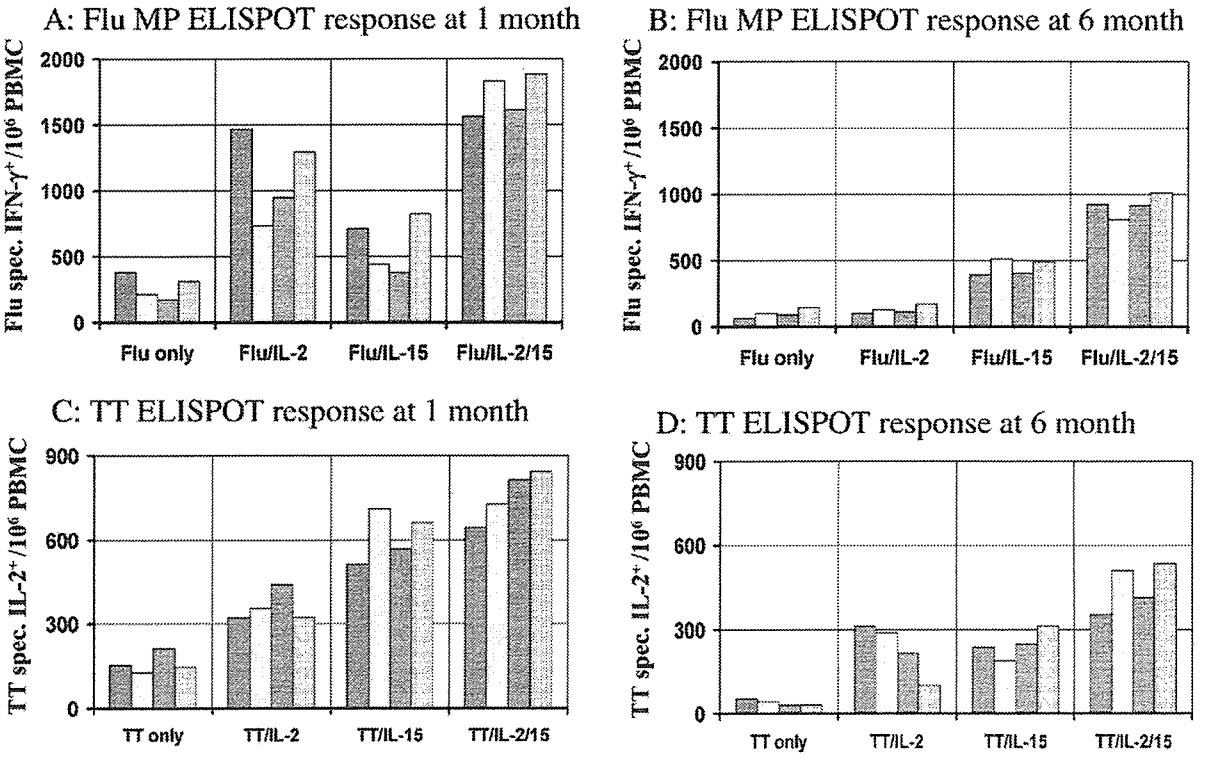

Fig. 4. Influence of IL-2 vs. IL-15 on primary antigen specific T cell expansion and retention. Groups of four rhesus macaques were administered a single TT and Flu immunization before being administered either 5 μg/kg IL-2, 10 μg/kg IL-15 or alternating doses of IL-2 and IL-15 for 4 weeks following the immunization. Flu-MP specific IFN-γ responding CD8 T cells (A and B) and TT specific IL-2 responding CD4 T cells (C and D) were enumerated by ELISPOT at the end of cytokine administration (1 month (A and C)) and 5 months later (6 months (B and D)). The control group only received the immunizations but no cytokines.

cells over primed but untreated controls, IL-15 treated animals exhibited significantly higher expansion of such TT specific effectors over the group treated with IL-2 (Fig. 4C). The combined administration of IL-2 and IL-15 appeared to further expand such TT specific effectors, however, the difference between this group and the group administered IL-15 was modest ($P > 0.05$). At 6 months post prime, all cytokine treated groups showed statistically higher levels of TT specific memory T cells over the primed untreated group (Fig. 4D, $P < 0.0001$). There was, however, no statistically relevant difference between the groups treated with IL-2 or with IL-15 for levels of TT specific effectors. Only the group that had received the combination of IL-2/IL-15 showed a higher level of TT specific memory cells over IL-2 and IL-15 treated groups ($P < 0.0001$).

3.4.1. Role of IL-2 and IL-15 in the expansion of secondary T cell responses

Next, the immunomodulatory role of IL-2 and IL-15 was evaluated following a booster immunization (secondary response) with Flu/TT in the same animals shown in Fig. 3. Thus, 9 months following the Flu/TT prime, each animal was administered a booster dose of Flu and TT as described in Section 2. Three days later, the animals previously administered IL-2 were again given 4 weeks of IL-2 treatment twice weekly while the animals previously administered IL-15 underwent another IL-15 treatment twice weekly and the animals administered the IL-2/15 combination received the same combination twice weekly for 4 weeks post booster immunization. The control group animals were boosted and left untreated. Comparison of the data obtained before and after the Flu boost showed a readily detectable anamnestic expansion of Flu-MP specific T cells in all animals (Fig. 5A and B). However, unlike the findings in these animals following the primary immunization, sequential administration of IL-2 did not appear to influence the magnitude of Flu-specific T cells, while IL-15 treatment of these monkeys (with already elevated memory levels following primary immunization) still appeared to enhance the expansion of Flu-MP specific T cells following booster immunization (Fig. 5B, $P < 0.0003$). In marked contrast, the group of monkeys administered the IL-2/IL-15 combination, in spite of exhibiting the highest levels of memory T cells before the booster immunization, yielded secondary responses slightly higher than monkeys boosted without cytokine administration or treated with IL-2, but markedly lower than monkeys administered IL-15 alone (Fig. 2C, $P < 0.0001$). While surprising, these results clearly suggest that administration of IL-2 with the aim of enhancing secondary antigen specific responses may be inappropriate, unless the lack of enhanced antigen specific T cells in PBMCs is secondary to increased homing of these cells to tissues, which remains to

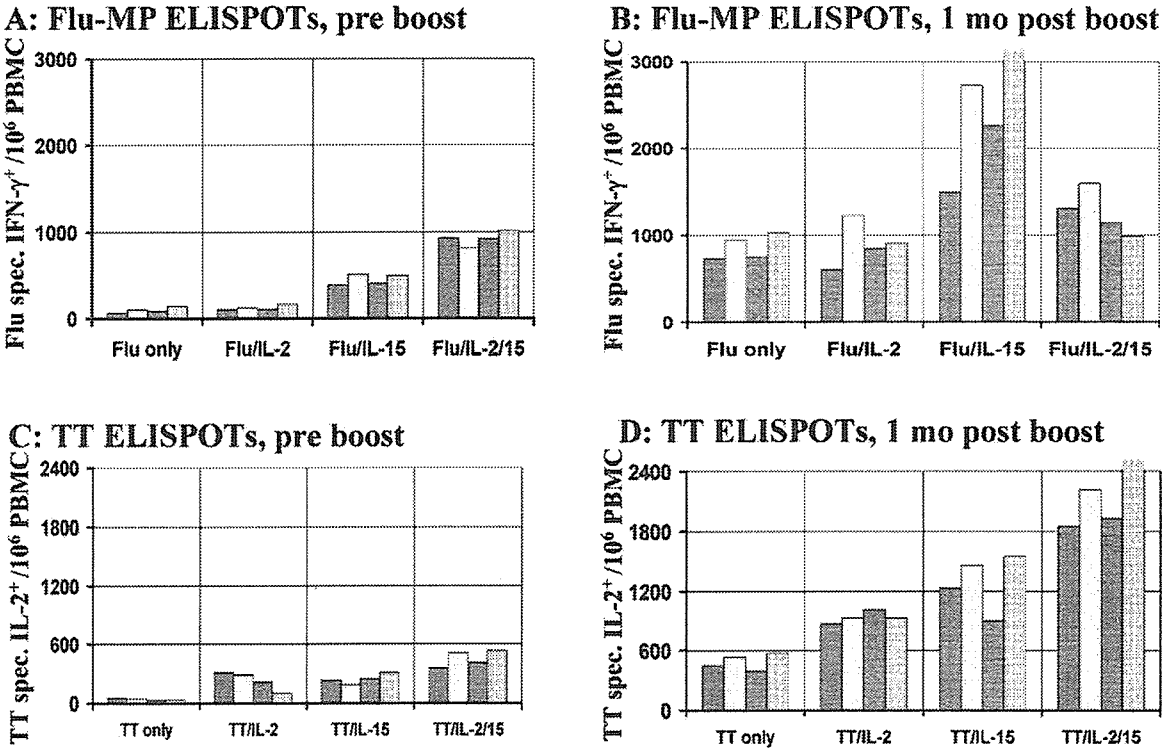

Fig. 5. Influence of IL-2 vs. IL-15 on secondary antigen specific T cell expansion. Groups of four rhesus macaques shown in Fig. 3 were administered a booster TT and Flu immunization before being re-administered the same cytokine regimen given after the primary immunization, either 5 μg/kg IL-2, 10 μg/kg IL-15 or alternating doses of IL-2 and IL-15 for 4 weeks following the immunization. Flu-MP specific IFN-γ responding CD8 T cells (A and B) and TT specific IL-2 responding CD4 T cells (C and D) were enumerated by ELISPOT before the booster ((A and C) corresponding to Fig. 3B and C, respectively) or at the end of cytokine administration (1 month post boost (B and D)). The control group again was administered booster immunizations but no cytokines.

be addressed. Nevertheless, monkeys administered IL-2 following the booster immunization exhibited decreased levels of circulating Flu-MP specific CD8 T cells in contrast with the results obtained from monkeys administered IL-15 alone.

In contrast to Flu specific CD8 T cell responses, TT specific CD4 mediated responses while markedly elevated by the administration of the booster immunogen in the untreated monkeys, showed enhanced responses following administration of IL-2 and even higher levels of TT specific T cells following IL-15 administration, similar to the memory responses observed following the primary immunization (Fig. 5C and D, $P < 0.0005$). Unlike the decrease observed in CD8 mediated responses in the IL-2/IL-15 treated group, the TT specific CD4 levels were markedly higher in this group as compared to the groups administered a single cytokine, suggesting functional differences in the maintenance of antigen specific CD4 versus CD8 T cells in the periphery.

To investigate whether the values obtained above using the ELISPOT assay truly represent levels of effector T cells, a comparison of samples from the three cytokine treated groups was performed using the p-CTL assay against Flu-MP and proliferative assay against TT. As seen in Fig. 6, analysis of Flu-MP specific pCTLs levels essentially reproduced the data obtained with the ELISPOT assays (Fig. 6A and B versus Fig. 5A and B), whereby IL-15 administration markedly enhanced Flu-MP p-CTL levels induced by the booster immunization over animals given IL-2 ($P < 0.0001$). In contrast, administration of the combination of IL-2 and IL-15 only modestly enhanced the Flu-MP specific p-CTL levels whereby the increase from pre booster level was not different from the IL-2 only treated group, since the levels of Flu-MP specific memory T cells were significantly higher than the ones measured in IL-2 treated monkeys at the same time point (Fig. 6A). This correlation was further confirmed by performing a scatter analysis between Flu-MP pCTL and ELISPOT values for each individual monkey and time point, showing a linear correlation, irrespective of cytokine treatments (data not shown).

