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Incorporation of CD40 Ligand into SHIV Virus-Like Particles (VLP) Enhances SHIV-VLP-Induced Dendritic Cell Activation and Boosts Immune Responses against HIV

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Abstract

Engagement of CD40 with CD40L induces dendritic cell (DC) maturation and activation, thereby promoting immune responses. The objective of this study was to investigate whether immunization with chimeric CD40L/SHIV virus-like particles (CD40L/SHIV-VLP) could enhance immune responses to SIV Gag and HIV Env proteins by directly activating DCs. We found that CD83, CD40, and CD86 were significantly up-regulated and significantly increased cytokines production were observed after hCD40L/SHIV-VLP treatment in human CD14⁺ monocyte-derived DCs as compared to SHIV-VLP treatment. Mice immunized with mCD40L/SHIV-VLP showed more than a two-fold increase in HIV Env-specific IgG antibody production, an increase in SIV Gag and HIV Env-specific IFN- γ and IL-4 producing cells, and an increase in HIV Env-specific cytotoxic activity compared to that in SHIV-VLP immunized mice. Furthermore, multifunctional CD4⁺ Th1 cells, which simultaneously produce IFN- γ , IL-2 and TNF- α triple cytokines, and CD8⁺ T-cells, which produce IFN- γ were elevated in the mCD40L/SHIV-VLP immunized group. These data demonstrate that chimeric CD40L/SHIV-VLP potently induce DC activation and enhance the magnitude of both humoral and cellular immune responses to the SIV Gag and HIV Env proteins in the mouse model. Therefore, incorporation of CD40L into VLP may represent a novel strategy to develop effective HIV vaccines.

1. Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) for initiating immune responses. DCs efficiently capture antigens at the site of invasion and subsequently migrate to lymphoid organs for the optimal presentation of the invading pathogen components [1]. Upon antigen encounter, DCs exhibit an increase in the cell surface expression of a broad range of co-stimulatory molecules critical for T-cell activation such as CD80, CD86, CD40, OX40-L and CD137 [2–4]; correlating with phenotypic maturation. DCs produce massive amounts of proinflammatory cytokines such as IL-1, IL-6, and IL-12

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[5–7]. Most importantly, efficient presentation of antigen peptides in conjunction with both major histocompatibility complex (MHC) class I and II [8] renders DCs capable of initiating T-cell activation. However, DCs at different stages of differentiation may exhibit various immunoregulatory functions that lead to different immunological responses such as stimulation or suppression of Th1/Th2 and Th17 cytokine induction [9–11]. Therefore, vaccine candidates that could efficiently activate DCs may represent an especially potent vaccine strategy.

DC maturation and activation can be induced by many factors, such as the bacterial component lipopolysaccharide (LPS), the inflammatory cytokine TNF- α , or receptor-mediated events [2,12–14]. Among those, the TNF receptor/TNF superfamily members CD40 and CD40L were reported to play a critical role in DC maturation [15–17] as well as cytotoxic T lymphocyte (CTL) priming [12,18]. CD40L, also known as CD154, is a transmembrane protein expressed on activated T-helper cells. The interaction of CD40L with CD40 on DCs promotes DC activation [18]. For example, DCs treated with soluble trimeric CD40L plus IFN- γ induced a potent T-cell proliferation to CASTA, a soluble protein from *C. albicans*, thereby increasing both antigen-specific lysis and the yield of antigen-specific IFN- γ -producing T cells [19]. Since the CD40-CD40L interaction can deliver DC maturation signals [20], T-cell-mediated maturation of DCs can be mimicked by artificial CD40 triggering through anti-CD40 antibodies [21], CD40L transfection-mediated expression [15], or soluble human CD40L-trimer molecules [19,22].

We previously reported that SHIV-VLP could bind and activate DCs [23] as well as induce humoral and cellular immune responses against HIV Env protein in mouse models [24,25]. Other reports also have shown that VLP alone were inefficient in inducing CTL responses, but could represent a very powerful vaccine strategy if applied together with substances that activate APCs, such as CpGs or anti-CD40 antibodies [26]. The CD40/CD40L interaction plays an important role in the therapeutic treatment of AIDS patients. Recent reports have shown that HIV functional envelope glycoproteins exposed on CD4⁺ T-cells fail to provide activation signals to autologous DCs, but this failure of HIV-exposed CD4⁺ T-cells to activate DCs can be reversed by restoration of the CD40/CD40L interaction [27]. Indeed, the CD40/CD40L interaction is essential for DC activation during bacterial or viral infection [28]. In addition, secretion of high levels of cytokines by DCs requires a second signal from T-cells that may be replaced by CD40L incorporated onto SHIV-VLP. In this study, we produced chimeric CD40L/SHIV-VLP in which CD40L was incorporated into the surface of SHIV-VLP, and studied the effects of the particulate chimeric SHIV-VLP on DC activation. Given the potential advantage of incorporating HIV Env antigen and the APC activation signaling molecule CD40L in the same particle, we hypothesized that CD40L/SHIV-VLP could augment the effectiveness of DC phenotypic and functional activation, which should enhance the humoral and cellular immune responses against HIV Env and SIV Gag *in vivo*; this strategy could lead to the development of chimeric CD40L/SHIV-VLP as a potent HIV vaccine candidate. Therefore, we investigated the immunomodulatory effect of chimeric hCD40L/SHIV-VLP on DC maturation, activation and antigen presentation *in vitro*. In addition, we also measured the production of anti-HIV Env antibodies and generation of SIV Gag or HIV Env-specific IFN- γ and IL-4-producing CD8⁺ T cells, and multifunctional CD4⁺ Th1 cells which simultaneously producing IFN- γ , IL-2 and TNF- α in mCD40L/SHIV-VLP immunized mice.

2. Materials and methods

2.1. Cells, antibodies and reagents

Spodoptera frugiperda Sf9 cells were grown at 27°C in the SF-900 II optimized serum-free medium (Invitrogen Life Technologies, Grand Island, NY). Antibodies to human CD11c-

PE, CD40-FITC, CD83-FITC, CD86-FITC, CD40L-FITC, anti-mouse CD40L-PE, isotype controls, purified mouse and human CD40L antibodies were obtained from BD PharMingen (San Diego, CA). Intracellular cytokine staining starter kit-mouse, BD™ ELISPOT mouse IL-4 pair, BD™ ELISPOT mouse IFN- γ pair, BD™ ELISPOT AEC substrate, anti-mouse IL-2-FITC, anti-mouse IFN- γ -PE, anti-mouse TNF- α -APC were purchased from BD Bioscience (San Diego, CA). Recombinant human (rhu) IL-4, GM-CSF, TNF- α , mCD40L, hCD40L and Quantikine® CD40L immunoassay kit were obtained from R & D Systems (Minneapolis, MN). LPS (*Escherichia coli* 026:B6) and human AB serum were purchased from Sigma-Aldrich (St. Louis, MO). The HIV III B gp160 protein was purchased from Advanced Biotechnologies Inc. (Columbia, Maryland). Purified mouse IgG for ELISA was purchased from Southern Biotechnology Associates (Birmingham, AL). HIV IIIB Env. peptides and SIV mac239 Gag peptides were obtained from NIH AIDS Research & Reference Reagent Program (Germantown, MD). SIVmac 239 Gag (15-mer/each) peptides complete set (catalog number: 6204) has total of 125 vials, SIV Gag pool I contains peptides 1-62 of 125, pool II contains peptides 63-125 of 125. HIV-1 Consensus Subtype B Env (15-mer/each) peptides complete set (catalog number: 9480) has total of 211 vials, HIV Env peptides pool I contains first half of 105 vials, and pool II contains second half of 106 vials. ELISPOT-MultiScreen 96-well filtration plates were purchased from Millipore Corporation (Bedford, MA). Magnetic cell sorting system and CD14 MicroBeads were purchased from Miltenyi Biotech Inc. (Auburn, CA, USA).

