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Conformational instability governed by disulfide bonds partitions the dominant from subdominant helper T-cell responses specific for HIV-1 envelope glycoprotein gp120

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Abstract

Most individuals infected with human immunodeficiency virus type 1 (HIV-1) generate a CD4⁺ T-cell response that is dominated by a few epitopes. Immunodominance may be counterproductive because a broad CD4⁺ T-cell response is associated with reduced viral load. Previous studies indicated that antigen three-dimensional structure controls antigen processing and presentation and therefore CD4⁺ T-cell epitope dominance. Dominant epitopes occur adjacent to the V1-V2, V3, and V4 loops because proteolytic antigen processing in the loops promotes presentation of adjacent sequences. In this study, three gp120 (strain JR-FL) variants were constructed, in which deletions of single outer-domain disulfide bonds were expected to introduce local conformational flexibility and promote presentation of additional CD4⁺ T-cell epitopes. Following mucosal immunization of C57BL/6 mice with wild-type or variant gp120 lacking the V3-flanking disulfide bond, the typical pattern of dominant epitopes was observed, suggesting that the disulfide bond posed no barrier to antigen presentation. In mice that lacked gamma interferon-inducible lysosomal thioreductase (GILT), proliferative responses to the typically dominant epitopes of gp120 were selectively depressed, and the dominance pattern was rearranged. Deletion of the V3-flanking disulfide bond or one of the V4-flanking disulfide bonds partially restored highly proliferative responses to the typically dominant epitopes. These results reveal an acute dependence of dominant CD4⁺ T-cell responses on the native gp120 conformation.

Keywords

T-helper; lymphocyte; cellular immune response; protein conformation; protein denaturation; antigen processing; antigen presentation; disulfide

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Introduction

The profile of CD4⁺ T-helper epitope specificities is likely to have a significant influence on the protectiveness of an immune response. In the case of HIV-infected patients, low viremia has been attributed to the presence of cytotoxic CD4⁺ T cells and breadth of CD4⁺ epitope specificity [1, 2]. Mechanisms controlling CD4⁺ epitope dominance have not been resolved. Peptide affinity for the MHC protein is an important factor, but it is not well correlated with epitope dominance [3-6]. Relatively few studies have addressed the relationship of antigen structure to CD4⁺ T-cell immunogenicity or epitope dominance. Conformational destabilization of immunogens has been associated with changes in immunogenicity, and this has been attributed to an increased susceptibility to proteolytic degradation [7, 8]. We and others have described the modulation of helper T-cell responses by the structure of the antigen, including that of HIV envelope glycoprotein gp120 [3, 6, 9-13].

We previously noted that gaps in the T-helper response coincided with the locations of the cysteines in the outer domain of HIV gp120, and we hypothesized that the substitution of alanines for disulfide-bonded cysteines would raise new helper T-cell epitopes by creating regions of flexibility that can serve as cleavage sites for antigen processing [14]. The recombinant wild-type and three disulfide variant proteins were expressed in insect cells, evaluated for native-like conformations, and compared in terms of their T-helper epitope maps. In contrast to expectations, BALB/c mice immunized with the HIV-1_{89.6} gp120 variants exhibited globally reduced helper T-cell responses compared to those immunized with wild-type gp120. We concluded that the disulfide-bond deletions destabilized the gp120 variants, leading to epitope destruction and low CD4⁺ T-cell responses.

We predicted that disulfide deletions would perturb the structure and stability of gp120 from any strain of HIV-1 because the nine disulfide bonds are highly conserved [15]. Initially we examined HIV-1_{89.6} gp120 because it was included in first recombinant simian-human immunodeficiency virus (SHIV), but this Env protein is unusual in its binding to coreceptor CXCR4. Since the majority of transmissible HIV strains use CCR5 [16], we next focused on HIV-1_{JR-FL} gp120 [17], which more accurately represents vaccine candidates currently under investigation [18]. Based on the findings with the HIV_{89.6} disulfide variants, we hypothesized that γ -interferon-inducible lysosomal thio reductase (GILT) aggravates the destabilizing effect of disulfide-bond deletions, leading to epitope destruction and a low CD4⁺ T-cell response. Since GILT-knock-out (GILT-KO) mice exhibit defects in presentation of antigens that contain disulfide bonds [19-25], we predicted that disulfide deletions would have the opposite effect in GILT-KO mice, in that they would allow the antigen to bypass a defect in presentation and enhance the T-cell response.

