Incorporation of GPI-anchored GM-CSF or CD40 ligand enhances immunogenicity of chimeric simian immunodeficiency virus-like particles

Ioanna Skountzou¹, Fu-Shi Quan¹, Sailaja Gangadhara¹, Ling Ye¹, Andrei Vzorov¹, Periasamy Selvaraj², Joshy Jacob¹, Richard W. Compans¹*, and Sang-Moo Kang¹,*

Running title: Enhanced Immunogenicity of Chimeric SIV VLPs

¹Department of Microbiology and Immunology and Emory Vaccine Center
Emory University School of Medicine,
1510 Clifton Rd, Atlanta, GA 30322
²Department of Pathology and Laboratory Medicine
Emory University School of Medicine.

*Co-corresponding authors

Richard W. Compans: compans@microbio.emory.edu
Sang-Moo Kang: skang2@emory.edu
ABSTRACT

The rapid worldwide spread of HIV mandates the development of successful vaccination strategies. Since live attenuated HIV cannot be used as a vaccine due to safety concerns, virus like particles (VLPs) offer an attractive safe alternative because they lack the viral genome yet they are perceived by the immune system as a virus particle. We hypothesized that adding immunostimulatory signals to VLPs would enhance their efficacy. To accomplish this we generated chimeric simian immunodeficiency virus (SIV) VLPs containing either GPI-anchored granulocyte-macrophage colony-stimulating factor (GM-CSF) or CD40 ligand (CD40L), and investigated their biological activity and ability to enhance immune responses in vivo. Immunization of mice with chimeric SIV VLPs containing GM-CSF induced SIV Env-specific antibodies as well as neutralizing activity at significantly higher levels compared to those induced by standard SIV VLPs, SIV VLPs containing CD40L, or standard VLPs mixed with soluble GM-CSF. In addition, mice immunized with chimeric SIV VLPs containing either GM-CSF or CD40L showed significantly increased CD4+ and CD8+ T cell responses to SIV Env, as compared to standard SIV VLPs. Taken together, these results demonstrate that the incorporation of immunostimulatory molecules enhances humoral and cellular immune responses. We propose that anchoring immunostimulatory molecules to VLPs can be a promising approach to augment the efficacy of VLP antigens.
INTRODUCTION

With HIV spreading worldwide, the development of an effective, safe, and affordable vaccine is a crucial goal for controlling the HIV pandemic. At present, there is no vaccine against HIV that has been approved for license. Chemically inactivated or attenuated live viruses have been developed for some traditional vaccines approved for use in humans. However, with HIV, there are potential safety concerns relating to either incomplete inactivation or to the potential reversion of an attenuated vaccine. Therefore, current approaches to develop HIV vaccines have been proposed based on recombinant vectors, recombinant proteins, or multi-protein assemblies such as VLPs.

Most vaccines depend on their capability to induce protective antibody responses. However, in contrast with other approved vaccines against infectious agents, replicating recombinant vector and DNA vaccines against HIV currently under study primarily induce cell mediated cytotoxic T lymphocytes (19, 30). Although a number of these vaccines prolong survival in primates, they do not prevent against infection. Thus it is a high priority to design alternative vaccines that are more effective in the induction of neutralizing antibodies with the potential to block the initial step of infection. In this respect, virus-like particles (VLPs) are an attractive type of recombinant protein vaccine. Expression of the HIV or SIV Gag and Env proteins results in the self-assembly of a core structure which is released by budding at the cell surface to produce particles containing Env, that are similar in size to viruses but lack viral genetic materials. VLP-based vaccines are currently under investigation for several families of human viruses, including hepatitis viruses, papillomavirus, rotavirus, parvovirus, and influenza virus (3, 8, 17, 21, 39). Several studies have demonstrated the induction of neutralizing antibodies by HIV VLP immunization using murine models (9, 13, 52) or primates (33). Importantly, VLP
Antigens can be processed to present antigens through the major histocompatibility class (MHC) II pathway as well as the MHC I endogenous pathway, inducing both CD4+ and CD8+ T cell mediated immune responses (4, 12, 40). Although VLPs are a promising candidate for HIV vaccines, it is highly desirable to develop approaches to enhance the immunogenicity of VLPs such that both efficacious humoral and cellular immune responses can be induced.

Here, we investigated the hypothesis that immunostimulatory molecules can be incorporated into chimeric VLPs to increase their efficacy. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is known to expand myeloid-derived dendritic cell (DC) populations (20, 47), to augment antigen-induced humoral and cellular immune responses and to affect the Th1/Th2 cytokine balance (45). It has been extensively used as an effective genetic or protein adjuvant to enhance immunogenicity of tumor or vaccine antigens (6, 14, 16, 28, 29, 31, 35, 42, 48, 50, 54, 56). Another immunostimulatory molecule is CD40 ligand (CD40L) which is a surface molecule primarily expressed on mature CD4+ T cells. Interaction between CD40L and CD40 is important for T cell dependent B cell activation and isotype switching (5, 49). Binding of CD40L to CD40 modulates the cellular immune responses by inducing IL-12 production and expression of co-stimulatory molecules residing on APCs. As a result of the upregulation of costimulatory molecules (51, 58) the APCs are activated, the CD4+ T cell responses are augmented by increased cytokine production (10) and CD4+ dependent naïve CD8+ T cells are activated in vivo (44). Genetic fusion of CD40L to DNA vaccines was demonstrated to be effective in enhancing the cellular immune responses to a vaccine antigen (11, 55).

In the present study, we produced a GPI-anchored form of GM-CSF and investigated its expression and assembly into SIV VLPs. Similarly, we expressed CD40L for production of chimeric VLPs containing the SIV Env and Gag proteins. We then investigated the immune
responses to these chimeric VLPs as well as the biological activities of these particles on cells of the immune system.

**MATERIALS AND METHODS**

**Protein and peptide antigens.** For ELISPOT and ELISA assays the following peptide stimulants were used: 1) Two peptides derived from SIVmac239 Env: Env amino acid (aa) 211-230 (CNTSVIQESCDKHYWD AIRF) and Env aa 231-250 (RYCAPPGYALLRCNDTNYS G) as Env MHC I peptide stimulants (at final conc 1 µg/ml), and 2) RQIINTWHKVGKNVYL Env (aa 435-450) as a MHC II peptide (final conc. 2.5 µg/ml). 3) SIVmac239 Env peptide pools; peptides are 15 amino acids in length, with 11-amino acid overlaps between sequential peptides. All peptides were obtained from the NIH AIDS Research and Reference Reagent program.