2.2. Virus-like particles production and characterization

Human and mouse CD40L cDNA constructs were a gift from Dr. Malcolm Brenner (Center for Cell and Gene Therapy, Baylor College of Medicine). Human or mouse full length CD40L cDNA was cloned in frame in the pVL1392 baculovirus vector. SIV-VLP (including SIV Gag only) and SHIV-VLP (including SIV Gag plus HIV BH10 envelop truncated protein, *env_t*) were produced as previously described [29]. Chimeric CD40L/SHIV-VLP was produced by using the similar method as SHIV-VLP. Briefly, Sf9 insect cells were co-infected with a baculovirus recombinant (rBV) expressing SIVmac239 gag at m.o.i. of 2, rBV expressing HIV *env_t* at m.o.i. of 10 and rBV expressing human CD40L at m.o.i. of 10. At 3 days post infection, the culture medium was collected and centrifuged at 2500 rpm for 20 min (Beckman GPR desktop centrifuge). The supernatant was then filtered through a 0.45 μ m filter, and VLP was pelleted at $120,000 \times g$ for 2 h at 4°C. The resuspended VLP was then purified through a 20–60% continuous sucrose gradient at $100,000 \times g$ for 16 h at 4°C. The VLP band was collected and dialyzed against PBS using a 10,000 MW cut-off membrane. VLP was then pelleted and resuspended overnight in PBS. The efficient incorporation of Env protein and CD40L was determined by Western blot and ELISA assay. Protein concentrations of purified SHIV-VLP and chimeric CD40L/SHIV-VLP were determined by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The endotoxin level in each SHIV-VLP and chimeric CD40L/SHIV-VLP preparation was quantitated with *Limulus* amoebocyte assay (Associates of Cape Cod, Inc., Woods Hole, Mass.), and was controlled below 0.004 μ g/ml.

2.3. ELISA assay for incorporation of CD40L protein in CD40L/SHIV-VLP

The incorporation efficiency of human or mouse CD40L protein in chimeric CD40L/SHIV-VLP was determined by ELISA assay. Briefly, 100 μ l of serial diluted chimeric CD40L/SHIV-VLP was added to the 96 well polystyrene microplate coated with a polyclonal antibody against human or mouse CD40L. Included were also a series of CD40L standard proteins for quantifying the amount of CD40L incorporated in the chimeric CD40L/SHIV-VLP. The plate was incubated for 2 h at room temperature on a horizontal orbital shaker. After washing 4 times, 200 μ l of monoclonal anti-CD40L antibodies with conjugated HRP was added to each well. The plate was then incubated for 2 h on the shaker at room

temperature. After washing 5 times, 200 μ l of substrate solution was added to each well and incubated for 30 min at room temperature. Finally, 50 μ l of stop solution was added to each well and the plate was read at 405 nm within 30 min by using EL 800 Universal Microplate reader (Bio-Teck Instruments, Inc.). The amount of CD40L was calculated against the CD40L standard curve.

2.4. CD14 positive monocytes isolation and DC generation

Fresh human buffy coat blood was obtained from Gulf Coast Regional Blood Center (Houston, TX). Therefore, no Baylor College of Medicine IRB approval or written informed consent was necessary when we purchased buffy coat from the blood center. Leukocytes were purified by Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden) and washed three times using PBS. About 1×10^7 PBMC in 80 μ l of PBS/0.5% BSA were incubated with 20 μ l of CD14 microbeads for 15 min at 4°C. The CD14 microbeads-labeled cells were washed with 20 times volume PBS/0.5% BSA and subjected to MACS separators (Miltenyi Biotech Inc., Auburn, CA, USA) for CD14 positive cells isolation. The purified CD14 positive cells were analyzed by FACS and showed more than 95% purity. CD14⁺ monocytes were plated in the RPMI 1640 medium supplemented with 5% human AB serum, 10 mM pyruvic acid, 10 mM nonessential amino acid, 100 μ g/ml kanamycin, 800 U/ml recombinant human GM-CSF and 500 U/ml IL-4 for 5 days at 37°C to generate DCs. At day 2 and day 4, one-half volume of the medium was replaced by the fresh medium supplemented with 800 U/ml GM-CSF and 500 U/ml IL-4.

2.5. Chimeric hCD40L/SHIV-VLP binding assay

Human CD14⁺ monocytes-derived DCs were incubated with 10 μ g of SHIV-VLP or chimeric hCD40L/SHIV-VLP per 1×10^6 DCs in 100 μ l at 4°C for 1 h. After extensive washing, rabbit anti-HIV-Env antibody (Harriet Robinson, Yerkes Primate Center, Emory University, Atlanta, GA) at 1:100 dilution was added and incubated for 30 min at 4°C. Secondary anti-rabbit antibody conjugated with FITC at 1:500 dilution was then stained for another 30 min at 4°C. Cells were then fixed with 2% paraformaldehyde and analyzed by FACSCalibur (Becton Dickinson, San Jose, CA).

2.6. DC activation assay

To evaluate DC maturation, DCs at day 6 were collected and incubated with VLP at a concentration of 10 μ g/ 10^6 cells in 1 ml of PBS/1% FBS for 1 h at 37°C. LPS at 5 μ g/ml was used for positive controls. Heat inactivated CD40L/SHIV VLP was also used as a negative control. To inactivate CD40L/SHIV VLP, the VLP was heated for 10 min at 95°C in PBS. After thorough vortex, supernatant was collected and used. Untreated DC culture was also used as negative controls. Cells were subsequently incubated for 2 days in 3 ml of RPMI 1640 medium supplemented with 1000 U/ml GM-CSF and 1000 U/ml IL-4. At the end of 2 days incubation, cells were harvested, washed with PBS/1% FBS, and subsequently stained with CD11c-PE and each of DC activation markers (CD40, CD83, CD86) labeled with FITC in 100 μ l of PBS/1% FBS for 30 min at 4°C. The cells were washed with 3 ml PBS and then fixed with 2% paraformaldehyde for FACS analysis (FACS Calibur). PE- and FITC-labeled DCs were acquired by CELLQuest software. CD11c⁺ cells were then gated for further analysis.

To block CD40L function of hCD40L/SHIV-VLP, 10 μ g of VLP and 5 μ g anti-human CD40L antibody were incubated for 1 h at 4°C. The Antibody-blocked VLP was incubated with 10^6 DCs in 1 ml for 2 h at 37°C, then 2 ml medium was added, and DCs were continuously cultured for 2 days. At the end of 2 days incubation, cells were analyzed as described above. LPS and hCD40L protein were used as positive controls, and unrelated antibody anti-SV5 was used as a negative control.

To confirm whether CD40L/SHIV VLP can bind to CD40 molecule through CD40-CD40L interaction, competitive assay by adding either SHIV VLP or CD40L/SHIV VLP was used in LPS activated human or mouse B cells. Human or mouse B cells was activated by 5 mg/ml LPS in complete RPMI1640 media containing 10% FBS overnight to induce high-expression of CD40 on cell surface. Then, SHIV VLP or hCD40L/SHIV or mCD40L/SHIV VLP was added to B cells that highly express CD40 at 10 µg/ml in PBS and then incubated on ice for 4 hrs. After thorough washing and Fc receptors blocking, the anti-CD40 antibodies were incubated with the cells for additional 30 minutes on ice. After washing, samples were acquired by BD FACSCalibur. Data were analyzed by Flowjo.

2.7. Bio-Plex cytokine assay

The concentration of cytokines secreted by different VLP-treated DCs including IL-2, IL-4, IL-5, IL-10, IL-12(p70), IL-13, GM-CSF, IFN- γ , and TNF- α were determined by using the Bio-Plex multiplex Human Cytokine Th1/Th2 Assay kit and the Cytokine Reagent kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA). Briefly, 50 µl of culture supernatants or cytokine standards were plated into a 96 well filter plate coated with a multiplex of beads coupled to antibodies against above mentioned cytokines and incubated on a platform shaker at 300 rpm for 30 min at room temperature. After a series of washes to remove the unbound proteins, a mixture of biotinylated detection antibodies against these cytokines, each specific for an epitope different with that of the primary antibody, was added to the reaction resulting in the formation of sandwiches of antibodies around the target proteins. Streptavidin-phycoerythrin (streptavidin-PE) was then added to bind to the biotinylated detection antibodies on the bead surface. Data from the reaction were then acquired and analyzed by using the Bio-Plex suspension array system (Luminex 100 system) from Bio-Rad Laboratories. For CD40L blocking experiment, the supernatants of DCs treated with hCD40L antibody-blocked hCD40L/SHIV-VLP were analyzed as described above.

