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Enzymatic removal of mannose moieties can increase the immune response to HIV-1 gp120 *in vivo*

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Abstract

The Env glycoproteins gp120 and gp41 are used in humoral immunity-based vaccines against human immunodeficiency virus (HIV-1) infection. One among many obstacles to such a vaccine is the structural defenses of Env glycoproteins that limit their immunogenicity. For example, gp120 mannose residues can induce immunosuppressive responses *in vitro*, including IL-10 expression, via mannose C-type lectin receptors on antigen-presenting cells. Here, we have investigated whether mannose removal alters gp120 immunogenicity in mice. Administering demannosylated gp120 (D-gp120) in the T_H2-skewing adjuvant **A**lum induced ~50-fold higher titers of anti-gp120 IgG, compared to unmodified gp120. While the IgG subclass profile was predominantly T_H2-associated IgG1, Abs of the T_H1-associated IgG2a and IgG3 subclasses were also detectable in D-gp120 recipients. Immunizing with D-gp120 also improved T-cell responses. Giving an IL-10 receptor blocking MAb together with unmodified gp120 in Alum increased the anti-gp120 IgG titer, implicating IL-10 as a possible mediator of auto-suppressive responses to gp120.

Keywords

AIDS; HIV-1; antibodies; T cells; vaccine; gp120; glycoproteins; mannose; immune suppression

Introduction

The envelope glycoproteins (Env) of human immunodeficiency virus type 1 (HIV-1) are the focus of attempts to make a preventative vaccine based, wholly or in part, on virus-neutralizing antibodies (NAbs) (Montefiori et al., 2007). The most common immunization method for Env vaccines involves administering soluble monomeric gp120 or trimeric gp140 proteins, in a suitable adjuvant (Pantophlet and Burton, 2006). This approach generally leads to the induction of significant titers of antibodies (Abs) against the immunizing antigen (e.g., anti-gp120 binding Abs), but little or no NAbs against primary isolates (Burton et al., 1991; Gilbert et al., 2005; Graham et al., 1998). Underlying this problem are the structural defenses that have

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evolved in Env proteins, which hinder the induction of NAbs and then restrict their binding to the functional Env trimer, notably by shielding critical epitopes (Grundner et al., 2004; Pantophlet and Burton, 2006). However, there are other adverse aspects of Env immunogenicity that may also impede the development of a NAb-based vaccine. For example, although Abs can clearly be induced by gp120 vaccines, these proteins are not particularly immunogenic compared to many other antigens (Pantophlet and Burton, 2006); relatively high gp120 concentrations are required to induce even binding antibodies (Connor et al., 1998; Gilbert et al., 2005; Graham et al., 1998; Grundner et al., 2005); the titers of these Abs decay fairly rapidly, necessitating repeat boosting (Connor et al., 1998); and a significant minority of gp120 vaccinees never develop any detectable Ab responses (Connor et al., 1998; Grundner et al., 2005). Moreover the IgG response to gp120 is unusually T_H2-polarized in both mice and humans (Gorse et al., 1999; Jankovic et al., 1997). We have suggested that at least some of these observations reflect the existence of Env-induced immunosuppressive responses (Shan et al., 2007).

HIV-1 gp120 is a biologically active protein that can activate signal transduction via multiple cell surface receptors such as CD4, CCR5 or CXCR4, and mannose C-type lectin receptors (MCLRs) when it is added in the μg/ml concentration range to various types of human cells *in vitro* (Pantophlet and Burton, 2006). It is uncertain which, if any, of these signaling events is relevant during HIV-1 infection *in vivo*, because the concentrations of soluble gp120 and/or virions in lymphoid tissues are hard to quantify (Klasse and Moore, 2004). However, during vaccination, 0.1–1 mg of gp120 or gp140 proteins is typically added in a small volume, directly into a muscle. The resulting concentrations are likely to easily exceed those that trigger signaling events *in vitro*, rendering these events relevant to understanding what may happen during the initial stages of a host response to Env vaccines.

We have recently described the effects of gp120 on human, immature monocyte-derived dendritic cells (MDDCs) *in vitro* (Shan et al., 2007). The immunosuppressive cytokine IL-10 is induced by some gp120 proteins (e.g., from HIV-1 JR-FL) in cells from ~50% of normal donors. Furthermore, MDDC maturation is adversely affected, independently of the IL-10 response, impeding their ability to stimulate $CD4^+$ T-cell proliferation (Shan et al., 2007). These responses to gp120 are dependent on interactions of mannose moieties on its N-linked glycans with MCLRs on the MDDC surface including, but perhaps not limited to, DC-SIGN; they can be prevented by treating gp120 with a mannosidase (Shan et al., 2007).

Here, we have investigated whether mannosidase digestion affects the immunogenicity of gp120 in mice, including the T_H2 bias of the IgG response. To do this, we immunized mice with JR-FL gp120 preparations that contained or lacked mannose moieties, in either Quil A or Alum adjuvant. We found that demannosylated gp120 (D-gp120), in contrast to unmodified gp120 (M-gp120), induced significantly higher titers of gp120-binding antibodies when administered in Alum adjuvant, but not in Quil A. In marked contrast to the IgG1-dominated T_H2 antibody response to M-gp120, the response to D-gp120 in Alum also involved the T_H1-associated subclasses IgG2a and IgG3. Furthermore, D-gp120 was also a superior immunogen for T cell responses. Because gp120 induces IL-10 expression *in vitro*, albeit with human MDDCs, we investigated whether IL-10 might play a role in suppressing the immunogenicity of gp120 under *in vivo* conditions. Accordingly, we administered a blocking MAb to the IL-10 receptor at the time of M-gp120 immunization, and found that this procedure also increased the titers of anti-gp120 binding antibodies. As expected for immunogens based on monomeric gp120, neither demannosylation nor the use of the IL-10 blocking MAb induced NAbs at detectable titers.

Results

Biochemical and antigenic characterization of demannosylated gp120

We have shown that JR-FL gp120 activates IL-10 production by human MDDCs *in vitro*, as well as impairing their maturation, as a result of interactions between its mannose moieties and an MCLR (Shan et al., 2007). In view of the role played by dendritic cells in priming immune responses *in vivo*, we wished to see whether gp120 that had been treated with a mannosidase enzyme to eliminate these effects might be a better immunogen. We have shown elsewhere that mannosidase treatment eliminates the mannose-dependent interactions of JR-FL gp120 with MAb 2G12 and DC-SIGN, but without compromising CD4-binding or conformational epitopes for other MAbs (Sanders et al., 2002; Shan et al., 2007). Here, we show the biochemical and antigenic properties of two different batches of demannosylated gp120 (D-gp120) that were used to immunize mice. These studies compared D-gp120 with unmodified gp120 and with gp120 that had been treated exactly as D-gp120, but without exposure to mannosidase (M-gp120).

Mannosidase digests were carried out as described in the Methods section. Demannosylation of gp120 reduced its molecular weight by ~10kDa, judged by migration on a 4–12% Bis-Tris NuPage gel (Fig. 1A). A ~10 kDa reduction corresponds to the removal of ~55 mannose residues, which are each of 0.18 kDa (Sanders et al., 2002). Batch #1 of D-gp120 contained some residual mannosidase enzyme, which is visible as two bands of 66 kDa and 44 kDa on a Bis-Tris NuPage gel (Fig. 1B). However, an enzyme activity assay showed that the contaminant enzyme had minimal activity (Table 1). Thus, the residual mannosidase activity in D-gp120 batch #1 was ~3% of what was present in the reaction mix at the start of the process (defined as 100%). For comparison, the residual enzyme activity was ~15% when mannosidase was processed under identical conditions to D-gp120 batch #1 but without gp120 present (Table 1). Unless otherwise specified, D-gp120 from batch #1 was used in subsequent studies.

Because of concerns about whether the residual mannosidase in D-gp120 batch #1 might have had an influence on the outcome of immunogenicity experiments (see below), perhaps by acting as an adjuvant, we devised a procedure to remove the enzyme prior to immunization of mice. To do this, the standard D-gp120 preparation was passed through a ceramic hydroxylapatite (CHT) column. The resulting D-gp120 batch #2 preparation was essentially free of mannosidase, as assessed using both the Bis-Tris NuPage gel system (Fig. 1A) and an enzyme activity assay (Table 1). The endotoxin levels of both batches of D-gp120 were below the detection limit of the LAL test (0.06 EU/ml).

We confirmed that mannosidase digestion was functionally effective by showing that both D-gp120 preparations were unable to bind MAb 2G12 (Fig. 2, and data not shown). The epitopes for other anti-gp120 Abs on D-gp120 were, however, unaltered. Thus HIVIG, CD4-IgG2 and MAbs b6, b12, 15e, A32, 17b (+sCD4), 447-52D and F425-B4e8 all bound comparably to the mannosidase-treated and control gp120s (Fig. 2, and data not shown). Hence mannose removal does not affect the antigenicity of JR-FL gp120, other than by eliminating the 2G12 epitope. Hence any differences in immune responses to the unmodified and mannose-depleted proteins would probably not be attributable to changes in antibody epitope accessibility. The antigenic profile of the mannosidase-free (batch #2) preparation of D-gp120 was similar to that of batch #1 (data not shown).

Enhanced IgG response in mice immunized with demannosylated gp120 in Alum adjuvant but not in Quil A

To explore the immunogenicity of D-gp120, we first conducted a pilot experiment in which BALB/c mice were primed and then twice boosted with gp120, M-gp120 or D-gp120 (batch

#1). Previous studies have indicated that the murine immune response to gp120 is strongly T_H2 polarized (Daly et al., 2005; Jankovic et al., 1997), so we used both a T_H1-polarizing and a T_H2-polarizing adjuvant, Quil A (Fernando et al., 1998) and Alum (Pollock et al., 2003) respectively, to explore whether any effect of gp120 demannosylation was adjuvant-dependent.

Compared to gp120 and M-gp120, the serum gp120-binding IgG titers were elevated in mice immunized with D-gp120 in Alum (Fig. 3A). The mean titers were increased by 30-fold at the 4-week time-point (p=0.0417 vs gp120 or M-gp120, one-tailed Student's t test), and by 6-fold at 6 weeks (p=0.0041 for D-gp120 *vs*. gp120 or M-gp120, one-tailed Student's t test). As expected, there were no differences between the anti-gp120 responses to gp120 and M-gp120 at any time point (week 4, p=0.4716; week 6, p=0.2825, one-tailed Student's t test).