For *in vitro* cultures, soluble recombinant murine GM-CSF, IL-4 and CD40L were purchased from Peprotech (Rocky Hill, NJ), and used at 50 ng/ml (500 U/ml). The final concentration of VLPs in culture supernatants ranged from 1 to 2 µg/ml.

**DNA constructs.** Plasmids containing cDNAs encoding GM-CSF with a glycosyl-phosphatidylinositol (GPI)-anchoring domain of CD59 and LFA3 were described previously (36). These plasmids were digested with Hind III and Apa I (for CD59 GPI) or Xba I (for LFA3 GPI) to obtain DNA fragments of GM-CSF fused to the GPI anchoring domain. After filling in with the Klenow polymerase fragment to obtain blunt ends, GM-CSF DNA fragments were ligated into the pSP72 vector with the T7 promoter, which was subsequently used to transform E. coli DH5α cells. pSP72 constructs isolated from bacterial clones were screened by restriction
enzymes and the correctness of the GM-CSF DNA constructs was confirmed by DNA sequencing and protein expression in HeLa T4 cells using the T7 recombinant vaccinia virus expression system as described (24). Sma I and Xba I DNA fragments of GM-CSF pSP72 plasmids were cloned into baculovirus expression vector pc/pS1 and used to produce recombinant baculoviruses (rBV). GM-CSF containing pc/pS1 plasmids were transfected into Sf9 insect cells using the Baculo-Gold transfection kit (BD Pharminogen) by following the manufacturer’s manual. Plaques of rBV were screened by their ability to express GM-CSF. The cDNA encoding mouse CD40L (obtained from Dr. Mark Feinberg, Emory University) was PCR-amplified using the following primers: F-CD40L-Sma I, 5’-CCTT CCCGGG ACC ATGATAGAAACATACAGC- 3’ and R-CD40L-Xba I, 5’- CTG CAG TCT AGA TCA GCG CAC TGT TCA G – 3’ (underline denotes restriction enzyme recognition sites). The Sma I and Xba I digested CD40L encoding DNA fragment was cloned into the pSP72 vector, and CD40L pSP72 constructs were screened by restriction enzymes (Sma I and Xba I) and confirmed by DNA sequencing. Similarly, the DNA segment for CD40L from the pSP72 construct was cloned into the pc/pS1 rBV shuttle vector, and an rBV expressing CD40L was generated using a Baculo-Gold transfection kit. The virus titer was determined with a Fast Plax titration kit according to the manufacturer’s instructions (Novagen, Madison, WI).

**Cell surface expression.** Sf9 insect cells were infected with rBV expressing SIV Gag (SIVG) (m.o.i. 2) as a negative control, GM-CSF (m.o.i. 2) or CD40L (m.o.i. 2) and cultured in suspension. One million cells were harvested and stained for flow cytometry. For GM-CSF, a rat anti-GM-CSF (A2/F17-107) monoclonal antibody was mixed with rBV-infected cells at final concentration 5 µg/ml and incubated at 4 °C for 30 min. As a secondary antibody, FITC-
conjugated goat anti-rat-IgG (Zymed) was incubated at a dilution of 50 in phosphate saline buffer (PBS) with 2% FBS for 30 min at 4°C. For CD40L, PE-conjugated hamster anti-mouse CD40L (BD-PharMingen) was used at a dilution of 200 in PBS with 2% FBS. After staining, cells were fixed in PBS with 1% paraformaldehyde, and analyzed with a FACS Calibur instrument (Becton Dickinson) and WINMDI 2.8 software (Scripps Research Institute Cytometry Software).

Production of VLPs. SIV VLPs were produced using a modification of a previously described method (57). For Gag VLPs Sf9 insect cells were infected with rBV expressing SIVmac239 Gag at a m.o.i of 2 and incubated at 27°C for 72 hr. SIV VLPs containing SIV Env and Gag were produced from Sf9 cells co-infected with rBVs expressing SIV Gag or SIV Env (SIVmac239) at m.o.i. ratios of 1:4. Chimeric SIV VLPs were produced from Sf9 cells co-infected with rBV expressing SIV Gag, SIV Env and CD40L or GM-CSF at m.o.i ratios 1:4:4. Three days post-infection the culture supernatants were collected and centrifuged at 1,500 x g for 20 min, filtered through a 0.45-µm-pore size filter, and the VLPs were pelleted at 100,000 x g for 1 hr at 4°C in a Beckman SW28 rotor. The pellets were resuspended in PBS at 4°C overnight and VLPs were further purified through a 20-35-60% discontinuous sucrose gradient at 28,000 rpm for 1 hr at 4°C. The VLP bands were collected, washed with PBS, pelleted, and resuspended overnight in PBS. To quantitate the yield of purified VLPs, the protein concentration of each sample was estimated with the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). For protein analysis, all samples were normalized to 1 µg/ml and they were loaded on SDS gels at the same concentration (5 or 10 µg), and SIV Gag and Env proteins, GM-CSF, and CD40L were probed using monkey anti-SIVmac239 sera (kindly provided by Dr. Silvija Staprans, Emory Vaccine Center), rabbit anti-mouse GM-CSF (Peprotech), and goat anti-mouse CD40L (Peprotech).
Electron microscopy. To analyze the quality and purity of VLP preparation, purified VLPs (5 µg) were applied to a formvar carbon coated grid at room temperature for 1 min. Excess VLP suspension was blotted with filter paper, and the grid was immediately stained with 1% uranyl acetate for 30 seconds. Excess stain was removed by filter paper, and the samples were examined using a transmission electron microscope.

Co-immunoprecipitation. To determine whether the targeting molecules (GM-CSF, CD40L) are efficiently incorporated into the same VLPs as the viral Env protein, samples from each VLP group were immunoprecipitated with 1:50 or 1:100 diluted anti-GM-CSF or anti-CD40L antibodies, run on SDS-PAGE and probed with anti-SIV antibody (1:5,000 in PBS-T with 1% skim milk). Other samples of chimeric VLP group were immunoprecipitated with anti-SIV sera (1:100 or 1:500) and then probed with anti-GM-CSF or anti-CD40L antibodies (1:1,000 and 1:7,000) after being separated on SDS-PAGE gels.

Quantitative ELISA for Env, Gag, GM-CSF and CD40L. To estimate the percentage of Env incorporation in VLPs, we used a sandwich ELISA; 96-well Nunc Maxisorb flat bottom plates were coated overnight with 1:1,000 dilution of SIV mac251 gp120 monoclonal antibody (NIH AIDS Research and Reference Reagent Program). The VLPs were pretreated with 0.1% RIPA buffer (1 M Tris buffer pH 8.0, 5 M NaCl, 10% Triton, 10% Sodium deoxycholate, 10% SDS) and added at a concentration of 400 ng/well. Goat anti-SIV gp120 (1:4,000) and rabbit anti-goat IgG HRP (1:4,000) diluted in PBS+0.05% Tween 20 supplied with 2% BSA were used as primary and secondary antibodies. Purified SIVmac239 gp130 was used to construct the standard
curve to detect the Env concentration was provided from the NIH AIDS Research and Reference Reagent Program (National Institutes of Health, Rockville, MD).