2.8. Allogeneic T cell proliferation assay

Human PBMC were incubated with RPMI 1640 for 2 h on petri-dishes, and non-adherent cells were collected and passed through 40 µm nylon cell strainer. CD3⁺ magnetic beads (BD Bioscience) labeled T cells were purified by MACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) two times to reach more than 96% purify of the T cells. Purified T cells were incubated with AIM V medium (Invitrogen corporation, Grand Island, NY) for one day. About 1×10^5 T cells were incubated with VLP or hCD40L antibody-blocked hCD40L/SHIV-VLP-treated DCs (irradiated at 5000 rad for 5 min) at ratios of 20:1, 40:1, 80:1, and 160:1. After co-culture of T and DCs for 4 days, 1 µCi/well of [³H] TdR was added for 18 h before harvesting. The cell mixture was harvested by Filtermate Harvester (Packard Instrument Company, Meriden, CT) and cell-associated radioactivity was determined by TopCount-NXT (Packard Instrument Company, Meriden, CT).

2.9. Mouse immunizations

C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME), five per group, were immunized intradermally (i.d.) with SIV-VLP (20 µg/mouse) or chimeric mCD40L/SIV-VLP (20 µg/mouse) or SHIV-VLP (20 µg/mouse and 100 µg/mouse) or chimeric mCD40L/SHIV-VLP (20 µg/mouse and 100 µg/mouse) at 0, 2, 4, and 6 weeks.

2.10. Mouse sample collection

Blood samples were collected by retro-orbital plexus puncture at 0, 2, 4, 6, and 8 weeks. After clotting and centrifugation, serum samples were collected. Samples were then spun in a microcentrifuge for 15 min and supernatants were collected. After collection, 1% (v/v) 100

mM PMSF in isopropanol was added to each serum sample as a protease inhibitor. All samples were stored at -20°C until analysis for antibody titration. Spleens were harvested at 8 weeks and ground in 40 μm Nylon cell strainers to make single-cell suspensions of splenocytes. All the animal experiments were done according to Baylor College of Medicine IACUC committee approved animal protocols. All the procedures abided by BCM institutional guidelines on animal husbandry, experimentation and care/welfare.

2.11. ELISA assay for antibody production

All sera were individually collected, and anti-HIV Env IgG antibody production was determined by ELISA. Immunolon-4 HBX 96-well microtiter plates (Nunc Life Technologies, Basel, Switzerland) were coated with 100 μl of purified HIV Env protein (2 $\mu\text{g}/\text{ml}$)/well in borate-buffered saline (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.6) at 4°C overnight. Plates were blocked with PBS containing 1% BSA at room temperature for 2 h. After three washes in PBS containing 0.05% Tween 20, 100-fold diluted sera, samples were added to the wells and incubated at 4°C overnight. After four washes, the wells were treated with goat anti-mouse IgG-peroxidase conjugates (Sigma-Aldrich) for 1 h at room temperature. After removal of the unbound conjugates, the substrate solution prepared in ABTS (Sigma), and hydrogen peroxide was added to the plates. ODs were read at 405 nm by using EL800 Universal Microplate reader (Bio-Teck Instruments, Inc.).

2.12. IFN- γ and IL-4 ELISPOT assay

The levels of HIV Env- and SIV Gag-specific CD8^+ T cells in VLP immunized mouse splenocytes were determined by IFN- γ and IL-4 ELISPOT assay. Splenocytes were suspended in the complete medium (RPMI with 10% fetal calf serum, 2 μM 2-mercaptoethanol, and 10 U/ml IL-2) at a concentration of 2×10^5 cells/100 μl and added to individual wells of 96-well hydrophobic polyvinylidene difluoride-backed plates (Millipore, Bedford, Mass.) previously coated with 50 μl of 10 $\mu\text{g}/\text{ml}$ anti-IFN- γ or anti-IL-4 monoclonal antibody (BD PharMingen, San Diego, CA) overnight at 4°C . HIV-1 Env or SIV Gag peptides and the positive control, phytohemagglutinin (Sigma, St. Louis, Mo.), were added to the well at a final concentration of 10 $\mu\text{g}/\text{ml}$. The wells with no added peptides served as negative controls. All responses were tested in triplicate. Plates were incubated for 36 to 48 h at 37°C in 5% CO_2 , washed with phosphate-buffered saline containing 0.05% Tween 20, and incubated at room temperature for 2 h with biotinylated 2 $\mu\text{g}/\text{ml}$ of anti-IFN- γ or anti-IL-4 monoclonal antibody (BD PharMingen, San Diego, CA). The avidin-horseradish peroxidase (HRP) from the BDTM ELISPOT AEC substrate set (BD PharMingen, San Diego, CA) was added at room temperature for 1 h, followed by the AEC peroxidase substrate. ELISPOT plates were evaluated independently in a blinded fashion (ZellNet Consulting, Fort Lee, NJ) using a KS ELISPOT reader (Carl Zeiss, Thornwood, NY) with KS 4.5.

2.13. Intracellular cytokine assay

IFN- γ -secreting CD8^+ T cells and triple cytokine secreting CD4^+ T cells were measured by intracellular cytokine staining and analyzed by flow cytometry. Spleens were harvested at 8 weeks and ground to make single-cell suspensions of splenocytes. Splenocytes (10^6) were pulsed with 2 μM HIV Env 15 mer peptide pool at 37°C for 2 h and then GolgiPlug (1: 500) in 200 μl of RPMI 1640 medium supplemented with 10% FCS, 2-mercaptoethanol (2 μM), and L-glutamine in the flat bottom 96 well plate for another 4 h. After the incubation, cells were transferred to round bottom 96 well plates. Anti-CD8 FITC antibody for IFN- γ -secreting CD8^+ T cells staining or anti-CD4 PerCP antibody for triple cytokine secreting CD4^+ T cells staining, was added to the cells and incubated for 30 min in ice. After washing for 3 times, cells were fixed with Cytotfix/CytoPerm solution (BD PharMingen, San Diego, CA). After washing with Permwash buffer (BD PharMingen, San Diego, CA), cells were

incubated with anti-IFN- γ PE (for IFN- γ -secreting CD8⁺ T cells staining) or with anti-IL-2 FITC and anti-IFN- γ PE and anti-TNF- α APC for 30 min on ice in Permashield buffer. Finally, cells were washed, resuspended in PBS containing 2% paraformaldehyde and analyzed by using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA).

2.14. Cytotoxic T lymphocyte activity assay

The CTL activity was measured by using the CytoTox 96® Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions (Promega, Madison, WI). Splenocytes were freshly isolated and stimulated with HIV Env peptide pool at 2 μ M for 5 days. These cells were used as effector cells in the CTL assay. EL-4 cells (H-2^b) pulsed with HIV Env peptide pool (2 μ M) for 1 h was used as target cells. EL-4 cells pulsed with no peptides were used as negative control target cells. Five different effector to target cell ratios (50:1, 25:1, 12.5:1, 6:1, and 3:1) were tested in the assay with 1×10^4 target cells for 4 h. The specific lysis of target cells was measured by LDH release and calculated using formula in the Cytotox 96 assay kit (Promega, Madison, WI).

2.15. Statistical analysis

Data from treated and control groups were analyzed using an unpaired Student's t test (two tail) for parametric data including CD40L incorporation (pico gram), [³H]thymidine incorporation (CPM), serum IgG (O.D. value), and ELISPOT (spots). Mann-Whitney test was used for non parametric data including CTL assay. Statistical software was used (Minitab software, Sigma Breakthrough Technologies, Inc., San Marcos, TX). P value < 0.05 was considered statistically significant. Data are reported as mean $\pm$ the standard deviation (SD).

3. Results

3.1. CD40L is incorporated efficiently into chimeric CD40L/SHIV-VLP

To determine if CD40L could be expressed efficiently on the cell surface, we constructed baculovirus recombinants expressing the full length sequence encoding human or mouse CD40L. After rBV hCD40L or rBV mCD40L transfection, the expression levels of CD40L on the surface of Sf9 cells were determined by FACS analysis. As shown in Figures 1A and 1B, both hCD40L and mCD40L were expressed by 70.8% and 81.5% of Sf9 cells, respectively.