In contrast to the CD8 responses and to the TT specific ELISPOT responses, TT specific proliferative responses were not significantly different between monkeys administered IL-2, IL-15 or the IL-2/15 combination post boost ($P > 0.05$), suggesting that the regulation and response of TT specific CD4 T cells by these cytokines invokes distinct pathways from the ones used by CD8 T cells, and that the proliferative response does not directly correlate with the frequencies of IL-2 producing CD4 T cells as measured by ELISPOT, although the reason for the latter difference is unclear at present.

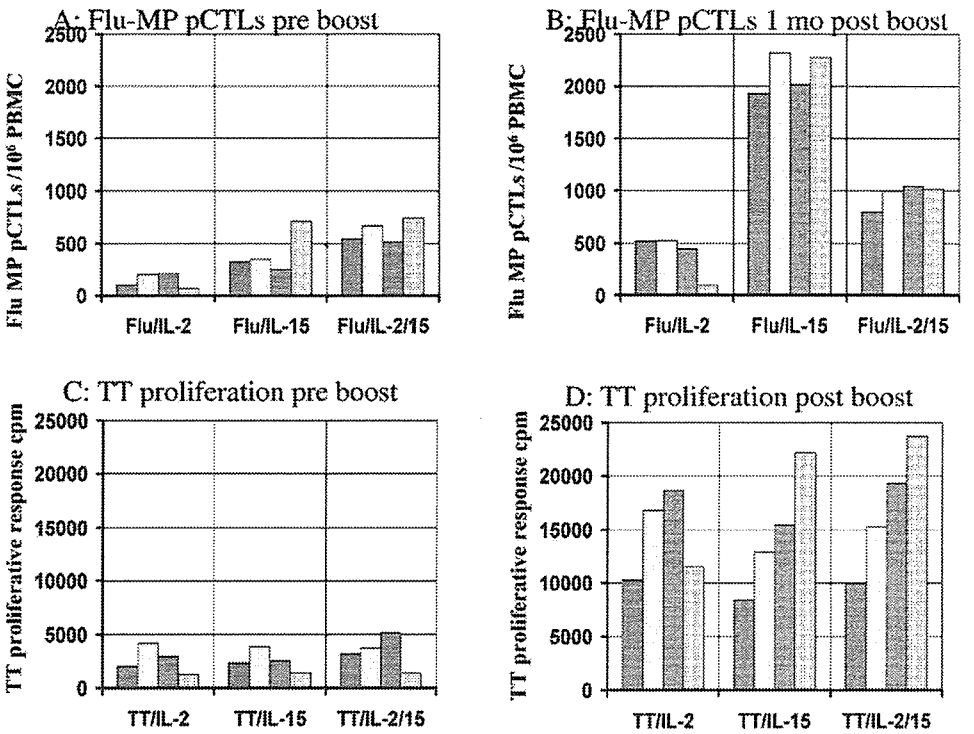

Fig. 6. Influence of IL-2 vs. IL-15 on secondary functional T cell expansion. Samples from the monkeys analyzed in Figs. 3 and 4 were tested for Flu-MP specific p-CTLs (A and B) and TT specific proliferation (C and D) before (A and C) and at 1 month following the booster immunizations and cytokine administration (B and D). Samples from the non cytokine treated control group were unavailable for these assays.

4. Discussion

The elaboration of an immune response to a novel immunogen represents the sum of a large variety of mechanisms both intrinsic and environmental contributing to the magnitude and the quality of such responses. Thus, while the genetic background of the host clearly plays a role, the route of antigen delivery, the subset of antigen presenting cells involved, the site of antigen presentation and ultimately the milieu in which such antigen presentation occurs all have the potential for modulating the initial expansion and maintenance of antigen specific T cell responses [38–40]. The recent technical progress in our capacity to more precisely enumerate antigen specific T cells has allowed for a better definition of the kinetics of antigen specific T cell expansion and contraction as well as the determination of levels of memory T cells. Thus, at least for some antigens, a direct correlation was found between the amplitude of the T cell pool being expanded post antigen recognition and the levels of long lived memory T cells being established in vivo [4,41], prompting the search for strategies to enhance the initial expansion of such T cells to achieve higher levels of antigen specific memory T cells following infection or immunization.

The second important finding was that long-term maintenance of memory T and B cells which was thought to require periodical re-exposure to the antigen, has recently been found to be effectively maintained via homeostatic mechanisms alone, most likely for the life of the host even in the absence of occasional antigen exposure [11]. Based on these findings, it appears reasonable to assume that increased homeostatic turnover of antigen specific memory T cells would also result in higher levels of antigen specific memory T cells in a particular host, thus, enhancing the effectiveness of immunization strategies and potentially decreasing the need for multiple booster doses.

Traditionally, improving the immunization efficacy required the use of adjuvants and/or special formulation of the antigen resulting in either improved antigen presentation and/or T cell recognition. With increasing understanding of the various pathways involved in antigen presentation and recognition resulting in T cell responses of specific quality as well as the mode of action of such adjuvants, it is now possible to dissect the sum of these mechanisms more effectively by administering only select agents or triggering only specific pathways among the multiple pathways normally activated by the administration of adjuvants. Thus, the use of cytokines in the clinical setting has been investigated for some time, including the role of GM-CSF in enhancement of antigen presentation, high doses of IL-2 in the mobilization of tumor infiltrating lymphocytes, low doses of IL-2 for immune restoration in HIV infected patients to name a few [42–45]. Along these lines, our lab also demonstrated that the use of IL-12 in the context of pathogenic SIV or plasmodium infection has profound effects on the course of infection with these pathogens [46,47]. The use

of such biological reagents is, however, problematic in the clinical setting due in large part to the pleiotropic nature of these factors, potentially resulting in a host of unwanted side-effects [48,49]. Clearly, switching to low dose administration of these factors has provided a far better clinical window of application in general [45,48], albeit the optimal administration schedule may vary significantly with regards to the endpoints targeted. In that regard, our studies clearly demonstrated that in terms of maintenance of enhanced antigen specific T cell levels, daily administration of IL-2 or IL-15 was counter productive, while spacing such administration appeared far more efficient for reasons that remain to be investigated. It also remains to be seen whether this administration optimized for memory T cell levels also represents an optimal administration schedule in terms of hematological homeostasis. Nevertheless, even though the data may only be representative of the model antigens used in our study, our findings are likely to prompt the re-evaluation of current clinical protocols whereby cytokine therapy trials such as those utilizing IL-2 administration given on a five daily schedules per week [43,50,51].

The next point brought up by our studies clearly highlight the short-term effect of IL-2 on effector CD8 T cells when compared to IL-15, suggesting differential rationale for the use of either cytokine in vivo. While again, it may be argued that the range of antigen and responses analyzed in our studies is rather restricted, the data obtained with outbred monkeys parallels findings reported for murine models [14,52,53]. Thus, while both cytokines target largely overlapping subsets of T cells due to the shared components of their respective receptors, the activating signals delivered by either factor appear to differ in the magnitude of the activation and in the ultimate longevity of the activated T cells. Thus, IL-2 has been shown to induce T cell activation and proliferation [52], however, such effect is also accompanied by the upregulation of apoptotic markers presumably as a negative regulatory feedback mechanism to limit the expansion and presence of such highly activated effector cells [14,52,53]. In contrast, IL-15 appears to induce a less potent effector response while still inducing marked levels of T cell proliferation, but without extensive induction of apoptotic mechanisms leading to higher levels of long-term expanded T cells in vivo. In addition, homeostatic re-stimulation with IL-15 still results in enhanced levels of antigen specific T cells while IL-2 appeared ineffective at enhancing levels of memory CD8 T cells post boost, when compared to animals given booster immunization without cytokines. The precise nature of the mechanistic differences remains to be fully elucidated as well as its potential implications on various effectors mechanisms as illustrated by the comparison of functional data presented in Figs. 5 and 6. In addition, several hypotheses may account for the decrease in antigen specific CD8 T cells following IL-2 administration including apoptosis induction, refractoriness of antigen specific T cells via anergy, difference in re-circulation patterns of CD4 versus CD8, central versus peripheral memory T cells and lastly the induction of regulatory T cells. Delineation of the contribution to each of these potential mechanisms will be important for the design of vaccine in future studies.

Acknowledgements

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Effect of partial and complete variable loop deletions of the human immunodeficiency virus type 1 envelope glycoprotein on the breadth of gp160-specific immune responses

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Abstract

Induction of cross-reactive cellular and humoral responses to the HIV-1 envelope (env) glycoprotein was examined after DNA immunization of BALB/c mice with $gp140_{89.6}$-derived constructs exhibiting partial or complete deletions of the V1, V2, and V3 domains. It was demonstrated that specific modification of the V3 loop (mV3) in combination with the V2-modified (mV2) or V1/V2-deleted (ΔV1/V2) region elicited increased levels of cross-reactive $CD8^+$ T cell responses. Mice immunized with the mV2/mV3 or ΔV1/V2/mV3 $gp140_{89.6}$ plasmid DNA were greater than 50-fold more resistant to challenge with recombinant vaccinia virus (rVV) expressing heterologous env gene products than animals immunized with the wild-type (WT) counterpart. Sera from mV2/mV3- and ΔV1/V2/mV3-immunized mice exhibited the highest cross-neutralizing activity and displayed intermediate antibody avidity values which were further enhanced by challenge with rVV expressing the homologous gp160 glycoprotein. In contrast, complete deletion of the variable regions had little or no effect on the cross-reactive antibody responses. The results of these experiments indicate that the breadth of antibody responses to the HIV-1 env glycoprotein may not be increased by removal of the variable domains. Instead, partial deletions within these regions may redirect specific responses toward conserved epitopes and facilitate approaches for boosting cross-reactive cellular and antibody responses to the env glycoprotein.

Keywords: HIV-1; DNA vaccine; Envelope glycoprotein; Cellular responses; Neutralizing antibodies

Introduction

The current challenge for the design of effective HIV-1 vaccines is to develop immunization strategies to elicit both stronger and broader immunity against diverse viral species (Chakrabarti et al., 2002; Schulke et al., 2002). The envelope (env) glycoprotein is an important component of an efficacious vaccine for AIDS because gp120 is the major viral surface glycoprotein and a key mediator of the entry process (Binley et al., 2000; Kwong et al., 2000; Moore and Binley, 1998; Moore and Sodroski, 1996; Parren et al., 1999; Reitter et al., 1998; Wyatt et al., 1998). However, to persist as a chronic infection, HIV-1 has evolved ways to escape from cytotoxic T lymphocyte (CTL) recognition and to limit the generation of neutralizing antibodies. This includes mutations arising within regions of the viral genome encoding immunodominant epitopes that contribute to viral spread and the inability of anti-HIV-1 immunity to prevent the onset of AIDS (Goulder et al., 1999). Additionally, there is extensive shielding of conserved regions by carbohydrates (Reitter et al., 1998) and hiding of the coreceptor-binding site by variable loops of gp120 until the CD4 interaction occurs, thereby minimizing the time and space available for the antibody to intervene against this stage of the fusion process (Kwong et al., 1998; Moore and Binley, 1998). Because the overall structure of gp120 must be at least partially conserved for the protein to function in

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receptor binding and cellular entry, therefore the receptor-binding sites may provide a target for structural modifications to induce broadly neutralizing antibodies.

