Soluble multi-trimeric TNF superfamily ligand adjuvants enhance immune responses to a HIV-1 Gag DNA vaccine

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ABSTRACT

Background: DNA vaccines remain an important component of HIV vaccination strategies, typically as part of a prime/boost vaccination strategy with viral vector or protein boost. A number of DNA prime/viral vector boost vaccines are currently being evaluated for both preclinical studies and in Phase I and Phase II clinical trials. These vaccines would benefit from molecular adjuvants that increase correlates of immunity during the DNA prime. While HIV vaccine immune correlates are still not well defined, there are a number of immune assays that have been shown to correlate with protection from viral challenge including CD8+ T cell avidity, antigen-specific proliferation, and polyfunctional cytokine secretion.
Methodology and principal findings: Recombinant DNA vaccine adjuvants composed of a fusion between Surfactant Protein D (SP-D) and either CD40 Ligand (CD40L) or GITR Ligand (GITRL) were previously shown to enhance HIV-1 Gag DNA vaccines. Here we show that similar fusion constructs composed of the TNF superfamily ligands (TNFSFL) 4-1BBL, OX40L, RANKL, LIGHT, CD70, and BAFF can also enhanced immune responses to a HIV-1 Gag DNA vaccine. BALB/c mice were vaccinated intramuscularly with plasmids expressing secreted Gag and SP-D-TNFSFL fusions. Initially, mice were analyzed 2 weeks or 7 weeks following vaccination to evaluate the relative efficacy of each SP-D-TNFSFL construct. All SP-D-TNFSFL constructs enhanced at least one Gag-specific immune response compared to the parent vaccine. Importantly, the constructs SP-D-4-1BBL, SP-D-OX40L, and SP-D-LIGHT enhanced CD8+ T cell avidity and CD8+/CD4+ T cell proliferation 7 weeks post vaccination. These avidity and proliferation data suggest that 4-1BBL, OX40L, and LIGHT fusion constructs may be particularly effective as vaccine adjuvants. Constructs SP-D-OX40L, SP-D-LIGHT, and SP-D-BAFF enhanced Gag-specific IL-2 secretion in memory T cells, suggesting these adjuvants can increase the number of self-renewing Gag-specific CD8+ and/or CD4+ T cells. Finally adjuvants SP-D-OX40L and SP-D-CD70 increased T_H1 (IgG2a) but not T_H2 (IgG1) antibody responses in the vaccinated animals. Surprisingly, the B cell-activating protein BAFF did not enhance anti-Gag antibody responses when given as an SP-D fusion adjuvant, but nonetheless enhanced CD4+ and CD8+ T cell responses.
Conclusions: We present evidence that various SP-D-TNFSFL fusion constructs can enhance immune responses following DNA vaccination with HIV-1 Gag expression plasmid. These data support the continued evaluation of SP-D-TNFSFL fusion proteins as molecular adjuvants for DNA and/or viral vector vaccines. Constructs of particular interest included SP-D-OX40L, SP-D-4-1BBL, SP-D-LIGHT, and SP-D-CD70. SP-D-BAFF was surprisingly effective at enhancing T cell responses, despite its inability to enhance anti-Gag antibody secretion.

1. Introduction

Classical vaccination approaches such as protein subunit vaccines have been successful in the control of viral infections by inducing neutralizing antibodies, but research over the past two decades suggest that an alternative strategy will be required to develop an effective HIV vaccine. DNA vaccination has been shown to induce both cellular and humoral responses against various

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antigens and protect animals against subsequent infection with microbial pathogens [1–4]. In addition, DNA vaccination has shown excellent safety profiles in clinical trials for HIV vaccines and other diseases [5–8].

Typically, HIV-1 vaccination strategies have sought to induce one or more of the following immune responses: neutralizing antibodies against a broad range of HIV primary isolates; cytotoxic T cell responses in a vast majority of recipients; and/or strong mucosal immune responses, such as IgA antibody secretion or the induction of memory CD4+ T cells. Though DNA vaccination can enhance the cellular and humoral immune response against an antigen, the response generated is often insufficient to protect from microbial challenge unless the DNA is given as part of a prime/boost vaccine [1,9,10]. One strategy to enhance the effectiveness of DNA vaccines encoding weakly immunogenic antigens is by co-delivering genes encoding molecular adjuvants. Several cytokines and chemokines have been tested successfully for their ability to augment DNA vaccine potency, for example, IL-12, Il-28, GM-CSF, and IL-15 [11–16].

TNF superfamily ligands (TNFSFL) are costimulatory molecules involved in DC and T cell activation and have also been tested as adjuvants to enhance immune responses in several vaccination studies [17–23]. A soluble form of the TNFSFL protein CD40L is currently in phase I/IIa clinical trials for bladder carcinoma and colorectal cancer [24,25]. TNFSFL molecules of particular interest include CD40L, GITRL, RANKL, OX40L, 4-1BBL, LIGHT, CD70, and BAFF [26,27]. CD40L induces effector T cell differentiation [28–30] and also stimulates production of a variety of cytokines, such as dendritic cell production of IL-12 [31]. Its receptor, CD40, is expressed on dendritic cells, B cells, monocytes, activated lymphocytes and endothelial cells [32,33]. GITRL induces expansion of both effector and regulatory CD4+ T cells [34] and its receptor GITR is expressed mainly on lymphocytes [35]. OX40L and 4-1BBL play a role during the late effector and memory stage of antigen-specific T cells [36,37]. Their receptors (OX40 and 4-1BB) are expressed on T lymphocytes and dendritic cells with the majority of expression of 4-1BB on CD8+ T cells [38]. LIGHT and CD70 have been shown to induce early T cell responses and proliferation [39–41]. CD27, the receptor for CD70, is expressed mainly on T cells while the receptors for LIGHT are expressed on T cells, dendritic cells, B cells, and NK cells [42,43]. BAFF plays an important role as a costimulator of B cell proliferation and function [44]. The BAFF receptors BAFFR and TACI are expressed on B cells but also on T cells and dendritic cells [45]. Finally RANKL is critically involved in the function of CD4+CD25+ regulatory T cells [46] and in osteoclast development [47].

Based on previously published data [26,48–52], 4-trimer soluble forms of the TNFSFL proteins CD40L, OX40L, and GITRL are effective as vaccine adjuvants and enhance CD8+ T cell specific immunogenicity. The 4-trimer soluble form of these TNFSFL was achieved by using the scaffold protein Surfactant Protein D (SP-D), a collectin protein that forms a plus-sign-shaped molecule with four trimeric arms, generating a multi-trimeric soluble form of the fusion proteins [26,53–55].

HIV-1 Gag antigen encoded within DNA or viral vector vaccines is known to induce measurable immune responses [56,57]. However, molecular adjuvants could enhance the overall immune response to this antigen, potentially inducing an effective immune response that can prevent infection or control viral pathology. Of particular interest are adjuvants that can enhance T cell correlates of immunity during the memory phase (>6 weeks post vaccination) [58]. Maintaining a population of virus specific memory T cells is crucial to protect against re-infection [59]. The current study aims to evaluate the efficacy of DNA plasmids encoding SP-D-TNFSFL (SP-D-CD40L, SP-D-GITRL, SP-D-CD70, SP-D-BAFF, SP-D-RANKL, SP-D-LIGHT, AND SP-D-4-1BBL) in combination with an HIV-1 Gag

DNA vaccine. We present evidence that a number of these constructs can enhance immune responses compared to the parent vaccine. Enhanced memory immune responses include a significant increase in T cell avidity, IL-2 secretion, and proliferation, factors that have previously been shown to correlate with effective T cell mediated immune control of viral infection [12,60–62].

2. Materials and methods

2.1. DNA plasmids

pscGag encodes a secreted, codon-optimized form of the HIV-1 Gag protein cloned into the pcDNA3.1 expression vector [26,50], fusing the HIV-1 gag coding sequence with the first 21 amino acids of human tissue plasminogen activator (t-PA). Gag protein secretion was confirmed by p24 ELISA assay on supernatant from transfected 293 T cells. Plasmids coding for the 4-trimer soluble form of murine SP-D-CD70, SP-D-GITRL, SP-D-41BBL, SP-D-OX40L, SP-D-BAFF, SP-D-LIGHT, and SP-D-RANKL were generated using pcDNA3.1 as previously described [26]. Constructs were cloned such that mouse SP-D protein from the N-terminus to amino acid sequence ALFPDG was fused directly to the mouse TNFSFL extracellular domain, starting from the N-terminal amino acid sequences RTEPRP (4-1BBL), LSKQQQ (CD70), SLKPTA (GITRL), SSSPAK (OX40L), AFQGPE (BAFF), LHQRLG (LIGHT), and RAQMDP (RANKL).