Anti-gp120 titers were 1000- and 100-fold greater at 4 and 6 weeks, respectively, when Quil A was used as the adjuvant, compared to Alum (Fig. 3B). In contrast to the beneficial effect of gp120 mannose removal seen with Alum adjuvant, there were no significant differences in the anti-gp120 titers between mice immunized with gp120, M-gp120 or D-gp120 in Quil A (Fig. 3B).

To assess the reproducibility of these observations and to extend them, we repeated the pilot experiments with C57BL/6 mice instead of BALB/c. In these C57BL/6 experiments, we omitted the gp120 arm, M-gp120 serving as the control for D-gp120 using both alum and Quil A adjuvants. Higher anti-gp120 IgG titers were again observed in the D-gp120 (Alum) recipients, compared to M-gp120 (Fig. 4). Thus, mannose removal consistently renders gp120 more immunogenic in the context of a T_H2 type adjuvant. During the early phase of the response (weeks 3–7), the titer differential between D-gp120 and M-gp120 in Alum was 40–50-fold (p<0.05, one-tailed Student's t test) although it narrowed over the next 3 months (20- and 7-fold at weeks 11 and 17) and had disappeared by week 22 (Fig. 4). In contrast, there was no difference in anti-gp120 titers in mice immunized with D-gp120 or M-gp120 in Quil A, except at week 3 when the titer in D-gp120 recipients was slightly higher than with M-gp120 (Fig. 4, inset).

Because monomeric gp120 proteins are poor inducers of NAbs (Burton et al., 2004; Gilbert et al., 2005; Graham et al., 1998) and NAb responses are generally weak in mice (Burton et al., 2004), we did not expect to induce any NAbs in these experiments; indeed, their purpose was to assess influences on gp120 immunogenicity in general, not on the induction of NAbs per se. However, for completeness, we did assess whether sera from the different groups of gp120-immunized mice differed in their ability to neutralize JR-FL or MN Env-pseudoviruses (Beddows et al., 2005). As anticipated, NAb titers to both viruses were extremely low even at the highest serum concentration used, and there was no consistent pattern between the different immunization groups (data not shown).

The D-gp120 (batch #1) preparation contained some mannosidase protein that survived processing, albeit with minimal enzymatic activity (Fig. 1B, Table 1). To assess whether the presence of this additional protein might have an adjuvant effect that increased the immunogenicity of D-gp120, we prepared a second batch of D-gp120 from which all detectable mannosidase was removed by column chromatography (Fig. 1B). The immunogenicity of this D-gp120 preparation (batch #2) was compared to M-gp120 in C57BL/6 mice, using Alum as the adjuvant (Fig. 5). Other than a minor difference in the kinetics of the response to M-gp120 between weeks 4 and 7, the anti-gp120 antibody profiles were similar to those seen in the previous experiment (see Fig. 4, and also Fig. 6). The anti-gp120 titers in the D-gp120-immunized mice were again significantly greater (60-fold, at week 8, p=0.0083 using a one-tailed Student's t test) than in the M-gp120 recipients (Fig. 5). Hence the increased

immunogenicity of D-gp120 results from removal of its mannose moieties, and not from any adjuvant effect of the enzyme contaminant.

Blocking the IL-10 receptor increases the antibody response to gp120 in mice

The mannose moieties on gp120 induce the production of IL-10 from human MDDCs (Shan et al., 2007). Despite the species difference (mouse vs humans) and the uncertainty of whether MDDCs resemble the types of dendritic cell that initiate immune responses to vaccine antigens, we considered it worthwhile to investigate a possible role for IL-10 in the differential antibody responses to D-gp120 and M-gp120 in mice. We therefore administered an IL-10 receptor blocking MAb (anti-mIL-10R) to mice that were immunized with gp120 in either Alum or Quil A (this experiment was carried out simultaneously with the one described in Fig. 4). An irrelevant MAb of the same isotype served as a control.

The effect of blocking IL-10 activity during immunization with gp120 in Alum was similar to that of using D-gp120 instead of gp120 (Fig. 6, compare with Fig. 4). Thus, during the early phase of the response (weeks 2–7), anti-gp120 IgG titers were approximately 10–30-fold higher ($p<0.05$ using a one-tailed Student's *t* test) in mice given the anti-mIL-10R MAb than in ones receiving the control MAb (Fig. 6). The titer differential narrowed beyond 7 weeks, presumably reflecting clearance of the anti-mIL-10R MAb after its last administration (at 5 weeks). In contrast, blocking IL-10 activity in mice receiving gp120 in Quil A had no consistent effect on the anti-gp120 titer (Fig. 6, inset). The localized immunosuppressive effects of gp120-induced IL-10 expression may, therefore, be responsible for reducing the antibody response to gp120 in Alum adjuvant, an effect overcome by removal of the mannoses from gp120, or by changing the adjuvant to Quil A.

IgG subclass responses to demannosylated and unmodified gp120s

We measured the endpoint titers of IgG subclasses in sera from the C57BL/6 mice at week 7, to gauge whether T_H1-type (IgG2a and IgG3) or T_H2-type (IgG1) responses were dominant under the different experimental conditions (Snapper et al., 1992; Stevens et al., 1988). We also measured IgG2b titers, for which class switching is induced by TGF-β (McIntyre et al., 1993). There was no evidence for the induction of gp120-specific serum IgA antibodies in any of the groups.

Alum-adjuvanted M-gp120 induced IgG1 antibodies consistently, but not IgG2a, IgG2b or IgG3 (Fig. 7A). This pattern is consistent with previous reports that the antibody profile to gp120 in mice is strongly T_H2-biased and IgG1-dominated (Daly et al., 2005;Jankovic et al., 1997;Moore et al., 1999). In addition to inducing ~60-fold higher IgG1 titers ($p=0.0079$ compared to M-gp120), D-gp120 was able to trigger the production of IgG2a, IgG2b and IgG3 antibodies. Similar results were obtained when sera from the gp120-, M-gp120 and D-gp120-immunized BALB/c mice were similarly analyzed (data not shown).

When Quil A was the adjuvant, both gp120s induced binding antibodies from all four subclasses, the IgG1 and IgG2b titers being markedly higher than those seen with Alum (Fig. 7B). However, only the IgG2a titers differed significantly between the D-gp120 and M-gp120 groups (10-fold higher for D-gp120; $p=0.00079$).

The IgG subclass responses to gp120 in Alum were also measured in the anti-mIL-10R and isotype control groups at week 7, this being the last time-point during the early phase at which total IgG titers were significantly elevated in the anti-mIL-10R group (27-fold higher median, $p=0.0079$). The median IgG1 titer to gp120 was 15-fold higher in the anti-mIL-10R recipients ($p=0.032$) and the IgG3 response was more consistent (3 of 5 mice responding compared to 0

of 4 controls). However, there were no differences in the IgG2a or IgG2b titers between the two groups (Fig. 8).

To assess whether the use of D-gp120 had changed the IgG subclass profile in the C57BL/6 mice, or had triggered a general increase in all the subclass titers, we calculated the T_H1:T_H2 index (IgG2a+IgG3 average titer rank divided by the IgG1 titer rank) for the anti-gp120 response to each protein–adjuvant combination (see Methods). This showed that the antibody response to D-gp120 in Quil A adjuvant was significantly less skewed towards a T_H2 profile, compared to the response to M-gp120 (p=0.0087) (Table 2). For the Alum groups, although T_H1-associated IgG2a and IgG3 anti-gp120 antibodies were detected consistently only in the mice given D-gp120 (Fig. 7A), the T_H1:T_H2 indexes were not significantly different between the D- and M-gp120 recipients (Table 2). The use of the anti-IL-10R MAb did not change the IgG subclass distribution compared to the isotype control MAb group (Table 2). Thus IL-10 may be only partially responsible for the normally T_H2-skewed IgG response to gp120.

Blocking IL-10 activity and demannosylating gp120 induce superior T cell responses

To measure functional T cell responses, we used secreted cytokine assays to assess the *in vitro*, gp120-specific recall responses of splenic T cells from the C57BL/6 mice. The total numbers of splenic T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$) isolated from the various test groups were comparable, as measured by flow cytometry (total splenocyte counts did not vary markedly between groups; data not shown).

Antigen (gp120)-specific T cell re-stimulation triggered secretion of IL-2 and IFN-γ but not IL-4 or IL-10 *in vitro*, more so in the mice immunized using Quil A adjuvant than when Alum was used (Figs. 9,10). The greater recall response seen using Quil A is consistent with the known properties of these adjuvants (Sun et al., 2009;Takahashi et al., 1990). Moreover, T cells from D-gp120-immunized mice secreted 2- to 4-fold and 2- to 100-fold more IL-2 and IFN-γ, respectively, upon gp120 re-stimulation than cells from mice given M-gp120, irrespective of the adjuvant (Fig. 9). An anti-CD3 antibody (polyclonal) stimulus also showed that the general responsiveness of T cells from D-gp120-immunized mice was significantly stronger than for M-gp120; thus, levels of IL-4, IL-10 and IFN-γ secretion were elevated by ~2-fold (Fig. 9). Similar experiments conducted in the pilot study with BALB/c mice also showed that splenic T cells from D-gp120-immunized mice (at week 6) secreted 2- to 8-fold more IL-2 and IFN-γ than cells from mice given either gp120 or M-gp120 (data not shown).

We also assessed whether inhibiting any IL-10 responses to gp120 *in vivo*, via use of the receptor-blocking MAb, could boost T-cell responses to gp120. T-cells from mice given gp120 in Alum together with the anti-mIL-10R MAb had a superior antigen (gp120)-specific response, producing significantly more IL-2 and IFN-γ than T-cells from mice receiving the control MAb (Fig. 10). Furthermore, T-cells from mice in the gp120 + anti-mIL-10R MAb (Alum) group also produced IL-2 (but not IFN-γ) in the absence of antigenic stimulation (Fig. 10; media). This observation suggests they had become activated *in vivo*. Anti-CD3 stimulation also generated stronger cytokine responses when the *in vivo* IL-10 response was blocked, irrespective of the adjuvant. Overall, these studies indicate that T cell responses in mice can be improved by immunizing with mannosidase-treated gp120, which does not induce IL-10 from human MDDC *in vitro*, or by blocking the IL-10 receptor via use of a MAb.

DISCUSSION

The purpose of this mouse immunization study was to extend observations that JR-FL gp120 can induce IL-10 expression from human MDDC *in vitro* and impair their ability to activate T-cell proliferation. Both events are triggered by interactions between gp120 mannose moieties and MCLRs on the MDDC surface, including, but perhaps not limited to, DC-SIGN (Shan et

al., 2007). Accordingly, removing the terminal mannoses from its N-linked glycans by use of a mannosidase eliminates these adverse effects of gp120 on MDDC functions *in vitro* (Shan et al., 2007). Because IL-10 can limit the generation of immune responses by affecting cells involved in antigen-presentation (APCs) and adaptive immunity (Couper et al., 2008), we felt it worth exploring whether mannosidase-treatment might improve the immunogenicity of gp120.

