendritic cells in vitro and enhance vaccine responses in vivo. In the previous vaccine studies, antigen and multi-trimer CD40L adjuvant were used as separate molecules and mixed together for immunization (Stone *et al.*, J Virol. 2006;80(4):1762-72). To further improve this vaccine design, an immunogen was developed that incorporated antigen (exemplified by HIV-1 Gag) and multi-trimer CD40L into a single polypeptide, SPD-Gag-CD40L. The p55 portion of Gag was inserted into protein sequence for the collagen-like trimeric “arm” of SPD, between amino acid 105 and 106 of mouse SPD within the SPD-CD40L construct (FIG. 5A). To show that SPD-Gag-CD40L has the expected structure, protein was produced by transfecting 293T cells with pSPD-Gag-CD40L plasmid DNA. Using reducing conditions, SDS-PAGE, and western blotting for CD40L, the resulting culture supernatant was found to contain a single protein of the expected size of 105 kDa (FIG. 6A). A single 105 kDa band was also observed using antibody to the p24 portion of Gag (data not shown). To confirm that SPD-Gag-CD40L forms a large protein complex, PAGE and western blotting were performed using a non-denaturing gel in the absence of reducing agents. Multiple bands were observed at >200 kDa molecular weight, demonstrating the formation of large multimeric complexes (FIG. 6B).

Biological activity of multi-trimer soluble SPD-Gag-CD40L

[112] To assess the ability of SPD-Gag-CD40L to stimulate the CD40 receptor, a CD40-bearing indicator cell line was used as described previously (Maurais *et al.*, Virology. 2009;385(1):227-32). In

this cell line, CD40 stimulation activates the NF- κ B pathway which in turn activates the κ B promoter driving the expression of secreted alkaline phosphatase (SEAP) that is measured by a colorimetric enzymatic assay. Supernatants from 293T cells transfected with pSPD-Gag-CD40L or parent pSPD-CD40L stimulated these CD40 receptor-bearing cells to produce SEAP (FIG. 7A). In contrast, supernatants from 293T cells transfected with pcDNA3.1 empty vector were inactive. To evaluate the biological activity of the soluble forms of CD40L, bone marrow-derived dendritic cells were treated with supernatants from 293T cells transfected with either pSPD-Gag-CD40L or pcDNA3.1 empty vector. A cytokine mix (TNF, IL-1 β , IL-6, and PGE2) was used to “mimic” an inflammatory environment and used as a positive control. As shown in FIG. 7B, CD80, CD86 and CCR7 were significantly upregulated by SPD-Gag-CD40L supernatant compared to pcDNA3.1 control supernatant. In contrast, CD40 expression was significantly reduced, consistent with endocytosis of CD40 following SPD-Gag-CD40L ligation.

As a DNA vaccine, multi-trimer soluble SPD-Gag-CD40L was more immunostimulatory than separate plasmids for Gag antigen and SPD-CD40L adjuvant

[113] Plasmid DNA for SPD-Gag-CD40L (pSPD-Gag-CD40L) was evaluated for its ability to enhance immune responses as a DNA vaccine. Mice were vaccinated three times at two-week intervals with an intramuscular injection of 100 μ g of pSPD-Gag-CD40L plasmid DNA. For comparison, 100 μ g of plasmid DNA encoding soluble secreted Gag antigen (pGag) was mixed with 20 μ g of separate plasmids encoding either SPD-CD40L or IL-12p70 adjuvants or pcDNA3.1 empty control vector. The vaccination schedule is outlined in FIG. 8A. Two weeks following the third vaccination, T cell responses were analyzed by IFN- γ and IL-2 ELISPOT assays using the Kd-restricted HIV-1 Gag peptide AMQMLKETI to stimulate mouse splenocytes. As shown in FIG. 8B, there was a significant increase in Gag-specific CD8+ T cell responses measured by IFN- γ ELISPOT in splenocytes from mice vaccinated with pSPD-Gag-CD40L compared to mice vaccinated with pGag alone or a mixture of separate plasmids for pGag antigen combined with either pSPD-CD40L or pIL-12p70 adjuvants. Comparing pSPD-Gag-CD40L to unadjuvanted pGag alone, mean IFN- γ ELISPOT responses increased >60-fold. In contrast, the responses to separate plasmids for pGag mixed with pSPD-CD40L or pIL-12p70 adjuvants were much less. Similarly, IL-2 ELISPOT responses were significantly increased for pSPD-Gag-CD40L compared to pGag alone or

separate plasmids for pGag antigen mixed with pSPD-CD40L or pIL-12p70 adjuvants (FIG. 8C). Comparing pSPD-Gag-CD40L to pGag alone, mean IL-2 ELISPOT responses increased >10-fold.

[114] To determine if high avidity CD8⁺ T cells were present, CD8⁺ T cell IFN- γ ELISPOT responses were tested at limiting AMQMLKETI peptide concentrations. As shown in FIG. 9A, pSPD-Gag-CD40L significantly increased IFN- γ ELISPOT responses compared to other vaccine groups at all peptide dilutions. At 10 pg/ml of AMQMLKETI peptide, IFN-gamma ELISPOT responses were only detectable from the splenocytes of mice vaccinated with pSPD-Gag-CD40L. Overall, these data show that pSPD-Gag-CD40L markedly enhanced anti-Gag CD8⁺ T cell immune responses and CD8⁺ T cell avidity levels compared to alternative vaccination approaches.

[115] To evaluate humoral immune responses, Gag-specific IgG antibody titers in mice serum were measured by ELISA assay two weeks following vaccination. As shown in FIG. 9B, all vaccine groups induced similar Gag-specific IgG responses compared to Gag vaccination alone and there were no significant differences between groups.

Single-trimer Gag-CD40L fusion protein failed to enhance immune responses compared to multi-trimer SPD-Gag-CD40L

[116] We next evaluated the role of multi-trimerization by the SPD scaffold on the immune response. The N-terminus of SPD is involved in disulfide bonding and is required to form 4-trimer complexes (Crouch *et al.*, J Biol Chem. 1994;269(25):17311-9). Deleting this N-terminal portion of SPD (amino acids 106-256 in murine SPD) results in a single-trimer form of Gag-CD40L (pTrimer-Gag-CD40L). A t-PA signal peptide was added at the N-terminus sequence to direct protein secretion, followed by HIV-1 Gag, amino acids 106-256 of murine SPD, and then amino acids 47-260 of murine CD40L. Lacking the multimerizing “hub” of SPD, this construct is expected to form single trimer molecules containing Gag and CD40L. To examine the biological activity of pTrimer-Gag-CD40L, protein was made by transfecting 293T cells with pTrimer-Gag-CD40L plasmid and testing the resulting supernatant in the CD40 NF- κ B SEAP indicator cell line assay described above. As expected, with only one trimer of CD40L, the pTrimer-Gag-CD40L-encoded protein had little or no activity in this assay (data not shown), confirming previous reports that single trimers of CD40L are essentially unable to stimulate CD40 receptor-bearing cells (Holler *et al.*, Mol Cell Biol. 2003;23(4):1428-40; Haswell *et al.*, Mol Cell Biol. 2003;23(4):1428-40). Mice were then vaccinated

with DNA vaccines encoding pGag (unadjuvanted antigen alone), pTrimer-Gag-CD40L (single trimer of Gag antigen fused to CD40L) or pSPD-Gag-CD40L (multi-trimer of Gag antigen fused to CD40L). Mice vaccinated with pSPD-Gag-CD40L showed a significant increase in IFN-gamma ELISPOT responses compared to unadjuvanted pGag alone or pTrimer-Gag-CD40L which contains Gag and CD40L but lacks the multi-trimer structure (FIG. 10A). Also observed was a significant increase in IL-2 ELISPOT responses for the pSPD-Gag-CD40L group vs. pTrimer-Gag-CD40L (FIG. 10B).

Vaccination with pSPD-Gag-CD40L protected mice from virus challenge by vaccinia-Gag

[117] To determine the protective efficacy of the CD8⁺ T cells induced by DNA vaccination with pSPD-Gag-CD40L, vaccinated mice were challenged by vaccinia virus expressing the HIV-1 Gag antigen (vP1287 or vaccinia-Gag) (Qiu *et al.*, J Virol. 1999;73(11):9145-52). Two weeks following final DNA vaccination, mice were challenged intraperitoneally with vaccinia-gag (10E7 PFU). As shown in FIG. 11A, mice vaccinated with pSPD-Gag-CD40L had a significantly less tissue virus in ovaries compared with unvaccinated animals ($p < 0.001$) or animals vaccinated with pGag DNA vaccine alone ($p < 0.05$) when vaccinia PFUs were measured on day 5 following vaccinia-Gag challenge. Overall, 4 out of 13 mice vaccinated with pSPD-Gag-CD40L had undetectable viral titers (less than 10 PFU in total ovary lysate).

[118] To determine the effect of CD40L multi-trimerization on the protection conferred by vaccination, mice were vaccinated with pcDNA3.1 empty vector, pGag antigen alone, pTrimer-Gag-CD40L, or pSPD-Gag-CD40L (FIG 11B). There were no significant differences in vaccinia-Gag titers between pGag and pTrimer-Gag-CD40L groups, with both groups reducing viral load by ~1 log compared to pcDNA3.1 treated mice. In contrast pSPD-Gag-CD40L reduced mean viral load by ~3 log in this experiment.

Mice vaccinated with an Ad5-SPD-Gag-CD40L viral vector were completely protected from vaccinia-Gag challenge

[119] While DNA vaccination is effective in mice, its translation to humans has proved difficult. Instead, most currently tested HIV-1 vaccines have used viral vectors, especially adenovirus-5 (Ad5). Consequently, the nucleic acid sequences for Gag alone (Ad5-Gag) or SPD-Gag-CD40L (Ad5-SPD-Gag-CD40L) were cloned into replication defective Ad5 and used to vaccinate mice twice at two-

week intervals with 1×10^9 viral particles (vp) i.m. Two weeks following the final vaccination, mice were challenged intraperitoneally with vaccinia-Gag (107 PFU). Remarkably, all 5 mice vaccinated with Ad5-SPD-Gag-CD40L had no detectable vaccinia virus in their ovaries (<10 PFU/mouse) (FIG. 12), which was statistically significant compared with either the Ad5-Gag or unvaccinated groups ($p < 0.01$). Overall there was a 7-log reduction in vaccinia virus titers when Ad5-SPD-Gag-CD40L was compared to Ad5-Gag. A repeat experiment gave similar results (data not shown). These data support the strategy of introducing SPD-Gag-CD40L into viral vector vaccines such as Ad5.

Discussion

[120] Stimulation through the CD40 receptor is important for generating CD8⁺ T cell responses under non-inflammatory conditions. Numerous studies in mice have shown that agonistic antibodies to CD40 can activate strong responses to vaccination. However, the translation of agonistic anti-CD40 antibody to the clinic has proved challenging due to concerns about toxicity, depletion of CD40-positive cells such as B cells, and the relatively limited effectiveness of agonistic anti-CD40 antibody in humans when compared to studies in mice.

[121] An important advance in the understanding of the CD40L/CD40 system has been the recognition that DC activation requires clustering of the CD40 receptor in order to stimulate the formation of an intracytoplasmic signaling complex. For agonistic anti-CD40 antibodies, clustering requires that the antibodies be mounted via FcRs on an adjacent cell. Under conditions where an adjacent FcR-bearing cell is absent, agonistic anti-CD40 antibodies are not effective.

[122] Keeping in mind this requirement for CD40 receptor clustering, we and others have examined various multi-trimer forms of CD40L as agonists for murine, macaque, and human DCs. These molecules were made as fusion proteins between a multimerization scaffold such as SPD and the extracellular domain of CD40L. SPD is an ideal scaffold because CD40L is a Type II membrane protein in which the C-terminus faces outward and SPD forms a plus sign-shaped structure where the N-terminus is at the central “hub” and the C-terminus faces conveniently outward. When used as a DNA vaccine, multi-trimer SPD-CD40L was an effective adjuvant when added to plasmid DNA encoding an antigen and led to significantly increased antigen-specific CD8⁺ T cell responses. However, we hypothesized that the vaccine response might be even stronger if the antigen and multi-trimer CD40L protein sequences were physically linked rather than being mixed together for

vaccination. Consequently, a tripartite fusion protein was constructed that combined the SPD multimerization scaffold, HIV-1 Gag as an antigen, and murine CD40L as the adjuvant (SPD-Gag-CD40L) (FIG. 5A and 5B).

[123] As a first step, non-denaturing PAGE was used to show that SPD-Gag-CD40L protein is indeed a high molecular weight multimer complex (FIGs. 6C and 6D). In vitro, this multi-trimer CD40L molecule could stimulate a CD40 receptor-bearing indicator cell line that reports out NF- κ B activation by releasing secreted alkaline phosphatase (SEAP) (FIG. 7A). As a control, a molecule was made in which the N-terminal “hub” of SPD was deleted, leading to a 1-trimer CD40L molecule that had little or no activating in this NF- κ B activation assay (data not shown). This control revealed the critical importance of the multi-trimer structure in forming a highly active form of CD40L, as previously demonstrated by Haswell et al. (Eur J Immunol. 2001;31(10):3094-100). As expected, SPD-Gag-CD40L stimulated murine bone marrow-derived DCs in vitro to express cell surface markers of activation (FIG. 7B). While these data do not present direct evidence that the SPD-Gag-CD40L constructs folds into the structure outlined in Figure 5B, we consider the ability of the construct to form biologically active trimers to provide initial evidence that functional trimers are being generated. In preliminary experiments we have also observed biological activity for SPD-CD40L fusions with alternative antigens including gp100 and HIV-1 Env gp120 (data not shown), supporting the concept that SPD-CD40L fusions with antigen is broadly applicable as a vaccine design strategy.

