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Independent Expansion of Epitope-Specific Plasma Cell Responses upon HIV-1 Envelope Glycoprotein Immunization

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Abs that bind the functional envelope glycoprotein (Env) spike are considered critical for a broadly effective prophylactic HIV-1 vaccine. The difficulty in eliciting such Abs by vaccination is partially attributed to the immunodominance of hydrophilic, surface-exposed variable protein regions of Env. However, little is known about the potential for competition between B cells that recognize distinct and distal epitopes on Env during protein subunit vaccination. In this study, we address this basic question at the level of Ab-secreting cells and serum IgG using a pair of isogenic soluble Env trimers, designated wildtype and gV3, which differ only in their potential to activate B cell responses against the highly immunogenic V3 region of Env. Immunization of mice with gV3 resulted in a markedly lower Ag-specific response compared with that induced by wildtype Env and could be explained by a loss of V3-directed reactivities. There was no redistribution of the response to other regions of Env in gV3-inoculated mice, suggesting that the epitope-specific Ab-secreting cell responses measured after boost are independently regulated rather than dictated by direct or indirect competition between B cells recognizing different structural elements of Env. This information is relevant for ongoing efforts in Env immunogen design to focus responses on conserved neutralizing determinants and for our general understanding of B cell responses to large-protein Ags that display numerous B cell epitopes.

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Infection with recombinant proteins in adjuvant generates T-dependent humoral immune responses that are characterized by the formation of germinal centers (GCs) where Ag-specific B cells undergo affinity maturation and differentiation into memory B cells and Ab-secreting cells (ASCs) (1–4). The diverse naive B cell repertoire allows the humoral immune system to generate Ab responses against a large number of distinct epitopes on each foreign-protein Ag (5). The number of epitopes that may be targeted depends on the capacity of the naive B cell repertoire to recognize epitopes on the Ag. Moreover, it is generally appreciated that Ab responses to different determinants on the same protein vary both in magnitude and affinity; however, it is not fully known how much of these differences are dictated by the biophysical properties of the individual epitopes, the binding/activation characteristics of the naive B cell repertoire, presentation of Ag to different B cell subsets, available T cell help, or the capacity of Ag-experienced B cells to expand and differentiate in response to Ag re-exposure.

Studies showed that, although both low- and high-affinity monoclonal B cell lineages specific for the hapten, 4-hydroxy-3-nitrophenylacetyl (NP) or for a well-defined B cell epitope on hen egg lysozyme epitope can persist in vivo, only the high-affinity lineages prevail in the GC if both are present at the time of infection (6–9). This demonstrates that B cell clones are competitively regulated in GCs if they share the same target. However, it remains unclear how these results relate to the polyclonal response elicited by complex, multiepitope protein Ags using common immunization regimens. An important question to address is whether B cells that recognize distinct, nonoverlapping epitopes or regions on the same protein Ag compete with each other during the generation of an immune response or if they are independently regulated. If the former were the case, it would suggest that the removal, or shielding, of highly immunogenic epitopes would redirect the B cell response to other determinants on the same protein, an approach that could be exploited for target-specific vaccine design.

In this study, we address this question using a well-characterized HIV-1 envelope glycoprotein (Env) immunogen (10). HIV-1 Env has received considerable interest as a component of a prophylactic HIV-1 vaccine; however, Abs capable of neutralizing a broad array of circulating HIV-1 variants have not been induced by vaccination using current Env-based vaccine candidates (11–13). One proposed reason for this deficit is that B cell responses against the variable regions of HIV-1 Env dominate the response and, thereby, hamper the elicitation of more broadly reactive Env Abs to cross-conserved epitopes (14–16). That this is the case is unknown, but it is a central question for HIV-1 Env vaccine design.

