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Vaccination with a Fusion Protein That Introduces HIV-1 Gag Antigen into a Multitrimer CD40L Construct Results in Enhanced CD8$^+$ T Cell Responses and Protection from Viral Challenge by Vaccinia-Gag

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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 multitrimer 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 multitrimer 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 major histocompatibility complex class I-restricted Gag peptide and improved vaccine-induced protection from challenge by vaccinia-Gag virus. The importance of the multitrimeric 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 multitrimer soluble form of CD40L, leading to strongly protective CD8$^+$ T cell responses.

DNA vaccination induces both cellular and humoral responses against an encoded antigen, protecting animals against subsequent infection with a microbial pathogen (1, 2). DNA vaccines are potent inducers of virus-specific T cell responses (3), 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 simian immunodeficiency virus (4–9). The HIV-1 Gag antigen encoded within DNA or viral vector vaccines is known to induce measurable immune responses (10, 11), providing a method to vaccinate against HIV-1. One strategy to enhance the effectiveness of DNA vaccines encoding weakly immunogenic antigens is by codelivering genes encoding molecular adjuvants. Tumor necrosis factor (TNF) superfamily ligands, 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 (12).

CD40L acts on DCs to induce or "license" CD8$^+$ T cell responses (13–15). CD40L also works on DCs to diminish the immune suppression due to CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cells (Tregs) and prevents the premature disappearance of vaccine-generated CD8$^+$ T cells (16). Consequently, we and others have examined the potential of CD40 stimulation as an adjuvant for vaccines designed to generate CD8$^+$ T cell responses.

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 (17–19). 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 (20), their toxicity for mice (21) and humans (22), and their depleting effect on CD40-bearing B cells in the blood (23). 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.

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

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trimer of CD40L is unable to provide clustering of CD40 receptors sufficient to generate a cell signal (24). Consequently, we (25) and others (24, 26) 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 four-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 (25, 27).

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 multitrimer 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. To construct an 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 (25, 28). A DNA construct encoding murine SPD-CD40L was also previously described (24, 25). 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 sequences GERGLSG and PPGLPGI of murine SPD) (Fig. 1). 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 dicysteine-containing "hub" region needed for self-assembly into a four-armed molecule. As a result, this construct is expected to form single trimers of Gag-SPD-CD40L (see Fig. 1). Plasmid pIL-12p70, encoding mouse single chain interleukin-12 (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 X-114 extractions as previously described (29). Plasmid endotoxin level was <0.2 endotoxin units/ml for all constructs, as confirmed by LAL endotoxin assay (Lonza, Inc.). Gag protein secretion for all Gag-containing constructs was confirmed by p24 enzyme-linked immunosorbent assay (ELISA) on supernatants from transfected 293T cells.

Transient-transfection and Western blotting of protein constructs. 293T cells were transiently transfected with plasmid constructs using Genjet-Plus transfection reagent (Signagen Laboratories, Iamsville, MD).