Although mechanisms responsible for induction of cross-protective antibody responses to the HIV-1 env glycoprotein remain to be elucidated, several lines of evidence indicate that exposure of certain neutralizing epitopes participating in the env-CD4 and -coreceptor binding can be increased by structural modification of the variable domains of gp120 (Barnett et al., 2001; Cao et al., 1997; Malenbaum et al., 2000; Stamatatos and Cheng-Mayer, 1998). Changes in the gp120 V2 loop or V1/V2 stem region that are responsible for both CD4-independent entry into cells and gp120 binding to CCR5 in the absence of CD4 (Kolchinsky et al., 1999, 2001) can alter immunogenicity of the single or double mutant. For example, deletion of the V1 and V2 loops from the env glycoprotein of the HIV-1_{HXBc2} was shown to increase susceptibility of the virus to neutralization by anti-V3 and certain CD4-induced monoclonal antibodies (Cao et al., 1997). Deletion of the central region of the V2 loop in gp120 of the HIV-$1_{SF162\Delta V2}$ isolate enhanced induction of cross-neutralizing antibodies by altering the immunogenicity of the V3 and V1 loops and rendering the C5 region immunogenic (Barnett et al., 2001; Srivastava et al., 2003). Deletion of the V1/V2 variable loop also leads to a significant reduction of the carbohydrate content (Reitter et al., 1998) that modulates the conformation of the V1/V2 stem-loop and affects the exposure of conserved, discontinuous structures on the HIV-1 gp120 glycoprotein (Wyatt et al., 1995). Studies in rhesus monkeys revealed that simian immunodeficiency virus mac239 variants lacking specific N-linked carbohydrate attachment sites within and around the V1 and V2 regions of gp120 were more sensitive to antibody-mediated neutralization and better elicitors of neutralizing antibody responses (Reitter et al., 1998).

The third hypervariable loop is another domain of gp120 that modulates immunogenicity of the env glycoprotein (Trkola et al., 1996). A number of studies based on the construction of chimeric env glycoproteins of X4 and R5 strains have demonstrated that the V3 loop is the primary determinant of tropism and coreceptor usage (Hung et al., 1999; Hwang et al., 1991), although other domains of gp120 are also involved (Basmaciogullari et al., 2002; Ross and Cullen, 1998). It has been reported that V3 loop-derived peptides can inhibit viral entry into target cells or syncytium formation between cells expressing the env glycoproteins of HIV-1 and cells that co-express CD4 and CCR5 or CXCR4 in a coreceptor-specific manner (Verrier et al., 1999). Recent studies have demonstrated that human monoclonal antibodies (mAbs) which recognize epitopes at the crown of the V3 loop derived from the HIV-$1_{JR\text{-}CSF}$ isolate showed cross-clade binding to native, intact virions of clades A, B, C, D, and F (Gorny et al., 2002). Additionally, the N-terminal V3 loop glycan was found to block access to the binding site for CD4 and modulated the chemokine receptor binding site of phenotypically diverse clade A and clade B isolates (Malenbaum et al., 2000). Because the majority of primary HIV-1 isolates reveals good exposure of the V3 region on the surface of intact virions (Nyambi et al., 2000a, 2000b), it has been suggested that antibodies which react with conformation-sensitive epitopes of the V3 loop may have potent cross-neutralizing activities (Park et al., 2000).

While "sterilizing" immunity mediated by broadly neutralizing antibody responses remains the ultimate goal for prophylactic HIV-1 vaccines, env-specific CTL responses may represent an important component of therapeutic vaccines. For example, the clearance of HIV-1 from plasma during the primary infection occurs before the appearance of neutralizing antibodies in newly infected individuals (Moore et al., 1994). In many studies, vaccinated macaques were able to efficiently control a virus challenge in the absence of detectable neutralizing antibodies, particularly those animals that were immunized with live, attenuated virus vaccines (Bogers et al., 1995; Cho et al., 2001; Dunn et al., 1997). Additional studies in pigtailed macaques demonstrated that animals immunized with a mixture of HIV-1 env glycoproteins exhibited lower levels of plasma virus than monkeys in the control group despite the absence of any detectable neutralizing antibody, suggesting induction of superior cellular responses by the polyvalent vaccine (Cho et al., 2001). Our previous studies have demonstrated that immunization of the HLA-A2/K^b transgenic mice with the ΔV3 env vaccine enhanced $CD8^+$ T-cell responses to conserved epitopes of gp160 and broaden cross-reactive CTL responses (Kiszka et al., 2002). However, the ability of the ΔV3 $gp140_{89.6}$ mutant to induce cross-reactive neutralizing antibody responses has not been examined. Therefore, it was of interest to analyze neutralization activities in ΔV3-immunized mice and compare them with responses induced by $gp140_{89.6}$-derived constructs exhibiting partial or complete deletions of the V1, V2, and V3 domains. This head-to-head analysis of cellular and humoral responses demonstrated that partial deletion of the V3 domain in combination with V2 or V1/V2 region modifications increased the breadth and potency of cellular, and to a lesser degree, protective antibody responses.

Results

Variable domain-modified env vaccines

To analyze the effect of specific modifications within the variable region of the env glycoprotein on the repertoire of cellular and humoral responses, we have generated DNA vaccines with partial or complete deletions of the V1, V2, and V3 domains on the background of gp140 of the HIV-$1_{89.6}$ isolate. Several reasons underlay this choice: (i) the HIV-$1_{89.6}$ virus is a primary, dual tropic virus (Doranz et al., 1996); (ii) it is a molecularly cloned virus with a well-

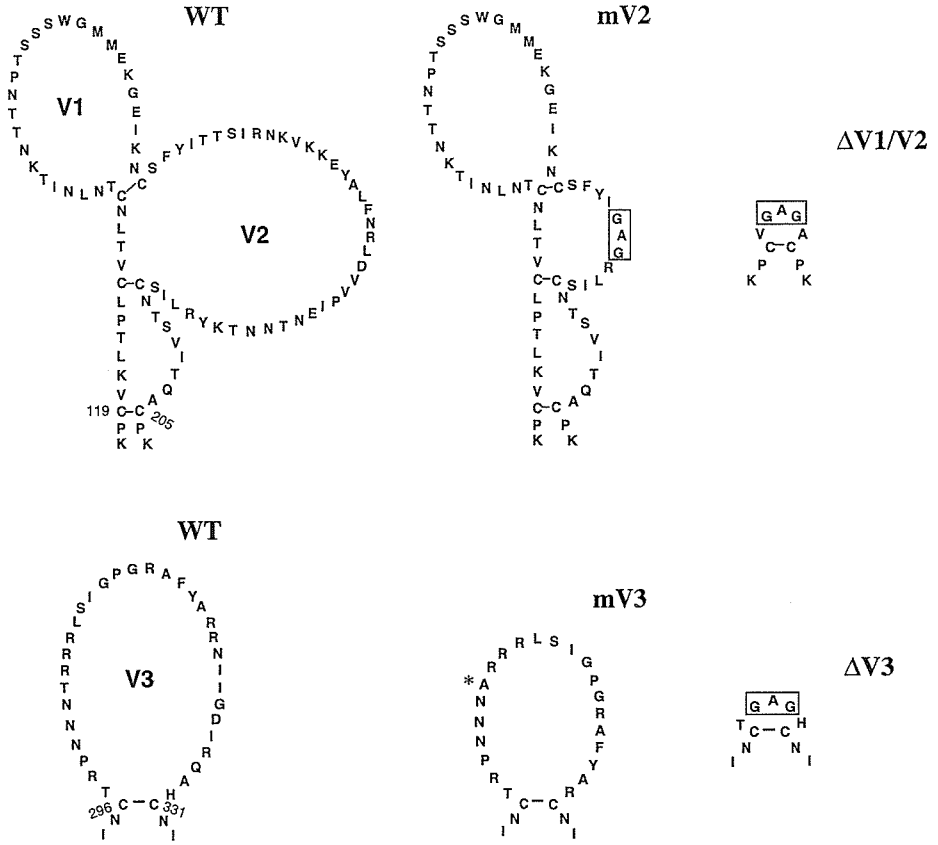

Fig. 1. Schematic representation of the variable domain-modified HIV-$1_{89.6}$ gp140 mutants. The amino acid sequences of V1 and V2 domains of the WT env glycoprotein and the mV2 and ΔV1/V2 mutants are shown in the upper panel. The lower panel depicts the amino acid sequence of the V3 loop of the WT env glycoprotein and the mV3 and ΔV3 mutants. In all mutants, the GAG sequence replaced the deleted residues. The 303T/A substitution in the mV3 domain is indicated by asterisk. The numbering of amino acids is based on the HxBc2 sequence available from the HIV Sequence Database.

characterized env gene (Kim et al., 1995); and (iii) we have previously expressed the gp140 protein from HIV-$1_{89.6}$ and analyzed its immunogenicity in mice (Kiszka et al., 2002).

To generate the variable region-modified $gp140_{89.6}$ mutants with deletion of the V1 and V2 domains (ΔV1/V2), residues 121 to 203 were replaced with a 3-aa sequence

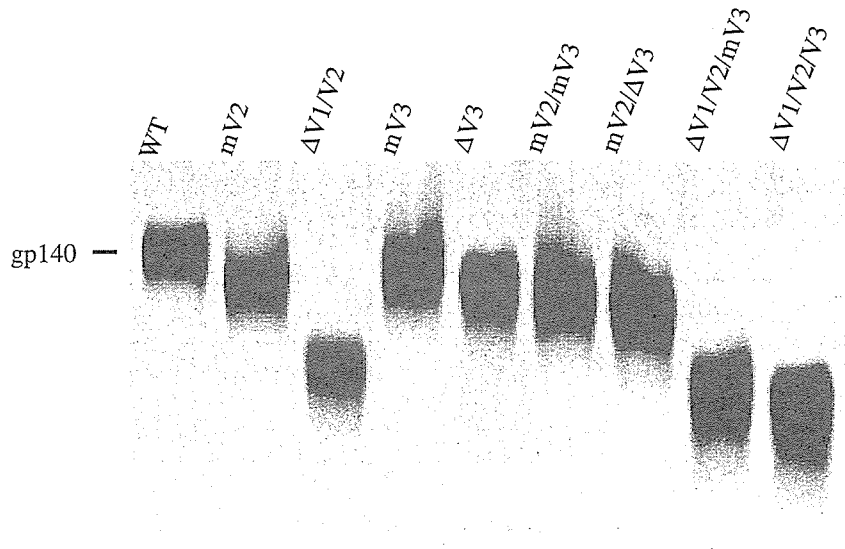

Fig. 2. Immunoprecipitation of WT and domain-modified HIV-$1_{89.6}$ gp140 glycoproteins. The 293T cells transiently transfected with plasmid DNA encoding the WT or the variable domain-modified env glycoproteins were radiolabeled with [^{35}S]methionine/cysteine and lysed in Nonident P-40 buffer. The env proteins from cell lysates were immunoprecipitated with a mixture of sera from HIV-1-infected individuals followed by polyclonal rabbit anti-human Ig and protein A-Sepharose CL-4B.

