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Chemical cross-linking of HIV-1 Env for direct TLR7/8 ligand conjugation compromises recognition of conserved antigenic determinants

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Abstract

Covalent conjugation of immune-stimulatory compounds to protein antigens is a potential means to self-adjuvant non-replicating subunit vaccines. Previously, it was demonstrated that covalent coupling of a Toll-like receptor (TLR) ligand to the exterior HIV-1 envelope glycoprotein, gp120, enhanced its immunogenicity. However, the consequences of chemical conjugation to gp120 on broadly neutralizing antibody (bNAb) epitopes were so far not examined. Here, we conjugated a TLR7/8 ligand to lysine residues on gp120 using NHS-PEO_8-maleimide linkers and investigated if this affected Ab recognition of the CD4 binding site (CD4bs), a highly conserved target for bNAbs. We demonstrate that the recognition of the CD4bs was reduced following coupling, especially at a higher coupling ratio. These results have implications for the coupling of ligands to vaccine antigens where elicitation of humoral immune responses to specific neutralizing determinants is desired.

Keywords

HIV-1 envelope glycoprotein; cross-linker; Toll-like receptor ligand; neutralizing antibody

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INTRODUCTION

Activation of the innate immune system is critical for the induction of strong adaptive immune responses against invading pathogens. In part, this is achieved by recognition of molecules that are directly or indirectly associated with the infection by specialized cellular receptors, leading to intra- and intercellular signaling cascades that promote inflammatory responses and immune activation. A plethora of pattern recognition receptors (PRRs) were described in the past decades, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and inflammasome-associated molecules (Akira, Takeda, and Kaisho, 2001; Blasius and Beutler, 2010; Henao-Mejia et al., 2012; Jacobs and Damania, 2012; Kawai and Akira, 2010; Loo and Gale, 2011; Rathinam, Vanaja, and Fitzgerald, 2012). Of the TLRs, several sense single-stranded or double-stranded RNA or DNA to alert the host of an invading pathogen. The increased definition of PRRs and their downstream signaling pathways has encouraged the development of molecular adjuvants that target these, such as synthetic analogues of TLR ligands (reviewed in (Akira, Takeda, and Kaisho, 2001; Carter and Reed, 2010; Coffman, Sher, and Seder, 2010; Guy, 2007; Lore and Karlsson Hedestam, 2009; Pulendran, 2004)). Studies in mice and non-human primates demonstrate that TLR ligands, when used together with purified protein antigens, stimulate enhanced humoral and cellular immune responses (Huleatt et al., 2007; Jackson et al., 2004; Tewari et al., 2010; Weiner et al., 1997; Wille-Reece et al., 2005a). Dendritic cells (DCs) are the primary target cells for priming naïve T cells. However, other antigen-presenting cells such as B cells are also activated by some TLR ligands, in particular by TLR7/8/9 ligands (Lanzavecchia and Sallusto, 2007), which may influence humoral immunity. The possibility to directly conjugate TLR ligands to protein-based vaccines to augment both cellular and humoral immune responses therefore represents an attractive approach to vaccine design. However, the consequences of such chemical modifications for the integrity of antigenic sites on target proteins that may be critical for the elicitation of broadly neutralizing antibodies are currently not well defined.

Here, we examined the effects of chemically cross-linking a synthetic TLR7/8 ligand to surface-exposed lysine residues on the external subunit of the HIV-1 envelope glycoprotein (Env), gp120. We selected HIV-1 gp120 as a suitable antigen to test this concept since the antigenic properties of this antigen are well described and because alternative approaches for HIV-1 Env-based vaccine design are critically needed to elicit effective Ab responses, including bNAbs (2010; Burton et al., 2004; Karlsson Hedestam et al., 2008; Mascola and Montefiori, 2010). Of the nucleic acid-based TLR ligands, we chose a TLR7/8 ligand for these studies as both myeloid DCs (MDCs) and plasmacytoid DC (PDCs) express TLR7 or TLR8, which upon activation mediate antigen presentation and production of innate cytokines critical for enhancing T and B cell responses (Lore et al., 2003). Importantly, since B cells also express TLR7/8 (Bekeredjian-Ding et al., 2005; Douagi et al., 2009) antigens that are covalently conjugated to such ligands have the potential to activate B cells both directly and indirectly via DCs. This makes TLR7/8 ligands particularly well suited for the studies performed here. Previous studies demonstrate that direct conjugation with this TLR ligand effectively enhance immune responses to another protein-based antigen, HIV-1 Gag, as assessed in both mice and non-human primates (Wille-Reece et al., 2005a; Wille-Reece et al., 2006; Wille-Reece et al., 2005b).

In our current study, we demonstrate that it was possible to control the amount of ligand that was covalently attached to the gp120 surface lysines by varying the ratio of linker to gp120 in the coupling reaction. We found that an increased ratio of TLR7/8 ligand to gp120 led to more effective in vitro activation of PDCs to produce IFN-alpha. However, high levels of coupling abrogated the binding by several Env ligands tested, including several bNAbs recognizing the conserved primary receptor binding site (CD4bs), a critical neutralizing

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determinant and vaccine target on Env. We therefore attempted to alter this outcome by structure-guided, conservative lysine to arginine substitutions on residues proximal to the CD4 binding region of gp120. However, these structure-based alterations did not preserve recognition of these epitopes following coupling.

Collectively, the data presented in this study demonstrate a hitherto unappreciated consequence of cross-linking ligands to HIV-1 Env, highlighting the need to carefully evaluate B cell recognition of conformational epitopes on antigens modified by this approach before attempting to elicit effective Ab responses *in vivo*.

MATERIALS AND METHODS

Recombinant gp120 production and site-directed mutagenesis

Adhesive 293F cells (ATCC), in the presence or absence of 20 g/L swainsonine, were transiently transfected, using 293 Fectin™, with a pcDNA3.1 plasmid encoding gp120 according to the manufacturer's instructions (Invitrogen, Life Sciences). After four days, the cell media was harvested, cleared of cells by centrifugation and filtered through a 0.2 m filter. Recombinant gp120 was then purified by 17b-affinity chromatography, concentrated with an Amicon spin column and dialysed against a buffer containing PBS, 0.3M NaCl and 5mM EDTA at pH 7.1. Non-conjugated and conjugated gp120 were deglycosylated with PNGase (New England BioLabs) at 37°C. In some experiments we used GnTI-293S cells (ATCC) for production of gp120 containing Man5 N-linked glycans. To eliminate the reaction of cross-linker with lysine residues proximal to or within the composite footprint of the CD4bs mAbs and CD4 itself, 5 lysine (K) codons at positions 121, 282, 357, 421 and 432, respectively were altered to Arginine (R) codons by QuikChange Mutagenesis (Stratagene). The gp1205K/R mutant was expressed and purified as described above for subsequent analysis.

