HIV-1 envelope glycoproteins with costimulatory domains for vaccine applications
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CHAPTER 2

A chimeric HIV-1 envelope glycoprotein trimer with an embedded granulocyte-macrophage colony-stimulating factor (GM-CSF) domain induces enhanced antibody and T cell responses

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Abstract

An effective HIV-1 vaccine should ideally induce strong humoral and cellular immune responses that provide sterilizing immunity over a prolonged period. Current HIV-1 vaccines have failed in such immunity. The viral envelope glycoprotein complex (Env) can be targeted by neutralizing antibodies to block infection, but several Env properties limit the ability to induce an antibody response of sufficient quantity and quality. We hypothesized that Env immunogenicity could be improved by embedding an immunostimulatory protein domain within its sequence. A stabilized Env trimer was therefore engineered with the granulocyte-macrophage colony-stimulating factor (GM-CSF) inserted into the variable loops 1 and 2 (V1V2) domain of gp120. Probing with neutralizing antibodies showed that both the Env and GM-CSF components of the chimeric protein were folded correctly. Furthermore, the embedded GM-CSF domain was functional as a cytokine in vitro. Mouse immunization studies demonstrated that chimeric Env \textsubscript{GM-CSF} enhanced Env-specific antibody and T cell responses compared to wild-type Env. Collectively, these results show that targeting and activation of immune cells using engineered cytokine domains within the protein can improve the immunogenicity of Env subunit vaccines.
Introduction

The HIV-1 pandemic continues to spread worldwide. It would be desirable to have a protective vaccine, but despite massive effort over the past 20 years such a vaccine remains elusive. Live attenuated vaccines have provided the most robust protection against viral challenge in animal models, but they are considered unsafe for human use (1-3). Moreover, the immune correlates of protection by live attenuated vaccines remain undefined, hampering the design of safer vaccines (4).

Ideally, a vaccine should induce strong humoral and cellular immune responses that are protective against the extensive variety of HIV-1 strains that are now circulating. T cell-based vaccines can partially control viral replication and reduce the viral set point in animal models (5), but have not yet protected humans in clinical trials (6, 7). HIV-1 Env gp120 subunit vaccines have induced antibody (Ab) responses that could inhibit neutralization-sensitive laboratory strains in monkeys, but no protection has been observed against primary HIV-1 strains (8, 9). Monomeric gp120 vaccines in humans also failed to induce protective Ab responses (10, 11). A recent vaccine trial in Thailand using a recombinant canarypox virus expressing HIV-1 proteins followed by a gp120 protein boost showed a modest reduction in HIV-1 acquisition (12).

Sterilizing protection can be achieved in nonhuman primates by passively immunizing with neutralizing Ab (NAb). Infusion of the NAb b12 directed against the CD4-binding site (CD4BS) on gp120 inhibited vaginal infection with SHIV in a dose-dependent manner (13, 14). Other passive immunization studies using broadly active NAb such as 2F5 or 2G12 also showed protection against SHIV challenge (15-18). Passive immunization with the b12 NAb 2-18 h after viral challenge did not block HIV-1 infection, showing that the time window of NAb to provide protection against HIV-1 acquisition is limited (19). Thus, preexisting NAb can provide sterilizing protection, but inducing them with current vaccines has been unsuccessful.

Formulating antigens in adjuvants can improve their immunogenicity. Commonly used adjuvants that stimulate the innate immune system include agonists of pattern recognition receptors such as are toll-like receptors, as well as inorganic molecules, emulsion surfactants, or other carrier molecules that have immune stimulating properties (20). An alternative, more specific approach is the use of costimulatory host-derived proteins that activate specific
immune cells. The adjuvant effects of such costimulatory molecules can be increased by covalently linking them directly to the antigen of choice (21-24).

One immune molecule that can serve as an adjuvant in vaccines is the granulocyte-macrophage colony-stimulating factor (GM-CSF). This cytokine is produced by fibroblasts, activated T lymphocytes, macrophages, tumor, endothelial, mesothelial and epithelial cells and functions as a hematopoietic growth factor. GM-CSF is secreted during various types of inflammation to stimulate proliferation and prevents apoptosis of various immune cells (25), such as antigen-presenting cells (APCs) (26). Its activity is mediated by the GM-CSF receptor (GMR) that is composed of a specific α-GMR domain and a commonly shared βc subunit that is also a part of the IL-3 and IL-5 cytokine receptor. Signaling is mediated by formation of a hexameric complex consisting of two GM-CSF molecules and the four receptor subunits. Ultimately, two of these GM-CSF-GMR hexamers can dimerize to form a dodecameric complex. Depending on the GM-CSF concentration and the nature of the ligand-receptor complex, GM-CSF can induce different downstream signaling cascades. Low picomolar concentrations of the cytokine promote cell survival, whereas higher GM-CSF concentrations can stimulate cell proliferation.

Here we investigated whether GM-CSF embedded within the HIV-1 trimeric gp140 protein can augment Env-specific immune responses. The rationale for integrating GM-CSF within the Env protein was to simultaneously activate immune cells that are targeted by the antigen, resulting in a selective and specific augmentation of the antigen-specific immune response. To do this, the first and second variable loop domain (V1V2) of Env was replaced by the GM-CSF. The resulting chimeric EnvGM-CSF was folded properly and had GM-CSF activity in vitro. Mice immunized with the chimeric protein had stronger Env-specific Ab and T cell responses than those given unmodified Env protein. Embedding cytokine domains is therefore useful for enhancing the immunogenicity of HIV-1 Env-based vaccines.

