Improving HIV-1 envelope glycoprotein vaccines

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Citation for published version (APA):

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Targeting HIV-1 envelope glycoprotein trimers to B cells enhances the induction of neutralizing HIV-1 antibodies

Mark Melchers, Ilja Bontjer, Dirk Eggink, Kenneth Kang, David Montefiori, William C. Olson, Ben Berkhout, John P. Moore, and Rogier W. Sanders

Manuscript in preparation
Abstract

Despite many years of research, no effective vaccine against HIV-1 has been developed. A vaccine aimed at inducing neutralizing antibodies would have to include the HIV-1 Env glycoprotein complex (Env), but the use of Env in vaccines is hampered by several factors that limit the quantity and quality of the antibodies raised against it. To address this issue we previously created a fusion construct consisting of Env and CD40 ligand (CD40L), the rationale being that the CD40L component would target Env to dendritic cells while at the same time activating these cells to initiate a potent anti-Env immune response. However, dendritic cells process antigens for antigen presentation while a potent neutralizing antibody response requires an interaction of intact antigen with B cells. In the current study we fused trimeric Env to two proteins that can target and activate B cells: B-cell Activating Factor (BAFF/BLyS/TNFSF13b) and A PRoliferation-Inducing Ligand (APRIL/TNFSF13). The resulting fusion constructs were expressed efficiently and well-folded. When tested in a rabbit immunization experiment, the Env-APRIL fusion protein induced the highest anti-gp120 and anti-trimeric gp140 antibody titers. Env-APRIL also induced the most efficient neutralizing responses against various HIV-1 strains in an IgG dependent matter. No upregulation of total antibody levels was observed, indicating that the APRIL activation effect was specific for Env. Env targeting and activation to B cells by fusion to APRIL or other cis-adjuvants may help to improve subunit vaccines against HIV-1.
Introduction

Despite over 25 years of extensive research, no effective vaccine against HIV-1, the causative agent of AIDS, has yet been developed. One important obstacle is the remarkable capacity of HIV-1 to first limit the induction of effective immune responses, and then to mutate and escape from those that are generated. So far, neither vaccines aimed at inducing neutralizing antibodies (NAbs) nor those intended to stimulate cell-mediated responses have been sufficiently successful (1-5). Both strategies continue to be pursued, both individually and as immunogen combinations (6-8).

The trimeric envelope glycoprotein complex (Env) that is responsible for HIV-1 entry into target cells is the only relevant target for the induction of NAbs. Multiple immune evasive properties hamper the design of engineered Env proteins for NAb induction. These defense mechanisms include surface loops that can vary in sequence in response to NAb selection pressure, without major effects on Env function (9-12). The more conserved regions of Env involved in receptor binding are shielded by these loops and by glycans that are generally not recognized as foreign by the immune system (13-15). Furthermore, both native and soluble recombinant Env trimers are unstable, exposing highly immunogenic, non-neutralizing decoy epitopes when they dissociate into their component subunits, gp120 and gp41 (16). As a result, most anti-Env antibodies induced during HIV-1 infection or by Env vaccination are non-neutralizing. Broadly neutralizing antibodies can sometimes be found in infected humans, but are generally not elicited in animals or humans vaccinated with Env proteins of various designs (1-5,10,17).

Various attempts have been made to overcome Env instability and create better mimics of the native complex, as soluble gp140 constructs. We have generated a soluble trimer, SOSIP gp140, that contains specific amino acid substitutions to stabilize the gp120-gp41 and gp41-gp41 interactions (18,19). The SOSIP gp140 trimer is a reasonably good, but still imperfect, mimic of the native Env complex (Binley 2000, Sanders 2002). Single particle cryo EM studies show that SOSIP gp140 and the native complex have similar structures and undergo comparable CD4-induced conformational rearrangements. SOSIP gp140 is slightly better than the corresponding gp120 monomer at inducing NAbs (20,21).

A general constraint of subunit protein and DNA plasmid vaccines is their poor immunogenicity compared to live-attenuated or inactivated viral vaccines, which is in part explained by their lack of components such as Toll like receptor activators that provide costimulatory signals. However, HIV-1 Env-based subunit vaccines appear to be particularly poor immunogens, even when compared to other subunit vaccines such as influenza A virus hemagglutinin (HA) (22). Thus, several high doses of gp120 or gp140 proteins are generally needed to induce reasonable antibody titers, and these titers wane quickly with a short half-life of 30-60 days (2). One among several contributory
explanations may be interference with dendritic cell function via the oligomannose N-glycans present on gp120 (23-25).

We have previously shown that we could activate dendritic cells (DCs) by fusing Env to the active domain of CD40 ligand (CD40L), which acts as a “cis-adjuvant” (26). CD40L (TNFSF5/CD154), a TNF superfamily (TNFSF) member, is an important immunostimulatory molecule that is mainly expressed by activated T helper cells. It stimulates DCs to increase their capacity to present antigens, secrete pro-inflammatory cytokines and prime naïve T cells (27-30). CD40L also induces naïve B cells to mature to memory or plasma cells, and promotes antibody affinity maturation and IgG or IgA class switching (28-30).