HIV-1 Env consists of a trimer of noncovalently associated heterodimers of the exterior glycoprotein, gp120, and the membrane-anchored glycoprotein, gp41 (reviewed in Ref. 17). Data from an Ab cross-competition study demonstrated the presence of distinct antigenic surfaces on Env (18). This was followed by structural
analyses of monomeric gp120 core fragments, with or without Ab ligands, by x-ray crystallography (19–23, reviewed in Refs. 24, 25) and by tomographic and single-particle cryo-electron microscopy studies describing the overall quaternary structure of the Env trimer (26–29). The extensive structural and antigenic information available for HIV-1 Env makes this a well-suited model Ag to address the role of B cell competition to different antigenic determinants during the generation of vaccine-induced immune responses to a protein immunogen.

Using a soluble version of the HIV-1 Env spike, the YU2-based gp140 trimers (10, 30, 31), we demonstrated previously that ~50% of the Ag-specific IgG+ ASCs are specific for the V3 region of Env after three inoculations in adjuvant, likely representing a preferential expansion of V3-specific ASCs from the Ag-specific memory B cell pool upon boosting (32). The ability of Env to tolerate substitutions in selected regions of V3 without compromising infectivity suggests that alterations designed to modify the immunogenicity of this region will have minimal impact on the overall conformation and function of the glycoprotein (33, 34). In this study, we investigate the consequence of masking the Env V3 region from B cell recognition to examine the contribution of this region to the total and epitope-specific ASC response induced by Env immunization. We demonstrate that hyperglycosylation of V3 leads to a significant reduction in total Env-specific ASC and IgG responses, as measured 5 d after the second boost with hyperglycosylated (gV3) trimer, compared with animals inoculated with the wildtype (wt) trimers; this reduction is directly associated with a loss of V3-directed ASCs in the gV3-inoculated animals. Of significance, responses to other common immunogenic regions outside of V3 were similar in wt and gV3-inoculated mice, indicating that responses to these determinants do not occupy, or “fill in,” the immunologic space vacated by the lack of the potentially competing V3-directed ASC response.

Materials and Methods

Ag design and protein production

The YU2-derived wt Env trimers and the probes used for the differential ELISPOT were described previously (10, 32). The gene encoding gV3 was designed using Vector NTI (Invitrogen) and a plasmid encoding wt trimers as a template (Fig. 1A). The gV3-encoding gene was synthesized (GenScript) and cloned into a pcDNA3.1+ expression vector (Invitrogen). Soluble recombinant protein was produced using the FreeStyle 293 expression system (Invitrogen), as previously described (31). The glycosylated trimer in processing wt and a total volume were purified in culture supernatants in a two-step process. First, proteins were captured via glycans with lentil-lecitin affinity chromatography (GE Healthcare). After extensive washing (PBS/0.5 M NaCl), the His-tag-containing proteins were eluted (1 M methyl-α-D-mannopyranoside, 10 mM imidazole [EM], 0.5 M NaCl, PBS [pH 7.4]) and subsequently captured in the second step via nickel-chelation chromatography (GE Healthcare). After extensive washing (PBS, 40 mM IM, 0.5 M NaCl, PBS [pH 7.4]), the proteins were eluted with a 300 mM IM-containing PBS buffer. Buffer exchange to PBS and concentration of eluted protein were performed with Amicon Ultra centrifugal filter units with a 30-kDa cut-off (Millipore), and the final concentration was measured using a NanoDrop system (Thermo Scientific). The relative m.w. of respective immunogen was resolved by SDS-PAGE on a 4–12% Tris–Bis gel (Invitrogen). Deglycosylation of wt and gV3 trimers was performed by PNGase F treatment, according to the manufacturer’s instructions (New England Biolabs). The Avi-tag motif of the ELISPOT probes gp140, gp120, gp120αV3, and gp120αV123 was biotinylated as previously described (32).

