

How Can HIV-Type-1-Env Immunogenicity Be Improved to Facilitate Antibody-Based Vaccine Development?

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Abstract

No vaccine candidate has induced antibodies (Abs) that efficiently neutralize multiple primary isolates of HIV-1. Preexisting high titers of neutralizing antibodies (NAbs) are essential, because the virus establishes infection before anamnestic responses could take effect. HIV-1 infection elicits Abs against Env, Gag, and other viral proteins, but of these only a subset of the anti-Env Abs can neutralize the virus. Whereas the corresponding proteins from other viruses form the basis of successful vaccines, multiple large doses of HIV-1 Env elicit low, transient titers of Abs that are not protective in humans. The inaccessibility of neutralization epitopes hinders NAb induction, but Env may also subvert the immune response by interacting with receptors on T cells, B cells, monocytes, macrophages, and dendritic cells. Here, we discuss evidence from immunizations of different species with various modified Env constructs. We also suggest how the divergent Ab responses to Gag and Env during infection may reflect differences in B cell regulation. Drawing on these analyses, we outline strategies for improving Env as a component of a vaccine aimed at inducing strong and sustained NAb responses.

The Neutralizing Antibody Response as a Block to HIV Type 1 Transmission

THE ENVELOPE GLYCOPROTEIN COMPLEX (Env) studs the HIV-1 virion and mediates entry into target cells. Env consists of trimers of heterodimers, with each unit of the transmembrane protein, gp41, noncovalently attached to a monomer of the receptor-binding surface glycoprotein, gp120. The Env complex is the only target for neutralizing antibodies (NAbs), i.e., Abs that block productive viral entry.^{1,2} Other structural components (e.g., Gag) are immunogenic during infection, but the resulting Abs are not neutralizing, because the target proteins are inaccessible within the virion. Passive immunization of macaques with NAbs protects against virus transmission,³⁻⁵ whereas Env-binding, nonneutralizing Abs, depending on the epitope to which they are directed, are completely inert or markedly less effective⁶ (D.R. Burton and J.P. Moore, unpublished observations). Although we consider NAbs crucial to protection, much of what we discuss below applies also to nonneutralizing Abs.

Antigenicity and immunogenicity are both important aspects of the Ab response to Env. *Antigenicity*, the capacity to be recognized by an immune response, is problematic for Env,

because many Abs bind to denatured, disassembled, or incompletely processed Env components but not to the functional trimer. Flexibility of the Env complex can impede interactions with Abs.⁷ In addition, the virus rapidly mutates its *env* gene, thereby escaping from NAbs by changing its antigenicity.^{8,9} *Immunogenicity*, the capacity to elicit an immune response, is also restricted for the Env complex: much of its surface is shielded by high-mannose glycans, rendering it largely immunosilent.² Furthermore, gp120 specifically binds to important immune-system molecules: CD4, chemokine receptors, mannose-binding C-type lectin receptors (MCLR), and the homing integrin $\alpha_4\beta_7$,¹⁰ thereby potentially perturbing key players in the immune response such as T and B lymphocytes, monocytes, macrophages, and dendritic cells. There are cross-reactive neutralization epitopes on gp41, but NAbs against them are rarely elicited during infection and never to date by engineered protein mimics.^{11,12} Other gp41 epitopes are immunodominant but bind nonneutralizing Abs.¹³ The gp41 subunit, like its counterparts from other retroviruses, has been implicated in immunosuppression, at least *in vitro*.¹⁴

How HIV-1 establishes infection in a new host poses particular challenges to vaccination. Immune reactions to

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incoming viruses normally benefit the host, but they can perversely favor HIV-1 by increasing target cell numbers.^{15,16} Furthermore, HIV-1 destroys large populations of T-helper (T_H) cells and inductive sites for B cells in the intestinal mucosa during acute infection.^{17,18} The damage might undermine any vaccine-primed anamnestic responses to an incoming virus. In the macaque model, passive immunization by NAb transfer is protective when started 6 h, but not 24 h, after virus inoculation.¹⁹ By analogy, anamnestic immune responses in vaccinees may not have time to outpace the incoming virus.²⁰ Together, the disadvantages of immune activation, the early pathogenic effects, and the time constraints emphasize the importance of eliciting preexisting high titers of NAbs (Fig. 1).

The NAb response that eventually matures in a minority of HIV-1-infected patients is stronger and broader than anything that has yet been accomplished by Env vaccination.²¹ Compared with cellular immune responses, NAbs do little to curb viral loads, but do they protect against superinfection? The evidence, though inconclusive, is not encouraging. The incidence of superinfection varies from 50% to 100% of the primary-infection rate in uninfected control cohorts,^{22–24} and superinfection occurs in the face of substantial cross-neutralizing titers, also specifically against early posttransmission forms of the virus.²² However, the minority of subjects with

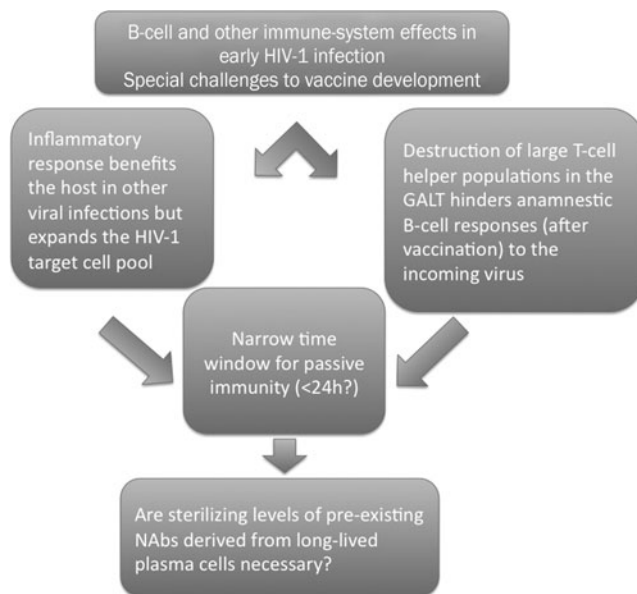


FIG. 1. The early effects of HIV-1 infection on the immune system pose atypical demands on a vaccine. On the one hand, the inflammatory response benefits the virus by providing an expanded pool of susceptible cells (e.g., lymphocyte activation leads to increased CCR5 expression). Conversely, CD4⁺ T lymphocytes are rapidly destroyed in the gut-associated lymphoid tissue (GALT), which deprives the B cell response of much early T cell help. Studies of the timing of postexposure passive immunization after SHIV challenge of macaques show that to prevent infection, the Ab must be given earlier than 24 h after the virus. The time window for immune prevention of the establishment of infection may be similar, and hence too short for an anamnestic response to be effective; the most feasible safety net would be preexisting high titers of neutralizing antibodies.

broad and strong NAb responses may be too small to make a detectable impact, and the NAb titers may even more rarely reach the high levels found experimentally to be required for protection.