Targeting an antigen to DC can promote its uptake and processing, thereby facilitating a T helper cell response and indirectly aiding the development of a B cell response (27). However, for the induction of NAbs against HIV-1, the Env antigen must be conformationally intact so that complex epitopes can be presented. We therefore wondered whether it would be possible to both target Env to B cells and activate them directly. B-cell Activating Factor (BAFF/BlyS/TNFSF13b) and A PRoliferation-Inducing Ligand (APRIL/TNFSF13) resemble CD40L in being homotrimeric type II transmembrane proteins with immunostimulatory functions, in this case for B cells (31-33). The principal sources of BAFF and APRIL are innate immune cells such as neutrophils, macrophages, monocytes, DCs and follicular DCs (FDCs) (33-39). The two proteins have similar effects on B cells because they share two receptors (BCMA/TNFRSF17 and TACI/TNFRSF13b), although they have different affinities and bind under different circumstances (33,40-42). Like CD40L, BAFF and APRIL stimulate B cells to mature into memory cells or antibody secreting plasma cells, in a T-cell independent manner, as well as activating B cell receptor affinity maturation and class switching to IgG or IgA (33,43,44). BAFF has also been suggested to counteract the tolerogenic effects of certain antigens such as gp120 (32). These properties of BAFF and APRIL seem particularly advantageous for HIV-1 Env vaccines because the production of broadly active NAbs may require extensive affinity maturation (45,46). Furthermore, the promotion of IgA class switching by BAFF and APRIL could help the development of mucosal immune responses that intervene against HIV-1 sexual transmission (47). IgA responses to HIV-1 infection and vaccines are notoriously poor (48,49).

In this study we investigated whether targeting trimeric HIV-1 Env proteins to B cells via fusion to APRIL, BAFF or CD40L would improve antibody responses. We found that the Env-APRIL construct did indeed induce improved NAb responses in rabbits compared to the unconjugated Env protein.
Results

**Design and construction of chimeric Env-APRIL/BAFF/CD40L fusion proteins**

To target Env to immune cells, we fused stabilized soluble trimeric gp140 proteins to the globular domains of APRIL, BAFF or CD40L. We used the rabbit version of these molecules to facilitate immunogenicity studies in this species. As the amino acid sequence of rabbit CD40L was not previously known, we sequenced the gene by amplifying cDNA from New Zealand White rabbit PBMCs (supplemental material and Suppl. Fig. 1). We subsequently generated codon-optimized versions of the three fusion partners.

The SOSIP.R6-IZ gp140 protein we used was based on the R5-using subtype B primary isolate JR-FL, which was modified to increase its stability (18,19,50,51) (Fig. 1A). A SOSIP.R6-IZ-CD40L fusion protein (termed Env-CD40L hereafter for simplicity), based on the human CD40L sequence and described elsewhere (26), served as the template for the new proteins. Thus, the rabbit versions of the Env-APRIL, Env-BAFF, Env-CD40L constructs were generated by exchanging the human CD40L moiety by the active domains of rabbit APRIL, BAFF and CD40L.

When SOSIP.R6 gp140 is expressed in 293T cells, proteolytic cleavage between gp120 and gp41 is incomplete, but near complete (>95%) cleavage can be achieved by co-transfection of a plasmid expressing the furin protease (18,50). As described in a previous study (26), adding C-terminal modifications such as an isoleucine zipper (IZ) domain to the gp41 ectodomain seems to have negative effects on the cleavage efficiency. Even in the presence of exogenous furin, the cleavage efficiency does not rise above 50%. For some reason C-terminal modifications influence proteolytic cleavage several hundred residues away. Because uncleaved Env trimers are known to be antigenically different from fully processed Env (18,21,52,53), we are actively studying the possible underlying causes. For this study we decided to not co-express furin and therefore all trimers can be considered uncleaved except when mentioned otherwise.

**Chimeric Env-APRIL/BAFF/CD40L proteins are expressed efficiently**

To determine whether the Env-APRIL, Env-BAFF and Env-CD40L proteins could be expressed efficiently, we transiently transfected HEK 293T cells. The secreted fusion proteins secreted into the supernatants were analyzed using reducing SDS-PAGE and western blotting. The fusion proteins and the unconjugated reference gp140 were all expressed at comparable levels (Fig. 1B). The slower migration of the fusion proteins is consistent with the extra mass (~17 kDa) associated with the domains of APRIL, BAFF or CD40L.
We performed a Blue Native PAGE (BN-PAGE) analysis to assess whether the presence of the APRIL, BAFF or CD40L domains affected gp140 trimerization. The unconjugated reference gp140 was expressed predominantly as trimers, as described previously (Fig. 1C) (26,51). The chimeric proteins did also form trimers, although significant amounts of monomers and dimers were also present (Fig. 1C). The APRIL, BAFF and CD40L moieties therefore affect trimer formation and stability, to at least some degree.

**Env fusion proteins bind CD4 and NAbs**

We next studied whether the fusion proteins were folded correctly by immunoprecipitating them from the supernatants using a CD4 mimetic protein (CD4-IgG2) and various Abs and NAbs to conformational epitopes (Fig. 1D), followed by western blot detection. Purified Ig from HIV-1 infected individuals (HIVIg), CD4-IgG2 and NAb b12 to the CD4 binding site on gp120 all recognized Env-BAFF efficiently. NAb 2F5 against the membrane proximal external region (MPER) of gp41, which is located adjacent to the trimerization domain and the BAFF sequence, also precipitated the Env-BAFF fusion protein. NAb 17b directed to a CD4-induced epitope overlapping with the coreceptor-binding site on gp120 bound constitutively to Env-BAFF, but did so more strongly in the presence of soluble CD4, indicating that the fusion protein can undergo conformational changes upon CD4 binding (Fig. 1D). Similar results were obtained when the same analyses were performed on the Env-APRIL and Env-CD40L proteins (data not shown). Overall, these studies show that the Env components of the Env-APRIL/BAFF/CD40L fusion proteins are properly folded and are able to present relevant neutralization epitopes.