Animals and immunizations

Male or female BALB/c mice, 7–9 wk old, were injected s.c. up to four times with 10 μg wt or gV3 trimer and 10 μg AbISCO-100 adjuvant (Baxter) in 100 μl phosphate-buffered saline and 100 μl of a 1:1 mixture of Freund’s complete adjuvant containing 5 μg/ml tuberculin and 5 μg/ml of Mycobacterium tuberculosis. The animals were bled at 5 d after the last injection, unless otherwise stated. All mice were kept at the animal facility at Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, under approved conditions, and all experiments were preapproved by the Committee for Animal Ethics (Stockholm, Sweden) and performed according to given guidelines.

Evaluation of binding Abs

High-protein–binding MaxiSorp plates (Nunc) were coated with 100 or 200 ng/well of recombinant wt trimer, gV3 trimer, or peptides spanning the V3 region of either wt or gV3 trimers at 4°C overnight. The coated plates were blocked with 2% fat-free milk in PBS. In some cases, wt or gV3 trimer–coated wells were preincubated with 40 μg/ml soluble 4-domain CD4 (Promega) prior to the addition of mAb 17b. After washing (PBS, 0.05% Tween-20), mouse serum or human-derived mAbs were added at different concentrations. In other instances, serum was preincubated with 100, 10, or 1 μg/ml wt or gV3 trimers before addition to wt or gV3 trimer–coated wells. The wells were then probed with HRP-conjugated anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, or IgM (Fc region) or anti-human IgG (Fc region) (Southern Biotech). The plates were then washed, and addition of a colorimetric HPA substrate containing 3,3′,5,5′-tetramethylbenzidine (Invitrogen) was used to detect binding. The enzymatic reaction was stopped by adding one volume of 1 M H2SO4, and OD was read at 450 or 450–620 nm. All incubations were performed at room temperature for 1 h, unless otherwise stated. To detect Ag binding to the primary receptor of HIV-1 Env, human CD4 or wt or gV3 trimers were coincubated with purified human CD4+ T cells for 30 min. The cells were then incubated with mAb 17b for an additional 30 min at 4°C, and Ag–Ab complexes bound to the cell surface were detected by flow cytometric analysis after the addition of FITC-conjugated anti-human IgG Ab (BD Pharmingen). The Env-directed mAbs were kindly provided by Dennis Burton (The Scripps Research Institute) (b12), Gary Nabel and John Mascola (National Institutes of Health) (VRC01), Marshal Posner and Lisa Cavacini (Harvard University) (Fl05), Susan Zolla-Pazner (New York University School of Medicine) (447–52D), and James Robinson (Tulane University School of Medicine) (48D, 17b, 39F).

Single-cell suspensions

Single-cell suspensions were achieved by passing spleen through a 70-μm nylon mesh. RBCs were subsequently lysed with hypotonic ammonium chloride solution for 1 min, and the remaining cells were washed and resuspended in complete RPMI 1640 medium (Sigma) containing 5% FBS, 50 μg/ml 2-ME, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Enumeration of cells was made in a Countess cell counter (Invitrogen).

Detection of IgG-producing ASCs or IFN-γ-producing CD4+ T cells

Detection of IgG-producing ASCs or IFN-γ-producing CD4+ T cells was performed using an ELISPOT assay, as previously described (32, 35). Briefly, 96-well MultiScreen-IP filter plates (Millipore) were pretreated with 70% ethanol and washed three times in sterile PBS. Plates were coated with 1 μg/well of polyclonal goat anti-mouse IgG Ab or anti-IFN-γ (both from both Medtech, Stockholm, Sweden) diluted in PBS and incubated overnight at 4°C. Plates were washed three times in sterile PBS and blocked in complete RPMI 1640 medium for 2 h at 37°C. Cells were added to triplicate or quadruplicate wells at 1–6 × 105 cells/well for detection of Env-specific ASCs or at 1 × 106 to 1 × 107 cells/ml for detection of total IgG-producing ASCs. For the detection of IFN-γ-producing CD4+ T cells, 2 × 105 cells and 6.67 μg/ml wt or gV3 trimer were added to each well. Plates were incubated with 70% ethanol and washed six times in water, and spots were developed by the addition of M2–M E, 2 μg/ml polyclonal goat anti-mouse IgG (AbISCO), biotinylated anti–IFN-γ (b12), Gary Nabel and John Mascola (National Institutes of Health) (VRC01), Marshal Posner and Lisa Cavacini (Harvard University) (Fl05), Susan Zolla-Pazner (New York University School of Medicine) (447–52D), and James Robinson (Tulane University School of Medicine) (48D, 17b, 39F).