Experimental Procedures

Plasmids

A mammalian codon-optimized wild-type gp120 from strain JR-FL, including a C-terminal His₆, was cloned into pFastBac-1 vector as previously described [3]. Variants gp120dss298, gp120dss378, and gp120dss385 were constructed from this original plasmid by mutating codons for paired cysteines into codons for alanines, using a Stratagene site-directed

mutagenesis kit. Each variant lacks a single outer-domain disulfide bond connecting wild-type gp120 residues 288-322 (gp120dss298), 368-431 (gp120dss378), or 375-404 (gp120dss385) and was given the same name as the corresponding variant from HIV-1_{89.6} (Fig. 1A).

Proteins

High Five insect cells expressed His₆-tagged versions of the wild-type gp120 from HIV-1 strain JR-FL and the three gp120 variants. The proteins were purified from the culture supernatant by lectin-affinity chromatography and nickel-affinity chromatography, and the concentration was assayed as previously described [14].

Peptides

The 46 peptides spanning residues 35-502 of the HIV-1_{JR-FL} *Env* gp120 sequence were designed to correspond to the set of HIV-1_{89.6} gp120 20-mers [3]. Most of the peptides are 20-mers overlapping by 10 residues (Table 1), and all peptides were synthesized by JPT.

Antibodies and ELISA

Monoclonal antibodies (MAbs) 17B, E51, 21E, 15E, C11, and 23G were prepared as previously described [26-28]. Monoclonal antibodies F425 A1g8, F425 B4e8, and F105 were provided by Marshall Posner and Lisa Cavacini through the NIH ARRR Program. IgG1 b12 was provided by Dennis Burton and Paul Parren through the NIH ARRR Program, while 2G12 was provided by Hermann Katinger through the same reagent program. All antibodies were used at a concentration of 5 µg/mL. Binding of MAb was detected with goat anti-human IgG conjugated with horseradish peroxidase (HRP). Binding of sCD4 was detected with biotinylated guinea pig anti-CD4 and HRP-streptavidin.

Limited proteolysis

Each reaction of 10 µL contained 5 µg of wild-type or variant gp120 and 0, 0.12, 0.18, or 0.24 µg of protease in phosphate-buffered saline (50 mM sodium-phosphate, 125 mM sodium chloride) and 10% glycerol and was incubated as follows. For elastase and trypsin, the reaction mixtures were incubated for 30 min at 37° C and terminated by addition of 1 µL of 100 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol. For cathepsin S (Sigma), reaction mixtures were incubated for 30 min at 25° C and terminated by addition of NuPAGE gel-loading buffer (Invitrogen). For proteinase K, reaction mixtures were incubated for 30 min at 4° C and terminated by addition of 1 µL of 100 mM PMSF as above. Samples were reduced with dithiothreitol, boiled for 2 min, and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a 4 to 12% NuPAGE SDS-morpholineethanesulfonic acid gel (Invitrogen).

Chemical denaturation with guanidine hydrochloride (GuHCl)

Protein was diluted to 0.5 µM with a solution of Tris-saline (125 mM NaCl, 50 mM Tris-HCl, pH), including variable amounts of guanidine hydrochloride (GuHCl). The fluorescence emission spectrum was recorded on a PTI Quantamaster. Proportion folded was calculated at a given wavelength according to the expression,

$$(F_{xM} - F_{6M}) / (F_{0M} - F_{6M}),$$

where F_{xM} is the fluorescence intensity at GuHCl concentration x . Proportion folded was fitted by nonlinear regression to the following expression:

$$F_{\text{unfolded:folded ratio}} = F_{\text{folded}} - \left((F_{\text{folded}} - F_{\text{unfolded}}) e^{-\Delta G_u / RT} \right) / \left(1 + e^{-\Delta G_u / RT} \right).$$

The expression,

$$\Delta G_u = \Delta G_{uw} - m_{eq} [GuHCl],$$

was used to calculate the free energy of unfolding at zero denaturant (ΔG_{uw}) for each protein [29].

Mice

GILT-KO mice were kindly provided by Peter Cresswell [19]. Re-derivation was performed by Charles River Laboratories. C57BL/6 mice were obtained from Jackson Laboratories.

Immunization

C57BL/6 mice (six- to eight-week-old females) were intranasally immunized by a single administration of 20 μ g gp120 protein plus 5 μ g of double mutant (R192G, L211A) heat-labile toxin as adjuvant [30]. The mice were sacrificed after 1 week. GILT-KO mice (six- to nine-week-old, 50% of each sex in each group) were immunized using the same protocol as for C57BL/6 mice.