We elected to conduct exploratory experiments in mice, both to compare mannosidase-treated gp120 (D-gp120) with unmodified gp120 and to assess whether the use of a blocking MAb to the IL-10 receptor could improve the immune response to unmodified gp120. The mouse is not an ideal animal for studying host restrictions on the immune response to gp120 because the receptor biology differs from humans; specifically, gp120 does not recognize murine CD4 or coreceptors (Clapham and McKnight, 2002; Moore et al., 2004). However, as gp120 can bind to various murine MCLRs (Koppel et al., 2005; Trujillo et al., 2007) and gp120-MCLR interactions are responsible for the immunosuppressive effects we studied *in vitro*, we considered that a mouse experiment was justified.

We observed that D-gp120 was indeed more immunogenic than unmodified gp120, particularly when administered in the T_H2-polarizing adjuvant, Alum. Thus anti-gp120 IgG titers were consistently and markedly (up to 50-fold) higher with the D-gp120 immunogen, and T-cell responses were also improved. The beneficial effects of mannose removal on IgG titers were not, however, seen when the adjuvant was switched to Quil A, which is much more T_H1-polarizing than Alum, although stronger T-cell responses to D-gp120 were seen even with Quil A. One other notable effect of mannose removal was to increase the titers of anti-gp120 antibodies of the less prevalent IgG subclasses (IgG2a, IgG2b and IgG3). The anti-gp120 response in mice is normally dominated by T_H2-associated, IgG1 antibodies (Daly et al., 2005; Jankovic et al., 1997; Moore et al., 1999), just as we saw in response to unmodified gp120. However, the T_H1-associated IgG2a and IgG3 subclasses were induced more consistently to D-gp120 than to unmodified gp120, particularly in Quil A (in Alum, all the IgG subclass titers were elevated). Serum gp120-specific IgA responses were undetectable in all groups, which was expected considering that we did not use a mucosal immunization protocol. The cellular immune response assays also showed that the use of D-gp120 improved T_H1-associated responses. Hence there are indications that removing the mannoses from gp120 may partially counteract the normally strong T_H2-dominance of the murine immune response to this protein. However, additional studies will be required to confirm this.

One explanation of the improved immune responses to D-gp120 is that mannose removal alleviates an auto-suppressive response that normally limits the immunogenicity of unmodified gp120. Because gp120 induces mannose-dependent IL-10 expression from human MDDCs, we investigated whether IL-10 expression might be involved in restricting the immune response to gp120. Thus, we co-administered a blocking MAb to the IL-10 receptor, to antagonize any localized IL-10-dependent immunosuppressive effects of the gp120 mannose moieties. This intervention did indeed increase the anti-gp120 titer during the primary phase of the immune response, an effect similar in kinetics and magnitude to that achieved by using D-gp120 instead of unmodified gp120. Hence, IL-10 induction by gp120 may suppress immune responses to this protein.

The beneficial effects of both mannose removal and the IL-10 receptor MAb on anti-gp120 IgG titers were eventually lost over time, suggesting that any IL-10-dependent immunosuppressive effect of gp120 might be restricted to the primary phase of the humoral response, and inactive during the memory phase. However, both interventions did influence memory T-cell responses to gp120, especially in Alum adjuvant, since we observed differences in IL-2 and IFN-γ expression several months after immunization. Removing the influence of

IL-10 during primary responses has also been found to benefit memory T-cell responses in other studies (Biswas et al., 2007; Foulds et al., 2006).

The anti-IL-10 receptor MAb did not, however, mimic gp120 mannose removal by broadening the anti-gp120 IgG subclass profile, suggesting that factors other than, or as well as, IL-10 may be involved in the T_H2 polarization of the immune response to gp120. Expressing IL-12 in mice immunized with gp120 in Alum was found to help balance the normally T_H2-biased response, probably by increasing the IL-12:IL-10 ratio (Gurney et al., 2005). T-cell responses to a gp120-expressing DNA vaccine construct were more T_H1-polarized in IL-10−/− mice than in controls, with modestly lower anti-gp120 IgG1 titers and no change in IgG2a (Daly et al., 2005). These differences between IL-10−/− and control mice were not observed with an influenza HA DNA vaccine that induces much more T_H1-polarized responses than gp120. Hence when a response is already skewed towards T_H1, the lack of IL-10 may have little additional effect (Daly et al., 2005). The T_H2-bias of the gp120 response may involve up-regulating members of the suppressor-of-cytokine-signaling (SOCS) family in local or infiltrating APCs. Thus, IL-10 activates SOCS-1 and SOCS-3 expression in murine B cells, and SOCS-1 restricts the immunostimulatory ability of murine DCs by interfering with IL-12-dependent responses (Ding et al., 2003; Evel-Kabler et al., 2006). As a result, SOCS-1 silenced DCs are superior stimulators of gp120-specific B and T cell responses in mice (Song et al., 2006). SOCS-3 expression in murine DCs also promotes T_H2-type immune responses (Li et al., 2006). This aspect of how the immune system sees gp120 warrants further study.

Although we have obtained suggestive evidence that IL-10 is involved in suppressing immune responses to gp120, we were unable to detect IL-10 in mouse serum post-vaccination (data not shown). However, it is more likely that IL-10 would be induced and act locally, something we now intend to investigate. DCs, macrophages and T-cells all express the IL-10 receptor and produce IL-10 *in vivo* (Couper et al., 2008; Moore et al., 2001). Furthermore, the absence of IL-10 receptor signaling on T-cells, but not DCs, improves T-cell responses to *Listeria monocytogenes* antigens in mice (Biswas et al., 2007). Hence, although our *in vitro* studies of the IL-10 response to gp120 have focused on DCs (Shan et al., 2007), other cell types might be involved *in vivo*, and IL-10 may act directly on B-cells and T-cells.

In this study, we have shown that the mannose moieties of N-linked glycans have immunosuppressive effects on responses to HIV-1 gp120 *in vivo*. There are other examples of mannoses suppressing the host immune response to pathogens that establish persistent infections, again mediated via MCLRs. For example, ManLAM and related mannosylated antigens from *M.Tb, Schistosoma spp.* eggs, *Leishmania spp* and *Helicobacter spp* all bind to DC-SIGN and, in several cases, induce IL-10 expression (Bergman et al., 2004; Geijtenbeek et al., 2003; Ghosh and Bandyopadhyay, 2004; van Liempt et al., 2007). There are also many reports from other viral infection or vaccination systems of an adverse role for IL-10 in the development of immune responses, including LCMV, HCV and CMV (Brady et al., 2003; Brooks et al., 2008; Brooks et al., 2006; Humphreys et al., 2007; Redpath et al., 1999; Roque et al., 2007). It may be valuable for persistent viruses and other intracellular pathogens to skew the host away from the T_H1 responses that are considered important for clearing newly established infections. Thus, although Alum-adjuvanted influenza virus antigens induced high antibody titers in mice, the T_H2-biased response was much less protective than a more T_H-balanced one when the mice were challenged with live virus (Bungener et al., 2008). Whether LCMV, HCV and CMV use mannose moieties and MCLRs for this purpose remains to be determined, as there may be multiple ways for the same or different viruses to achieve the same ends. Thus, various HIV-1 proteins have been reported to induce IL-10 expression *in vitro*: Tat suppresses gp120-specific T cell responses through an IL-10-dependent mechanism (Gupta and Mitra, 2007); Nef impairs DC functions (Quaranta et al., 2007) and stimulates IL-10 release from monocytes (Tangsinmankong et al., 2002); and the gp41 immunosuppressive peptide

region induces IL-10 expression by monocytes (Barcova et al., 1998). It is controversial whether immunosuppressive effects of IL-10 arise during natural HIV-1 infection, although elevated IL-10 levels can be detected (Orsilles et al., 2006; Srikanth et al., 2000). However, dysfunctions of T_H-cells from HIV-1-infected individuals can be reversed by *ex-vivo* treatment with anti-IL-10 antibodies (Clerici et al., 1994). Moreover, a high IL-10:IL-12 ratio has been suggested to promote HIV-1 mucosal transmission by upregulating DC-SIGN expression on local antigen presenting cells (Gurney et al., 2005).

Our goal in these studies was to explore influences on the overall immunogenicity of Env glycoproteins, using monomeric gp120 as a simple model antigen. Monomeric gp120 is not an effective inducer of NAbs against primary isolates, particularly in mice; we were, therefore, not surprised when no NAbs were detected in the mouse sera. Mannose depletion alone would be unlikely to overcome the many limitations to the immunogenicity of monomeric gp120, which have a basis in Env structure (Karlsson Hedestam et al., 2008; Pantophlet and Burton, 2006). Complex carbohydrates are unaffected by mannosidase treatment. Thus, most of the carbohydrate chains that occlude the underlying protein epitopes and which contribute to the limited immunogenicity of gp120 are still intact.

Of note is that the ~10kDa reduction in the m.wt. of gp120 after mannosidase treatment corresponds to the removal of ~55 mannose residues. Although the number of high-mannose glycan structures on JR-FL gp120 is unknown, there are 11 such structures on IIIB gp120 (Leonard et al., 1990). Each of these oligomannose glycans contain 4–8 α1–2, α1–3 and α1–6-linked mannose moieties that are susceptible to removal by α(1–2,3,6)-mannosidase. Hence there are probably between 44 and 88 theoretically vulnerable mannose residues on IIIB gp120, and the number on JR-FL gp120 is likely to be similar. Hence our observed m.wt reduction of ~10 kDa (~55 mannoses) implies that a majority of the vulnerable mannoses are removed, but some may remain. However, here and elsewhere, we show that mannosidase treatment abolishes the binding of 2G12 and DC-SIGN to gp120 (Sanders et al., 2002; Shan et al., 2007). Hence the surface-exposed mannose residues most involved in ligand binding have been removed; investigating the consequences for the immunogenicity of gp120 was principal goal of the present study.