What Env vaccine could then induce better NAb responses than infection does? The critical structural aspects of Env vaccine design have been well addressed elsewhere,^{2,25} as have adjuvant strategies based on innate immunity.^{26,27} Here, we discuss which features of Env may limit its immunogenicity. We compare Env with the nonglycosylated core protein Gag, as well as with viral proteins used in other vaccines, and we outline how Env may suppress and divert precisely the kind of Ab responses that an effective vaccine must elicit. Many, often adverse, effects of gp120 and gp41 components on immune-system cells have been studied *in vitro*. The required concentrations of the proteins or peptides can be so high that the observed effects are of questionable relevance to pathogenesis.²⁸ However, the local Env concentrations soon after vaccination could be in a range that makes some *in vitro* studies pertinent to immunization strategies.

The B Cell Response to Env in HIV-1 Infection

B cell responses have many dimensions, including thymus-dependent (TD) vs. thymus-independent (TI) stimulation; Ab-isotype profiles, partly reflecting T-helper (T_H) polarization; memory B cells vs. long-lived plasma cells; and high vs. low antigen-concentration dependence. In discussing the Ab response to HIV-1 infection, we focus on how these characteristics relate to vaccine research. For a more detailed discussion of HIV-1-induced B cell abnormalities and pathogenic mechanisms, other reviews should be consulted.^{29,30}

Hypergammaglobulinemia (hyper-GG) is a hallmark of untreated HIV-1 infection.^{31,32} The excess IgG is directed against Ags from various microorganisms, including HIV-1, and some of it cross-reacts with autoantigens.^{17,33–35} HIV-1 also causes B cell depletion.^{30,36} Both the hyperstimulation of B cells and their loss can hinder NAb production. Inductive anatomical niches and survival factors may be scarce commodities — not least for long-lived plasma cell differentiation³⁷ — easily squandered on the nonprotective hyperproliferation that contributes to hyper-GG. Losing naive B cells may remove NAb-producing precursors at an early stage, while depleting memory B cells could reduce somatic hypermutation (SHM), thereby compromising the development of broadly active NAbs.^{38–41}

Differential responses to Gag and Env

Within weeks of HIV-1 infection, humans seroconvert to multiple HIV-1 Ags, most prominently to Gag, Pol, and Env. While Ab titers to gp120 and gp41 remain high, those to the Gag protein p24 decline with disease progression^{42,43} (Table 1). Why do anti-Gag and anti-Env titers diverge? Although p24 antigenemia rises concomitantly, absorption of Abs by Ag does not explain the loss of anti-Gag Abs. First, the molar excess of Ab over Ag is vast.^{28,42} Second, Ag drives Ab production,^{44–46} e.g., NAb titers are higher in people with elevated Ag levels than in long-term nonprogressors and effectively treated patients.^{29,47,48} Third, Gag can be less abundant than Env, at least in lymph nodes.⁴⁹

An alternative explanation is that Env and Gag Ab responses are stimulated differently. The Gag response may be

TABLE 1. PARADOXES OF THE ANTIBODY RESPONSE TO HIV-1

In spite of immune pathology, the neutralization response is generally higher and broader during infection than after immunization with gp120.
Cross-neutralizing responses are slow to develop (months, years), slower than other Abs to both Gag and Env, but often directed to single, dominant epitopes.
NAb titers are generally lower in patients with undetectable than in those with high viral loads.
During clinical progression Ab levels to Gag, but not Env, decline. Absorption by Ag is not responsible. Are Gag and Env responses regulated differently?

Ab, antibody; NAb, neutralizing antibody; Ag, antigen.

strongly TD and therefore particularly sensitive to CD4⁺ T cell losses.^{42,50} In contrast, the Env response may have a TI component, may depend strongly on Ag levels, and could be enhanced by continuous sequence evolution, the latter mechanism being less applicable to the more conserved Gag protein.^{23,36} In other words, the Env-directed Ab response, which is sustained in the face of declining T cell help, may reflect continual stimulation by evolving antigenic variants of Env rather than the continued production of Ab elicited by the Env protein of the originally infecting virus. How do these hypotheses agree with clinical and experimental observations?

When CD20⁺ B cells were depleted to treat a lymphoplasmacytoid lymphoma in an HIV-1-positive patient, the autologous-virus NAb titer dropped 3-fold over the following 3 months while viral loads increased 50-fold.⁵¹ During this period, the virus became more neutralization sensitive; NAb titers were eventually restored and viral loads were again suppressed. Although we cannot generalize from a single case, the dynamic changes suggest that NAbs can suppress HIV-1 replication. Of particular note is that plasma cells do not express CD20. Hence, long-lived plasma cells may not have been responsible for the NAb titers in this patient. The generation of long-lived plasma cells is typically a TD process. Overall, in this clinical case, the Ab response to virus-derived Env may have resembled the transient responses to Env protein immunization (see below).

Only 17 cases of seronegative HIV-1 infection have been described, all with otherwise normal Ig levels but high viral loads.⁵² It seems likely that these patients never developed an Ab response to the virus, rather than doing so early on and then seroreverting.⁵³ In principle, early viral damage to T_H cells could account for the failure to seroconvert, but so could more direct adverse effects on B cells. Thus, severe losses of activated memory B cells during acute SIV infection of macaques are associated with lack of seroconversion; blocking the Programmed-Death-1 (PD-1) pathway of apoptosis prevents these losses and restores the suppressed Ab titers to both Gag and Env.^{54,55} When antiretroviral therapy (ART) suppressed viremia in six of the seronegative humans, four seroconverted to the major antigens within 1–9 months, one did so only to Env, and one remained completely seronegative.⁵² The increase in Ab production could reflect the restoration of T_H function, which might indicate that both the Gag and Env responses are TD. Alternatively, recovery of B cells may be directly responsible, since ART normalizes the numbers of naive and resting memory B cells.⁵⁶ Overall, these

various studies are inconclusive as to the relative TD and TI contributions to the Gag and Env Ab responses. More direct evidence came from a study in which T_H cell costimulation of B cells was blocked in SIV-infected macaques. The intervention delayed the Ab response to viral antigens, including NAbs against a sensitive test virus.⁵⁷ This suggests that at least the initial anti-Env Ab response is largely TD, although a TI component cannot be excluded.