[124] In vivo, plasmid DNA (pSPD-Gag-CD40L) was tested as a vaccine (FIG. 8A) and compared to vaccination with plasmid DNA for Gag alone (pGag) or an mixture of separate pGag antigen plasmid with pSPD-CD40L adjuvant plasmid. Strikingly, pSPD-Gag-CD40L elicited the strongest CD8⁺ T cell responses as judged by the number of IFN- γ and IL-2 producing cells in an ELISPOT analysis (FIGs. 8B, 8C, 10A and 10B). pSPD-Gag-CD40L elicited CD8⁺ T cells with remarkably increased avidity for the Gag peptide antigen (FIG. 9A). However, as we and others have previously described, multi-trimer CD40L is not a good adjuvant for antibody responses (FIG. 9B), which emphasizes the special effects of CD40L on DCs and subsequent CD8⁺ T cell responses. While CD40L plays a role in promoting B-cell proliferation and immunoglobulin class switching, several reports have shown that strong CD40 stimulation can also prevent the movement of B cells into germinal centers, block the development of memory B cells, and impair B-cell differentiation into

antibody-secreting plasma cells. We have also observed similar responses by SPD-CD40L in previous studies. We propose that SPD-Gag-CD40L is unable to enhance antibody responses through one or more of these mechanisms.

[125] In addition, these CD8⁺ T cell responses were protective as judged by the 2-3 log reduction in tissue viral load after challenging the mice with vaccinia-Gag (FIGs. 11A and 11B). However, we note that viral titers following SPD-Gag-CD40L vaccination were not significantly different than viral titers following vaccination with Gag plus SPD-CD40L, despite a large difference in interferon gamma and IL-2 ELISPOT responses between the two groups. Partly this may reflect the inherent variability of DNA vaccine immune responses, given that 4/13 mice given SPD-Gag-CD40L were able to clear virus while Gag plus SPD-CD40L was unable to reduce titer below 104 pfu/mouse. Overall, Gag plus SPD-CD40L gave a similar mean viral titer to Gag plus empty vector. Since DNA vaccination is a relatively inefficient way to deliver a genetic construct, an adenoviral vector (Ad5) was also used to vaccinate mice. Very remarkably, there was a ~7 log reduction in tissue viral load in mice vaccinated with Ad5-SPD-Gag-CD40L and no challenge virus could be detected (FIG. 12).

[126] To account for the effectiveness of the SPD-Gag-CD40L vaccine design, three factors should be considered: (1) Use of multi-trimer CD40L to cluster the CD40 receptor and thereby activate DCs; (2) Role of CD40L in targeting antigen to CD40 receptor-bearing DCs; and (3) simultaneous delivery of both the Gag antigen and CD40L adjuvant to the same DC at the same time.

[127] (1) Regarding the multi-trimer nature of CD40L in SPD-Gag-CD40L, it is worth noting that others have previously made antigen-CD40L fusion proteins. Xiang *et al.* (J. Immunol. 2001;167(8):4560-5) fused a tumor antigen to the C-terminal end of CD40L in a position that could conceivably impair binding of the ligand to the CD40 receptor. No data were presented to rule out this concern, but the vaccine's effectiveness was modest. Similarly, Zhang *et al.* fused a tumor antigen onto the N-terminus of the CD40L extracellular domain and delivered this construct using an adenovirus vector. In this case, the molecular design allowed for CD40L to bind unimpaired to its receptor. Even so, the effectiveness of this vaccine was relatively modest (Proc Natl Acad Sci U S A. 2003;100(25):15101-6). This is expected when a 1-trimer form of CD40L is used rather than a receptor-clustering multi-trimer construct such as SPD-Gag-CD40L.

[128] (2) Regarding the targeting of antigen to CD40 on DCs, this has emerged as a very desirable property for vaccine design. Barr *et al.* showed that antigen conjugated to anti-CD40 antibody elicited strong vaccine responses, although toxicity and anti-idiotypic antibody development are drawbacks to this approach (Barr *et al.*, Immunology. 2003;109(1):87-92). In vitro, Flamar *et al.* showed that anti-CD40 antibody conjugated to five HIV antigenic peptides could be taken up by human DCs in vitro and the antigens were then presented to T cells from the blood of HIV-infected subjects (Flamar *et al.*, AIDS. 2013 Aug 24;27(13):2041-51). In vivo, Cohn *et al.* found that conjugating antigen to anti-CD40 antibody broadened the types of DCs that crosspresent antigen to T cells to include BDCA1(+) DCs in addition to standard crosspresentation by BDCA3(+) DCs (J Exp Med. 2013;210(5):1049-63. PMID: 3646496). However, DC crosspresentation alone does not generate CD8+ T cell responses. As shown by Bonifaz and Steinman, antigen conjugated to anti-DEC205 antibody was targeted to DCs but the unactivated DCs lead to abortive T cell responses and subsequent tolerance. As they showed, the induction of CD8+ T cell responses by the anti-DEC205 antibody/antigen vaccine also required the addition of a DC-activating CD40 stimulus ((Bonifaz *et al.*, J Exp Med. 2002;196(12):1627-38). Thus, targeting of antigen to CD40 is helpful but not sufficient for DC-mediated T cell activation and expansion. Indeed, targeting a vaccine antigen to unactivated DCs could be counterproductive and lead to tolerance rather than augmented vaccine responses.

[129] (3) Regarding the need for delivery of both antigen and adjuvant to the same DC at the same time, this issue was recently examined by Kamath *et al.* (J Immunol. 2012;188(10):4828-37). When antigen was delivered to DCs in the absence of adjuvant, antigen-specific T cells were induced to proliferate but did not subsequently differentiate into effector cells. Instead, effective immunity was only induced when the test vaccine provided antigen and adjuvant to the same individual DCs within a short window of time. These parameters are fulfilled by the design of SPD-Gag-CD40L because the antigen and adjuvant are linked in time and space as parts of the very same molecule.

[130] In conclusion, a vaccine was developed that combines multi-trimer CD40L as an adjuvant covalently linked to HIV-1 Gag antigen. Extremely strong and highly protective CD8+ T cell responses were induced by this vaccine, especially when the construct was incorporated into an Ad5 vector. Since other antigens can be substituted for HIV-1 Gag in SPD-Gag-CD40L, this immunogen

design suggests a general method for constructing an effective preventative and/or therapeutic vaccine for infections and tumors for which a strong CD8⁺ T cell response is required.

EXAMPLE 2: Cancer Immunotherapy

[131] Previous studies have shown that plasmid DNA vaccination using an exogenous gene encoding tumor associated antigens can induce cancer-specific CTLs with antitumor activity. A second-generation improvement on this approach is the targeting of antigen to dendritic cells (DC) by fusion to antibodies or natural ligands that bind dendritic cell receptors. Recently it has been shown that targeting of antigen to DC via CD40 is particularly effective at inducing cross presentation of targeted antigens.

[132] In this example we explored the use of CD40 ligand to target tumor antigen to DC. A DNA vaccine was generated encoding a single fusion protein composed of the spontaneously multimerizing gene Surfactant Protein D (SPD), gp100 tumor antigen, and the extracellular domain of CD40L. This “third generation” antigen-CD40L approach was developed to both target antigen to DC and optimally activate dendritic cells by clustering CD40 on the cell membrane. SPD-gp100-CD40L was expressed as a single 110 kDa protein strand that self-assembles inside cells into a molecule with four trimeric arms containing 4 trimers of CD40L. The protein was biologically active on dendritic cells and able to induce CD40-mediated signaling. SPD-gp100-CD40L was evaluated in a B16-F10 melanoma DNA vaccine model either alone or in combination with plasmids encoding IL-12p70 and GM-CSF. Vaccination with SPD-gp100-CD40L + IL-12p70 + GM-CSF significantly increased survival and inhibited tumor growth compared to all other treatments. Expression of gp100 and SPD-CD40L as separate molecules did not enhance survival, suggesting incorporation of gp100 within the SPD-CD40L polymer is required for activity. These data support a model where gp100 antigen incorporated within SPD-CD40L multi-trimers targets antigen to DC in vivo, induces activation of these DC, increases cross-presentation of gp100 antigen, and generates a protective anti-tumor T cell response when given in combination with IL-12p70 and GM-CSF molecular adjuvants.

[133] Cancer vaccination has attracted renewed attention as a therapy for the treatment of tumor growth and metastasis. The use of Tumor Associated Antigens (TAA) is particularly promising. Therapeutic effects specific to cancer cells can be generated through the careful selection of TAA

preferentially expressed on tumor cells. In particular, it has been reported that DNA vaccination using an exogenous plasmid encoding a TAA can induce cancer-specific cytotoxic T lymphocytes (CTL) with antitumor activity. However, optimal CTL activity requires that the antigen be selectively and efficiently presented by antigen presenting cells (APC) such as dendritic cells (DC), which play a pivotal role in the initiation, programming and regulation of cancer-specific immune responses. One strategy to enhance the effectiveness of DNA vaccines encoding weakly immunogenic antigens is by co-delivering genes encoding molecular adjuvants that stimulate DC. TNF superfamily ligands (TNFSF) are costimulatory molecules involved in DC and T cell activation and have previously been tested as adjuvants to enhance immune responses in several vaccination studies, in particular the DC activating molecule CD40L, the cognate ligand for CD40.

[134] Melanoma-specific antigen gp100, encoded within DNA or viral vector vaccines, is known to induce measurable immune responses and suppress tumor growth. However, molecular adjuvants could enhance the overall immune response to this antigen, inducing an effective immune response able to prevent tumor growth. As important, targeting of tumor antigens directly to DC using the DC receptor DEC-205 has previously been shown to increase immune responses. Similarly, it has also been shown that delivery of antigens to DC via CD40 can enhance cross-presentation of antigen to CD8+ T cells via MHC I.

[135] CD40L stimulation increases effector T cell differentiation and also induces the production of a variety of cytokines, such as IL-12p70. Based on previously published data, a 4-trimer soluble form of CD40L has been shown to be particularly effective as a vaccine adjuvant. This 4-trimer soluble form was achieved using the scaffold protein Surfactant Protein D (SPD), a collectin family member that spontaneously forms a plus-sign-shaped molecule with four trimeric arms, generating a 4-trimer soluble complex.

[136] In addition to CD40L, other adjuvants previously tested in cancer vaccine models include GM-CSF and IL-12p70. Systemic co-administration of IL-12p70 or GM-CSF have been shown to induce antitumor immunity. Studies have also evaluated these cytokines as DNA-encoded adjuvants for DNA vaccines where they have shown modest efficacy.

[137] In the present study, the fusion protein SPD-gp100-CD40L was generated encoding murine CD40L extracellular domain fused to the collagen-like domain of murine SPD, with gp100 antigen

inserted within the SPD coding region. We reasoned that these soluble CD40L multi-trimers would deliver gp100 to DC while simultaneously activating the DC, thereby inducing an enhanced CD8+ T cell CTL response. As we report, SPD-gp100-CD40L protein was stable, formed large polymeric complexes, and was biologically active on DC, suggesting proper assembly of CD40L trimers. Co-delivery of SPD-gp100-CD40L, GM-CSF, and IL-12p70 plasmids by intramuscular injection enhanced survival of mice challenged with B16-F10 and significantly suppressed tumor growth. This response was not observed with any other DNA vaccine combination, and was not observed when gp100 and SPD-CD40L were delivered as separate molecules, either in presence or absence of GM-CSF and IL-12p70. Overall, these data support the hypothesis that SPD-gp100-CD40L, when augmented with GM-CSF and IL-12p70 cytokines, targets gp100 antigen to DC in situ, activates these DC via CD40 stimulation, and induces an immune response that controls tumor growth and enhances survival.

Materials and Methods

Construction and preparation of DNA plasmids

[138] Plasmid encoding human glycoprotein 100 (pgp100) was a gift of Dr. Patrick Hwu. Plasmid encoding the 4-trimer soluble form of murine SPD-CD40L was generated as previously described (Stone *et al.*, J Virol. 2006;80(4):1762-72). To construct pSPD-gp100-CD40L, DNA encoding amino acids 25 to 596 (sequence KVPRNQD to EAGLGQV) of human gp100, incorporating the full extracellular domain or gp100, was inserted between amino acids 105 and 106 of mouse SPD within construct SPD-CD40L (*i.e.* between peptide sequences GERGLSG and PPGLPGI of murine SPD). Murine IL-12p70 plasmid pIL-12 was purchased from Invivogen and encodes a single chain dimer of IL-12 p35 and p40 (InvivoGen). Murine GM-CSF plasmid was constructed using a codon-optimized gene encoding murine GM-CSF inserted into plasmid pcDNA3.1. Clone pgp100-IRES-SPD-CD40L was generated by placing an IRES sequence between human gp100 (amino acids 1-594) and murine SPD-CD40L (Zhou *et al.*, Proc Natl Acad Sci U S A. 2008;105(14):5465-70). All plasmids were propagated in *Escherichia coli* strain TOP10™. Highly purified, endotoxin-free DNA plasmid preparations were produced using the Qiagen™ endofree GIGA™ plasmid kit. Plasmids were further purified using Triton-X114™ purification method as previously described (Stone *et al.*, J Virol. 2006;80(4):1762-72). All plasmid endotoxin levels were <0.2 EU/ml as confirmed by LAL endotoxin assay (Lonza Inc.).