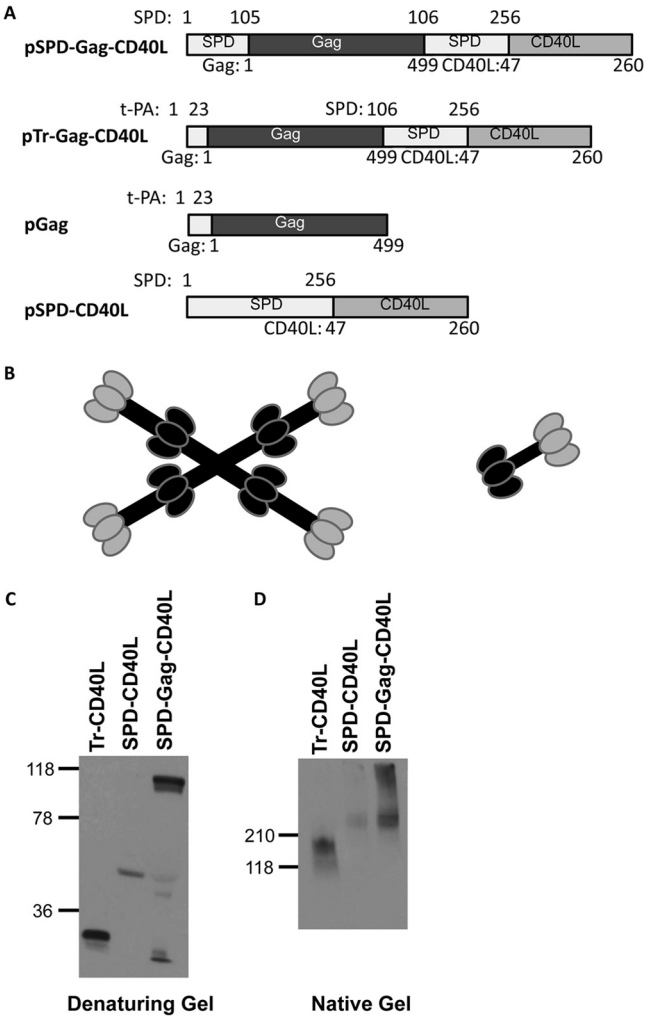

FIG 1 Construction of SPD-Gag-CD40L fusion protein. (A) Cloning strategy. For pSPD-Gag-CD40L, a nucleic acid sequence was constructed that fused amino acids 1 to 105 of murine surfactant protein D (SPD) to amino acids 1 to 499 of HIV-1 HXB2 Gag, followed by amino acids 106 to 256 of murine SPD, followed by amino acids 47 to 260 of murine CD40L. For pTrimer-Gag-CD40L, the N-terminal portion of SPD was deleted and replaced with the t-PA signal peptide sequence to direct protein secretion. pSPD-CD40L is a multitrimer CD40L adjuvant molecule that has been previously described (see the text). pGag is an antigen-only construct that contains t-PA signal peptide fused to the HIV-1 Gag sequence. (B) Schematic of proposed four-trimer complex of SPD-Gag-CD40L. CD40L trimers are shown as gray circles, Gag trimers are shown as black circles, and SPD collagen-like domain are shown as black bars. For pTrimer-Gag-CD40L, the N-terminal portion of SPD is absent. Since this N-terminal portion of SPD contains the disulfide-linked "hub" needed for the assembly of the natural four-"arm" SPD structure, the resulting trimer-Gag-CD40L protein only forms a one-trimer molecule that was used as a control for the complete multitrimer SPD-Gag-CD40L structure. (C) 293T cells were transfected with DNA plasmid cytomegalovirus (CMV) promoter expression vectors encoding soluble trimeric CD40L (25), SPD-CD40L, or SPD-Gag-CD40L. After 48 h of culture, supernatant was collected and run on an SDS-PAGE gel in the presence of reducing agent. Western blots were performed with polyclonal antibody to murine CD40L. (D) To confirm the presence of multitrimer complexes, a Western blot was performed on the 293T supernatants as in panel A except using nondenaturing PAGE in the absence of a reducing agent.

A control transfection with green fluorescent protein (GFP) plasmid was used to confirm transfection efficiency of each reaction. After 48 h, supernatants were centrifuged and filtered with a 0.45-μm-pore-size filter to remove debris. Filtered supernatant was reduced with 2-mercaptoethanol, loaded onto sodium-dodecyl sulfate–10% polyacrylamide gels (SDS–10% PAGE; Bio-Rad), electrophoresed, and blotted onto polyvinylidene difluoride membranes (Pierce). The membranes were blocked using 5% (wt/vol) 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 nondenaturing PAGE was performed in the absence of SDS and reducing agent.

CD40 *in vitro* activity assay. 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 the control of NF-κB (30). Briefly, 80,000 CD40-293-SEAP reporter cells, grown in Dulbecco modified Eagle medium with 10% fetal bovine serum (FBS), were plated in each well of a 96-well plate. A total of 100 μl 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 μl of the supernatants was added to each well of a 96-well assay plate, together with 100 μl of QUANTI-Blue alkaline phosphatase substrate (InvivoGen)/well. The plates were incubated for 20 min at 37°C, and the optical density at 650 nm (OD_{650}) was read.

DC activation and maturation assay. Bone marrow-derived murine DCs were generated by standard methods (31) with the following modifications. Bone marrow cells were obtained from C57BL/6 mice and washed in RPMI 1640 medium. The cells were then placed in tissue culture-treated T75 flasks at a concentration of 10^6 cells per ml in 20 ml of complete RPMI (RPMI 1640 with 10% FBS, 20 μg of gentamicin sulfate/ml, 50 μM 2-mercaptoethanol), and 20 ng of murine recombinant granulocyte-macrophage colony-stimulating factor/ml, and 10 ng of murine recombinant IL-4/ml [Peprotech, Rocky Hill, NJ]. Cells were cultured at 37°C and 5% CO_2 and, on day 3, the medium was replaced with fresh complete RPMI containing cytokines. On day 5, the cells were harvested, washed, and resuspended in complete RPMI at 5×10^5 cells/ml. A total of 2 ml was added to each well of six-well tissue culture-treated plates. Subsequently, 300 μl of supernatant containing SPD-Gag-CD40L or DC activation cytokine mix (containing IL-1β, IL-6, and prostaglandin E_2 [PGE_2]) was added, and the cells were incubated for 36 h. 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 major histocompatibility complex (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 $CD11c^+$ DCs 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. 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 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). After 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 absorbances at 260 nm and 280 nm, and calculated using the formula $vp/ml = OD_{260} \times \text{viral dilution} \times 1.1 \times 10^{12}$. To determine infectious units, virus titers were determined using an Adeno-X rapid titer kit (Clontech).