Materials and methods

Plasmid construction
The pPPI4 plasmid (Progenics Pharmaceuticals, Tarrytown, NY) encoding stabilized SOSIP.R6-IIZ-H8 gp140 (Env) using optimized codons has been described elsewhere (27-31). The Env construct is based on the subtype B, CCR5 primary isolate JR-FL and is shown in Fig. 1. We also used plasmids encoding Env coupled to B cell activation factor (Env-BAFF)
or CD40 ligand (Env-CD40L). These constructs were generated by exchanging the C-terminal His-tag for a codon-optimized mouse CD40L or BAFF sequence. Codon-optimized DNA encoding either human GM-CSF (amino acids 26-139) or mouse GM-CSF (amino acids 26-136) flanked by HindIII and BmgBI restriction sites was synthesized (Mr. Gene GmbH, Regensburg, Germany). The V1V2 domain of Env was exchanged with the GM-CSF sequences using the HindIII and BmgBI sites. The Env construct containing human GM-CSF was used for Ab probing and in GM-CSF activity assays, whereas the constructs containing mouse GM-CSF were used to immunize mice.

**Reagents**

DC-SIGN-Fc was purchased from R&D Systems (Minneapolis, MN). HIV-Ig was obtained through the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, National Institutes of Health. Monoclonal Abs (mAb) 2F5 and 2G12 were a gift from Hermann Katinger through the ARRRP. CD4-IgG2, soluble CD4 (sCD4) and anti-V3 gp120 mAb PA1 were gifts from Bill Olson (Progenics Pharmaceuticals, Tarrytown, NY). mAb b12 was donated by Dennis Burton (The Scripps Research Institute, La Jolla, CA). mAbs 17b, 48d, 412d and 39F were gifts from James Robinson (Tulane University, New Orleans, LA). Goat D7324 Ab was purchased from Aalto Bioreagents, Dublin, Ireland. Peter Kwong and John Mascola (Vaccine Research Center, Washington) donated VRC01. Anti-GM-CSF Ab (clone Ab54429) was purchased from Abcam (Cambridge, UK). Recombinant human GM-CSF (rhGM-CSF) was obtained from Schering-Plough (Brussels, Belgium). Labeled mouse-specific T cell Ab TCR-APC (clone H57-597), CD3-APC (clone 145-2C11), CD4-PerCP (clone RM4-5), CD8-PE (clone 53-6.7) were obtained from BD Biosciences.

**Mouse immunizations**

Plasmid DNA amplified in DH5α bacteria was isolated using the EndoFree plasmid giga kit (Qiagen, Venlo, the Netherlands). Five outbred NMRI mice/construct were immunized on days 0, 14, 28 and 42 with 20 μg of DNA in the abdominal dermis using gene gun technology. Blood samples were obtained on days 0, 14, 28, and 42. Immunizations were carried out by Genovac (Freiburg, Germinay) at the facilities of MfD Diagnostics (Wendelsheim, Germany). All animals were kept according to DIN EN ISO 9001:2000 standards, the regulations of the German Welfare Act of 19 May 2006 (BGBl. I S. 1206), the regulations of the European Union guidelines 86/609/EWG of November 24, 2006, and the European Agreement of
March 18, 1986 for the protection of animal trials and other scientific studies using vertebrates (Act of December 11, 1990 (BGBI. II S. 1486)). All protocols dealing with animal manipulations are in accordance with guidelines published by the Federation of European Animal Science Association and the German Society of Laboratory Animal Science and are carefully reviewed by the MfD Diagnostics animal care committee. The study was approved by the Landesuntersuchungsamt (Koblenz, Germany), permit number 23 177-07/A 10-15-001.

Cells
293T were maintained in Dulbecco’s modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Perbio, Etten-Leur, the Netherlands), MEM nonessential amino acids (0.1 mM; Invitrogen, Breda, the Netherlands) and penicillin/streptomycin (P/S; both at 100 U/ml). TZM-bl cells, which express the HIV-1 receptors CD4, CCR5 and CXCR4, were cultured and maintained in DMEM supplemented with 10% FCS, 0.1 mM MEM and P/S. These cells contain the luciferase gene under control of the HIV-1 LTR promoter and were used to measure HIV-1 neutralization. TF-1 cells, a gift from Paul Coffer (Universiteit Utrecht), were cultured in RPMI 1640 (Invitrogen) with 10% FCS, supplemented with 50 U/ml of rhGM-CSF (Schering-Plough). Splenic lymphocytes were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS, HEPES, glutamine, sodium pyruvate, P/S, MEM and 2-mercaptoethanol.