T-cell proliferation assay

Briefly, spleens were dissociated using a 40 μ m cell strainer (Falcon BD). The cells were pelleted via centrifugation, and red blood cells were lysed using RBC lysing buffer (Sigma) per the manufacturer's instructions. The purified splenocytes were isolated by centrifugation and immediately plated in a 96-well plate at a density of 4×10^5 cells in 170 μ L. Peptide was added in a volume of 30 μ L to achieve a concentration of 2 μ g/mL. Proliferation was assayed by adding 1 μ Ci of [3 H] thymidine on day three of culture and harvesting the cells after 18 h. Cell proliferation was measured using a scintillation counter.

Statistics and bioinformatics

Significance of differences between variant and wild-type gp120s was established by 1-way ANOVA with Dunnet's post-test for multiple comparisons, except where otherwise indicated. Percent solvent accessibility data for gp120 were extracted from the CD4-bound crystal structure of HIV-1_{JR-FL} gp120 (PDB ID: 2B4C) using MolMol [31]. The Shannon sequence entropy for gp120 was calculated from the alignment of 40 HIV-1 clade B gp120 sequences, including strains HXB2, YU2, 89.6, and JR-FL. The consensus sequence and the H(x) for each residue were generated using BioEdit [32].

Results

The wild-type HIV-1_{JR-FL} gp120 and three disulfide-bond variants (gp120dss298, gp120dss378, and gp120dss385) were purified at comparable levels (approximately 4 mg/L of culture) from the supernatants of insect-cell cultures infected with recombinant baculoviruses.

Limited proteolysis

Preferred sites of proteolysis occur within flexible, solvent-accessible regions on the protein; well-structured regions of a protein are not susceptible to limited proteolysis [33].

Susceptibility to proteolysis was monitored by the disappearance of the intact variant or wild-type gp120 following treatment with individual proteases (Fig. 1B). All three of the disulfide variants were more digestible than wild-type gp120 when treated with elastase or cathepsin S. The variant gp120dss298 was more digestible than wild-type gp120 when treated with any of the four proteases. We were unable to distinguish variants gp120dss378 or gp120dss385 from wild-type gp120 in digestibility by trypsin or proteinase K.

Monoclonal antibody and CD4 binding

The conformations of gp120dss298 and gp120dss385 were most perturbed by the disulfide deletions, as inferred from changes in the binding of well-characterized monoclonal antibodies (mAb) or soluble CD4 (sCD4). Only the mAbs that bind linear epitopes, the V3-specific (F425 B4e8 or 2.1E) and 2.3G mAbs, bound to these variants at wild-type gp120 levels (Fig. 1C). Binding of sCD4 to gp120dss298 and gp120dss385, respectively, was 11% and 16% of that to the wild-type gp120 (Fig. 1D). The conformation of gp120dss378 was least perturbed. Binding of sCD4 to gp120dss378 was 76% that of the wild-type gp120.

Chemical Denaturation

The free energies of unfolding gp120 and its variants were determined by denaturant-induced unfolding monitored by intrinsic fluorescence. All three disulfide-deletion variants had significantly lower free energies than wild-type gp120 (Figs. 1E and 1F).

Helper T-cell epitope mapping in C57BL/6 mice

Groups of 10 C57BL/6 mice were immunized by a single intranasal administration of HIV-1_{JR-FL} gp120 or gp120dss298 in order to examine the impact of a disulfide deletion for gp120 in animals with normal expression of GILT before proceeding to GILT-KO mice that share the same class II MHC protein (I-A^b). The gp120dss298 was selected to represent the disulfide variants because its conformation was most perturbed. Proliferative responses were analyzed following restimulation with individual 20-mer peptides spanning gp120 in 10-residue steps (Table 1). Stimulation in a single assay per peptide was recorded in terms of cpm in excess of background proliferation, as log(net cpm). Stimulation index (SI) was also calculated as cpm for a stimulated well divided by cpm for the average of unstimulated wells. A significant response was identified by cpm in excess of $2 \times \text{SD}$ of background proliferation (7 replicates, each mouse). A peptide was identified as containing an immunogenic epitope if the median cpm for the 10-mouse group was greater than the median unstimulated cpm, according to the Wilcoxon signed rank test.