2.2. Plasmid preparation

Plasmids were propagated in *Escherichia coli* strain TOP10. Highly purified, endotoxin-free DNA plasmid preparations were produced using the Qiagen endofree Giga plasmid kit. The plasmids were further purified using a Triton-X114 purification method as previously described [49]. The plasmid endotoxin level was <0.2 EU/ml as confirmed by LAL endotoxin assay (Lonza Inc.).

2.3. Transient transfections and Western blotting of fusion protein constructs

293 cells (AD-293, Stratagene) were transiently transfected with the plasmid constructs using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, supernatants were centrifuged and filtered. Supernatants were loaded onto sodium-dodecyl sulfate–10% polyacrylamide gels (Bio-Rad), electrophoresed, and blotted onto PVDF membrane (Pierce). The membrane was blocked using 5% (w/v) dry milk and then probed with goat anti-mouse for CD70 or 4-1BBL (R&D Systems), or rat anti-mouse for OX40L, BAFF, LIGHT, or RANKL (R&D Systems), followed by incubation with either anti goat or anti-rat horseradish peroxidase-conjugated antibodies (Jackson Immunoresearch). The protein band was developed onto X-ray film using chemiluminescence. To evaluate SP-D complexes a non-denaturing PAGE was performed on the SP-D-TNFSFL constructs, including SP-D-CD40L. Rat anti-mouse for CD40L (R&D Systems) was used for immunoblotting. Supernatants were run on a 7.5% Tris–HCl polyacrylamide gel in the absence of SDS.

2.4. CD40 in vitro activity assay

A CD40 reporter cell line 293-CD40-SEAP was generated to measure CD40L mediated activation. This 293-derived cell constitutively expresses murine CD40 on its surface, along with the secreted alkaline phosphatase (SEAP) gene under control of an NFκB promoter. Briefly, 80,000 293-CD40-SEAP reporter cells grown in DMEM medium with 10% FBS were plated in each well of a 96-well plate. A total of 100 μl of SP-D-CD40L or pcDNA3.1 transfected 293 T supernatant was added to the reporter cells for

24 h in triplicates at various dilutions. On the next day, 10 μl/well of the supernatants was added to a 96-well assay plate together with 100 μl/well of QUANTI-Blue Alkaline Phosphatase substrate (InvivoGen). Wells were incubated for 20 min at 20 °C and OD was read at 650 nm.

2.5. B cell in vivo proliferation assay

10–12 weeks old C57BL/6 mice were injected with 100 μg pSP-D-BAFF plasmid DNA in a final volume of 100 μl in PBS, given intramuscularly into the hind quadriceps muscle, 50 μg per limb. The pcDNA3.1 empty vector (100 μg) was injected as a negative control. 72–75 h post injection, mice were sacrificed and single cell suspensions were prepared from the spleen and inguinal lymph nodes. RBC Lysis Buffer (Biolegend) was used to deplete RBC from the splenocyte prep, according to manufacturers instructions. Total spenocytes and lymph node cells were enumerated and stained with an antibody directed against B220 (BD Biosciences) to determine the B cell population. Data was acquired on a BD LSR II flow cytometer and analyzed using the FlowJo software package (Tree Star). The absolute numbers of B cells were calculated and data were compared using Student's *t* test. Values of $p \leq 0.05$ were considered statistically significant.

2.6. Mice and immunization schedule

Female BALB/c mice (7–8 week 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). 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.

Immunization Schedule. pscGag was combined with either pcDNA3.1 or each SP-D-TNFSFL adjuvant plasmid and injected intramuscularly in the quadriceps muscle of both hind limbs. Vaccinations were given three times at two-week intervals with 80 μg of Gag plasmid mixed with either 20 μg of pcDNA3.1 or 20 μg of SP-D-TNFSFL adjuvant. Doses were administered in a total volume of 100 μl PBS (50 μl per limb). To ensure that mice do not spontaneously induce an anti-Gag response, control mice were injected with 100 μg of pcDNA3.1.

Splenocyte preparation. Two weeks or seven weeks following the third immunization mice were euthanized and spleens 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).

2.7. In vitro CD4+ T cells proliferation assay

To determine whether T cell proliferation could be induced by SP-D-TNFSFL in vitro, CD4+ T cells were positively selected from naïve splenocytes using anti-mouse CD4 MACS Microbeads (Miltenyi Biotec) following the manufacturers instructions. The isolated mouse CD4+ T cells (2×10^5/well) were cultured in 96-well round bottom plates containing plate-bound anti-CD3 antibody (1 μg/ml) in 100 μl complete R10 medium plus 100 μl of supernatant obtained from 293 cells transfected with pcDNA3.1 plasmid (negative control) or the various SP-D-TNFSFL genes. Soluble anti-CD28 antibody (1 μg/ml) was added as a positive control. The CD4+ T cells were cultured for 72 h at 37 °C in 5% CO$_2$. Proliferative response of the CD4+ T cells was determined by incorporation of [^{3}H]-thymidine. Each well was pulsed with 1 uci [^{3}H]-thymidine for the final 19 h of incubation. Cells were harvested onto fiberglass

filters and radioactivity was measured in a liquid scintillation counter (Wallac Inc.). The results were calculated as cpm (mean ± SD of triplicate cultures). The proliferation of CD4+ T cells induced by supernatant from SP-D-TNFSF constructs was directly compared to proliferation induced by pcDNA3.1.

2.8. Enzyme linked immunospot (ELISPOT) assay

IFN-γ and IL-2 ELISPOT assays were performed to determine antigen specific cytokine secretion from immunized mice splenocytes. ELISPOT assays were carried out per the manufacturer's protocol (R&D Systems) using 96-well MAIP plates (Millipore). Freshly prepared vaccinated mouse splenocytes (5×10^5 cells/well) were added to each well of the plate, and stimulated for 18 h at 37 °C, 5% CO$_2$, in the presence of HIV-1 Gag peptide AMQM-LKETI (10 μg/ml or as described). A c-myc peptide (negative control) and PMA/Ionomycin (positive control) were also included to calculate the number of antigen-specific ELISPOTS. After 18 h, spots were developed with AEC substrate kit (Vector Laboratories), according to manufacturer's instructions. The membrane was read by automated 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. *Avidity Assay.* The number of IFN-γ secreting high avidity CD8+ T cells was determined by stimulating the cells with 1 μg/ml, 1×10^{-3} μg/ml, or 1×10^{-5} μg/ml of Gag peptide (AMQMLKETI) to evaluate the number of T cells able to secrete IFN-γ at low peptide concentrations.

2.9. [^{3}H]-thymidine incorporation proliferation assay

Proliferative responses of T cells isolated from immunized mice two weeks post vaccination were determined using a standard [^{3}H]-thymidine incorporation assay. 100 μl aliquots of the spleen cell suspension (2×10^5 cells/well) were plated into triplicate wells in 96-well round-bottom microtiter plate and stimulated using Gag protein (5 μg/ml), media control or 10 μg/ml Concanavalin A (positive control). The cells were incubated for 72 h before the addition of 1 μCi/well [^{3}H]-thymidine. After 19 h, thymidine incorporation was counted as described above. The proliferative response was calculated as the fold increase in radioactivity with gag antigen over that for cells cultured in the media alone and is represented as the stimulation index (SI) [26]. The response to antigen is considered positive when the SI values were 2 or greater and twice the value for the media control in the same experiment.

2.10. CFSE based CD4+ and CD8+ T cell proliferation assay

A CFSE proliferation assay was used to determine Gag specific proliferation of CD4+ and CD8+ T cells in mice. Splenocytes obtained from immunized mice were suspended at a concentration of 5×10^6 cells/ml in PBS plus 10% FBS. Cells were stained by rapidly mixing equal volume of cell suspension with 10 μM CFSE in PBS with 10% FBS for 5 min at 37 °C. The labeled cells were washed three times with R10 complete medium. The cells were then incubated for 4 days in the presence of Gag protein (5 μg/ml), medium alone (negative control), or Concanavalin A (positive control). On day 5, cells were washed with FACS buffer (composition) and stained with PacBlue-anti CD3, PerCP-anti-CD8, and APCcy7-anti-CD4 (BD Biosciences). Cells were washed and fixed in 200 μl of 1% formalin in PBS. Flow cytometric data were analyzed using an LSRII flow cytometer (BD Biosciences). For analysis, lymphocytes were first gated for CD3+ T cells, then for CD4+/CD8− or CD8+/CD4− populations. Analysis was performed using FlowJo software (Treestar Inc.).