Env production
293T cells were transiently transfected (32) with plasmids expressing recombinant Env using linear polyethylenimine (PEI, MW 25000; Polysciences Europe GmbH, Eppelheim, Germany) or Lipofectamine 2000 transfection reagent according to standard manufacturer’s protocol (Invitrogen). Briefly, plasmid DNA was diluted in one-tenth of the final culture volume of DMEM and mixed with PEI (0.15 mg/ml final concentration). After incubation for 20 min, the DNA-PEI mix was added to the cells for 4 h before being replaced with normal culture medium. Env-containing supernatants were harvested 48 h after transfection and frozen in aliquots.
gp140 trimer ELISA
Env trimer ELISAs were performed as described previously (29, 30). Supernatants containing His-tagged Env gp140 proteins were diluted 1:3 in TBS, 10% FCS and added for 2 h to pre-blocked nickel-nitrilotriacetic (Ni-NTA) HisSorb (Ni-NTA) 96-well plates (Qiagen). After three washes using TSM (20 mM Tris, 150 mM NaCl, 1 mM CaCl$_2$, 2 mM MgCl$_2$), serially diluted polyclonal HIV-Ig, Env specific mAbs, DC-SIGN-Fc or CD4-IgG2 in TSM, 5% BSA was then added for 2 h, with or without 1 µg/ml sCD4, followed by three washes with TSM, 0.05% Tween 20. HRP-labeled goat anti-human immunoglobulin G (0.2 µg/ml; Jackson Immunoresearch) was used as secondary Ab and the absorption at 450 nm was measured after the colorimetric reaction was stopped using H$_2$SO$_4$.

gp120 ELISA
Anti-gp120 Ab titers were measured by ELISA (30). In short, Microlon 96-well plates (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) were coated with anti-gp120 Ab D7324 (10 µg/ml). The plates were washed twice with TBS and residual protein-binding sites were blocked with 2% skim milk powder (Sigma-Aldrich) in TBS for 30 min. gp120 from transiently transfected 293T cells was added to D7324-coated wells for 2 h at room temperature. Mice serum serially diluted in 20% sheep serum (Biotrading, Mijdrecht, the Netherlands), 2% skim milk powder in TBS was applied for 2 h. gp120-specific mouse IgG was detected with HRP-labeled goat anti-mouse IgG (Jackson Immunoresearch), used at 1:5000 (0.2 µg/ml), followed by colorimetric detection. Endpoint titers were calculated using Graphpad Prism version 5.03 by determining the serum dilution at which the OD$_{450}$ signal was three times above the OD$_{450}$ background signal obtained without mice sera.

SDS-PAGE, blue native PAGE and western blotting
SDS-PAGE and western blotting were performed as previously described (30). Env was detected using the primary mAb PA1 (0.2 µg/ml) and a secondary HRP-labeled goat anti-mouse IgG (1:5000 dilution) followed by Western Lightning ECL solution (PerkinElmer Life Sciences). Blue native PAGE was carried out as previously reported (30). Briefly, purified protein samples or cell culture supernatants were diluted with an equal volume of a buffer containing 100 mM MOPS, 100 mM Tris with HCl, pH 7.7, 40% glycerol, and 0.1% Coomassie blue, immediately prior to loading onto a NuPAGE 4-12% bis-Tris gel.
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(Invitrogen). Typically, gel electrophoresis was performed for 2 h at 150 V (~0.07A) using 50 mM MOPS, 50 mM Tris, pH 7.7, as running buffer.

**HIV-1 neutralization**

TZM-bl cells, used to measure HIV-1 infection and were cultured to 70-80% confluency in a 96-well plate. HIV-1 SF162 or JR-FL (derived from peripheral blood mononuclear cell supernatants) was mixed and incubated for 1 h with serially diluted pooled sera from five Env<sub>wt</sub> or five Env<sub>GM-CSF</sub> immunized mice. The target cells were washed once with PBS prior to addition of virus (5 ng/ml CA-p24) with or without mouse sera. The protease inhibitor saquinavir (400 nM; Hoffmann-La Roche) was added to block replication, as well as 40 µg/ml DEAE-dextran (Sigma-Aldrich), in a total volume of 200 µl. The medium was removed 2 days post-infection and the cells were washed once with PBS before lysis with reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a luciferase assay kit (Promega) and a Glomax luminometer according to the manufacturer’s instructions (Turner BioSystems, Sunnyvale, CA). All infections were performed in duplicate. The luciferase activity measured in uninfected cells was taken as background and was subtracted from the signals derived from the experimental samples.

**V3 peptide competition assay**

Pooled sera from five Env<sub>wt</sub>- or Env<sub>GM-CSF</sub>-immunized mice were incubated with a mix of three peptides (Env V3-1: NNNTRKSIHIGPGRA, Env V3-2: SIHIGPGRAFYTTGE, Env V3-3: GRAFYTTGEIIGDIR (30 µg/ml each)) overlapping the V3 sequence of HIV-1 JR-FL, or with an unrelated peptide (QAPKPRKQ; 90 µg/ml) for 1 h at room temperature. The peptide-serum mixtures were then tested in the HIV-1 neutralization assay. To control for non-specific inhibition by the peptides, we also performed neutralization assays using mAb b12 (6 µg/ml) in the presence or absence of the three V3 peptides.