To estimate the percentage of incorporation of growth factors into the chimeric VLPs we used a direct ELISA assay. VLPs were used to coat each well (5 µg per well) of a 96-well Nunc Maxisorb flat bottom plate. For the determination of GM-CSF, rabbit anti-mouse GM-CSF (1:2,000) and goat anti-rabbit IgG coupled to HRP (1:2,000) were used. For the determination of CD40L goat anti-mouse CD40L (1:3,000) and rabbit anti-goat IgG HRP (1:5,000) were used. For the standard curves we used soluble recombinant murine GM-CSF and CD40L (PeproTech, Inc, Rocky Hill, NJ). The substrate tablets O-Phenylenediamine (OPD) (Zymed, San Francisco, CA) dissolved in citrate buffer pH 5.0 were used to develop color in all aforementioned assays. Optical density was read at 450 nm.

**Functional characterization of GM-CSF and CD40L incorporated into VLPs.**

*Cell proliferation.* Bone marrow cells were prepared as described (23). 1x10^6 bone marrow cells were labeled with CFSE (carboxyfluorescein diacetate, succinimidyl ester) (Molecular Probes, Eugene, OR) at a final concentration of 1 µM and CFSE was quenched by further incubation in serum containing medium, and extensively washed in RPMI media. To determine the effect of chimeric VLPs on cell proliferation, the CFSE labeled bone marrow cells were cultured in RPMI media in the presence of 1 µg/µl VLPs. Following incubation at 37°C in 5% CO2 for 4 days, cells were harvested and analyzed by FACS. As a separate experiment to identify the phenotypes of cells, bone marrow cultures expanded in the presence of VLPs or rGM-CSF plus rIL-4 were stained with PE conjugated anti-CD11c and APC conjugated anti-CD11b for 20 min at 4°C and fixed in PBS with 1.5 % paraformaldehyde, and analyzed with a FACS Calibur instrument.
(Becton Dickinson). Viable cells were counted by light microscopy after staining with trypan blue.

**B cell activation and isotype switching.** To measure antibody production, 2\times10^6 spleen cells were cultured in triplicate in 48-well round-bottom plates in 500 µl media with or without VLPs or growth factors. VLPs were added at 1 or 2 µg/ml final concentrations. After incubation at 37°C in 5% CO2 for 3 to 5 days, 100 µl of supernatant were collected at days 3, 4, and 5 for measurements of IgG, IgG1, IgG2a, IgG2b, IgG3, IgM and IgA on ELISA plates coated with 4 µg/ml of Ig(H+L) as described (22). To analyze the phenotype of activated cells, spleen cells were cultured as above and collected on day 4. One million cells were stained with PE-conjugated anti-CD69, PerCP-conjugated anti-B220, APC-conjugated anti-CD8 or FITC-conjugated anti-CD4 (eBioscience) for 20 min at 4°C. After staining, cells were washed and fixed with 1 % paraformaldehyde and analyzed with a FACSCalibur.

**Immunizations.** Female Balb/c mice (6-8 weeks of age, 6 mice per group) (Charles River Laboratory, Wilmington, MA), were immunized subcutaneously (s.c.) with VLPs or chimeric VLPs to assess immune responses. All immunizations were performed with one VLP preparation that met all the quality control requirements (Western blot, electron microscopy, co-immunoprecipitation, and quantitative ELISA of proteins incorporated). All animals received a priming immunization (s.c.) of VLPs (50 µg/dose) followed by two s.c. boosts with the same dose at weeks 4 and 8. At 2 weeks after each immunization the animals were bled from the retro-orbital plexus and the sera were used for the determination of SIV Env-specific antibodies with ELISA and neutralization assays.
Evaluation of humoral immune responses. All sera were individually collected, and SIV Env-specific antibody levels for IgG, IgG1, IgG2a, IgG2b and IgG3 SIV Env were quantitatively determined by enzyme-linked immunosorbent assay (ELISA) as described (22). The substrate O-Phenylenediamine (OPD) (Zymed, San Francisco, CA) dissolved in citrate buffer pH 5.0 was used to develop color. Optical density was read at 450 nm and antibody concentrations were determined based on standard curves of each subtype antibodies. Neutralization activity was determined using SMAGI cell assays as described (22). Briefly, pre-immune and immune sera were heat-inactivated at 56 °C for 30 min, serially diluted, incubated with SIVmac1A11 virus (100 pfu) for 1 hr at 37 °C, and then added to the SMAGI cells. β-gal-expressing blue foci indicating infectious spots were counted. Neutralization titers were expressed as reverse values of dilution factors giving 50% reduction of β-gal stained infected cell foci as compared to control wells without serum samples.

Evaluation of cellular immune responses. Spleens were collected from individual mice at 2 weeks after the final immunization and a single cell suspension was prepared, and used for enzyme-linked immunospot (ELISPOT) and cytokine ELISA as described (22). Spleen cells or mesenteric lymph node cells (0.2x10^6/200 µl complete RPMI medium) were prepared from immunized mice at 2 weeks after the last immunization, and stimulated in vitro with Env peptide pools at a final concentration of 1 µg/ml in complete RPMI medium. After 72 hr the cells were centrifuged and the supernatant was collected and stored at –80°C until assayed. The ELISA reagents for IL-6, IL-10, IL-12 and TNF-α were purchased from eBiosciences (San Diego, CA) and for IFN-γ and IL-4 were purchased from BD-PharMingen. Cytokines were determined according to the manufacturer’s instructions. For ELISPOT assay, freshly isolated splenocytes
(0.5-1.0x10⁶/200 µl complete RPMI) from immunized mice were cultured for 36 hours in the presence of peptide stimulants in complete RPMI medium, as previously described (22). All ELISPOT reagents were purchased from BD-PharMingen.

**Statistical analysis.** Results are expressed as the means ± standard errors of the means (SEM). Statistical comparisons were performed by a two-tailed paired test and p<0.05 was considered statistically significant.