An effective vaccine will require the induction of NAbs to primary isolates, not just the gp120-binding antibodies we have measured here (Walker and Burton, 2008). Similarly, the detection of IL-2 or IFN-γ expression by T-cells does not prove that these cells have functional T-helper or CTL activity (Walker and Burton, 2008). However, if and when new immunogens, perhaps ones based on Env trimers, are developed that can induce NAbs efficiently, knowing how to present them effectively to the human immune system will be important. All things being equal, if an Env antigen is developed that can induce NAbs, increasing its overall immunogenicity, for example by mannose removal, could be advantageous. This may be particularly so if the intervention has a dose-sparing effect on an Env antigen that is difficult to make in bulk. Using effective adjuvants can help, but some adjuvants (e.g., those containing detergents) may be incompatible with preserving the appropriate Env antigen configuration. Hence, it is relevant that the most substantially improved humoral and cellular responses to demannosylated gp120 were seen with Alum, a routinely used adjuvant. Moreover, the auto-suppressive effects of gp120 mannoses on anti-gp120 responses might extend to any other co-administered antigens in the context of a multivalent vaccine, particularly those intended to induce T-cell responses. We are presently assessing how to remove the mannose moieties from Env trimers without compromising trimer stability or conformation, with the goal of performing immunogenicity studies to determine whether mannose-mediated suppression of the immune response also occurs with these more vaccine-relevant forms of HIV-1 Env antigens.

Materials and Methods

Mannosidase treatment of gp120 proteins

Recombinant, Chinese hamster ovary (CHO) cell expressed monomeric gp120 from HIV-1 JR-FL was prepared under pyrogen-free conditions and was enzymatically demannosylated (to make D-gp120) as previously described, with minor modifications (Sanders et al., 2002; Shan et al., 2007). Briefly, gp120 was treated with Jack bean α(1–2,3,6)-mannosidase (ProZyme Inc, San Leandro, CA) for 18h at 37°C in the presence of protease inhibitors, before being exchanged into half-strength PBS using a 30kDa molecular weight cutoff spin filter (Sartorius, NY). The outcome of the digests was analyzed using a non-reducing 4–12% Bis-Tris NuPage gel system (Invitrogen, CA), and an enzyme activity assay (see below). A control preparation (M-gp120) was made by processing gp120 under identical conditions to D-gp120 batch #1, but without the addition of the mannosidase enzyme. To remove mannosidase when making D-gp120 batch #2, the standard D-gp120 preparation was passed through a ceramic hydroxylapatite (CHT) column (BioRad Laboratory, CA).

Endotoxin was removed from the various gp120 preparations using the Endofree Red 5/1 kit (Profos AG, Regensberg, Germany); the resulting endotoxin levels were below the detection limit of the LAL test (0.06 EU/ml). To specifically determine gp120 protein concentrations in various preparations (after the enzyme reactions, and before and after the CHT column purification), we used both a bicinchoninic acid assay (Pierce Protein Research Products, Thermo Fisher Scientific, Rockford, IL) and SDS-PAGE densitometry.

Analysis of mannosidase activity

The residual Jack bean α(1–2,3,6)-mannosidase activity in D-gp120 preparations was determined using an enzymatic activity assay, following the manufacturer's protocol (Prozyme) with some modifications. Briefly, 0.4 ml of substrate solution, pre-warmed to 37° C, was added to each tube. Then 5 μl of samples, or different concentrations of α(1–2,3,6)-mannosidase standards, were added for 5 min before termination of the reaction with 0.6 ml of 1 M sodium carbonate and measurement of the absorbance at 400 nm using a spectrophotometer. The residual enzymatic activity in the D-gp120 preparations was determined using the standard curve derived using known enzyme concentrations. One unit of α(1–2,3,6) mannosidase is defined as the amount of enzyme required to hydrolyze 1 μmol of *p*-nitrophenol (Sigma, MO) in 1 min at 37°C, pH 5.0. The activity of enzyme added to the reaction mixture at the start of the demannosylation procedure was defined as 100% for normalization purposes; this corresponded to 14.2 unit/mg gp120 protein or 1.42 unit/ml (see Table 1). The assay detection limit was 0.01 unit/ml.

Monoclonal antibodies (MAbs)

MAb 2G12 to a mannose-dependent gp120 epitope (Buchacher et al., 1994; Scanlan et al., 2002; Trkola et al., 1996) was obtained from Dr. Hermann Katinger, University of Natural Resources and Applied Life Sciences, Vienna. MAbs 17b to the CD4i site on gp120 (Thali et al., 1993), A32 to a gp120 C1–C4 epitope (Moore et al., 1994; Wyatt et al., 1995), and 15e (Robinson et al., 1990), b6 (Parren et al., 1997) and b12 (Burton et al., 1991) to CD4BS-associated gp120 epitopes were all obtained from the reagent repository of IAVI's Neutralizing Antibody Consortium. MAbs F425-B4e8 (Cavacini et al., 2003) and 447-52D (Gorny et al., 1992) to gp120 V3 epitopes (contributed by L. Cavacini and S. Zolla-Pazner respectively) and pooled serum from HIV-1 infected individuals (HIVIG) were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. CD4-IgG2 (PRO 542) and soluble CD4 were manufactured at Progenics Phramceuticals. Inc. (Allaway et al., 1993). All the above MAbs are of human origin, and CD4-IgG2 contains a human Ig Fc region.

MAb reactivity with gp120 and demannosylated gp120

The gp120-capture enzyme-linked immunosorbent assay (ELISA) procedure using the sheep Ab D7324 to the gp120 C-terminus has been described previously (Moore and Jarrett, 1988; Moore and Sodroski, 1996) (see below). The starting concentrations for 3-fold serial dilutions of the test MAbs were: 1μg/ml (2G12, 17b, A32, 15e, b6, b12, CD4-IgG2); 0.75μg/ml (F425-B4e8); 2μl/ml (447-52D). To each dilution of 17b, 300ng/ml of soluble CD4 was added. For HIVIG, the starting concentration was 10μg/ml. All assays were performed using duplicate wells. The data were plotted using Graph Pad Prism software (sigmoidal curve with variable slope, bottom constraint zero). Wells receiving serum but no gp120 were used to derive background OD values that were subtracted from test values.

Immunization of mice with gp120, and use of anti-mIL-10R MAb

Eight-week old, female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories, Wilmington, MA. All experimental procedures were conducted in accordance with protocols approved by the relevant Institutional Animal Care and Use Committees.

In the pilot experiment, BALB/c mice (5 per arm) were immunized at weeks 0, 2 and 4 and bled at weeks 2, 4 and 6. In the later experiments, C57BL/6 mice (5 per arm) were immunized at weeks 0, 2, 5 and 20, and bled at weeks 1, 2, 3, 5, 7, 11, 17 and 22. In all experiments, a 130μl formulation containing 5μg of gp120, M-gp120 or D-gp120, and either 10μg of Quil A (saponin derived from the bark of *Quillaja saponaria* (Aguilar and Rodriguez, 2007), Accurate Chemical, Westbury, NY) or 250μg of Alum adjuvant (Aguilar and Rodriguez, 2007; Eisenbarth et al., 2008; Kool et al., 2008; Seubert et al., 2008)(Alhydrogel; Accurate Chemical, Westbury, NY) was injected subcutaneously into the groin region. Primary and boosting immunizations were carried out identically.

In the IL-10 blocking studies, the mice were injected intra-peritoneally with 500μg of an anti-mIL-10R MAb or an isotype control MAb (Brooks et al., 2008; Brooks et al., 2006), one day prior to the gp120 immunizations at 0, 2 and 5 weeks, but not prior to the fourth immunization at week 20. The purified, no-azide/low-endotoxin (NA/LE), rat anti-mouse IL-10 receptor blocking MAb (anti-mIL-10R, Clone 1B1.3a, Cat# 550012) and the isotype-matched control MAb (Clone R3-34, Cat#553922) were purchased from BD Biosciences.

After blood was drawn, it was allowed to clot for 1h at room temperature. Serum was then collected, and stored in aliquots at −20°C. Splenocytes were obtained post mortem, pooled for each group and processed to make single cell suspensions in RPMI plus 10% FCS and 10% DMSO, and stored in liquid nitrogen.

Enzyme linked immunosorbent assays (ELISA) for anti-gp120 total IgG, IgA and IgG subclasses in mouse sera

The gp120-capture ELISA procedure has been described previously (Moore and Jarrett, 1988; Moore and Sodroski, 1996). Briefly, the plates were coated with 10μg/ml of sheep Ab D7324 to the gp120 C-terminus (Cliniqa Corp, San Marcos, CA, Cat. #6205). Recombinant, CHO-cell expressed JR-FL gp120 (300ng/ml in TBS) was then captured onto the bound D7324. In an alternative procedure (used for gp120-binding IgG2a Abs, see below), ELISA wells were directly coated with gp120 (5ug/ml in carbonate buffer overnight). Mouse serum (3-fold serial dilutions from 1:100) was then added, followed by a suitable dilution of an alkaline phosphatase (AP)-conjugated detection antibody. The AMPAK Enzyme Amplification Kit (Dakocytomation, Ely, UK) was used to provide a colorimetric end-point. The plates were read at 490nm on an *E* max precision microplate reader (Molecular Devices, Sunnyvale, CA).

The detection antibodies for total IgG, IgG2a and IgA were AP-conjugated polyclonal goat anti-mouse IgG and anti-mouse IgG2a (1:2000; AbD Serotec, Oxford, UK; Cat# STAR117A and STAR 82A, respectively) and goat anti-mouse IgA (1:500; Southern Biotech, Birmingham, AL; Cat#1040-04). To detect IgG1, IgG2b and IgG3 Abs, we first used unconjugated rat anti-mouse IgG1 (AbD Serotec, Cat#MCA1289), IgG2b (BD Biosciences, Cat#553392) and IgG3 (AbD Serotec, Cat#MCA1292). The gp120-bound IgG1, IgG2b and IgG3 Abs were then detected using a 1:30,000 dilution of AP-conjugated rabbit anti-rat IgG (Sigma, Cat #A6066), a reagent we confirmed to be reactive with neither mouse IgG nor the sheep D7324 capture Ab.

Prior to testing mouse serum samples, the anti-mouse IgG1, IgG2a, IgG2b and IgG3 detection antibodies were assessed for cross-reactivity and specificity using authentic mouse IgG1, IgG2a, IgG2b and IgG3 MAbs (BD Biosciences). A preliminary test was conducted by directly coating these MAbs (1μg/ml) onto ELISA plate wells. The plates were washed with PBS plus 0.1% Tween. Well-blocking and antibody dilution procedures were carried out using PBS plus 10% FCS. The anti-mouse IgG1 was tested at 0.5, 1 and 2μg/ml, anti-mouse IgG2a at dilutions of 1:1000, 1:2000 and 1:4000, anti-mouse IgG2b at dilutions of 1, 2 and 4μgml, and anti-mouse IgG3 at dilutions of 2.5, 5 and 10μg/ml. The optimal concentrations that provided acceptable sensitivity and minimal cross-reactivity were found to be: 1μg/ml of anti-mouse IgG1, a 1:2000 dilution of anti-mouse IgG2a, 2.5μg/ml of anti-mouse IgG2b and 2.5μg/ml of anti-mouse IgG3.