**GM-CSF activity assay**

TF-1 cells (50000 in 50 µl) were seeded in a 96-well plate. Serially diluted supernatants from Env<sub>wt</sub>, Env<sub>GM-CSF</sub>, mock transfected 293T cells, or rhGM-CSF (75 U/ml) diluted in mock transfected medium were added in quadruplicate. The cells were harvested on day 5 and the number of living cells was measured by flow cytometry. The number of living TF-1 cells from wells treated with mock medium (~15000 cells/75 µl) served as a background and was subtracted from test values.
**T cell responses**
The number of splenic T cells from immunized mice was determined by LSR-II flow cytometry (BD Biosciences) using standard cell surface staining protocols. Data was analyzed using FlowJo software. In short, prior to staining, Fc receptors were blocked using an anti-mouse CD16/CD32 antibody (clone 2.4G2). Cell surface staining of TCRβ, CD3, CD4 and CD8 was carried out in PBS, supplemented with 10% FBS. *In vitro* restimulation of T cells (CD4+ and CD8+ combined) in unfractionated splenocyte cultures was carried out by culturing 5 x 10^5 cells/well with JR-FL gp120 (10 µg/ml) in a final volume of 200 µl. Stimulation by an anti-CD3e antibody (2 µg/ml; BD Biosciences, clone 145-2C11, Cat 553057) served as a positive control, stimulation by culture medium as a negative control. Supernatants were collected after 96 h at 37°C in 5% CO₂ and stored at -80°C until further analysis. The concentrations of IL-2, IL-4, IL-5, IL-10, IL-21, and IFN-γ in the supernatants were measured by a sandwich ELISA, according to the manufacturer’s instructions (OptEIA mouse ELISA kits, BD Biosciences), with the use of a 3, 3’, 5, 5’ tetramethylbenzidine substrate kit (BD Biosciences). The assay sensitivity limits were approximately 3 pg/ml for IL-2, IL-21, 8 pg/ml for IL-4, 16 pg/ml for IL-5 and 30 pg/ml for IL-10 and IFN-γ.

**Modeling chimeric gp120_hGM-CSF trimers**
Structural models for the gp120-hGM-CSF within a trimeric spike were generated as follows. The trimeric configuration of gp120 in an unliganded spike was obtained by fitting the b12-bound conformation of the gp120 HxBC2 core (PDB ID:2NY7; (33)) into the cryoelectron tomography density of unliganded HIV-1 BaL strain using the program Chimera (34,35). RosettaDesign (36, 37) was used to thread the sequence of JR-FL core gp120 onto the 2NY7 gp120 structure from residue 83 to 492. The structure of hGM-CSF (PDB ID:2GMF, chain A, residues 9-122 (38)) was inserted at the V1V2 stem between residues 127 and 195 (gp120 2NY7 numbering), flanked by Gly-Ser-Gly (GSG) linkers on both sides. The conformations of the connecting segments and the rigid body orientation of hGM-CSF relative to the gp120 trimer were modeled using RosettaRemodel (P.H. and W.R.S., manuscript in preparation). Briefly, the backbones for the connecting segments (2NY7 residues 112-127 and 195-212 plus the GSG linkers) were generated using an *ab initio* fragment insertion protocol (39), cyclic-coordinate descent was used to maintain proper backbone connectivity (40, 41), and rebuilt segments were further optimized by cyclic-coordinate descent refine (40, 41) and side-chain repacking. Modeling of hGM-CSF on the gp120 V1V2 stem bound to the b12 Fab fragment
was carried out to determine functional steric constraints in the model. Disulfide bonds in the V1V2 (2NY7 residues 119-205 and 126-196) were approximated by C-C distance constraints. The V3 loop was left truncated as in 2NY7. Possible isoforms (~1000) of Env<sub>hGM-CSF</sub> were generated and models (~100) with the lowest energy state were further filtered for (a) clash-free binding of b12, CD4 (33) or VRC01 Fab (42) to the gp120 trimer (b) maintenance of solvent exposure of the three glycosylation sites in hGM-CSF and the V1V2 stem. The resulting set of models was grouped into three classes: up, down and side conformation. A representative model of each class was chosen for the figures.

Statistical analysis
Statistical significance is indicated in Figs. 6 and 7 (*, p≤0.05; **, p≤0.01; ***, p≤0.001). The specific statistical tests performed depended on the nature of the experiments and are indicated in the figure legends.

Results

Design of an HIV-1 Env trimer with an embedded GM-CSF domain
To generate a trimeric HIV-1 Env immunogen that could be targeted to immune cells and simultaneously stimulate immune activation, we deleted the V1V2 domain of gp120 and replaced it with almost the complete sequence of the GM-CSF cytokine. We used the stabilized SOSIP.R6-IZ gp140 protein backbone, hereafter called Env (27, 28, 30, 31). The mouse or human GM-CSF (mGM-CSF or hGM-CSF) sequence was inserted after the second cysteine bridge in the V1V2 stem between amino acids 127 and 195. To facilitate flexibility at the junctions between GM-CSF and Env, Gly-Ser-Gly linkers were added to the N and C termini of the GM-CSF sequence (Fig. 1A and 1B). In total, 120 and 116 amino acids were introduced for hGM-CSF and mGM-CSF, respectively, at the expense of 65 amino acids of the V1V2 domain.