To confirm whether CD40L could be incorporated efficiently into the SHIV-VLP, the amount of CD40L incorporation was determined by ELISA. After co-infection with rBV SIV Gag and rBV HIV Env with or without rBV CD40L, VLP were collected from the culture supernatants and purified. High levels of CD40L incorporation on chimeric CD40L/SHIV-VLP are shown in Figure 1C for hCD40L and Figure 1D for mCD40L. There was an increasing amount of CD40L detected in the samples that corresponded with increasing total amounts of VLP ($p < 0.05$, $n = 3$). CD40L incorporation was estimated to make up 0.1–0.2% of the total mass of the chimeric CD40L/SHIV-VLP. As expected, there was no CD40L detected in SHIV-VLP samples. These results indicated that heterologous CD40L can be incorporated efficiently into the chimeric CD40L/SHIV-VLP.

3.2. Incorporation of CD40L into SHIV-VLP enhances binding of SHIV-VLP to DCs

To determine if the incorporation of CD40L into chimeric hCD40L/SHIV-VLP could enhance the binding of SHIV-VLP to DCs, we examined the binding activity of hCD40L/SHIV-VLP with human CD14⁺ monocyte-derived DCs by FACS analysis using an antibody against HIV Env. Figures 2A and 2B show representative data of the VLP-DC binding rate

from three independent experiments using different donors. The average binding efficiency of SHIV-VLP and hCD40L/SHIV-VLP to DCs gated on the CD11c⁺ cell population was approximately 50.4±3.1% and 62.2±4.3%, respectively, indicating that hCD40L/SHIV-VLP had a higher binding capacity on DCs compared with SHIV-VLP.

3.3. Incorporation of CD40L into SHIV-VLP enhances SHIV-VLP-induced DC maturation

To determine whether chimeric hCD40L/SHIV-VLP had greater potential than SHIV-VLP to activate DCs, human CD14⁺ monocyte-derived DCs at day 6 were treated with chimeric hCD40L/SHIV-VLP, SHIV-VLP, heated SHIV-VLP, LPS, or no treatment for 48 h. The phenotypic maturation then was evaluated via expression of CD83, CD40, CD86, CD54, and MHC class I and II by FACS analysis. As shown in Figure 2C, there was a dramatic up-regulation of CD83 on DCs treated with chimeric CD40L/SHIV-VLP (from 1.6% to 72.1%). Specifically, there was approximately a two-fold increase in the numbers of CD83-positive cells within the population of chimeric hCD40L/SHIV-VLP-treated DCs compared with that in SHIV-VLP-treated DCs (72.1% vs. 42.9%), although there were no significant changes in mean fluorescence intensity (MFI) in CD83 between hCD40L/SHIV-VLP and SHIV-VLP treatment groups. Since CD83 is a specific marker for matured DCs, increased CD83 cells may indicate enhanced induction of DC maturation by chimeric hCD40L/SHIV-VLP compared with SHIV-VLP. There was no increase in up-regulation of CD86 (94.5 to 95.8%) and marginal up-regulation of CD40 (80.3 to 87.6%) between the hCD40L/SHIV-VLP and SHIV-VLP treatment groups. Both chimeric hCD40L/SHIV-VLP- and SHIV-VLP-treated DCs also exhibited increased MFI for CD86, CD54, HLA-A, B, C and HLA-DR, DP, DQ on DCs (data not shown). These results indicated that VLP could up-regulate the expression of CD40, CD83, CD86, CD54, HLA-A, B, C and HLA-DR, DP, DQ on DCs, which promoted phenotypic maturation; CD40L-containing VLP induced a greater level of DC maturation according to the phenotypic expression of these surface molecules. Heated hCD40L/SHIV-VLP had only a limited effect on the expression of DC maturation markers, indicating that the intact virion structure of SHIV-VLP is important for DC activation. Thus, chimeric hCD40L/SHIV-VLP possesses a greater potential to induce phenotypic maturation of human CD14⁺ monocyte-derived DCs compared to SHIV-VLP.

To determine whether the enhanced effect of hCD40L/SHIV-VLP on DC maturation could be blocked by hCD40L antibody, hCD40L/SHIV-VLP were pre-incubated with hCD40L antibody for 1 h then incubated with DCs for 48 h (treatment #6 in Figures 2D and 2E). The effect on DC phenotypic maturation was evaluated by changes of the expression of CD83, CD40 and CD86. The expression of CD40 was reduced slightly by CD40L antibody blocking treatment (Figure 2D) ($p < 0.05$). However, CD83 and CD86 expression was reduced significantly by two- to three-fold by hCD40L antibody blockade (Figures 2D and 2E) but not the irrelevant antibody control ($p < 0.01$). The expression of those markers in the cells with hCD40L Ab-blocked hCD40L/SHIV-VLP treatment was even lower than in cells with SHIV-VLP treatment. These results indicated that potent activation of DCs by hCD40L/SHIV-VLP may be due to hCD40L incorporation.

To confirm whether CD40L/SHIV VLP can physically bind to CD40 molecule through CD40-CD40L interaction, we did a competitive assay by addition of either SHIV VLP or CD40L/SHIV VLP on LPS activated B cells. As shown in Fig. 2F, after LPS treatment of human (subpanels a–c) or mouse B cells (subpanels d–f), CD40 was highly expressed on the B cell surface as detected by anti-CD40 antibody in the FACS analysis (subpanels a and d). With addition of either SHIV VLP (subpanels b and e) or hCD40L/SHIV (subpanel c) or mCD40L/SHIV VLPs (subpanel f), binding of anti-CD40 antibody to the SHIV VLP treated LPS activated B cells is not affected (subpanels b and e), however, the binding of anti-CD40 antibody to CD40L/SHIV VLP treated LPS activated human B cells (subpanel c) and mouse

B cells (subpanel f) were greatly diminished, indirectly indicating the interaction of CD40L/SHIV VLP with CD40 on CD40 overexpressing cells.

3.4. Incorporation of hCD40L into SHIV-VLP enhances the potency of induction of DC maturation compared with soluble hCD40L protein

To compare the potency of soluble hCD40L protein mixed with SHIV-VLP and hCD40L incorporated into SHIV-VLP in inducing DC maturation, DCs were treated with soluble hCD40L protein, SHIV-VLP, hCD40L/SHIV-VLP, hCD40L Ab-blocked hCD40L/SHIV-VLP and SHIV-VLP plus soluble hCD40L protein, respectively. As shown in Figures 2D and 2E, the soluble CD40L alone failed to activate DCs, but both SHIV-VLP and hCD40L/SHIV-VLP treatments showed two- to four-fold increases of expression of CD40, CD83, and CD86 on DCs compared with soluble hCD40L or untreated immature DC. These data also demonstrate that CD40L/SHIV-VLP is more potent than SHIV-VLP in promoting DC activation. Although SHIV-VLP plus soluble hCD40L protein and hCD40L/SHIV-VLP produced similar expression of CD40 and CD86, hCD40L/SHIV-VLP was more potent than SHIV-VLP plus soluble hCD40L protein in inducing expression of CD83, which is only highly expressed in mature DCs.

3.5. Chimeric hCD40L/SHIV-VLP significantly stimulate Th1 type cytokine production by activated DCs

To evaluate the functional activation of DCs, we assessed the production of Th1/Th2 cytokines from DCs upon exposure to VLP using a Bio-Plex cytokine assay. As shown in Table 1, there was a substantial increase in the levels of TNF- α , IFN- γ , and IL-12 in the supernatant of hCD40L/SHIV-VLP-treated DCs compared with SHIV-VLP-treated DCs. hCD40L/SHIV-VLP-treated DCs showed a seven-fold increase in TNF- α , a two-fold increase in IFN- γ , and a 12-fold increase in IL-12 compared with SHIV-VLP-treated cells (Table 1). Only a slight enhancement of IL-10 and negligible IL-2 or IL-5 secretion were observed in hCD40L/SHIV-VLP-, SHIV-VLP-, and LPS-treated cells (data not shown). We found that the stimulatory action of hCD40L/SHIV-VLP depended on their native structure since heated hCD40L/SHIV-VLP had almost no effect on cytokine secretion (i.e., similar to the basal level of untreated DCs). These results indicate that hCD40L/SHIV-VLP is more potent than SHIV-VLP to induce DCs to release Th1-type cytokines.