There is indeed evidence that the anti-Env Ab response may have a TI element. Thus, Env-expressing virus-like particles can induce anti-Env Ab production in CD4 knockout mice,⁵⁸ and so can gp120-pulsed DCs in CD4⁺ T cell-depleted mice.⁵⁹ HIV-1, and Env in particular, can activate B cells in both an Ag-specific manner and by polyclonal, nonspecific mechanisms.^{33,60–62} These reactions are reminiscent of TI-1 responses, which involve both mature and immature B cells. Pathogen-associated molecular patterns (PAMPs), including those on viral envelope glycoproteins, often elicit TI-2 responses, which activate only mature B cells.⁶³ Some PAMPs directly activate B cells through interactions with pattern-recognition receptors and stereotyped B cell receptors (BCRs).^{64,65} If soluble or virion-bound gp120 interacts with B cells in this way, there could be synergy with its binding to MCLR. Together these stimuli may elicit secretion of interleukin (IL)-10 and B cell-activating factor of the TNF family (BAFF) and then CD40-independent class switch recombination (CSR) as well as Ab secretion⁶⁵ (Fig. 2). Overall, however, the relative contributions of TI and TD mechanisms to the anti-Env response remain unknown. As there are implications for Env vaccine responses (see below), this gap in our knowledge should be filled.

How do the Ab responses to Gag and Env vary with Ag concentration and T-helper cell levels? When people who have already seroconverted begin ART, both anti-Gag and anti-Env Ab responses generally decline as viremia is suppressed, although on rare occasions they rise.^{42,44,46,50,66,67} Both Ab responses seem, therefore, to be more responsive to Ag concentration changes than they are to the restored T cell help. A unique case for comparison is the possible eradication of HIV-1 through transplantation of bone marrow from a CCR5-Δ32-homozygous donor. Before transplantation, ART had suppressed the recipient's viral load to undetectable levels, whereas 20 months afterward the virus remained undetectable in the absence of ART. At that time, his previously strong Ab response to Gag had disappeared but anti-Env Abs, although declining, were still detectable.⁶⁸ Whether this difference was due to a longer half-life of Env- than Gag-specific plasma cells, or a slower turnover of Env than Gag Ag, cannot be determined.

Stores of nonreplicating Ag may help maintain HIV-1-specific Ab responses after ART suppresses viral replication. Both Gag and Env proteins can persist for months on follicular dendritic cells in lymph node germinal centers, in the absence of detectable viral RNA.⁶⁷ MCLR expressed by these cells might trap Env, but other mechanisms such as Fc-receptor-mediated capture of immune complexes must also contribute to the maintenance of Ag depots, particularly for non-glycosylated Gag proteins that are often present at higher levels than Env.^{49,67} Hence, individual differences in the amounts of trapped Gag and Env protein may contribute to the variation in the corresponding Ab levels in ART recipients.^{44,67}

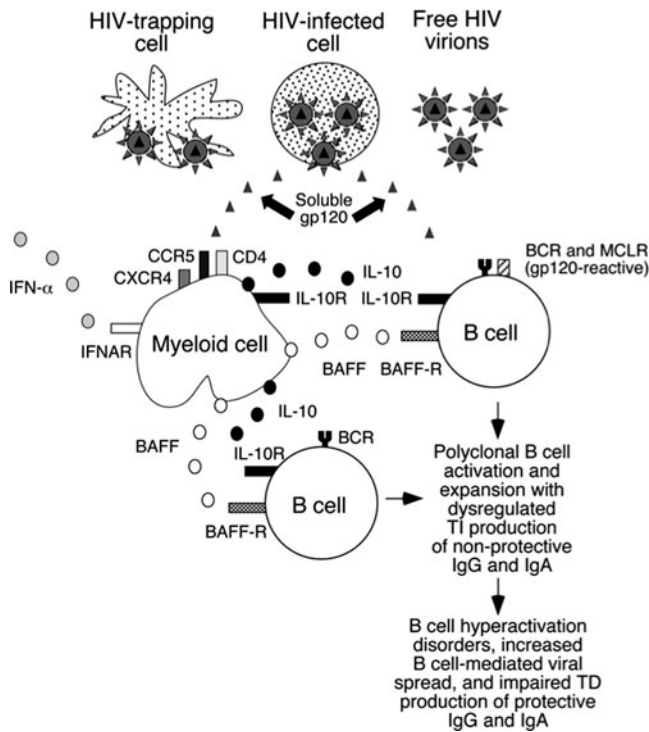


FIG. 2. The interplay among gp120, interferon (IFN)- α , interleukin (IL)-10, B cell-activating factor of the TNF family (BAFF), and the receptors CD4, CXCR4, CCR5, IFN- α -R, BAFF-R, mannose-binding C-type lectin receptors (MCLR), IL-10R, and B cell receptors (BCRs). gp120 (or other forms of Env) can be present on infected cells, on the virion surface, as a soluble protein, or attached to MCLR- or CD4-expressing cells. gp120 and IL-10 together promote IgG and IgA class switch recombination (CSRs), but Ab secretion requires BAFF and BCR engagement; gp120 may induce IL-10 secretion from B cells, monocytes, and dendritic cells via MCLR binding, and it triggers BAFF secretion from monocytes and macrophages. IL-10 and IFN- α also induce BAFF, which can upregulate MCLR. Env proteins may also engage stereotypical BCRs. The net result of this CD40L-independent circuitry may be impaired development of high-affinity Abs to Env, particularly of the rare cross-neutralizing specificities. (Copyright 2006. The American Association of Immunologists, Inc.)

The cross-neutralizing Ab response during infection appears to be Ag concentration dependent in that it correlates with viremia,^{47,48,69–71} particularly with the early levels of antigenemia.⁷² This broad neutralization response takes 2–3 years to develop,⁷¹ during which time multiple Env variants are presented to the immune system. Paradoxically, the substantial heterogeneity in Env may by default boost the response to the most conserved epitopes. Similarly, superinfection, which presents new sets of Env variants, is also followed by increased cross-neutralization.^{22,23} However, it is not only the amount of Ag or how much it varies that may favor the development of broad neutralization responses. Some variants appear to have a stronger propensity to induce such responses: amino acid signatures in Env, particularly in the CD4-binding site, have been identified as predictors of the strength and breadth of the responses that will be elicited by infection with a particular HIV-1 variant.⁷³ Furthermore,

neutralization breadth correlates with the degree of activation of CD4⁺ T cells, which suggests that this component of the Ab response to Env is TD.⁷¹ It is important to ascertain whether the broad neutralization response is more T-helper cell dependent than the general Ab response to Env.