Chimeric Env<sub>hGM-CSF</sub> is expressed efficiently and forms trimers
To determine whether the chimeric Env<sub>hGM-CSF</sub> protein was produced and folded properly, we transiently transfected 293T cells with gp140 (Env<sub>wt</sub>), gp140 lacking the V1V2 domain (Env<sub>ΔV1V2</sub>) (29) and Env<sub>hGM-CSF</sub>. The expression of all constructs was determined using SDS-PAGE and western blotting (Fig. 2, left panel). The expression of Env<sub>hGM-CSF</sub> was slightly
Figure 1. Design of chimeric Env<sub>GM-CSF</sub>. (A) Linear representation of Env<sub>wt</sub>, Env<sub>ΔV1V2</sub> and Env<sub>GM-CSF</sub>. Clade B JR-FL gp140 (amino acids 31-681) contains several modifications that have been described elsewhere (29). The amino acid sequences of the Env-GM-CSF junctions are shown. The linker residues are indicated in italics, GM-CSF residues in bold type. A potential site for N-linked glycosylation is underlined. (B) Schematic of Env<sub>GM-CSF</sub> compared to Env<sub>wt</sub> and Env<sub>ΔV1V2</sub>. The V1V2 loop present in Env<sub>wt</sub> was replaced by amino acids 26-139 of hGM-CSF or amino acids 26-136 of mGM-CSF.
lower than Env<sub>wt</sub>. The Env<sub>hGM-CSF</sub> protein had an apparent molecular mass of 150 kDa, Env<sub>wt</sub> of 140 kDa and Env<sub>∆V1V2</sub> of 120 kDa, consistent with the expected sizes (Fig. 2, left panel). The Env<sub>wt</sub>, Env<sub>∆V1V2</sub> and Env<sub>hGM-CSF</sub> proteins were all predominantly expressed as trimers when analyzed by blue native PAGE (Fig. 2, right panel), showing that replacing the V1V2 domain with hGM-CSF did not markedly affect trimer formation or stability.

**Figure 2. Env<sub>GM-CSF</sub> is expressed as a trimer.** Shown as reducing SDS-PAGE (left panel) and blue native PAGE (right panel) analyses of Env<sub>wt</sub>, Env<sub>∆V1V2</sub> and Env<sub>GM-CSF</sub> proteins secreted from transiently transfected 293T cells. Recombinant purified JR-FL gp120 (50 ng) was included for comparison.

**Chimeric Env<sub>hGM-CSF</sub> is recognized by neutralizing antibodies**

To determine whether Env<sub>hGM-CSF</sub> had an antigenic structure comparable with Env<sub>wt</sub>, we used a trimer ELISA and assessed its binding to a panel of Abs and receptor mimics (30). Polyclonal Ig from pooled HIV-positive patient sera (HIV-Ig) bound similarly to Env<sub>wt</sub>, Env<sub>∆V1V2</sub> and Env<sub>GM-CSF</sub> (Fig. 3A). The 2F5 mAb, directed to the gp41 epitope located far from the GM-CSF insertion, also bound identically to the three proteins (Fig. 3A). Similar findings were made using the human DC-SIGN-Fc protein and the 2G12 mAb that both recognize oligomannose N-glycans on gp120 (Fig. 3B).

We then tested the conformation of Env epitopes closer to the GM-CSF insertion. V3 mAb 39F recognized the three Env proteins equally well, as did CD4-IgG2, a receptor mimic for CD4 (Fig. 3C). NAbs VRC01 and b12 against discontinuous epitopes associated with the CD4-binding site (CD4BS) recognized Env<sub>hGM-CSF</sub> (Fig. 3C), but the binding was subtly less efficient compared with that to Env<sub>wt</sub> (by 2-fold for VRC01, 4-fold for b12). Thus, the CD4BS on Env<sub>hGM-CSF</sub> is intact, but the accessibility and/or conformation of the b12 and, to a lesser extent, VRC01 epitopes is subtly altered by the replacement of the V1V2 domain by GM-CSF.
CD4 binding induces a conformational change in Env involving rearrangement of the V1V2 domain, which exposes the coreceptor binding site and the overlapping CD4-induced (CD4i) epitopes. In the absence of CD4, the CD4i epitope mAb 17b, 48d and 412d bound poorly to Env\textsubscript{wt} and Env\textsubscript{hGM-CSF} but efficiently to Env\textsubscript{V1V2}, as expected (Fig. 3D, left panels). Adding soluble CD4 substantially increased the binding of these mAb to Env\textsubscript{V1V2} and especially to Env\textsubscript{wt}, but binding to Env\textsubscript{hGM-CSF} was improved only modestly (Fig. 3D, right panels). Hence the presence of GM-CSF in the V1V2 region either limits the accessibility of the CD4i epitopes or blocks the conformational changes that expose them. Collectively, the Env conformational analyses show that the overall antigenic structure of Env\textsubscript{hGM-CSF} is similar to that of Env\textsubscript{wt}, but some epitopes located close to the insertion site are affected by the GM-CSF insertion.

Figure 3. Chimeric Env\textsubscript{hGM-CSF} is recognized by conformational Env Abs and receptor mimics. ELISA analysis of the binding of Env variants to: (A) HIV-Ig and 2F5, (B) glycan dependent DC-SIGN-Fc and 2G12, (C) 39F (V3), CD4-IgG2, b12 and VRC01 (CD4Bs), and (D) 17b, 48d and 412d (CD4i) in the absence and presence of soluble CD4 (sCD4). Culture supernatant from mock transfected 293T cells was used as a negative control.
**The GM-CSF domain of chimeric Env<sub>hGM-CSF</sub> is functional**

To probe the integrity of the GM-CSF domain, we performed an ELISA using a conformation-dependent neutralizing GM-CSF mAb (Fig. 4A). The Env<sub>hGM-CSF</sub> protein was efficiently recognized by this mAb, whereas Env<sub>wt</sub> and Env<sub>ΔV1V2</sub> were not. Hence the GM-CSF domain embedded within the Env backbone assumes a similar conformation as native GM-CSF.