In this study, a peptide was *dominant* if the peptide stimulated a response from a majority of mice (6 or more) that was greater than $2 \times \text{SD}$ of background proliferation. A peptide was *subdominant* if it stimulated a response from fewer than a majority of mice but the median cpm across mice was significantly higher than the median background cpm by the Wilcoxon signed rank test. A peptide was *non-immunogenic* if the median cpm was indistinguishable from the median background cpm. Of the 46 peptides tested for proliferative responses in gp120-immunized C57BL/6 mice, 8 peptides were dominant, 25 were subdominant, and 13 were non-immunogenic. The average stimulation index for each group of peptides was as follows: dominant, 3.8; subdominant, 1.5; and non-immunogenic, 1.1.

The number of mice responding to each peptide was not significantly different for gp120dss298 in comparison to gp120 (Fig. 2A). Of the eight dominant epitopes in gp120, seven were also dominant in gp120dss298 (Table 2). Peptide 13 elicited a larger average log(net cpm) but was not immunogenic by the criteria listed above (Figs. 2B and 2C).

In order to analyze changes in the strength of CD4+ responses, peptides were organized according to rank-order of proliferative response (Figs. 2D and 2E). The shape of the distribution for wild-type gp120 in C57BL/6 mice indicates a discontinuity near $\log(\text{net cpm}) = 2.8$ that divides the peptides into two groups of distinct proliferative intensity, which we characterized as highly proliferative, $\log(\text{net cpm}) > 2.8$, and moderately proliferative, $\log(\text{net cpm}) < 2.8$. In general, the eight dominant peptides identified in gp120-immunized mice elicited the highly proliferative responses in both groups of C57BL/6 mice. The eight dominant peptides in gp120 accounted for 50% and 53% of the total response (cpm above background) in gp120- and gp120dss298-immunized mice, respectively. Most of the remainder of the response (39% and 34%, respectively) was distributed over the 25 subdominant peptides.

Helper T-cell epitope mapping in GILT-KO mice

Groups of ten GILT-KO mice were immunized by a single intranasal administration of wild-type gp120 or one of three variants, gp120dss298, gp120dss378, or gp120dss385; and proliferative responses were mapped as described above.

The magnitudes of peptide-specific responses were approximately 50% lower in the gp120-immunized GILT-KO mice than in the gp120-immunized C57BL/6 mice discussed above. The reduction in response to the eight dominant peptides in C57BL/6 mice accounted for most of the decrease.

Using the criteria noted above, 7-9 peptides were identified as dominant for each of the immunogens (Table 2). In comparison to the epitope-dominance pattern identified in C57BL/6 mice, the dominance pattern in GILT-KO mice was least similar for wild-type gp120, intermediate for gp120dss378, and most similar for gp120dss298 and gp120dss385.

Using a multiple comparison across all peptides (2-way ANOVA), only peptides 13 and 36 were significantly increased in the gp120dss298- and gp120dss385-immunized groups (Figs. 3C and 3E). In spite of the increase, peptide 13 remained non-immunogenic using the above criteria. Peptide 36 rose from non-immunogenic to subdominant.

Significant differences between GILT-KO mouse groups were revealed when peptides were ranked by strength of response. As described for the C57BL/6 mice, a discontinuity was observed in the log(net cpm) range of 2.5-3.0. In the gp120dss298- and gp120dss385-immunized GILT-KO mouse groups, the highly proliferative and moderately proliferative peptide sets were readily distinguishable (Figs 3G and 3I). For both of these mouse groups, the highly proliferative peptide sets contained most of the dominant epitopes identified in gp120-immunized C57BL/6 mice. In a multiple comparison of responses to the eight dominant peptides identified in C57BL/6 mice (paired 1-way ANOVA), the gp120dss298- and gp120dss385-immunized GILT-KO groups responded more strongly than the gp120-immunized GILT-KO group ($p < 0.001$). For gp120 these eight peptides accounted for 22% of the total response, and for gp120dss298 and gp120dss385 this percentage rose to 33% and 29% of the total, respectively.

Discussion

Similarities in T-cell response between immunized mice and humans suggest that robust features of glycoprotein conformation shape the T-helper epitope dominance pattern. Six of the eight dominant peptides observed in C57BL/6 mice (peptides 17, 18, 19, 27, 30, and 31) have been observed as dominant in humans exposed to the HIV envelope glycoprotein. The dominant peptide 17 corresponds to the second most frequently identified epitope in the Thai (RV144) vaccine trial [34]. An epitope corresponding to dominant peptide 19 was the most frequently observed gp120 epitope in a treatment-naïve cohort of HIV+ individuals [35]. Epitopes corresponding to or overlapping dominant peptides 27, 30, and 31 were observed in at least four of seven HIV+ subjects on anti-retroviral therapy [36]. Efficient proteolytic cleavage in the V2 and V3 loops during antigen processing most likely accounts for the dominance of these six epitopes [3, 12, 36, 37].