### RESULTS

**Generation of chimeric SIV VLPs containing membrane-anchored immunostimulatory molecules.** We have previously produced SIV VLPs by expression of the Gag and Env proteins in insect cells using recombinant baculoviruses (rBVs) as expression vectors (22, 57). Here, we designed membrane-anchored forms of GM-CSF to enable its incorporation into VLPs containing SIV Env and Gag as an approach to induce enhanced immune responses against SIV antigens. Since GM-CSF is a secreted protein, we used glycosyl-phosphatidylinositol (GPI)-anchored forms of GM-CSF constructs (36) as described in Fig. 1A. To anchor GM-CSF to VLPs we used the GPI signal sequences from CD59 and LFA3. Since CD40L is normally expressed in a membrane bound form there was no need to attach any additional membrane anchoring sequences. To express GM-CSF and CD40L in insect cells, we cloned these constructs into a baculovirus shuttle vector and generated rBVs. We then confirmed the cell surface expression of GPI-anchored GM-CSF fusion proteins and CD40L by infecting insect cells with these rBVs and analyzing them by flow cytometry. Both CD59- and LFA3-GPI anchored GM-CSFs (GM-
CSF_{CD59}, GM-CSF_{LFA3} as well as CD40L were found to be expressed on the cell surface (data not shown).

Chimeric SIV VLPs containing membrane-anchored GM-CSF or CD40L were produced by co-infecting insect cells with rBVs expressing SIV Env, Gag, and GM-CSF_{CD59}, GM-CSF_{LFA3}, or CD40L. We harvested VLPs from the culture supernatants and purified them using sucrose gradient ultracentrifugation. The purified VLP preparations were routinely tested for integrity and homogeneity by electron microscopy; the particles were about 90-100 nm in diameter (Fig. 2A). SIV Env proteins were found to be present at similar levels among various SIV VLP preparations (Fig. 2B), which were estimated to be 1.5+/−0.2 % of total VLP proteins as determined by quantitative ELISA. We determined the incorporation of immunostimulatory molecules by Western blot analysis of purified VLPs using antibodies specific to GM-CSF or CD40L (Fig. 2C and 2D). Both GM-CSF_{CD59} and GM-CSF_{LFA3} were found to be incorporated into SIV VLPs at similar levels. Also, CD40L was incorporated efficiently into SIV VLPs. The levels of GM-CSF and CD40L were quantitated by ELISA and determined to be approximately 0.1 % and 0.14 % of total VLP proteins, respectively.

To determine whether viral proteins and cytokines are directly incorporated into the same VLP structures, we used co-immunoprecipitation assays. Chimeric SIV VLPs containing GM-CSF were immunoprecipitated with anti-GM-CSF antibody, and the proteins were probed with anti-SIV antibody after separation by SDS-PAGE. SIV Env and Gag proteins were found to be co-precipitated using these antibodies, indicating that GM-CSF and SIV antigens are present in the same VLP structures (Fig. 2E). Similarly, when chimeric SIV VLPs were first immunoprecipitated with anti-SIV antibody and the blots were subsequently probed with α-GM-CSF, the growth factor was found to be co-precipitated (Fig. 2F). Analogous experiments
confirmed the incorporation of Env, Gag and CD40L into the same VLP structures (data not shown).

**GM-CSF or CD40L incorporated into VLPs is biologically active.** GM-CSF is a potent activator of hematopoietic progenitor cells and induces their differentiation and expansion into myeloid DC populations when supplemented with rIL-4 (25, 53). In order to determine whether GM-CSF incorporated into VLPs maintained this activity, we tested whether these VLPs could induce proliferation of bone marrow cells. We found that GM-CSF<sub>CD59</sub> and GM-CSF<sub>LFA3</sub> anchored to SIV VLPs increased the overall cell numbers of bone marrow cells, cultured for 4 days in the presence of 1 µg/ml SIV VLPs, by 4 and 3 fold, respectively, compared to the medium control (Fig. 3A). In contrast, SIV VLPs did not induce a significant increase in number of viable cells compared to the medium control. We then labeled bone marrow cells with 1 µM CFSE and incubated them for 4 days in the presence of 1 µg/ml SIV VLPs or GM-CSF anchored SIV VLPs. As controls, we set up analogous cultures with spleen cells. As expected, we observed massive expansion of bone marrow cells in the presence of GM-CSF incorporated into VLPs as compared to the SIV VLP control (Fig. 3B). In contrast, neither VLPs induced proliferation of spleen cells (data not shown). We then analyzed the expanded bone marrow cell cultures using flow cytometry for the presence of dendritic cells. We observed that bone marrow cells cultured in the presence of either GM-CSF<sub>CD59</sub> or GM-CSF<sub>LFA3</sub> anchored on SIV VLPs contained significantly higher numbers of CD11c<sup>+</sup>CD11b<sup>+</sup> myeloid DCs as compared to cultures treated with control SIV VLPs (Fig. 3C). As expected CD40L incorporated into VLPs had no effect in stimulating proliferation of bone marrow cells (data not shown).
We also examined bone marrow and spleen cell cultures incubated in the presence of chimeric GM-CSF or CD40L VLPs, SIV VLPs and controls such as recombinant soluble GM-CSF by light microscopy. We observed distinct morphological changes in bone marrow cultures, which were more pronounced after incubation with chimeric VLPs containing GM-CSF and were similar to those observed with rGM-CSF plus IL-4, indicating cell activation and differentiation into DCs (Fig. 4b, 4c, 4d). SIV VLPs and CD40L SIV VLPs induced only minimal changes in cell morphology of bone marrow cells (Fig. 4a). In contrast to the bone marrow cultures, we observed a very characteristic circular clustering of spleen cells when cultured with VLPs containing CD40L (Fig. 4h) and random amorphous clustering in the presence of rGM-CSF and IL-4 (Fig. 4f) or VLPs containing GM-CSF (Fig. 4g). These results suggest that although the target cell populations and the mechanisms of action of GM-CSF or CD40L are different, both molecules retain biological activities when incorporated into SIV VLPs.

**GM-CSF and CD40L incorporated into VLPs activate B lymphocytes.** Both GM-CSF and CD40L play a critical role in the activation of B cells. To determine whether GM-CSF or CD40L-bearing chimeric VLPs are capable of activating B cells, we cultured splenocytes for 4 days in the presence of GM-CSF or CD40L anchored to VLPs, or soluble GM-CSF or CD40L. We then analyzed B cell activation by checking the expression of the activation marker, CD69 (Fig. 5A). We found increased numbers of activated CD69^+^B220^+^ cells when splenocytes were cultured with chimeric VLPs but not with soluble GM-CSF, CD40L or SIV VLP (Fig. 5A). GM-CSF\_LFA3 anchored to VLPs doubled the number of CD69^+^B220^+^ cells (p=0.0052) when compared to the SIV VLP control. The addition of VLPs containing CD40L (V/CD40L) in the culture tripled the number of CD69^+^B220^+^ cells when compared to the SIV VLPs (p=0.0152) and significantly
enhanced the numbers of double positive CD69+ B220+ cells when compared to the GM-CSF VLPs (p=0.0019 for VLPs containing GM-CSF\textsubscript{CD59} and p=0.05 for V/GM-CSF\textsubscript{LFA3}). However, no significant increases were observed in numbers of CD4+CD69+ and CD8+CD69+ T cells (data not shown).