2.11. ELISA assay for anti-Gag IgG responses

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. Mice serum at different dilutions (1:30, 1:120, 1:480 and 1:1920) was added to the plates and incubated at room temperature for 2 h with shaking. The Gag antigen specific IgG, IgG1 and IgG2a antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Jackson Immunoresearch Inc.), respectively. Signal was developed using BluePhos substrate (KPL, Inc.). Plates were analyzed by using a 96-well plate absorbance reader at 650 nm. Endpoint titers were calculated as the highest dilution that was twice the baseline value.

2.12. Statistical analysis

Statistical analysis was evaluated using a two-tailed Student's t test, comparing mice vaccinated with Gag antigen + empty vector to mice vaccinated with Gag antigen + SP-D-TNFSFL construct. In all figures, p values were labeled by asterisks for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Statistical analysis was performed using Graphpad Prism 4.0 software.

3. Results

3.1. Co-stimulation of CD4+ T cells using supernatants of 293 cells transfected with SP-D-TNFSFL

Based on previous studies with recombinant SP-D-CD40L and SP-D-GITRL, we generated DNA plasmids expressing the fusion of SP-D protein with the TNF superfamily ligands OX40L, BAFF, LIGHT, RANKL, CD70, and 4-1BBL. As shown in Fig. 1A, all 293 supernatants generated a band at the expected molecular weight of approximately 55 kDa when Western blotted with their respective anti-TNFSFL antibodies. To confirm that SP-D-TNFSFL are able to form a multi-trimer complex, Western blots were performed with the constructs on a non-denaturing gel in the absence of SDS and DTT. Bands were observed that correspond to SP-D-TNFSFL trimers (~200 kDa) and multi-trimer complexes (>400 kDa).

To assess the in vitro activity of these SP-D-TNFSF constructs, enriched naïve CD4+ T cells from BALB/c mice were cultured with supernatant from the SP-D-TNFSFL constructs for 4 days on anti-CD3 antibody-coated 96-well plates. The proliferation of CD4 T cells was determined by [³H]-thymidine incorporation during the final 19 h of culture (Fig. 1B). The proliferation of CD4+ T cells induced by SP-D-BAFF and SP-D-OX40L supernatants was similar to that of pDNA3.1 supernatant, suggesting these constructs do not stimulate naïve CD4+ T cell proliferation. In contrast, proliferation of CD4 T cells was significantly enhanced by SP-D-CD40L ($p = 0.0022$), SP-D-CD70 ($p = 0.0002$), SP-D-41BBL ($p = 0.0002$), SP-D-RANKL ($p = 0.004$), and SP-D-LIGHT ($p = 0.0005$). Proliferation induced by these constructs was comparable to proliferation induced by the anti-CD28 antibody positive control (Fig. 1B, right hand column).

To assess the ability of SP-D-CD40L to directly stimulate CD40, an SEAP cell line reporter system was used. This assay was performed using a 293-derived reporter cell line engineered to express CD40, the natural counter-receptor of CD40L. The cell line also expressed secreted alkaline phosphatase (SEAP) under the control of an NF-κB promoter. These 293-CD40-SEAP cells express SEAP following CD40 stimulation. We monitored the ability of SP-D-CD40L supernatants to drive reporter gene activity. Empty vector pcDNA3.1 transfected 293 T cells conditioned medium were used as a negative control. As shown in Fig. 1C, SP-D-CD40L increased the level of SEAP enzyme in a dose dependent manner compared to empty vector.

Next we evaluated the in vivo activity of pSP-D-BAFF to confirm that this construct is biologically active. The plasmid construct was injected intramuscularly into C57BL/6 mice and spleen and inguinal lymph nodes were evaluated for any changes in total number of B cells. Recombinant BAFF protein induces NF-κB activation and increases B cell numbers in vivo [63,64]. Consistently, injection of recombinant or pSP-D-BAFF significantly increased total B cell numbers in spleen and lymph node when compared to empty vector (Fig. 1D and data not shown).

3.2. Comparing the adjuvant activity of SP-D-TNFSFL in a HIV-1 Gag vaccine model

SP-D-TNFSFL constructs were evaluated in vivo in a DNA vaccine model using plasmid encoding soluble secreted Gag antigen (pscGag) and plasmid encoding each SP-D-TNFSFL recombinant gene [26]. Mice were vaccinated three times at two-week intervals with an intramuscular injection of 80 µg pscGag plasmid in combination with 20 µg of either pcDNA3.1 or SP-D-TNFSFL plasmid. The vaccination schedule is outlined in Fig. 2A. Two weeks following the third vaccination, T cell responses were analyzed using IFN-γ ELISPOT, stimulating mouse splenocytes with the BALB/c K^d immunodominant Gag peptide AMQMLKETI. This peptide is specifically recognized by CD8+ T cells following MHC I presentation, allowing us to evaluate CD8+ T cell responses alone. As shown in Fig. 2B, we saw a significantly enhanced IFN-γ ELISPOT response when Gag was combined with adjuvants SP-D-CD40L, SP-D-GITRL, SP-D-CD70, SP-D-41BBL, and SP-D-LIGHT compared to Gag vaccination alone. Vaccination with pscGag + pcDNA3.1 induced a moderate IFN-γ response compared to control animals vaccinated with pcDNA3.1 alone.

In addition to IFN-γ ELISPOT we also examined Gag specific T cell proliferation by stimulating vaccinated mouse splenocytes with Gag protein (5 µg/ml) for 4 days. Proliferation of Gag antigen-specific T cells was analyzed by [³H]-thymidine incorporation as detailed in Section 2. Control pcDNA3.1 and pscGag vaccination induced minimal proliferation of T cells (Fig. 2C). In contrast, immunization with pscGag plus SP-D-CD40L ($p < 0.01$) or SP-D-GITRL ($p < 0.05$) induced a significant increase in proliferation of Gag specific T cells. While not statistically significant, the remaining SP-D-TNFSFL constructs showed a trend toward increased proliferation compared to Gag vaccination alone. These data suggest that SP-D-TNFSFL, and specifically CD40L and GITRL constructs, enhance Gag specific T cell proliferation, previously characterized as a potential correlate for the immune control of SIV infection [65].

Next, we determined humoral immune responses for vaccines adjuvanted with SP-D-TNFSFL constructs. As shown in Fig. 2D, adjuvants SP-D-LIGHT, SP-D-CD40L, SP-D-4-1BBL, SP-D-GITRL, and SP-D-CD70 increased anti-Gag antibody titers compared to the pscGag at 1:30 or 1:120 serum dilutions. However, this increase was not statistically significant. Surprisingly, certain adjuvants such as SP-D-OX40L and SP-D-RANKL generated lower antibody responses compared to pscGag alone, but again this did not reach statistical significance. Adjuvants SP-D-LIGHT, SP-D-GITRL, and SP-D-CD40L generated antibody responses at titers as low as 1:2000 and these ELISA values remained above responses from Gag vaccination alone.