We conclude that T-cell epitope dominance receives relatively little influence from the quaternary structure or carbohydrate trimming of the glycoprotein, or from differences in MHCII-peptide binding specificity of the immunized organism. The gp120 expressed from baculovirus in insect cells is predominantly monomeric, and it differs from natural HIV gp120 in carbohydrate trimming but not in the sites of glycosylation [38]. It is possible that the trimeric structure of the native glycoprotein has little effect on T-cell epitope dominance because the trimer dissociates in the antigen-processing compartment or because the monomeric gp120 shed by the virus is responsible for most of the response. Insofar as the glycoforms are part of the gp120 structure, they may influence antigen processing and thereby epitope dominance [39]; but glycoforms do not appear to play a major role at the step of recognition by TCR. We have previously noted that T-cells primed by glycosylated gp120 sequences can be recalled by non-glycosylated peptides, presumably because the carbohydrates were removed from gp120 during processing [40]. The affinity of peptides for the class II MHC protein (I-A^b), as predicted by the NETMHCII server [41], can explain the dominance of only peptides 18 and 19. For these two peptides, the affinities were among the top 20% of gp120 peptides (Fig. 4).

Sequences corresponding to peptides 4 and 5 were not reported to be dominant in humans, although the immediate N- and C-terminal sequences contain dominant epitopes in the

treatment-naïve cohort mentioned above. In immunized mice we previously noted greater influence from MHC class II alleles on selection of epitopes in the metastable inner domain of gp120 [3]. Thus, epitope dominance in humans could be more diffuse in the region of peptides 4 and 5 because this region of the protein is broadly accessible for MHC binding. Both peptides 4 and 5 were among the top 20% of predicted I-A^b-binding peptides (Fig. 4).

The reduced response to gp120 of GILT-KO mice compared to C57BL/6 mice is most likely due to inhibition of processing and/or presentation by one or more disulfide bonds during T-cell priming, but other explanations cannot be ruled out. The response to gp120 could be affected through other mechanisms affected by lack of GILT, such as maturation or stability of proteases and DM [42, 43]. In addition, the naïve T-cell repertoire in GILT-KO mice could be substantially different from that in C57BL/6 mice. Although the mechanism(s) for different immune responses in C57BL/6 and GILT-KO mouse strains is unresolved, the difference in response between gp120 and its disulfide variants in GILT-KO mice can be interpreted with far greater confidence.

Responses to dominant, highly proliferative epitopes observed in C57BL/6 (GILT+/+) mice were selectively diminished in GILT-KO mice. The percentage of the total response to gp120 in the “GILT+/+” dominant epitopes decreased from 50% in C57BL/6 mice to 22% in GILT-KO mice. Although the GILT-KO mice responded with increased frequency to some newly dominant epitopes (Table 2), the responses were not highly proliferative (Fig. 3F). We note that rigorous, yet sensitive, statistical tests of T-cell response (in 10-mouse groups) were necessary to analyze immunogenicity and dominance GILT-KO mice. A singular SI-cutoff for dominance or immunogenicity would not have been appropriate for both C57BL/6 and GILT-KO mice, and it would not have supported the differentiation of subdominant from non-immunogenic epitopes.

Deletion of the V3-flanking or the proximal V4-flanking disulfide partially restored the “GILT+/+” pattern of dominant epitopes (Table 2), and it restored highly proliferative responses to these epitopes in GILT-KO mice (Fig. 3G and 3I). Deletion of these disulfides presumably removed the conformational barrier to presentation caused by the disulfide bonds and restored the GILT+/+ dominance pattern. In contrast, deletion of the distal V4-disulfide (to form gp120dss378) did not restore the GILT+/+ dominance pattern (Fig. 3H). Thus, presentation of the dominant epitopes depends on reduction of either the V3-flanking disulfide bond or the proximal V4-flanking disulfide bond. Since the deletion of these disulfide bonds caused a much greater disruption of gp120 conformation than the distal V4-flanking disulfide (Fig. 1), we conclude that efficient processing and presentation of the dominant epitopes requires local unfolding of gp120.