The same MAbs were then tested for cross-reactivity with the sheep D7324 capture Ab, coated onto ELISA plate wells as described above The anti-mouse IgG1, IgG2b and IgG3 antibodies did not cross-react significantly with D7324, but the anti-mouse IgG2a did react significantly, precluding its use in the standard gp120-capture ELISA. The alternative procedure of coating the ELISA wells directly with gp120 was therefore used for detection of anti-IgG2a Abs (see above).

To calculate end-point titers, background OD values (wells without gp120 but with the lowest serum dilution) were subtracted from OD values derived from test wells that contained both gp120 and serum. The resulting values were plotted on Graphpad Prism software (sigmoidal curve with variable slope, bottom constraint zero). The formula used for titer calculations was $X=\log EC_{50}-([\log(TOP/Y-1)/Hillslope])$ where Y=5 SD of 3 background wells receiving gp120 but no serum and X=log[serum dilution] ($\log EC_{50}$, TOP and Hillslope calculated by the Graphpad Prism software). Sera with extrapolated reciprocal titer values below 1.0×10^2 (1:100 serum dilution) or ones generating graphs with poor R^2 values (below 0.7) were considered negative for the Ab being detected.

Calculation of T_H1:T_H2 index

To determine whether gp120 demannosylation or IL-10-receptor blockade affected the relative amounts of anti-gp120 Abs from IgG subclasses that are linked to T_H1 (IgG2a and IgG3) and T_H2 (IgG1) polarization, we proceeded as follows. The reciprocal IgG subclass titers for each mouse were ranked from one to four in ascending order and then summed across the mice of each test group (titers $< 1.0 \times 10^2$ were considered negative and excluded from the analysis). Based on the summed values, the T_H1:T_H2 index was then calculated as ([IgG2a rank + IgG3 rank]/2)/(IgG1 rank). The higher the resulting value, the more polarized the antibody response towards T_H1-associated subclasses. Although we also measured IgG2b titers, we excluded them from the titer index calculation because of controversies about the precise relationship between this IgG subclass and T_H-polarization (McIntyre et al., 1993; Weiner, 2001).

HIV-1 neutralization assay

A single round Env-pseudovirus assay involving the U87/CD4.CCR5 and U87/CD4.CXCR4 cell lines was used to test the ability of mouse sera to neutralize HIV-1, as described previously but with minor modifications (Binley et al., 2004). Luciferase-expressing, Env-pseudotyped virus stocks based on the homologous, neutralization-resistant R5 Env JR-FL and the neutralization sensitive X4 Env MN were made as described previously (Beddows et al., 2005). The pseudoviruses were then incubated for 1h at 37°C with serial fourfold dilutions of heat-inactivated (56°C for 30 min) mouse serum, starting at a 1:20 dilution. CD4-IgG2 (1μg/ml) served as a positive control. Virus and sera were then added to the target cells (plated 24h earlier in flat-bottom, 96-well plates at 3×10^3 cells/well). Inhibition of infectivity was determined 72h post-inoculation by measuring luciferase activity (in counts-per-second, cps). Inhibition of infection for a given dilution of serum (normalized to pre-bleed sera from the same mouse) was calculated using the formula $\{[1-(a-c)/(b-c)]\}\times100$, where a =cps for cell +virus+serum from immunized mouse, b = cps for cell+virus+serum from pre-bleed mouse, c= cells only (Beddows et al., 2005).

T cell functional assays

In vitro re-stimulation of T cells ($CD4^+$ and $CD8^+$ combined) in unfractionated splenocyte cultures was carried out by culturing 2.5×10^5 cells/well (in triplicate) with JR-FL gp120 (10μg/ml) in a final volume of 200μl/well. Positive control wells received an anti-CD3e antibody (2μg/ml, BD Biosciences, Clone 145-2C11, Cat 553057), negative control wells received media. Supernatants were collected after a 72h culture at 37°C in 5% CO_2 and stored at −80°C till further use. Concentrations of IL-2, IL-4, IL-10 and IFNγ in the supernatants were measured by a sandwich ELISA, according to the manufacturer's instructions (OptEIA mouse ELISA kits, BD Biosciences), with a TMB substrate kit (BD Biosciences) used to provide a colorimetric endpoint at 405nm. The assay sensitivity limits were approximately 4pg/ml for IL-2, 10pg/ml for IL-4, and 30pg/ml for IL-10 and IFNγ. Cytokine levels were expressed in pg/ml above the respective assay detection limits and graphs plotted to show the mean value ± SD for triplicate wells.

Flow cytometry

The numbers of T-cells in spleens of immunized mice were determined using standard cell surface staining protocols and an LSR-II flow cytometer (BD Biosciences). The resulting data were analyzed using FlowJo software (Version 7.2, Tree Star Inc. Ashland, OR). All antibodies were purchased from BD Biosciences. Surface staining was carried out in a PBS + 10% FBS buffer for 20 min, followed by 2 washes with the same buffer. Prior to staining for specific cell surface markers, Fc receptors were blocked with an anti-mouse CD16/CD32 antibody (clone 2.4G2). The following labeled antibodies specific for mouse T cell markers were used: TCRβ-APC (clone H57-597), CD3 APC (clone 145-2C11), CD4 PCP (clone RM4-5), CD8 PE (clone 53-6.7).

Statistical analyses

A one-tailed Mann-Whitney U test was employed for statistical analysis of data unless otherwise mentioned.

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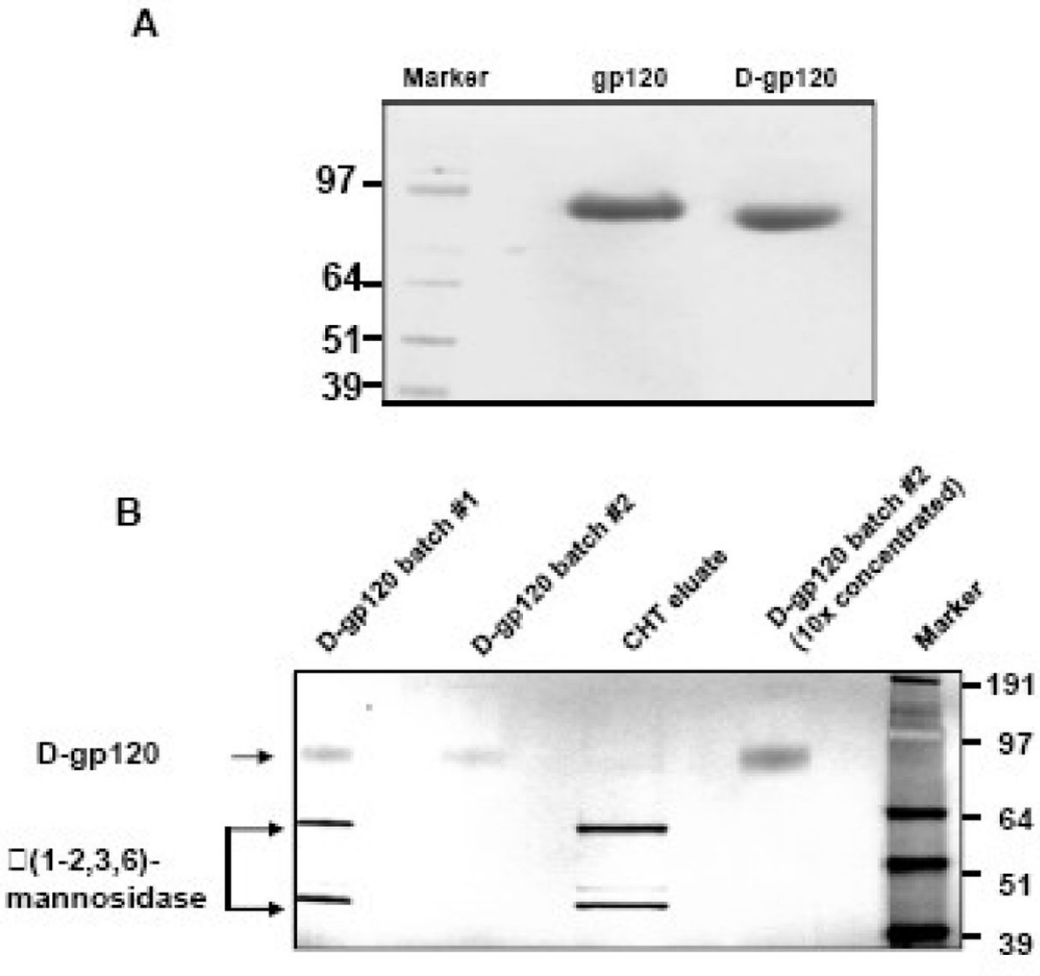

Figure 1. Biochemical properties of demannosylated gp120

Preparations of gp120 were assessed for (A) m.wt. changes, (B) the presence of α(1–2,3,6)-mannosidase using a non-reducing 4–12% Bis-Tris NuPage gel. The D-gp120 preparation shown in (A) is from batch #2 after CHT column purification.