Analysis of intracellular cytokine production in CD4+ T cells

Single-cell suspensions of splenocytes were incubated with a pool of 15-mer peptides overlapping by 9 aa spanning all but the V3 region of the wt Ag (Env-V3), the V3 region (V3), PMA (10 ng/ml); ionomycin (5 μM), or...
complete RPMI 1640 medium. Brefeldin A was added to a working concentration of 10 μg/ml after 1 h of incubation, and cells were harvested for flow cytometric analysis after an additional 5 h of incubation. Posttranslational modifications were performed by incubation of splenocytes with 0.5 μg/ml wt or gV3 trimers. Brefeldin A was added after 2 h of incubation, and the cells were harvested 15 h later for flow cytometric analysis. IL-2- and IFN-γ-producing CD4+ T cells were detected by incubation of stimulated splenocytes with FITC-conjugated anti-CD4 (BD Pharmingen) and PE-conjugated anti-CD8 (BD Pharmingen) for 30 min at 4˚C, followed by permeabilization of cells by Cytofix/Cytoperm, according to the manufacturer’s instructions (BD Biosciences), and subsequent incubation with allophycocyanin-conjugated anti–IL-2 and PerCP-conjugated anti–IFN-γ (both BD Biosciences). Cells were collected on a BD FACSCalibur, and data were analyzed using FlowJo software (TreeStar).

Mitogenic stimulation of splenocytes

Single-cell suspensions of splenocytes from naive female BALB/c mice were incubated in complete RPMI 1640 medium in the presence or absence of 2 μg/ml LPS (Sigma) for 5 d at 37˚C in 5% CO2. Supernatants were harvested and cleared of debris by centrifugation, and supernatant IgM-binding to Ag was measured by ELISA, as described above.

Statistical analysis

Statistical analysis was performed using GraphPad Prism V5.04 (GraphPad Software). Data sets were first analyzed with the D’Agostino and Pearson omnibus normality test. Sets conforming to normal distribution were then analyzed further using a paired or nonpaired two-tailed Student t test to determine the significance of observed differences. Data sets not exhibiting normal distribution were analyzed using a nonparametric Mann–Whitney U test or the Wilcoxon matched-pairs signed-rank test.

Results

Design, production, and biochemical characterization of an isogenic protein pair

Posttranslational modifications by N-linked glycosylation are not only critical for the folding of solvent-exposed glycoproteins, they also act to shield antigenic regions of viruses from B cell recognition (34). The feasibility of introducing additional sites for N-linked glycosylation to simultaneously dampen the immunogenicity to V1, V2, and V3 on gp140 trimers was demonstrated previously (36, 37). In this study, we used this approach to specifically dampen the V3 regions of soluble YU2 Env trimers by targeted introduction of three (NXT/S) sites per gp120 protomer. This created an isogenic glycoprotein pair differing only in their potential to stimulate V3-specific B cell responses (Fig. 1A). The glycosylation sites were spaced evenly in V3 to allow efficient shielding of the antigenic region, while leaving the proline in the GPG motif unaltered to minimize disruption of the V3 conformation (38, 39). The introduced modifications increased the number of NXT/S motifs on the trimers from 84 to 93, and the modified and potentially hyperglycosylated trimers were designated as gV3. The production yields of both gV3 and wt trimers were similar, suggesting that the mutations in gV3 were well tolerated (data not shown).