In previous studies we have shown that human CD40L is functional when fused to Env, as it can be bound by CD40 and a NAb to CD40L and the fusion protein induced immunostimulatory effects. Because APRIL and BAFF are known to have a structure similar to CD40L, we assume that APRIL and BAFF are also functional, but this was not tested in this study.

**Env-APRIL/BAFF/CD40L induce stronger antibody responses than unmodified Env**

We assessed whether targeting Env to B cells via APRIL, BAFF or CD40L could improve antibody responses in vivo. Since the mouse model is generally a poor choice for studying neutralization responses to HIV-1 Env, we used rabbits. Groups of 4 animals were primed with DNA plasmids encoding unconjugated Env (group A), Env-APRIL (group B), Env-BAFF (group C), Env-CD40L (group D) via gene gun immunization, or received gp120 protein in Alum adjuvant (group E). We chose this experimental design because the DNA plasmids are taken up by dermal DCs, which then mature and migrate to draining lymph nodes, where the fusion protein can be expressed in an environment rich in B cells. This provides the opportunity for the B cells to be stimulated both by the Env component
through the B cell receptor (BCR) and via the costimulatory component (APRIL, BAFF, or CD40L). The gp120 protein arm (group E) allows a comparison with an immunization regimen that has been more widely used in both animals and humans (1,17,54).

All the rabbits received the same boosting agent, the cleaved JR-FL SOSIP.R6 gp140 protein in Quil A, a saponin-based adjuvant. The rationale for this choice was that, as noted above, the gp140 fusion proteins expressed from the DNA plasmids in vitro, and presumably in vivo, are largely uncleaved. By boosting with cleaved trimers, we hoped to promote responses that recognize fully processed Env and hence might have neutralization activity. Since the boosting agent was the same for all groups, any differences in boosting efficiency can be attributed to the memory response induced by the priming agents, which allows a comparison of the three different immunostimulatory moieties. Priming immunizations were performed at weeks 0, 2, 4, 8, the single protein boost at week 16, and the animals were bled for serological analyses at weeks 0, 2, 4, 6, 8, 12, 16, 18 and 20 (Fig. 2A).

We measured anti-gp120 IgG midpoint titers by ELISA as our initial assessment of the antibody responses to the different immunogens. The Env-APRIL, Env-BAFF and Env-CD40L proteins all induced higher titers than unconjugated Env (Figs. 2B,C). The

**Figure 1.** Chimeric Env-APRIL, Env-BAFF and Env-CD40L proteins are properly folded. (A) Schematic of the Env, Env-APRIL, Env-BAFF and Env-CD40L proteins. The clade B JR-FL gp140 (amino acids 31-681) protein contains several modifications for stabilization that have been previously described (see Materials and Methods). Codon-optimized sequences encoding the active domains of rabbit APRIL, BAFF or CD40L were added to the open reading frame encoding the gp140 C-terminus. (B) Reducing SDS-PAGE and (C) Blue Native-PAGE analysis of Env, Env-APRIL, Env-BAFF and Env-CD40L proteins secreted from transiently transfected 293T cells. Recombinant purified JR-FL gp120 (50 ng) and Env from a control transfection were included for comparison. (D) Recognition of Env-APRIL and Env-BAFF by antibodies and CD4. The Env-APRIL and Env-BAFF proteins were immunoprecipitated by CD4-IgG2, HIVIg, antibodies to gp120 (b12) or gp41 (2F5), or via a CD4-induced epitope (17b) on gp120 in the absence of presence of soluble CD4 (sCD4), followed by reducing SDS-PAGE and western blot analysis.
differences were significant (p<0.05) at weeks 6, 18 and 20 for Env-APRIL compared to Env (4-, 4- and 3-fold, respectively) and at weeks 4, 6, 18 and 20 for Env-APRIL compared to gp120 (8-, 4-, 11- and 13-fold, respectively) when using the Mann-Whitney test. When using the more stringent Kurskal-Wallis test with Dunn's post test Env-APRIL significantly enhanced gp120-binding titers than gp120 at weeks 18 and 20 (p<0.05). A similar trend was observed for Env-BAFF and Env-CD40L, but this was not significant. To verify whether the *cis*-adjuvants specifically enhance the antibody levels for Env only, we also measured total IgG levels (Fig. 2D). At each time point IgG levels were found to be equal for all sera, indicating that the observed enhancement is specific for the anti-Env responses.

Measuring the changes in anti-gp120 titer after week 16, when all the animals received the same booster immunization with trimeric SOSIP.R6 gp140 protein, allows a comparison of the memory responses that had been induced in the priming phase by the different plasmids. The anti-gp120 IgG titers increased by 48-fold between weeks 16 and 18 in the Env-APRIL primed rabbits, while the corresponding increases in the Env, Env-BAFF and Env-CD40L groups were 25-, 23-, and 23-fold, respectively (Fig. 2E). In the animals primed with gp120 protein, the titer increase during the same 2-week period was

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**Figure 2:** Env-APRIL induces higher antibody titers than Env. (A) Rabbit immunization scheme. (B) Midpoint anti-gp120 IgG titers over the course of the immunization experiment as determined by ELISA. The mean values ± standard error of the mean (SEM) of 4 rabbits per group are given. (C) Midpoint anti-gp120 IgG titers at week 18. The mean values ± standard error of the mean (SEM) of 4 rabbits are given. (D) Total IgG midpoint titers at week 0, 12 and 18. Mean values ± SEM of four rabbits per group are given. (E) Fold induction of IgG anti-gp120 titers. The mean relative increase ± SEM of 4 rabbits at week 18 compared to week 16 is given. * indicates p<0.05 as determined by one-tailed Mann-Whitney test.
only 6-fold. The differences between Env-BAFF and gp120 and between Env-APRIL and gp120 were significant (p<0.05) (Fig. 2E). Overall, the data suggest that the Env-APRIL construct induced the best memory response.