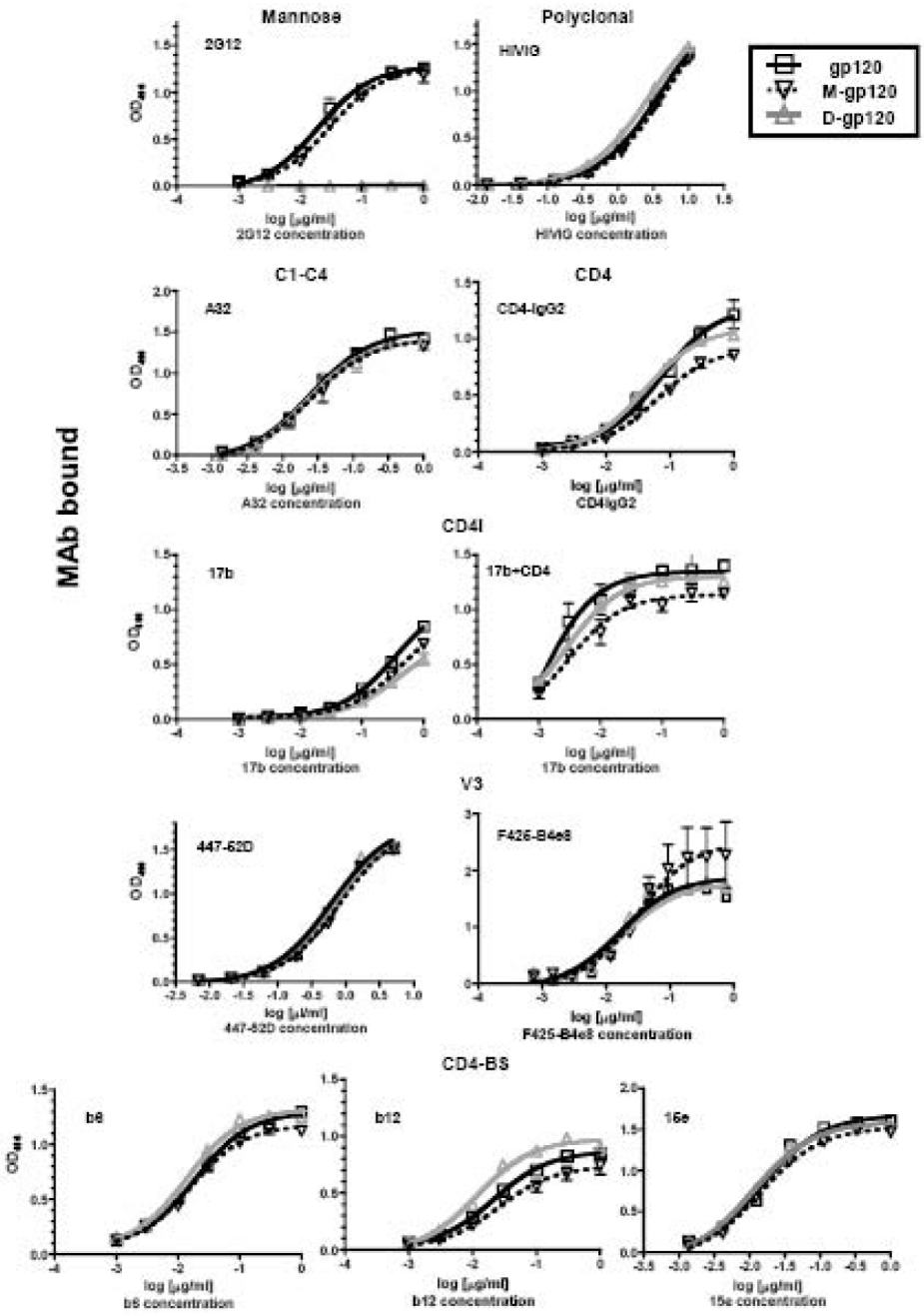

Figure 2. Antigenic properties of demannosylated gp120

Unmodified gp120, mannosidase-treated gp120 (D-gp120, batch #1) or gp120 processed in the absence of mannosidase (M-gp120) were captured onto ELIS plates via Ab D7324, then reacted with serial 3-fold dilutions of the antibodies indicated on each panel. The OD_{490} values displayed are the means from duplicate wells. The test MAbs recognize terminal mannoses (2G12); a discontinuous C1–C4 epitope (A32); a CD4-induced (CD4i) epitope (17b ± sCD4); V3 epitopes (447-52D, F425-B4e8); or epitopes overlapping the CD4 binding site (CD4BS) (b6, b12, 15e). CD4IgG2 binds the CD4Bs and HIVIG is a polyclonal antibody preparation derived from the sera of HIV-1-infected people.

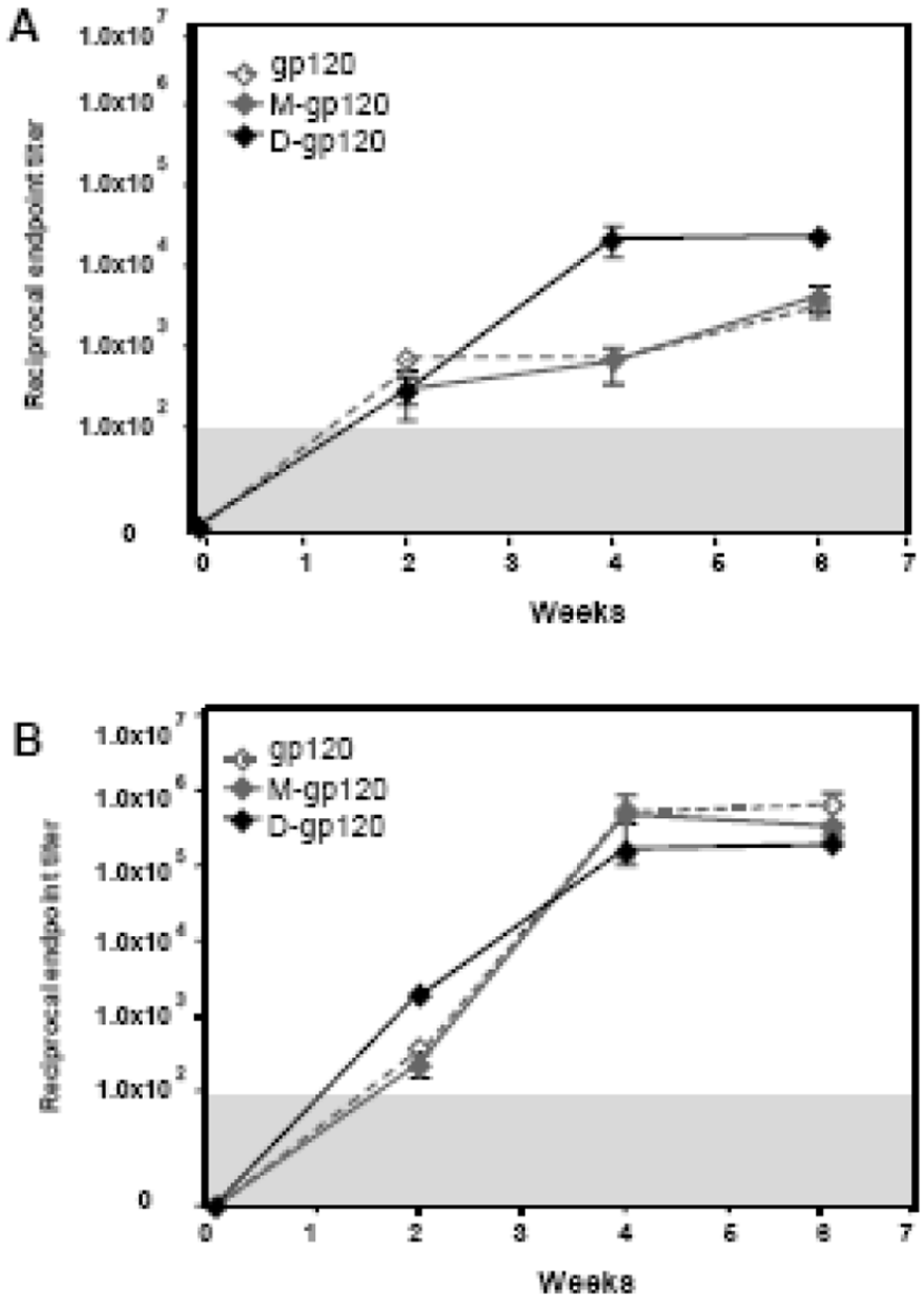

Figure 3. Comparative immunogenicity of D-gp120 in BALB/c mice

Mice (5 per group) were immunized with gp120, M-gp120 or D-gp120 (batch #1) in Alum (A) or Quil A (B) adjuvants, as indicated. Anti-gp120 IgG titers were determined using a gp120 capture ELISA. Each symbol represents the mean (± SEM) reciprocal endpoint anti-gp120 titer for each group of mice (n=5). Titers <100 (grey shaded area) are considered negative. The mean reciprocal end-point titer values for prebleed samples were all $< 1.0 \times 10^2$ (data not shown).

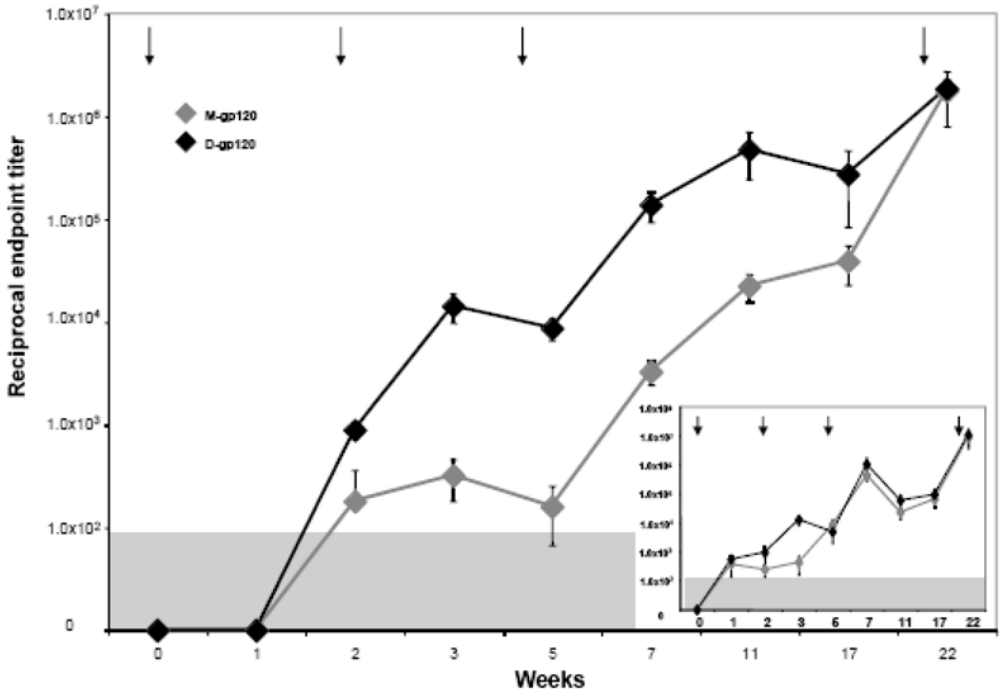

Figure 4. Comparative immunogenicity of D-gp120 in C57BL/6 mice

Mice were immunized with M-gp120 (grey symbols) or D-gp120 batch #1 (black symbols) at the times indicated by the arrows. Main figure: Alum adjuvant; inset, Quil A adjuvant. Each symbol represents the mean (± SEM) reciprocal endpoint anti-gp120 titer for each group of mice (n=5 initially, but n=4 for M-gp120/Alum from week 7 due to the death of one mouse from an unrelated cause). Titers <100 (grey shaded area) are considered negative.