To assess glycan occupancy of the introduced NXT/S sites, we resolved the gV3 and wt trimers by reducing SDS-PAGE and analyzed their relative mobility in a gradient gel before and after treatment with PNGase F (Fig. 1B). Consistent with a higher content of N-linked glycans in gV3, this glycoprotein variant migrated more slowly than did the unmodified wt trimers, suggesting that the introduced NXT/S sites on gV3 were used. In support of this, the deglycosylated forms of wt and gV3 trimers migrated similarly (Fig. 1B). The bands visible at ~36 kDa in the gel are composed of the PNGase F enzyme.

Upon binding of Env to the primary receptor, primate CD4, the variable regions of gp120 are displaced, and the coreceptor binding site (CoRbs) is formed or exposed (40, 41). The conformation induced by CD4 binding is associated with an increased binding capacity of CoRbs-directed mAbs to gp120; we previously demonstrated that this also was the case for the YU2-derived wt trimers (31). To assess the conformation of the wt and gV3 trimers, we directly compared their capacity to bind human CD4+ T cells and to undergo the CD4-induced conformational change at the CoRbs. By coincubating wt or gV3 with purified human CD4+
T cells and subsequently detecting bound trimers with the CoRbs-directed mAb 17b, we found that both immunogens bound CD4+ T cells with a similar capacity and in a concentration-dependent manner (Fig. 1C). After preincubation of both trimeric glycoproteins with soluble human CD4, a similar increase in 17b binding was observed, demonstrating that the gV3 trimers retained the capacity to undergo a CD4-induced conformation change that was analogous to wt trimers (Fig. 1D).

To determine whether the additional N-linked glycans affected the overall antigenicity or conformational integrity of gV3, we probed wt and gV3 trimers with mAbs (b12, VRC01, and FI05) directed against discontinuous epitopes overlapping the conserved CD4 binding site and an additional mAb (48D) directed against the CoRbs (Fig. 1E). The similarity in binding of these mAbs to the wt and gV3 trimers confirmed that the major functional regions of Env remained structurally intact following introduction of the additional glycans in the gp120 V3 region. In contrast, the V3-directed mAbs, 447D and 39F, were unable to bind gV3 (Fig. 1E). Because some of the amino acid substitutions introduced into gV3 to create the N-linked glycosylation sites would perturb the epitopes of these mAbs, this result was expected, even without the additional glycosylation.

**Immuno**-gen-specific IgM production

The antigenic similarity between wt and gV3 trimers of all regions, except V3, allowed us to design an immunization experiment to investigate how the ASC response against distal epitope regions of Env would evolve following boosting in the absence of the highly immunogenic V3 region (Fig. 2A). Because immunosilencing of an immunodominant region on Env may reduce the number of cognate naive B cells with potential to respond to the Ag after immunization, we first assessed the capacity of IgM produced from naive, Ag-inexperienced B cells to bind the respective Ag (Fig. 2B). Significant wt- and gV3-specific binding was detected after a 5-d in vitro culture of splenocytes, isolated from naive mice, in the presence LPS; however, the average IgM binding to gV3 was only 57% of that of the wt trimers ($p < 0.0001$). This suggested that immunosilencing of V3 would result in a decreased potential of the naive B cell pool in BALB/c mice to respond to gV3 compared with the wt trimers. We next compared the IgM response to wt and gV3 in serum 9 d after a primary immunization and detected no difference, regardless of whether the response was measured against the wt or gV3 trimers (Fig. 2C). In contrast, 5 d after a second immunization, the wt trimers stimulated higher Ag-specific IgM responses than did gV3 at several serum dilutions, whereas the response against gV3 was similar for wt and gV3-inoculated animals at this time point (Fig. 2D). These results suggest that V3-directed IgM responses are not detectable until after a second immunization with the wt trimers.