Env-APRIL improves neutralization responses

Since Env-based immunogens induce mostly non-neutralizing antibodies, anti-gp120 binding antibody titers rarely correlate with virus neutralization titers. We therefore measured the ability of the sera to neutralize HIV-1 SF162 and JR-FL Env-pseudotyped viruses in a single cycle assay using TZM-bl cells. SF162 is a highly neutralization sensitive virus classified as tier 1 (55,56); JR-FL, which is homologous to the used Env immunogens, is relatively resistant and is in tier 2 (55,56). Although the relevance of tier 1 neutralization for vaccines is questionable, it provides a first low bar measure of a functional antibody response. The pre-bleed sera were inactive against SF162 (50% neutralization titers <10), but post-immunization samples from most animals were able to neutralize this virus (1). The most consistent response at week 18, after the gp140 protein boost, was seen in the group primed with Env-APRIL. Sera from all 4 animals in this group neutralized SF162 with 50% titers >50, compared to 1/4 in the Env group, 2/3 in the Env-BAFF group, 3/4 in the Env-CD40L group and 2/4 in the gp120-primed group. At the earlier time points (week 6 and 12) SF162 was also more frequently neutralized by Env-APRIL sera.

Neutralization of tier 2 strain JR-FL by 50% was only observed sporadically (Table 1). Only 5 animals had titers >30: #B294 (Env-APRIL), #C297 and #C299 (Env-BAFF), #D303 and #D304 (Env-CD40L). No neutralization was observed in the Env or gp120-primed groups. Neutralization of SF162 and JR-FL did not correlate, indicating that different NAb specificities are responsible.

The Duke Central Immunology Laboratory for AIDS Vaccine Research and Development performed an independent neutralization analysis using the three tier 1 viruses SF162.LS, MN and BaL.26, and several tier 2 viruses. Although several of the sera were active against the tier 1 viruses, none of them neutralized any of the tier 2 viruses by 50% at dilutions of more than 20-fold (Table 2 and data not shown). The titers against SF162 derived in the reference laboratory were generally considerably higher than those we had generated, but the overall neutralization pattern was similar. The most consistent and strongest response was observed in the Env-APRIL group. A similar pattern was seen when MN was used. Although still a tier 1 virus, BaL.26 is generally more neutralization resistant than SF162.LS and MN (57). Accordingly, the rabbit sera neutralized BaL.26 at lower titers and less consistently. Nonetheless, the best responses were again seen in the Env-APRIL group, where 3/4 rabbits neutralized BaL.26 at titers >100, while the responses in the other DNA-primed groups were less consistent. None of the sera from animals primed and boosted with gp120 protein could neutralize BaL.26.
Neutralization is mediated by IgG

To assess whether anything other than antibody, such as interferons, chemokines or factors influencing cell viability, might be affecting the outcome of the neutralization assays, we further analyzed selected sera with particularly potent activity. IgG antibodies from the sera of rabbits #B296 (Env-APRIL), #C297 (Env-BAFF), and #D304 (Env-CD40L) were depleted to <5% of the original level using protein G-coupled agarose beads. The depleted sera as well as the purified IgG recovered from the columns and the unfractionated sera were then tested against SF162 using our in-house assay. IgG was recovered for ~75% on average as determined by quantitative IgG ELISA (data not shown). Depletion of IgG almost completely eliminated the neutralizing activity of the sera (data not shown). In contrast, the purified IgG was at least as active as the original sera when compensating for the different volumes after purification (Fig. 3).

### Table 1: Env-APRIL induces enhanced neutralizing responses as shown by in-house assay

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Table 2: Enhanced neutralization induced by Env-APRIL is confirmed by NIH reference lab

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ND: Not determined. Rabbit died during experiment
The neutralizing antibody response matures over time

During natural infection, the initial Env-specific response is dominated by nonneutralizing antibodies; NAbS appear only later in the course of infection (13). Here, we have studied whether the response to our Env immunogens also matures over time by measuring neutralization of SF162 at weeks 0, 6, 12 and 18 post-immunization. As expected, there was no neutralization at week 0, but the average titer increased over time, particularly in the Env-APRIL arm (Fig. 4A). To assess whether this titer increase reflected an increase in the quantity or quality of neutralizing antibodies, we divided the midpoint SF162 neutralization titers by the midpoint gp120 binding antibody titers (Fig. 4B). The resulting ratios increased over time, with median values of 0.013, 0.042 and 0.067 at weeks 6, 12 and 18 respectively. Hence, the increase in SF162 neutralization over time is attributable to more than just an increase in the quantity of anti-gp120 antibodies produced; there is also a qualitative increase, perhaps due to antibody affinity maturation or changes in antibody specificity over time. When the neutralization/binding ratios were compared for the various experimental groups, there was no statistically significant difference among them.