T_H1 or T_H2 polarization is not clearly reflected in the human IgG subclass profile of the response to HIV-1 since IgG1 Abs, which are not associated with either T-helper-cell subset,⁷⁴ dominate the response to all the viral antigens.^{75–78} IgG2 responses can be TD, and when they are, they are linked to T_H1 polarization.⁷⁴ However, IgG2 Abs can also be elicited as a TI response to repetitive carbohydrate epitopes. Among asymptomatic HIV-1-positive patients, IgG2 Abs specific for gp41 in Western blot were associated with low viral load and weak neutralizing titers, whereas IgG1 Env reactivity correlated positively with viral load.⁷⁹ Together, HIV-1-specific T_H1 T cell responses and anti-gp41 IgG2 Abs were reported to provide an excellent prognostic marker for lack of clinical progression.⁸⁰ In a cross-sectional study, IgG2 reactivity with glycosylated gp41 was also linked to asymptomatic infection, but IgG2 reactive with nonglycosylated gp41 showed no such association.⁷⁸ In line with the latter finding, when gp41-specific IgG2 was detected with nonglycosylated peptides, no difference in reactivity between viremic and virus-controlling patients was seen.⁷⁵ To what extent IgG2 can be elicited against glycosylation-dependent epitopes on Env in a TI fashion remains to be determined. IgG4 Abs, associated with T_H2 polarization in humans, are rarely reactive with HIV-1 proteins, although more frequently with Gag than Env.^{76–78}

Overall, the B cell responses to Gag and Env are not easily categorized. Both responses fluctuate with Ag concentration; they may both be largely TD, but it remains a viable hypothesis that Env also elicits responses with prominent TI components (Fig. 2). Any TI responses that Env may trigger are highly relevant to vaccination and deserve further scrutiny. Perhaps the most striking difference between the two viral proteins, the extent to which they vary in sequence, provides the best clue to how they differ as immunogens during infection. The extraordinary variation in Env, partly driven by NAb selection pressure,⁸¹ may repeatedly prime new Ab responses rather than boost the already formed specificities. The repeated stimulation by Env neopeptides could be what sustains anti-Env Ab levels when anti-Gag Abs are lost during untreated HIV-1 infection. As a complication, the extreme and prolonged antigenic stimulation may also, in the end, lead to B cell exhaustion. Of course any stimulatory mechanisms of the Ab response that depend on Env hypervariability will not apply when sequence-invariant Env vaccines are used. Nevertheless, the transience of the Ab responses to Env vaccines, by analogy to what was seen in a case of iatrogenic B cell depletion during HIV-1 infection,⁵¹ may be attributable to a deficiency in the generation of long-lived plasma cells.

The Ab Response to Env Vaccines

How do Ab responses to Env differ between infection and vaccination? The lack of protection by Env subunit vaccines allows vaccine- and infection-induced Ab responses to be compared in the same individuals. Thus, 12 people who had been immunized at least three times with gp120 (eight with MN and four with SF-2) had a low range of peak mid-point

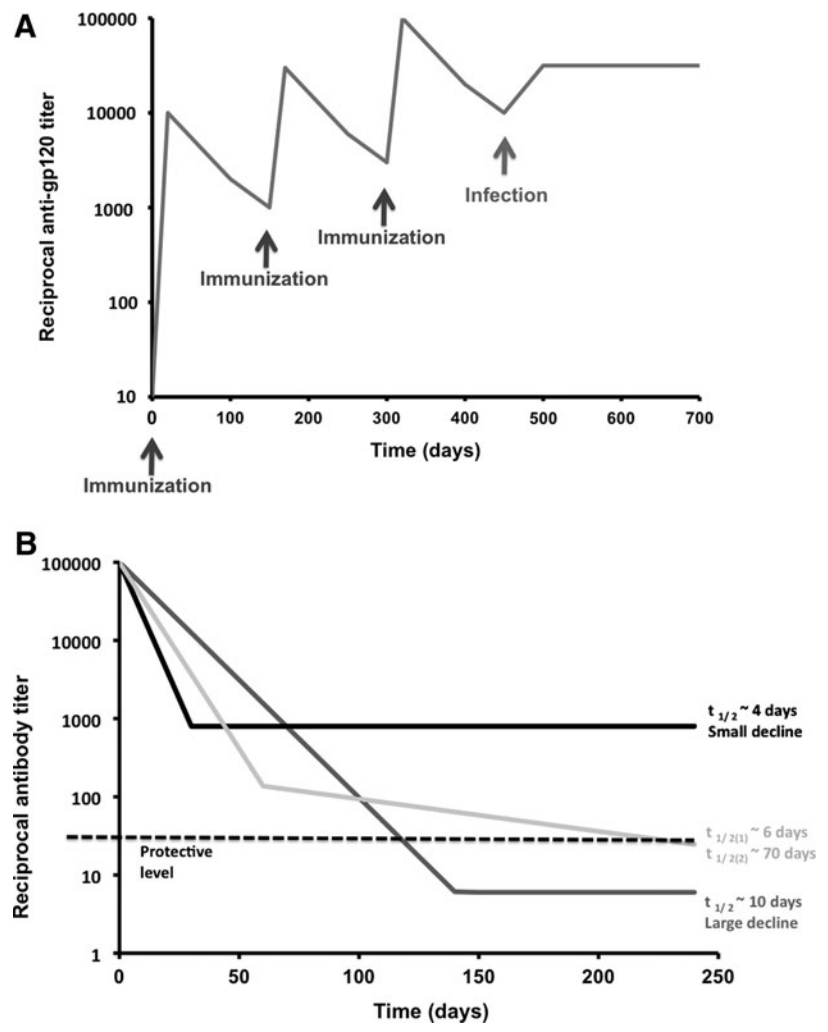
titers of Ab to heterologous gp120 (100–13,000 as reciprocal titers, also given henceforth). Stable titers against the same antigens in the same individuals after infection were higher (10,000–1,000,000), as were the titers in 16 other infected individuals (62,000–94,000).⁸² Another comparison found end-point titers to gp120 of 300–200,000 in gp120-vaccinated volunteers and 500–30,000,000 in infected people.⁸³ The wide spread of titers could reflect genetic influences on the gp120-specific responses to infection as well as to immunization.⁸⁴

The kinetics of the Ab response to Env vaccines

Both gp120-protein-only and DNA-prime with gp120-protein-boost immunizations of humans induce Ab responses that fade away within 2 years. Anti-gp120 titers increase after every injection, but less each time, and then drop rapidly with initial half-lives of ~50 days^{82,85–87} (Fig. 3A). In one study, titers fell during the year after a prime-boost regimen as rapidly as they rose during the immunization process and although the decline decelerated, the titers trended toward undetectable levels⁸⁸ (Fig. 3B). The titer kinetics in nonhuman

primates given HIV-1 or SIV Env proteins resemble those of the human response.^{89,90} In contrast, when infection occurs in gp120 vaccinees, anti-gp120 titers jump to high levels that are sustained throughout infection.⁸² The decay in the anti-gp120 titer when ART suppresses HIV-1 replication in infected people is biphasic; a rapid but small decline is followed by a more stable phase⁴⁴ (Fig. 3B). When cynomolgus macaques were simultaneously immunized with HIV-1 Env trimers (in one leg) and influenza HA monomers (in the other leg), IgG titers to each antigen declined at similar rates after the second immunization. The half-lives were around 2 weeks for both IgG responses during an initial phase, which was followed by a more stable second phase (C. Sundling and G. Karlsson Hedestam, personal communication). After gp120 vaccination of humans, this second phase is usually either absent or the initial response is too weak to allow its detection. Whether vaccine recipients have any detectable anti-Env plasma Abs several years after immunization is not known: Long-term follow-up studies have not been described. Nor is it known whether the magnitude of the peak response to Env vaccines (which varies substantially among individuals) predicts the