Mice and immunization schedule. Female BALB/c mice (7 to 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 Ad5s for immunological and vaccinia virus challenge experiments.

DNA immunization schedule. DNA was injected intramuscularly into the quadriceps muscles of both hind limbs. Vaccinations were given three times at 2-week intervals with 100 μg of SPD-Gag-CD40L or 100 μg of Gag 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 of phosphate-buffered saline (PBS; 50 μl per limb). Control mice were injected with 100 μg of pcDNA3.1 empty vector.

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

Adenovirus immunization schedule. Five mice per group were immunized by intramuscular injection with Ad5 constructs twice at a 2-week interval. Viral vector was injected in a total volume of 100 μl of PBS (50 μl per limb) in the quadriceps muscles of both hind limbs.

ELISPOT assay. Gamma interferon (IFN-γ) and IL-2 enzyme-linked immunospot (ELISPOT) assays were performed to determine antigen-specific cytokine secretion from immunized mouse splenocytes. ELISPOT assays were carried out according to the manufacturer's protocol (R&D Systems) using 96-well MAIP plates (Millipore). Freshly prepared vaccinated mouse splenocytes (10^5 cells/well) were added to each well of the plate and stimulated for 18 h at 37°C and 5% CO_2 in the presence of HIV-1 Gag peptide AMQMLKETI (10 μg/ml or as described). A c-myc peptide (negative control) and phorbol myristate acetate/ionomycin (positive control) were evaluated to calculate the minimum and maximum numbers, respectively, of antigen-specific ELISPOT assays. After 18 h, spots were developed with an AEC substrate kit (Vector Laboratories) according to the 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 spot-forming counts (SFC) per million cells plated, subtracting negative-control values.

T cell receptor avidity ELISPOT assay. ELISPOT analysis was performed as described above, stimulating the cells with 1 μg, 10^{-3} μg, or 10^{-5} μg of Gag peptide (AMQMLKETI)/ml to evaluate the number of T cells able to secrete IFN-γ at limiting peptide concentrations.

ELISA for anti-Gag IgG responses. Anti-Gag antibody production was measured by ELISA. HIV-1 p55 Gag protein (10 μg/ml) was coated onto 96-well ELISA plates overnight at 4°C. Mouse sera at various 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. Two weeks following DNA or Ad5 immunization, mice were challenged intraperitoneally with 10^7 vp of vaccinia-

Gag virus vP1287 as described previously (28). At 5 days after challenge, mice were sacrificed and ovaries were removed and homogenized in 500 μl of 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. After 48 h of incubation, the plates were stained with 0.1% (wt/vol) crystal violet in 20% ethanol. Plaques were counted and expressed as the PFU of virus in total lysate volume (PFU/mouse).

Statistical analysis. All error bars represent the standard errors of the mean. GraphPad Prism 6.0 software was used to calculate significance by one-way analysis of variance for multiple comparison or by two-tailed Student *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 multitrimer SPD-Gag-CD40L. 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 multitrimer form of CD40L is needed since single trimers of CD40L do not provide an effective stimulus (reviewed in reference 32). Consequently, multitrimer 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 DCs *in vitro* and enhance vaccine responses *in vivo*. In the previous vaccine studies, antigen and multitrimer CD40L adjuvant were used as separate molecules and mixed together for immunization (25). To further improve this vaccine design, an immunogen was developed that incorporated antigen (exemplified by HIV-1 Gag) and multitrimer 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 acids 105 and 106 of mouse SPD within the SPD-CD40L construct (Fig. 1A). 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. 1C). 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 nondenaturing gel in the absence of reducing agents. Multiple bands were observed at >200 kDa, demonstrating the formation of large multimeric complexes (Fig. 1D).

Biological activity of multitrimer soluble SPD-Gag-CD40L. To assess the ability of SPD-Gag-CD40L to stimulate the CD40 receptor, a CD40-bearing indicator cell line was used as described previously (30). In this cell line, CD40 stimulation activates the NF-κB pathway, which in turn activates the κB promoter driving the expression of 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. 2A). 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 DCs were treated with supernatants from 293T cells transfected with either pSPD-Gag-CD40L or pcDNA3.l empty vector. A cytokine mix (IL-1β, IL-6, and PGE_2) was used to "mimic" an inflammatory environment and used as a positive control. As shown in Fig. 2B, 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, a finding consistent with endocytosis of CD40 following SPD-Gag-CD40L ligation.