Transient transfections and Western blotting of fusion protein constructs

[139] 293T cells were transiently transfected with plasmid constructs using **Genjet-plus™** transfection reagent (Signagen Laboratories). Forty-eight hours later, supernatants were centrifuged and filtered. Supernatants were loaded onto a sodium-dodecyl sulfate–10% polyacrylamide gel (BioRad) in the presence of DTT, electrophoresed, and blotted onto PVDF membrane (Pierce). The membrane was blocked using 5% (w/v) dry milk and then probed with goat anti-mouse CD40L antibody (R&D Systems), followed by incubation with anti-goat horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch). The protein band was developed onto X-ray film using chemiluminescence. For analytical light scattering analysis, 293T cells were transiently transfected with the pSPD-gp100-CD40L construct and supernatant was collected and then concentrated 10-fold using an Amicon™ centrifugal filtration system with 100 kDa cutoff (Millipore).

CD40 SEAP in vitro activity assay

[140] The CD40 receptor bearing reporter cell line CD40-293-SEAP was used to monitor CD40L mediated activation. This 293-derived cell line constitutively expresses human CD40 receptor along with the gene for secreted alkaline phosphatase (SEAP) under the control of NF- κ B [59]. Briefly, 80,000 CD40-293-SEAP reporter cells grown in DMEM medium with 10% FBS were plated in each well of a 96-well plate. A total of 100 μ l of SPD-gp100-CD40L, SPD-CD40L or pcDNA3.1 transfected 293T cell supernatant was added to the cells in triplicate at various dilutions. After 18 hours, 10 μ l/well of the supernatant from each well was added to a 96-well assay plate together with 100 μ l/well of **QUANTI-Blue™ Alkaline Phosphatase substrate (InvivoGen)**. Wells were incubated for 20 min at 20 °C and read at 650 nm in a 96-well plate reader.

DC activation and maturation assay

[141] Bone marrow derived DC were generated by standard methods with the following modifications. Bone marrow cells were obtained from C57BL/6 mice and washed in RPMI 1640 media. The cells were then placed in a non-tissue culture treated T75 flask at a concentration of 1×10^6 cells per ml in 20 ml complete RPMI (RPMI 1640 with 10% FBS, 20 μ g/ml gentamycin sulfate, 50 μ M Mercaptoethanol), 20 ng/ml murine recombinant GM-CSF and 10 ng/ml murine recombinant IL-4 (Peprotech, Rocky Hill, NJ)). Cells were cultured at 37°C, 5% CO₂ and on day 3, media was replaced with fresh complete RPMI containing cytokines. On day 5, cells were harvested,

washed and resuspended in complete RPMI at 5×10^5 cells/ml. A total of 1×10^6 cells were added to each well of 6-well non-tissue culture treated plates. Subsequently, 300 μ l of supernatant containing SPD-gp100-CD40L, pcDNA3.1 control supernatant, or cytokine mix positive control (15 ng/ml IL-1 β , 5 ng/ml TNF α , and 1 μ g/ml PGE2 final concentration) was added and cells were incubated for 36 hours. Cells were harvested and stained with Hamster anti-mouse CD11c clone N418 PE-Cyanine7 conjugate (eBioscience, San Diego, CA) combined with each of the following antibodies: anti-mouse CD80 clone 16-10A1, anti-mouse CD86 clone GL1, anti-mouse CD40 clone 1C10, anti-mouse CD83 clone Michel-17, anti-mouse MHC Class II (I-A/I-E) clone M5-114.15.2, and anti-mouse CCR7 clone 4B12 (all from eBioscience). After flow cytometry analysis, the mean fluorescence intensity was calculated for gated CD11c⁺ dendritic cells under each experimental condition. FlowJo 7.6.4, flow cytometry analysis software, (FlowJo, Ashland, OR) was used for analysis. Three independent wells were analyzed for each condition.

Tumor immunotherapy studies

[142] Female C57BL/6 mice (7–8 weeks old) were used in all experiments. Animals were housed at the University of Miami under the guidelines of the National Institutes of Health (NIH, Bethesda, MD). Animal experiments were performed in accordance with national and institutional guidance for animal care and were approved by the IACUC of the University of Miami. A total of 50,000 B16-F10 cells were injected i.d. into the left flank. Mice were then injected i.m. with plasmid DNA on day 3, 10, and 17 following tumor challenge into both hind quadriceps muscles. Mice received a mixture of from one to three plasmid constructs. Empty vector pcDNA3.1 was used as filler to ensure all groups received the same total micrograms of plasmid. Tumor volume was measured 3 times per week using a digital caliper, measuring the longest diameter (a) and shortest width (b) of the tumor. Tumor volume was calculated by the formula $V \text{ (mm}^3\text{)} = 0.5 \times ab^2$. Animals were euthanized when tumors reached $>1500 \text{ mm}^3$. For GVAX vaccination, B16-F10 tumor cells expressing GM-CSF, kindly provided by Dr. Glenn Dranoff, were irradiated (5,000 rad) and 1×10^6 cells were injected subcutaneously on the right flank on day 3, 6, and 9.

Histology

[143] Tumors were harvested for histological analysis on day 15-20, fixing the tissue overnight at 4°C in 10% formalin prior to embedding in paraffin. Serial 4 µm sections were then stained with hematoxylin and eosin (H&E) to evaluate for the presence of lymphocyte infiltration.

Statistical analysis

[144] Graph pad Prism 6.0 software was used to calculate significance by two-tailed Student's t test. In all figures, p values were labeled by asterisks for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Results**Construction and expression of multi-trimeric soluble SPD-gp100-CD40L**

[145] Previous studies have shown that CD40L-mediated signaling is required for functional CTL memory development against tumors. Similarly, we have previously shown that injection of plasmid DNA expressing SPD-CD40L into B16-F10 tumors can slow tumor growth when combined with TLR agonists. CD40L mediates the co-stimulation, activation, and maturation of dendritic cells (DC), and this function is critical for the induction of an effective T cell mediated immune response. Previous research has shown that monoclonal antibodies targeting DC surface protein DEC-205 can target cancer antigens to DC in vivo, inducing a protective immune response. We surmised that SPD-CD40L could similarly be used as a carrier to transport tumor associated antigens (TAA) to DC in vivo by incorporating the antigen within the SPD collagen-like domain of SPD-CD40L. We constructed the plasmid pSPD-gp100-CD40L, where human gp100 is fused between amino acids 105 and 106 of the collagen-like domain of murine SPD-CD40L (FIG. 13A) and SEQ ID NO 5 and SEQ ID NO 6. A model of the expected 4-trimer complex is shown in FIG. 13B. Following transfection of pSPD-gp100-CD40L into 293T cells, secreted SPD-gp100-CD40L was detected at the expected size of 110 KDa by SDS-PAGE Western blot in the presence of DTT (FIG. 13C).

Biological activity of SPD-gp100-CD40L

[146] To confirm that SPD-gp100-CD40L retains biological activity, an SEAP cell line reporter assay was performed as described previously. We monitored the ability of SPD-gp100-CD40L supernatant to drive NF-kappaB-mediated expression of the SEAP reporter enzyme. Empty vector pcDNA3.1 transfected 293T cell supernatant was used as a negative control. As shown in FIG. 14A,

both SPD-CD40L and SPD-gp100-CD40L induced SEAP activity at a similar level in a dose-dependent manner when compared to empty vector.

[147] Next, we evaluated the ability of SPD-gp100-CD40L to activate bone marrow derived DCs. DCs were cultured with supernatant from 293T cells transfected with either empty vector pcDNA3.1 or pSPD-gp100-CD40L. A cytokine mix containing recombinant IL-1beta, TNFalpha, and PGE2 (Mimic) was used as a positive control. We observed a significant increase in CD80, CD86 and CD83 MFI (comparing pcDNA3.1 to pSPD-gp100-CD40L supernatant). SPD-gp100-CD40L was moderately active compared to the Mimic positive control.

SPD-gp100-CD40L DNA alone did not inhibit B16-F10 tumor growth in mice

[148] We next investigated the anti-tumor efficacy of pSPD-gp100-CD40L plasmid, using a B16-F10 melanoma therapeutic vaccination model (FIG. 15). Mice were divided into three vaccination groups: (i) PBS, (ii) pSPD-gp100-CD40L, and (iii) GVAX therapy. Group (ii) received 100 µg of pSPD-gp100-CD40L i.m. per vaccination. We did not observe a statistical difference in tumor sizes and survival between groups (FIG. 15B and 15C), suggesting that pSPD-gp100-CD40L alone is insufficient to induce an anti-tumor activity.

The combination of pSPD-gp100-CD40L, pGM-CSF, and pIL-12p70 inhibited tumor growth and enhanced survival following B16-F10 tumor challenge

[149] Next, we investigated whether SPD-gp100-CD40L activity could be enhanced using the molecular adjuvants GM-CSF and IL-12p70. We hypothesized that DC chemoattraction induced by GM-CSF and T cell costimulation induced by IL-12p70 would synergize with the CD40L-mediated DC activation induced by SPD-gp100-CD40L, increasing the overall anti-tumor immune response. Mice were divided into 5 vaccination groups: (i) PBS, (ii) pSPD-gp100-CD40L + pGM-CSF, (iii) pSPD-gp100-CD40L + pIL-12, (iv) pSPD-gp100-CD40L + pGM-CSF + pIL-12, and (v) GVAX. Empty vector pcDNA3.1 was used as filler to ensure all DNA vaccine groups received the same quantity of total plasmid (120 µg). All DNA vaccinations contained 80 µg of pSPD-gp100-CD40L and 20 µg each of pGM-CSF, pIL-12, and/or pcDNA3.1. The mean tumor size for group (iv) (SPD-gp100-CD40L + GM-CSF + IL-12) was significantly lower compared to groups (i), (ii), and (iii) on days 15, 17, and 20 (FIG. 16B). We observed a statistically significant difference in survival between group (iv) and groups (i), (ii) and (iii) ($P < 0.05$) (FIG. 16C), and a statistically significant difference

in tumor-free survival between group (iv) and groups (i), (ii), and (iii) ($p < 0.01$). As shown in FIG. 16D, five out of five mice in group (iv) were free of palpable tumors on day 11 while five out of five mice in groups (i) (ii) and (iii) had palpable tumors on day 11. GVAX “gold standard” vaccination slowed tumor growth compared to untreated animals, however neither tumor growth nor survival reached statistical significance when comparing GVAX to other groups (FIG. 16B and 16C).

Alternative combinations of gp100, SPD-CD40L, IL-12, and GM-CSF fail to control of B16-F10 tumor growth

[150] The previous experiments did not evaluate all possible combinations of gp100, SPD-CD40L, GM-CSF, and IL-12. We therefore wished to confirm that physically linking gp100 and SPD-CD40L was required for activity. Six groups were evaluated: (i) PBS, (ii) pgp100, (iii) pgp100 + pGM-CSF, (iv) pgp100 + pIL-12, (v) pgp100 + pGM-CSF + pIL-12, and (vi) pgp100-IRES-SPD-CD40L (gp100 and SPD-CD40L expressed as separate molecules) + pIL-12 + pGM-CSF. Empty vector pcDNA3.1 was used as filler to ensure all DNA vaccine groups received the same quantity of plasmid (120 μ g total, including 80 μ g of the gp100-containing plasmid and 20 μ g each of pGM-CSF, pIL-12, and/or pcDNA3.1). We observed no statistical difference in mean tumor sizes between any of the six groups (FIG. 17B). We also failed to observe a statistical difference in survival between groups (FIG. 17C).

Discussion

[151] Recent advances in cancer immunotherapy support the concept that the immune system can induce effective antitumor responses. In this context it has been reported that DNA vaccination is effective for the prevention of metastasis and relapse. In particular, the application of DNA vaccination against melanoma has shown promise following the identification of tumor associated antigens (TAA) including gp100, MART-1 and TRP2. For the most part, melanoma DNA therapeutic vaccines are based on the expression of full length antigen following intramuscular injection or electroporation of plasmid DNA. The antigen is secreted from the vaccination site and taken up by APC at the vaccine site or the local draining lymph node. However, it is becoming recognized in the field that targeting cancer antigens directly to APC (in particular dendritic cells) induces a more effective immune response compared to untargeted tumor antigens. We hypothesized that fusing melanoma antigen gp100 within the SPD collagen-like domain of SPD-CD40L multi-trimeric clusters would: 1) target gp100 to DC expressing CD40 in situ, 2) induce cross

presentation of gp100 by these DC, possibly via delivery of gp100 to the early endosome, and 3) activate and mature the DC via CD40 crosslinking with CD40L multi-trimers on the DC membrane surface. The SPD-gp100-CD40L fusion protein is a single gene 3.1 kb in size that can be easily encoded within DNA, RNA, or viral vector cancer vaccines. Initially, we determined that SPD-gp100-CD40L was efficiently secreted from transfected cells and formed large multimeric complexes. Western blotting showed that SPD-gp100-CD40L was expressed and secreted into the culture supernatant at the expected molecular weight of 110 kDa. We also confirmed the biological activity of SPD-gp100-CD40L protein using an NF- κ B reporter system and DC activation assay. Together these data suggest that SPD-gp100-CD40L is forming a biologically active trimeric CD40L headgroup, in a manner similar to the previously characterized SPD-CD40L protein, and these trimers are forming spontaneous 4-trimer complexes, consistent with the native SPD protein.