Env-APRIL induces improved anti-trimer responses that correlate with neutralization

Neutralizing antibodies should be able to recognize the functional, trimeric Env spike. We determined whether antibodies in the rabbit sera could bind recombinant gp140 trimers in an ELISA that we have described elsewhere (26,51,58). This ELISA makes use of a construct that is similar to the Env used for the control immunization group, SOSIP.R6-IZ-His, in which the His-tag was replaced by a D7324 epitope tag. All week 12 and 18 sera contained trimer-reactive antibodies (Fig. 5A). The highest titers were found in

Figure 4: The neutralizing antibody response matures over time. (A) Mean SF162 mid-point (50%) neutralization titers are shown for each group of four rabbits. (B) The ratios of the SF162 mid-point neutralization titers to the anti-gp120 midpoint binding antibody titers at weeks 6, 12 and 18 are plotted for each rabbit. ** and *** indicate p<0.01 and p<0.001, respectively as determined by one-tailed Mann-Whitney test.
the Env-APRIL group, while the lowest titers of anti-trimer antibodies were found in the group of animals primed with gp120, which was significant on week 18. A similar but non-significant trend was observed for Env-BAFF. Moreover, unlike the other groups, the anti-trimer titers in the gp120-primed rabbits did not increase after gp140 trimer booster immunization at week 16 (Fig. 5A).

To investigate whether the anti-trimer antibodies correlated with neutralization, we plotted the midpoint neutralization titers for SF162 and JR-FL against either the anti-gp120 or the anti-trimer binding antibody titers (Fig. 5B-E). There was a strong and significant correlation between the SF162 neutralization titers and both the anti-gp120 (Spearman coefficient \( r = 0.51; p = 0.0008 \)) and anti-trimer (\( r = 0.52; p = 0.0007 \)) titers. For JR-FL neutralization, the correlation was poor and not significant (\( r = 0.19; p = 0.25 \) and \( r = 0.26; p = 0.10 \)).

**Figure 5:** The neutralizing antibody response correlates with anti-trimer binding antibody levels (A) Mean midpoint IgG trimer-binding titers ± SEM of the rabbit sera as determined by ELISA at weeks 0, 12 and 18. (B-E) The mid-point SF162 neutralization titers are plotted for each individual rabbit at week 12 (grey symbols) and week 18 (black symbols) against (B) the mid-point anti-gp120 binding antibody titer and (C) the mid-point anti-trimer binding antibody titer. (D,E) as (B,C) but with JR-FL neutralization titers. (F) Ratio of the anti-trimer/anti-gp120 binding antibody titers at weeks 0, 12, 18. ** indicates \( p < 0.01 \) as determined by one-tailed Mann-Whitney test. (G-I) Mid-point neutralization titers for MN (G), SF162.LS (H) and BaL.26 (I), as determined by the Duke Central Immunology Laboratory for AIDS Vaccine Research and Development, are plotted against the ratio of the anti-trimer/anti-gp120 binding antibody titers. The Spearman r-values and two-tailed p-values are shown. Where possible, the appropriate immunization group is indicated by the relevant symbol as explained in (B). * indicates \( p < 0.05 \).
We also calculated the ratios of the anti-trimer and anti-gp120 binding antibody titers, to gauge what proportion of the latter antibodies could also recognize trimers. There were no significant differences in this ratio between the Env, Env-APRIL, Env-BAFF and Env-CD40L groups (data not shown), which is consistent with the use of the same Env moiety in all these groups. Thus, conjugation of Env to, e.g., APRIL increases the antibody responses to both monomeric gp120 and trimeric gp140, but it does not change the relative proportion of the two categories of Env-reactive antibodies. In contrast, sera from the gp120-primed rabbits contained a significantly lower proportion of trimer-reactive antibodies (Fig. 5E). Thus, the anti-trimer to anti-gp120 ratio in this group was 0.26 at week 12, while the average ratio across the gp140-primed rabbits (i.e., the Env, Env-CD40L, Env-BAFF and Env-APRIL groups combined) was 0.71 (p<0.01). The anti-trimer to anti-gp120 ratio was ~2-fold higher in all the groups after the gp140 trimer protein boost at week 16, but the differential between the gp140-primed and gp120-primed animals remained (1.27 vs 0.51, p<0.01). Thus, gp140 trimers are more effective than gp120 monomers at priming the production of antibodies that can bind to a trimeric Env protein in an ELISA.

To assess whether the trimer/monomer binding antibody ratio correlated with neutralization activity, we plotted the neutralizing titer against this binding ratio for each serum (Fig. 5G-I). There was a strong correlation between the trimer/monomer binding antibody ratio and neutralization of MN, SF162.LS or BaL.26 (Spearman correlation coefficients (r) of 0.58, 0.53 and 0.80) at statistical significant levels (p=0.002, p=0.006 and p=0.002 respectively). Thus, sera that contain a high proportion of trimer-reactive antibodies are better able to neutralize HIV-1 infection, compared to sera that contain lower levels of such antibodies.

Discussion

To try to improve the immunogenicity of Env-based vaccines, we fused a trimeric gp140 protein to protein domains (”cis-adjuvants”) that can target and activate B cells. Most previous immunotargeting strategies have focused on DCs, since these cells play a critical role in the induction of immunity, particularly T cell responses. Targeting antigens to DCs via lectins such as DEC205 and Clec9A has proven to be useful in this regard (59-62), although the addition of costimulatory signals such as CD40L may be necessary to overcome tolerance (62). Although antigen targeting to DC followed by antigen processing and presentation is beneficial for T cell responses, neutralizing B cell responses require interaction of intact antigen with B cells. We considered that targeting Env directly to B cells could be a useful alternative way to increase the performance of protein-based vaccines intended to induce NAbs. We used the TNFSF members CD40L, APRIL and BAFF to both target the Env antigen to immune cells and to provide an activation signal for the same cells.
The chimeric fusion proteins Env-APRIL, Env-BAFF and Env-CD40L could be produced efficiently, with their Env components folded correctly and presenting appropriate NAb epitopes. When these fusion proteins were tested in rabbits, the Env-APRIL construct performed best at inducing Env-binding and virus-neutralizing antibodies. The Env-APRIL immunized rabbits performed best at neutralizing the tier 1 virus SF162, MN and BaL, but tier 2 viruses were infrequently neutralized. This may be related to the immunization protocol, since the rabbits received only one protein immunization.