**Induction of Env-specific IgG+ Ab-producing cells after immunization**

Using a differential B cell ELISPOT assay, we next enumerated splenic IgG+ ASCs to four different Env-derived probes that were previously described: wt trimers (gp140), wt trimers lacking the gp41 ectodomain (gp120), wt trimers lacking the gp41 ectodomain and V3 (gp120ΔV3), and wt trimers lacking the gp41 ectodomain and variable regions 1, 2, and 3 (gp120ΔV123) (32). After two immunizations, the numbers of total IgG-producing ASCs elicited by the wt and gV3 trimers were similar (Fig. 3A). These Ags also induced similar numbers of total Env-specific ASCs and, consistent with our previous data (32), there were significantly more ASCs reactive with the gp140 probe than with the gp41-lacking gp120 probe (Fig. 3B). When analyzing serum IgG derived from the two groups of animals after two inoculations, we did not observe a significant difference in binding to the wt or gV3 trimers, suggesting that the V3-directed response was still a minor component of the total Env-directed response (Fig. 3C). However, V3-specific IgG was detectable in mice inoculated twice with wt trimers, but not in mice inoculated with gV3, when directly analyzing serum reactivity to a peptide spanning the wt-derived V3 sequence ($p = 0.0002$) (Fig. 3D). This confirmed that V3-directed Abs were elicited by the wt trimers, but not the gV3 trimers, at this time point but that their contribution to the overall response was relatively low. Importantly, there was no significant difference in serum reactivity to a gV3-derived V3 peptide between wt and gV3-inoculated mice, suggesting that the V3 region of gV3 did not contain any highly immunogenic neoepitopes (Fig. 3D).

After one additional injection with each trimeric immunogen, the mean IgG-producing ASCs from wt- and gV3-immunized mice (665 and 527, respectively) remained statistically similar (Fig. 3E). In contrast, there were significantly more ASCs recognizing the V3-containing probes, gp140 (326 versus $167$, $p = 0.003$) and gp120 (316 versus $144$, $p = 0.001$), in mice immunized with the wt trimers compared with those immunized with gV3 (Fig. 3F). Furthermore, the wt and gV3 trimers induced similar numbers of ASCs against both probes lacking V3, gp120ΔV3 and gp120ΔV123, respectively. The serological responses were consistent with this interpretation, because the average IgG ELISA titers against the wt trimers were significantly higher in wt trimers–immunized mice than in gV3 trimers–immunized mice (3869 versus 1653, $p = 0.004$) (Fig. 3G). In contrast, both immunogens induced similar IgG ELISA titers when binding to gV3 was measured, indicating that other specificities did not change, and there was no detectable reactivity against potential gV3-derived neoepitopes in gV3-inoculated mice. If present, such neoepitopes would consist of glycan and peptide moieties, a type of...
hybrid epitope described to evolve at relatively low frequency in individuals chronically infected with HIV-1 (42). So far, however, such glycopeptide-directed Abs have not been described to be induced by subunit Env immunogens.

The potential presence of neoepitopes in gV3 was investigated further by preincubating serum from wt- and gV3-inoculated mice, diluted to yield similar gV3-binding titers, as shown in Fig. 3G, with different concentrations of soluble wt or gV3 trimers and then assessing the resulting reduction in binding to wt- or gV3-coated ELISA plates (Fig. 3H). These ligand-adsorption experiments demonstrated that adsorption with wt trimers, but not gV3 trimers, potentially reduced binding of serum from wt-inoculated mice to wt-trimer–coated ELISA plates (Fig. 3H, left panel). This was consistent with strong V3-directed responses after wt injection. In contrast, serum reactivity of gV3-inoculated mice to wt-trimer–coated ELISA plates was similarly reduced, irrespective of competing soluble trimer. In a complementary set of experiments, we assessed the capacity of soluble wt and gV3 trimers to reduce serum reactivity from wt or gV3-inoculated mice to ELISA plates coated with gV3 trimers (Fig. 3H, right panel). These experiments showed a similar level of reduction, regardless of immunization regimen, consistent with the lack of strong neoepitopes in gV3. Taken together, these data demonstrate that the wt trimers induced a significantly more potent Ag-specific B cell response than did the gV3 trimers after a third inoculation, and this difference could be attributed to a difference in the V3-directed response.