Since splenic B cells were activated by chimeric SIV VLPs containing either GM-CSF or CD40L, we then determined whether these VLPs could induce B cells to produce antibodies. Briefly, we cultured spleen cells for 5 days in the presence of VLPs, soluble GM-CSF or CD40L. At days 4 and 5 we collected culture supernatants and analyzed them for Ig levels by ELISA. GM-CSF\textsubscript{CD59} or GM-CSF\textsubscript{LFA3} VLPs or CD40L VLPs stimulated the production of Ig isotypes compared to the SIV VLP controls (Fig. 5B and 5C). Only marginal differences were observed in IgG1 and IgG2a subclasses when comparing GM-CSF VLPs and CD40L VLPs (Fig. 5B and C). The GM-CSF\textsubscript{CD59} VLPs enhanced production of IgG1 (Fig. 5B) whereas CD40L VLPs showed enhanced IgG2a levels at day 5 (Fig. 5C). The most pronounced effect by CD40L VLPs was observed in the total IgM levels which were at least 2-fold higher than those induced by other VLPs (Fig 5D). Neither soluble GM-CSF nor CD40L activated splenic B cells to secrete Igs. These results demonstrate that GM-CSF or CD40L incorporated into VLPs can activate splenic B cells to secrete antibodies specific to Env antigen on VLPs.

**GM-CSF anchored to VLPs induces enhanced humoral immune responses.** To investigate whether GM-CSF or CD40L incorporated into SIV VLPs can enhance humoral immune responses to the SIV Env protein, groups of mice were immunized subcutaneously (s.c.) with SIV VLPs (Env plus Gag), chimeric SIV VLPs (Env plus Gag plus GM-CSF or CD40L) or control VLPs, Gag VLPs (Env-negative VLPs). We also included an additional control of SIV VLPs...
administered with soluble rGM-CSF. We used 10 ng soluble rGM-CSF because the VLP immunization dose of 50 µg contained approximately 10 ng of GPI-anchored GM-CSF. We measured serum levels of SIV Env-specific IgG at 2 weeks after each immunization (Fig. 6A), and isotypes, IgG1, IgG2a, IgG2b and IgG3 in sera after the last immunization by ELISA (Fig. 6B). The IgG levels induced by the chimeric GM-CSF<sub>CD59</sub> and GM-CSF<sub>LFA3</sub> VLPs were found to be 2.3 (p=0.0104) and 2.8 fold (p=0.0001) higher than those with SIV VLPs, two weeks after the last immunization. In contrast, CD40L incorporated into SIV VLPs did not induce a comparable enhancement of serum antibody responses. When compared with the groups that received a mixture of SIV VLPs and soluble GM-CSF, the chimeric GM-CSF SIV VLPs induced significantly higher levels of antibody responses (2.5 to 3.0-fold) (p=0.03 for GM-CSF<sub>CD59</sub> and p=0.0012 for GM-CSF<sub>LFA3</sub>). Sera from mice that received the negative control, Gag VLPs exhibited minimal background titers (data not shown). In addition, the data show that incorporation of GM-CSF into VLPs did not alter the Th1 vs. Th2 profiles of the antibody responses; the SIV Env-specific IgG2a/IgG1 ratio was similar in all groups (Fig. 6B).

We were surprised that CD40L containing VLPs did not induce strong IgG responses following immunization because these VLPs had shown profound effects on in vitro cultures of splenocytes (Figs. 4, 5). One possibility is that CD40L VLPs fail to induce class switching from IgM to IgG. Therefore we analyzed sera from immunized mice for SIV Env-specific IgM titers. We found that while the IgM levels of mice immunized with GM-CSF VLPs decreased after the second boost, they remained the same in CD40L VLP immunized mice following the primary and secondary boost (Fig. 6C). Taken together these data suggest that chimeric CD40L VLPs fail to induce IgM to IgG class switching in Env-specific B cells.
Next, we assessed the neutralizing activity of the induced antibodies by determining ability of sera to neutralize live SIV 1A11 virus. Sera from the control, SIV Gag VLP immunized mice showed very low neutralizing activity (titer <10) similar to levels found in unimmunized mice (data not shown). Sera from mice immunized with SIV VLPs, SIV VLPs co-immunized with soluble rGM-CSF, or chimeric CD40L VLP exhibited neutralizing antibody end-point titers of 80. In contrast, sera from mice immunized with chimeric GM-CSF VLPs demonstrated a significant increase in neutralization activity with an end-point titer of 320 (Fig. 7A, B). Based upon these results, we conclude that chimeric VLPs containing a membrane-anchored form of GM-CSFs are significantly more effective in inducing neutralizing antibodies than VLPs containing only the Gag and Env proteins, and that anchoring the adjuvant molecule to the VLP is important for the enhancement of this response.

Both GM-CSF and CD40L incorporated into SIV VLPs enhance CD4+ and CD8+ T cell responses. To compare T cell responses to VLPs, we measured cytokine production by ELISA and ELISPOT assays as an indicator of cellular immune responses. Briefly, we isolated spleens from mice immunized with various VLPs and stimulated them with Env-specific MHC I- or MHC II- restricted peptides to quantitate Env-specific CD4 and CD8 cells secreting IL-2, IL-4, IFN-γ, IL-5, IL-6, IL-10, and IL-12 (Fig. 8 A-G). Splenocytes from GM-CSF and CD40L-bearing chimeric VLP-immunized mice demonstrated a 4 to 8-fold increase of CD4 and CD8 T cells producing IL-4 when compared to the SIV VLP group, with the highest numbers observed in the GM-CSF_LFA3 group (Fig. 8A). GM-CSF SIV VLPs induce significantly higher levels of IFN-γ secreting CD4 T cells than CD40L SIV VLP or control SIV VLPs (p=0.0015 for GM-CSF_LFA3 and p=0.05 for GM-CSF_CD59) (Fig. 8B). Both CD40L as well as GM-CSF bearing SIV VLPs
induced significantly higher numbers of Env-specific, IFN-γ secreting CD8 T cells than conventional SIV VLPs (p=0.0016 for GM-CSF<sub>LFA3</sub>, p=0 for GM-CSF<sub>CD59</sub>, and p=0.0185 for CD40L) (Fig. 8B).