3.3. Generation of long-term Gag-specific immune responses with SP-D-TNFSFL adjuvants

While increased short-term immunity (two weeks post vaccination) is beneficial, it will be critical for HIV vaccines to maintain
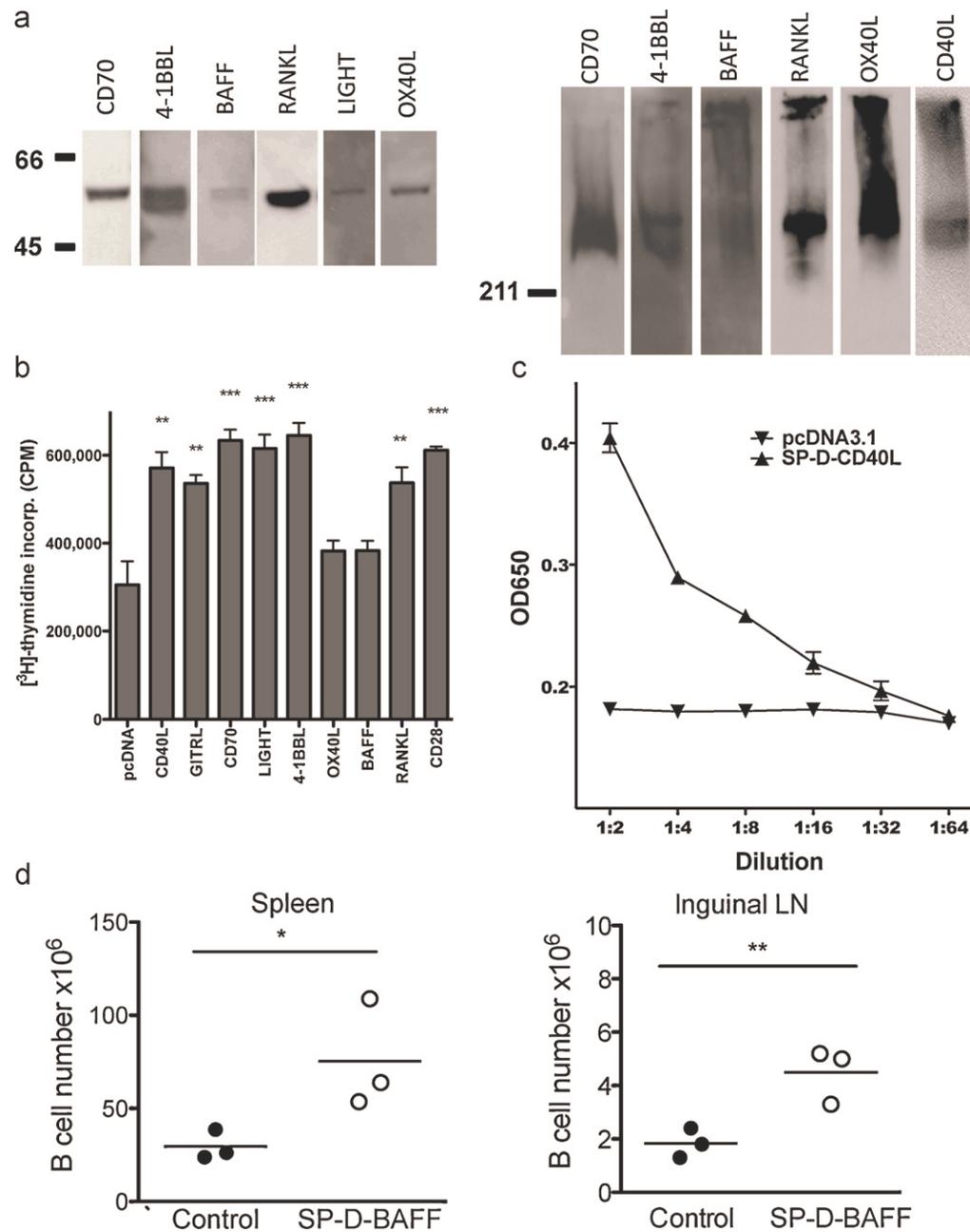
Fig. 1. Expression and activity of murine SP-D-TNFSF ligands in vitro. (A) 293 T cells were transfected with DNA plasmid expression vectors encoding various SP-D-TNFSF ligand fusion proteins. After 48 h culture, supernatant was collected and run on an SDS-PAGE gel in the presence of reducing agent. Western blots were performed using polyclonal antibody to murine OX40L, BAFF, LIGHT, RANKL, CD27L AND 4-1BBL proteins (left panel). To confirm the presence of multi-trimer complexes a Western blot was performed on the SP-D-TNFSFL as above but using non-denaturing PAGE in the absence of reducing agent (right panel). (B) Costimulation of CD4+ T cells with anti-CD3 antibody and supernatant from 293 T cells transfected with the various SP-D-TNFSF ligand plasmids. An equal amount of each SP-D-TNFSFL protein was added based on ELISA assay. Transfected supernatants were mixed with control pcDNA3.1 supernatant so that each well received the same total volume of 293 T supernatant. Representative data from two independent experiments is shown. Error bars represent multiple tests within the same experiment. Supernatant from SP-D-CD40L, SP-D-CD70, SP-D-GITRL, SP-D-LIGHT, SP-D-41BBL, and SP-D-RANKL induced a significant increase in the proliferation of CD4+ T cells. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ by Student's t test compared to pcDNA3.1 transfected 293 T cell supernatant control). (C) In vitro activity of pSP-D-CD40L using an SEAP CD40 reporter system. An equivalent amount of 293 T supernatant from pcDNA3.1 or pSP-D-CD40L was incubated with 293-CD40-SEAP NF-κB reporter cells. Activity was dose dependent in response to dilution of the supernatant. (D) In vivo activity of pSP-D-BAFF. C57BL/6 mice (3 mice per group) were injected intramuscularly with 100 μg of either pcDNA3.1 or pSP-D-BAFF plasmid. SP-D-BAFF significantly increased the total number of B220+ B cells in both spleen and lymph node of animals. *$p < 0.05$, **$p < 0.01$ by Student's t test compared to control mice.

antigen-specific immune activity long-term following vaccination. Therefore we compared SP-D-TNFSFL adjuvants for their ability to enhance immune responses during the memory phase. Animals were vaccinated every two weeks a total of three times. Splenocytes and serum were collected 7 weeks post vaccination. In addition to the assays outlined above, recent publications propose a correlation between CD8+ T cell avidity to a viral or tumor antigen and the control of virally infected cells or tumors [12]. One or more SP-D-TNFSFL adjuvants may increase the number of high avidity Gag-specific T cells, suggestive of enhanced control of HIV infection. In addition, it will be critical to maintain these high avidity T cells long-term as memory cells in order
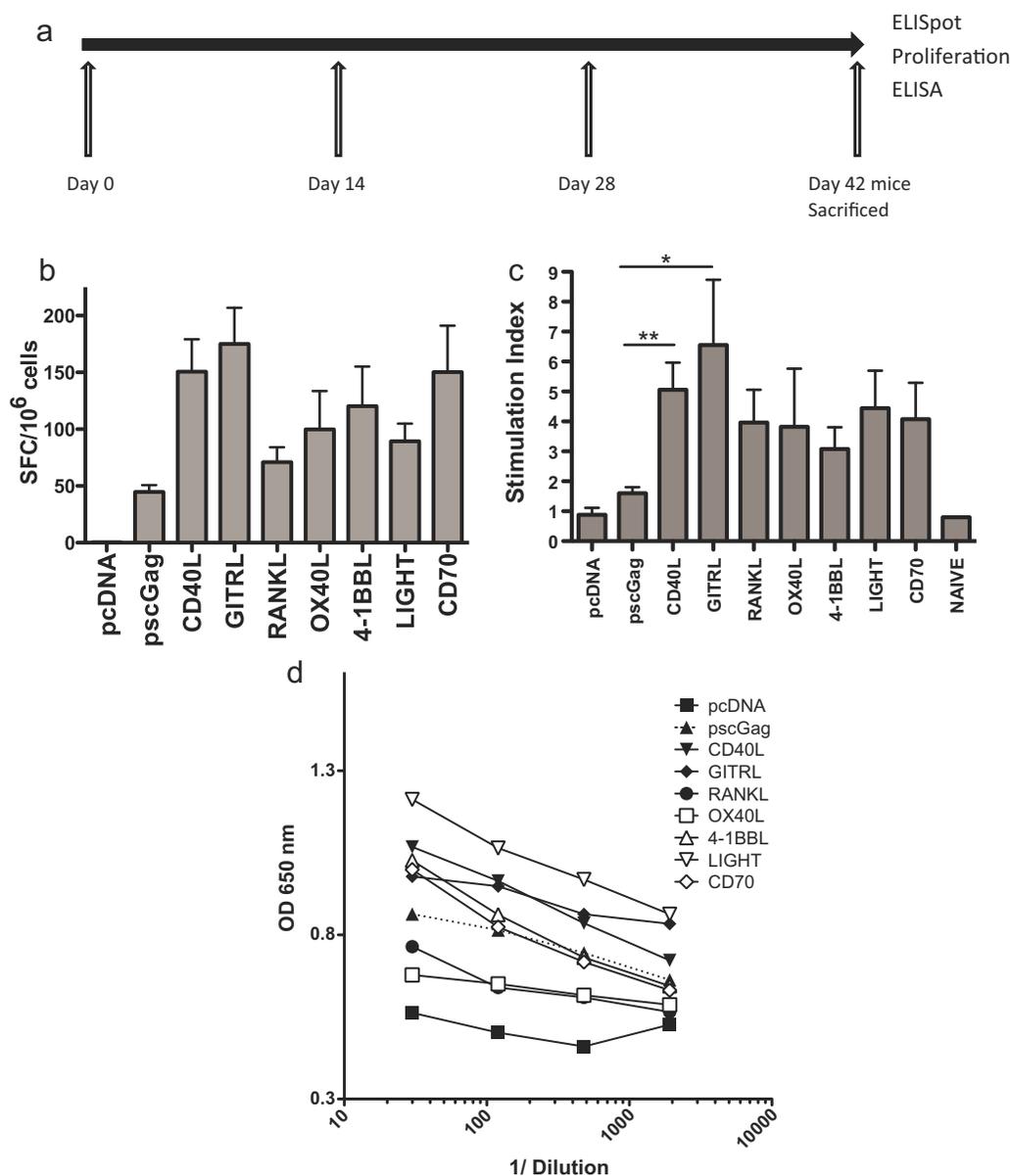
Fig. 2. Comparison of in vivo immune responses to SP-D-TNFSFL adjuvants. (A) Immunization schedule. BALB/c mice (5 per group) were immunized intramuscularly with pscGag plasmid either alone or in combination with SP-D-TNFSFL adjuvants on days 0, 14, and 28. A total of 100 µg of plasmid was injected (80 µg pscGag + 20 µg SP-D-TNFSFL or pcDNA3.1), 50 µg into each quadriceps muscle. 2 weeks later mice were sacrificed and splenocytes analyzed for Gag specific immune responses. (B) IFN-γ ELISPOT assay to measure Gag specific CD8+ T cell responses. Splenocytes were collected and cultured for 18 h in the presence of 10 µg/ml HIV-1 Gag CD8+ specific peptide AMQMLKETI. The adjuvants SP-D-CD40L, SP-D-GITRL, SP-D-4-1BBL, SP-D-LIGHT, and SP-D-CD70 significantly increased Gag-specific IFN-γ secretion compared to pscGag + pcDNA3.1 vaccination. (C) Induction of Gag-specific proliferation. Splenocytes were cultured for 5 days in the presence of Gag protein. Cultures were pulsed with [^{3}H]-thymidine overnight before cell harvesting onto glass filters and scintillation counting to calculate stimulation index. The adjuvants SP-D-CD40L and SP-D-GITRL induced a significant increase in stimulation index compared to pscGag + pcDNA3.1. (D) IgG antibody responses to Gag DNA vaccination. Total IgG specific for Gag was measured by ELISA assay from mouse serum collected on day 42. The adjuvant SP-D-LIGHT showed increased absorbance compared to pscGag + pcDNA3.1, but did not reach statistical significance. Adjuvants SP-D-OX40L and SP-D-RANKL reduced total IgG levels. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to pscGag + pcDNA3.1 vaccination.