Covalent coupling of TLR7/8 ligand to YU2 gp120

The imidazoquinoline compound (3M-012) referred to as the TLR7/8 ligand modified to contain an SH group was provided by 3M Pharmaceuticals. The contaminating endotoxin levels were 0.125 EU/ml in the TLR ligand preparations as measured using a Limulus Amebocyte Lysate assay, in which 10 EU equals approximately 1 ng endotoxin. The TLR7/8 ligand was covalently linked to gp120 in PBS containing 0.3M NaCl and 5mM EDTA at pH 7.1 in a two-step process. First, purified gp120 was reacted with a DMSO-dissolved cross-linker at various molecular ratios for 1 hour. The linker contains a primary amine-reactive N-hydroxysuccinimide ester (NHS) group, which reacts with surface-exposed lysines and the N-terminus on gp120, a polyethylene oxide$_8$ (PEO_8 = polyethylene glycol) and a thiol-reactive maleimide group (Pierce). Excess linker was removed by a PD-10 column (GE-Healthcare). The maleimide group of the gp120-PEO_8-maleimide intermediate was then reacted with the thiol group on the TLR7/8 ligand, at a 4-fold molar excess, for 1 hour. To quench the reaction, 100 mM free L-Cys was added and the gp120-TLR7/8 conjugates were purified with a superdex200 10/500GL gel filtration column using an ÄKTA system. Aliquots of the intermediate gp120-linker product were retained for analysis. In some experiments, we coupled gp120 using a PEO_2 cross-linker by similar methods (not shown).

Quantification of cross-linker attachment to gp120

The migration pattern of the different known MW bands of a PreSieve® protein ladder (Lonz Rockland, Inc.) on an reducing SDS-page gel was used to approximate an equation describing the log MW of a protein as a function of its relative migration distance (R_f) by using linear curve-fit in Prism 5 (y=- 0.8803ln(x)+2.411, r^2=0,97). Since gp120 is a

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relatively large, heavily glycosylated protein, with N-linked carbohydrate comprising roughly 50% of its molecular mass, we reasoned that deglycosylated gp120 might enable us to better estimate the apparent MW. The non-conjugated and conjugated gp120 were treated with PNGase F (New England Biolabs, Inc.) according to the manufacturer's instructions. In brief, samples were denatured in the Glycoprotein Denaturing Buffer at 100°C for 10 minutes. Deglycosylation was then performed by incubating the protein in Reaction Buffer at 37°C for 1 hour in the presence of PNGase F. The amount of linker attached to each gp120 under different conditions was estimated by subjecting deglycosylated gp120-$(PEO_8)_X$ to gel electrophoresis under reducing conditions. The PNGase F protein band in each lane on the gel also served as an internal reference to normalize the migration of the gp120 in different lanes. Each covalently attached PEO_8 accounts for an approximate 0.69 kDa increase in MW when compared to non-conjugated gp120. From the increase in kDa, the approximate cross-linker stoichiometry per gp120 was determined.

Env antigenicity analysis by ELISA

The following antibodies were kindly obtained from James Robinson (17b), Susan Zolla-Pazner (447-52D), Marshal Posner (F105), Dennis Burton (b6 and b12), Herman Katinger (2G12), Davide Corte (HJ16) and HIVIG (the NIH AIDS Reagent Program). High-protein-binding MaxiSorp plates (Nunc) were coated with 200 ng/well of lentil lectin in 100ul PBS at 4°C overnight and then blocked with PBS-2% fat-free milk for 2 hours at room temperature. After washing by PBS, 200 ng/well of gp120 or gp120-$(PEO_8)_X$ was added into plate and incubated for 1 hour. The plate was then probed with mAbs VRC01, PGV04, HJ16, b6, 447-52D, 2G12, b12, F105, 17b (+/- soluble CD4, sCD4) or CD4-Ig for 1 hour and subsequently washed in PBS, containing 0.05% Tween. Addition of HRP-conjugated anti-human-Ig (Jackson) followed by the colorimetric peroxide enzyme immunoassay substrate (3,3′,5,5′- tetramethylbenzidine; Bio-Rad) was used to induce a colorimetric change, based on the level of mAb:gp120 complexes in each well, and the reaction was stopped by adding 1 M H_2SO_4. OD was read at 450 nm.

Isolation and stimulation of primary human dendritic cell subsets

This study was approved by the Institutional Review Boards at the NIH and Karolinska Institutet. Our sorting procedures for direct isolation of subsets of DCs from peripheral blood mononuclear cells (PBMCs) have been described previously (Douagi et al., 2009; Lore et al., 2003). Briefly, PDCs and MDCs were isolated from elutriated monocytes using the BDCA-4 and the CD1c DC isolation kits, respectively (Miltenyi Biotec). Following this purification, the subsets of DCs were highly enriched (on average >90% PDCs and >80% MDCs) as determined by staining for the Lineage markers CD3, CD14, CD15, CD20 and CD56 plus HLA-DR, CD123 and CD11c. DCs were cultured in complete medium (RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supplemented with 2 mmol/l L-glutamine, 1% streptomycin and penicillin, 10% FCS (Invitrogen, Carlsbad, CA). To maintain viability, the media for PDCs and MDCs were supplemented with the recombinant human cytokines IL-3 (1 ng/ml, R&D Systems, Minneapolis, MN) and GM-CSF (2 ng/ml, PeproTech Inc, Rocky Hill, NJ), respectively. The sorted DC populations were cultured at 1×10^6 cells/ml at 37°C in polystyrene round-bottom tubes (Becton Dickinson Labware, Franklin Lakes, NJ). The DCs were stimulated with unconjugated gp120, gp120-Lo and gp120-Hi (10, 25 or 50 g/ml) as indicated for 24 hrs and the supernatants were analysed for secretion of IFN-alpha and IL-12 (p40 and p70) ELISA. Both ELISA kits were purchased from (Biosource International, Camarillo, CA) and the assays were performed according to the manufacturer's instructions.

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Binding kinetics by Bio-Layer Interferometry

Experiments were performed on the Octet-Red® device (FortéBio, Inc., Menlo Park, CA). Buffer or serially diluted samples of gp120 or conjugates were dispensed into 96-well microtiter plates at a volume of 200 μL per well. Operating temperature was maintained at 30 °C. Anti-Human IgG Fc biosensors (FortéBio, Inc., Menlo Park, CA) were used to capture VRC01, F105 or 447-52D antibodies which were then dipped into solutions of the gp120 or conjugates at selected concentrations with agitation for 6 min at 1,000 rpm to measure association constants. Dissociation was detected following transfer of sensors into wells containing buffer alone for 6 min. Data were collected and analyzed by the Octet® User Software (version 6.4). For data analysis, different concentration ranges were used: 800 nM, 200 nM, 50 nM and 13 nM for gp120-Hi binding with VRC01 and F105, gp120-Lo binding with VRC01 and F105; 800 nM, 200 nM and 50 nM for gp120-Hi binding with 447-52D; 200 nM, 50 nM, 13 nM and 3 nM for gp120-Lo binding with 447-52D and gp120 binding with VRC01; 50 nM, 13 nM, 3 nM and 0.78 nM for gp120 binding with F105 and 447-52D.