[152] In a cancer model, therapeutic immunization with SPD-gp100-CD40L DNA vaccine failed to control tumor growth or improve survival of B16-F10 melanoma (FIG. 16). This is not surprising, given the aggressive nature of established B16-F10 tumor. One possibility is that secretion of immunosuppressive cytokines such as VEGF, IL-10 and TGF- β by B16-F10 prevents activated cytotoxic T lymphocytes (CTL) induced by SPD-gp100-CD40L from entering into the tumor bed. Alternately, these and other immunosuppressive cytokines suppress cytotoxic activity once the CTL enters the tumor tissue. Previous studies have evaluated cytokines IL-12 and GM-CSF for their ability to enhance T cell mediated immune responses. We hypothesized that SPD-gp100-CD40L combined with cytokines IL-12 and GM-CSF would enhance antigen cross-presentation (via SPD-gp100-CD40L) and immune activation (via GM-CSF and IL-12), overcoming tumor-mediated immune suppression. Consistent with this hypothesis, we observed that vaccination with all 3 genes significantly slowed tumor growth, delayed tumor onset, and improved mouse survival (FIG. 17). Only the triple combination was effective, and all other combinations failed to significantly suppress tumor growth or enhance survival (FIG. 16), including separate expression of gp100 and SPD-CD40L (together with IL-12 and GM-CSF). All animals received the same amount of plasmid (120 μ g), allowing us to control for immune stimulation provided by plasmid DNA itself. Based on the literature and our data we propose a model where the effectiveness of SPD-gp100-CD40L is due to the targeting of gp100 to DC, enhanced cross-presentation through CD40-mediated delivery to the early endosome, and the capacity of CD40L multi-trimers to enhance DC activation and maturation. In this model, SPD-gp100-CD40L-mediated DC cross-presentation and activation, coupled with IL-

12-p70-mediated T cell stimulation and GM-CSF-mediated chemoattraction of DC, generated an enhanced CD8⁺ T cell response that was able to overcome immune tolerance at the tumor site. Our results also suggest that CD40L stimulation is a critical component of this vaccine. We did not observe any reduction in tumor growth kinetics when gp100 alone was combined with IL-12 and GM-CSF, despite higher levels of gp100 protein expression in pgp100 transfected cells compared to pSPD-gp100-CD40L transfected cells (FIG. 13C). In addition, the separate delivery of gp100 and SPD-CD40L molecules (using an IRES construct) was unable to replicate the effect of SPD-gp100-CD40L (FIG. 17), consistent with the requirement that gp100 be physically linked to the CD40L multi-trimers for optimal activity. Additional research will be required to determine whether multi-trimerization of CD40L plays a role in the activity of this construct. Of interest, recent studies have shown that delivery of antigen via CD40 can enhance cross presentation to DC. Both enhanced cross-presentation and the simultaneous antigen delivery and DC activation to the same cell may explain the ability of SPD-gp100-CD40L to induce a robust anti-tumor immune response.

[153] In conclusion, this study demonstrates that the fusion of gp100 within SPD-CD40L multi-trimers induces a response against B16-F10 melanoma when combined with IL-12p70 and GM-CSF molecular adjuvants. Overall, SPD-gp100-CD40L is a novel cancer DNA vaccine reagent that provides CD40-mediated APC activation in the context of efficient targeting and cross-presentation of cancer antigen. Future studies will explore alternative SPD-TAA-CD40L fusion proteins using tumor-associated antigens other than gp100. This will allow us to determine whether this strategy can be expanded to a wider range of cancers and TAA. In summary, this study presents a novel reagent for use in cancer therapeutic vaccines, exploiting the unique properties of CD40L on the activation of DC and using CD40L for the targeting and enhanced cross presentation of antigen on APC.

Claims

1. A fusion protein comprising:
 - (a) a multimerization scaffold, composed of two or more trimeric collectin-like arms consisting each of three individual polypeptide strands, joined at their N-terminus by a disulfide-linked hub region, wherein the multimerization scaffold is selected from the group consisting of SPD, Acrp30, C11, HIB27, SPA, conglutinin, collectin-43, MBL1, and MBL2, and
 - (b) two or more complete Tumor Necrosis Factor SuperFamily (TNFSF) receptor binders, comprising three receptor binder protein chains, wherein the two or more complete TNFSF receptor binders bind a complete TNFSF receptor to activate a cell, and wherein the TNFSF receptor binders are selected from the group consisting of CD40L (TNFSF5), CD27L (TNFSF7), CD137L (TNFSF9), OX40L (TNFSF4), GITRL, 4-1BBL, RANKL, LIGHT, CD70, and BAFF, wherein each of said receptor binder protein chains is operatively linked by a peptide bond to the C-terminus of one of said individual polypeptide strands; and
 - (c) one or more antigens recognized by the immune system, wherein the amino acid sequence of the one or more antigens is contained within each of the individual polypeptide strands within the multimerization scaffold.
2. The fusion protein of claim 1, wherein the amino acid sequence of the one or more antigens is positioned on each of the individual polypeptide strands from 10 to 140 amino acids C-terminal to the disulfide-linked hub region of the multimerization scaffold and 40 to 167 amino acids N-terminal to the TNFSF receptor binders, and wherein the one or more antigens are from an infectious disease agent selected from the group consisting of viruses, bacteria, fungi, protozoa, and parasites; or wherein the one or more antigens are from a malignant cell or cancer-causing virus.

3. The fusion protein of claim 1 or claim 2, wherein a natural C-terminal domain has been removed from the individual polypeptide strands of the trimeric collectin-like arms and replaced by the TNFSF receptor binders.
4. The fusion protein of claim 1 or claim 2, wherein the multimerization scaffold is composed of two trimeric collectin-like arms, wherein the three individual polypeptide strands are coupled to each other by a trimerizing component and the two trimeric collectin-like arms by a dimerizing component.
5. The fusion protein of claim 4, wherein the dimerizing component is the Fc portion of an immunoglobulin; or wherein the trimerizing component is selected from the group consisting of the trimerizing components of GCN4, TRAF2, thrombospondin 1, Matrilin-4, CMP, HSF1, cubilin, and the neck region of surfactant protein D.
6. The fusion protein of claim 1, comprising the sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, or 18.
7. The fusion protein of claim 1, wherein the multimerization scaffold is a C1q family protein or a collectin.
8. The fusion protein of any one of claims 1 to 7, wherein the TNFSF receptor binders are an extracellular domain of one of CD40L (TNFSF5), CD27L (TNFSF7), CD137L (TNFSF9), OX40L (TNFSF4), GITRL, 4-1BBL, RANKL, LIGHT, CD70, and BAFF.
9. A nucleic acid encoding the fusion protein according to any one of claims 1 to 8.
10. The nucleic acid of claim 9, comprising the sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, or 17.
11. A viral vector comprising the nucleic acid of claim 9 or 10.

12. The viral vector of claim 11, wherein the vector is selected from the group consisting of adenoviruses, poxviruses, alphaviruses, arenaviruses, flaviviruses, rhabdoviruses, retroviruses, lentiviruses, herpesviruses, paramyxoviruses, and picornaviruses.

13. A pharmaceutical composition comprising the fusion protein of any one of claims 1 to 8, the nucleic acid of claim 9 or 10 or the viral vector of claim 11 or 12 and a pharmaceutical acceptable carrier.

14. The use of the composition of claim 13 in activating an antigen-presenting cell (APC), wherein the APC is treated with said composition for inducing the maturation of the APC,

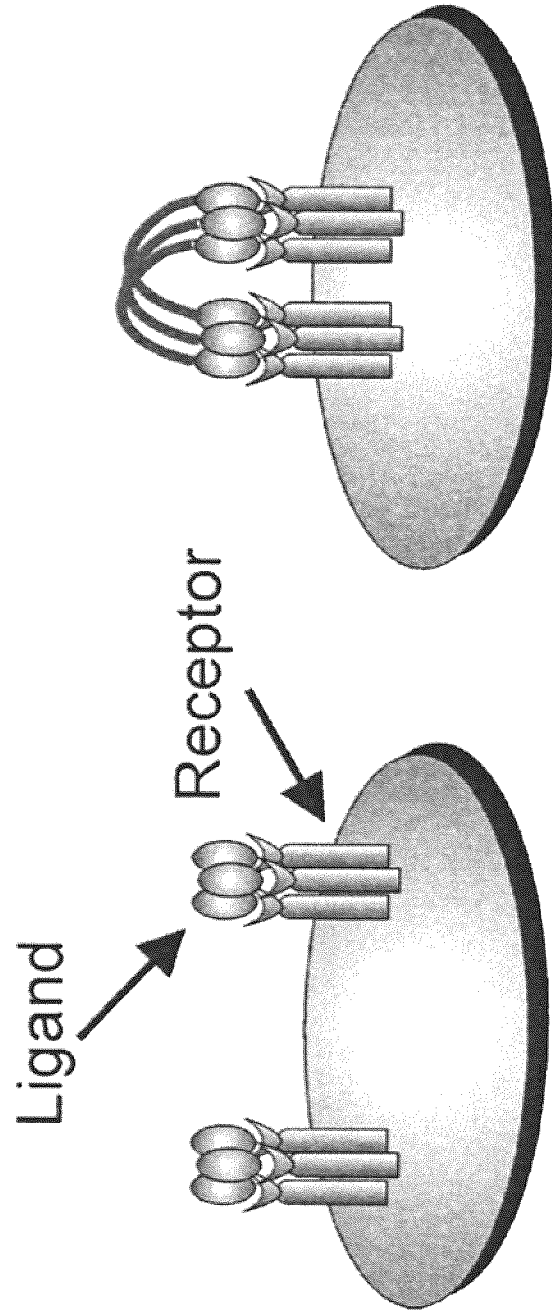
such that the treated APC is for administration to a subject as a vaccine or in immunotherapy, and

wherein the APC is selected from the group consisting of dendritic cells, monocytes, macrophages, and B cells.

15. The use of the composition of claim 13 as a vaccine or in immunotherapy to elicit an immune response against the one or more antigens in a subject in need of this immune response, wherein the composition is for administration to said subject.

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TNFSF activity often requires ≥ 2 trimers
 Receptor clustering is needed for activation

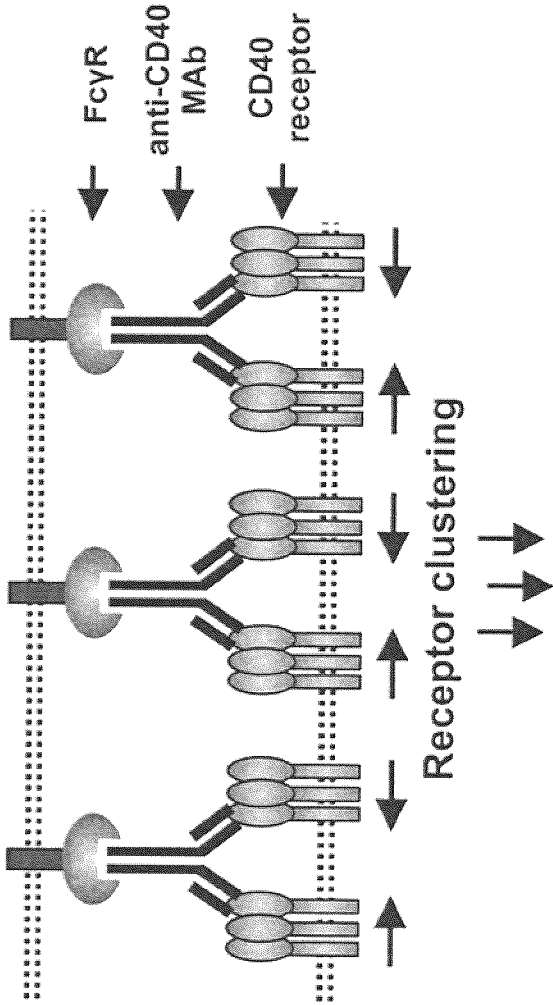


no activation strong activation

FIG. 1

Clustering of TNFSF receptors
by “agonistic” anti-TNFSF receptor antibodies

Agonistic anti-CD40 antibodies must bind to Fc receptors on adjacent cells in order to stimulate CD40-bearing cells