to generate an effective immune response soon after viral challenge.

To determine if high avidity CD8+ T cells are present and can be maintained long-term post vaccination, mice were vaccinated with pscGag vaccine alone or in combination with SP-D-TNFSFL adjuvants. Splenocytes were evaluated 7 weeks following the third vaccination (Fig. 3A). We analyzed CD8 T cells avidity using the CD8+ T cell specific Gag peptide AMQMLKETI, directly comparing the number of IFN-γ secreting CD8+ T cells produced by different combinations of Gag vaccine plus SP-D-TNFSFL adjuvant (Fig. 3B). Two separate experiments were performed to evaluate constructs

SP-D-CD40L, SP-D-LIGHT, SP-D-RANKL, SP-D-OX40L, SP-D-CD70, SP-D-4-1BBL, and SP-D-BAFF. As shown in Fig. 3B, all adjuvants induced a significant increase in IFN-γ ELISPOTs 7 weeks post vaccination when stimulated with gag peptide AMQMLKETI at the concentration of 1 µg/ml. When peptide was added at a lower concentration of 1 ng/ml, only adjuvants SP-D-CD40L, SP-D-LIGHT, SP-D-OX40L, and SP-D-4-1BBL still significantly increased the number of IFN-γ ELISPOTS when compared to pscGag alone. Together these data showed that at 7 weeks post-vaccination SP-D-TNFSFL can adjuvant HIV Gag vaccines and are able to maintain HIV-Gag specific immune responses even at low peptide concentrations,
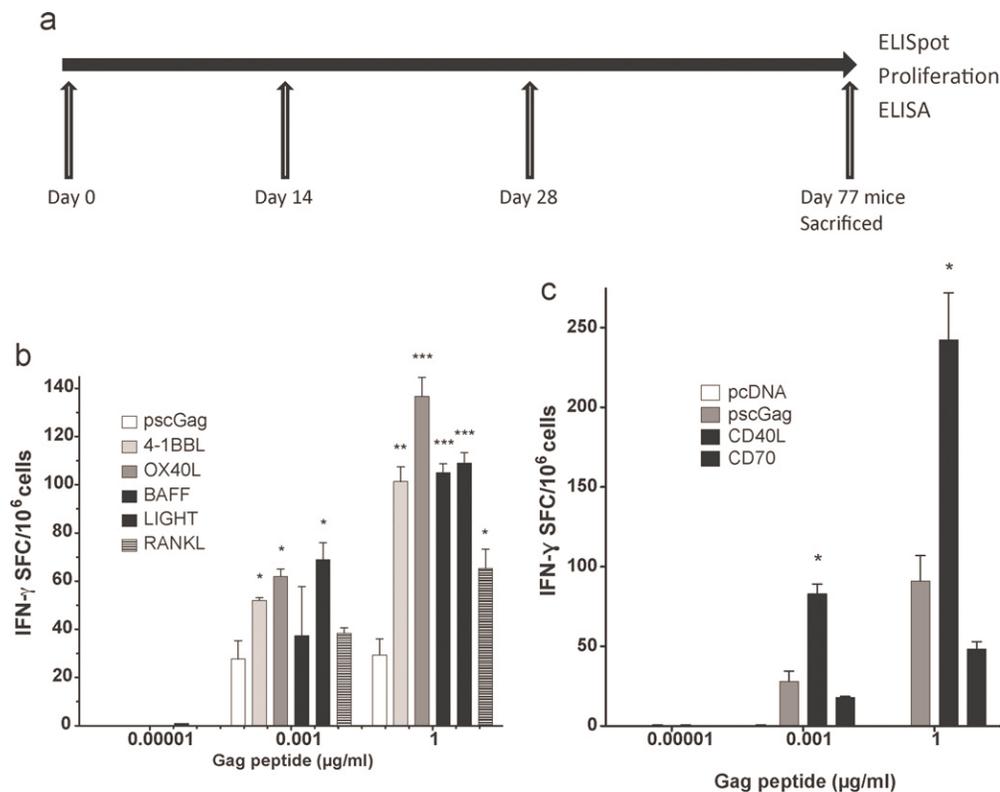
Fig. 3. Adjuvants SP-D-4-1BBL, SP-D-OX40L, SP-D-LIGHT, and SP-D-CD40L enhance memory CD8+ T cell avidity to Gag. (A) Immunization schedule. BALB/c mice were immunized intramuscularly with pscGag plasmid either with pcDNA3.1 or SP-D-TNFSFL adjuvants on day 0, 14 and 28. Mice were sacrificed on day 77 and splenocytes analyzed for Gag-specific memory T cell responses. (B) and (C) Peptide dilution ELISPOT assay. Splenocytes were cultured with serial dilutions of CD8+ T cell specific peptide AMQMLKETI for 18 h. At 1 μg/ml plasmid, all adjuvants with the exception of SP-D-CD70 induced a greater IFN-γ ELISPOT response compared to pscGag. Adjuvants SP-D-4-1BBL, SP-D-OX40L, SP-D-LIGHT, and SP-D-CD40L also induced a greater ELISPOT response at 1 ng/ml peptide compared to pscGag + pcDNA3.1. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared to pscGag vaccination.

suggesting they enhance CD8+ T cell avidity during the memory phase.