The functional activity of the inserted GM-CSF domain was tested using TF-1 cells, which require GM-CSF signaling for proliferation. The number of living TF-1 cells was measured by flow cytometry after a 5-day culture with 293T cell supernatant containing the Env<sub>hGM-CSF</sub>, Env<sub>wt</sub> or Env<sub>ΔV1V2</sub> protein. Supernatant from untransfected 293T cells was used as a negative control, and rhGM-CSF protein, diluted in 293T culture medium, as a positive control. TF-1 cells exposed to Env<sub>hGM-CSF</sub> medium proliferated in a dose-dependent manner, as did cells stimulated with authentic rhGM-CSF (Fig. 4B). In contrast, apoptosis was observed in TF-1 cell cultures treated with Env<sub>wt</sub>, Env<sub>ΔV1V2</sub>, or the mock medium. The hGM-CSF protein tethered to the Env backbone was not as potent as soluble rhGM-CSF at stimulating TF-1 cell proliferation. The maximal response of Env<sub>hGM-CSF</sub>, extrapolated from the curve trend, was calculated at ~32000 living cells. rhGM-CSF had an maximal response of 41000 living TF-1 cells. Based on the half-maximal response levels for the secreted Env<sub>GMC</sub> and the authentic rhGM-CSF, we estimated the GM-CSF activity of Env<sub>GMC</sub>. The half-maximal stimulation response of Env<sub>hGM-CSF</sub>-containing supernatant was reached at a 3-fold dilution, whereas the half-maximal response to rhGM-CSF occurred at 1.9 U/ml. Env<sub>hGM-CSF</sub> was therefore estimated to have a GM-CSF activity equivalent to 5.7 U/ml.

![Figure 4. The GM-CSF domain of chimeric Env<sub>hGM-CSF</sub> is functional.](image)

(A) ELISA analysis of the binding of a neutralizing, conformation-dependent anti-hGM-CSF antibody. (B) Proliferation of TF-1 cells in response to either culture supernatant containing Env<sub>GMC</sub> or Env<sub>wt</sub> or rhGM-CSF. The numbers of cells present after 5 days of culture in a volume of 75 µl are given. Culture supernatant from mock transfected 293T cells was used as a negative control (~15000 cells) and deducted from the test values.
Model of an Env<sub>hGM-CSF</sub> trimer

Based on the atomic structure of hGM-CSF (43), the structures of gp120 in complex with either b12, CD4 (33), or VRC01 (42), a gp120 trimer model (44), and additional information provided by the antigenicity and functional data (Fig. 3 and Fig. 4), we generated a structural model of the Env<sub>hGM-CSF</sub> trimer. Because of the flexibility of the linkers, GM-CSF can be modeled on the gp120 core in multiple orientations. Three symmetric Env<sub>hGM-CSF</sub> trimers (“down”, “side”, and “up”) are depicted in Fig. 5 to illustrate the diversity of potential low energy conformations. We note that hGM-CSF will likely sample a range of conformations around and between the “down”, “side” and “up” conformations, and that the orientation of GM-CSF may not be symmetrical in the three gp120 subunits. All the models in Fig. 5 are compatible with VRC01, b12 and CD4 binding. The model with hGM-CSF in the upper position may be the most accurate since in that orientation the accessibility of the CD4i epitope is restricted by GM-CSF, as observed with the CD4i Ab-binding data (Fig. 3).

Environ<sub>mGM-CSF</sub> induces enhanced Env-specific Ab responses in mice

To test whether incorporating the GM-CSF protein within Env would improve Env-specific immune responses, we immunized mice with plasmids expressing Env, with or without embedded murine GM-CSF, according to the schedule present in Fig. 6A. In addition we immunized mice with plasmids encoding the Env protein (again, with or without embedded mGM-CSF) coupled to mouse BAFF (mBAFF) or mouse CD40L (mCD40L). BAFF and CD40L are costimulatory molecules that were linked C-terminally to the Env to provide an
additional stimulus for immune activation. The mice were immunized on days 0, 14, 28 and 42, and the gp120-specific IgG response was measured by ELISA. The gp120-specific Ab titer for mice immunized with plasmids encoding Env-mBAFF or Env-mCD40L was not significantly greater than found with Env (Student’s t-test) (Fig. 6B). Thus, the Env-mBAFF and Env-mCD40L constructs did not improve the immune response against Env whether mGM-CSF was present or not. To increase the statistical power of subsequent analyses, we therefore pooled all of the mice groups that received Env and all of the groups given Env containing embedded mGM-CSF, irrespective of the additional presence of mBAFF or mCD40L sequences (Fig. 6C). Anti-Env Ab were detected at low levels after the first boost on day 14. The endpoint titers from Env- and Env_mGM-CSF-immunized mice were significantly different after the second boost on day 28. The corresponding titers for the Env_mGM-CSF group were 3-fold higher than for the Env control group (471 versus 174, p=0.03). The titer difference between Env and Env_mGM-CSF further increased at day 42 (1357 versus 4918, p=0.0002). Collectively, inserting GM-CSF within an Env immunogen enhanced the Ab response to the Env component of the chimeric protein.