RESULTS

Cross-linking and biochemical characterization of HIV-1 gp120:cross-linker and TLR7/8 ligand conjugates

In the present study, we used heterobi-functional NHS-PEO$_8$-maleimide linkers to conjugate a TLR7/8 ligand to purified, monomeric gp120 derived from the HIV-1 strain, YU2. We attempted as well cross-linker modification of soluble YU2 gp140 trimers, however this treatment resulted in very high molecular weight oligomers, which we deemed unsuitable immunogens (not shown). For gp120, we adjusted the reaction conditions so that different coupling ratios of cross-linker or TLR ligand to gp120 were obtained and we investigated the biochemical and biological consequence of these different coupling conditions. YU2 gp120 contains 30 lysines on its molecular surface with 20 of these charged residues located in the gp120 core-region (Fig. 1A), 5 residues in variable regions 1, 2 and 3 (V1V2V3) and 5 additional in the N and C-terminal conserved regions (not shown). The primary amines of each solvent-exposed lysine residue (and the N-terminus) are potentially highly reactive with N–hydroxysuccinimide (NHS) esters, leading to the formation of an amide bond. Use of NHS esters is a common method to form covalent bonds between a protein and a small molecule of choice, either directly or indirectly via a NHS-containing linker intermediate. To covalently link a sulfhydryl-containing TLR7/8 ligand to gp120, we first produced gp120-maleimide intermediates by reacting gp120 with different molar ratios of an amine-to-sulfhydryl linker (NHS-PEO$_8$-maleimide) (Fig. 1B). The resulting gp120-maleimide intermediate was then incubated with an excess of sulfhydryl-containing TLR7/8 ligand to allow cross linking of the active maleimide with the reactive sulfhydryl on the ligand (Fig. 1B).

To investigate the approximate coupling stoichiometries achieved following conjugation of the cross-linker and the TLR7/8 ligand to gp120, we performed analysis by SDS-PAGE and size exclusion chromatography. Accordingly, we first examined the apparent molecular weight (MW) of gp120 and the cross-linked gp120 conjugates on reducing gels. Since gp120 is a relatively large, heavily glycosylated protein, with N-linked carbohydrate comprising roughly 50% of its molecular mass, we performed gel analysis on both untreated and deglycosylated gp120 (Fig. 2A, left panel). We reasoned that the lower MW bands following deglycosylation of gp120 might enable us to better estimate the average cross-linker conjugation levels per gp120 molecule. To generate deglycosylated gp120, we first expressed the protein from 293 cells either in the presence of swainsonine, or from GnTI-293S cells. Both these treatments of the cells theoretically render all N-linked glycans on the

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recombinant gp120 as forms of high mannose and thereby sensitive to both Endo H and PNGase digestion. For the MW estimates, we were unable to fully deglycosylate full-length gp120 by the relatively mild Endo H reaction conditions (not shown) so we performed deglycosylation with PNGase under denaturing conditions. Following PNGase digestion, we estimated the MW of untreated and cross-linked gp120 using the center of the Coomassie-blue stained glycoprotein bands on the gel. A representative analysis of three independent digestions and gel analyses is shown in Fig 2A. Based upon the protein MW standards, we derived a graph plotted from the log MW versus relative migration distance of the gp120 bands [Rf = (protein migration)/(migration of the 38 kDa band from the MW standards)] (Fig 2B). The approximate apparent MW of the PNGase-digested gp120 and the gp120-maleimide intermediates was calculated by interpolation using this graph. By averaging values derived from the three independent gel runs, the difference in MW between unconjugated gp120, and the gp120-maleimide intermediates at different ratios of 1:5, 1:20 and 1:120, was determined to be 9.0, 9.7 and 14.5 kDa, respectively, corresponding to the coupling of 13, 14 and 21 NHS-PEO8-maleimide linkers (MW = 0.69 kDa) to each molecule of gp120, on average (Fig 2B). We observed that, although untreated gp120 was reduced to a tight protein band consistent with full deglycosylation by PNGase, the bands of the cross-linked gp120 were broader. This is likely due to heterogeneity of cross-linker coupling to the population of gp120 molecules in the reaction mixture. Consistent with this interpretation, when estimating the number of cross-linker molecules per gp120 using both the front and trailing edge of the protein:adduct bands on the gel shown in Fig 2B, we derived ranges that did not exceed the limit of 30 conjugated lysines per gp120, even at the highest cross-linker concentration. For the NHS-PEO8-maleimide linkers, the value were 13 (range 5-19), 14 (range 5-19), and 21 (13-27) crosslinkers per gp120, respectively.

Besides the predominant monomeric TLR-ligand-gp120 species, we also observed a band with a higher MW in the gel lanes loaded with gp120-maleimide moieties, which likely corresponds to covalently attached dimers of gp120. For our subsequent analysis of the biochemical and *in vitro* biological properties of gp120-TLR7/8 conjugates, we chose to use the conjugates defined by the gel analysis containing approximately 14 linkers per gp120 (designated gp120-Lo) and 21 linkers per gp120 (designated gp120-Hi). Because of the heterogeneity of the gp120:cross-linker reaction products, and because previously we had observed that during crystallization trials deglycosylation of gp120 by PNGase would result in aggregation to higher molecular weight species (not shown), we did not to attempt confirmation of the gp120:cross-linker stoichiometry by mass spectroscopy.

To achieve a homologous population of monomers for the biological analysis, and to remove unreacted TLR7/8 ligand, we subjected the reaction products to size exclusion chromatography (SEC). As expected, gp120, gp120-Lo and gp120-Hi were resolved by SEC in a manner consistent with their relative MW estimated in parallel by the SDS-PAGE analysis (Fig. 2 and not shown). Pure monomers were isolated by excluding the first 25% of the elution peak for each ligand-protein conjugate. Additional peaks were detected after resolution of the main gp120-conjugate peaks. Since, such peaks were not detected when unconjugated gp120 was subjected to the same SEC analysis it is likely that the peaks were not contaminants in the gp120 preparation and represented elution of non-gp120-bound forms of the TLR7/8 ligand in different aggregation states. After subsequent buffer exchange by dialysis into PBS, using a 30 kDa cut-off to remove potential trace amounts of free TLR7/8 ligand, we confirmed the homogeneity of gp120, gp120-Lo and gp120-Hi by gel electrophoretic analysis (Fig. 2D).

In vitro stimulation of primary human DC subsets with the gp120-TLR7/8 conjugates

To analyze the biological activity of the gp120-TLR7/8 Hi and Lo conjugates on primary human DCs, we used a sorting procedure for isolation of $CD11c^+$ MDCs and $CD123^+$

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PDCs, isolated directly from blood. This protocol results in highly pure populations of viable MDCs and PDCs (Fig. 3A). We have previously characterized the stimulatory effects of TLR ligands on these DC subsets as well as their capacity to act as antigen-presenting cells (Lore et al., 2003). These and other studies show that MDCs produce IL-12 in response to TLR3 and TLR7/8 ligation and are potent antigen-presenting cells, while PDCs produce high levels of IFN-alpha in response to TLR9 and TLR7/8 stimulation, which in turn can impact B cell activation and differentiation in co-culture systems (Bekeredjian-Ding et al., 2005; Douagi et al., 2009), but are less efficient as antigen-presenting cells.