3.4. Generation of Gag-specific IL-2 secreting T cells

Studies have shown that IL-2 secreting CD8+ T cells can proliferate in a CD4-independent manner [66]. In addition, evaluation of polyfunctional T cell responses suggests that the size of the IL-2+ T cell population (whether single IL-2 secreting or multiple cytokine secreting) correlates with the overall polyfunctional T cell response and viral control [60,67]. We observed in previously published polyfunctional flow analysis of HIV vaccines that polyfunctional T cells tend to secrete IL-2, while cells that secrete one or two cytokines do not secrete IL-2, irrespective of the other cytokines secreted. These observations suggest that we can use a standard single-color IL-2 ELISPOT assay as a marker of polyfunctionality. We initially performed an IL-2 ELISPOT by stimulating the splenocytes using Gag 9-mer peptide (AMQMLKETI) at 10 μg/ml for 18 h (Fig. 4A and B). SP-D-OX40L ($p < 0.005$), SP-D-BAFF ($p < 0.0005$), SP-D-CD40L ($p < 0.003$), and SP-D-LIGHT ($p < 0.001$) significantly increased the IL-2 ELISPOT responses of Gag specific CD8 T cells, while SP-D-41BBL and SP-D-RANKL were unable to significantly increase IL-2 ELISPOT responses when compare to Gag vaccination alone. Overall, IL-2 secretion by CD8+ T cells was relatively low compared to IFN-γ secretion (Compare Figs. 3C and 4A & B). We also performed an IL-2 ELISPOT assay using whole Gag protein (2.5 μg/ml recombinant p55 Gag) in order to induce both CD4+ and CD8+ T cell IL-2 secretion (Fig. 4C and D). Adjuvants SP-D-OX40L ($p < 0.05$), SP-D-BAFF ($p < 0.05$), SP-D-LIGHT ($p < 0.001$), and

SP-D-RANKL ($p < 0.01$) all significantly increased IL-2 ELISPOT responses. In contrast, there was no difference in IL-2 ELISPOT results for SP-D-CD40L, SP-D-CD70, and SP-D-4-1BBL compared to Gag vaccine alone.

3.5. Expression of TNFSF ligands increases Gag-specific CD4 and CD8 T cell proliferation in mice

Ideally a molecular adjuvant for HIV vaccination should induce a strong T cell proliferative response, given the relationship between T cell proliferative capacity and protection from viral challenge [68]. To test whether SP-D-TNFSFL adjuvants enhance the proliferation of Gag specific memory CD4+ and CD8+ T cells, we performed a CFSE-based proliferation assays on splenocytes collected 7 weeks post-vaccination in the presence of 5 μg/ml Gag antigen. Cells were incubated for 5 days prior to FACS analysis. As shown in Fig. 5A, mice immunized with pcDNA3.1 or Gag vaccine alone induced proliferation of approximately 5–10% of CD4+ T cells. However, the combination of Gag vaccine with a number of SP-D-TNFSFL adjuvants increased the proportion of proliferating Gag antigen-specific T cells. Proliferation of CD4+ T cells was significantly increased with adjuvants SP-D-4-1BBL ($p < 0.01$), SP-D-OX40L ($p < 0.01$), SP-D-BAFF ($p < 0.05$) and SP-D-LIGHT ($p < 0.01$) in the presence of Gag protein (Fig. 5A). In contrast, SP-D-CD70, SP-D-CD40L and SP-D-RANKL did not induce a significant increase in CD4 T cell proliferation compared to Gag antigen alone. Interestingly, SP-D-4-1BBL ($p < 0.05$), SP-D-OX40L ($p < 0.01$) and SP-D-BAFF ($p < 0.05$) also significantly enhanced proliferation of Gag antigen-specific CD8+ T cells (Fig. 5B). Taken together, these data demonstrate that TNFSFL,
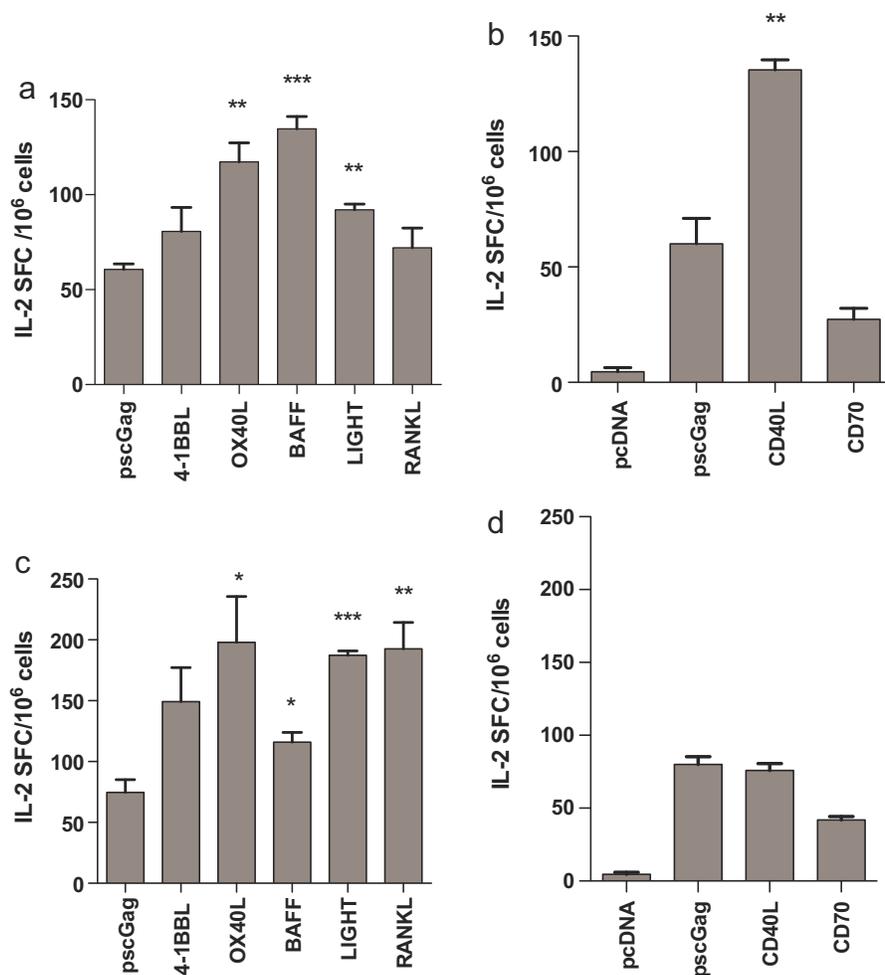
Fig. 4. Gag-specific IL-2 secretion by memory T cells. (A) ELISPOT assay measuring the number of Gag specific CD8+ T cells secreting IL-2 in response to AMQMLKETI peptide. BALB/c mice ($n = 5$) were immunized with HIV-1 Gag with SP-D-TNFSFL plasmids or empty vector pcDNA3.1, as indicated. 7 weeks after the third vaccination, mice splenocytes from each group were pooled and assayed in triplicate. SP-D-OX40L, SP-D-BAFF, and SP-D-LIGHT induced a significant increase in CD8+ T cell IL-2 secretion. (B) ELISPOT assay for SP-D-CD40L and SP-D-CD70 adjuvants. Construct SP-D-CD40L increased IL-2 secretion from CD8+ T cells. (C) IL-2 secretion during incubation of splenocytes with whole Gag protein. To evaluate both CD8+ and CD4+ T cell responses, splenocytes were incubated with Gag p55 protein. SP-D-LIGHT and SP-D-RANKL displayed enhanced IL-2 secretion using protein for stimulation. (D) Whole Gag ELISPOT for SP-D-CD40L and SP-D-CD70. These two adjuvants did not increase IL-2 secretion when using Gag protein to at both CD4+ and CD8+ T cell responses. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared to pscGag + pcDNA3.1 vaccination.

when used as a soluble multimeric adjuvant, can enhance antigen specific proliferation of 7 week memory T cells in a HIV vaccine mouse model.

3.6. Anti-Gag antibody responses in mice immunized with pscGAG plus SP-D-TNFSF ligands

Ideally a DNA-based HIV vaccine should enhance both cellular and humoral immune responses to protect against viral challenge. To evaluate humoral immune responses, we tested the effect of SP-D-TNFSFL on Gag specific antibody titers in mice serum, as measured by ELISA assay. Seven weeks after the third DNA vaccination, serum from mice was collected and tested for various subtypes of IgG, including IgG1 and IgG2a. The SP-D-TNFSFL adjuvants had various effects on antibody titer. As shown in Fig. 6A, immunization of mice with pscGag in combination with SP-D-OX40L and SP-D-BAFF induced higher Gag-specific IgG responses at 1:30 and 1:120 dilutions compare to Gag vaccination alone. We also determined specific titers of IgG1 and IgG2a. SP-D-OX40L and SP-D-CD70 enhanced IgG2a antibody titers at 1:30 and 1:120 dilutions, suggesting a T_H1 type response (Fig. 6B). No anti-Gag antibodies were detected in mice immunized with pcDNA3.1 alone.