As a DNA vaccine, multitrimer soluble SPD-Gag-CD40L was more immunostimulatory than separate plasmids for Gag antigen and SPD-CD40L adjuvant. 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 2-week intervals with an intramuscular injection of 100 μg of pSPD-Gag-CD40L plasmid DNA. For comparison, 100 μg of plasmid DNA encoding soluble secreted Gag antigen (pGag) was mixed with 20 μg of separate plasmids encoding either SPD-CD40L or IL-12p70 adjuvants or pcDNA3.1 empty control vector. The vaccination schedule is outlined in Fig. 3A. Two weeks after the third vaccination, T cell responses were analyzed by IFN-γ and IL-2 ELISPOT assays using the K^d-restricted HIV-1 Gag peptide AMQMLKETI to stimulate mouse splenocytes. As shown in Fig. 3B, there was a significant increase in Gag-specific $CD8^+$ T cell responses measured by an IFN-γ ELISPOT assay 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. 3C). Comparing pSPD-Gag-CD40L to pGag alone, mean IL-2 ELISPOT responses increased >10-fold.

To determine whether 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. 3D, pSPD-Gag-CD40L significantly increased IFN-γ ELISPOT responses compared to other vaccine groups at all peptide dilutions. At 10 pg of AMQMLKETI peptide/ml, IFN-γ 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.

To evaluate humoral immune responses, Gag-specific IgG antibody titers in mice serum were measured by ELISA 2 weeks after vaccination. As shown in Fig. 3E, 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 multitrimer SPD-Gag-CD40L. We next evaluated the role of multitrimerization by the SPD scaffold on the immune response. The N terminus of SPD is involved

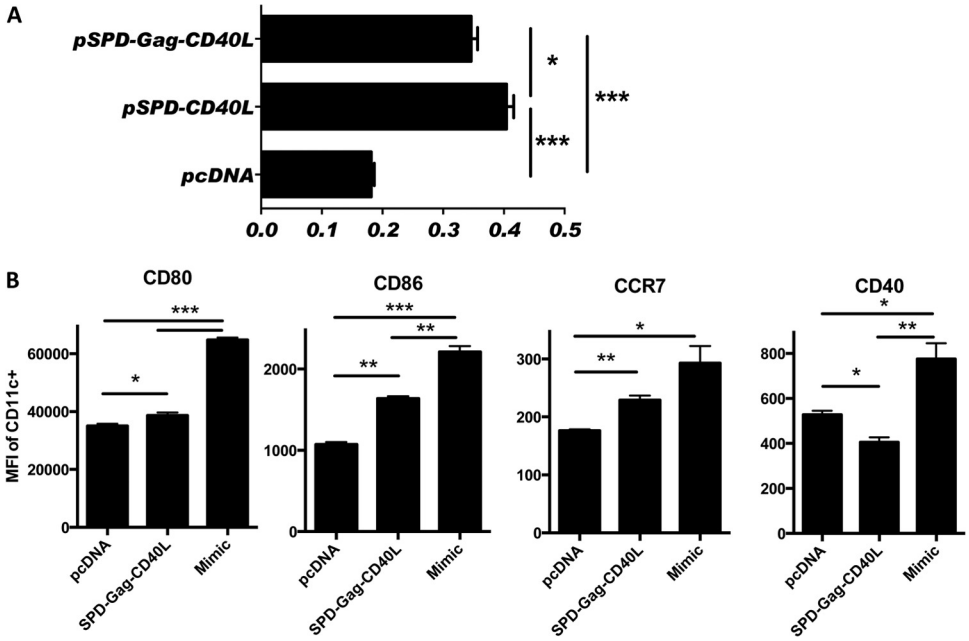

FIG 2 Biological activity of SPD-Gag-CD40L *in vitro*. (A) *In vitro* activity using a CD40 receptor NF-κB indicator cell line. Equivalent amounts of culture supernatants from 293T cells transfected with pcDNA3.1, pSPD-CD40L, or pSPD-Gag-CD40L were incubated with CD40-293-SEAP reporter cells. NF-κB-driven SEAP production was measured by a colorimetric enzyme assay at OD_{650}. In this assay, both the pSPD-CD40L adjuvant protein and the new SPD-Gag-CD40L protein were active as CD40 receptor activators. (B) Stimulating activity on mouse bone marrow-derived DCs (BMDDCs). Equivalent amounts of culture supernatants from 293T cells transfected with pcDNA3.1 or pSPD-Gag-CD40L were incubated with BMDDCs for 18 h. 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. As expected, the CD40 receptor was downregulated by exposure to SPD-Gag-CD40L. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to pcDNA3.1 supernatant). The data represent independent wells in the same experiment.

in disulfide bonding and is required to form four-trimer complexes (34). Deleting this N-terminal portion of SPD (amino acids 106 to 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 to 256 of murine SPD and then amino acids 47 to 260 of murine CD40L (see Materials and Methods). 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-κB 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 (24, 26). 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 (multitrimer of Gag antigen fused to CD40L). Mice vaccinated with pSPD-Gag-CD40L showed a significant increase in IFN-γ ELISPOT responses compared to unadjuvanted pGag alone or pTrimer-Gag-CD40L that contains Gag and CD40L but lacks the multitrimer structure (Fig. 4A). Also observed was a significant increase in IL-2 ELISPOT responses for the pSPD-Gag-CD40L group versus pTrimer-Gag-CD40L (Fig. 4B).