**Figure 6.** Env_mGM-CSF induces better anti-Env Ab responses in mice. (A) Mouse immunization scheme. (B) Endpoint anti-gp120 IgG titers obtained from mice immunized with different Env immunogens at day 42 as determined by ELISA. (C) Endpoint anti-gp120 IgG titers over the course of the immunization experiment as determined by ELISA. The individual endpoint titer is shown for each mouse. Note that both comparison groups (Env versus Env containing embedded mGM-CSF) contain fifteen mice. Within these groups of fifteen, five mice were immunized with Env, Env-BAFF or Env-CD40L, or for comparison, with Env GM-CSF, Env_GM-CSF-BAFF or Env_GM-CSF-CD40L. The mean endpoint titers of the 15 mice/group are shown ± standard error of the mean. Statistical significance was determined using a one-tailed Mann-Whitney test, and significant p values are represented with asterisk: *, p≤0.05; ***, p≤0.001.
Env_{mGM-CSF} induces improved Env-specific T helper responses

GM-CSF is known to activate a variety of cells but not B cells, implying that the enhanced Ab response was probably mediated indirectly through other cell types. We therefore investigated the Env-specific T cell response in spleen cells from mice immunized with Env or Env_{mGM-CSF}, focusing on the induction of cytokines that are important for B cell responses. Spleens were harvested on day 56 and their cytokine production profile was measured by restimulating the splenocytes with gp120 protein or anti-CD3 compared with a mock stimulation.

gp120-induced IL-2 secretion was 37 pg/ml for Env-immunized mice and 54 pg/ml for Env_{mGM-CSF} mice (p=0.06)(Fig. 7A). IL-2 secretion in response to anti-CD3 Ab could not be detected (Fig. 7B). IL-4 production by splenic T cells from the Env_{mGM-CSF} group was 6-fold higher than the Env group (41 pg/ml versus 7 pg/ml, p<0.0001; Fig. 7A), and IL-4 secretion induced by the anti-CD3 Ab was also higher (147 pg/ml versus 75 pg/ml, p=0.02; Fig. 7B). Env-specific IL-5 production was similarly increased in the Env_{mGM-CSF} group compared to the Env group (45 pg/ml versus 14 pg/ml, p=0.02), whereas IL-5 secretion in response to the

![Figure 7. Env_{mGM-CSF} induces enhanced Env-specific T helper responses. Splenocytes were incubated with control media, gp120, or anti-CD3 stimuli for 96 h in vitro. Release of cytokines in the supernatant of (A) gp120- or (B) anti-CD3-stimulated splenocytes was measured. Cytokine responses from splenocytes treated with media provided background values that were subtracted from the values obtained with anti-CD3 or gp120 stimulation. Statistical significance was determined using a two-tailed Student’s t-test, and significant p values are represented with asterisks: *, p≤0.05; **, p≤0.01; ***, p≤0.001.](image)
anti-CD3 Ab was comparable. We could not detect Env-specific IL-21 production, but production of this cytokine in response to the anti-CD3 mAb was significantly higher in the Env_{GM-CSF}-immunized mice than in the Env group (63 pg/ml versus 23 pg/ml, p=0.003). There were no differences in IFN-γ or IL-10 production between the Env and Env_{mGM-CSF} immunized mice in response to either gp120 or anti-CD3 mAb restimulation.

Collectively, these results indicate that Env_{mGM-CSF} is better than Env_{wt} at inducing Env-specific T cell responses to supply T help for B cells. The cytokine data are thus consistent with the enhanced Ab responses.

**Env_{mGM-CSF} induces enhanced neutralizing antibody responses**

The mouse model is not an ideal system to induce NAb responses against HIV-1 (45). Nonetheless, we tested whether the sera from mice immunized with Env_{wt} or Env_{mGM-CSF} DNA constructs contained Ab with neutralizing activity against HIV-1. Day 42 sera from 5 mice of each of the Env_{wt} and Env_{mGM-CSF} groups were pooled and tested in a single-cycle neutralization assay using TZM-bl reporter cells. Ab neutralization was tested on the highly sensitive tier 1 isolate SF162 and the more resistant tier 2 isolate, JR-FL. The pooled sera had no neutralization activity against JR-FL (data not shown). Sera from both groups of mice were active against SF162, but the 50% titer was significantly higher for the pooled Env_{mGM-CSF} sera (368 versus 110, p<0.0001; Fig. 8A). Although neutralization of the sensitive SF162 has little relevance to the development of a useful vaccine, the results confirm the outcome of the Ab assays and show that replacing the V1V2 domain with GM-CSF improves the Ab response to Env.

Neutralization of SF162 by immune sera is predominantly mediated by V3-specific Abs (46). To assess whether Env_{mGM-CSF}-immunized mice also predominantly generate V3-directed NAb, we performed a V3 peptide competition assay. Mice sera of day 42 were preincubated with an excess of three overlapping V3 peptides to inhibit the neutralization by V3-directed NAbs (Fig. 8B). Env_{wt} sera inhibited SF162 infection by 51% and the preincubation with V3 peptides restored infection to ~100%, while addition of an unrelated control peptide did not restore infection. These results show that SF162 neutralization by Env_{wt} sera was mediated exclusively by anti-V3 specificities. In contrast Env_{mGM-CSF} sera reduced SF162 infection by 72% and adding V3 peptides only partially reversed the inhibition (to 59%), implying that NAb with specificities other than V3 were present. As a control, we showed that neutralization of SF162 by NAb b12 against the CD4BS was not affected by
addition of the V3 peptides (Fig. 8B). Thus, replacing the V1V2 domain with GM-CSF may divert the Ab response away from V3 and towards other epitopes on Env that may be more relevant to broadly active neutralization.

**Discussion**

To improve the immunogenicity of HIV-1 Env vaccines, we constructed a chimeric gp140 trimer in which the V1V2 region of gp120 was replaced by the GM-CSF cytokine. We selected GM-CSF because it has a well defined adjuvant activity and usage in humans has been shown to be safe. We found that the chimeric Env<sub>GM-CSF</sub> protein was more immunogenic than the unmodified Env trimers in mice, with improvements of both Ab and T helper responses.