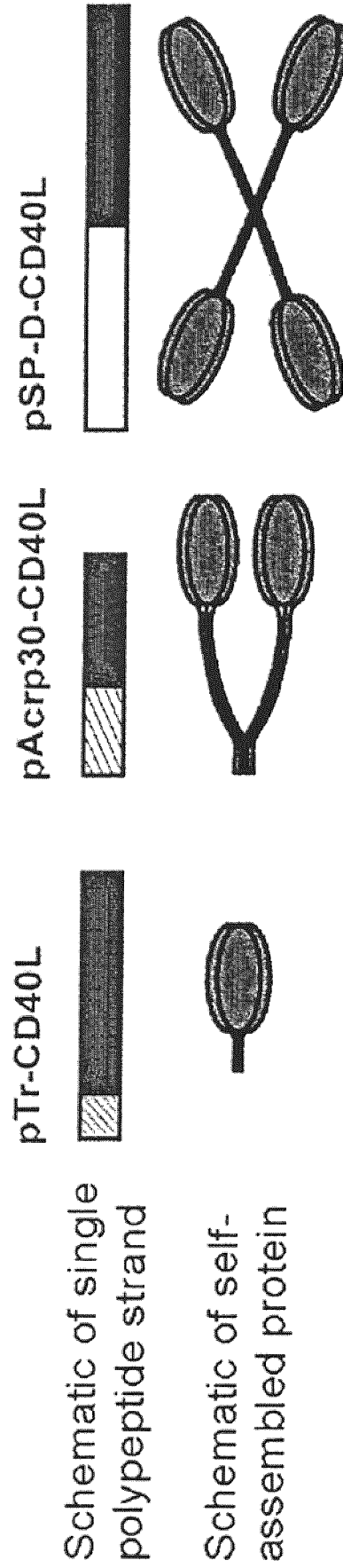


Downstream signaling
Wilson et al., An Fcγ receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells. *Cancer Cell* 19:101-113, 2011.
White et al., Interaction with FcγRIIb is critical for the agonistic activity of anti-CD40 monoclonal antibody. *J Immunol* 187:1754-1763, 2011

FIG. 2

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CD40L as a vaccine adjuvant

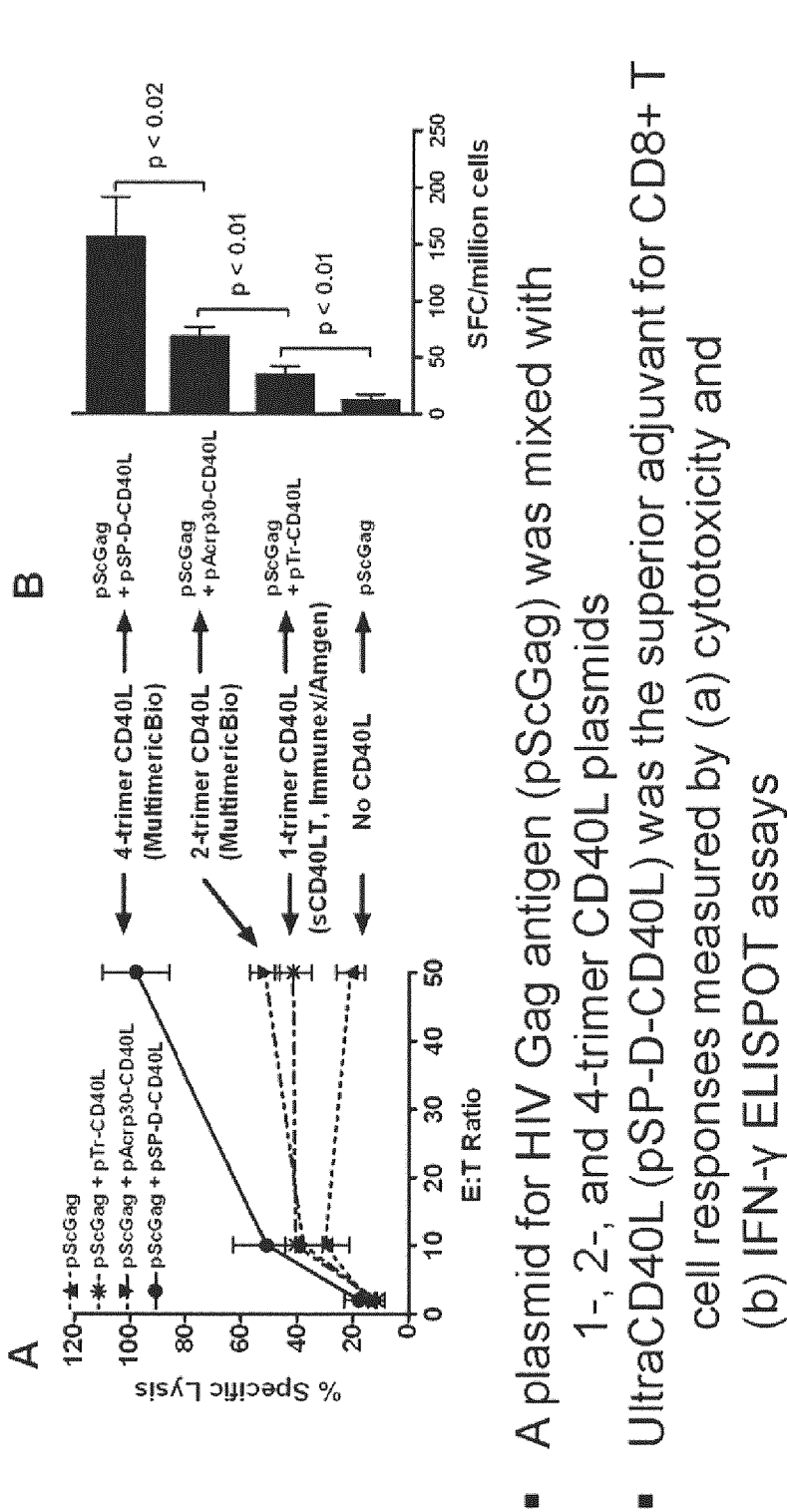


To examine the effect of valency on adjuvant activity, 1-trimer, 2-trimer, and 4-trimer DNA constructs were tested in a DNA vaccine

FIG. 3

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CD8+ T cell response to DNA vaccination is enhanced by an adjuvant plasmid with 2 or more linked CD40L trimers



Stone, G.W. et al (Kornbluth group). Multimeric soluble CD40 ligand and GITR ligand as adjuvants for human immunodeficiency virus DNA vaccines. J Virol 80:1762-1772, 2006.

FIG. 4

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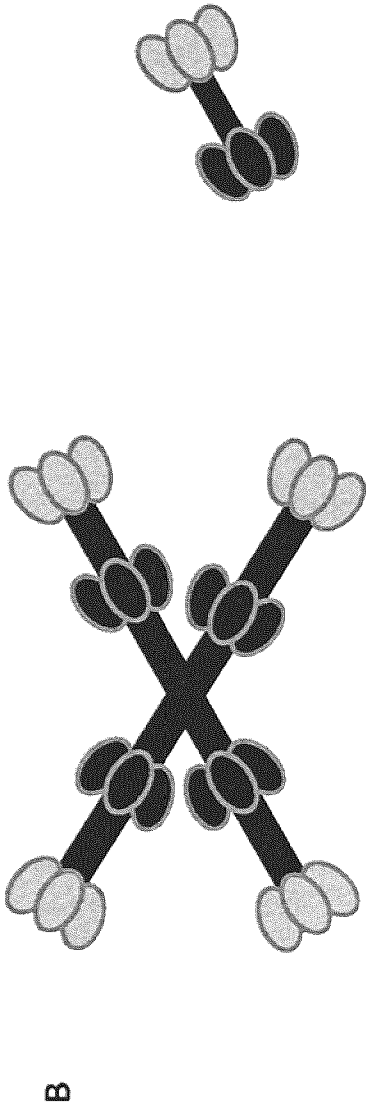
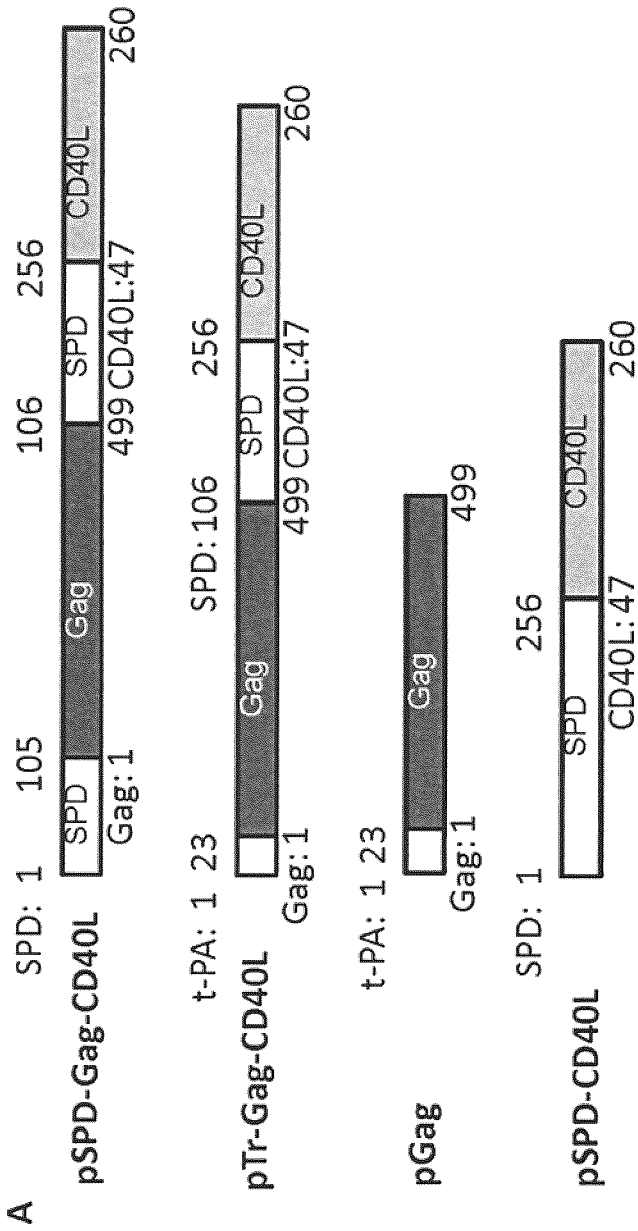


FIG. 5

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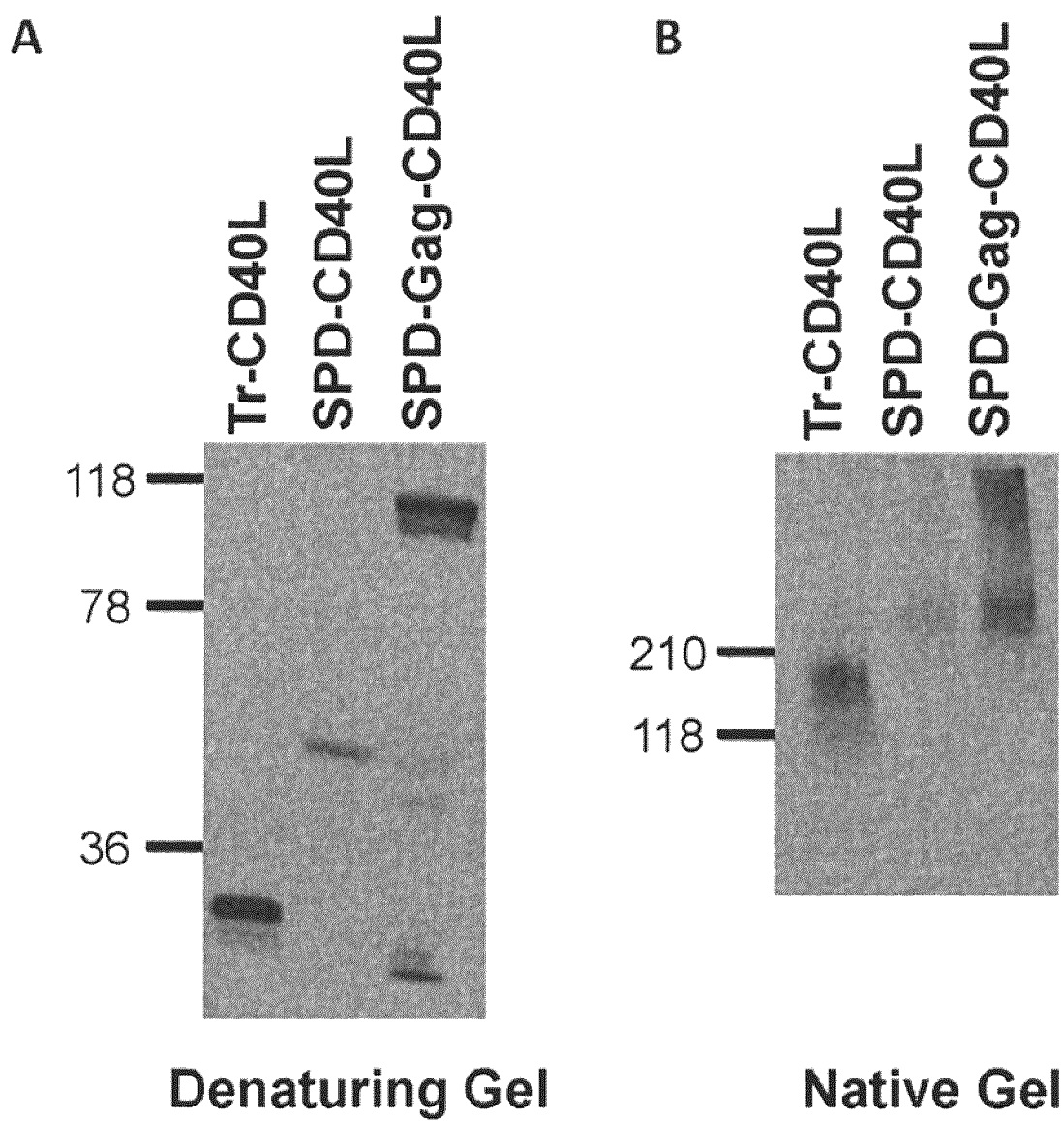
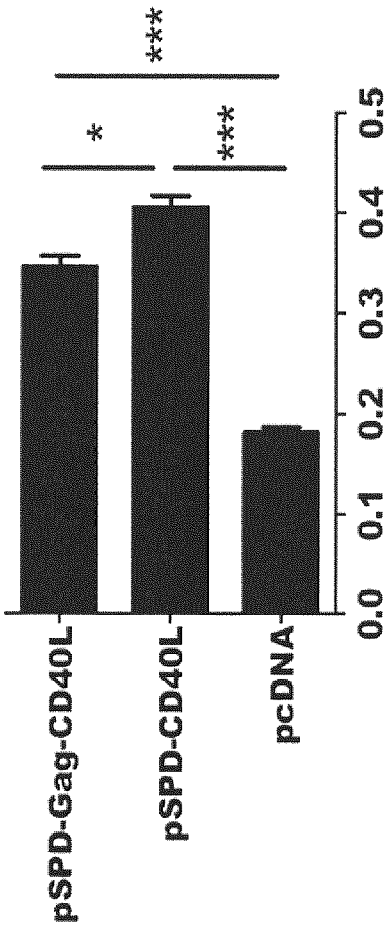