To determine whether the elevated cytokine production in hCD40L/SHIV-VLP-treated DCs could be blocked by hCD40L antibody, hCD40L antibody was added to hCD40L/SHIV-VLP before addition to DCs, and cytokine production in the supernatant was determined by a Bio-Plex cytokine assay. As shown in Table 1, the levels of TNF- α , IFN- γ and IL-12 from hCD40L antibody plus hCD40L/SHIV-VLP-treated DCs were reduced to the levels of SHIV-VLP-treated DCs. Although irrelevant antibody anti-SV5-treated hCD40L/SHIV-VLP also showed slightly reduced cytokine production, this nonspecific effect was much less than that of specific blocking by hCD40L Ab. These data confirm hCD40L incorporated in VLP is involved in the enhanced induction of Th1-type cytokines by DCs.

3.6. Chimeric hCD40L/SHIV-VLP-activated DCs have enhanced T-cell allostimulatory activity compared with SHIV-VLP

The most characteristic feature that discriminates DCs from other APCs is their ability to induce primary T-cell responses [30], which can be determined by the allostimulatory assay. To examine and compare the functionality of phenotypically matured DCs after SHIV-VLP or chimeric hCD40L/SHIV-VLP stimulation, we used an allogeneic mixed lymphocyte reaction (MLR) assay. DCs treated with or without chimeric hCD40L/SHIV-VLP, SHIV-VLP, heated hCD40L/SHIV-VLP, hCD40L Ab-treated hCD40L/SHIV-VLP, LPS, soluble hCD40L protein, or a mixture of SHIV-VLP plus soluble hCD40L protein were co-

cultivated with purified allogeneic CD3⁺ T-cells for four days. As shown in Figure 3A, immature DCs (iDCs) exhibited low allostimulatory capacity. However, LPS-matured DCs acquired potent allostimulatory capacity. The different VLP-treated DCs possessed greater allostimulatory capacity than iDCs, a finding that corresponded with the phenotypic results. As expected, co-culture of T-cells with untreated iDCs or heated hCD40L/SHIV-VLP-treated DCs resulted in low levels of T-cell proliferation as shown in Figures 3A and 3B. In contrast, chimeric hCD40L/SHIV-VLP-treated DCs had the highest and most significantly enhanced T-cell proliferation and stimulation capacity compared with DCs treated with LPS, SHIV-VLP, hCD40L soluble protein, or a mixture of SHIV-VLP plus hCD40L soluble protein ($p < 0.05$, $n = 3$). SHIV-VLP-treated DCs induced an approximately twofold increase in T-cell proliferation compared to chimeric hCD40L/SHIV-VLP-treated DCs, which showed about a three-fold increase in T-cell proliferation versus untreated iDCs. In addition, when DCs were treated with hCD40L antibody-treated hCD40L/SHIV-VLP, the capacity of hCD40L/SHIV-VLP to stimulate T-cell proliferation was reduced significantly; however, the irrelevant antibody anti-SV5 did not have any effect. These results confirm that the incorporation of hCD40L in hCD40L/SHIV-VLP potentially enhances the capacity of DCs to induce allogeneic T-cell proliferation.

3.7. Chimeric mCD40L/SHIV-VLP enhances anti-HIV Env IgG production in immunized mouse serum

To investigate whether chimeric CD40L/SHIV-VLP could enhance anti-HIV Env antibody production *in vivo* compared with SHIV-VLP, C57BL/6J mice were immunized through i.d. route with low (20 μ g) and high (100 μ g) doses of SHIV-VLP, mCD40L/SHIV-VLP, SIV-VLP, or mCD40L/SIV-VLP as described in Table 2. Two weeks after final immunization, blood samples were collected and serum anti-HIV Env IgG levels were measured by an enzyme-linked immunosorbent assay (ELISA). As shown in Figure 4, the negative control mouse group immunized with SIV-VLP showed nearly undetectable levels of HIV Env specific IgG antibody and no response to either low or high dose SIV-VLP immunization. On the other hand, the mouse group immunized with SHIV-VLP or mCD40L/SHIV-VLP revealed high levels of HIV Env-specific IgG. Both groups showed a seven-fold increase in HIV Env-specific IgG production resulting from low to high dose VLP immunization. The mCD40L/SHIV-VLP significantly enhanced the HIV Env-specific IgG production by two-fold as compared with SHIV-VLP ($p < 0.01$, $n = 5$). These data indicate that both SHIV-VLP and mCD40L/SHIV-VLP can induce a humoral immune response in a dose-dependent manner, and incorporation of mCD40L into SHIV-VLP significantly enhances HIV Env-specific IgG production compared with SHIV-VLP. Therefore, mCD40L could be a potent adjuvant for SHIV-VLP immunization.

3.8. Enhanced cellular immune response to SIV Gag in chimeric SIV VLP or mCD40L/SIV-VLP immunized mice

The ideal HIV vaccine would induce both humoral and cellular immune responses. To evaluate the cellular immune response elicited by chimeric CD40L/SHIV-VLP, splenocytes from the same immunization groups as in Table 2 were used and analyzed by ELISPOT assay to detect IFN- γ and IL-4-secreting lymphocytes in response to stimulation by SIV Gag peptides. As shown in Figures 5A and 5B, mCD40L/SIV-VLP significantly increased the numbers of both IFN- γ and IL-4 producing cells by two- to five-fold in response to stimulation by SIV Gag peptides pool 1 and by two-fold in response to stimulation by SIV Gag peptides pool 2 ($p < 0.01$, $n = 5$). These results indicate that mCD40L incorporation significantly increases both Th1-type immune response (IFN- γ producing) and Th2-type (IL-4 producing) cellular immune responses.

3.9. Enhanced cellular immune response against both HIV-Env and SIV-Gag in response to chimeric SHIV-VLP and CD40L/SHIV-VLP immunization

Enhanced HIV Env and SIV Gag-specific CD8⁺ T-cell responses also were found in mice immunized with low (20 µg) and high (100 µg) doses of mCD40L/SHIV-VLP. As shown in Figure 6, immunization with the high dose of chimeric CD40L/SHIV-VLP increased the number of IFN-γ producing cells by four-fold in response to stimulation with SIV Gag peptides pool 1; an 80% increase was measured in response to Gag peptide pool 2 as compared with those in SHIV-VLP immunized mice ($p < 0.05$, $n = 5$). At a higher dose of VLP (100 µg/mouse), both SHIV-VLP and mCD40L/SHIV-VLP could induce elevated SIV Gag-specific IFN-γ or IL-4 producing T-cell numbers in a dose-dependent manner ($p < 0.05$, $n = 5$). However, a higher dose of mCD40L/SHIV-VLP only induced enhanced HIV Env-specific IFN-γ producing T-cells but not IL-4 producing T-cells. Taken together, mCD40L incorporation in SHIV-VLP can enhance HIV Env and SIV Gag-specific cellular responses at the higher dose.

To further confirm the enhanced cellular immune response elicited by chimeric mCD40L/SHIV-VLP, we evaluated Th1-type cytokine IFN-γ production by intracellular cytokine staining assay. Splenocytes from VLP-immunized mice were stimulated with the HIV Env peptide pool and the numbers of IFN-γ secreting CD8⁺ T-cells were analyzed by flow cytometry. As shown in Figure 7A, both SHIV-VLP and mCD40L/SHIV-VLP enhanced the number of IFN-γ secreting CD8⁺ T-cells compared with the PBS immunized mouse group. The percent-positive HIV Env-specific IFN-γ secreting CD8⁺ T-cells ranged from 2.82 – 4.76% in mCD40L/SHIV-VLP immunized mice versus 1.79 – 2.41% in SHIV-VLP immunized mice, respectively, as compared with PBS-treated control mice (0.91 – 1.21%). These data indicate that mCD40L incorporated onto SHIV-VLP can augment the Th1-type cellular response against HIV.