(GAG) as indicated in Fig. 1 (upper panel). Similarly, the GAG sequence replaced the deleted residues 298 to 329 in the ΔV3 mutant (Fig. 1, lower panel). We have also prepared an additional set of V2 and V3 domain-modified $gp140_{89.6}$ constructs wherein the variable loops were only partially deleted (Fig. 1). In the V2 loop-modified mutant (mV2), the residues 162 to 191 were replaced with GAG, leaving the RLISC sequence in V2 as it represents a part of the CTL epitope RLISCNTSV with the A2-binding motif (Korber et al., 1999). The V3 loop-modified construct (mV3) retained the N-terminal 23-aa fragment from cysteine 296 to arginine 320. This segment contained the GPGR sequence recognized by the broadly neutralizing 447-52D human mAb (Conley et al., 1994) and the RRLSIGP sequence, in the N-terminal side of the V3 loop, that shares antigenic feature recognized by human mAbs with intra- and interclade cross-reactivity (Gorny et al., 1997). The mV3 loop also contained several CTL epitopes with multiple HLA-binding motifs (Korber et al., 1999), and had the N-linked glycan at residue 303 eliminated by the NNT to NNA amino acid replacement. This N-linked glycan was shown to block access to the binding site for CD4 and modulated the chemokine receptor binding site of phenotypically diverse clade A and clade B isolates (Malenbaum et al., 2000).

The variable region deletions were introduced in the HIV-1 $gp140_{89.6}$ construct together or separately, and cloned in frame with the human tissue plasminogen activator (tPA) signal sequence for expression in the pNGVL-7 plasmid as DNA vaccines. The expressions of the WT and domain-modified gp140 proteins were analyzed by immunoprecipitation of transiently transfected 293 cells. The env glycoproteins were immunoprecipitated from cellular lysates of [^{35}S]methionine/cysteine-labeled transfectants with a mixture of sera from HIV-1-infected individuals and resolved by SDS-PAGE. Because the HIV-$1_{89.6}$ gp140 was originally

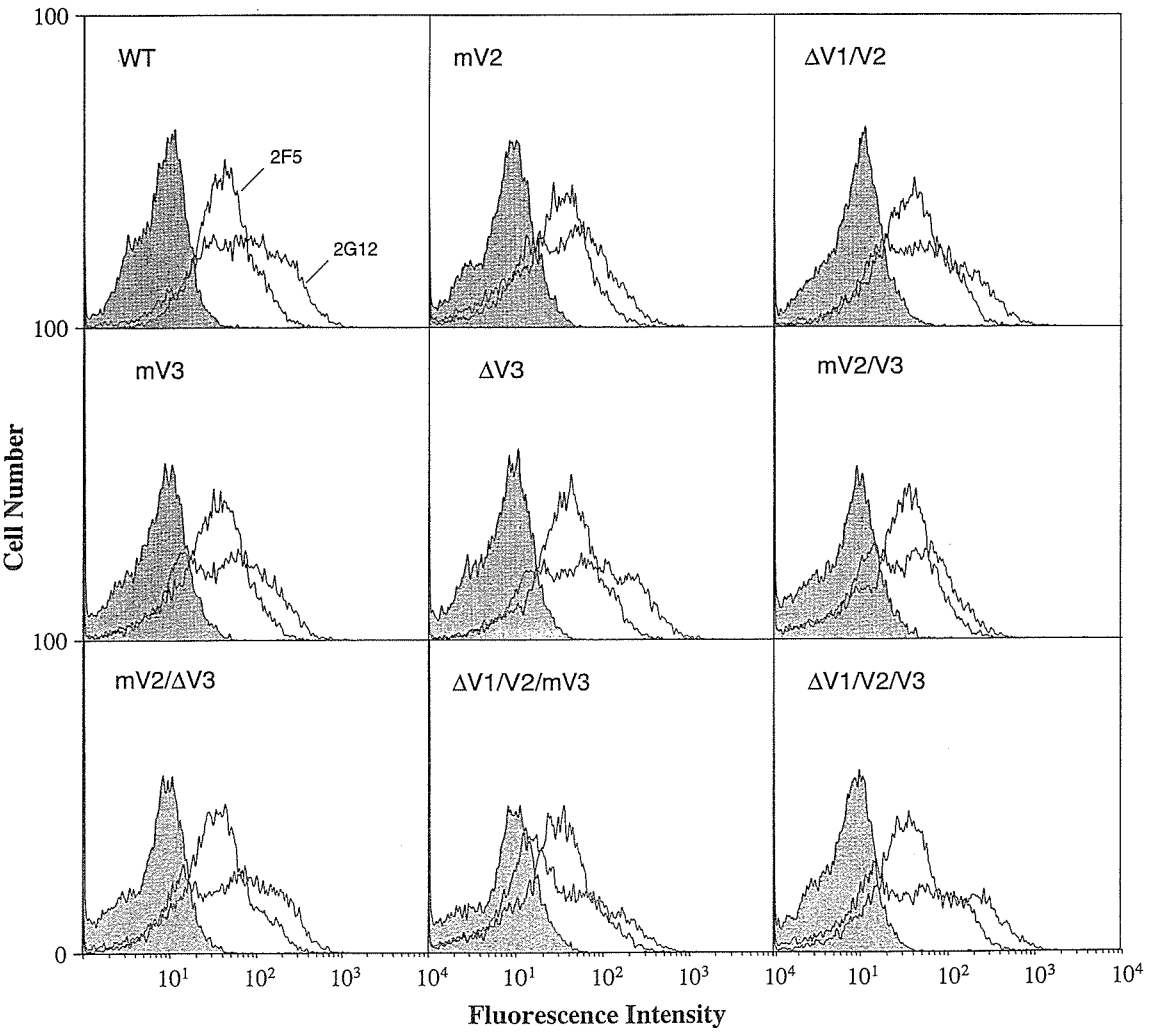

Fig. 3. Immunofluoresce staining and flow cytometry analysis of the full-length and variable domain-modified HIV-$1_{89.6}$ gp140 proteins expressed in 293T transfectants. The expression of the env glycoproteins was analyzed 48 h after transfection by intracellular staining with 2F5 and 2G12 human mAbs followed by the FITC-conjugated F(ab′)$_2$ portion of goat anti-human Ig. Cells were analyzed by flow cytometry on FACScan. The light gray areas denote transfectants stained only with the secondary antibody. Continuous and dotted lines indicate transfectants stained with 2F5 and 2G12 mAbs, respectively.

generated as a gp120–gp41 cleavage site mutant wherein the furin cleavage motif was replaced with a hexameric Leu–Arg motif (Binley et al., 2000), the full-length and domain-modified env glycoproteins were immunoprecipitated as single bands (Fig. 2). The mutants could be distinguished based on differences in the molecular weight due to complete or partial deletions of the variable regions. Variations in the expression levels between the WT and the env mutants were less than 30%, suggesting that the expression of the gp140 mutants was not largely altered by the variable loop modifications. Furthermore, immunostaining of intracellularly expressed env mutants in transiently transfected 293 cells with broadly neutralizing antibodies revealed that the gp140 mutants retained native antigenic determinants similar to the unmodified env protein (Fig. 3). The broadly neutralizing mAbs 2G12 and 2F5 used for staining recognize a carbohydrate-dependent epitope located on the gp120 outer domain and the NEQELLSLWN epitope near the C-terminal end of the gp41 ectodomain, respectively (Parker et al., 2001; Trkola et al., 1996).

Breadth of cellular responses induced by immunization with the WT and domain-modified mutants of HIV-$1_{89.6}$ gp140 protein

In the first set of experiments, we compared the ability of domain-modified env vaccines to induce cross-reactive $CD8^+$ T cell responses. Groups of BALB/c mice ($n = 5$) were immunized intramuscularly (i.m.) with DNA vaccines expressing the complete or domain-modified $gp140_{89.6}$ glycoproteins. Three weeks after the last immunization, frequencies of IFN-γ-secreting splenocytes were analyzed by ELISPOT assay against P815 (H-2^d) target cells infected with recombinant vaccinia virus (rVV) expressing homologous (vBD3) or heterologous (vPE16 or vV1) env gene products. Cells infected with vSC8 virus expressing β-galactosidase served as negative control. This analysis permitted detection of env-specific $CD8^+$ T cells because the P815 mastocytoma cell line expresses only MHC class I molecules (Gherardi and Esteban, 1999).

As shown in Fig. 4, immunization with WT and domain-modified env mutants induced comparable frequencies of env-specific IFN-γ-secreting $CD8^+$ splenocytes against the homologous gp160 gene products. However, the profile of $CD8^+$ T cell responses directed to heterologous gp160 varied among mice immunized with different env vaccines. For example, $CD8^+$ T cells derived from mice immunized with the WT $gp140_{89.6}$ construct exhibited a rather restricted pattern of reactivity, in which the heterologous responses were at approximately 35% (vPE16) and 15% (vV1) of those directed to the homologous env gene products. Immunization with the mV2 or ΔV1/V2 mutant had no effect on the profile of cross-reactive IFN-γ responses in cultures stimulated with vPE16 or vV1-infected target cells. On the other hand, the ΔV3 and mV3 env-induced cellular immunity showed a broader pattern of reactivity responding to vPE16- and vV1-infected target cells with approximately 40% higher efficiencies than responses elicited by the WT env vaccine. Among the double deletants, the highest levels of cross-reactive responses were induced with the mV2/mV3 and ΔV1/V2/mV3 $gp140_{89.6}$ vaccines. In mice immunized with the mV2/mV3 $gp140_{89.6}$ mutant, the responses to vPE16- and vV1-infected cells represented approximately 75% and 60% of those directed to target cells expressing the homologous env gene products, respectively. They were also significantly higher than the heterologous responses in animals immunized with the WT $gp140_{89.6}$ vaccine ($P = 0.003$ and $P = 0.0002$, respectively). A similar profile of