Here, we stimulated MDCs and PDCs for 24 hours with unconjugated gp120, gp120-Lo or gp120-Hi. Secreted IL-12 and IFN-alpha were analyzed by ELISA from MDC or PDC supernatants respectively. We found that incubation of MDCs with either gp120-Lo or gp120-Hi resulted in IL-12 production with an increase of approximately 3-fold for gp120-Lo and 2-fold for gp120-Hi when the concentration of protein was raised from 10 μg/ml to 25 μg/ml (Fig. 3B). No detectable IL-12 was measured from MDC cultures stimulated with the unconjugated gp120 control protein. In contrast, for PDCs, only the gp120-Hi conjugate, at concentrations of 25 or 50 μg/ml induced IFN-alpha at detectable levels. From these *in vitro* data, we conclude that the more biologically active conjugate was the gp120-Hi TLR7/8 derivative which thereby became the more critical conjugate to evaluate by further analysis.

Antigenic characterization of conjugate proteins

A critical event to activate the humoral immune response by vaccine antigens is the recognition of surface exposed antigenic surfaces by the naïve B cell receptor (BCR). This subsequently leads to the maturation of the humoral immune response and the elicitation of high-affinity antibodies against all immunogenic sites on the antigen. While the covalent attachment of TLR ligands to an antigen may lead to a more effective activation of the innate immune response through TLRs expressed by immune cells, such coupling may adversely alter the antigenic surface of the target protein. The alteration of the antigenic surface could either activate B cells bearing BCRs not capable of binding to the wild-type antigen or misdirect B cell affinity maturation to an altered epitope, thereby ablating of B cell responses directed toward neutralizing Ab epitopes. Therefore we sought to determine whether the initial coupling reaction with the NHS-maleimide linker interfered with the recognition of important antigenic surfaces on gp120.

We initially assessed the antigenic preservation of the highly conserved CD4bs and the co-receptor binding site (CoRbs) of Env using antibody probes. The naïve BCR generally has a relatively low affinity (μM) for its cognate binding surface, in contrast to a mature Ab, which can possess extremely high affinity for the same antigenic surface. To investigate Ab recognition of the CD4 binding region of gp120, we probed this surface with the high-affinity, broadly neutralizing CD4bs-directed monoclonal Abs (mAbs) VRC01 and PGV04 (Fig. 4). We also included the less broadly neutralizing CD4bs-directed mAbs, b12, HJ16, the non non-broad F105 and b6 mAbs, as well as the co-receptor binding site (coRbs)-directed mAb 17b with or without pre-incubation with soluble CD4 (sCD4). This analysis demonstrated that conjugation affected the recognition of gp120 by both broad and non-broad CD4bs-directed mAbs, as well as of the CoRbs-directed 17b mAb. These effects were especially pronounced at the higher coupling ratio (Fig. 4). For example, the 50% binding concentration (BC_{50}) of VRC01 to wt gp120 was 0.0094 μg/ml while binding at the low conjugation concentration (gp120-Lo) was 0.0351μg/ml and at the high concentration (gp120-Hi) 0.143 μg/ml (Supplementary Table. S1). The effect of the conjugation was not global as binding of 447-52D, an Ab specific for the third major variable region (V3) and 2G12, an Ab specific for a highly conserved cluster of high mannose glycans on the outer domain of gp120, were not affected. Furthermore, neither of these structurally defined Abs

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possess a lysine residue in their epitope. Collectively this analysis demonstrates that covalent coupling of ligands to gp120 using the approach described here severely compromises the recognition of important epitopes by the humoral immune system.

Structure guided lysine-to-arginine substitution proximal to the CD4 binding site

Due to the loss of recognition of the CD4 binding site by the broadly neutralizing mAbs, we inspected the gp120 molecular surface from the available gp120 core crystal structure to perform conservative substitutions of CD4bs-proximal lysine (K) residues to arginine (R). As shown in Fig. 5A, we selected 5 K residues most proximal to the composite footprint of the CD4bs broadly neutralizing ligands and CD4 itself and introduced substitutions to R (termed gp120 K/R). These conservative substitutions would render these residues non-reactive with the cross linker and also should not alter the electrostatic surface of gp120 as determined by modeling (Fig. 5B). Therefore, electrostatic changes should not be a factor altering the protein biochemically. Following production and purification of the 5Kgp120R variant, we performed the lysine-specific reactions again using the NHS-maleimide linker adduct at the low and high conditions. Following conjugation, we performed ELISAs as before with the set of six mAbs. As shown in Fig. 5C, even with the 5 K to R substitutions there was still a modest loss of recognition by the CD4bs mAbs and CD4-Ig under the 20:1 linker:gp120 reaction conditions and a greater loss of recognition under the higher 120:1 linker: gp120 reaction conditions. We also performed conjugation with a cross-linker possessing a shorter spacer (PEO_2) to determine if perhaps steric occlusion by the longer linker was impeding mAb recognition of the CD4bs. However, as for the longer linker, conjugation of the shorter linker to gp120 also reduced recognition of all antibodies specific for the CD4bs.

Binding kinetics of antibodies to gp120-conjugates following coupling

Since we saw decreases in recognition of the gp120-conjugates by ELISA relative to unmodified gp120, we sought to determine binding kinetics to determine one-to-one changes in affinity rather than the avidity changes determined by ELISA using bivalent IgG. Therefore, we attached selected antibodies to the solid phase and determined kinetics to monomeric gp120 and to cross-linker conjugated gp120 in solution by Bio-Layer Interferometry (ForteBio). As shown in Fig 6, on-rates of all antibodies were slightly reduced at the Lo conjugation conditions. By this more direct assessment of affinity, effects on the on-rate, even for 447-52D at the Lo conditions were detectable. The 447-52D mAb recognizes a continuous epitope in the V3 region of gp120. However, at the Hi conditions, the on-rates of all the antibodies were affected. Most critically, for the broadly neutralizing CD4bs there was almost no detectable binding for CD4bs-directed mAbs VRC01 and F105. The off-rate of 447-52D was not greatly affected under either of the conjugation conditions, implying that for this Ab and epitope, the results are consistent with steric occlusion caused by the cross-linker. This occlusion apparently is manifest with less than full occupancy of the surface lysines, which are not directly part of the 447-52D epitope. For the CD4bs-directed Abs, the data do not clearly distinguish between steric occlusion imposed by the cross-linker coupling or by more direct effects on the conformational CD4bs epitopes recognized by VRC01 and F105.

DISCUSSION

TLR ligands are small bioactive molecules known to act as immune-enhancers and/or immune-modulators in a variety of contexts. The *in vivo* effect of co-administering TLR ligands and protein antigens in the absence of conjugation or formulation may be limited due to diffusion of the small agonists from the site of injection (Wille-Reece et al., 2006). However, addition of TLR ligands to vaccines containing MF59 or Alum, two adjuvants in

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clinical use, markedly affects the quality and sometimes the magnitude of the induced response (Baldwin et al., 2012; Cooper et al., 2004a; Cooper et al., 2004b; Crompton et al., 2009; Mullen et al., 2006; Wack et al., 2008). In some settings the inclusion of a TLR ligand in the formulation skews the response toward a Th1-type of response or shifts it toward a balanced Th1/Th2 type of response, which may be more desirable than a predominant Th2-biased response such as that elicited by alum alone. The more balanced response afforded by inclusion of TLR ligands may increase the effectiveness of vaccine-induced immune responses against many pathogens, including HIV-1 (Baldwin et al., 2012; Darrah et al., 2007; Lousada-Dietrich et al., 2011; Pattacini et al., 2012).