To determine whether multifunctional HIV Env-specific CD4⁺ T-cells can be induced by VLP immunization, IFN-γ, TNF-α, and IL-2 triple cytokine staining was performed with splenocytes from mice immunized with the different VLPs; the splenocytes had been stimulated with HIV Env peptide pool. As shown in Figure 7B, there was an increase in the numbers of triple cytokine-positive CD4⁺ T-cells induced in the VLP immunized mice. In contrast, the PBS-immunized negative control group had 0.5 – 0.64% of triple cytokine-positive CD4⁺ T-cells. SHIV-VLP immunization induced 2.5 – 2.89%, while mCD40L/SHIV-VLP immunized group had 3.24 – 3.72% triple cytokine-positive CD4⁺ T-cells. Therefore, not only does mCD40L/SHIV-VLP immunization induce HIV Env-specific CD4⁺ T-cell activation, it also induces multifunctional CD4⁺ T-cells.

3.10. Enhanced cytotoxic T-cell responses against HIV-Env in chimeric mCD40L/SHIV-VLP immunized mice

To study the effector functions of T-cells from mCD40L/SHIV-VLP immunized mice, the HIV-Env-specific cytotoxic T-cell assay was performed eight weeks after initial immunization. As shown in Figure 8, splenocytes obtained from mice immunized with chimeric mCD40L/SHIV-VLP elicited higher percentages (28%) of Env-specific lysis of haplotype-specific target cells (H-2^b restricted) than those obtained from mice immunized with SHIV-VLP (19%) at an E:T ratio of 50:1. A decrease in cell specific lysis correlated with a decrease in the ratios of E:T used. However, mice given mCD40L/SHIV-VLP immunization showed higher CTL activity at all E:T ratios used as compared to the SHIV-VLP immunized group (* $p < 0.05$). A high E:T ratio may be necessary since the splenocytes used here were unfractionated and contained heterogeneous T-cell populations. These results indicate that incorporation of mCD40L into SHIV-VLP also enhances HIV Env-specific MHC-restricted CTL immune responses.

4. Discussion

The present study showed that incorporation of CD40L into SHIV-VLP provided enhanced immunogenicity over SHIV-VLP as evidenced by the induction of both humoral and cellular immune responses against HIV Env and SIV Gag *in vivo*. The augmented immunogenicity may have resulted from the enhanced ability of CD40L/SHIV-VLP to induce phenotypic and functional maturation of DCs. The induction of large amounts of cytokines by chimeric hCD40L/SHIV-VLP-treated DCs suggest that CD40L incorporated into SHIV-VLP mimics the natural interaction between the CD40L-expressing CD4⁺ T-cells and CD40-expressing DCs. The unique aspect of this study is that both the antigenic components (SIV Gag and HIV Env) and the DC activation molecule (CD40L) in their native structural state reside simultaneously in the same particles, thus inducing more potent activation of DCs and stronger humoral and cellular responses against HIV Env and SIV Gag than SHIV-VLP. Similar results obtained by incorporation of CD40L on VLP to enhance the immunogenicity have been reported by Skountzou et al. Although the *in vivo* outcome of the current study is confirmatory to this previous paper, the current study extends the results and demonstrated the mechanistic details in the *in vitro* system that activation of DCs plays a major role in increasing the immunogenic efficacy of the CD40L incorporated VLPs. Specifically, we further studied the mechanism of action of CD40L/SHIV VLPs with two different systems- *in vitro* interactions with human monocyte derived dendritic cells (MDDC), and *in vivo* immunogenicity using VLPs expressing the murine ortholog of CD40L presented also on SHIV-VLPs. We believe this study further elucidated the mechanisms of this costimulatory molecule CD40L in DC activation and enhanced immunogenicity to SHIV VLP.

We found that CD40L was successfully expressed using a baculovirus expression system and efficiently incorporated into SHIV-VLP. The chimeric CD40L/SHIV-VLP could bind effectively to human DCs. There have been reports of HPV-VLP that can bind and activate DCs [32–34]. However, there are only a few reports of HIV VLP interacting specifically with DCs. We previously found that SHIV-VLP can bind and activate DCs efficiently [23]. Consistent with other reports of CD40L function, we found that chimeric SHIV-VLP with addition of the immune co-stimulatory molecule hCD40L is an efficient vector to stimulate both humoral and cellular immune responses [35,36]. For human CD14⁺ monocyte-derived iDCs, chimeric CD40L/SHIV-VLP treatment led to an increase in the expression of costimulatory molecules and cytokine production by DCs. The increase in CD40, CD83, and CD86 expression in CD40L/SHIV-VLP-treated DCs was comparable or even slightly higher than those in the positive control (LPS), or SHIV-VLP treatment groups. Although previous reports showed that ligation of CD40 could induce DC maturation and enhance expression of adhesion and costimulatory molecules including CD40, CD80, CD86, this interaction also could increase secretion of proinflammatory cytokines and chemokines by matured DCs [2,12,37–39]. One unique feature of chimeric CD40L/SHIV-VLP treatment is that it significantly increased CD83 expression on DCs (a marker of maturation) compared with soluble CD40L protein or a mixture of SHIV-VLP with soluble CD40L protein treatment. In addition, blocking of CD40L by anti-CD40L antibody in CD40L/SHIV-VLP also blocked the up-regulation of CD83 expression. This result is consistent with the effect of human CD40L trimer on DC maturation, which also demonstrated that a small subpopulation of DCs expressed high levels of CD83 [40]. Our results showed that the native conformation of CD40L in chimeric CD40L/SHIV-VLP is more efficient in inducing DC maturation and activation compared with soluble CD40L. Using mAb against CD40L could raise a concern that there could be a possibility of antibody-bound CD40L/SHIV VLP taken up by Fc receptors on DC. Therefore, the best approach to exclude this possibility is by blocking the CD40/CD40L interaction using a non-activating antibody against CD40. However, there is no commercial available antibody against CD40 which do not activate CD40 signaling. Alternatively, pretreating the DCs with an antibody against the Fc-gamma receptor could

also address the concern. However, since Fc-gamma receptors on DC also involve activation signaling, Fc blocking antibody may also have DC activation effect. Nevertheless, we believe the current approach is the best we can do to prove that addition of CD40L is important in CD40L/SHIV VLP involved DC activation.

CD40L binding to CD40 on DCs leads to increased expression of surface MHC, adhesion, and costimulatory molecules, facilitating antigen processing and presentation by MHC class II molecules, and promoting secretion of various pro-inflammatory cytokines such as TNF- α , IL-1, and IL-12 [15,41–45]. Whether IFN- γ can be secreted by DCs is controversial [46,47]. Recent evidence clearly showed IFN- γ secretion by human blood monocyte-derived DCs [48–52]. We found that higher levels of IL-12 p70, IFN- γ , and TNF- α were produced by DCs treated with CD40L/SHIV-VLP compared with LPS or SHIV-VLP. In contrast, levels of IL-12, IFN- γ , and TNF- α production were reduced when CD40L was blocked by anti-CD40L antibody. These results indicate that enhanced secretion of IL-12, IFN- γ , and TNF- α is due to CD40L incorporation into the SHIV-VLP. Our data are consistent with a previous report that CD40L could deliver both tumor-derived antigens (Ag) and maturation stimuli simultaneously to DCs while also leading to the production of proinflammatory chemokines and pro-Th1 cytokines like IL-6, IL-8, IL-12, IFN- γ , and TNF- α [53]. Another report showed that HIV-1-infected DCs had up-regulated cell surface markers but failed to produce IL-12 in response to CD40L stimulation, suggesting that HIV-1 infection disables DC function [54]. However, our results show that without virus-infection, CD40L VLP induce high level IL-12 production, which polarizes CD4⁺ T helper 1 (Th1) cells, enhances proliferation of CD8⁺ T-cells, and activates NK cells. These findings also demonstrate that CD40L incorporated into VLP is effective in activating DCs to secrete Th1 type cytokines. Therefore, a CD40L/SHIV-VLP vaccine might be of therapeutic benefit to HIV-1 patients.