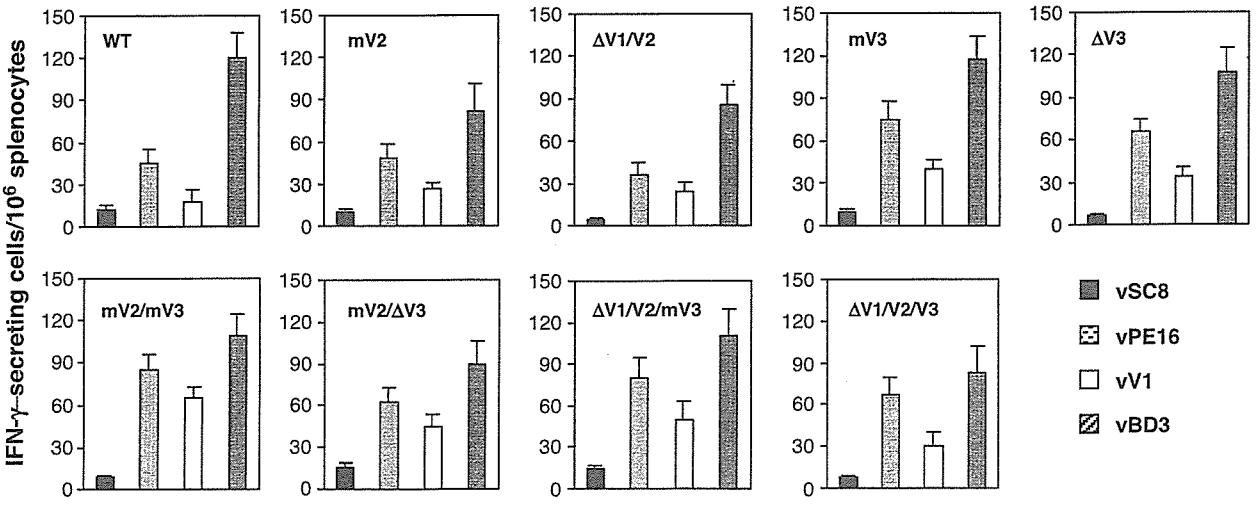

Fig. 4. Induction of cross-reactive cellular responses by DNA immunization with WT and variable domain-modified HIV-$1_{89.6}$ gp140 mutants in BALB/c mice. The numbers of IFN-γ-secreting $CD8^+$ T cells in splenocyte cultures established from mice immunized with the env vaccines were determined by ELISPOT assay with P815 cells infected with vPE16, vV1, and vBD3. Control cells were infected with vSC8 expressing β-galactosidase. Results are presented as the means ± SD of at least three independent experiments. The frequencies of IFN-γ-secreting cells were determined by regression analysis from a curve generated by plotting the number of spots versus the number of effector cells.

reactivity was detected in ΔV1/V2/mV3-immunized mice whose cross-reactive $CD8^+$ T cell activities directed to vPE16- and vV1-infected targets were 2- and 3-fold higher compared to those elicited by the unmodified vaccine ($P = 0.02$ and $P = 0.009$, respectively). The latter responses represented 70% and 45% of IFN-γ-secreting $CD8^+$ splenocytes directed to vBD3-infected cells, respectively.

Protection against challenge with rVV expressing homologous and heterologous env glycoproteins

The significance of WT and domain-modified env vaccine-induced cellular responses was further examined in protective studies against challenge with vPE16, vV1, and vBD3 as surrogates for HIV-1 (Belyakov et al., 1998). BALB/c mice immunized with the full-length or variable loop-modified mutants of the $gp140_{89.6}$-derived DNA constructs were challenged on day 28 by intraperitoneal (i.p.) injection with 5×10^6 PFU of vPE16, vV1, or vBD3 as described (Kiszka et al., 2002). To determine whether any protection induced by the vaccines was env-specific, additional groups of immunized and control mice were challenged with vSC8 virus expressing β-galactosidase. Because the vaccinia virus replicates most efficiently in ovaries (Belyakov et al., 1998), ovaries were removed 5 days after the challenge and tested for vaccinia titer on a monolayer of $HuTK^-$ 143B cell line.

As shown in Table 1, the vaccinia titers in ovaries of mice immunized with the full-length env or the domain-modified mutants and challenged with vBD3 were approximately 3 $\log_{10}$ lower than in vSC8-challenged animals. The protection levels against the homologous challenge with vBD3 were comparable in animals immunized with the unmodified or domain-modified vaccines. Consistent with the profile of $CD8^+$ T cell responses, immunization with the WT env gene products elicited approximately 10- and 100-fold lower protection levels against heterologous challenge with vPE16 and vV1, respectively. A similar level of protection was detected in mice immunized with the mV2 or ΔV1/V2 deletant. Animals immunized with the ΔV3 or mV3 mutant and challenged with the heterologous vPE16 and vV1 viruses had approximately 10-fold lower viral titers in ovaries than WT env-vaccinated mice. The highest level of protection against the heterologous challenge was detected in mV2/mV3- and ΔV1/V2/mV3-immunized mice. In these animals, resistance to heterologous challenge with vPE16 and vV1 was similar and approximately 50-fold higher compared with mice immunized with WT $gp140_{89.6}$ ($P < 0.01$). There was no significant difference between vSC8 titers in control mice and those immunized with the WT env or any of the domain-modified mutants, indicating that the protection elicited by the mV2/mV3 and ΔV1/V2/mV3 $gp140_{89.6}$ mutants could not be mediated by nonspecific inflammatory responses.

Development of env-specific antibodies in mice immunized with the domain-modified $gp140_{89.6}$ vaccines

The full-length and domain-modified $HIV\text{-}1_{89.6}$ gp140-derived immunogens induced antibodies capable of binding to the homologous env glycoprotein expressed on the surface of vBD3-infected cells (Table 2). Although variations in the antibody titers were measured throughout the immunization schedule, no statistically significant differences were recorded among groups of mice vaccinated with different env constructs. The endpoint binding antibody titers in animals immunized with the unmodified or the modified env vaccines were in the range of 2×10^3 at the end of the vaccination schedule. These results suggest that, based on the assay used here to determine antibody titers, the domain-modified gp140 vaccines were as effective as the unmodified env in eliciting antibody responses even if

Table 1
Env vaccine-induced protection against challenge with rVV expressing homologous and heterologous gp160 of primary HIV-1 isolates

Envelope vaccine	Challenge[a]						
	vSC8	vBD3		vPE16		vV1	
	Virus titer[b] ($\log_{10}$/ovaries)	Virus titer[b] ($\log_{10}$/ovaries)	Protection[c] ($\Delta\log_{10}$)	Virus titer[b] ($\log_{10}$/ovaries)	Protection[c] ($\Delta\log_{10}$)	Virus titer[b] ($\log_{10}$/ovaries)	Protection[c] ($\Delta\log_{10}$)
WT	9.1 ± 0.8	6.0 ± 0.4	3.1	7.4 ± 0.5	1.7	8.0 ± 0.6	1.1
mV2	9.2 ± 0.6	6.3 ± 0.6	2.9	7.2 ± 0.7	2.0	7.6 ± 0.5	1.6
ΔV1/V2	9.0 ± 0.8	6.2 ± 0.5	2.8	7.1 ± 0.7	1.9	7.7 ± 0.6	1.3
mV3	8.7 ± 0.8	5.6 ± 0.4	3.1	6.6 ± 0.4	2.1	6.8 ± 0.5	1.9
ΔV3	9.1 ± 0.5	6.1 ± 0.4	3.0	6.8 ± 0.2	2.3	7.0 ± 0.4	2.1
mV2/mV3	8.8 ± 0.8	5.3 ± 0.2	3.5	5.7 ± 0.3	3.1	5.9 ± 0.2	2.9
mV2/ΔV3	9.2 ± 0.6	5.6 ± 0.2	3.6	6.2 ± 0.3	3.0	6.4 ± 0.3	2.8
ΔV1/V2/mV3	8.8 ± 0.7	5.4 ± 0.3	3.4	5.9 ± 0.2	2.9	6.1 ± 0.4	2.7
ΔV1/V2/V3	8.6 ± 0.7	5.8 ± 0.2	2.8	6.3 ± 0.2	2.3	6.5 ± 0.4	2.1

[a] On day 28 after immunization with the WT or domain-modified $gp140_{89.6}$ DNA vaccines, BALB/c mice were challenged i.p. with 5×10^6 PFU of vPE16, vV1, vBD3, or vSC8. Five days later, mice were sacrificed, ovaries were removed, homogenized, sonicated, and assayed for vaccinia virus titers by plating serial 10-fold dilutions on human $HuTK^-$ 143B cells, staining with crystal violet, and counting plaques at each dilution.

[b] The rVV titers are presented as the mean $\log_{10}$ ± SD of PFU per ovaries of four mice per group.

[c] Protection is reduction of vBD3, vPE16, or vV1 titers ($\Delta\log_{10}$ PFU) in ovaries compared with the value for the control group challenged with vSC8 virus.

Table 2
Env-specific antibody responses and neutralization activities induced by the domain-modified HIV-$1_{89.6}$ gp140 vaccines

Domain-modified HIV-$1_{89.6}$ gp140 vaccines	Antibody titer to the homologous envelope glycoprotein (endpoint titer ± SD)[a]	Specific neutralization (% inhibition ± SD)[b]	
		HIV-$1_{89.6}$	HIV-1_{SF162}
WT	2100 ± 461	36 ± 16	17 ± 8
mV2	2933 ± 562	70 ± 19	49 ± 15
ΔV1/V2	1600 ± 800	48 ± 44	28 ± 26
mV3	2000 ± 565	28 ± 11	ND
ΔV3	1867 ± 661	24 ± 9	26 ± 22
mV2/mV3	2400 ± 800	91 ± 19	86 ± 9
mV2/ΔV3	2240 ± 661	81 ± 10	67 ± 11
ΔV1/V2/mV3	1800 ± 546	95 ± 13	88 ± 14
ΔV1/V2/V3	3133 ± 1194	67 ± 34	60 ± 16

[a] The env-specific antibody responses to native viral env glycoprotein expressed in vBD3-infected 293 cells were analyzed in sera of mice immunized with the WT or the variable domain-modified gp$140_{89.6}$ vaccine using the ELISA assay. Sample dilutions were considered positive if the optical density recorded for that dilution was at least 2-fold higher than the optical density recorded for a naive sample at the same dilution (Staats et al., 1996). Results are from three independent experiments.

[b] The neutralizing antibody responses induced by the WT and variable domain-modified env vaccines were examined using the homologous HIV-$1_{89.6}$ isolate and CEMx174 cells, or the heterologous HIV-1_{SF162} virus and PHA-stimulated PBMC as target cells. Neutralizing activity was evaluated at 1:20 dilution, taking into consideration the nonspecific neutralization recorded with sera collected from animals vaccinated with the DNA vector alone. Results represent means ± SD from three independent experiments.

the former immunogens lack up to 86 amino acids from the V1/V2 and 31 amino acids from the V3 region.

Next, we investigated the ability of unmodified and domain-modified HIV-$1_{89.6}$ gp140 vaccines to generate neutralizing antibodies to the functional env glycoprotein on the HIV-1 virus. In the initial set of experiments, the neutralizing assay was performed using the homologous HIV-$1_{89.6}$ isolate and the susceptible CEMx174 cells as a target. Analysis of p24-antigen levels in culture supernatants of CEMx174 cells infected with HIV-$1_{89.6}$ in the presence or absence of the immune sera revealed that immunization with the unmodified gp$140_{89.6}$ vaccine was not very effective in inducing neutralizing antibodies (Table 2). The neutralizing activity of the WT gp$140_{89.6}$-induced antibody responses measured at a serum dilution of 1:20 revealed 36 ± 16% inhibition of infection. A similar profile of neutralizing activity was observed with sera from animals immunized with the single mV3 or ΔV3 deletant. A trend toward higher neutralization levels was recorded in sera collected from animals vaccinated with the mV2 mutant, and the neutralizing activities of the serum antibodies were further augmented in mV2/mV3- and ΔV1/V2mV3-immunized mice ($P < 0.02$).