FIG. 3. (A) The characteristic saw-tooth pattern of anti-gp120 titers after vaccination is shown on a log scale as a function of time. After each immunization (black arrows) with typically 200 μ g of purified, recombinant gp120 in Alum, the binding-Ab titer rises by a log. The titers then decline rapidly. When infection occurs (gray arrow), the titers tend to stabilize at a high plateau. The kinetics of neutralization titers against a hypersensitive HIV-1 strain such as a TCLA virus or SF162 would look similar to the saw-tooth pattern of the first part of the curve, but there would be little or no cross-neutralization of primary isolates. However, several months after infection, significant titers of primary virus-neutralizing Abs do often develop. In **(B)**, three schematic curves illustrate distinct features of immune responses to viral vaccines and infections. Time 0 can represent the time of completion of an immunization series or the initiation of antiretroviral therapy (ART) during infection. In the latter case phases with different rates of decline have been observed; in the former they are hypothetical. The magnitude of the antibody titer decline to a stable level is at least as important as the half-life of the early response. The black curve shows the response with the shortest half-life in the initial phase, ~4 days, but it also has the smallest decline. The dark gray curve shows a longer initial half-life (10 days), but the decline is large. The light gray curve shows a biphasic decline with an initial intermediate half-life of ~6 days, and ~70 days in the second phase. If a minimal protective level equal to a reciprocal titer of 30 (dashed black line) is postulated, the plateau of the black curve falls above that cut-off, the dark gray curve below it. Thus, only the black curve with the initial shortest half-life remains safely above minimal protective levels (although it should be noted that no vaccine-induced Ab titer has been proven to confer protection against HIV-1). This diagram illustrates that the maximum degree of decline can be more important than the initial half-life in determining whether residual Ab levels are protective.



long-term outcome. Are strong initial responders more likely to sustain their responses for longer? Or does everyone's response decay at much the same rate and to the same eventual extent? Does the Ab response to some viral envelope glycoproteins decline more rapidly than to other soluble protein immunogens? The long-term response to Env vaccines might reflect specific aspects of the underlying B cell biology.

The average half-life of IgG titers in human serum after passive immunization is ~21 days,⁹¹ but it can vary widely among recipients of the same IgG preparation.⁹² The half-life of anti-gp120 IgG after immunization, ~40–60 days, is 2- to 3-fold longer,^{85,88} which suggests some continuous production of Abs occurs. In the short term, the declining Ab levels presumably also reflect how Ag concentrations diminish over time after vaccination. Long-lived plasma cells, which have down-modulated both BCR and MHC class II, are no longer sensitive to fluctuations in Ag levels, and they have substantially longer half-lives, of several months.⁹³ The Ab response to gp120 vaccination thus conforms to a pattern in which Ag-concentration-sensitive memory B cells convert to short-lived plasma cells with half-lives of days.⁹⁴ The pattern of titer decay suggests that Env-specific, long-lived plasma cells are not generated, which would hinder the creation of a protective vaccine that requires only a few immunizations rather than regular boosting. Hence, there is a need to investigate whether these very important long-lived plasma cells are elicited by Env vaccination and, if not, why not.

A recent model of *imprinted lifespan* of plasma cells suggests how to prolong humoral responses.⁹⁵ The model proposes that the duration of humoral immunity is determined only at the priming stage of the immune response, and not by subsequent boosting, by the persistence of trapped Ag after the germinal-center reaction,⁹⁶ by intermittent polyclonal activation, nor by the mere availability of bone marrow niches for plasma cell survival. Accordingly, different immunogens may elicit responses of characteristically distinct durations.^{95,97} The lifespan of the plasma cell would be proportional to the cumulative signals received by B cells during the critical priming phase of the immune response. This signaling would be mediated by T cell help, the cross-linking of B cell receptors, TLR activation by PAMPs, and stimuli from cytokines such as BAFF and APRIL. If an immunogen possesses only some of these features, responses might be improved by supplying missing elements. Given the brevity of the anti-Env vaccine response, this new model is well worth considering.

How much Env does an optimal immune response require?

The gp120 dose used in human trials is 200–600 μ g, the number of immunizations ranging from two after DNA priming to seven over 30 months in a protein-only regimen.^{86,98,99} When three 200- μ g immunizations were followed by a fourth of either 200 or 800 μ g, the higher dose elicited a 10-fold higher titer.⁹⁸ However, the functional activity of gp120, defined as CD4-binding capacity, varies several-fold from one affinity-purified preparation to another.¹⁰⁰ All gp120 preparations contain misfolded or denatured protein and the proportion of nonnative forms may be greater with trimeric Env, which tends to dissociate and aggregate.¹⁰¹ After immunization, Env could be degraded further, a process

that might be exacerbated by some adjuvants. Native Env molecules may, therefore, constitute only a small fraction of what the immune system encounters after vaccine administration; their quantity could indeed be suboptimal, for it is the functional activity rather than the total gp120 concentration that most influences the NAb response.¹⁰⁰ The nonnative forms of Env may compromise the induction of NABs by competing for T cell help and B cell survival factors.^{102,103}

Immunosuppression through Env–receptor interactions?

gp120, like some other viral vaccine immunogens, specifically binds receptors on cells that have key immune functions. When 300 μ g of gp120 in 1 ml is injected, the local concentration is initially 2.5 μ M, which would, e.g., give >95% occupancy of CD4. For comparison, the highest level of gp120 found in lymph nodes of HIV-1-infected patients is 70 pM, i.e., ~40,000-fold lower. Moreover, no gp120-specific Abs are present immediately after immunization to block gp120–receptor interactions, whereas they are usually in vast excess over gp120 during infection. It is therefore possible that gp120 could have effects near the injection site similar to those observed in cell culture systems,²⁸ such as inducing apoptosis of T cells, arresting the differentiation of dendritic cells, reducing IL-12 production, and increasing IL-10 and BAFF secretion.^{65,104–107} By interacting with CD4 and coreceptors on monocytes, gp120 elicits the secretion of BAFF, which cooperates with gp120 in TI B cell activation⁶⁵ (Fig. 2). The resulting TI cascades could harm the vaccine response by terminally differentiating and exhausting B cells, thereby reducing the number available for the TD responses that yield high-affinity Abs and long-lived plasma cells, which sustain high levels of Ab secretion. Furthermore, both the low-affinity Env-specific and the polyclonally activated B cells might compete with the few NAB-producing B cells for survival factors.