In the current study, we showed that soluble CD40L protein alone did not induce expression of DC maturation markers such as CD40, CD83, and CD86. Two other reports [6,55] showed that recombinant human soluble CD40L alone only induced IL-12 p40 but failed to induce IL-12 p70 production by DCs since IL-12 p70 secretion is IFN- γ dependent. Another report [22] showed that trimeric recombinant human CD40L enhanced the cell surface expression of CD80, CD83, and CD86 and secretion of TNF- α and IL-12 p40 by DC; however, the expression of DC maturation marker CD83 was not demonstrated in a dose-dependent manner [56,57]. Consistent with the current study, only when CD40L was delivered with different viruses, such as vaccinia virus, adenovirus, and lentivirus-expressed CD40L, could CD40L stimulate DCs to produce IL-12 [56–59]. Virus-induced IFN- γ secretion by monocytes may play an important role in enhancing CD40L-triggered DC maturation. Thus, the incorporation of CD40L in CD40L/SHIV-VLP could efficiently enhance immune modulation of DCs by SHIV-VLP. Vaccination strategies based on the activation of DCs through CD40 may be especially critical in eliciting CTL responses in conditions such as AIDS where the number or activity of CD4⁺ T_H cells is limiting.

From a technical aspect, we found that measuring positively stained cell populations (percentages) by flow cytometry is more sensitive than measuring MFI to study the increase of cell surface protein expression in response to certain treatments; however, this approach is appropriate only if this protein is initially at relatively low levels, such as CD40 and CD83. However, if a protein is initially at relatively high levels (such as CD86), measuring MFI is more sensitive than measuring percentages to monitor its expression increase after treatment [22]. Since CD40 expression was low in iDC (31%), the change of percentage of positive cells is a sensitive method to compare this marker expression resulting from different VLP treatments, while change in MFI was marginal in this case. In contrast, we observed MFI changes in CD86 expression were more sensitive for comparing different

treatments because changes in the percentage of positive cells in this case was masked by the high overall percentage of CD86 positive cells (>90% in iDC) before VLP treatment.

DCs treated with chimeric hCD40L/SHIV-VLP had increased expression of costimulatory molecules and production of inflammatory cytokines, which correspond to the enhanced ability of DC to activate T-cells compared with DCs treated with SHIV-VLP, soluble CD40L protein, or a mixture of SHIV-VLP and soluble CD40L protein. Previous studies showed TNF- α or CD40 triggering can promote DC maturation and enhance allostimulatory activity [15,46,48,60,61]. Other reports indicated that enhanced surface marker expression was not accompanied by increased activation of allo- or antigen-specific T-cells [22,62]. These discrepancies in both aspects of the results suggest that the sensitivity of the assays for functional and phenotypic characterization differ considerably. Our study indicates that both antigenic components (SIV Gag and HIV Env) and the DC activation molecule (CD40L) in their native structural state residing simultaneously in the same particles can lead to more effective antigen presentation by DCs to induce T-cell proliferation more potently.

The titer of IgG in response to specific antigens may be critical for desired therapeutic effects in clinical applications of a vaccine. In the current study, we found that mice i.d. immunized with SHIV-VLP or mCD40L/SHIV-VLP, or even SHIV-VLP alone could induce high titer anti-HIV Env specific IgG. mCD40L/SHIV-VLP significantly enhanced the production of anti-HIV Env-specific IgG, which was due specifically to the incorporation of CD40L into the VLP. Our anti-HIV Env IgG production data are consistent with our previous studies that mice could had enhanced anti-HIV Env IgG production when immunized by intranasal route with SHIV-VLP and HA/SHIV-VLP [25]. Mucosal immunization of HA/SHIV-VLP also resulted in high titers of anti-HA IgG in the serum. The effect of an enhanced mucosal immune response against HIV Env by foreign influenza hemagglutinin (HA) incorporation could be caused by either production of anti-HA IgG to universally enhance the immune responses or by binding of HA to promote cell-to-cell interactions [24]. However, enhancement of the humoral immune response against HIV Env by CD40L incorporation is not well understood. Other reports have shown that fusion of the TNF- α protein to VLP increased IgG titers against TNF- α by 1000-fold compared with fusion proteins alone, but there was no significant difference in the response to self and foreign epitopes after vaccination [63]. We also found high titers of anti-mouse self CD40L IgG in the serum as measured by ELISA (data not shown). Originally, we hypothesized that the CD40-CD40L interaction would enhance the immunogenicity of SHIV-VLP, therefore, CD40L protein was successfully incorporated into SHIV-VLP as a potent adjuvant and costimulator. Interestingly, high titer CD40L antibody was observed after mCD40L/SHIV-VLP immunization. It is possible that the high titer antibody against CD40L could neutralize CD40L, and therefore reduce the immunogenicity of SHIV-VLP. Paradoxically, we found potent enhancement of immunogenicity of mCD40L/SHIV-VLP despite high titers of CD40L antibodies. This result presents a dilemma to explain the enhancement in the immune response by CD40L protein incorporated VLP. More recent reports have shown that injecting mice with recombinant IL-2 and IL-2-specific monoclonal antibodies, previously designated as cytokine-neutralizing antibodies, augmented the proliferation of CD8⁺ T-cells [64]. Monoclonal antibodies that bind to different sites on IL-2 can enhance, rather than neutralize, the potency of the cytokines *in vivo* and stimulate the proliferation of the different populations of lymphocytes [65]. Therefore, if the cytokine/antibody complex plays an additional role here, the potent enhancement of the immune response against HIV Env by chimeric mCD40L/SHIV-VLP could be caused by both anti-CD40L IgG production and CD40L-CD40 ligation on antigen presenting cells. This could be another interesting point for further investigation.

In conclusion, exposure of chimeric CD40L/SHIV-VLP to human CD14⁺ monocyte-derived iDCs induces phenotypic maturation of DCs as evaluated by the expression of costimulatory molecules and the production of proinflammatory cytokines. Subsequently, these DCs greatly enhanced T-cell allostimulatory activity as compared with DC that matured in response to LPS. In addition, immunization by mCD40L/SHIV-VLP induces strong humoral and cellular immune responses against HIV Env. The results presented here indicate that chimeric CD40L/SHIV-VLP could be a potent HIV vaccine candidate. Furthermore, chimeric CD40L/SHIV-VLP matured-DCs could be used as an immunotherapy strategy. Thus, the capacity of a chimeric CD40L/SHIV-VLP-based vaccine to elicit broad immune responses may have clinical applications for both preventive and therapeutic vaccines against AIDS.

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Figure 1.

Expression and incorporation of CD40L into chimeric CD40L/SHIV-VLP. Sf9 cells were infected by rBV CD40L at an m.o.i. of 10. At 24 h post infection, the expression of CD40L was measured at the surface of Sf9 cells by FACS analysis with monoclonal antibody to hCD40L FITC (A) or mCD40L PE (B). Serially diluted hCD40L/SHIV-VLP or mCD40L/SHIV-VLP was added to a 96 well polystyrene microplate coated with a polyclonal antibody against hCD40L or mCD40L, respectively. Subsequently, monoclonal antibody against the respective CD40L conjugated with HRP was added. After addition of the substrate and chromogen, the plate was read at 405 nm within 30 min by using a microplate reader. CD40L concentration was calculated against a standard curve of the purified CD40L protein. Human CD40L incorporation amount is shown in (C) and mouse CD40L incorporation amount is shown in (D). Data are presented as mean $\pm$ standard deviation. P values (**p<0.01) were calculated by Student t-test. n=3.

Figure 2.