To examine whether domain-modified gp140 vaccines were effective in eliciting cross-reactive neutralizing antibody responses, we tested the HIV-1_{SF162} isolate whose susceptibility to neutralization by sera collected from animals immunized with the ΔV2 gp140_{SF162} mutant had previously been examined (Barnett et al., 2001; Srivastava et al., 2003). At a 1:20 serum dilution, the sensitivity of the HIV-1_{SF162} virus to neutralization by sera obtained from mice immunized with the unmodified gp$140_{89.6}$ vaccine was at least 2-fold lower than that detected using the homologous HIV-$1_{89.6}$ virus (17 ± 8% inhibition of infection, Table 2). At this dilution, a mixture of sera from HIV-1-infected individuals, used as a positive control in the assay, inhibited the infection by >95%. Removal of the entire V1/V2 region or the V3 loop did not augment the neutralizing responses. The antibody-neutralizing potency was 2–3-fold enhanced in sera derived from animals immunized with the mV2, mV2/ΔV3, or ΔV1/V2/V3 mutant ($P < 0.03$). The highest, approximately 5-fold, increases in susceptibility of the HIV-1_{SF162} virus to neutralization were elicited by mV2/mV3 and ΔV1/V2/mV3 gp$140_{89.6}$ vaccines ($P = 0.0006$ and $P = 0.002$, respectively), indicating that specific modifications of the variable loops might be capable of redirecting antibody responses toward conserved epitopes on the env glycoprotein.

Env-specific antibody avidity

Previous studies with attenuated SIV vaccines in rhesus macaques demonstrated that the establishment of long-term protective immunity was associated with maturation of antibody responses (Cole et al., 1997; Montelaro et al., 1998). These results prompted us to evaluate the nature of anamnestic responses to rVV challenge in mice immunized with domain-modified mutants that elicited the highest cross-reactive neutralizing antibody responses after DNA priming. Because variations in priming specificity by the variant env immunogens could be reflected in differences in the protective immune responses to the viral challenge, we measured the association between env-specific antibody avidity and neutralizing activities in sera of mice immunized with the WT, mV2/mV3, or ΔV1/V2/mV3 vaccine after challenge with vBD3 virus expressing homologous env gene products. Mice immunized with the env-specific DNA vaccines and challenged with vSC8 virus expressing β-galactoside served as controls. The antibody avidity was determined by measuring the relative stability of the native env antigen–antibody complexes to an 8 M urea wash in concanavalin A (ConA) ELISA assay as described (Cole et al., 1997).

Table 3
Env-specific antibody responses after rVV challenge in mice immunized with the WT, mV2/mV3, or ΔV1/V2/mV3 $gp140_{89.6}$ vaccine

Envelope vaccine	Challenge[a]	Avidity index[b] (%)	Specific neutralization[c]			
			HIV-$1_{89.6}$		HIV-1_{SF162}	
			50%	90%	50%	90%
WT	ND	25	–	–	–	–
WT	vBD3	37	+	–	–	–
mV2/mV3	vSC8	43	+	+	+	–
mV2/mV3	vBD3	56	+	+	+	+
ΔV1/V2/mV3	vSC8	34	+	+	+	–
ΔV1/V2/mV3	vBD3	49	+	+	+	–

[a] BALB/c mice were immunized with plasmid DNA expressing the WT, mV2/mV3, or ΔV1/V2/mV3 mutant of $gp140_{89.6}$ and challenged i.p. with 5×10^6 PFU of vBD3 or vSC8 3 weeks after DNA vaccine. The env-specific antibody responses to the native env glycoprotein were analyzed in the ConA ELISA with or without an 8 M urea wash. ND, not determined.
[b] The avidity index was determined by measuring the relative stability of the native viral env antigen–antibody complexes to an 8-M urea wash in ConA ELISA, and values were calculated by using the equation (urea-washed wells/PBS-washed wells) × 100. The avidity index values $x \geq 50\%$, $50\% > x > 30\%$, and $x \leq 30\%$ have been defined high, intermediate, and low avidity, respectively. Results represent average values from three independent experiments.
[c] Neutralizing activity was calculated at 1:20 dilution, taking into consideration the nonspecific neutralization recorded with sera collected from animals vaccinated with the DNA vector alone. –, 50% specific neutralization was not recorded; +, 50% or 90% specific neutralization was recorded. Results are from three independent neutralization experiments.

The results summarized in Table 3 demonstrated that antibody avidity values in mice immunized with the env-specific DNA constructs showed approximately 30% increases after challenge with vBD3 vaccinia virus compared to responses in vSC8-challenged mice. In the latter group of mice, the antibody avidity index varied between 25% and 43% with the lowest and highest values detected in animals immunized with the WT and mV2/mV3 vaccines, respectively. The antibody responses progressed from low to intermediate or to high avidity in vBD3-challenged mice at 11 days postinfection. The increases in antibody avidity after vBD3 challenge were also associated with small enhancement of neutralization activities against the HIV-$1_{89.6}$ and HIV-1_{SF162} isolates in animals immunized with the WT and mV2/mV3 vaccines, respectively (Table 3). However, this association was not observed in ΔV1/V2/mV3-vaccinated mice whose sera displayed similar neutralizing activities after challenge with vBD3 or vSC8 virus.

Discussion

Potent neutralizing antibody responses that prevent primary infection or reduce the viral load at the site of HIV-1 entry remain the ultimate goal for prophylactic AIDS vaccines. However, the same vaccines should also induce cross-protective cellular responses capable of increasing the level of immune control and inhibit spreading infection in HIV-1-positive individuals. Thus, efforts to improve the cross-reactivity of env-specific vaccine might require the creation of a polyvalent vaccine composed of env glycoproteins from multiple primary isolates or a vaccine in which the structure of the env is modified to elicit cellular and humoral responses to conserved epitopes that are otherwise poorly immunogenic. Previously, we have demonstrated that immunization of the HLA-A2/K^b transgenic mice with the ΔV3 env mutant induced qualitative changes at the level of env peptide-specific $CD8^+$ T cell responses that were associated with an increased recognition of target cells expressing heterologous env gene products (Kiszka et al., 2002). In these studies, we showed that the ΔV3 env vaccine was not effective in eliciting neutralizing antibody responses. These findings are consistent with previous studies which demonstrated that immunization strategies using variable loop-deleted env constructs failed to generate cross-reactive neutralizing antibodies (Kim et al., 2003; Lu et al., 1998). The experiments presented here also indicated that partial deletions within the V3 loop in combination with the V2-modified or V1/V2-deleted region were more effective in eliciting broader levels of cross-reactive $CD8^+$ T cell and neutralizing antibody responses than the single V3 loop-deleted mutant or the WT $gp140_{89.6}$ vaccine.

Using the challenge experiments with rVV-expressing homologous or heterologous env gene products, we demonstrated that modifications within the V3 domain that increased the level of cross-reactive cellular responses in vitro also elicited higher protection levels against challenge with rVV expressing heterologous gp160 in the immunized mice. Although immunization with the mV2 or the ΔV1/V2 mutant affected neither the level of cross-reactive $CD8^+$ T cell responses nor the protection against the heterologous rVV challenge, adding mV3 into these constructs significantly increased their immunogenic efficacies. Our findings in the murine model together with the data available from the HIV Molecular Database, which demonstrated the presence of only three CTL epitopes with the HLA-A2- and Cw8-binding motifs in the V1–V2 region (Korber et al., 1999), indicate that this region might not be targeted by $CD8^+$ T cell responses. However, specific deletions within the V1–V2 region could result in structural changes that indirectly affected the immunogenicity of the mV2/mV3 and ΔV1/V2/mV3 mutants at the level of antigen processing or presentation. Because the quantity of the epitope–MHC complex on the cell surface may regulate CTL responses (Yewdell and Bennink, 1992), it is possible that increases in cross-reactive cellular responses induced by mV2/mV3 and ΔV1/V2/mV3 vaccines could be associated with a higher pool of conserved epitopes available for presentation in cells expressing the double mutants than the WT env gene products. This can be due to the appearance of new CTL epitopes that are more frequently present on heterologous envelopes than are those presented by unmodified $gp140_{89.6}$ vaccine. Alternatively, the modified gp140 immunogen may increase the relative concentration of conserved epitopes by reducing a competition with less desirable epitopes during

antigen processing and presentation. Although further studies are required to determine the mechanism that contributes to the increased immunogenicity of the conserved epitopes in mV2/mV3 and ΔV1/V2/mV3 vaccines, results presented here suggest that one possible approach to broaden the repertoire of env-specific cellular responses is by modifications of the variable sequences within the env glycoprotein.

Consistent with the notion that both cellular and humoral responses will be required for an effective AIDS vaccine, understanding of the immunogenicity of env glycoproteins from several primary HIV-1 isolates will be necessary for the development of broadly protective immune responses. Our results with the gp140 mutants of the HIV-$1_{89.6}$ isolate demonstrated that despite different constrains for the induction of cross-reactive cellular and humoral responses, structural modifications of the env glycoprotein can be introduced to redirect both types of immune responses toward conserved epitopes that can be recognized on more than one strain of HIV-1. It is also noteworthy that the variable loop modifications had a more pronounced effect on induction of cross-reactive cellular than neutralizing antibody responses. This could be due to the inherent difficulties of generating cross-neutralizing antibodies related to the highly variable structure and glycosylation of gp160 as well as the poor exposure and immunogenicity of the receptor-biding sites on the env molecules (Kim et al., 2003; Kwong et al., 1998; Wyatt et al., 1998). In addition, the single immunization approach based on a DNA vaccine that elicited low- to intermediate-avidity antibodies might have reduced efficacy of the domain-modified vaccines. The use of mouse sera in our assay and that of Kim et al. (2003), which suffers from low neutralizing activities against primary isolates, could also contribute to the weak cross-neutralizing responses induced by the variable domain-deleted mutants. However, regardless of the differences in the experimental models and HIV-1 isolates, in both systems complete removal of the variable regions was not effective in inducing cross-reactive cellular and antibody responses, suggesting that partial deletions within these regions may facilitate more effective approaches for boosting the breadth of protective immunity.

In summary, we demonstrated that specific modifications of the variable domains of the env glycoprotein could be introduced with favorable antigenic properties. Additional studies that would examine in detail the existence and epitope-specificity of the cross-protective cellular and antibody responses elicited by mV2/mV3 and ΔV1/V2/mV3 $gp140_{89.6}$ mutants may help to establish to what extent the specific deletions differentially alter the structure of certain env regions that are already immunogenic or render certain regions immunogenic. Similarly, further studies with a panel of heterologous primary isolates are also required to determine susceptibility of these HIV-1 isolates to neutralization by antibodies elicited by mV2/mV3 and ΔV1/V2/mV3 $gp140_{89.6}$ vaccines. These results will help to establish whether the cross-reactive epitopes present on those $gp140_{89.6}$ mutants are absent from the heterologous isolates that are resistant to neutralization or whether they are more efficiently masked on these particular isolates than on the viruses susceptible to neutralization. As such, this model system will provide a comprehensive means for selecting the most effective envelope vaccines for future vaccine trials.