To evaluate an alternative approach to harness the potent biological activity of TLR ligands, we directly coupled a TLR7/8 ligand to gp120 by targeting the secondary amines typically found on solvent exposed lysine residues. The possibility to covalently couple TLR ligands to protein antigens is advantageous in that it diminishes systemic dissemination of the ligand upon inoculation, which might cause bystander effects. Covalent coupling also greatly increases the likelihood that the antigen and adjuvant are taken up by the same antigen-presenting cell, which may enhance antigen presentation to CD4+ T cells (Blander and Medzhitov, 2006). An early study evaluated the effect of direct conjugation of the TLR9 ligand to HIV-1 gp120 and found that coupling resulted in improved immunogenicity of gp120, however a detailed examination of the antigenic properties of the gp120-CpG conjugates was not performed in that study (Tighe et al., 2000). Here, we used a TLR7/8 ligand for covalent coupling to gp120 as, in addition to stimulating MDCs and PDCs, TLR7/8 ligands are potent polyclonal B cell stimulators (Douagi et al., 2009; Wille-Reece et al., 2005a).

Chemical conjugation of TLR7/8 or TLR9 ligands to HIV-1 Gag, a T cell antigen, was previously shown to increase the immunogenicity of this protein antigen (Wille-Reece et al., 2005a; Wille-Reece et al., 2005b). Subsequent studies showed that conjugation to the antigen was also critical for effective crosspresentation to CD8+ T cells (Oh and Kedl, 2010). However, a potential draw-back when coupling ligands to proteins is that the antigenic surface of the protein may be altered, either by direct disruption of specific B cell epitopes, or by steric effects imposed by the hetero-bifunctional cross-linkers used for coupling. Coupling, as performed here, is often done by lysine-directed chemistry. Furthermore, depending on the biochemical properties of the protein and the coupling chemistry used, the process may result in protein aggregation and subsequent disruption of epitope recognition (Kastenmuller et al., 2011). Aggregation may be less critical for immunogens aimed to elicit potent T cell responses, and might even enhance processing and cross-presentation of peptides to CD8+ T cells, but for B cell immunogens aimed to elicit bNAbs epitopes aggregation is best avoided. For gp120 this is especially pertinent as many broadly neutralizing anti-gp120 Abs recognize relatively poorly exposed, conformation-sensitive epitopes, which contain lysines near or within their binding footprint. Thus, preservation of the integrity of these epitopes is likely critical for the elicitation and antigen drive of CD4bs-directed B cell responses that would affinity mature to this region and be capable of neutralizing HIV-1. However, if this region is antigenically altered, as we demonstrate here, then maturation of the antibody response will be to a surfaces not present on the wild type gp120 present on the virus, thereby reducing the likelihood of eliciting neutralizing CD4bs-directed antibodies.

Here, we show that the effective coupling of the TLR7/8 ligand to the heavily glycosylated gp120 was achievable, rendering it more immune activating compared to unconjugated gp120 when assessed on primary human DCs, in vitro. By our estimates of cross-linker units coupled to lysines on gp120 we observed that the coupling process did not go to completion, especially under the lower ratios of cross-linker to gp120. Even at the highest ratio, which

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was in vast molar excess of cross-linker to gp120, approximately two thirds of the surface lysines were conjugated. This relative inefficiency reflects the challenges of subjecting a complex carbohydrate-laden glycoprotein such as gp120 to this coupling approach.

In the studies presented here, we attached a heterobifunctional cross-linker to gp120 surface lysines and we estimated cross-linker occupancy on the heavily glycosylated gp120 by a combination of PNGase deglycosylation and SDS gel analysis. We had observed previously during crystallization trials that the use of PNGase to deglycosylate gp120 resulted in aggregation of gp120 to higher molecular weight species. We therefore did not attempt to determine gp120:cross-linker stoichiometry by mass spectroscopy of the PNGase treated products, but instead relied on the analysis of MW shifts as determined by the gel analysis. We found that the cross-linker conjugation process severely affected recognition by several bNAbs, illustrating the potential limits of this direct coupling approach for the generation of effective B cell immunogens.

For the CD4bs mAbs, not all footprints are structurally defined, but for those that are, proximity of lysines to this critical neutralizing determinant was a concern for linker-mediated ligand coupling. Indeed, following the cross-linker conjugation procedure, reduction in recognition of the CD4bs was observed for most antibodies directed to this region. Although some of reductions in affinity resulted in reasonable affinities of the mature CD4bs mAbs to gp120, decreases in affinity are indicative of an altered CD4bs or partial steric occlusion of this region. Alterations in optimal B cell recognition will alter affinity maturation pathways, and are likely to impact on the elicitation of optimal antibodies to the CD4bs due to the cross-linking. Effects on antibody binding to gp120 by addition of multiple cross-linkers may not be so surprising, as the cross-linkers are 40 Å in length and core gp120 is only 100 Å across in several orientations. So although the cross-linkers do not greatly increase the overall mass of gp120, their presence, along with the abundant N-linked glycans on the surface of gp120, may occlude multiple antigenic sites.

Due to the effects of the cross-linker on antibody recognition at the CD4bs, we performed conservative arginine shifts to eliminate coupling to lysines proximal to this site. However, this did not circumvent loss of antigenic recognition by CD4bs-directed ligands or by the CoRbs-directed ligand, 17b. The coupling-dependent decrease in recognition by 17b under all conditions was consistent with its structurally defined footprint, which is known to contain several unmodified lysine residues. That arginine substitution did not alter loss of recognition by the CD4bs-directed bNabs following coupling was somewhat unexpected. This result might be due to distal conformational effects caused by the cross-linker or by steric interference with recognition of the CD4bs. That CD4 requires a conformational change for high affinity binding and that CD4-Ig was markedly affected following cross-linker coupling suggests that the former explanation might partially account for the observed effects. To assess the latter possibility, we also performed coupling using a cross-linker (PEO_2) possessing a shorter backbone linker length, but we found that the shorter linker also altered recognition of ligands to the CD4 binding site (not shown). The binding kinetics indicated steric occlusion might be responsible for the observed decrease in recognition by the V3-directed antibody 447-52D, but this analysis was less definitive in regards to effects on recognition by the CD4bs-directed mAbs. These data suggest that other direct or indirect linker strategies might be considered when coupling TLR ligands to proteins possessing critical and conformational immune determinants.