Several observations presented here suggest that the presentation of dominant peptides is partitioned to a *high-priming* phase of processing and presentation that precedes complete unfolding of gp120 (Fig. 5). First, the dominant peptides obtained exceptionally high proliferative responses. Second, the responses to “GILT+/+” dominant peptides of gp120 were selectively reduced in GILT-KO mice. Third, the responses to “GILT+/+” dominant peptides were partially restored to dominance and high proliferation in the GILT-KO mice by elimination of the V3- and proximal V4-flanking disulfide bonds. Thus, these disulfide-

bond deletions removed a barrier to processing and presentation of dominant gp120 epitopes.

We and others have put forward a processing-directed model for epitope dominance in which the dominant epitopes emerge from the newly generated termini of antigen fragments generated by initial proteolytic cleavage events [10, 12, 37, 44-46]. This model provides a satisfying explanation for the exceptional dominance of epitopes that have only moderate affinity for the MHCII and for the striking absence from the immune response of many epitopes that are predicted (or observed) to have very high affinity for the MHCII [47-50]. Immediately after cleavage, epitopes near the cleavage site become much more available for MHCII binding, whereas other epitopes continue to be encrypted by the antigen 3D structure. Thus, the high-priming phase could be an early stage of antigen processing, characterized by active GILT, sufficient protease activity for an initial cleavage, and the presence of MHCII.

Strong T-cell epitope dominance could have a profound effect on the potential cross-reactivity of T-cell responses between HIV strains and between HIV and non-HIV antigens [51]. With regard to cross-reaction between strains of virus, this issue was previously considered in the context of antigenic “drift” and “shift” in the influenza hemagglutinin [52]. Amino-acid substitutions associated with antigenic drift should preserve epitope immunodominance, but deletions and insertions associated with antigenic shifts are likely to change epitope immunodominance. Thus, viral strains are likely to cross-react with T cells if the antigens are separated by antigenic drift, but possibly not cross-react if they are separated by an antigenic shift. Recent studies have highlighted the remarkably low specificity of T-cell recognition of presented peptides [53, 54]. This is due to an underlying weak specificity of MHCII-peptide binding and a modest degree of specificity imposed by the 4-5 peptide sidechains that point toward the TCR. Such a low level of specificity is expected to allow many TCRs to cross-react with host antigens and/or the vastly more diverse microbiome, potentially resulting in negative selection of naïve T cells or positive selection of memory T cells, respectively. However, the structure of the cross-reacting antigen also shapes the T-cell response to that antigen. Dominant epitopes are likely to be responsible for significant cross-reaction. In HIV gp120, a large fraction of gp120 peptides (72%) individually recalled a measurable T-cell response, but the subset of dominant peptides (17% of gp120 peptides) was responsible for most of the cumulative response. If cross-reacting antigens were similar in this respect to gp120, dominance would limit the universe of cross-reacting epitopes to 17% of the available sequences. Thus, the dependence of cross-reactivity on a similar conformational context may severely limit the ability of self-epitopes to shape naïve repertoires or of microbiomes to prime cross-reactive memory responses. This conclusion is consistent with the low (albeit significant) correlation of naïve T-cell populations with the number of self-similar epitopes [54].

We had expected each disulfide deletion to cause local increases in T-cell response that were unique to the particular variant. However, elimination of either the V3-flanking disulfide or the proximal V4-flanking disulfide bond allowed the presentation of peptide 36 as one of the subdominant epitopes. The N-terminus of peptide 36 is in the most buried part of gp120 (Fig. 6A). The N-terminus of peptide 36 is also highly conserved, according to the profile of

sequence entropy obtained from an alignment of 40 HIV-1 clade B gp120 sequences (Fig. 6B). This result shows that it is possible to reveal buried, conserved CD4⁺ T-cell epitopes through destabilization of the antigen. Nevertheless, it may not be possible to generate a response any stronger than moderately proliferative for such an epitope because the structure cannot be remodeled to produce a dominant epitope without wrecking the overall gp120 conformation.

In summary, presentation of dominant epitopes is partitioned to an early phase of gp120 processing, wherein the initial proteolytic nicks allow local unfolding and exposure of adjacent sequences for MHCII binding. At a later phase of gp120 processing, more extensive proteolysis exposes additional sequences for MHCII binding but also causes destruction of epitopes. Disulfide bonds can block the early phase but not the later phase of antigen processing.

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- Highly proliferative responses to dominant T-helper epitopes were selectively reduced in the absence of the lysosomal enzyme that cleaves disulfide bonds.
- Disulfide deletions restored the dominant T-helper responses.
- We concluded that the dominant T-helper epitopes were processed from a native-like conformation of the reduced intact protein.