The number of CD8 T cells secreting IL-5 was dramatically increased in the GM-CSF<sub>LFA3</sub> VLPs and GM-CSF<sub>CD59</sub> VLPs groups when compared to SIV VLP-immunized mice (Fig. 8C). For CD4 T cell responses, only the GM-CSF<sub>CD59</sub> was significantly higher than SIV VLP and CD40L SIV groups. GM-CSF VLPs induced statistically significant differences in the levels of IL-6 secreting CD8 cells when compared either to SIV VLP (p=0.0008 for the GM-CSF<sub>LFA3</sub> group and p=0.0003 for the GM-CSF<sub>CD59</sub> group, respectively) or to CD40L VLP (p=0.0065 for GM-CSF<sub>LFA3</sub> and p=0.0013 for GM-CSF<sub>CD59</sub>) (Fig. 8D). In the case of IL-10, which is known to have a dual role as a regulatory cytokine and as a Th2-inducing cytokine (27) all VLP groups demonstrated a 2 to 6-fold increase in IL-10 production by CD8 upon stimulation with MHC Class I-restricted peptides as compared to the unimmunized group but only the chimeric GM-CSF VLP groups exhibited a significant difference from the SIV VLP group (p=0.0042 for GM-CSF<sub>LFA3</sub> and p=0.0449 for GM-CSF<sub>CD59</sub>) (Fig. 8E). Mice immunized with chimeric GM-CSF VLPs and CD40L VLPs showed similar increases in CD4 cell numbers secreting IL-2 and IL-12. However, we observed significant differences in the CD8 cells secreting IL-2 between the GM-CSF VLPs and the SIV VLPs (p=0.011 for GM-CSF<sub>LFA3</sub> and p=0.0046 for GM-CSF<sub>CD59</sub> groups) (Fig 8F). In contrast to the results obtained with GM-CSF VLPs, the CD40L VLP-immunized group did not show any effect on IL-6 or IL-2 production but exhibited a 3-fold increase in IL-12-producing CD8 T cells (p=0.0008) (Fig. 8G).

Taken together these data demonstrate that SIV VLPs are capable of inducing Th1 and Th2 type cytokine production. They also demonstrate a potent stimulatory effect of chimeric GM-
CSF VLPs on the production of Th2 type cytokines IL-4, IL-5, and IL-10 and the pro-inflammatory cytokine IL-6. In contrast, the CD40L VLP group was more potent in enhancing the secretion of IL-12, IFN-γ and IL-10. Both GM-CSF and CD40L VLPs were highly effective in inducing IL-4 producing CD4 T cells whereas CD40L VLPs were more efficient at inducing IFN-γ producing CD8 T cells.

**DISCUSSION**

In this study, we have demonstrated for the first time that membrane-bound forms of immunostimulatory molecules, GM-CSF and CD40L can be incorporated into VLPs in a functionally active form to enhance immune responses to viral antigens. Both CD40L and GM-CSF proteins were incorporated into SIV VLPs when expressed in insect cells co-infected with rBVs expressing SIV Gag, Env, and CD40L or GPI-anchored GM-CSF. Further, we demonstrated that these GM-CSF and CD40L molecules incorporated into VLPs maintain their biological activities, and that immunization with chimeric SIV VLPs enhanced antibody as well as CD4 and CD8 T cell responses. GM-CSF-containing chimeric SIV VLPs not only enhanced the levels of SIV-specific antibodies but the SIV neutralization activity of these antibodies was significantly greater than that induced by conventional SIV VLPs.

In order to incorporate GM-CSF into VLPs we initially generated a membrane-bound GM-CSF by adding the transmembrane region of HIV gp160 to GM-CSF (GM-CSF<sub>TM-gp160</sub>). This chimeric GM-CSF was expressed on the cell surface and it exhibited biological activity as determined by the assays described in Figs 3 and 4. Surprisingly these chimeric VLPs with GM-CSF<sub>TM-gp160</sub> were not very effective in enhancing immune responses in mice (data not shown). As
an alternative, we generated membrane-bound GM-CSF by tethering with GPI anchors from CD59 or LFA3. Interestingly, the GPI-anchored GM-CSF was found to be incorporated at higher levels into the VLPs than GM-CSF_{TM-2p160} presumably because GPI-anchored proteins associate with lipid rafts, which are used as sites for HIV viral assembly (34, 41). In addition, the enhanced activity of SIV VLPs with GPI-anchored GM-CSF in vivo could possibly be attributed to greater flexibility and less steric constraints of the GPI anchor.

Although we could easily detect the incorporation of GPI-anchored GM-CSF into VLPs by Western blot, the levels of GM-CSF incorporated were estimated to be low. In the chimeric VLPs, GM-CSF accounted for approximately 0.1 % of the total VLP proteins. However, based upon quantitative ELISA analysis of SIV Env and GM-CSF incorporated into VLPs, the molar ratio of SIV Env trimers vs. GM-CSF is approximately 1:1, suggesting that GM-CSF is incorporated into VLPs as efficiently as SIV Env. Interestingly, the wild type murine CD40L which contains its own transmembrane domain could be effectively incorporated into the SIV VLPs. The level of CD40L in VLPs was 0.14 % of total VLP proteins, somewhat higher than those of GPI-anchored GM-CSF. Although the exact mechanism governing the process of protein incorporation into the budding retrovirus particles is not understood, it is clear that the membrane-anchored immunostimulatory expressed on the cell surface were incorporated into VLPs.

CD40L chimeric SIV VLPs were very efficient in stimulating robust CD4^+ and CD8^+ T cell responses. Our findings on the induction of T cell responses by CD40L are consistent with previously published studies (32, 46, 49). However, CD40L SIV VLPs did not induce enhanced SIV-Env specific serum IgG levels even after three immunizations; the levels were comparable to those found with control SIV VLPs. The lack of enhanced IgG levels was not due to a failure of
CD40L SIV VLPs to activate the B cell compartment since we observed significantly high levels of Env-specific IgM antibodies in CD40L VLP-immunized mice. Instead, the diminished serum IgG levels most likely reflect an absence of IgM→IgG class switching. It is known that cytokines play a critical role in immunoglobulin class switching. Schilizzi et al. have shown that simultaneous B-cell antigen receptor cross-linking along with CD40 engagement in the presence of IL-10 or IL-4 reduced IgG secretion in vitro (43). Thus the decreased IgM→IgG could presumably be explained by the high levels of IL-4 and IL-10 induced by CD40L SIV VLPs combined with CD40-CD40L interactions.