In sum, we show efficient TLR ligand conjugation to gp120 that enhanced the capacity of the antigen to stimulate primary DC subsets resulted in a marked loss of Ab recognition of broadly reactive B cell epitopes on HIV-1 Env. These results reveal a limitation to this

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conjugation approach, which should be taken into consideration in future Env vaccine design efforts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A TLR7/8 ligand was efficiently coupled to the gp120 surface by lysine chemistry
- The gp120-TLR ligand conjugate activated innate immune dendritic cells in vitro
- Cross-linker coupling affected antibody binding at the gp120 receptor binding site

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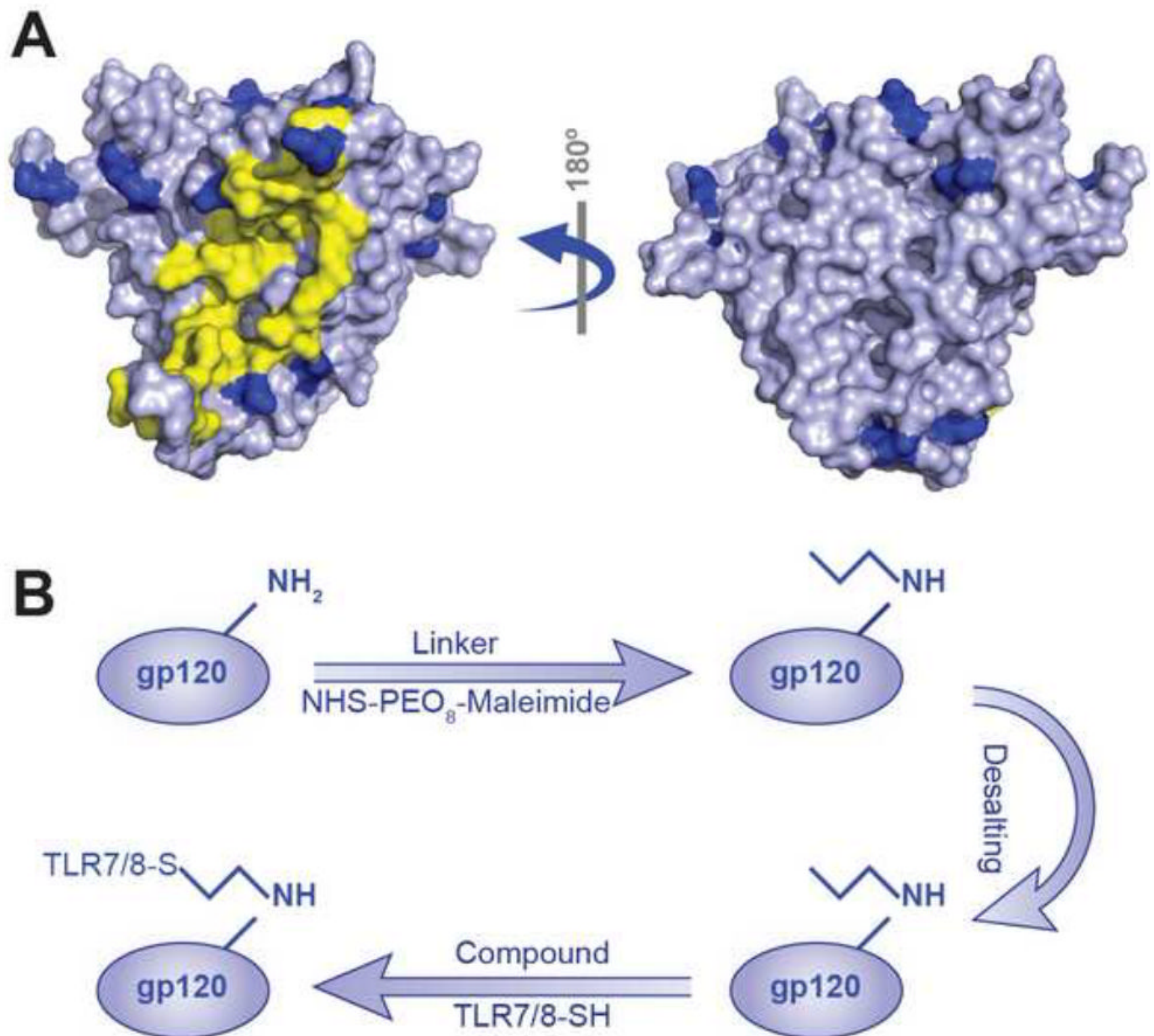

Figure 1. Molecular surface of the gp120 core and schematic of the coupling reaction

A. The gp120 surface in light blue with solvent accessible lysine residues in dark blue. Left, the primary receptor CD4 binding site is in yellow and is also the target for broadly neutralizing antibodies. Right, the surface of the core rotated 180 degrees from the CD4 binding site orientation. B. The *N*-hydroxysuccinimde (NHS) ester of the NHS-PEO_8 maleimide linker reacts with primary amine side chain of the lysines residues on the gp120 surface and the N-terminus. Excess linker is removed by a desalting column. The maleimide group on the gp120-linker intermediate reacts with a thiol group present on the TLR7/8 ligand, which then achieves covalent attachment of the TLR ligand to gp120.

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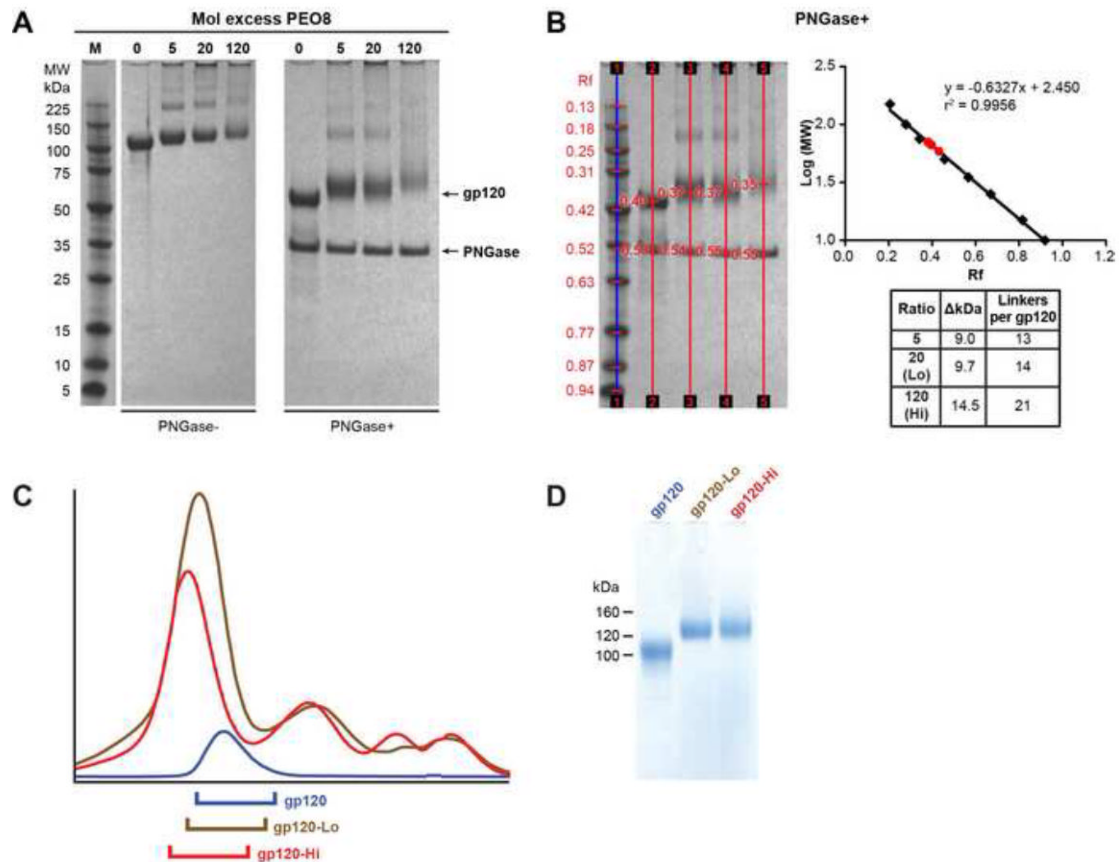