Adjuvants can be more powerful when they are coupled directly to antigen rather than when the two components are supplied as a mixture. For example, conjugating oligodeoxynucleotides (CpG ODN) to a Gag protein enhances the magnitude and quality of Gag-specific T cell responses (21). In earlier studies, the immune response was improved by linking HIV-1 Env antigens directly to costimulatory molecules such as C3d, TNF-α, Fms-like tyrosine kinase receptor-3 ligand, or Fas Ligand (FasL) (23, 24, 47-49). The advantage of
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Chimeric adjuvant fusion proteins over chemically linked adjuvant antigen complexes is that the former can be produced easily directly from DNA at a constant antigen to adjuvant ratio.

Instead of linking the costimulatory adjuvant molecules such as FasL or TNF-α to the N or C termini of Env, we replaced the V1V2 loop domain with the costimulatory GM-CSF molecule. The V1V2 loop was selected because this region can be deleted from Env without compromising the structure and, to some extent, the function of the protein (29,50). Flexible linkers were added at the Env-GM-CSF junctions to enable the cytokine to fold independently into a native structure and to reduce the possibility of a steric clash between the two components of the chimera. The chimeric Env\textsubscript{GM-CSF} protein was expressed efficiently in 293T cells as a secreted trimer (Fig. 2). Ab binding to most Env epitopes was very similar to that seen with the corresponding, unmodified Env, with the exception of the b12 and CD4i epitopes close to the GM-CSF insertion site, which were affected modestly (Fig. 3).

Although GM-CSF embedded into Env was functional, it did not stimulate TF-1 cell proliferation to the same level as recombinant, soluble GM-CSF (Fig. 4). It is possible that, despite the use of flexible linkers, GM-CSF binding to its receptor is partially blocked by proximal regions of Env. Alternatively, one GM-CSF molecule may be able to bind to the GMR normally, but formation of a ternary dodecameric complex is affected, limiting downstream signaling (51-53). In theory, binding of Env to CD4 on TF-1 cells could counteract the proliferative activity of GM-CSF (54). This explanation however seems unlikely, since addition of Env\textsubscript{wt} did not affect the activation of TF-1 cells by rhGM-CSF (data not shown).

The serum half-life of recombinant GM-CSF is short, varying from 0.9 to 2.5 h (55,56). This factor could adversely influence the adjuvant effect of GM-CSF, particularly when the immunogen and cytokine are either mixed or transcribed from separate plasmids. How fusion of Env with GM-CSF affects the half-life of both proteins requires further study.

Chimeric proteins such as those described here have not been described before, but GM-CSF has been used as an adjuvant in vaccine studies with a variety of results. Co-delivering GM-CSF in peptide-based vaccines, either as recombinant protein or plasmid DNA, augments HIV-1-specific T cell responses to various extents (57-59). Co-delivery of rhesus macaque GM-CSF expressed from DNA with Gag, Pol, and Env DNA followed by an MVA boost results in higher avidity anti-Env Ab in rhesus macaques (60). Mice immunized with a bicistronic plasmid encoding gp120 and GM-CSF have stronger gp120-specific CD4\textsuperscript{+} T cell responses than mice immunized with gp120 and GM-CSF on two separate plasmids.
(61). Hence, the method of GM-CSF co-delivery affects the outcome of the immune response. The timing of GM-CSF co-delivery also affects the outcome of the response. In one study, delivery of a GM-CSF plasmid prior to vaccination with a plasmid encoding a vaccine antigen yielded a Th2-type response, whereas GM-CSF delivery after vaccination with the antigen resulted in a Th1-type response. Both Th1 and Th2 responses are generated when GM-CSF and antigen are administered simultaneously (62). Although Ab to recombinant human GM-CSF protein (rhGM-CSF) arose when it was used in a human vaccine trial, they caused neither adverse hematologic events nor a loss of GM-CSF function (58).

We initially chose to introduce GM-CSF as a costimulatory molecule, but other molecules could be incorporated as well. A number of cytokines can increase the immunogenicity of HIV vaccines. Co-administration of murine IL-12 with HIV-1 Env pDNA markedly enhances the cell-mediated immune response in mice (59). Other cytokines such as IL-2, IL-4, IL-6, IL-10, IL-15, and IL-18 can augment the humoral and/or cell-mediated immune responses to HIV and non-HIV antigens (63-68).

However, some limitations need to be recognized when incorporating cytokines into the Env backbone. First, the length and molecular weight of the inserted protein should preferably be similar to the replaced V1V2 region, which spans 60 to 80 amino acids, including 6 glycans (in total ~20 kDa). Moreover, the N and C termini of the folded cytokine preferably should be close together such that the bulk of the protein protrudes away from the Env protein. Another constraint is the oligomeric state of the cytokine; it is unlikely that dimeric molecules, such as IL-10 or IL-12 (69, 70), could be embedded as a single functional unit within the trimeric Env complex. Taking these constraints into account, we have recently succeeded in replacing the V1V2 region with a functional human IL-4 (T. van Montfort, G. Isik and R.W. Sanders, unpublished observations).

In conclusion, we have shown that embedding a cytokine within an HIV-1 Env trimer improves the immunogenicity of the Env moiety. This approach may be useful when considering how to develop an HIV-1 vaccine aimed at inducing protective humoral immunity.

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