Although immunization with chimeric SIV VLPs containing GM-CSF or CD40L induced comparable responses in the CD4 T cell population, we observed significant differences between these groups in their CD8+ T cell responses. For instance, GM-CSF SIV VLPs induced significantly higher number of CD8+ T cells producing IL-2, IL-5 and IL-6 and significantly lower numbers producing IL-12 than those induced by CD40L SIV VLPs. These observations on the action of CD40L and GM-CSF molecules are consistent with previous studies and may result from activation of different subpopulations of APCs, particularly DCs activated by GM-CSF and CD40L (18, 37). GM-CSF expands the myeloid-related DC subset which induces large amounts of the Th2 cytokines IL-4 and IL-10, in addition to IFN-γ and IL-2 (38). CD40L in combination with CpG DNA has been shown to stimulate plasmacytoid DCs which induce high amounts of IL-12 (26).

In a pre-clinical study using GM-CSF as an adjuvant, 300 to 400 µg GM-CSF administered in combination with a vaccine showed enhanced immune responses without toxicity (7). A therapeutic regimen study also demonstrated that 300 µg daily administration subcutaneously for 3 to 11 days was moderately effective in recovering granulocytes, monocytes,
and polymorphonuclear cells in children with malignant brain tumors or in improving the clinical manifestations (1, 2). Regarding CD40L, a phase I dose-escalation study in patients with advanced solid tumors or high-grade non-Hodgkin's lymphoma demonstrated that a dose of 100 µg CD40L /kg body weight subcutaneously daily for 5 days significantly improved antitumor activity. Considering these previous clinical studies, the levels of GM-CSF or CD40L incorporated into VLPs (approximately 25 µg/kg body weight) should not be a limitation for vaccine application to humans.

In summary, we provide evidence supporting the hypothesis that immunostimulatory molecules can be incorporated into VLPs in their functionally active form resulting in enhancement of immunogenicity of viral antigens. We found that GPI-anchored GM-CSF upon incorporation into SIV VLPs induced significantly high levels of SIV Env specific antibodies, neutralizing activity, and cytokine secreting lymphocytes. This study, as well as our previous work (15), demonstrates that the surface of VLPs can be decorated with various biologically active molecules or immunogenic viral antigens. Further studies are needed to better understand the process of incorporation and to increase these levels of incorporation into VLPs as well as to determine how this affects the immune responses generated.

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REFERENCES


dendritic cells generated by Flt3L or GM-CSF/IL-4 and matured with immune stimulatory agents on the in vivo induction of antileukemia responses. Blood 100:4169-76.


FIGURE LEGENDS

Fig. 1. Design of immunostimulatory molecules. GM-CSF was incorporated into VLPs by generating recombinant GM-CSF constructs that contain the GPI-anchoring domain from either CD59 or LFA3. The resulting constructs were designated as GM-CSF$_{\text{LFA3}}$ and GM-CSF$_{\text{CD59}}$. CD40 ligand (CD40L) was used in its membrane-anchored form. The signal sequences, GPI anchors and transmembrane (TM) domains are shown.

Fig. 2. Characterization of chimeric SIV VLPs containing immunostimulatory molecules. SIV VLPs negatively stained with uranyl acetate (A). We analyzed purified VLPs for the incorporation of SIV Env, Gag proteins and the immunostimulatory molecules, by Western blot analysis. (B) Western blot of purified VLPs (5 µg per well) probed with monkey anti-SIV antibody. Lanes 1: SIV VLPs containing GM-CSF$_{\text{CD59}}$, 2: SIV VLPs containing GM-CSF$_{\text{LFA3}}$, 3: SIV VLPs containing CD40L, 4: SIV VLPs. (C) Western blot analysis of GM-CSF anchored to SIV VLPs using rabbit anti-mouse GM-CSF antibody. Lanes 1: SIV VLPs, 2: GM-CSF$_{\text{CD59}}$ anchored to SIV VLPs, 3: GM-CSF$_{\text{LFA3}}$ anchored to SIV VLPs, 4: Sf9 cell lysate infected with rBV expressing GM-CSF$_{\text{CD59}}$. (D) Western blot of CD40L SIV VLPs probed with goat anti-mouse CD40L antibody. Lanes 1: CD40L SIV VLPs, 2: Sf9 cell lysate infected with rBV expressing CD40L, 3: SIV VLPs. (E) Co-immunoprecipitation showing the incorporation of targeting molecules (GM-CSF, CD40L) into the same VLPs as viral Env protein. Chimeric GM-CSF SIV VLPs and SIV VLPs were immunoprecipitated with 1:50 dilution of anti-GM-CSF antibody and then probed with anti-SIV sera; 1: GM-CSF$_{\text{CD59}}$ anchored to SIV VLPs, 2: purified SIV VLP preparation, used as positive control, 3: SIV VLP. (F) Chimeric GM-CSF SIV VLPs...
and SIV VLPs were immunoprecipitated with anti-SIV sera and then probed with anti-GM-CSF antibody; 1: GM-CSF\textsubscript{CD59} anchored to SIV VLPs immunoprecipitated with 1:100 dilution of anti-SIV sera, 2: GM-CSF\textsubscript{CD59} anchored to SIV VLPs immunoprecipitated with 1:500 dilution of anti-SIV sera, 3: SIV VLPs immunoprecipitated with 1:100 dilution of anti-SIV sera, 4: SIV VLPs immunoprecipitated with 1:500 dilution of anti-SIV sera.

**Fig. 3.** GM-CSF anchored to VLPs stimulates cell proliferation in vitro. A) 10\textsuperscript{6} BM cells were cultured with 1 µg/ml of various VLPs or 50 ng of soluble murine rGM-CSF for four days. Four days later, we determined the absolute number of viable cells in each of the cultures. Medium indicates RPMI media alone, V/SIV denotes SIV VLPs; V/GM\textsubscript{CD59} denotes GM-CSF\textsubscript{CD59} VLPs; V/GM\textsubscript{LFA3} denotes GM-CSF\textsubscript{LFA3} VLPs; V/CD40L denotes CD40L VLPs. This experiment was done six independent times and the error bars denote standard error of the mean (S.E.M). B) Single cell suspensions of bone marrow cells from naïve Balb/c mice were labeled with 1 µM CFSE and cultured in vitro in the presence of 1 µg/ml of VLPs. Four days later, the cells were harvested and the extent of cellular proliferation was judged by CFSE dilution. Representative flow cytometric plots from three independent experiments are shown and numbers indicate percentage of gated populations in each quadrant (average+/standard error). C) In vitro cultures were set up as described in (A) and the cells were stained with APC-conjugated anti-CD11c and PE-conjugated anti-CD11b antibodies. A representative flow cytometric plot from three independent experiments is shown, and numbers indicate percentage of gated populations in each quadrant (average+/standard error). V/GMCSF denotes GM-CSF SIV VLPs.
**Fig. 4.** Chimeric VLPs containing GM-CSF or CD40L induce morphological changes of splenocytes or BM cells upon *in vitro* co-culture. Bone marrow (a-d) and spleen (e-h) cells isolated from naïve Balb/c mice were cultured in vitro from 4-5 days in the presence of 1 µg/ml of various SIV VLPs or recombinant soluble cytokine, GM-CSF (50 ng/ml) and photographed under light microscopy. Representative photomicrographs (40x magnification) are shown. a to d) Bone marrow cultures: (a) V/SIV denotes SIV VLPs; (b) rGM-CSF, recombinant GM-CSF + IL-4; (c) V/GM\textsubscript{LFA3}, SIV VLPs containing GM-CSF\textsubscript{LFA3} (d) V/GM\textsubscript{CD59}, SIV VLPs containing GM-CSF\textsubscript{CD59} VLPs. e to h) Spleen cell cultures: (e) V/SIV, (f) rGM-CSF, (h) V/GM\textsubscript{CD59}, SIV VLPs containing GM-CSF\textsubscript{CD59} VLPs, (g) V/CD40L denotes CD40L VLPs.