Figure 2. Analysis of gp120-linker intermediates and gp120-TLR7/8 conjugates at different coupling ratios

A. YU2 gp120, produced in 293S cells was reacted with different molar ratios of NHS-PEO8-maleimide linker. MW standards are shown (left panel). The gp120:cross-linker intermediates were resolved by SDS-PAGE before (middle panel) or after deglycosylation with PNGase (right panel). B. Protein standards were used to estimate MW shifts based upon migration distance in the gel. On this gel image, the measurements from the origin of the wells to the center of each band are shown in red using the gel analysis software Quantity One® (Bio-Rad Laboratories, Inc). Right top, the equation of the calibration curve generated by plotting log MW versus relative migration distances (Rf) of the standards in the gel is shown. Red dots indicate the gp120 values with or without cross-linker plotted onto the curve. The change in apparent MW generated by each reaction condition was determined using the calibration curve. Right bottom, approximate apparent MW shift differentials and cross-linker-to-gp120 stoichiometries are shown. C. Following conjugation, the proteins were purified by SEC. The FPLC profiles of gp120 (blue), gp120-Lo (brown, gp120-TLR7/8 at coupling ratio 1:20) and gp120-Hi (red, gp120-TLR7/8 at coupling ratio 1:120) are shown. D. By collecting and concentrating approximately 75% of the latter portion of the gp120-containing fractions, we could exclude the majority of gp120 dimers, as can be seen on the post-FPLC gel image.

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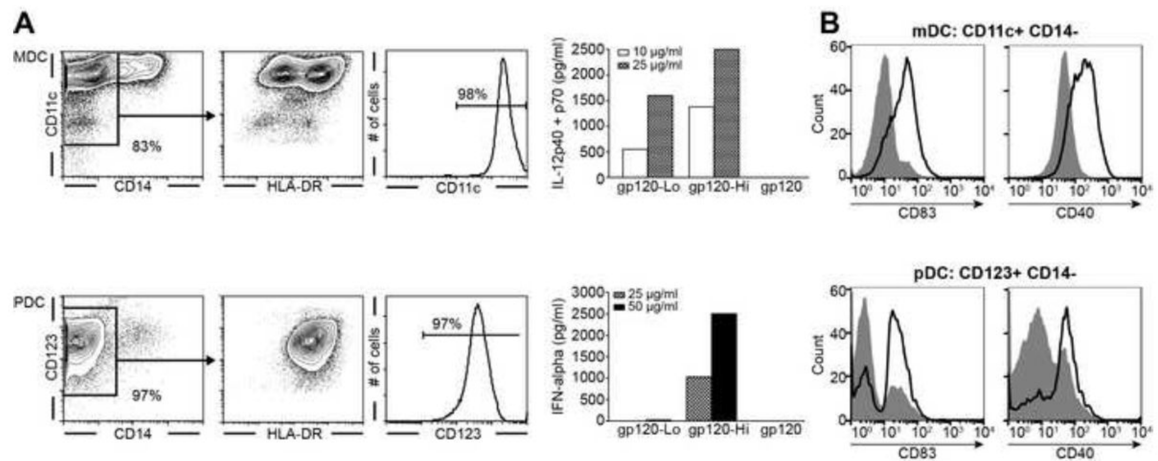

Figure 3. Purity of human PDCs and MDCs and*in vitro*stimulation with gp120 conjugates

A. Primary DCs were isolated from elutriated monocytes by immunomagnetic bead positive selection (AutoMacs, Miltenyi Biotec) using BDCA-4 followed by CD1c DC isolation kits (Miltenyi Biotec). The purities of the populations were 98 and 97% for MDCs and pDCs, respectively. PDCs and MDCs cultured at 1×10^6 cells/ml in 0.2ml in round bottom tubes were stimulated for 24 hours with gp120, gp120-Lo or gp120-Hi. Secreted IL-12p40+p70 and IFN-alpha from stimulated MDCs and PDCs, respectively, were measured in the supernatants by ELISA. Representative data from one of three donors are shown. B. MDCs and PDCs were stimulated for 24 hours with 25 g/ml gp120-Hi (black, unfilled histogram) and the surface expression of CD83 and CD40 was compared to that in unstimulated control cultures (gray, filled histograms).

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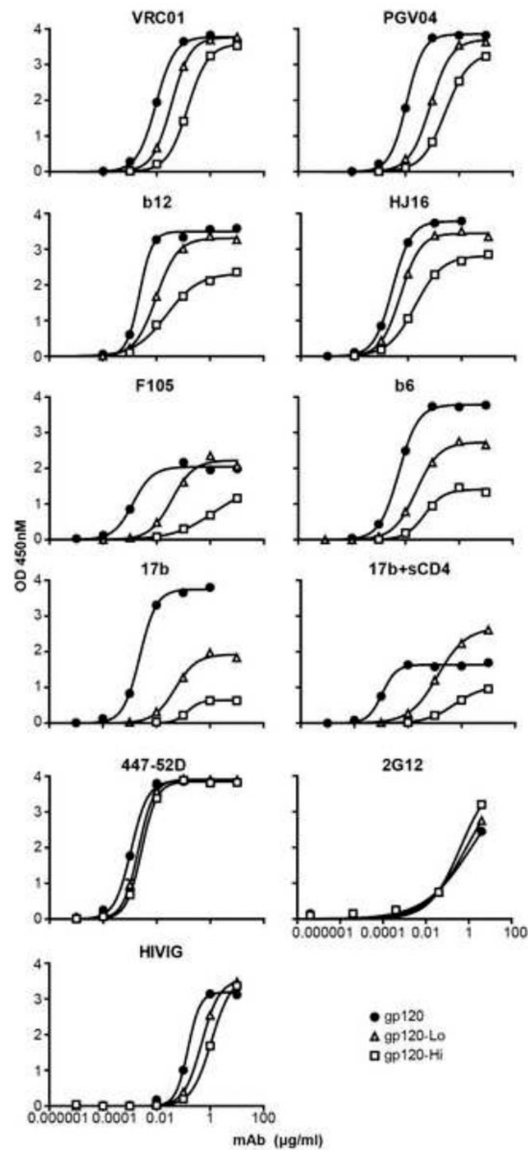

Figure 4. The antigenic surface of gp120 probed with monoclonal antibodies to the receptor binding sites before and after coupling with cross-linker

Binding curves for the broadly neutralizing CD4bs-directed mAbs VRC01, PGV04 and the less broad b12, HJ16, b6 and F105 CD4bs-directed antibodies. 17b is directed against the CoRbs. The V3-specific 447-52D, glycan-specific 2G12 mAbs, which do not possess lysines in their epitopes, and HIVIG, consisting of pooled polyclonal sera from HIV-infected individuals, served as controls to ensure that similar amounts of protein were analyzed by ELISA before and after cross-linking. The results are representative of two independent experiments.