FIG. 6

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A



B

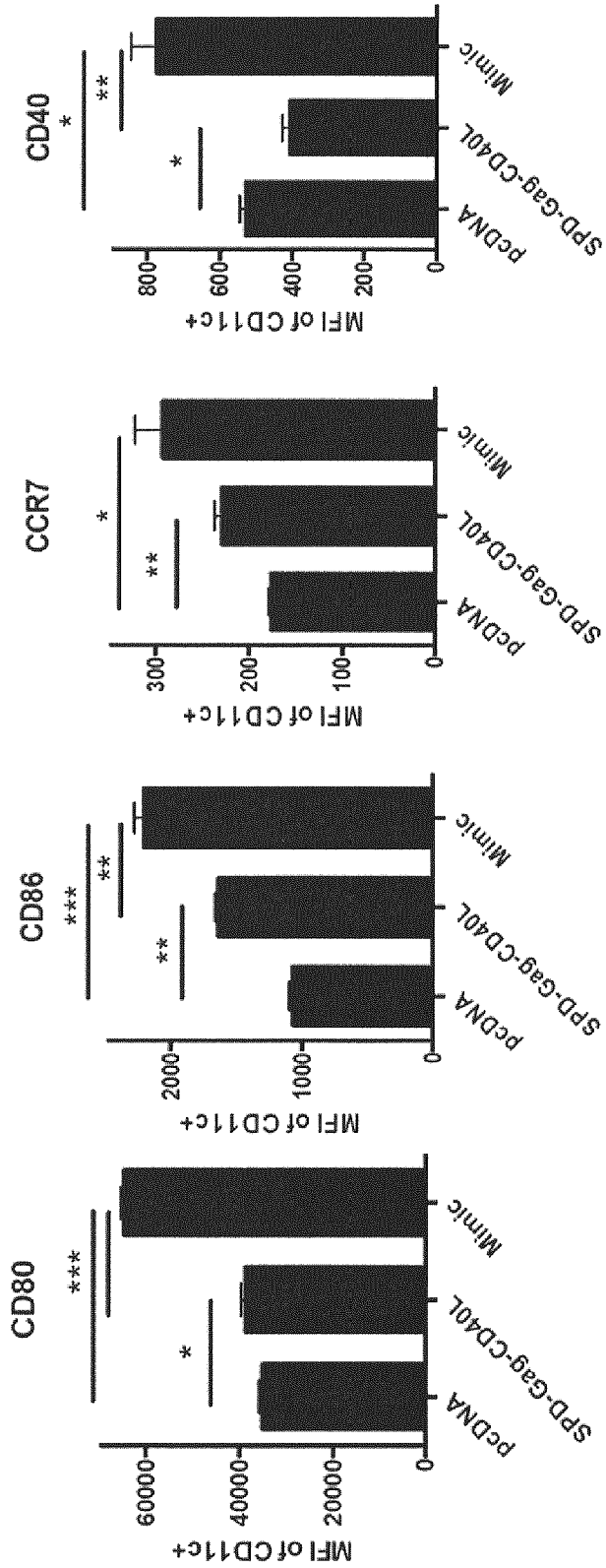


FIG. 7

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A

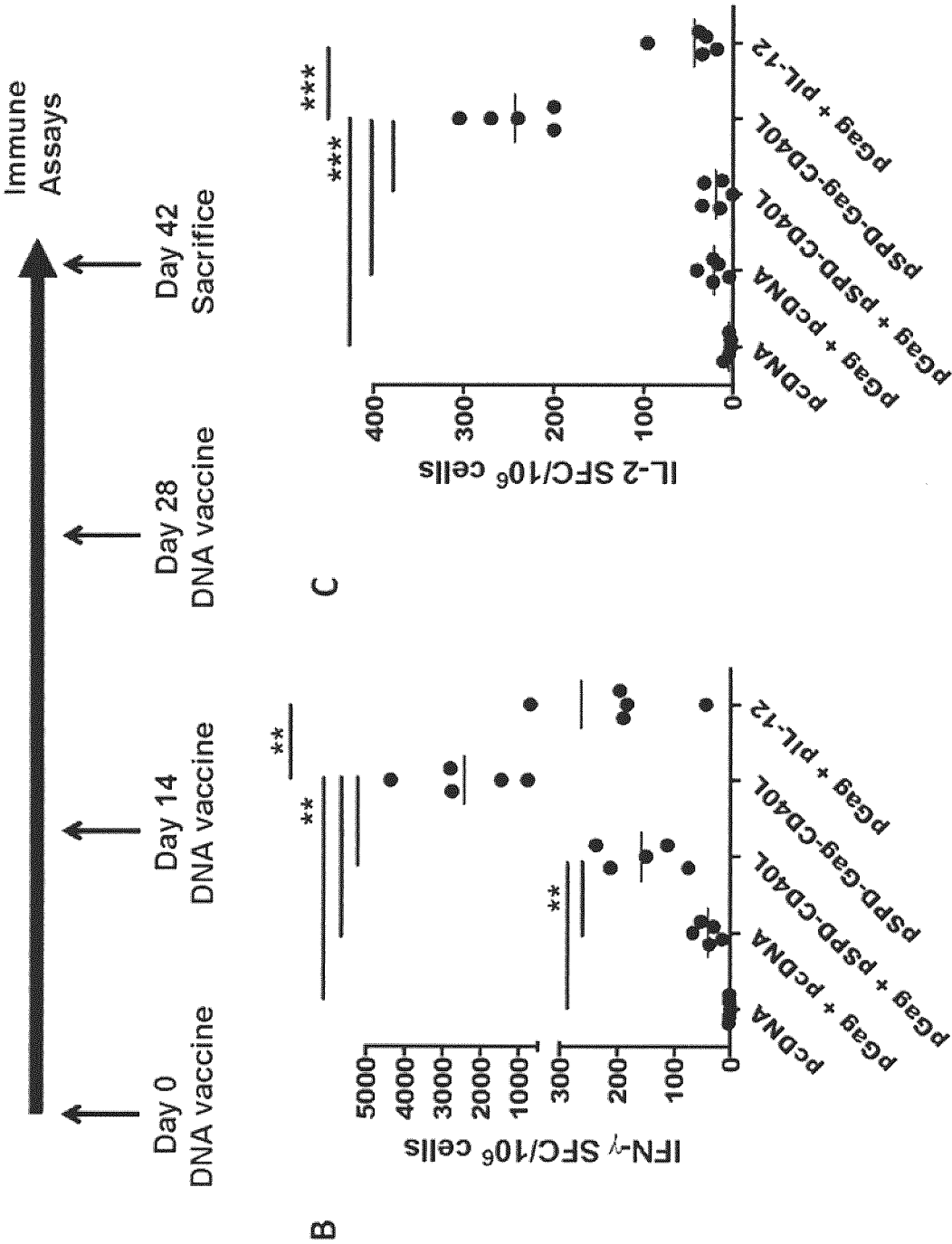


FIG. 8

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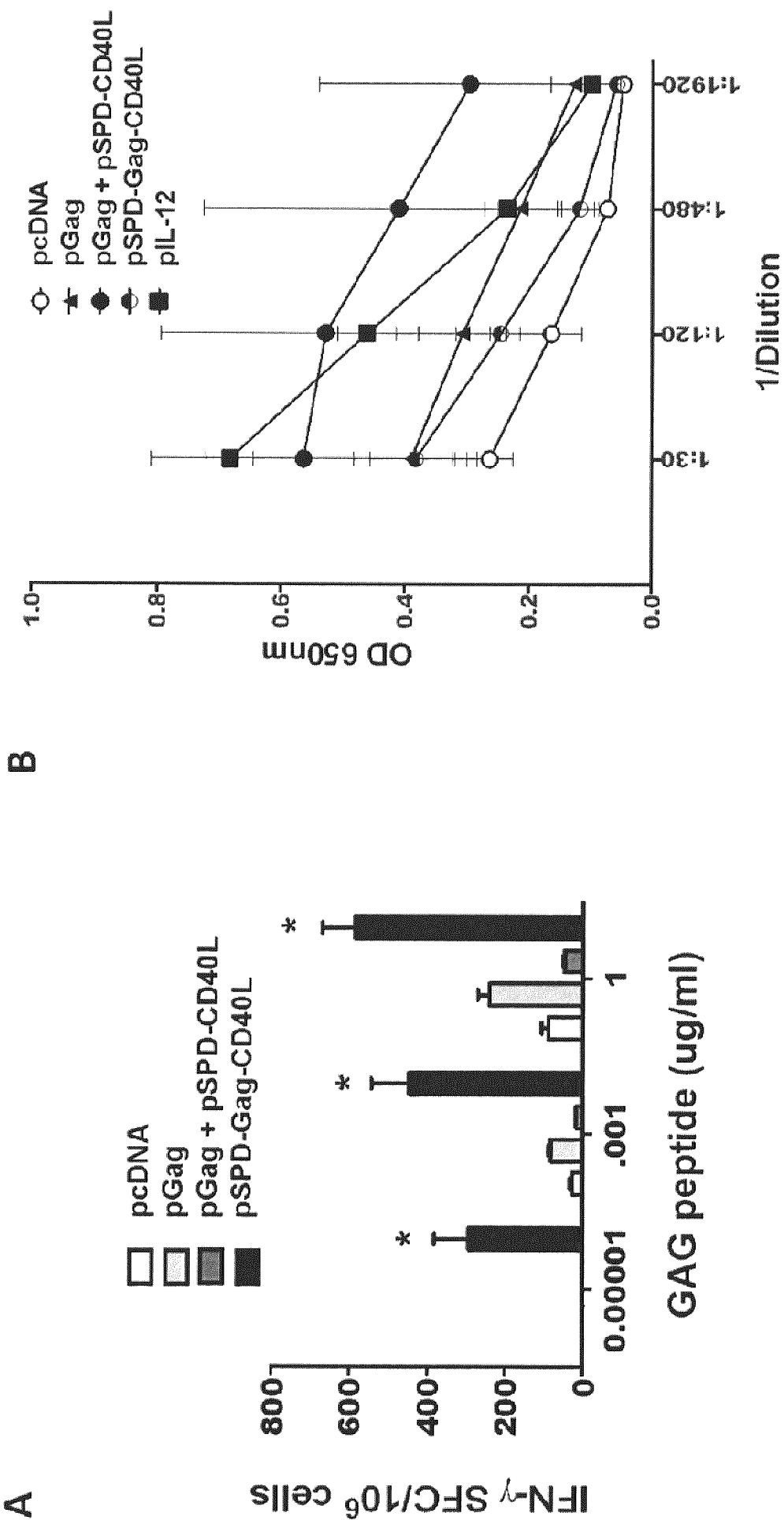