Importantly, no aspecific immune activation could be detected. Total IgG levels were comparable among the immunization groups during the length of the immunization study. This shows that the cis-adjuvants specifically improve anti-Env responses and do not cause general immune activation.

We assume that the stronger antibody responses induced by Env-APRIL arise because B cells are activated by the APRIL component of the chimeric construct, although we cannot exclude the possibility that stimulation of other cell types, such as T cells or DCs, also plays a role. Some DC subsets reportedly express TNFRSF13b/ transmembrane activator and CAML interactor (TACI), which should make them sensitive to BAFF and APRIL, although detailed information is presently lacking (33,63). However, if DCs were the prime targets, a more pronounced effect of CD40L might have been expected given how potently it activates these cells. Various antigens, including Env, fused to CD40L can activate DCs in a way that improves immunogenicity (26,64-71).

Here, we observed only moderate effects of CD40L on the humoral response to Env. One possible explanation could be that previous studies used mice and our previous study was performed with human cells, whereas this study was performed in rabbits (26,64-71). It is very well possible that the rabbit immune system responds differently to CD40L stimulation than the mouse or human immune system does.

The inferiority of CD40L as cis-adjuvant compared to APRIL might also be related to the immunization route that was used. The cells targeted by gene gun immunization include keratinocytes, Langerhans cells (LC) and dermal DC (72), which should not be activated by APRIL. However, LC and DC can migrate to draining lymph nodes, where produced Env-APRIL protein could activate B cells. We therefore speculate that de novo Env synthesis and secretion inside the lymph node is crucial in inducing the Env-specific antibody response in this immunization protocol. APRIL seems to be superior as cis-adjuvant in this step.

The gp140-encoding DNA constructs were better than monomeric gp120 at inducing antibodies able to recognize trimeric Env, as demonstrated by the higher trimer/monomer binding antibody ratio at week 12 (0.71 and 0.26). The good correlation between trimer/monomer binding ratios and the SF162 neutralization titers is consistent with the need
for Env vaccines to induce trimer-binding antibodies that are also capable of interacting with functional Env complexes on virions.

The denominator in the trimer/monomer binding antibody ratio (i.e., the anti-gp120 monomer titer) includes antibodies that recognize only gp120 as well as ones that recognize both monomeric gp120 and the gp120 component of trimers. In the former category are antibodies to gp120 epitopes that are occluded within the trimer. The numerator (i.e., the anti-trimer titer) again involves antibodies that recognize both monomeric gp120 and the gp120 component of trimers, but also includes antibodies that bind only to trimeric gp140 and not to gp120. The latter would include antibodies directed against quaternary epitopes bridging two or three protomers, epitopes involving components from both gp120 and gp41, and epitopes contained exclusively within gp41. Neutralizing antibodies in the first and third of these categories have been identified, although most anti-gp41 antibodies are not neutralizing (73). No antibodies to epitopes spanning gp120 and gp41 have yet been described. A better understanding of how to induce trimer-reactive antibodies, and the specificities involved, would be useful to further guide the design of vaccines intended to induce NAb.

The concept of targeting vaccine antigens to B cells using cis-adjuvants such as APRIL could prove useful for Env-based subunit vaccines as well as other vaccines based on proteins. Because TNFSF members can target a variety of cell types, and the type of response can be adjusted by selecting different members of the superfamily, this approach might be a useful addition, or alternative, to antigen targeting strategies based on lectins such as DEC205 and Clec9A.

**Materials and methods**

**Plasmids**

We have previously described modifications that improve the stability of soluble, cleaved gp140 trimers based on the R5 subtype B isolate JR-FL (18). The amino-acid sequence of gp120 and the gp41 ectodomain was modified as follows (Fig. 1A). We introduced: (i) a disulfide bond between residues 501 in gp120 and 605 in gp41 (A501C, T605C; (18)); (ii) a trimer-stabilizing substitution in gp41 (I559P; (19)); (iii) a sequence-enhanced site for furin cleavage (RRRRRR; (50)). We further modified the JR-FL SOSIP.R6 gp140 construct to include a C-terminal GCN4-based trimerization domain (isoleucine zipper; IZ) (26,51). We have shown that this domain further improves trimer stability. A C-terminal octa-Histidine tag (HHHHHHHHHH; H8) was also added. The SOSIP.R6-IZ-H8 construct is abbreviated here as “Env”.

To compare trimeric Env with monomeric gp120, we used a variant of SOSIP. R6-IZ-H8 in which the H8-tag was replaced by the epitope for the D7324 antibody (APTKAKRRVVQREKR), generating SOSIP.R6-IZ-D7324. Note that the internal D7324
epitope in the C5 domain of SOSIP constructs is mutated by the introduction of the cysteine at residue 501 that forms the intramolecular disulfide bond with gp41, and it is also occluded by the proximity of the gp41 subunit; hence unmodified SOSIP gp140s do not react efficiently with Ab D7324 (18,19). Because of the addition of C-terminal sequences to SOSIP gp140, SOSIP.R6-IZ-D7324 trimers are poorly cleaved (26).

Codon-optimized rabbit sequences of APRIL, BAFF and CD40L were synthesized (Mr. Gene, Regensburg, Germany) containing the restriction sites for Asp718I and SfuI. The APRIL, BAFF and CD40L sequences (corresponding to amino acids 115-250, 149-290, and 119-261, respectively) were then cloned C-terminally to SOSIP.R6-IZ, using Asp718I and SfuI.