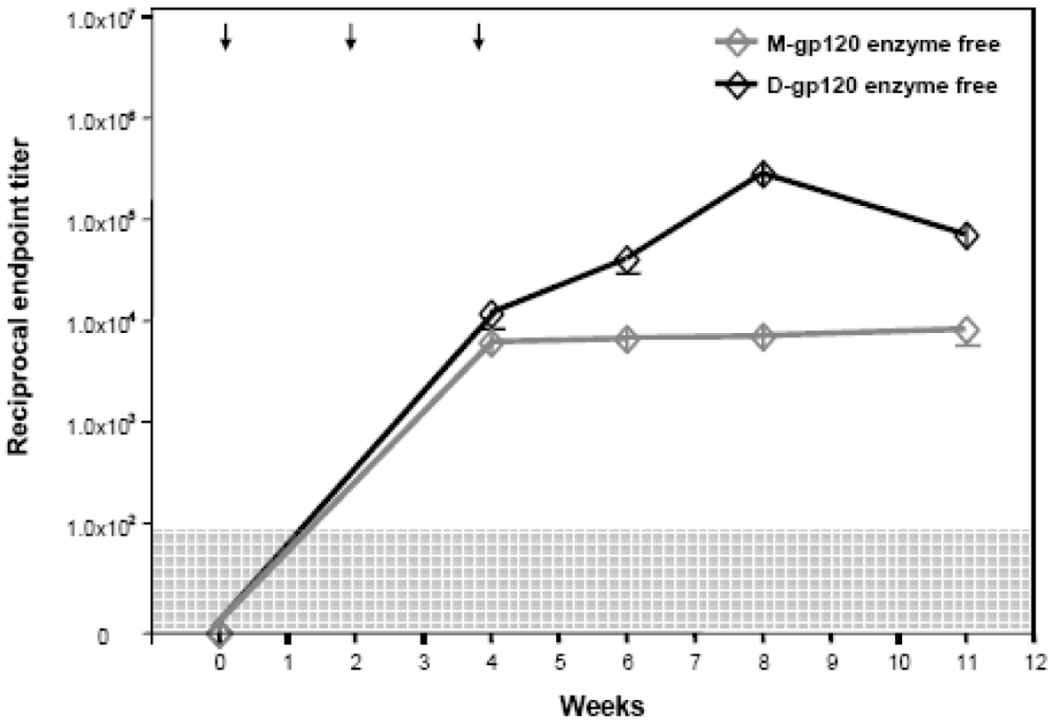

Figure 5. The increased immunogenicity of D-gp120 is not due to contaminant mannosidase

Mice (C57BL/6, 5 per group) were immunized with M-gp120 (grey symbols) or D-gp120 (batch #2; enyzme free; black symbols) using alum adjuvant, at the times indicated by the arrows. Each symbol represents the mean (± SEM) reciprocal endpoint anti-gp120 titer for each group of mice. Titers <100 (grey shaded area) are considered negative.

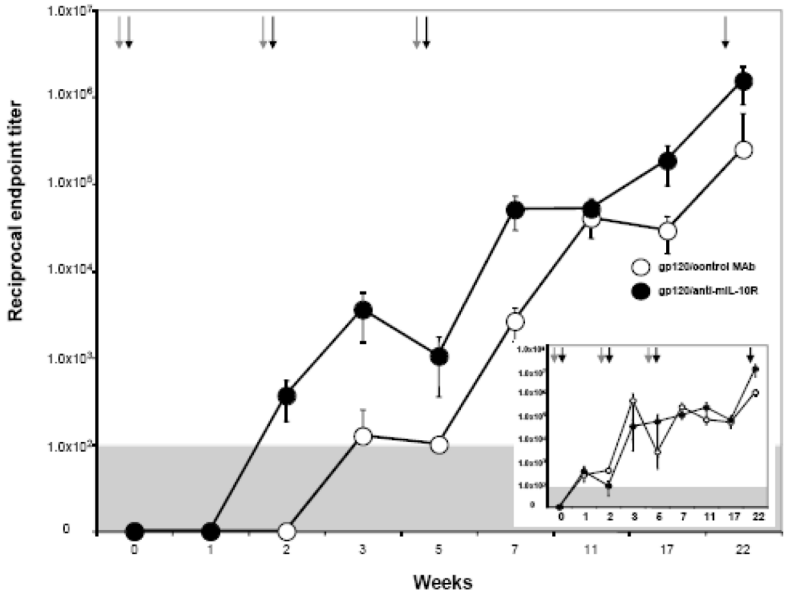

Figure 6. Blocking IL-10 activity during immunization with gp120 in Alum adjuvant, but not Quil A, increases the anti-gp120 titer in C57BL/6 mice

Mice were immunized with gp120 in the presence of an anti-mIL-10R MAb (black symbols) or an isotype control MAb (white symbols) at the times indicated by the arrows (black, gp120; grey, MAbs). Main figure: Alum adjuvant; inset, Quil A adjuvant. Each symbol represents the mean (± SEM) reciprocal endpoint anti-gp120 titer for each group of mice (n=5 initially, but n=4 for gp120 + control MAb in Alum from week 2 due to the death of one mouse from an unrelated cause). Titers <100 (grey shaded area) are considered negative.

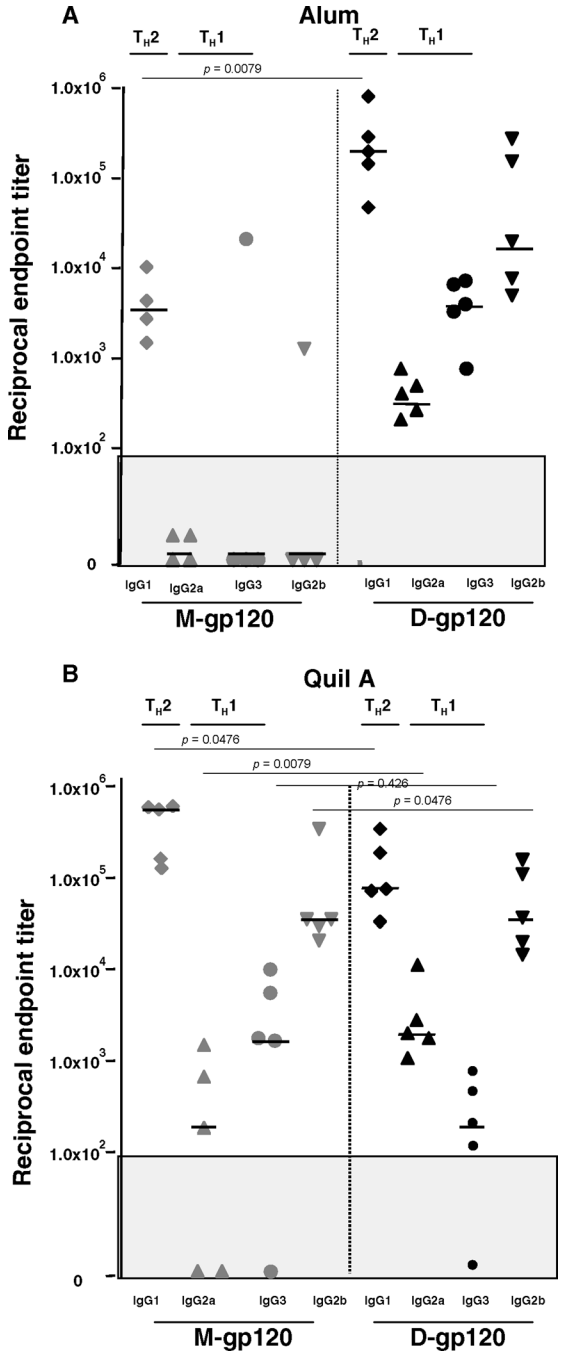

Figure 7. gp120-specific IgG subclass responses in C57BL/6 mice immunized with M-gp120 or D-gp120

Serum IgG subclasses were determined 7 weeks after primary immunization. Shown are reciprocal endpoint titers for each individual mouse (horizontal bar indicates median values) that received M-gp120 (grey symbols) or D-gp120 batch #1 (black symbols) in either Alum (panel A) or Quil A (panel B) adjuvants. Negative responders (titer < 100) are shown in the grey boxed area.

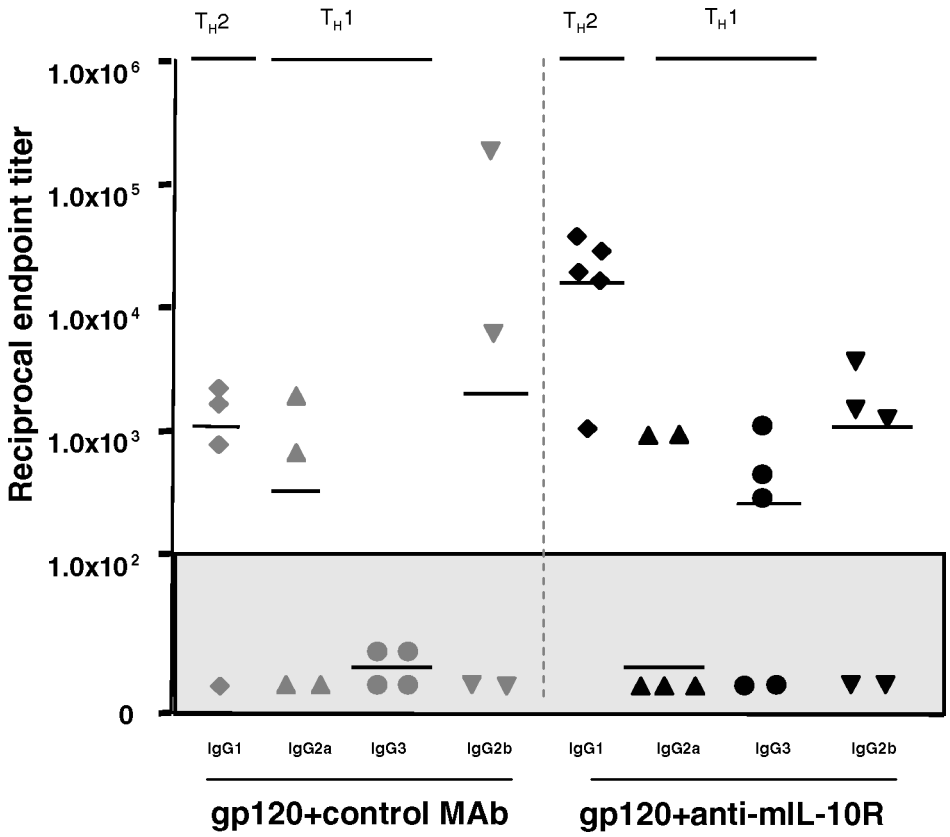

Figure 8. gp120-specific IgG subclass responses in C57BL/6 mice immunized with gp120 in the presence or absence of an IL-10 receptor Mab

Serum IgG subclasses were determined 7 weeks after primary immunization. Shown are reciprocal endpoint titers for each individual mouse (horizontal bar indicates median values) that received gp120 together with an anti-IL-10 receptor MAb (grey symbols) or an isotype control MAb (black symbols) in Alum adjuvant. Negative responders (titer < 100) are shown in the grey boxed area.