4. Discussion

Previous studies have shown that fusions of SP-D with the TNF superfamily ligands CD40L and GITRL can enhance DNA vaccines in mouse models [26,48,49]. Similarly fusions of SP-D with OX40L or CD40L can enhance canarypox viral vector vaccines [51,52]. The present study confirms the immune activating capacity of these fusion constructs. Initially we determined the effect of SP-D-TNFSFL co-stimulatory activity in vitro by studying their proliferation induction capability. Supernatants were collected from 293 cells transfected with different SP-D-TNFSF plasmids. The Western blot data clearly shows that the SP-D-TNFSFL were expressed and secreted into the culture supernatant and can form multimeric complexes as shown by Western blot. The induction of T cell activation requires both antigen and co-stimulatory molecules. Supernatants of SP-D-TNFSFL, as co-stimulators, induced proliferation of CD4 T cells in the presence of anti-CD3 antibody in vitro, suggesting these constructs are immunologically active. This T cell proliferation induction was similar to that of anti-CD28 plus anti-CD3 stimulation, suggesting these SP-D-TNFSFL constructs are potent T cell co-stimulators. The result of these in vitro proliferation assays suggest that SP-D-TNFSFL can be used to co-stimulate
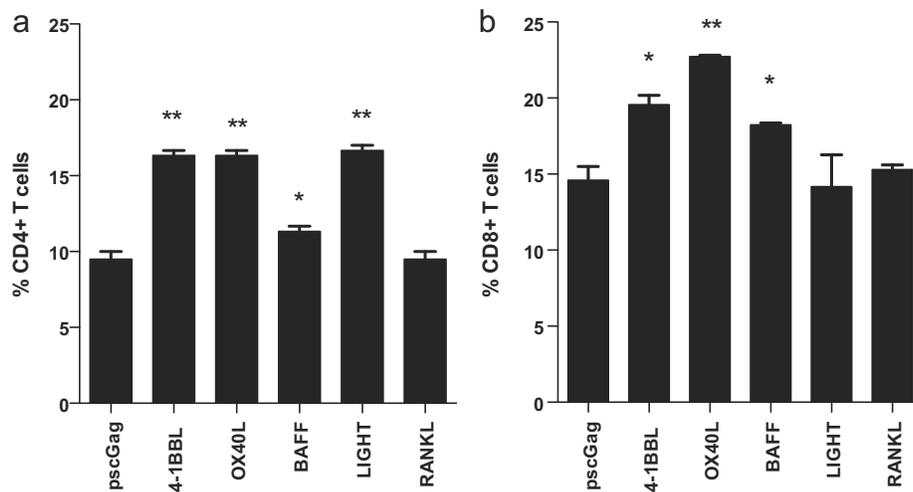
Fig. 5. CD4+ and CD8+ T cell memory proliferation responses induced by SP-D-TNFSFL adjuvants. BALB/c mice ($n = 5$) were immunized with HIV-1 Gag plus empty vector pcDNA3.1 or SP-D-TNFSFL plasmids, as indicated. 7 weeks after the third vaccination, mice splenocytes from each group were pooled, labeled with CFSE, and cultured with p55 Gag protein for 5 days. Cells were fluorescently labeled with anti-mouse CD3, CD4, and CD8 antibodies to differentiate CD4+ and CD8+ T cell populations. Percent proliferation was measured as the ratio of total CFSE^{low} cells to non-proliferating CFSE^{hi} cells. Samples were pooled and assayed in triplicate. (A) Significant CD4+ T cell proliferation was observed in the presence of SP-D-4-1BBL, SP-D-OX40L, SP-D-BAFF, and SP-D-LIGHT when compared to pscGag + pcDNA3.1. (B) CD8+ T cell proliferation was induced by SP-D-4-1BBL, SP-D-OX40L, and SP-D-BAFF. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to pscGag + pcDNA3.1 vaccination.

the proliferation of T cells in the presence of HIV antigen. Two constructs were unable to induce statistically significant CD4+ T cell proliferation: SP-D-BAFF and SP-D-OX40L. BAFF induces the proliferation of T cell indirectly by activating B-Cells [69]. Since B cells were not present in this assay, proliferation was not influenced by SP-D-BAFF. SP-D-BAFF activity was confirmed by an increase in the number of total B cells in the spleen and lymph node of mice treated with pSP-D-BAFF plasmid. Construct SP-D-CD40L activity was confirmed using a CD40 mediated NF-κB reporter system. OX40 expression is low on naïve T cells and is induced following the primary immune response [43,70]. Therefore we would not expect OX40 expression on the naïve CD4+ T cells used in this assay. Instead, SP-D-OX40L would be expected to enhance the secondary immune response and immunological memory, as was observed in our 7 week post vaccination study. A critical question is whether

human versions of these constructs also show activity in vitro. Biochemical analysis of human SP-D-CD40L has shown that this construct is functional (Stone and Kornbluth, unpublished data). In addition, we have previously explored the activity of non-human primate versions of SP-D-CD40L and SP-D-GITRL, both of which are functional in vitro [50].

To determine the in vivo effect of SP-D-TNFSFL as adjuvants in a Gag DNA vaccine we evaluated CD8+ T cell IFN-γ secretion, proliferation, and antibody secretion two weeks following three DNA vaccinations. Secretion of IFN-γ is a standard method to evaluate antigen specific CD8+ T cell responses. As shown previously [26,50], SP-D-CD40L and SP-D-GITRL induced a robust CD8+ T cell response. At this timepoint two weeks post-vaccination other SP-D-TNFSFL enhanced IFN-γ responses, but were not shown to be superior to SP-D-CD40L. Adjuvant SP-D-RANKL was not particularly effective,
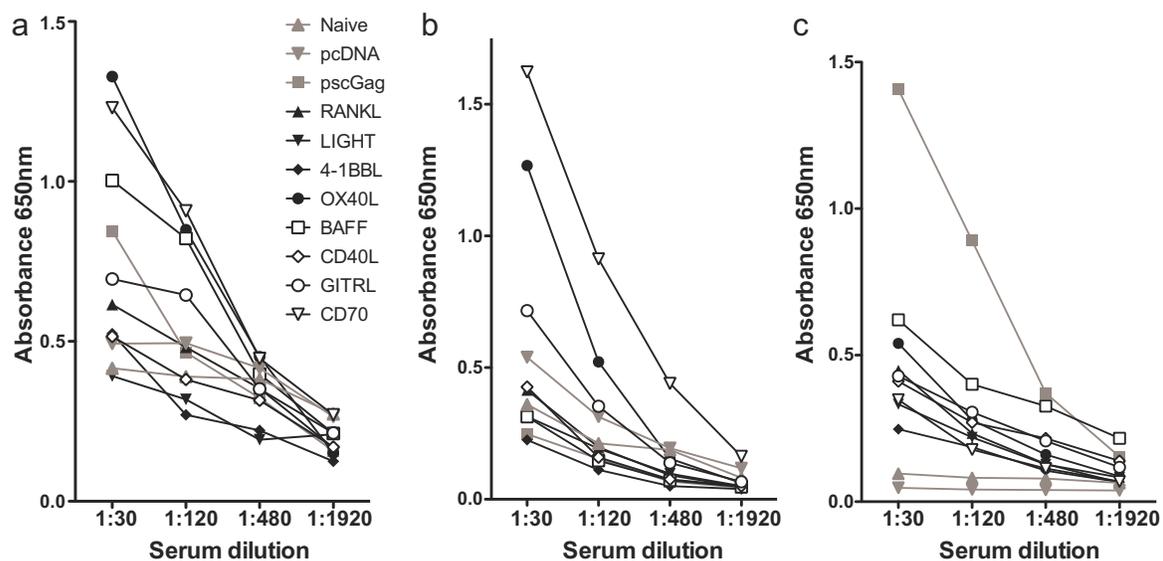

Fig. 6. SP-D-TNFSFL adjuvants increase IgG2a antibody responses to Gag. Mice were immunized with HIV-1 Gag alone or combination of SP-D-TNFSFL plasmids, as indicated. 7 weeks after the third vaccination, mice were bled and serum from each group was serially diluted and assayed for IgG binding to p55 Gag antigen. Total IgG, IgG1, and IgG2a titers were measured by ELISA. (A) Total IgG response. (B) IgG2a response. (C) IgG1 response. SP-D-CD70, SP-D-OX40L, and SP-D-BAFF enhanced total IgG binding at 1:30 and 1:120 dilutions. SP-D-CD70 and SP-D-OX40L enhanced IgG2a binding at titers as low as 1:480.