The sequence integrity of all clones was confirmed prior to use. The amino acid numbering of SOSIP.R6 gp140 is based on HXB2 Env.

**Cell culture and transient transfection**

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml) as previously described (74). HEK293T cells were transfected using polyethyleneimine (PEI), as described elsewhere (75). Briefly, DNA encoding Env protein was diluted in DMEM (Invitrogen, Breda, The Netherlands), to 1/10 of the final culture volume and mixed with PEI (0.12 mg/ml final concentration). After incubation for 20 min, the DNA–PEI mix was added to the cells for 4h before replacement with normal culture medium containing 10% FCS (HyClone, Perbio, Etten-Leur, The Netherlands), penicillin, streptomycin and MEM nonessential amino acids (0.1 mM, Invitrogen). Culture supernatants were harvested 48h after transfection.

**SDS-PAGE, BN-PAGE and western blotting**

SDS-PAGE, blue native (BN)-PAGE, and western blot analysis were performed as described elsewhere (19,26,76,77), using the JR-FL V3-specific mouse MAb PA-1 as an Env probe (78) (Progenics Pharmaceuticals).

**Immunoprecipitation assays**

100 µl of 20X concentrated 293T cell supernatant was incubated overnight at 4°C, with rotation, with MAbs or related reagents (HIV1g, b12, CD4-IgG2, 2F5 at 4 µg/ml, 17b at 1.5 µg/ml), and when appropriate sCD4 (10 µg/ml), in 500 µl of RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 0.25 % sodium deoxycholate, and protease inhibitors (Complete protease inhibitor tablets, Roche, Almere, The Netherlands). Next, Protein G-coated agarose beads (Pierce/Thermo Fischer, Etten-Leur, The Netherlands) were added and incubated for 2h at 4°C with rotation. The beads were then washed six times with RIPA buffer (supplemented with 0.01% Tween 20), after which the bound
proteins were eluted by heating at 100°C for 5 min in 50 µl of 2X SDS-loading buffer containing 100 mM dithiothreitol (DTT). The immunoprecipitates were fractionated by SDS-PAGE (8% polyacrylamide) at 125 V for 1.5 h. Env detection was performed using MAb PA-1 and standard Western blot techniques.

**Gene gun DNA and protein immunizations**

The pPPI4 plasmid encoding fusion proteins was amplified using DH5α cells and isolated using the EndoFree Plasmid Giga Kit (Qiagen, Venlo, The Netherlands). The immunizations were carried out at Genovac (Freiburg, Germany), under contract. The facilities at Genovac comply with the European Community guidelines for animal housing and in vivo experiments. New Zealand white rabbits (4 rabbits per group) were immunized at weeks 0, 2, 4 and 8 with 125 µg of endotoxin-free DNA, via the abdominal dermis, using gene gun technology. One group was immunized at weeks 0, 2, 4 and 8 with 30 µg JR-FL gp120 in Imject Alum (40 mg/ml aluminum hydroxide and 40 mg/ml magnesium hydroxide. The injection mixture was made according to the manufacturer's instructions (Pierce) and delivered subcutaneously at six sites: two each in the shoulder, abdomen, and hind limb. At week 16, all rabbits were injected with 1 ml PBS containing 30 µg of JR-FL SOSIP.R6 gp120 protein (18,19,50) mixed with 60 µg of Quil A (Brenntag, Frederikssund, Denmark). The injections were performed as follows: 300 µl intradermally (50 µl into each of the above 6 sites), 400 µl intramuscularly (200 µl into each hind leg) and 300 µl subcutaneously (into the neck region). Blood samples were obtained at weeks 0, 2, 4, 6, 8, 12, 16, 18, the final bleed at week 20.

**Env-specific and total immunoglobulin ELISAs**

Anti-gp120 antibody titers were measured by D7324-capture ELISA, essentially as described previously (51). Anti-trimeric gp140 titers were determined using SOSIP.R6-IZ-D7324, which was engineered to contain the D7324 epitope at its C-terminus (26,51).

For measuring total serum immunoglobulin levels, goat anti-rabbit IgG (Jackson ImmunoResearch, Newmarket, UK) was coated overnight (10 µg/ml) in 0.1 M NaHCO3, pH 8.6 (100 µl/well) onto microplate wells. After blocking, serially diluted serum was added for ~2h. Bound mouse IgG was detected with HRP-labeled goat anti-rabbit IgG (Jackson ImmunoResearch, Suffolk, England; used at 1:5000 (0.2 µg/ml)), followed by quantification in a luminometer. Midpoint titers were calculated using Graphpad Prism (version 5.03) by determining the dilution of the serum at which the optical density was 50% of the maximum value. For quantification a standard curve using known amounts of purified rabbit IgG (Jackson) was used.
Neutralization assays

The TZM-bl reporter cell line stably expresses high levels of CD4 and HIV-1 co-receptors CCR5 and CXCR4 and contains the luciferase and β-galactosidase genes under the control of the HIV-1 long-terminal-repeat promoter. The line was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health (John C. Kappes, Xiaoyun Wu, and Tranzyme Inc. (Durham, NC)). Single-cycle infection and inhibition experiments using TZM-bl cells were performed as described (79,80). The percentage of neutralization was determined by measuring how much of the luciferase signal was reduced by each serum dilution, compared to when serum was absent (defined as 100%). The 50% (midpoint) neutralization titers were determined. The viruses tested at the Duke Central Immunology Laboratory for AIDS Vaccine Research and Development were the tier 1 strains MN, SF162.LS and BaL.26 and the tier 2 strains JR-FL, 6535.3, QH0692.42, PVO.4 and RHPA4259.7. The sera were heat inactivated (30 min 56°C) before use.