Vaccination with pSPD-Gag-CD40L protected mice from virus challenge by vaccinia-Gag. 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) (28). Two weeks after the final DNA vaccination, mice were challenged intraperitoneally with vaccinia-Gag (10^7 PFU). As shown in Fig. 5A, mice vaccinated with pSPD-Gag-CD40L had significantly less tissue virus in ovaries compared to unvaccinated animals ($P < 0.001$) or animals vaccinated with pGag DNA vaccine alone ($P < 0.05$) when vaccinia virus PFU were measured on day 5 after vaccinia-Gag challenge. Overall, 4 of 13 mice vaccinated with pSPD-Gag-CD40L had undetectable virus titers (<10 PFU in total ovary lysate).

To determine the effect of CD40L multitrimerization 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. 5B). 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 the 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. Although 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 2-week intervals with 10^9 vp i.m. At 2 weeks after the final vacci-

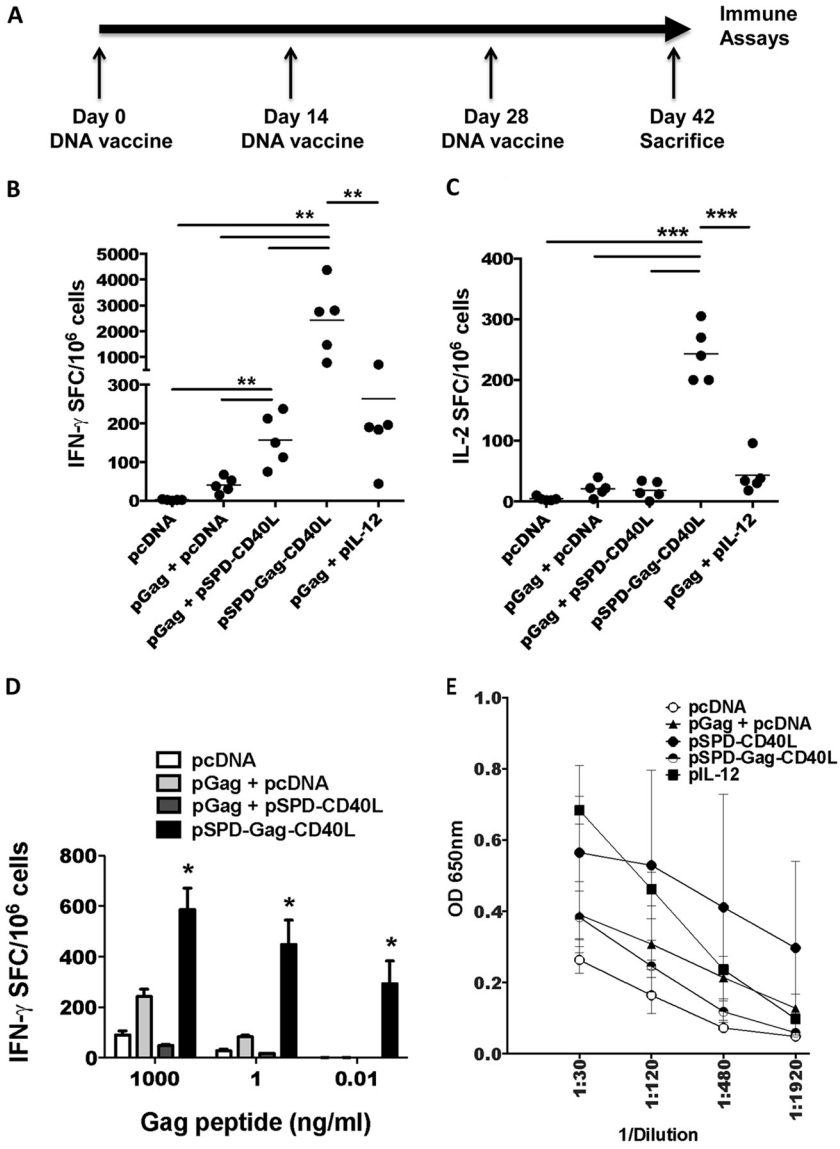

FIG 3 Vaccine-induced responses to SPD-Gag-CD40L *in vivo*. (A) Immunization schedule. BALB/c mice (five mice per group) were immunized intramuscularly with either pcDNA3.1 (empty vector), pGag mixed with pcDNA3.1 empty vector, pGag antigen plasmid mixed with either pSPD-CD40L or pIL-12p70 antigen plasmids, or pSPD-Gag-CD40L. Mice were vaccinated on days 0, 14, and 28. Mice received a total of either 100 μg of DNA (pSPD-Gag-CD40L) or 120 μg of DNA (100 μg of pGag plus 20 μg of pcDNA3.1, pSPD-CD40L, or pIL-12p70) by intramuscular injection, half into each hind-limb quadriceps muscle. Two weeks later, mice were sacrificed, and splenocytes were analyzed for Gag-specific immune responses. (B and C) IFN-γ ELISPOT (B) and IL-2 ELISPOT (C) assays for $CD8^+$ T cell responses. Splenocytes were collected 2 weeks after vaccination and cultured for 18 h in the presence of 10 μg of HIV-1 Gag $CD8^+$ specific peptide AMQMLKETI/ml. SPD-Gag-CD40L significantly increased the number of Gag-specific IFN-γ-secreting cells compared to pGag antigen alone or pGag mixed with separate plasmids for pSPD-CD40L or pIL-12 adjuvants. SPD-Gag-CD40L also significantly increased Gag-specific IL-2 secretion compared to pGag antigen plasmid mixed with separate plasmids for pSPD-CD40L or pIL-12 adjuvants. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (D) 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 h. Splenocytes from mice vaccinated with pSPD-Gag-CD40L induced a significant increase in IFN-γ ELISPOT assays after stimulation with Gag peptide AMQM LKETI at concentrations of 1 ng 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 ppSPD-CD40L adjuvant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to pGag alone or pGag plus SPD-CD40L vaccination). (E) IgG antibody responses against Gag antigen. Total IgG specific for Gag was measured by ELISA from mouse serum collected on day 42. Plasmid pGag in combination with pSPD-CD40L or pIL-12 induced higher Gag-specific IgG responses at various dilutions compared to pGag plus pcDNA3.1, but the differences were not statistically significant ($P > 0.05$ between all groups). IgG antibody levels after vaccination with pSPD-Gag-CD40L were similar to Gag antigen alone.

nation, mice were challenged intraperitoneally with vaccinia-Gag (10^7 PFU). Remarkably, all five mice vaccinated with Ad5-SPD-Gag-CD40L had no detectable vaccinia virus in their ovaries (<10 PFU/mouse) (Fig. 6), which was statistically significant compared to 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 strat-

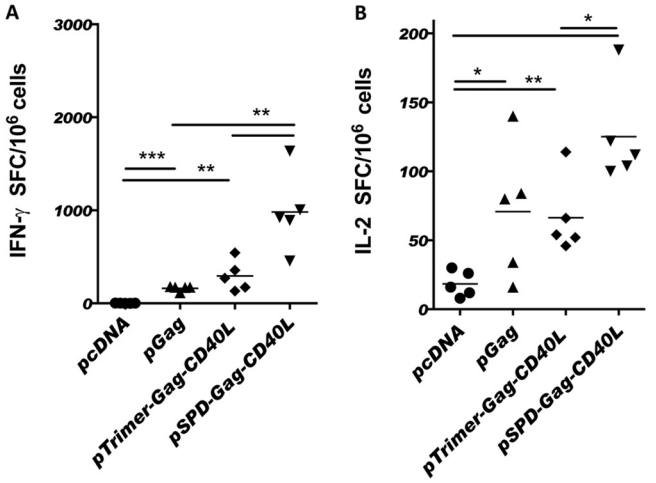

FIG 4 The multitrimer structure of SPD-Gag-CD40L is necessary for the improved vaccine effect. Vaccinations were performed as detailed in Fig. 3, comparing multitrimer pSPD-Gag-CD40L to a single-trimer Gag-CD40L construct missing the N-terminal disulfide-bonding domain of SPD (pTrimer-Gag-CD40L). IFN-γ (A) and IL-2 (B) ELISPOT assays were performed 2 weeks after the third DNA vaccination. As shown, the number of Gag-specific splenocytes was significantly greater using the multitrimer pSPD-Gag-CD40L vaccine compared to a one-trimer form of an analogous protein.