Materials and methods

Vector construction

The HIV-$1_{89.6}$ gp140 plasmid (accession number U39362) was used for the construction of env mutants with complete or partially deleted V1/V2, V2, and V3 domains using a two-step PCR protocol using as described (Kiszka et al., 2002). For each mutant, two fragments of the env gene were synthesized with an overlap. One fragment, with a specific deletion within the variable domains, was synthesized with primer A containing the *KpnI* cleavage site (5′-AAT*GGTACC* TGTGTGGAGA-GAAGCAACCAC-3′) together with anti-sense primer B that contains overlapping sequences with primer C that was used for synthesis of the second fragment (Table 4). The second fragment was synthesized with sense primer C in conjunction with the anti-sense primer D (5′-CCT*CCTGAGG* ATTGATTAAAGGCTATTGTT-3′) containing the *Bsu36I* restriction site. The nucleotide sequences for the restriction enzyme cleavage sites are underlined. These two fragments were then used together in a second reaction along with primers A and D to generate the indicated domain-modified env fragments. The final product was digested with *KpnI* and *Bsu36I* and exchanged with the corresponding segment of WT $gp140_{89.6}$ fused in frame with the tPA signal sequence under the control of human cytomegalovirus immediate early promoter in the pNGVL-7 plasmid (University of Michigan, Ann Arbor, MI) as described (Kiszka et al., 2002). Rev was not included in the gp140-expressing plasmids as expression of env with the tPA signal sequence in pNGVL-7 allows env expression without Rev (Lu et al., 1998). The integrity of the plasmid was verified by restriction enzyme cleavage and DNA sequence analysis. After DNA sequence verification, the expression of the env gene products was confirmed by immunoprecipitation from radiolabeled 293T cells transfected with the respective env-pNGVL-7 plasmid.

Transfection, labeling, and immunoprecipitation

Transient transfection of 293T cells was performed by calcium phosphate precipitation with 50 μg of plasmid DNA encoding the respective env protein. For radioimmunoprecipitation analysis, [^{35}S]methionine/cysteine (400 μCi per plate; DuPont-New England Nuclear, Boston, MA)

Table 4
Nucleotide sequence of partially overlapping primers B and C used for generation of the variable domain-modified HIV-$1_{89.6}$ gp140 vaccines

V1/V2 and V3-modified HIV-$1_{89.6}$ gp140[a]	Modified V1 and V2 domains		Modified V3 domain	
	B	C	B	C
WT	N/A	N/A	N/A	N/A
mV2	5′-TAACCTACCGGCACC GATATAGAAAGAGCA-3′	5′-GGTGCCGTTAGGTTA ATAAGTTGTAACACC-3′	N/A	N/A
ΔV1/V2	5′-ACAGGCACCGGCACC TACACATGGCTTTAG-3′	5′-GGTGCCGGTGCCT GTCCAAAGGTATCC-3′	N/A	N/A
mV3	N/A	N/A	5′-TCTCCCTGGTCCTATA GATAACCTTCTTCTTGC ATTGTT-3′	5′-ATAGGACCAGGGAGA GCATTTTATGCAAGATGT AACATTAGTAGAGCA-3′
ΔV3	N/A	N/A	5′-ACAATGGCCGGCACC TGTACAATTAATTAC-3′	5′-GGTGCCGGCCATTGT AACATTAGTAGAGCA-3′
mV2/mV3	5′-TAACCTACCGGCACC GATATAGAAAGAGCA-3′	5′-GGTGCCGGTAGGTTA ATAAGTTGTAACACC-3′	5′-TCTCCCTGGTCCTATA GATAACCTTCTTCTTGC ATTGTT-3′	5′-ATAGGACCAGGGAGA GCATTTTATGCAAGATGT AACATTAGTAGAGCA-3′
mV2/ΔV3	5′-TAACCTACCGGCACC GATATAGAAAGAGCA-3′	5′-GGTGCCGTTAGGTTA ATAAGTTGTAACACC-3′	5′-ACAATGGCCGGCACC TGTACAATTAATTAC-3′	5′-GGTGCCGGCCATTGT AACATTAGTAGAGCA-3′
ΔV1/V2/mV3	5′-ACAGGCACCGGCACC TACACATGGCTTTAG-3′	5′-GGTGCCGGTGCCT GTCCAAAGGTATCC-3′	5′-TCTCCCTGGTCCTATA GATAACCTTCTTCTTGC ATTGTT-3′	5′-ATAGGACCAGGGAGA GCATTTTATGCAAGATGT AACATTAGTAGAGCA-3′
ΔV1/V2/V3	5′-ACAGGCACCGGCACC TACACATGGCTTTAG-3′	5′-GGTGCCGGTGCCT GTCCAAAGGTATCC-3′	5′-ACAATGGCCGGCACC TGTACAATTAATTAC-3′	5′-GGTGCCGGCCATTGT AACATTAGTAGAGCA-3′

[a] The HIV-$1_{89.6}$ gp140 vaccines with complete or partially deleted V1, V2, and V3 regions were constructed using a two-step PCR method and partially overlapping primers B and C as described in Materials and methods section.

was added on the second day of transfection for an additional 12-h period. At the end of the incubation period, the cells were pelleted and lysed using Nonidet P-40 buffer (0.5% Nonidet P-40, 0.5 M NaCl, and 10 mM Tris–HCl, [pH 7.5]). Equal amounts of radiolabeled cell lysates, based on the protein concentration, were immunoprecipitated with a mixture of sera from HIV-1-infected individuals followed by polyclonal rabbit anti-human Ig (ICN Biomedicals, Inc. Costa Mesa, CA) and protein A-Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ). Immunoprecipitates were separated by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis (SDS-PAGE). The gels were fluorographed to visualize [^{35}S]methionine/cysteine-labeled proteins, and the intensity of individual bands was quantified with a LabImage Program (Copyright 1999–2001, Kapelan GmbH).

Flow cytometry analysis of the env glycoprotein expression

For intracellular staining of transfectants expressing the WT and domain-modified env glycoproteins, 293T cells were harvested 48 h after transfection, washed twice with PBS containing 2% FCS and 0.1% sodium azide, and stained with env-specific human mAbs 2F5 and 2G12 (Buchacher et al., 1994) (NIH AIDS Research and Reference Reagent Program, Rockville, MD). After washing, the cells were incubated with a 1:40 dilution of FITC-conjugated F(ab′)$_2$ of goat anti-human Ig (ICN Biomedicals, Inc.) and analyzed by flow cytometry (FACScan). The intracellular staining was carried out using BD Cytofix/Cytoperm kit (Pharmingen, San Diego, CA) according to the manufacturer's protocol.

Recombinant vaccinia viruses

The rVV's expressing the full-length gp160 of HIV-1_{IIIB} (vPE16) (Earl et al., 1991) or β-galactosidase (vSC8) (Earl et al., 1991) were provided by Dr. B. Moss (Laboratories of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD). The rVV's expressing the env glycoprotein of the primary VI-06 (vV1) and 89.6 (vBD3) HIV-1 isolates were provided by Drs. K. Luzuriaga (University of Massachusetts Medical Center, Worcester, MA) and R. Collman (University of Pennsylvania, Philadelphia, PA), respectively. VI-06 is a "transmitted" primary isolate derived from an infant with perinatal HIV-1 infection (Pikora et al., 1997) that exhibits 8–10% sequence variation compared with the env glycoprotein of the HIV-1_{IIIB} isolate. The second env glycoprotein derives from a dual-tropic HIV-$1_{89.6}$ primary isolate which uses CXCR4 and CCR5 as well as many other chemokine co-receptors for entry (Doranz et al., 1996). The HIV-$1_{89.6}$ isolate exhibits 82% identity with HIV-1_{IIIB} gp160 sequence with the highest (92%) and the lowest (64%) sequence homology in the C1 region and the V3 loop, respectively.

Mice and immunization

Six-week-old BALB/c (H-2^d) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free microisolator environment. Mice were injected i.m. in the quadriceps with 100 μg of plasmid encoding WT or domain-modified gp140 mutants on days 0, 14, 28, and 42. Control mice were immunized with a sham plasmid. To augment the level of envelope-specific immune

responses, plasmid DNA encoding IL-2/Ig (10 μg) was administered i.m. 2 days after immunization with the env-specific vaccine as described (Barouch et al., 1998; Wierzbicki et al., 2002).

ELISPOT assay

The number of IFN-γ-secreting T cells specific for the env gene products in splenocytes of the immunized mice was determined by the ELISPOT method 3 weeks after the last immunization as described (Gherardi and Esteban, 1999). Briefly, 96-well nitrocellulose plates (Millipore multiscreen-MAIP; Millipore, Bedford, MA) were coated with 15 μg/ml of rat anti-mouse mAb directed to IFN-γ (MAB785, R and D Systems Inc., Minneapolis, MN) in 0.05 M carbonate-bicarbonate buffer pH 9.6. After overnight incubation at 4 °C, the wells were washed with PBS containing 0.05% Tween 20 and blocked for 1 h with RPMI 1640 medium containing 10% FCS. For the analysis of frequencies of IFN-γ-secreting cells specific for the env gene products, splenocytes were combined with target cells infected with vPE16, vBD3, vV1, or vSC8 at a ratio of 3:1 and placed in 2-fold dilutions into the antibody-coated wells. For each dilution duplicate samples were used. P815 cells were used as antigen-presenting cells (Gherardi and Esteban, 1999). After 24 h of incubation at 37 °C, the plates were washed six times with PBS containing 0.05% Tween 20 and incubated for 2 h with 50 μl of 1 μg/ml of biotinylated mAb directed to mouse IFN-γ (MAB485, R&D Systems Inc.). The plates were washed and incubated for 1 h with 50 μl of 1:1000 diluted streptavidin-conjugated alkaline phosphatase (SA-5100, Vector Laboratories, Burlingame, CA). After a final wash with PBS, spots were developed with an alkaline phosphatase BCIP/NBT (5-bromo-4-chloro-3-indolyl-1-phosphate/nitroblue tetrazolium) substrate (SK-5400, Vector Laboratories) and counted under a stereomicroscope. The frequencies of IFN-γ-secreting cells were determined by regression analysis from a curve generated by plotting the number of spots versus the number of effector cells.

Protection against challenge with rVV expressing gp160 proteins of HIV-1 isolates

On day 28 after the last immunization with the env-specific vaccines, mice were challenged i.p. with 5×10^6 PFU of vPE16, vBD3, vV1, or vSC8. Five days later, mice were sacrificed, ovaries removed, homogenized, sonicated, and assayed for vaccinia virus titer by plating serial 10-fold dilutions on human HuTK$^-$ 143B indicator cells, staining with crystal violet and counting plaques at each dilution as described (Kiszka et al., 2002).