which is surprising given the expression of RANK on dendritic cells and the similarity in TRAF mediated signaling between CD40 and RANK [71]. Similar to IFN-γ secretion, T cell proliferation is a crucial parameter to measure the recall response after viral challenge. Mice vaccinated with Gag antigen and SP-D-CD40L or SP-D-GITRL adjuvants enhanced lymphocyte proliferation at a significantly greater level than mice vaccinated with Gag antigen alone. While the other SP-D-TNFSFL adjuvants induced a trend toward higher proliferation, they were unable to show a significant change. Given the role of CD4+ T cells in inducing antibody responses [72,73], TNFSFL such as 4-1BBL, OX40L, CD70 and LIGHT would be expected to enhance humoral responses. Surprisingly, ELISA assays for Gag antigen specific IgG indicate that while mice vaccinated with SP-D-TNFSFL constructs encoding LIGHT, CD40L, GITRL enhanced IgG antibody secretion, constructs encoding 4-1BBL, OX40L, and CD70 did not. However, our subsequent experiment (Fig. 6) suggests that OX40L and CD70 shift the humoral response to IgG2a (T_H1) rather than increasing the overall IgG levels. Given the role of T_H1 responses in the control of viral infection, this data suggests that adjuvants such as SP-D-OX40L and SP-D-CD70 may be particularly effective at inducing high titer T_H1 humoral immune responses if used in a DNA prime/protein boost or DNA prime/viral vector boost, where the boost will be able to increase the overall antibody titer. Overall, the results obtained for IFN-γ ELISPOT, proliferation and IgG antibody secretion demonstrate that particular SP-D-TNFSFL can enhance one or more cellular and humoral immune responses. While none appeared to be superior to SP-D-CD40L or SP-D-GITRL, they each displayed unique characteristics that may be particularly effective for control of HIV infection. For this reason studies are underway in our laboratory to determine if there is synergy when two or more SP-D-TNFSFL constructs are used in combination.

Given that SP-D-TNFSFL enhanced cellular and humoral responses 2 weeks after the final immunization, we next evaluated memory immune responses in vaccinated mice 7 weeks after the third vaccination. First we analyzed the activation of CD8+ T cells specific for 9-mer gag peptide using IFN-γ ELISPOT analysis. Recent evidence suggests that higher avidity CD8+ T cells correlate with antiviral immunity, so a peptide dilution assay was performed. As shown in the results, certain combinations of SP-D-TNFSFL with Gag vaccine significantly enhanced IFN-γ secretion of CD8+ T cells in the presence of Gag antigen at concentrations as low as 1 ng/ml. The avidity of CD8+ T cells specific for Gag antigen is significantly increased when the mice were vaccinated in the presence of adjuvants SP-D-4-1BBL, SP-D-OX40L, SP-D-LIGHT, and SP-D-CD40L. Data for SP-D-LIGHT is particularly intriguing, as LIGHT has not previously been characterized as a HIV vaccine adjuvant. Studies have shown that LIGHT expression within the tumor environment can eradicate established tumors [74,75], highlighting the potential of this adjuvant to enhance anti-HIV immunity. Our data suggests that one of the mechanisms of LIGHT-enhanced immunity in vivo may be to increase the avidity of antigen-specific CD8+ T cells.

Next, we looked at IL-2 secretion by CD8+ T cells (using 9 mer Gag peptide) or both CD4+ and CD8+ T cells (using whole Gag protein). Mice vaccinated with SP-D-CD40L enhanced IL-2 secretion of Gag specific CD8+ T cells. ELISPOT assays with total Gag protein showed that SP-D-OX40L, SP-D-BAFF, SP-D-RANKL and SP-D-LIGHT enhanced IL-2 secreting lymphocytes, but SP-D-CD40L did not show a difference in IL-2 secretion compared to Gag vaccine alone. These data are more or less consistent with the avidity data shown in Fig. 3. SP-D-CD40L, SP-D-OX40L, and SP-D-LIGHT enhanced both avidity and IL-2 secretion. Other adjuvants such as SP-D-4-1BBL enhanced avidity, but do not show a significant increase in IL-2 secretion from T cells, despite a trend toward enhanced IL-2 secretion using either CD8+ T cell specific peptide or whole protein. Surprisingly,

SP-D-CD70, which significantly enhanced CD8+ T cell IFN-γ responses two weeks post vaccination, was unable to enhance T cell mediated immune responses at 7 weeks post vaccination. Gag protein IL-2 ELISPOT numbers reflect both CD8+ and CD4+ T cell responses. Given that SP-D-RANKL showed enhanced IL-2 ELISPOT data with Gag protein but not Gag 9-mer peptide, this highlights the potential role of CD4+ T cells in RANKL mediated immune induction. As detailed in previous published data [26,48–50], CD40L enhanced the CD8+ T cell response, but it failed to increase the ELISPOT count when whole Gag protein was used to induce both CD4+ and CD8+ T cells. This has implications for HIV vaccines where selective induction of CD8+ but not CD4+ T cell responses may be desirable [76,77]. As we have shown in previous studies, SP-D-CD40L appears to be particularly effective at inducing CD8+ T cell responses without a simultaneous induction of antigen-specific CD4+ T cells.

The proliferation of T cells by CFSE labeling showed that CD4+ and CD8+ T cell proliferation was enhanced when mice were vaccinated with DNA encoding SP-D-41BBL, SP-D-OX40L, SP-D-BAFF and SP-D-LIGHT. Again, this is consistent with the ability of these costimulators to enhance T cell immune responses. Given the correlation between avidity, proliferation, and IL-2 secretion with T cell mediated immunity to viral infection, these results suggest that SP-D-OX40L and SP-D-LIGHT are particularly promising adjuvants, while SP-D-4-1BBL and SP-D-BAFF may also be effective at inducing T cell responses, but have limitations in enhancing all aspects of the immune response.

In addition to cellular responses, we also looked for antibody response induced by the SP-D-TNFSFL adjuvants, looking at gag specific total IgG, IgG1 (T_H2 mediated) and IgG2a (T_H1 mediated) titers by ELISA. Mice given SP-D-CD70, SP-D-OX40L, and SP-D-BAFF adjuvants enhanced total IgG antibody responses at titers as low as 1:480 dilution. The ELISA data for IgG2a indicates that SP-D-OX40L enhances anti-Gag IgG2a secretion, whereas SP-D-CD40L, SP-D-GITRL, and SP-D-BAFF enhanced IgG1 secretion, a T_H2 mediated immune response. Surprisingly, SP-D-CD70, which failed to show enhanced cellular responses in ELISPOT and proliferation assays, clearly showed an increase in both IgG1 and IgG2a anti-Gag antibody titers, suggesting that SP-D-CD70 enhances both T_H1 and T_H2 responses against Gag antigen. In addition, all SP-D-TNFSFL adjuvants tested appeared to reduce IgG1 antibody responses (Fig. 6C), suggesting that these SP-D-TNFSFL adjuvants induce a T_H1 biased response.

In conclusion, this study demonstrates that particular TNF superfamily ligands fused to the scaffold protein SP-D can enhance the immune response to HIV antigens when combined with secreted Gag antigen in a DNA vaccine. SP-D-TNFSFL enhanced cellular responses by increasing both IL-2 and IFN-γ secretion of CD8 T cells. Certain SP-D-TNFSFL also enhanced proliferation of lymphocytes. Finally, certain SP-D-TNFSFL enhanced anti-Gag antibody secretion. Maintaining long-term memory responses to HIV antigens is crucial to protect against HIV infection. Our study shows that SP-D-TNFSFL can maintain T cell responses to HIV-1 up to 7 weeks following vaccination, providing initial evidence that one or more of these adjuvants can enhance the memory T cell pool following DNA vaccination. Comparing data from 2 week and 7 week experiments SP-D-OX40L generated a significant T cell response and IgG2a antibody response at 7 weeks when compared to 2 weeks, possibly reflecting the role of OX40 stimulation in T cell memory development REF. In contrast SP-D-CD70 enhances the T cell response at 2 weeks post vaccination, but T cell responses are no longer significant by 7 weeks post vaccination, suggesting that the SP-D-CD70 adjuvant may not be particularly effective at inducing a memory response.

Overall these data suggest that particular SP-D-TNFSFL adjuvants may be uniquely effective for particular vaccine strategies. For example, SP-D fusions with CD40L, GITRL, 4-1BBL, OX40L, BAFF, and

LIGHT may be particularly effective as T cell vaccines, while OX40L and CD70 constructs are the most effective at inducing antibody responses at 7 weeks post vaccination. Particular combinations may also be effective. For example, recent studies suggest that combinations of 4-1BBL, GITRL, CD70, and CD40L are effective at enhancing dendritic cell vaccines when used in pairwise combinations [78]. Future studies will explore combinations of SP-D-TNFSFL constructs to determine if similar combinations are effective in this context. In summary, this family of molecular adjuvants provides a new series of reagents for evaluation in gene-based vaccines against HIV and other diseases.

Acknowledgements

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