Env immunogens can be modified to eliminate potentially adverse receptor interactions, although a complication is the overlap between receptor-binding sites and important neutralization epitopes. For example, the V2 region of gp120 interacts with the activated form of the homing integrin $\alpha_4\beta_7$ and overlaps the epitope for the potent cross-neutralizing Abs PG9 and PG16.¹⁰⁸ Preserving the latter structures is important, and yet Env interactions with homing receptors could be detrimental to immunogenicity. Thus, gp120 may block the intestinal homing of CD4⁺ T cells by binding to integrin $\alpha_4\beta_7$ ¹⁰ and of B cells by interacting with CCR5 and CXCR4.¹⁰⁹ If this occurs *in vivo*, intestinal B cells would be deprived of essential T cell help, and IgA-switched B cells diverted from the intestine, thereby compromising NAB-mediated protection of mucosal surfaces. The gp120–CD4 interaction has been proposed to impair T-helper responses.¹¹⁰ Mutating the CD4-binding site of gp120, to avoid such effects, reduced NAb titers in primates to that site and to CD4-induced epitopes. However, neither the overall anti-gp120 titers nor the Env-specific CD4 T cell responses were affected, arguing against a suppressive effect of CD4 binding *in vivo*.¹¹¹ Similarly, gp120 does not induce markedly superior Ab responses in rodents, despite being unable to bind CD4 and coreceptors in those species. It is therefore doubtful whether the CD4-binding capacity needs to be eliminated from Env vaccines.

The mannose residues on gp120 mediate binding to MCLR on dendritic cells, thereby potentially affecting T_H1 , T_H2 , T_H17 , and T_{reg} responses and tolerance.^{112,113} gp120 also binds to BDCA-2 (CD303), an MCLR specifically expressed on plasmacytoid dendritic cells, thereby suppressing tumor necrosis factor (TNF)- α and interferon (IFN)- α secretion.¹¹⁴ In addition, mannose residues can enhance clearance of proteins by the liver.¹¹⁵ Demannosylating mammalian-cell-expressed gp120 for immunization of mice increased Ab titers by a factor of 10^{83} and blocking the mannose moieties by a lectin has a similar effect (K. Banerjee, K. Palmer, J.P. Moore, and P.J. Klasse, unpublished observations). How countering mannose residues increases Env immunogenicity remains to be explained, but one possible mechanism is by eliminating gp120–MCLR interactions that trigger IL-10 secretion, which in turn selectively suppresses effector and memory $CD4^+$ T cell responses.^{107,116} Whether the specific mannose-mediated interactions or the mimicry of self-antigens by the glycan shield^{117,118} most reduce Env immunogenicity remains to be understood.

The epitope for the HIV-1 cross-neutralizing NAb 2G12 encompasses mannosyl residues, i.e., components of the glycan shield.^{119–122} Although the 2G12 epitope is present on gp120 variants from many divergent HIV-1 isolates, it is immunogenic only exceptionally. Of note is that if NAb responses to the 2G12 epitope were frequent, the plasticity of the glycan shield would probably allow facile viral escape, and as a result this particular epitope would have disappeared from the HIV-1 strains of the pandemic.^{123,124} Hence the poor immunogenicity of the 2G12 epitope during HIV-1 infection, combined with its widespread antigenicity, makes it an important target for vaccine design.¹²⁵ Arguably, the same might apply to other, hypothetical epitopes formed by components of the glycan shield.

The block to the immunogenicity of the 2G12 epitope is not explained merely by its similarity to self-antigens: Abs recognizing the same Man α 1-2 Man motif are abundant in human sera, and even greater numbers of such Abs can be induced by mannose-rich immunogens resulting from blocked carbohydrate processing.¹²⁶ Unfortunately, these Abs do not neutralize HIV-1.^{118,127} What, then, is the origin of the rare 2G12 MAb specificity that does efficiently neutralize many HIV-1 strains? 2G12 has as high an affinity for mannose-dependent epitopes on two species of the fungus *Candida* (*albicans* and *tropicalis*) as it does for gp120, an affinity that is unusually high for an anticarbohydrate Ab.¹²³ It is possible that the 2G12 specificity was elicited by *Candida* and not HIV-1 Env.¹²³ Whether this hypothesis is correct or not, the fungal molecules might be better immunogens than Env, if their other epitopes are less immunodominant than some on gp120, and if they are less immunosuppressive.

Broadly neutralizing Abs to another cluster of epitopes on the HIV-1 Env complex also show some cross-reactivity with autoantigens. NAb 2F5 and 4E10, both recognizing amino acid motifs in the membrane-proximal external region (MPER) of gp41, have shown different degrees of cross-reactivity with endogenous phospholipids, such as cardiolipin.^{128–131} The cross-reactivity is more marked for 4E10 than 2F5.^{130,131} It has been suggested that this recognition of “self” would eliminate B cells with such specificities through tolerance mechanisms.¹²⁸ However, similar polyspecificity occurs in several viral infections, and this particular cross-reactivity

with phospholipids resembles those antiphospholipid responses of infections more than they do the autoimmune counterpart.^{38,130,132} The phospholipid epitopes on living, as opposed to apoptotic or necrotic, cells are largely occluded, which means that Abs with these specificities may not mediate pathogenic, autoimmune effects. Indeed, the host could benefit from Ab-mediated clearance of cellular and microbial debris.¹³² If elimination of autoreactive Abs is not the explanation for the poor immunogenicity of the MPER epitopes, the contribution of the contiguous lipids to the MPER epitopes may nevertheless explain why linear MPER peptide sequences, even when presented on optimized scaffolds, fail to induce strong NAb responses.^{11,12}

Hypermannosylated gp120 from the YU2 primary isolate, made in insect cells, induced only weak Ab responses in mice and rabbits.¹³³ In contrast, gp120 produced from the T cell line-adapted clone HXBc2 in the same cells yielded high titers and a strong T_H response. The immunogenicity of the primary-isolate gp120 was improved in various ways: by making the protein in mammalian instead of insect cells, thereby increasing carbohydrate processing and reducing its mannose content; by using Freund’s complete adjuvant instead of Ribi; and by denaturing the protein. The last two strategies, which disrupt gp120 structure and are therefore counterproductive, may facilitate protein processing for presentation of internal T_H epitopes.¹³⁴ The resulting T cell help would promote Ab responses to both the native and denatured proteins that are present in any gp120 preparation. A final strategy to improve the B cell response was grafting a universal MHC class II T_H epitope onto the gp120 C-terminus. This strengthened the T_H2 response at the expense of T_H1 , as assessed by the IgG subclass profile and cytokine induction.¹³³