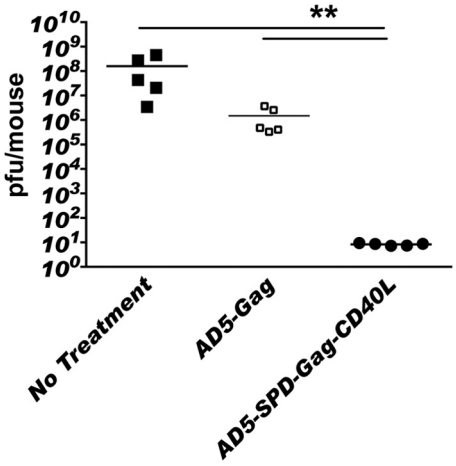

FIG 6 SPD-Gag-CD40L as part of an Ad5-vectored vaccine. BALB/c mice were immunized intramuscularly with Ad5-GAG or AD5-SPD-Gag-CD40L on days 0 and 14. Two weeks after the final vaccination, mice were challenged intraperitoneally with vaccinia-Gag virus (10^7 PFU). Mice were sacrificed on day 5 after vaccinia virus challenge, and ovaries were harvested for vaccinia virus PFU determinations using indicator Vero cells. Ad5-SPD-Gag-CD40L vaccination reduced viral load by ~7 logs after vaccinia-Gag challenge. No detectable virus could be found in the mice that had received this vaccine, indicating complete protection (sterilizing immunity).

egy of introducing SPD-Gag-CD40L into viral vector vaccines such as Ad5.

DISCUSSION

Stimulation through the CD40 receptor is important for generating $CD8^+$ T cell responses under noninflammatory conditions (13–16, 35). 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 $CD40^+$ cells such as B cells, and the relatively limited

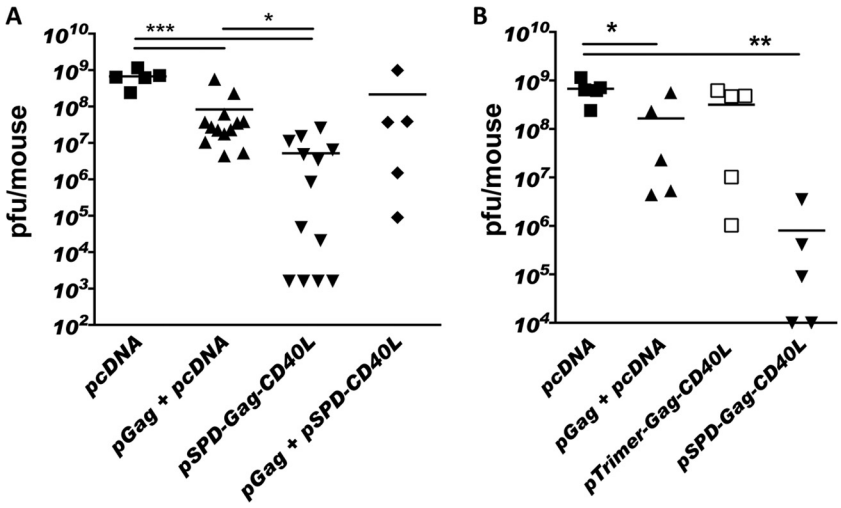

FIG 5 Protective effects of pSPD-Gag-CD40L vaccination measured by vaccinia-Gag viral challenge. BALB/c mice were immunized intramuscularly with pcDNA3.1 empty vector as a control, pGag mixed with pcDNA3.1 empty vector, pSPD-Gag-CD40L, a mixture of separate plasmids for pGag antigen plus pSPD-CD40L adjuvant, or pTrimer-Gag-CD40L that encodes a one-trimer protein. Immunizations were given on days 0, 14, and 28. At 2 weeks after the final vaccination, the mice were challenged intraperitoneally with vaccinia-Gag (10^7 PFU). Mice were sacrificed on day 5 after vaccinia virus challenge, and the ovaries were harvested. The vaccinia virus titer was determined on ovary lysates using Vero cells and is expressed as PFU. (A) pSPD-Gag-CD40L vaccination resulted in a significantly lower tissue viral load compared to animals vaccinated with pcDNA3.1 alone (empty vector) or pGag (antigen only). Vaccination with a mixture of pGag antigen plus pSPD-CD40L adjuvant as separate plasmids induced a modest reduction in viral loads that were not significantly reduced compared to pGag antigen alone. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The data represent two independent experiments. (B) Evaluation of single trimer pTrimer-Gag-CD40L construct. Mice were vaccinated with either pcDNA3.1 empty vector (control), pGag antigen only, pTrimer-Gag-CD40L, or pSPD-Gag-CD40L plasmid DNAs. Vaccination with pTrimer-Gag-CD40L did not significantly reduce tissue viral load compared to either or pcDNA3.1 empty vector control or pGag antigen alone.

effectiveness of agonistic anti-CD40 antibody in humans compared to studies in mice.