Figure 1. Properties of HIV-1_{JR-FL} gp120 disulfide-bond deletion variants

(A) Ribbon diagrams illustrate the positions of disulfide bonds (black space-filled atoms) that were deleted by replacing paired cysteines with alanines (at the indicated residue numbers). (B) Limited proteolysis. The indicated amount of elastase, cathepsin S, trypsin, or protease K was incubated with each gp120 protein for 30 minutes at room temperature prior to analysis by SDS-PAGE. Only the portion of the gel containing intact gp120 is presented. (C) Mean binding of monoclonal antibodies. Reactions with monoclonal antibodies (mAb) for well-known epitopes on HIV gp120 were grouped as indicated. Additional antibodies included 2G12 (glycan), C11 (discontinuous N- and C-terminal segments), and 23G (C-terminal segment). Mean reactions (n=3) that were significantly different from the wild-type gp120 ($P < 0.05$) are indicated with asterisks. Error bars indicate standard error. (D) Mean binding of sCD4. (E) Chemical denaturation of HIV-1_{JR-FL} gp120 with guanidine hydrochloride (GuHCl). The fluorescence emission spectrum of tryptophan was measured for each protein in the presence of increasing concentrations of GuHCl (n = 2 or 3). The ratio of the intensity at x GuHCl to zero GuHCl was plotted for each concentration, and the data were fitted to equations that solve for the free energy of unfolding at zero GuHCl (ΔG_{uw}). (F) The ΔG_{uw} for each disulfide variant. Error bars indicate standard error.

Figure 2. Epitope profiles for C57BL/6 mice immunized with gp120 or gp120dss298
Mice received a single administration of immunogen and were sacrificed after one week. Splenocytes were restimulated by overlapping peptides that span the gp120 sequence, and proliferation was analyzed by incorporation of ^3H -thymidine. The number of mice responding to each peptide is indicated. (A) The number of responding mice. (B) Mean proliferative response as log of net radioactive counts per minute (cpm) *versus* peptide number. (C) Difference in proliferation between the gp120dss298-immunized gp120-immunized and mouse groups. (D and E) Mean proliferative responses for significantly immunogenic peptides ranked by strength of response. Two sets of peptides were distinguishable by a discontinuity in the strength of response. The set of highly proliferative peptides is composed of dominant epitopes, and the moderately proliferative set is composed of subdominant peptides (see text for criteria for dominance and subdominance). The discontinuity was identified by excluding highly proliferative peptides until the remaining (moderately proliferative) peptides could be well-fit to a line by regression ($p < 0.05$, *Runs* test). For gp120, the least proliferative peptide was also excluded. For clarity, the points for only four of the eight dominant peptides are labeled.

Figure 3. Epitope profiles of GILT-KO mice immunized with gp120 or a disulfide-bond variant
 Immunization and analysis was as described for C57BL/6 mice in the legend for Fig. 2. (A) The number of responding mice. Results for only gp120dss298 are provided as example of a disulfide-bond variant. (B) Mean proliferative response as log(net cpm) *versus* peptide number. Results for only gp120dss298 are provided as example of a variant. (C, D, E) Difference in proliferation between the variant-immunized and gp120-immunized mouse groups. (F, G, H, I) Mean proliferative responses for significantly immunogenic peptides

ranked by strength of response. Highly proliferative and moderately proliferative peptides were distinguished as described in the legend for Fig. 2.

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Figure 4. Profile of predicted affinity for the I-A^b molecule and frequency of response to gp120 peptides in C57BL/6 mice (A) and GILT-KO mice (B)
Responses are as presented in Figs. 2A and 3A, respectively. Predicted affinities were obtained from the NETMHCII server [41].

A. gp120 in C57BL/6 mice

B. gp120 in GILT-KO mice

C. gp120dss298 in GILT-KO mice

Figure 5. Models for how disulfide-bonds affect gp120 processing and abundance of peptide presentation

(A) gp120 in C57BL/6 (GILT+/+) mice. Step 1: flexible loops are proteolytically nicked. Step 2: in the high-priming compartment (broken line), loop-associated disulfide bonds are cleaved; the gp120 locally unfolds; and MHCII binds to epitopes that will be dominant and highly proliferative (filled rectangles). Step 3: in a more degradative compartment, the gp120 is extensively proteolyzed; and MHCII binds epitopes destined to be subdominant and moderately proliferative (open rectangles). (B) gp120 in GILT-KO mice. Step 1: gp120 is proteolytically nicked as in (A). Step 2: disulfide bonds are not cleaved; local unfolding is blocked; and MHCII cannot reach the normally dominant epitopes. Step 3: in a more degradative compartment, the MHCII gains access to both the normally dominant and subdominant epitopes, and both types of peptide become moderately proliferative. (C) gp120dss298 in GILT-KO mice. Step 1: gp120 is proteolytically nicked, and gp120 partially unfolds around the deleted disulfide bond. Step 2: MHCII binds to epitopes that will be dominant and highly proliferative. Step 3: in a more degradative compartment, MHCII binds to epitopes that will be subdominant and moderately proliferative.