Evaluation of epitope-specific ASC responses following wt and gV3 trimer inoculation

To address region-specific responses in more detail, we subtracted ASC responses to common structural elements within the set of probes used and assessed the contribution of four epitope regions by differential subtraction. We found similar responses to gp41 (calculated as the difference between ASCs specific for the gp140 and gp120 probes), V12 (calculated as the difference between ASCs specific for the gp120 and gp120ΔV123 probes after subtracting V3 responses), and core determinants (calculated as the remaining gp140 response after subtracting gp41, V12, and V3 ASCs) after both two and three Env inoculations, regardless of the immunogen used (Fig. 4A). Consistent with our interpretation of probe-specific ASC responses and serology above, only the wt Env induced V3-specific responses (calculated as the difference between the gp120 and gp120ΔV3 probes, p < 0.0001). To determine whether immunogenic silencing of V3 affected the generation of memory B cells, we assessed region-specific responses after recall by additional injections with trimers at 35 and 188 d following the third injection (Fig. 4B). Of the four epitope regions analyzed by differential subtraction, only the response to V3 was different between the two immunogens because the wt trimers induced a higher magnitude of V3 responses at both recall time points (day 35, p = 0.008 and day 188, p = 0.04). Taken together, these data demonstrate no detectable difference in B cell responses to immunogen-common determinants outside of V3 when elicited by the isogenic trimer pair presenting wt or glycan-masked V3. Thus, the space vacated by the lack of V3-directed ASC in gV3-boosted mice is not occupied by an expansion of ASCs recognizing other antigenic regions of Env as measured at this time point.

Analysis of Ag-specific CD4+ T cells after wt and gV3 inoculation

The number and strength of CD4+ T cell epitopes within an Ag can influence the magnitude of vaccine-elicited B cell responses to protein Ags, including Env (43). Therefore, we wondered whether the
wt and gV3 trimers were able to stimulate differential CD4+ T cell responses as a possible explanation for their differences in immunogenicity. We found no peptide matching the YU2 V3 region that was compatible with MHC class II I-Ad presentation, based on searches in the comprehensive HIV databases (http://www.hiv.lanl.gov). Prediction of peptide binding to the BALB/c MHC class II I-Ad allele using NetMHCII 2.2 (44, 45) was consistent with this. We also investigated the capacity of wt and gV3 trimers to elicit CD4+ T cell responses experimentally by assessing frequencies of Ag-specific IL-2– and/or IFN-γ–producing CD4+ T cells 5 d following two injections of either wt or gV3 trimers. In vitro stimulation of splenocytes with 15-mer peptides spanning Env-V3 induced detectable cytokine production by intracellular staining (Fig. 5A, 5B). In contrast, peptides spanning V3 did not induce cytokine production, consistent with a lack of CD4+ T cell epitopes in this region. Similarly, we detected no significant difference between wt- and gV3-immunized mice with regard to Ag-specific CD4+ T cell cytokine production after overnight stimulation with respective Ags (Fig. 5C). Control stimulation with culture medium (--) is shown. (D) IFN-γ production from splenocytes, 5 d after two injections with wt or gV3 trimers (n = 10/group), was assessed with ELISPOT after overnight stimulation with respective protein Ag or media control. This assay mainly enumerates IFN-γ production from CD4+ T cells (35). (E) Isotype switching of Abs targeting common regions within gV3 and wt trimers after injection, as assessed by binding ELISA to gV3 trimers (n = 6/group). Error bars represent SD. *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon matched-pairs signed-rank test or Mann–Whitney U test.

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trimers, differing only in the potential to stimulate V3-specific B cells. Because our previous studies showed that approximately half of the ASC response was directed against V3 after three immunizations with the wt trimers (32), we envisioned two possible outcomes after immunization with gV3: a reduction in the total Env-specific response corresponding to the lost V3 response or a similar overall Env-specific response resulting from a redistribution of the response to other epitope regions of Env.