IgG depletion

Serum (120 µl) was mixed with 200 µl of a 50% (vol/vol) slurry of Protein G plus agarose (Pierce) and 780 µl of phosphate buffered saline, pH 7.4. The mixture was incubated overnight at 4°C, and the beads were then washed thoroughly with 1X RIPA buffer containing 0.05% Tween 20, as for the immunoprecipitation assay. Rabbit IgG was eluted from the beads using 550 µl of IgG elution buffer (Pierce) and the solution was immediately neutralized with 50 µl of 1 M Tris-HCl, pH 9.5.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 5.03. One-tailed Mann-Whitney U tests were carried out to analyze the immunogenicity experiments. Kurskal-Wallis tests were also performed to compare the same groups, followed by Dunn's multiple comparison test if the medians were found to be significantly different, unless indicated otherwise. Nonparametric Spearman tests (two-tailed) were performed to determine correlations.

Acknowledgements

We are grateful to Dennis Burton and James Robinson for reagents. This research was supported in part by AIDS fund (Amsterdam) grants #2005021 to BB and #2008013 to RWS, and by NIH grants R01 AI45463 and R37 AI36082 to JPM, and P01 AI82362 to WC Olson. RWS is a recipient of an Anton Meelmeijer fellowship, a VENI fellowship from the Netherlands Organization for Scientific Research (NWO) - Chemical Sciences, and a Mathilde Krim research fellowship from the American Foundation for AIDS Research (amfAR).
Peripheral blood mononuclear cells (PBMCs) were isolated from New Zealand White rabbit blood using Ficoll Paque (GE Healthcare, Diegem, Belgium) as described (81). Briefly, the blood was diluted 3X in Hanks Balanced Salt Solution (HBSS, Invitrogen) after which Ficoll Paque was added, followed by centrifugation for 30 min at 2200 rpm without braking. The serum was then removed and the cells were separated from the gradient. The cells were washed 3 times using HBSS, with homologous serum added after the first wash. The cells were then cultured in RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen), 40 IU/ml IL-2 (Chiron, Arnhem, The Netherlands), 10% FCS, 10% homologous rabbit serum and 2 µg/ml PHA (Oxoid, Badhoevedorp, The Netherlands) at 37°C. After three days, RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions.

Initially, first strand synthesis was performed using the Thermoscript RT-PCR System (Invitrogen) and a 3’ primer based on mouse CD40L (primer1: 5’-TCAGAGTTTGAGTAAGC-3’). The same primer and a mouse-based 5’ primer (primer2: 5’-ATGATAGAAAACATACAGCCAACCTTCC-3’) were then used for amplification, with an Expand High Fidelity PCR system (Roche, Almere, The Netherlands). The resulting PCR product was cloned into a TOPO vector using the TOPO TA Cloning Kit (Invitrogen). Subsequently, a PCR reaction was performed using primers again based on the mouse sequence of CD40L mRNA (primer 3: 5’-GACCCTCAAATTGCAGCACACGT-3’ and primer 4: 5’-TCAGAGTTTGAGTAAGCAAAAGATG-3’). These primers were designed to recognize an inner sequence relative to the first two. This PCR product was cloned into a TOPO vector after which sequencing revealed the inner part of rabbit CD40L mRNA.

In order to confirm that we indeed identified rabbit sequence as opposed to a contaminating sequence, we designed a 3’ primer based on the identified rabbit sequence (primer 5: 5’-CAAACACCGAAGCATCCGCTTGC-3’) to perform first strand synthesis and subsequently amplified it using primer 5 and primer 2. Cloning of this PCR product into a TOPO vector and subsequent sequence analysis revealed the same sequence as was originally found, confirming that the sequence was correct.

Analysis of a protein BLAST using the Blastp algorithm showed the closest known sequence to the amplified rabbit sequence to be a sequence that was 89% homologous to human CD40L; there was no closer match to any known nucleotide sequence. Blasting both the identified rabbit cDNA and corresponding amino acid in the database with all rabbit sequences showed there was no significant similarity to any previously identified sequence from this species (at the time, the rabbit genome identification project had not been completed).
The rabbit CD40L sequence was, however, incomplete at its 5’ and 3’ ends. We therefore determined the location and size of the exons in the mouse CD40L mRNA coding sequence and blasted the corresponding putative rabbit sequence, exon by exon, into the database with rabbit genome shotgun sequences. Each individual exon could be found within the shotgun rabbit sequences and was an exact match with the sequence we had identified. The rabbit exons were located in four pieces of the whole genome shotgun sequence (AAGW01417529 for exons 1 and 2, AAGW02046866 for exon 3, AAGW01715933 for exon 4, AAGW01620827 for exon 5). The spread and distribution of the exons over the genomic DNA was similar to what is in the mouse and human genomes. This confirmed that the sequence we found was of rabbit origin. Furthermore, it allowed us to determine the outer 5’ and 3’ end of the rabbit CD40L mRNA coding sequence. These ends had high homology with the corresponding mouse sequence. An alignment of rabbit, mouse and human CD40L sequences is shown in Supplemental figures 1A and 2. The rabbit CD40L sequence has been deposited in Genbank. A Thorbecke inbred rabbit derived sequence has since been deposited in the NCBI protein database as reference sequence XM_002720328 after automated annotation of rabbit genomic sequence NW_003159324. This sequence is 100% identical with the one we identified.
Supplemental figure 1: Alignment of the rabbit CD40L mRNA sequence with its human and mouse homologues.
Supplemental figure 2: Alignment of the rabbit CD40L amino acid sequence with its human and mouse homologues

References