When DNA vaccine encoding soluble HIV-1 gp120 or membrane-anchored influenza hemagglutinin was given to mice, the IgG titers against the two immunogens were similar but the gp120 response was more T_H2 biased. IL-10- or IL-4-defective mice responded to gp120 with a cytokine profile and an IgG subclass pattern that were more T_H1 polarized.¹³⁵ Likewise, demannosylation of mammalian-cell-expressed gp120 tipped the balance modestly toward T_H1 responses.⁸³ Another aspect of carbohydrate processing that can affect both gp120 antigenicity and immunogenicity is its sialic acid content; nonsialylated gp120 exposed CD4bs and CD4i epitopes better and induced stronger Ab responses than its sialylated counterpart.¹³⁶ Sialyl residues on the Fc portion mediate the binding of IgG to SIGN-R1, the murine orthologue of DC-SIGN, thereby initiating an antiinflammatory cascade.¹³⁷ If the sialyl residues on gp120 have similar effects, a certain redundancy is noteworthy since gp120 mannosylation also confers DC-SIGN binding.

Although interactions with DC-SIGN and other MCLR may hamper the immunogenicity of an Env-based immunogen, directing it to dendritic cells by other means could be beneficial. For example, tagging multiple α -galactose (α -Gal) epitopes to either p24 or gp120 increased their immunogenicity 10–100 times in mice that raise Abs to α -Gal because their α -1,3-galactosyltransferase gene has been knocked out.¹³⁸ Humans, like these engineered mice, do produce α -Gal Abs.¹³⁹ Natural Abs like these may help direct immunogens to follicular dendritic cells via CR2 and FcR γ .¹⁰² Similarly, precomplexing gp120 with nonneutralizing Abs increased anti-gp120 titers in rabbits (S. Phogat and W. Koff, personal

communication). This particular strategy could promote Env presentation to T_H cells while reducing unwanted interactions with BCRs specific for selected nonneutralization epitopes.

A recent study involving gp120 immunization of mice showed that silencing the ubiquitin-editing enzyme A20 offered several advantages: a preferential activation of T-helper over T_{reg} responses, an increased expression of the gut-homing receptors integrin $\alpha_4\beta_7$ and CCR9 on T and B lymphocytes in secondary lymphoid tissues, and increased titers of gp120-specific Abs, both serum IgG and mucosal sIgA. The mechanism of these changes is suggested by the role of A20 as an Ag-presentation attenuator that acts through feedback inhibition of the TNFR-, retinoic acid-inducible-gene I-, and TLR-signaling pathways.⁵⁹ Previously, improved Ab responses to gp120 and resistance to its immunosuppressive effects were achieved by silencing another Ag-presentation attenuator, the suppressor of cytokine signaling 1 (SOCS-1).¹⁴⁰ Even when these natural systems of brakes are not specifically activated by HIV-1 Env, countering them might be warranted to improve the problematic immunogenicity of that protein. Overall, safe pharmacological means of arousing the immune system temporarily by inhibiting the inhibitors could assist vaccination.¹⁴¹

In summary, the capacity of Env to signal via immunologically important receptors does not seem to compromise its immunogenicity, with the possible exception of MCLR interactions. However, a caveat is that usually only the short-lived, peak anti-gp120 response is measured in human and animal studies of Env immunizations. We noted above how the limited ability of gp120s to induce the TD responses that are important for the generation of long-lived plasma cells may be a severe hindrance to the development of sustained, high-level NAb responses. Whether the various receptor-binding capacities of Env play a role here is not known. It is also unknown whether the putative immunosuppressive motif in gp41¹⁴ affects the short- or longer-term immunogenicity of any Env immunogens that include this element. A systematic elimination of receptor-binding and other potentially immunosuppressive motifs from both gp120 and gp41 might elucidate whether these features influence the transient or sustained Ab responses to Env immunogens.

How and Why Anti-Env Responses Differ from Those to Other Immunogens

In a rare side-by-side comparison, volunteers were primed by four immunizations at one of four different mucosal sites, or both mucosally and intramuscularly, with a canarypox vector expressing either HIV-1 pseudovirus particles or the rabies virus glycoprotein.⁹⁹ They were then boosted twice intramuscularly with gp120 or the rabies vaccine Imovax. The serum IgG and IgA and the mucosal IgA responses to gp120 were all rarer than the corresponding responses to the rabies glycoprotein and canarypox proteins after priming, although a caveat is that expression levels and Ab detection sensitivities may have differed among the antigens. The study showed that combined systemic and mucosal priming was better than mucosal priming alone, and that IgA responses can be induced by a prime-boost strategy. But a more fundamental observation is that gp120 was less able to induce IgG and IgA responses than similarly delivered canarypox and rabies proteins.

The contrast between the responses to the human papilloma virus (HPV) vaccine and to HIV-1 vaccine candidates may be instructive. The HPV vaccine has shown 96–100% efficacy in preventing infection in a 5-year follow-up of women.¹⁴² It consists of the L1 protein from each of the four most pathogenically important strains (HPV-6, -11, -16, and -18), produced in yeast and forming virus-like particles. The Ab response is mostly of the IgG class with negligible IgA levels in the female genital tract.¹⁴³ IgG normally dominates at that site, unlike in other mucosae where IgA preponderates.¹⁴⁴ At least in women then, the HPV-protective Abs are likely to be IgG that transudates from plasma, although some may be actively transported or locally produced.¹⁴⁵ In the vaccine, 120 μ g of HPV L1 (30 μ g per variant) is adsorbed onto a variant of Alum adjuvant in 0.5 ml, and is given three times over 6 months. Thus, lower amounts of L1 are administered less often than gp120 (viz., 200–600 μ g per immunization, up to six times). The titers to all four HPV antigens drop ~10-fold over 30 months after the last immunization but then stabilize above baseline. For comparison, anti-gp120 titers fall 5- to 10-fold over 1 year, becoming undetectable.⁸⁸ Furthermore, the Ab responses to HPV vaccination and infection are equally strong,¹⁴² again in contrast to gp120 and HIV-1. The range of titers among the HPV vaccinees, <2-fold within the 95% confidence interval around the geometric mean, is also narrower than the corresponding range of anti-gp120 titers, ~10-fold.^{75,82} In addition, there may be a small group of particularly poor responders to gp120.^{82,146}

Immunization with hepatitis B virus (HBV) protein induces rapidly declining Ab responses but provides long-lasting protection. Boosting 5–22 years after initial immunization can raise titers ~100-fold, through a vigorous memory response.¹⁴⁷ The half-life of Ab levels is around 1 month, i.e., at least as short as for gp120. And, as with gp120, the range of titers among vaccinees can be wide.¹⁴⁸ Nonetheless, the majority of healthy, actively vaccinated subjects are protected even if they only develop weak Ab responses.¹⁴⁷ In contrast, after passive immunization, protection is rapidly lost as the anti-HBV Ab levels decline. To conclude, the successful HBV vaccine appears to rely on anamnestic responses, a mode of protection that is implausible in HIV-1 vaccination.