As shown in the current study, immunosilencing of V3 significantly reduced the total Env-specific serum IgG response measured after three homologous Env trimer inoculations, and this reduction was directly associated with the loss of V3 reactivity in gV3-inoculated animals. A correlation between Ag-specific naive CD4+ T cell frequency and the resulting CD4+ effector cell response after immunization was proposed previously (46). Similarly, the resulting T cell–independent ASC response against NP is related to the number of B cells recruited into the response after priming (47). Our data suggest a model in which differences in naive B cell precursor frequency influence the peak ASC responses to wt and gV3 trimers. However, detection of a significant difference in response to wt and gV3 required two (IgM) or three injections (IgG) before it constituted a measurable fraction of the total Env-specific response, suggesting that additional factors contribute to the maturation of V3-specific ASC responses to Env.

The expansion and differentiation of V3-specific B cells into IgG+ ASCs after three injections with wt trimers was demonstrated previously using a differential B cell ELISPOT assay (32). When applying this assay in the current study, we did not observe a redistribution of the response to gp41, V12, or core determinants in gV3-immunized mice at time points coinciding with the development of dominant V3-directed ASC responses in wt trimer-immunized mice. Entry of B cells into GCs after immunization with NP-coupled carrier protein is regulated via an affinity-based T cell–dependent checkpoint (48). However, we found no evidence that the difference in ASC induction was due to differential priming of specific CD4+ T cells or to a difference in Ab isotype switching, because these properties were similar for both immunogens. Instead, our data suggest that ASC responses to different Env determinants are regulated independently of each other, such that responses to non-V3 determinants do not compete with V3-specific ASCs, because the non-V3 responses were not increased in gV3-immunized mice.

One possibility for this apparent lack of competition between different B cell specificities is that the responses we measured 5 d after boost primarily arose from the stimulation of memory B cells and their subsequent differentiation into plasma cells, without further selection in the GC. In such a scenario, V3-specific cells may be preferentially amplified by homologous boosting, resulting in a significant fraction of V3-specific ASCs and V3-directed serum reactivity in wt trimer–immunized mice at the time points measured. That this “space” is not filled in gV3-inoculated animals suggests a lack of redistribution within this ASC response. The dominant V3-specific ASC responses measured after three injections are not reflected in the memory B cell compartment where the V3-specific fraction is detectable but smaller (Fig. 4B). This is consistent with memory B cells being generated within the first weeks after immunization (49–51) when V3-directed specificities represent a smaller fraction of the total Env-specific response. In this study, we did not investigate the wt- and gV3-induced response in the long-lived bone marrow plasma cell compartment, thus we cannot draw any conclusions about possible redistribution of responses in this compartment. From our data, we also cannot exclude the presence of subtle changes in Ab specificities elicited by each respective immunogen or definitively rule out a different outcome if a different epitope region of Env was hyperglycosylated. However, it is worth noting that reduced specific serum IgG responses were also observed after immunization with rationally modified therapeutic proteins (52–55). Although no direct investigation of the induction of specific ASCs was undertaken in those studies, the investigators suggested a loss of overall reactivity to the modified protein, similar to the results obtained in our studies. Thus, the observations described in this article are likely not limited to HIV Env–based immunogens; they may also apply to other proteins under preclinical or clinical evaluation as therapeutic proteins (reviewed in Ref. 56) or vaccine Ags.

Our data suggest that vaccine-induced responses against undesired determinants, such as the V3, may not be at the cost of responses targeting conserved determinants of Env, if such desired responses can be elicited as part of the polyclonal Ab response (57). Therefore, we suggest that our results have general implications for vaccine design where the aim is to focus B cell responses on specific subdeterminants of a protein immunogen. In summary, our data illustrate the need to use large, multiepitope proteins as models Ags to gain an improved understanding of B cell responses to real-world vaccine targets.

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