Enhancement of phenotypic activation of DCs by chimeric hCD40L/SHIV-VLP and blocking of hCD40L/SHIV-VLP-induced DC activation by hCD40L specific antibody. Chimeric hCD40L/SHIV-VLP was incubated with human CD14⁺ monocytes-derived DCs at 4°C for 1 h. Primary antibody against HIV Env then was added the samples were incubated at 4°C for 30 min. Secondary antibody against rabbit Fc, conjugated with FITC, was used and incubated at 4°C for 30 min at a 1:500 dilution. Binding of SHIV-VLP with DCs is shown in (A) and binding of hCD40L/SHIV-VLP is shown in (B). (C). Activation of DCs by chimeric hCD40L/SHIV-VLP. DCs were treated with or without VLP for two days before analyzing surface marker up-regulation by FACS. FITC-conjugated primary antibodies to different molecules were used. Dotted lines represent isotype controls and solid lines represent expression of specific molecules indicated. Numbers inside each insert represent percentage of positive cells in M1 region. Data represent one of three independent experiments with different donors. (D, E). Blocking of hCD40L/SHIV-VLP-induced DC activation. DCs were treated with SHIV-VLP, chimeric hCD40L/SHIV-VLP, soluble hCD40L protein, SHIV-VLP plus soluble hCD40L protein, chimeric hCD40L/SHIV-VLP plus anti-hCD40L antibody, and chimeric hCD40L/SHIV-VLP plus anti-SV5 irrelevant antibody, respectively, for two days. Untreated DCs were used as a negative control and cells treated with LPS as a positive control. DC surface molecules (D). CD40 and CD83, and (E). CD86 was analyzed by FACS. Data are presented as mean $\pm$ standard deviation based on three separate experiments. These experiments also were repeated in cells obtained from three different donors with similar results. Statistical analysis calculated by Student t-test in between hCD40L/SHIV VLP treatment with either anti-hCD40L pre-treated or irrelevant antibody treatment control group was indicated as P values (** $p < 0.01$ and * $p < 0.05$). (F). Physical interaction of CD40L/SHIV VLP with CD40 on LPS activated CD40 highly expressed B cells. Subpanels a-c represents human LPS activated B cells and subpanels d-f represents mouse LPS activated B cells. Subpanels a and d showed high levels of CD40 expression in the LPS activated B cells without any other treatment. Subpanels b

and e showed no blocking of anti-CD40 antibody binding to the LPS activated B cells in the presence of SHIV VLP. Subpanels c and f showed blocking of anti-CD40 antibody binding to the LPS activated B cells in the presence of CD40L/SHIV VLP. hCD40L/SHIV or mCD40L/SHIV VLP was added to LPS activated B cells at 10 µg/ml in PBS at 4°C for 4 hrs. After thoroughly washing and Fc receptors blocking, the anti-CD40 antibodies were incubated with the cells for additional 30 minutes on ice. Light grey line represents isotype control antibodies and dark line represents anti-CD40 antibodies.

Figure 3.

Enhanced allogeneic T-cell-stimulatory activity of chimeric CD40L/SHIV-VLP-activated DCs. (A). Different VLP-activated DCs were co-cultured with purified allogeneic T-cells at the ratio of 1:20 for five days. (B). DCs activated by different VLP or hCD40L antibody-treated hCD40L/SHIV-VLP were co-cultured with purified allogeneic T-cells at the ratio of 1:40, 1:80, 1:160 for five days. [³H] TdR (1 μ Ci/well) was added for 18 h before harvesting. The cell mixture was harvested by Filtermate Harvester and cell-associated radioactivity was determined by TopCount-NXT. Data are presented as mean $\pm$ standard deviation. P values (* p <0.05 and ** p <0.01) were calculated by Student t-test.

Figure 4.

Enhanced anti-HIV Env antibody production in chimeric mCD40L/SHIV-VLP immunized mice. VLP were used to immunize mice at 0, 2, 4 and 6 weeks. Low 20 µg and high 100 µg doses were used for immunization. C57BL/6J mice (5 per group) were immunized intradermally with SIV gag-VLP, SHIV-VLP, or mCD40L/SHIV-VLP. One week after the final boost, blood serum samples were collected and IgG antibodies were analyzed by ELISA at a dilution of 1:10,000. Data are presented as mean $\pm$ standard deviation. P values (** $p < 0.01$) were calculated by Student t-test. $n = 5$.

Figure 5.

Increased levels of cytokine production in mCD40L/SIV-VLP immunized mouse splenocytes after SIV Gag peptides pulse. Splenocytes were isolated from mice immunized with SIV-VLP or mCD40L/SIV-VLP (20 μ g) two weeks post final boost. Splenocytes then were stimulated with SIV Gag peptide pool 1 (1-259 a.a.) and pool 2 (249-510 a.a.) at 10 μ g/ml. IFN- γ (A) and IL-4 (B) production were determined by ELISPOT assay. Numbers of IFN- γ or IL-4-producing cells were determined by enumeration of spots in specific peptide pool-stimulated wells and subtracting the number of spots in irrelevant peptide-stimulated wells. Data are presented as mean $\pm$ standard deviation. P values (* p <0.05) were calculated by Student t-test. n =5.

Figure 6.

Elevated cytokine production in mCD40L/SHIV-VLP immunized mouse splenocytes after stimulation with Gag or Env peptides. Splenocytes were isolated from mice immunized with SHIV or mCD40L/SHIV-VLP (20 and 100 μ g) one week after the final boost. Isolated splenocytes then were stimulated with SIV Gag or HIV Env peptide pools at 10 μ g/ml. IFN- γ (A) and IL-4 (B) production were determined by ELISPOT assay. Numbers of IFN- γ or IL-4-producing cells were determined by enumeration of spots in specific peptide pool-stimulated wells and subtracting the numbers of spots in irrelevant peptide-stimulated wells. Data are presented as mean $\pm$ standard deviation. P values (* p <0.05 and ** p <0.01) were calculated by Student t-test. n =5.

Figure 7.

Increased CD8⁺ T cells and IFN- γ production and triple cytokine-positive CD4⁺ T cell numbers in chimeric mCD40L/SHIV-VLP immunized mouse splenocytes as measured by intracellular cytokine staining. Splenocytes were isolated from mice immunized with PBS, SHIV-VLP or mCD40L/SHIV-VLP one week after the final boost. Splenocytes then were stimulated with an HIV Env peptide pool at 2 μ M. IFN- γ producing CD8⁺ T-cells and IL-2, IFN- γ , and TNF- α -producing CD4⁺ T-cells were measured by intracellular cytokine staining. (A). CD8⁺ IFN- γ producing T-cells. Gated on CD8⁺ T cells and Y-axis represents IFN- γ production. Numbers shown are percentage of active CD8⁺ IFN- γ -producing T-cells; (B). Triple cytokine IL-2, IFN- γ , and TNF- α cell population in CD4⁺ T-cells among three immunization groups. Numbers shown on Y-axis are percentage of active triple cytokine positive CD4⁺ T-cells. Samples were analyzed using the FACSCalibur. Data analysis was

performed using Flowjo, and the results are presented as dot plots. The data represents one of five independent experiments with different mice.

Figure 8.

Increased HIV Env-specific CTL activity elicited by chimeric mCD40L/SHIV-VLP immunization. At eight weeks post initial immunization, splenocytes isolated from PBS, SHIV-VLP, and mCD40L/SHIV-VLP immunized groups were stimulated with an HIV Env peptide pool at 2 μ M for five days to generate effector cells. EL-4 cells pulsed with HIV Env peptide pool (2 μ M) for 1 h was used as target cells. Different effector-to-target cell ratios (50:1, 25:1, 12.5:1, 6:1, and 3:1) were mixed for 4 h. The specific lysis of target cells was measured by LDH release and calculated using formula in the Cytotox 96 assay kit (Promega, Madison, WI). Data are presented as mean $\pm$ standard deviation. The data represent one of five independent experiments with different mice. P values (* p <0.05) were calculated by Mann-Whitney test. n =5.

Table 1

Effect of hCD40L antibody on hCD40L/SHIV-VLP-induced secretion of Th1 type of cytokines.

	TNF-α (pg/ml)	IFN-γ(pg/ml)	IL-12 p70 (pg/ml)
Untreated	18	81	3
SHIV-VLP	428	105	4
hCD40L/SHIV-VLP	3200	194	49
hCD40L Ab+hCD40L/SHIV-VLP	339	103	6
SV5 Ab+hCD40L/SHIV-VLP	2277	164	47
LPS	2701	157	11

* Data shown are representative one of the three independent experiments with different donors.

Table 2

Mouse immunization schedule

Immunized Ag	Dose (μg)	Route	Boost (wk)	Sacrifice (wk)
SF9 sup.	20	i.d.	2, 4, 6	8
SIV VLP	20	i.d.	2, 4, 6	8
mCD40L/SIV-VLP	20	i.d.	2, 4, 6	8
SHIV-VLP	20	i.d.	2, 4, 6	8
SHIV-VLP	100	i.d.	2, 4, 6	8
mCD40L/SHIV-VLP	20	i.d.	2, 4, 6	8
mCD40L/SHIV-VLP	100	i.d.	2, 4, 6	8