FIG. 9

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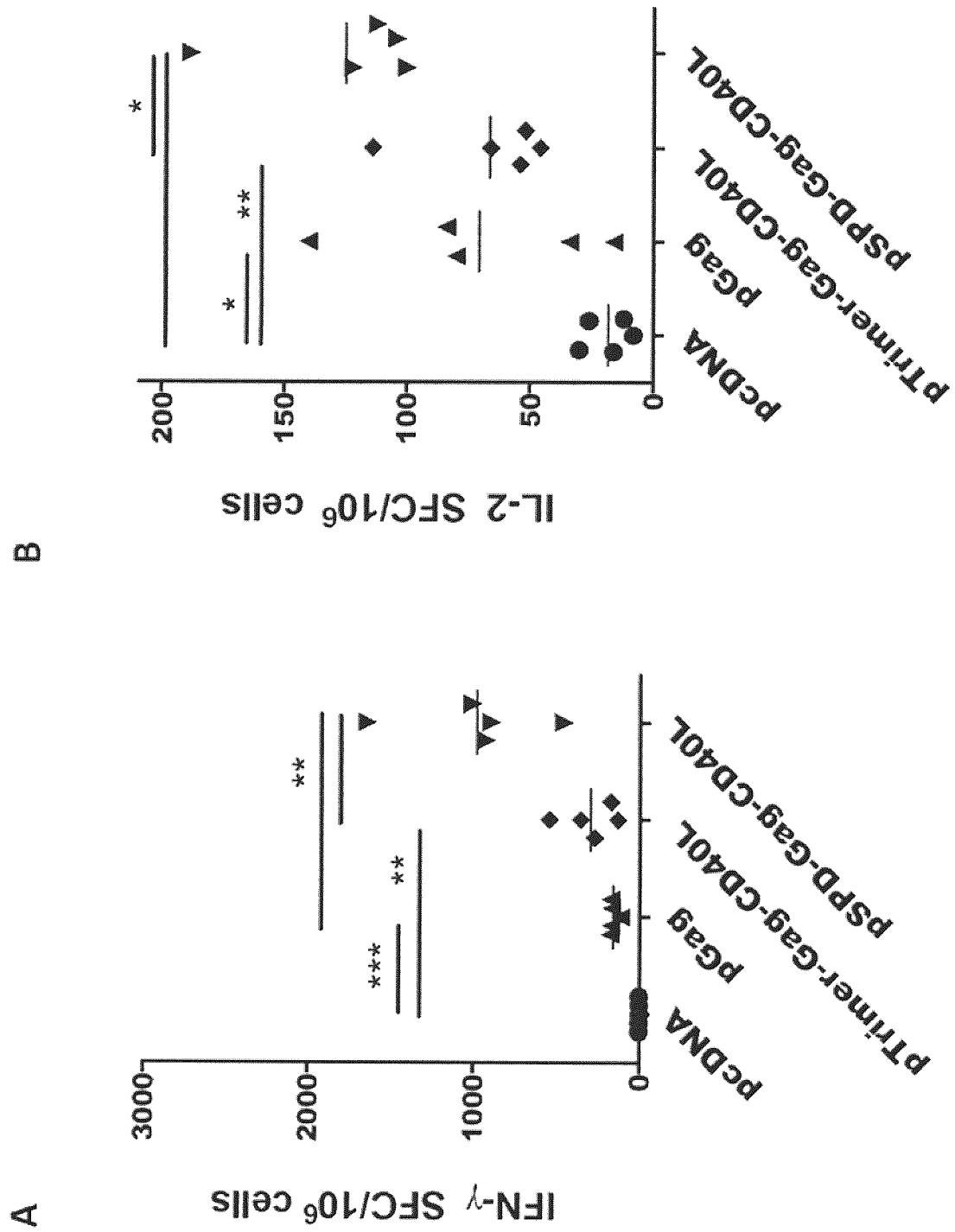


FIG. 10

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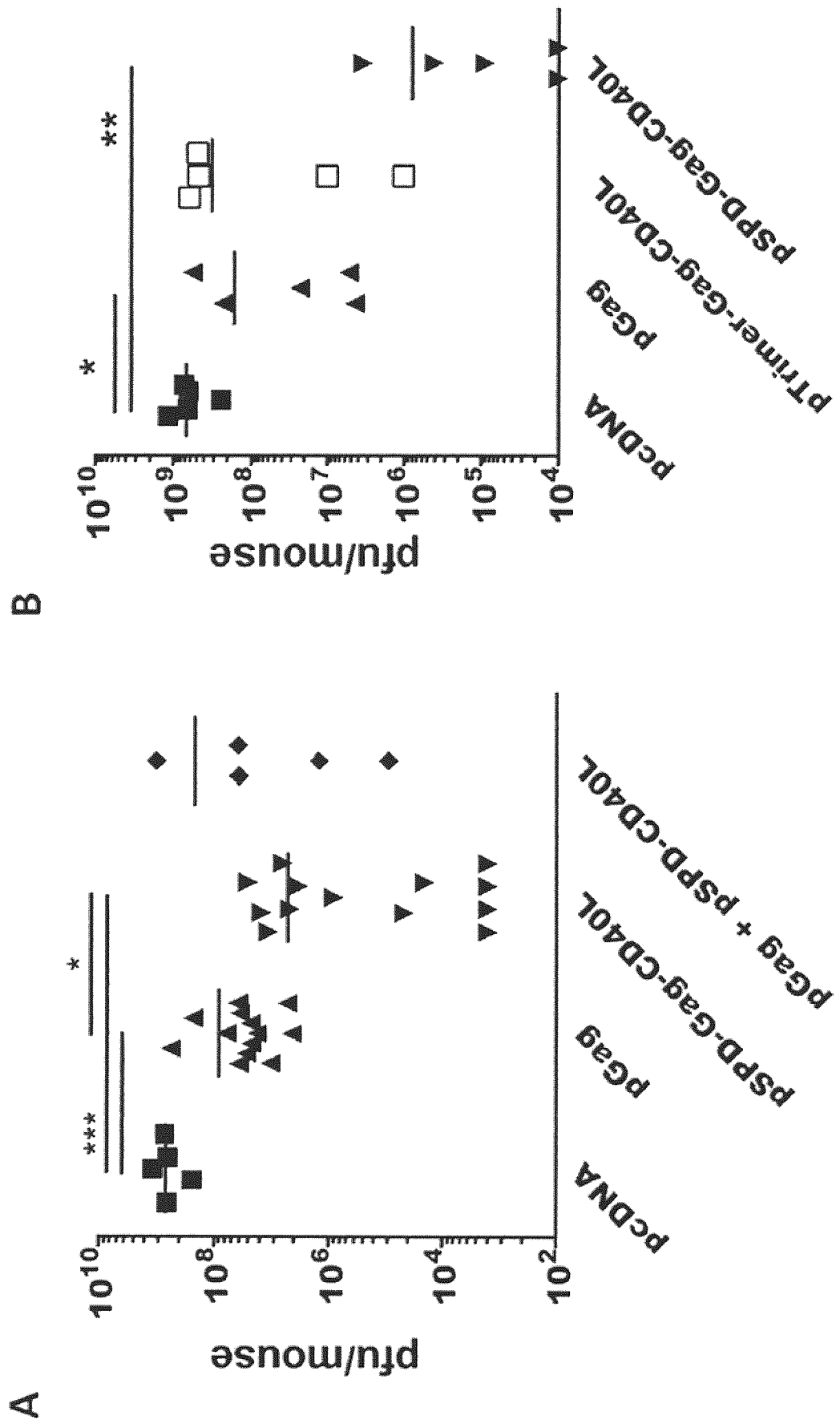


FIG. 11

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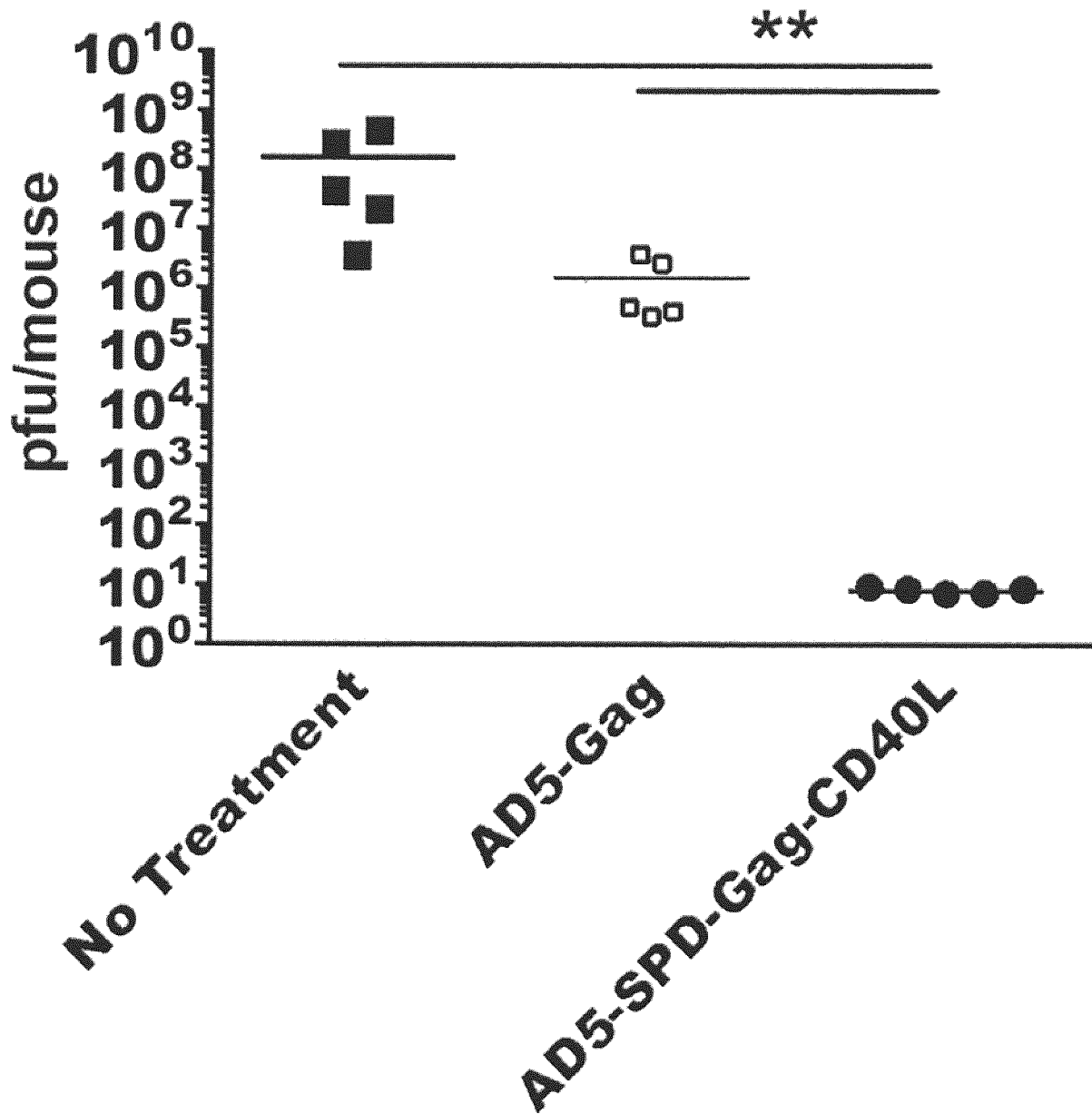