Figure 6. The N-terminus of peptide 36 is conserved and located in the least solvent-accessible region of gp120, where its immunogenicity was limited by disulfide bonds

(A) An alignment of gp120 sequences was created from forty gp120 sequences, including those from HIV-1_{89.6}, HIV-1_{HXB2}, HIV-1_{YU2}, and HIV-1_{JR-FL}. The Shannon Sequence Entropy was calculated with BioEdit. On the right, the percent solvent accessibility was extracted from the HIV-1_{JR-FL} gp120 crystal structure (PDB ID: 2B4C) using MolMol. The arrows indicate the N-terminus of peptide 36.

Table 1

Peptides for HIV-1(JR-FL) gp120

Peptide	Sequence
2	GVPVWKEATTTLFCASDAKA
3	TLFCASDAKAYDTEVHNVWA
4	YDTEVHNVWATHACVPTDPN
5	THACVPTDPNPQEVVLENVTE
6	PQEVVLENVTEHFNMWKNNM
7	EHFNMWKNNMVEQMVEDIIS
8	VEQMVEDIISLWDQSLKPCV
9	LWDQSLKPCVKLTPLCVTLN
10	KLTPLCVTLNCKDVNATNT
11	CKDVNATNTTNDSEGT
12	TNDSEGTMERGEIKNCS
13	MERGEIKNCSFNITTSIRDE
14	FNITTSIRDEVQKEYALFYK
15	VQKEYALFYKLDVVPIDNN
16	LDVVPIDNNNTSYRLISCD
17	NTSYRLISCDTSVITQACPK
18	TSVITQACPKISFEPIPIHY
19	ISFEPIPIHYCAPAGFAILK
20	CAPAGFAILKCNDKTFNGKG
21	CNDKTFNGKGPCKNVSTVQ
22	PCKNVSTVQCTHGIRPVVST
23	THGIRPVVSTQLLLNGSLAE
24	QLLLNGSLAEDEVVIRSDNF
25	EEVVIRSDNFTNNAKTIVQ
26	TNNAKTIVQLKESVEINCT
27	LKESVEINCTRPNNNTRKSI
28	RPNNNTRKSIHIGPGAFYT
29	HIGPGAFYTTGEIIGDIRQ
30	TGEIIGDIRQAHCNISRAKW
31	AHCNISRAKWNTLQIVIK
32	NTLQIVIKLREQFENKTI
33	LREQFENKTIVFNHSSGGD
34	VFNHSSGGDPEIVMHSFNCG
35	EIVMHSFNCGGEFFYCNSTQ
36	GEFFYCNSTQLFNSTWNNNTE
37	LFNSTWNNNTEGSNNTEGNT
38	GSNNTEGNTITLPCRIKQI
39	ITLPCRIKQIINMWQEVGKA
40	INMWQEVGKAMYAPPIRGQI

Peptide	Sequence
41	MYAPPIRGQIRCSSNITGLL
42	RCSSNITGLLLTRDGGINE
43	LTRDGGINENGTEIFRPGG
44	NGTEIFRPGGGDMRDNRSE
45	GDMRDNRSELYKYKVVKIE
46	LYKYKVVKIEPLGVAPTKAK
47	PLGVAPTKAKRRVVQREK

Table 2

Peptides identified as immunodominant in C57BL/6 or GILT-KO mice.

gp120 in C57BL/6	gp120dss298 in C57BL/6	gp120 in GILT KO	gp120dss298 in GILT KO	gp120dss378 in GILT KO	gp120dss385 in GILT KO
		2 *	2		2
4	4	4	4	4	4
5	5		5	5	5
		8			
17	17		17	17	17
18	18				
19	19	19	19	19	19
27					
30	30	30	30	30	30
31	31	31	31		31
				33	
		40			
		43		43	
			45	45	
		47		47	

* Highlighted cells indicate peptides that deviate from the dominance pattern in gp120-immunized C57BL/6 mice