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 (reviewed in reference 32). For agonistic anti-CD40 antibodies, clustering requires that the antibodies be mounted via FcRs on an adjacent cell (17–19). Under conditions where an adjacent FcR-bearing cell is absent, agonistic anti-CD40 antibodies are not effective.

Keeping in mind this requirement for CD40 receptor clustering, we and others have examined various multitrimer forms of CD40L as agonists for murine, macaque, and human DCs (24, 25, 27, 33, 36). 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, multitrimer 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 (25, 33). However, we hypothesized that the vaccine response might be even stronger if the antigen and multitrimer 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. 1A and B).

As a first step, nondenaturing PAGE was used to show that SPD-Gag-CD40L protein is indeed a high-molecular-weight multimer complex (Fig. 1C and D). *In vitro*, this multitrimer CD40L molecule could stimulate a CD40 receptor-bearing indicator cell line that reports out NF-κB activation by releasing SEAP (Fig. 2A). As a control, a molecule was made in which the N-terminal "hub" of SPD was deleted, leading to a one-trimer CD40L molecule that had little or no activating in this NF-κB activation assay (data not shown). This control revealed the critical importance of the multitrimer structure in forming a highly actively form of CD40L, as previously demonstrated by Haswell et al. (24). As expected, SPD-Gag-CD40L stimulated murine bone marrow-derived DCs *in vitro* to express cell surface markers of activation (Fig. 2B). Although these data do not represent direct evidence that the SPD-Gag-CD40L constructs fold into the structure outlined in Fig. 1B, we consider the ability of the construct to form biologically active trimers as initial evidence that functional trimers are being generated. In preliminary experiments, we have also observed biological activity for SPD-CD40L fusions containing alternative antigens, including gp100 and HIV-1 Env gp120 (data not shown), supporting the concept that SPD-Antigen-CD40L fusions are broadly applicable as a vaccine design strategy.

In vivo, plasmid DNA (pSPD-Gag-CD40L) was tested as a vaccine (Fig. 3A) and compared to vaccination with plasmid DNA for Gag alone (pGag) or a 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 (Fig. 3B, 3C, 4A, and 4B). pSPD-Gag-CD40L elicited $CD8^+$ T cells with remarkably increased avidity for the Gag peptide antigen (Fig. 3D). However, as we and others have previously described, multitrimer CD40L is not a good adjuvant for antibody responses (Fig. 3E), which emphasizes the special effects of CD40L on DCs and subsequent $CD8^+$ T cell responses. Although CD40L plays a role in promoting B-cell proliferation and immunoglobulin class switching (37), 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 (38–41). We have also observed similar responses by SPD-CD40L in previous studies, with low antibody responses after vaccination (25). We propose that SPD-Gag-CD40L is unable to enhance antibody responses through one or more of these mechanisms.

In addition, these $CD8^+$ T cell responses were protective as judged by the 2- to 3-log reduction in tissue viral load after challenging the mice with vaccinia-Gag (Fig. 5). However, we note that virus titers after SPD-Gag-CD40L vaccination were not significantly different than virus titers after vaccination with Gag plus SPD-CD40L, despite a large difference in IFN-γ and IL-2 ELISPOT responses between the two groups. In part, 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, whereas Gag plus SPD-CD40L was unable to reduce titer below 10^4 PFU/mouse. Overall, Gag plus SPD-CD40L gave a similar mean virus 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 an ~7-log reduction in tissue viral load in mice vaccinated with Ad5-SPD-Gag-CD40L, and no challenge virus could be detected (Fig. 6).

To account for the effectiveness of the SPD-Gag-CD40L vaccine design, three factors should be considered: (i) the use of multitrimer CD40L to cluster the CD40 receptor and thereby activate DCs, (ii) the role of CD40L in targeting antigen to CD40 receptor-bearing DCs, and (iii) the simultaneous delivery of both the Gag antigen and the CD40L adjuvant to the same DC.

(i) Regarding the multitrimer nature of CD40L in SPD-Gag-CD40L, it is worth noting that others have previously made antigen-CD40L fusion proteins. Xiang et al. (42) 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 (43). This is expected when a one-trimer form of CD40L is used rather than a receptor-clustering multitrimer construct such as SPD-Gag-CD40L.

(ii) Regarding the targeting of antigen to CD40 on DCs, this has emerged as a very desirable property for vaccine design. Heath 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 (20). *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 (44). *In vivo*, Cohn et al. found that conjugating antigen to anti-CD40 antibody broadened the types of DCs that cross-present antigen to T cells to include BDCA1(+)

DCs in addition to standard cross-presentation by BDCA3(+) DCs (45). However, DC cross-presentation 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 these authors 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 (46). 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.

(iii) 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. (47). 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.

In conclusion, a vaccine was developed that combines multitrimer 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.

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