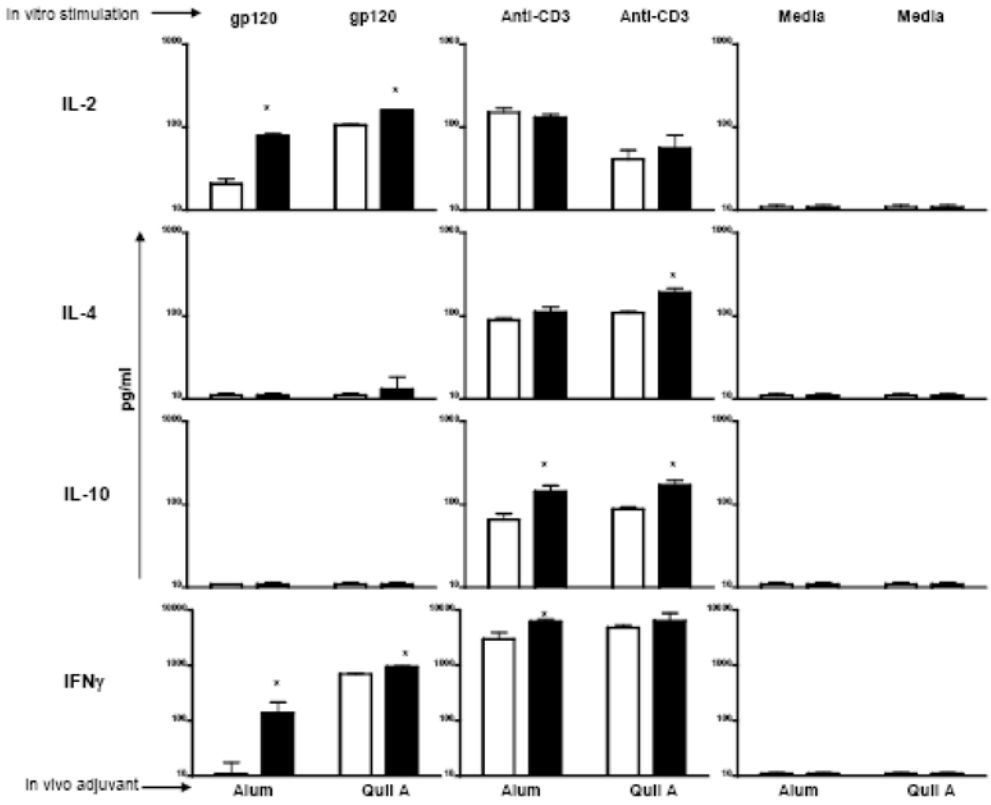

Figure 9. D-gp120 immunization improves antigen specific T cell responses

Unfractionated splenocytes (2.5×10^5 cells/well) derived from the indicated groups of C57BL/6 mice were cultured for 72h with 10μg/ml of recombinant gp120 (left panel), 2μg/ml of anti-CD3 (center panel) or media (right panel). Cytokine levels in supernatants from triplicate wells were measured by ELISA and are expressed in pg/ml as the mean value ± SD above the respective assay detection limits. The mice were immunized with M-gp120 (white bars) or D-gp120 batch #1 (black bars) in the adjuvant indicated at the foot of the figure. * $p < 0.05$ (one-tailed Student's *t* test).

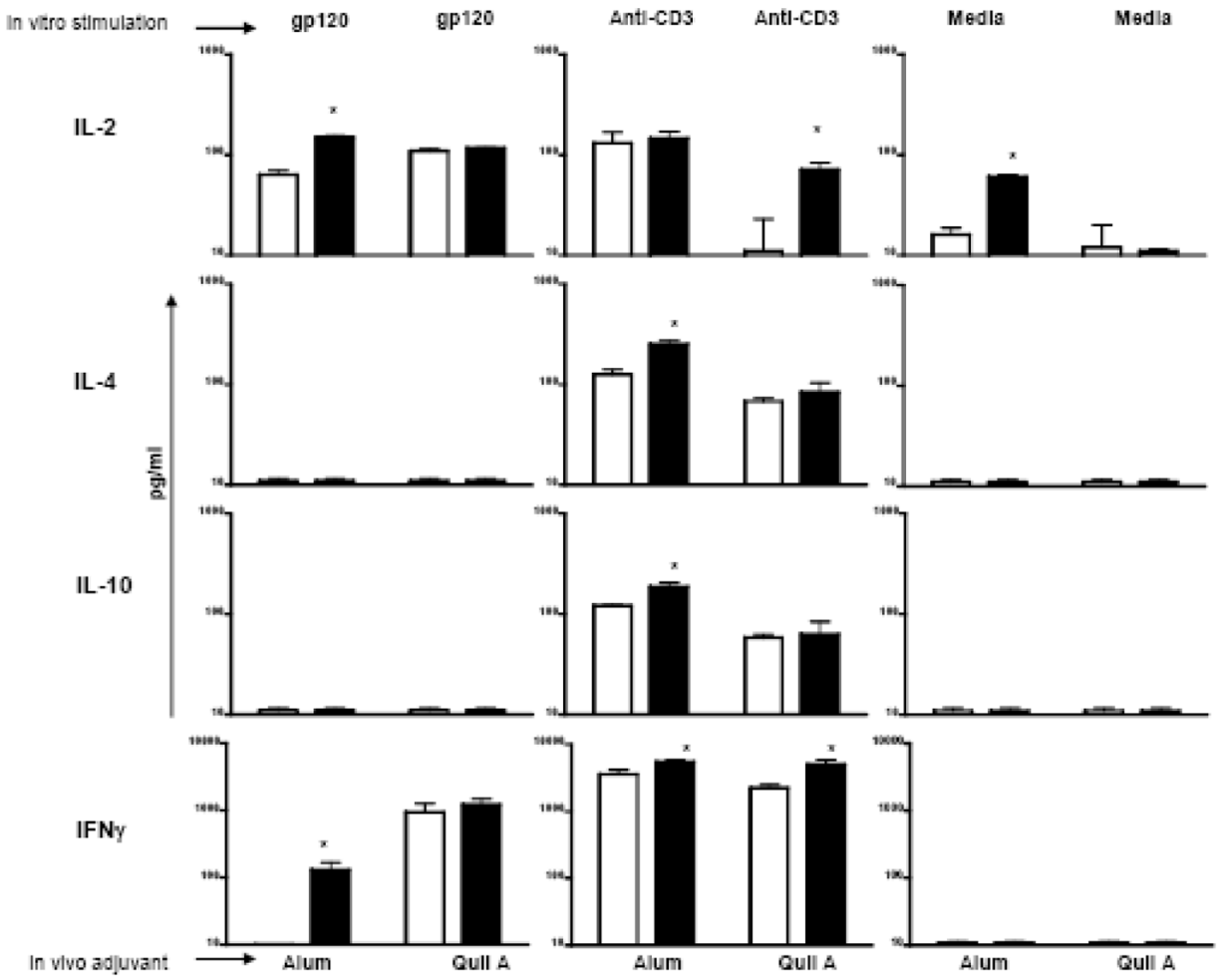

Figure 10. Antigen-specific T cell responses from gp120-immunized C57BL/6 mice

Unfractionated splenocytes (2.5×10^5 cells/well) derived from the indicated groups of C57BL/6 mice were cultured for 72h with 10μg/ml of recombinant gp120 (left panel), 2μg/ml of anti-CD3 (center panel) or media (right panel). Cytokine levels in supernatants from triplicate wells were measured by ELISA and are expressed in pg/ml as the mean value ± SD (the y-axis starts at the manufacturer specified detection limit for each cytokine). The mice were immunized with gp120 in the adjuvant indicated at the foot of the figure, and in the presence of an isotype control MAb (white bars) or the anti-mIL-10R MAb (black bars). * $p < 0.05$ (one-tailed Student's *t* test).

Table 1

Measurement of mannosidase activity

Sample	Condition	Enzyme activity (units/ml; %)
Demannosylation reaction mixture	fresh enzyme added to gp120 (time zero)	1.420 (100%)
Enzyme (mock treated)	treated similarly to that of D-gp120 batch #1	0.212 (14.9%)
D-gp120 batch #1	before CHT purification	0.045 (3.2%)
D-gp120 batch #2	after CHT purification	ND
Enzyme (inactivated)	boiling (100°C, 10min)	ND

The activity of α(1–2,3,6)-mannosidase in various preparations was determined according to the manufacturer's recommendation (see Methods). The activity of enzyme added to the reaction mixture at the start of the demannosylation procedure was 14.2 units/mg gp120 protein or 1.42 unit/ml. This was defined as 100%, for normalization purposes. ND, Not detectable; i.e., below the assay detection limit of 0.01 unit/ml.

Table 2

IgG subclass response to gp120 immunizations

Reciprocal titers in each group were ranked in ascending order when the endpoint titers were ≥ 100. The T_H1:T_H2 index was calculated as described in Materials and Methods

Adjuvant	Protein	MAb	T_H1:T_H2 index ([IgG2a+IgG3]/2)/IgG1[a]	Mann-Whitney U test (*p* values)
Alum	M-gp120	-	1.3[b]	0.082
	D-gp120	-	0.9	
Quil A	M-gp120	-	0.4	0.0087[c]
	D-gp120	-	1.7	
Alum	gp120	Isotype control	1.8	0.18[d]
	gp120	Anti-mIL-10R	0.6	
Quil A	gp120	Isotype control	1.3	0.24
	gp120	Anti-mIL-10R	1.0	

[a] Ranks of reciprocal subclass titers within each adjuvant category (n=11–12) were entered into the formula to calculate an index for each serum.

[b] Numbers are medians in each subgroup of mice, n=5–6.

[c] Significant differences by two-tailed comparison are marked in bold.

[d] Non-parametric comparison, hence the fold difference in absolute medians is not indicative of, and less important than how much the distributions overlap.