**Fig. 5.** Chimeric VLPs induce activation of B cells and enhance immunoglobulin secretion. (A) Spleen cells from naïve mice were cultured, in triplicate, with 2 µg/ml of VLPs, collected 5 days later and stained with PE-conjugated anti-CD69, PercP-conjugated anti-B220 mAbs and analyzed by flow cytometry. The frequency of activated CD69\textsuperscript{+} B220\textsuperscript{+} B cells for each group is shown. The experiment was done three independent times and the error bars denote S.E.M. (B to C) Splenocytes from naïve Balb/c mice were cultured as in (A) and the culture supernatants were collected on days 3, 4 and 5 and assayed for Env-specific IgG1 (B), IgG2a (C) and IgM (D) by ELISA (B-D). The mean and S.E.M. for each experimental group from two independent experiments run in triplicate are shown. The groups are as described in Fig. 3. sCD40L denotes soluble CD40L. * p<0.05 when compared to the V/SIV. ** p<0.05 when compared to the GM-CSF group.
**Fig. 6.** Chimeric GM-CSF SIV VLPs induce robust Env-specific antibody responses. Cohorts of Balb/c mice (6 mice per group) were immunized subcutaneously with 50 µg of purified SIV VLPs either alone or combined with 10 ng of rGM-CSF, chimeric GM-CSF SIV VLPs or chimeric CD40L SIV VLPs. Four and eight weeks later, mice were boosted with the same antigen dose. Serum samples were collected at 2 weeks after each immunization and Env-specific Ig levels (ng/ml) were determined using ELISA. The Env-specific total IgG (A), IgG isotypes (B), and IgM for sera each group are shown. Groups are as described in Fig. 3. V/SIV+rGM-CSF denotes SIV VLPs plus recombinant GM-CSF. Data shown are averages and standard errors from 6 mice per group. * p<0.05 when compared to the SIV group. ** p<0.05 when compared to the V/SIV+rGM-CSF group. * p<0.05 when IgM levels after 2nd boost are compared to antibody after primary immunization.

**Fig. 7.** Assay of virus neutralization activity. Virus-neutralization was done using sera collected at two weeks after the third immunization as detailed in Materials and Methods. (A) Neutralization assay showing the percentage of reduction of plaque forming units (p.f.u) of SIVmac1A11 virus by sera from immunized mice. Data are averages from six individual mice per group, the error bars denote S.E.M. (B) Neutralization titers, expressed as reverse values of dilution factors giving 50% reduction of β-gal stained infected cell foci as compared to positive control. Groups are as described in the Fig. 3 legend.

**Fig. 8.** GM-CSF or CD40L chimeric VLPs induced robust CD4 and CD8 T cell responses in vivo. Briefly, spleen cells from the different groups of immunized Balb/c mice, two weeks after the second booster immunization, were processed individually and cultured in the presence MHC
I and MHC II restricted SIV Env peptides and analyzed for cytokine production by either ELISPOT or ELISA assay. (A) IL-4, (B) IFN-γ, (C) IL-5, (D) IL-6, (E) IL-10, (F) IL-2 and (G) IL-12. For each plot, the closed and striped bars denote CD4 and CD8 T cell responses, respectively. Results are presented as the mean ± SEM from 6 mice per group. Cytokines assayed by ELISA were quantitated (pg/ml) whereas cytokines determined with ELISPOT are shown as number of spots formed per 1x10^6 of cultured cells. Groups are as described in the Fig. 3 legend. * p<0.05 when compared to the V/SIV. ** p<0.05 when compared to the V/CD40L, CD40L VLP group.
Fig. 1

- **GPI-GM-CSF_{LFA3}**
  - Signal sequence
  - GM-CSF (140 aa)
  - LFA3-GPI (34 aa)

- **GPI-GM-CSF_{CD59}**
  - GM-CSF (140 aa)
  - CD59-GPI (30 aa)

- **CD40L**
  - Signal sequence
  - TM
  - CD40L (220 aa)
  - COOH
Fig. 2

A. SIV VLP

B. SIV Gag and Env

C. GM-CSF

D. CD40L

E.

F.
A. Bone marrow cells (x10^6/ml)

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<th>Cells</th>
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<tr>
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<td>V/GM&lt;sub&gt;LFA3&lt;/sub&gt;</td>
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B. Fluorescence Intensity

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C. CD11b vs CD11c

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<tr>
<td>V/GM-CSF</td>
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Fig. 3
Bone marrow

a. V/SIV
b. rGM-CSF
c. V/GM<sub>CD59</sub>

e. Spleen
f. rGM-CSF
g. V/GM<sub>CD59</sub>

Fig. 4
Fig. 6A

Serum SIV Env-specific IgG (ng/ml)

- Pre-immune
- Priming
- 1st boost
- 2nd boost

B.

Serum SIV Env-specific IgM (ng/ml)

Pre-immune
- Priming
- 1st boost
- 2nd boost

C.

Serum SIV Env-specific IgG (ng/ml)

- Prime
- 2nd boost

IgG1
- IgG2a
- IgG2b
- IgG3

V/SIV, V/CD40L, V/SIV + rGM-CSF, V/GMCD59, V/GMLFA3

Fig. 6

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Fig. 7

A. Percentage reduction of SIVmac1A1 p.f.u.

B. Neutralization titers
**Fig. 8A, B**

A. **IL-4 Elispots/10^6 splenocytes**

- CD4
- CD8

B. **IFN-γ Elispots/10^6 splenocytes**

- Naïve
- V/SIV
- V/CD40L
- V/GM_{CD59}
- V/GM_{LFA3}
Fig. 8C,D,E,F,G

C. 

D. 

E. 

F. 

G.