FIG. 12

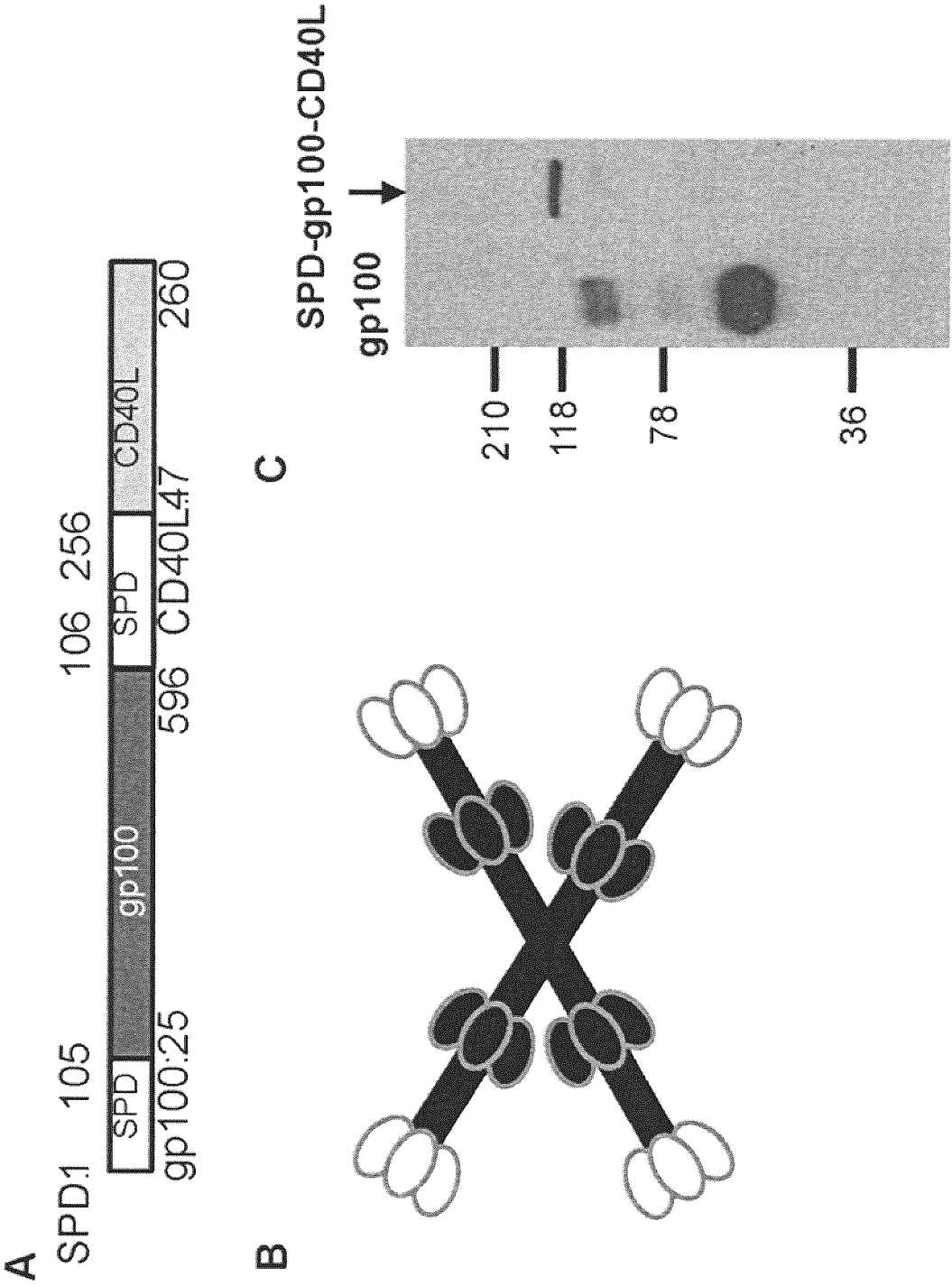
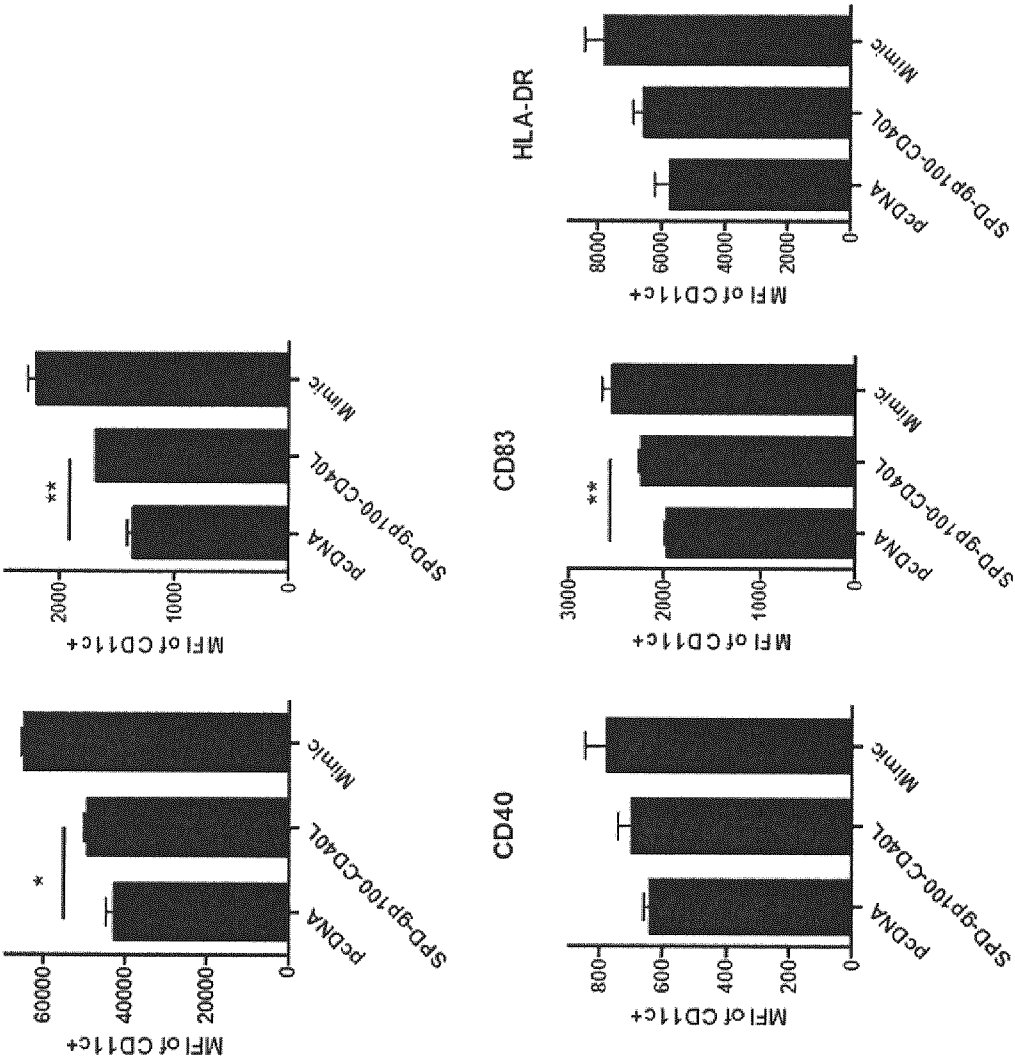


FIG. 13

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B



A

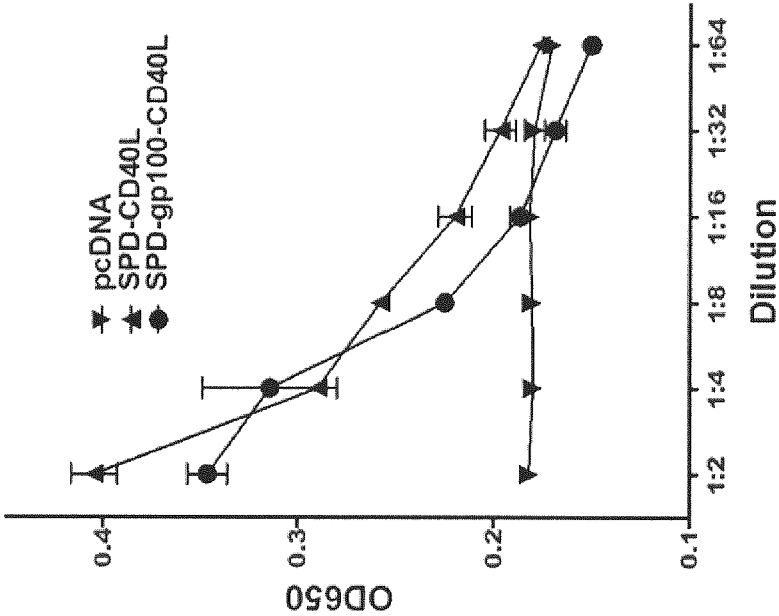


FIG. 14

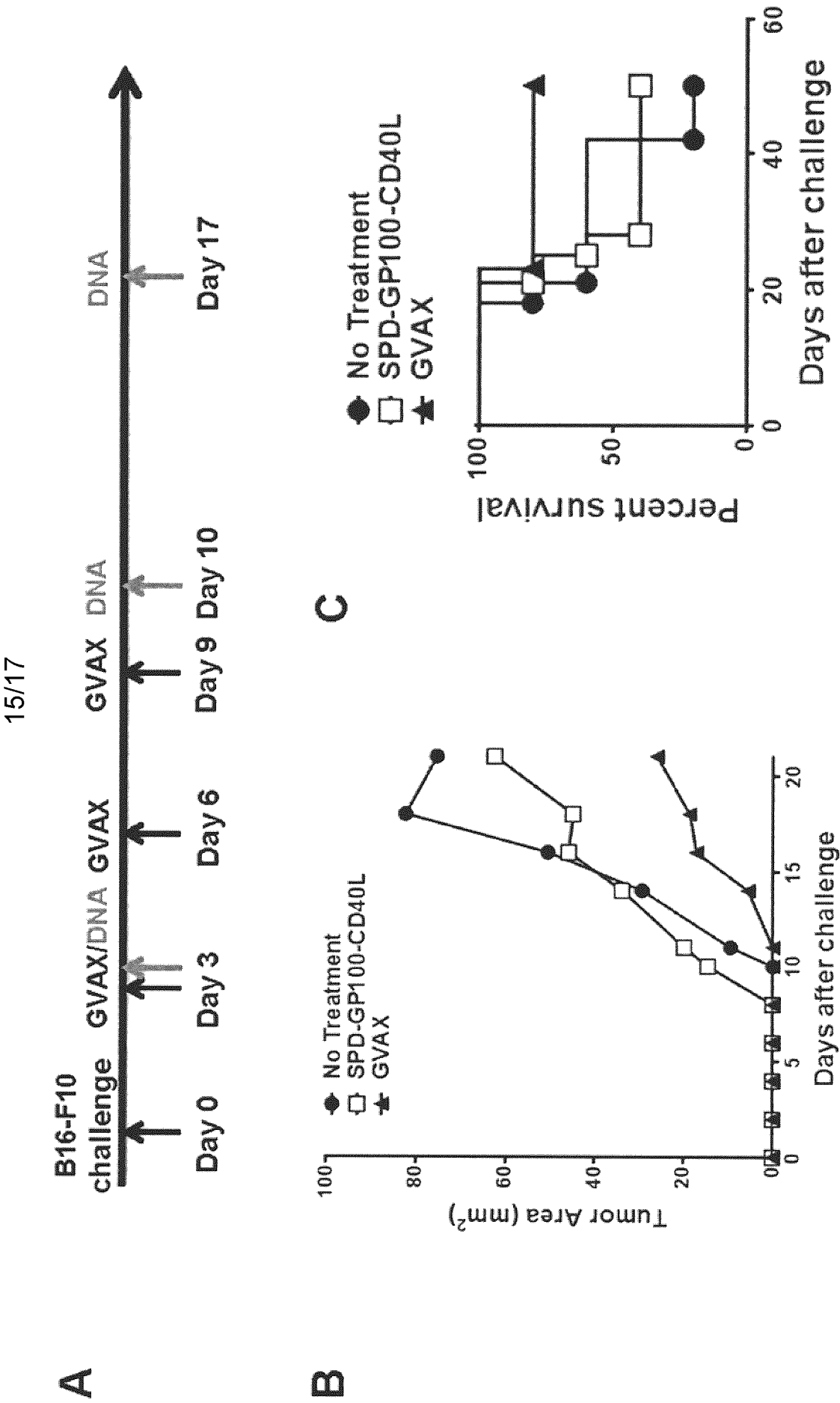


FIG. 15

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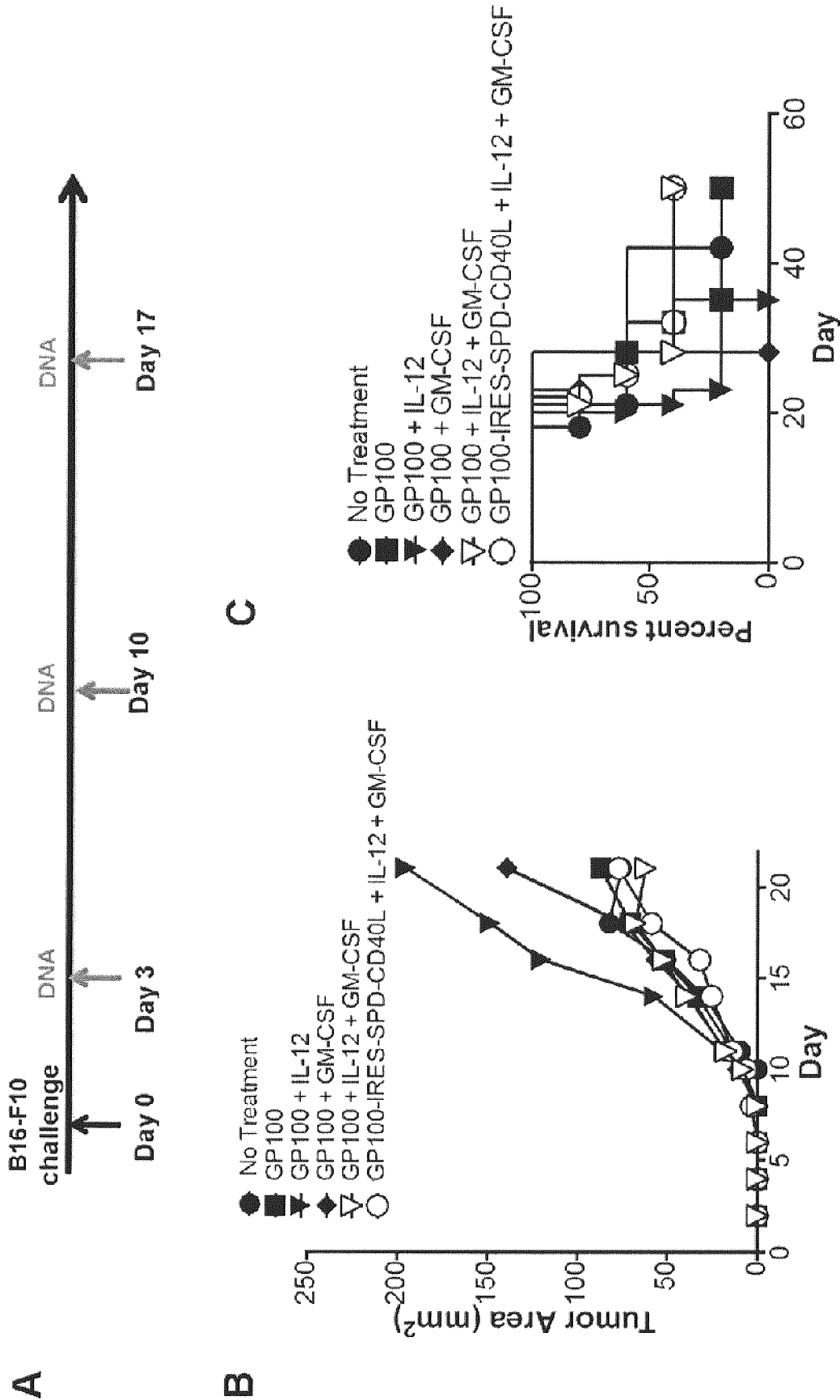


FIG. 17

TNFSF activity often requires ≥ 2 trimers
Receptor clustering is needed for activation