The long-term kinetics of protective Ab levels have been comprehensively studied for other pathogens. For example, Ab titers in response to a replicating vaccine (vaccinia), naturally infecting viruses (measles, mumps, rubella, varicella-zoster, and Epstein–Barr virus), and two nonreplicating bacterial vaccines (tetanus and diphtheria proteins) were monitored in 45 individuals over 26 years. A few recipients of vaccines against the nonpersistent measles, mumps, and rubella viruses were also included and resembled the infected subjects in Ab kinetics. The half-lives, reflecting second, slower phase of titer decay, were impressively long for the various viruses: 50 years for varicella-zoster and much longer than human life spans for the others, but they were shorter, around a decade, for the two bacterial antigens.⁹⁷ A separate study found that protective Ab levels to vaccinia were stable over 75 years, while the T cell responses to the virus declined with half-lives of 8–15 years.¹⁴⁹ Still, generalization about the types of immunization and the kinetics of the responses is not warranted: a comparison of multiple studies on replicating and inert viral immunogens revealed no associations between the rate of Ab decline and the vaccine type.¹⁵⁰ However, the

new theory of *imprinted lifespan* points to the totality of different B cell signals at the inductive stage of the immune response as the principal determinant of plasma-cell longevity and, hence, of Ab-titer kinetics.⁹⁵

Effective vaccines are available against flaviviruses causing yellow fever, Japanese encephalitis, and tick-borne encephalitis. Natural adaptive immunity and promising steps toward vaccine development have been recorded for other flaviviruses, such as the hepatitis C, dengue, and West Nile viruses.¹⁵¹ In other words, the obstacles to protective NAb responses collectively appear less insurmountable for this virus family than for lentiviruses such as HIV-1. Yet the neutralization targets on flaviviruses are also proteins that interact with various important surface molecules on immune system cells, some of which serve as entry receptors: CD81, CD14, MCLRs including DC-SIGN and the mannose receptor, and integrins.¹⁵¹ The attenuated yellow fever vaccine confers protection to 80–90% of human vaccinees. Neutralization titers vary without a clear demarcation of what is protective. A gene-expression signature that predicts a strong NAb response with 100% accuracy has been identified. This signature includes elevated expression of the tumor-necrosis-factor-receptor-superfamily member 17 (TNFRSF17), which is a receptor for the B cell growth factor BlyS-BAFF¹⁵² that potentiates Ab responses (see above). The immunogenicity of this receptor-binding viral protein thus strongly correlates with the host's expression of a B cell-regulating factor. HIV-1 Env might be similar in this regard, in that BAFF may heighten the amount of Env-specific Abs produced,⁶⁵ although it is uncertain whether this would promote or hinder protection. With the yellow fever vaccine, in contrast, even some of the weaker immune responses are protective.

Concluding Remarks

The demands on B cell responses for preventing HIV-1 infection are formidable. Anamnestic responses would arise too late to protect, which places the onus squarely on high, preexisting NAb titers, preferably at mucosal sites; although cytotoxic T cell responses may also contribute by eliminating cells that get infected when virus slips through the neutralizing barrier. Anti-Env Abs elicited by HIV-1 infection and by vaccination differ in both quantity and quality. During infection, Ab responses to Env can be strong, and somatic hypermutation over years sometimes leads to high cross-neutralizing Ab titers.^{38–41} This affinity maturation occurs despite widespread pathological effects of the virus, such as B cell hyperactivation, losses of naive and memory B cells, and impaired T cell helper functions. Despite these immune defects, the NAb response to HIV-1 infection overshadows what Env vaccines have elicited to date. Not only is NAb induction by Env vaccination difficult, but mucosal Ab responses to Env are particularly hard to elicit.⁹⁹ Also during chronic infection, only low amounts of HIV-1-specific IgA can be found in either sera or external secretions.¹⁵³ Given that IgA is normally the dominant Ab class in the gastrointestinal and some other mucosae, the deficient anti-gp120 IgA response in those locations is significant.^{144,154,155} The goals of long-lasting responses and mucosal targeting may even be linked in that long-lived plasma cells often express IgA and mucosal-homing factors.^{37,156,157} Such cells appear to be inefficiently induced in response to Env vaccines, and yet they are critical

TABLE 2. OUTSTANDING QUESTIONS IN NEUTRALIZING ANTIBODY INDUCTION AND THE IMMUNOGENICITY OF ENV

- Is Env immunosuppressive, specifically for B cell responses, either directly or indirectly?
- To what extent is the Env response TD or TI? What kind of response should be the aim of vaccination?
- Is Env a B cell mitogen? Are PAMP properties beneficial or harmful?
- How can T_H, particularly T_{FH} (IgA), T cell responses to Env be harnessed?
- How can the conditions in infection sometimes leading to high affinity and cross-neutralization through SHM be mimicked by vaccination?
- How can long-lived plasma-cell responses be induced by Env?

TD, thymus dependent; TI, thymus independent; PAMP, pathogen-associated molecular pattern; T_H, T-helper; T_{FH}, T-follicular helper; SHM, somatic hypermutation.

to the production of stable, high levels of NAbs. We need to understand how to generate these cells in large numbers (Table 2).

Env vaccine candidates require antigenic optimization, but whatever forms of Env are finally developed, their immunogenicity may also need to be improved. Thus, Env administered at high doses could conceivably compromise its own immunogenicity by interacting with cell-surface receptors on key immune cells. This may not affect the initial, transient response that is most commonly measured in vaccine trials, but the longer-term effects have not been studied. The hyperglycosylation of Env may have adverse effects on immunogenicity that go beyond the shielding of peptidic epitopes. Env glycans could be problematic by interacting with MCLRs, by mimicking self-antigens, or by creating PAMPs that elicit short-lived, nonneutralizing TI responses, which would compete with the desired generation of NAbs via TD processes. Our task must be to pinpoint the rare conditions during infection that yield strong, broad neutralization responses, and then to mimic them by engineering Env constructs on which nonneutralization epitopes are minimally immunogenic and from which immunosuppressive functions have been eliminated. Such immunogens may need to be complemented by TLR stimulators and other adjuvants, as well as inhibitors of Ag-presentation attenuators.

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Author Disclosure Statement

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