Measurement of HIV-1 env-specific antibody endpoint titers

Serum samples from control or env-vaccine immunized mice were analyzed for their reactivity to native viral env glycoprotein on vBD3-infected 293T cells in the ELISA assay. Briefly, 293T cells (4×10^4 cells/well) cultured on 96-well plates were infected with vBD3 at a multiplicity of infection of 10. After 12 h of infection, cells were fixed by 10-min treatment with 3.7% paraformaldehyde in PBS. Following a wash with PBS containing 0.05% Tween 20 (PBS/Tween 20), the wells were blocked for 2 h with a solution containing 2% BSA (Sigma) and 0.05% Tween 20 in PBS. Sera were prepared from murine blood samples, serially diluted in PBS/Tween 20, and added to the wells. For each dilution, duplicate wells were used. After 1-h incubation at room temperature, the plates were washed three times and incubated with a 1:500 dilution of a peroxidase-conjugated goat anti-mouse Ig (IgG, IgM, and IgA; Sigma) in PBS/Tween 20. After washing, the reaction was developed with *O*-phenylenediamine (0.4 mg/ml; Sigma) in 0.05 M phosphate-citrate buffer containing 0.03% sodium perborate (Sigma), stopped with 0.4 N sulfuric acid, and analyzed at 450 nm with an ELISA plate reader (Dynatech MRX, Chantilly, VA). Sample dilutions were considered positive if the optical density recorded for that dilution was at least twofold higher than the optical density recorded for a naive sample at the same dilution (Staats et al., 1996).

Neutralizing antibody responses induced by the unmodified and variable domain-modified env vaccines

The ability of env vaccines to generate neutralizing antibodies was tested to determine which of these env mutants could elicit a response to the functional env glycoprotein on the homologous and heterologous HIV-1 viruses. The assay was performed using the homologous HIV-$1_{89.6}$ isolate and CEMx174 cells obtained from Dr. R. Collman (University of Pennsylvania) or the heterologous HIV-1_{SF162} virus and PHA-activated human peripheral blood mononuclear cells (PBMC) as targets (Wierzbicki et al., 2002). The HIV-$1_{89.6}$ isolate exhibits 82% identity with HIV-1_{SF162} gp160 sequence with the highest (90%) and the lowest (62%) sequence homology in the C1 region and the V1 loop, respectively.

The HIV-$1_{89.6}$ and HIV-1_{SF162} isolates were grown and titrated in CEMx174 cells and in PHA-activated human PBMC, respectively. The stocks were aliquoted and kept at −80 °C until further use. For antibody-mediated neutralization, the virus stock diluted to one hundred 50% tissue culture infectious doses ($TCID_{50}$) in 50 μl of complete RPMI medium was preincubated with an equal volume of heat-inactivated (35 min at 56 °C) sera for 1 h at 37 °C in 96-well tissue culture plates. For each sample, triplicate wells were used. To account for the relatively high background and variability frequently observed with mouse antisera, samples were collected from control animals vaccinated with the sham vector and served as controls for nonspecific neutralization. To each well, 0.1 ml of medium containing 10^5 target cells was added. Following 4-h incubation at 37 °C, half of the

volume of each well was replaced with fresh, complete RPMI medium. Following centrifugation of the plate (5 min at 1500 rpm), half of the volume of each well was again replaced with fresh medium. This procedure was repeated twice. The p24 antigen concentration in each well was evaluated 6 days after infection by a p24-antigen-capture assay (NEN Life Science Products, Inc., Boston, MA) according to the manufacturer's procedures. Each assay plate contained uninfected and infected cell controls and a serum from an individual with AIDS as a positive control (Kmieciak et al., 1998). Because the infection was reduced in the presence of control sera, we calculated the difference between the percent of inhibition recorded with the immune sera minus that recorded with control sera. The 1:20 serum dilution indicated in the text refers to that present during the entire period of virus-cell incubation.

Measurement of HIV-1 env-specific antibody avidity

The avidity index values of serum antibodies to the native viral env were determined by measuring the resistance of antibody–env glycoprotein complexes to an 8 M urea wash in ConA ELISA (Cole et al., 1997). For avidity assays, the ConA-anchored native viral env glycoprotein substrate was purified from cell culture supernatants of vBD3-infected 293T cells by *Galanthus nivalis* lectin affinity column (Vector Laboratories) as described (Trkola et al., 1996). For the ConA ELISA assay, all test sera were diluted in 5% nonfat dry milk in PBS (BLOTTO) and plated in two sets of duplicate wells. After the serum incubation, one set of wells was treated in parallel for 5 min with either PBS or a solution of 8 M urea in PBS. Following this treatment, the wells were thoroughly washed with PBS and incubated with a 1:500 dilution of a peroxidase-conjugated goat anti-mouse Ig in PBS. After washing, the reaction was developed with *O*-phenylenediamine (0.4 mg/ml; Sigma) in 0.05 M phosphate-citrate buffer containing 0.03% sodium perborate (Sigma), stopped with 0.4 N sulfuric acid, and analyzed at 450 nm with an ELISA plate reader. The avidity index was then calculated from the ratio of the absorbance value obtained with urea treatment to that observed with PBS treatment multiplied by 100. Antibodies with avidity index values of $\leq$30% were designated low avidity, those with index values between 30% and 50% were designated intermediate avidity, and those with values $\geq$50% were designated high avidity. While measurements of antibody avidity were performed at the dilution producing an OD at 450 nm of about 1.0 in the endpoint titer ConA ELISA procedure, experiments using several different dilutions within this linear range were performed to ensure that the variation in actual values was within 10%.

Data analysis

Amino acid sequences of gp160 proteins of primary HIV-1 isolates were obtained through accession numbers available from the HIV Sequence Database (http://hiv-web.lanl.gov). The percentage of amino acid sequence identity within immunogenic regions of gp160 proteins of primary HIV-1 isolates was determined by the BLAST 2 program (http://www.ncbi.nlm.nih.gov/BLAST). The numbering of amino acids was based on the HxBc2 sequence available from the HIV Sequence Database (http://hiv-web.lanl.gov) using the HIV/SIV Sequence Locator Tool.

The significance of differences in the number of IFN-γ-secreting $CD8^+$ cells specific for the env gene products and the percentage of inhibition of HIV-1_{SF162} infection induced by the full-length and domain-modified gp140 vaccines was determined by the unpaired Student's *t* test using JMP software (SAS Institute Inc., Cary, NC). Mixed model analysis of variance (Winer, 1971) was used to compare mean values of the rVV titers between control mice and those immunized with the unmodified and domain-modified vaccines after challenge with vPE16, vBD3, vV1, or vSC8.

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Update on HIV/AIDS in Japan, 2003

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INTRODUCTION

In Japan, the first case of HIV-1 infection was discovered in 1983 (Miyoshi *et al.*, 1983) and its incidence continued to increase among Japanese hemophiliacs through 1985 by repeated inoculations of coagulation factor products VIII and IX imported from the United States into Japan. Approximately 2000 hemophiliacs were estimated to be infected with HIV, demonstrating a 40% infectivity rate of HIV among a total of 5000 hemophiliacs treated with the contaminated blood products. Furthermore, 12 non-hemophiliacs were retrospectively defined to be infected with HIV-1 (hereafter referred to simply as HIV) by their treatment with the contaminated products.

Surveillance of HIV/AIDS in Japan began in 1984 on a voluntary basis and become mandatory by the AIDS Prevention Law in 1989. According to this law, all HIV/AIDS individuals except those infected through the use of coagulation factor products are to be reported to the National AIDS Surveillance Committee, Ministry of Health and Welfare, as the repository of National HIV/AIDS Surveillance in Japan. Six AIDS patients were initially reported in 1985 and 55 HIV-positive individuals were detected in 1987. The number of HIV/AIDS cases has since increased every year. As of October 28, 2003, 5573 HIV infections had been reported, along with 2776 cases of AIDS, for a total of 8349 reported cases. This number corresponds to 4.05 HIV cases and 2.014 AIDS patients per 100,000 population in Japan (Infectious Disease Surveillance Center, 2003). Since sexual behavior among young people has been changing, there is concern over the prediction that the number of HIV-positive individuals may reach 100,000 by the end of the year 2010 (Kihara, 2003).

INITIAL DETECTION OF HIV IN JAPAN

The first report of HIV infection was detected in a non-hemophiliac co-infected with adult T cell leukemia virus in 1983 (Miyoshi *et al.*, 1983), followed by the identification of two additional AIDS cases and one HIV infection in Japan (Aoki *et al.*, 1984; Kobayashi

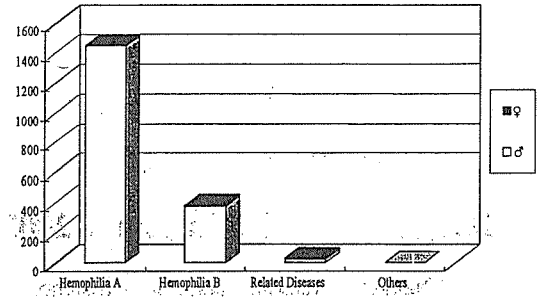

FIGURE 1. Total numbers of HIV-positive hemophiliacs treated with coagulant factor products prior to May 1996 in Japan. HIV-infected individuals treated with coagulant factors were categorized into 4 groups: hemophilia A, hemophilia B, related diseases, and others.

et al., 1984), suggesting that there had already existed an unidentified HIV-1 reservoir among non-hemophiliacs prior to 1983.

Since hemophiliacs seropositive for HIV had been reported in the United States from 1982, Abe *et al.* (1985) screened 50 samples of sera from Japanese hemophiliacs through collaboration with Dr. Gallo of the US National Cancer Institute and the National Institutes of Health in 1985. Surprisingly, 46% (23 hemophiliacs) were seropositive for HIV, including two hemophiliacs who had developed AIDS (Abe *et al.*, 1985). These hemophiliacs had been injected with imported and domestic coagulant factor VIII or IX for over six years. Based on these results, researchers recommended preparation of virus-free blood products for the prevention of HIV infection.

To establish a profile of HIV infection among hemophiliacs in Japan, the National Survey for HIV-positive Hemophiliacs began in 1987 .()(Yamada, 1996). Through May of 1996, 1862 hemophiliacs had been defined as HIV-positive, 1448 cases were among hemophilia A patients, and 382 cases were among hemophilia B patients (Figure 1). In 1996, 630 cases (33.7%) progressed to AIDS—33.4% of the hemophilia A patients and 35.1% of the hemophilia B patients; approximately 70% of the cases from both groups died in the same year (Figure 2).

The hemophiliac population provided an opportunity to study and identify the risk factors for HIV infection and its disease progression. Interestingly, the rate of long-term non-progressor status (more than 10-years maintenance of $CD4^+$ T-cells above 500 cells/mm^3) was as high as 14% among HIV-infected Japanese hemophiliacs (Yamada, 1996).

SURVEILLANCE OF HIV IN NON-HEMOPHILIACS IN JAPAN

Since HIV was not detected in hemophiliacs treated with coagulant factor products after 1986, due to the increased safety of the blood supply, the National AIDS Surveillance Committee examined analyses of reported cases of HIV infection and released its updated HIV/AIDS surveillance information twice a year.

In the most recent report in 2003 (Infectious Disease Surveillance Center, 2003), newly reported HIV infections and AIDS cases numbered 614 and 308, respectively (Figure 3A) and Japanese males dominated