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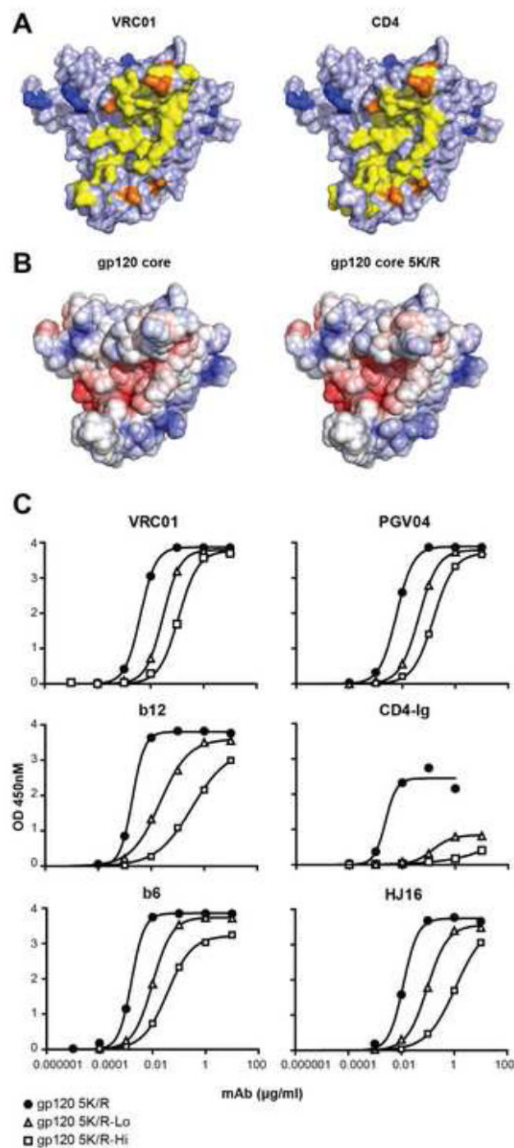

Figure 5. The gp120 molecular surface after modification of selected lysines to arginines and antigenic analysis of the conjugates

A. The gp120 core is shown with the lysines near the VRC01 epitope (left, yellow) or CD4bs (right, yellow) highlighted in burnt orange. These five lysine residues were altered to arginine (gp120 core 5K/R) by genetic means. These conservative changes did not affect the electrostatic surface as shown in B. C. Recognition by a subset of the CD4bs-directed mAbs and CD4-Ig by ELISA of unmodified gp120 and the gp1205K/R variant at Hi and Lo coupling ratios.

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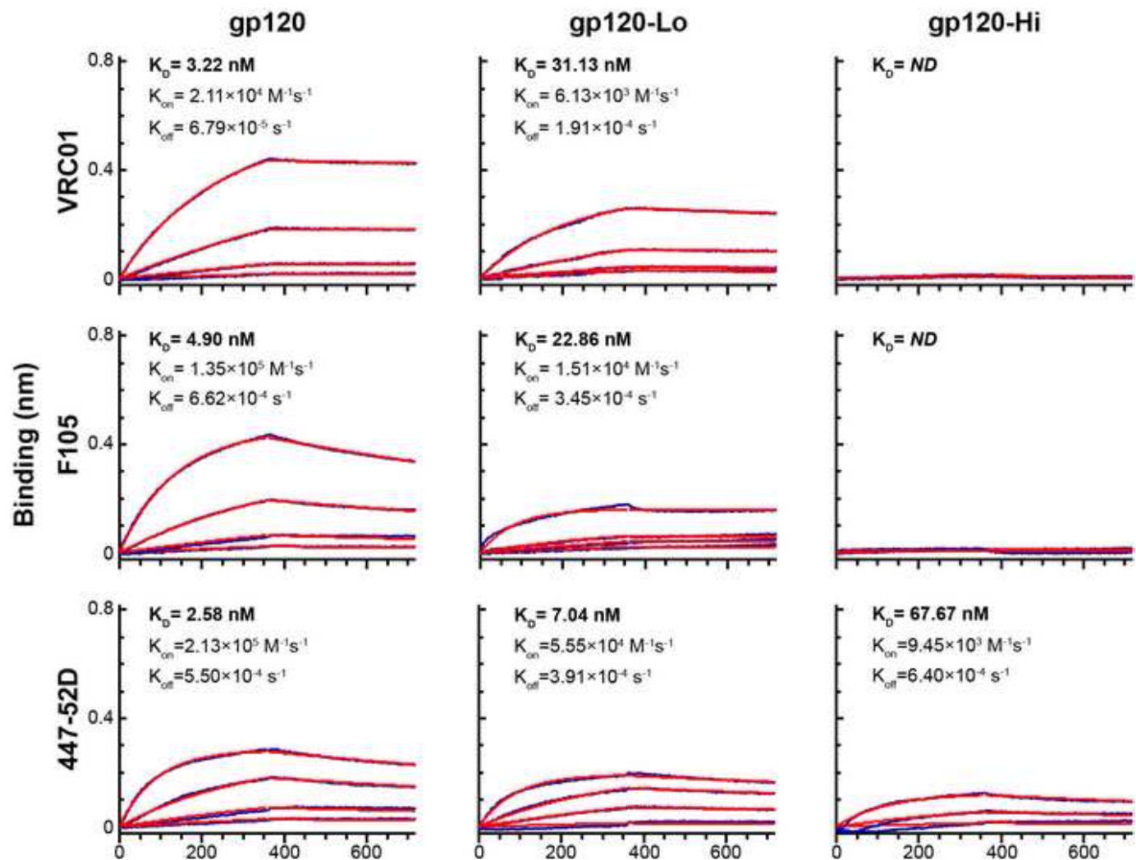

Figure 6. Binding kinetics of mAbs to unmodified and cross-linker conjugated gp120

Unmodified and cross-linker conjugated gp120 were used for the kinetics analysis. Shown in blue are the binding curves of VRC01, F105 and 447-52D to representative preparations of gp120, gp120-Lo and gp120-Hi glycoproteins at various concentrations of 800 nM, 200 nM, 50 nM, 12.5 nM, 3.13 nM and 0.78nM respectively (highest concentrations, top to bottom). Red curves depict the theoretical Langmuir fits generated by 1:1 binding kinetics (seconds (s), minutes (M) and non-detectable binding (ND)). K_D, K_{on} and K_{off} values are shown for which there was detectable binding as determined by Bio-Layer Interferometry (ForteBio). The data are representative of three independent experiments.