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Autoreactivity in HIV-1 broadly neutralizing antibodies: implications for their function & induction by vaccination

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Abstract

Purpose of Review—This review discusses progress in understanding the impact of immune tolerance on inducing broadly neutralizing antibodies (BnAbs), and how such knowledge can be incorporated into novel immunization approaches.

Recent Findings—Over 120 BnAbs have now been isolated, all of which bear unusual features associated with host tolerance controls, but paradoxically, may also be required for their function. Evidence that poly-/autoreactivity of MPER$^+$ BnAbs can invoke such controls has been demonstrated by knock-in (KI) technology, highlighting its potential for studying the impact of tolerance in the generation of BnAb lineages to distinct Env targets. The requirement for extensive affinity maturation in developing neutralization breadth/potency during infection is being examined, and similar studies in the setting of immunization will be aided by novel vaccine approaches and KI models that either selectively express reverted V(D)J rearrangements, or unrearranged germline segments, from BnAb lineages.

Summary—It is increasingly apparent that immune tolerance, sometimes invoked by self-reactivity that overlaps with BnAb epitope specificity, adds to a formidable set of roadblocks impeding BnAb induction. The path to an effective HIV-1 vaccine may thus benefit from a deeper understanding of host controls, including categorizing which are unique or common at distinct BnAb targets, and ranking those most feasible to overcome by immunization. Ultimately, such emerging information will be critical to incorporate into new vaccine approaches that can be tested in human trials.

Keywords

broadly neutralizing antibodies; polyeractivity; autoreactivity; immune tolerance; somatic hypermutation

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Conflicts of interest

There are no conflicts of interest to declare.

Introduction

Despite major advances in treatment and preventative measures, the HIV-1 pandemic has not slowed down significantly, with now over 35 million people infected. Thus, the development of a transmission-preventative HIV-1 vaccine is crucial, but continues to be elusive. Why is this? A correlate of most successful viral vaccines is generating potently neutralizing antibodies (1). HIV-1, however, differs from other viruses for which FDA-approved vaccines have been made, because as an integrating, rapidly mutating retrovirus, it is resistant to immune responses upon establishment of a latently-infected pool of $CD4^+$ T-cells (2). Therefore, it is critical for an HIV vaccine to rapidly induce neutralizing antibodies reactive to a broad spectrum of HIV-1 strains (BnAbs). Studies demonstrating robust protection by passive infusion of BnAbs preceding SHIV challenge in rhesus macaques (3,4) or their transduction in humanized mice prior to HIV-1 infection (5) lend support to this strategy. Furthermore, serum profiling of HIV-1 chronically infected subjects reveal that BnAbs can eventually develop, although only years after transmission, too late to avert disease course, and only in a minority of patients (6–9).

Unfortunately, efforts to elicit significant titers of BnAbs by immunization have failed. Thus, identifying the obstacles impeding BnAb induction by existing immunization regimens and devising strategies capable of overcoming them, is key to HIV-1 vaccine development. Defining the origins and precise characteristics of BnAbs from HIV-1 infected individuals has been facilitated by comprehensive serum profiling assays, improved antigen-specific memory B-cell sorting (10,11) and culture methodologies (12,13), and by high-throughput recombinant Ab cloning techniques (14,15). These efforts led to the discovery of new BnAbs with remarkable breadth/potency, and helped define structural characteristics for four vulnerable areas of the HIV-1 Envelope (Env) targeted by BnAbs: the gp120 CD4-binding site (CD4bs), the gp41 membrane proximal external region (MPER), and two novel peptide-glycan epitope-rich regions in either the gp120 V1/V2 or V3 hypervariable loops gp120 (reviewed in 16••). Furthermore, it is now known that BnAbs targeting more than one region are frequently co-produced in some individuals (8,17,18), passively-administered combinations of two BnAbs to distinct targets can confer near-complete breadth (19,20), and increased selection for escape mutants occur when individual BnAb lineages are produced during infection (21••). These data thus suggest that immunization regimens capable of eliciting more than one class of BnAb may not only be a desirable, but possibly necessary.

Despite this remarkable progress, the field's best efforts at engineering immunogens capable of presenting Env epitopes remain unsuccessful at inducing BnAbs (16••). Thus, it is becoming clear that "structure-based" approaches (see 22–28) will likely not solve the HIV-1 vaccine problem alone. Attention has now shifted to the host for insight into why BnAbs are difficult to elicit. This review provides a perspective on the potential association of BnAbs with self-reactivity and its role in generating subdominant BnAb responses to vaccines. It is not exhaustive; we will not cover structural considerations, which have been reviewed elsewhere (16••, 29–33). Furthermore, since all BnAbs isolated thus far originate from chronically-infected individuals, wherein secondary B-cell dysregulation/dysfunction effects may have impacted the magnitude/quality of BnAb responses (reviewed in (34•)), we

will focus on host factors we predict impact BnAb production not only during infection, but also in vaccination of healthy individuals.

Autoreactivity, polyreactivity, and other unusual BnAb traits associated with tolerance induction

Between 1993–2009, only a handful of BnAbs were identified, of which only three, 2F5, 4E10, and 2G12 were isolated directly from chronically infected patients. In 2005, two of these, 2F5 and 4E10, specific for linear, adjacent epitopes in the MPER, were reported to have elongated, hydrophobic CDRH3 residues, and to exhibit poly-/autoreactivity *in vitro* (35). Because both sets of traits are potential predictors of negative B-cell selection, based on numerous studies (reviewed in 36), this led to the hypothesis that BnAbs like 2F5 and 4E10 are rarely generated because the B-cells which produce them are subjected to immune tolerance (37). A corollary of this hypothesis, that BnAbs are more readily generated in autoimmune subjects (with defective tolerance) was also indirectly supported by reports of disproportionate infrequency of SLE^+ subjects with HIV-1 infection (38–42).

This hypothesis has been independently investigated by three groups, that monitored B-cell development in knock-in (KI) mice expressing the original (mutated) VDJ rearrangements of 2F5 and 4E10 (43,44,45••,46••,47•). 2F5/4E10 (VDJ or VDJ+VJ) KI mice share a striking blockade in immature B-cell generation, a phenotype characteristic of central clonal deletion, and similar to KI mice expressing BCRs with high affinities to well-defined self-antigens (48–50). Furthermore, residual 2F5 and 4E10 KI B-cell populations are under additional tolerance mechanisms including poorly expressing/signaling through, their BCRs (44,45••,46••), thus resembling anergic (unresponsive) B-cells (51) and immature 4E10/2F5 B-cells undergo extensive LC receptor editing that mitigates MPER epitope-associated self-reactivity (44) and apoptotic deletion (44,45••,46••). Thus, these results indicate 2F5 and 4E10 poly-/autoreactivities are indeed sufficient to induce profound negative B-cell selection.

Conserved vertebrate host antigens bound by 2F5 and 4E10 have now been identified: for 2F5, kynureninase (containing a motif identical to the ELDKWA neutralization epitope) and for 4E10, splicing factor-3b subunit-3 (SF3B3) (52••) and type-1 inositol triphosphate (IP_3R1) (47•). However, since affinity is only one aspect of an autoantigen's ability to effect tolerance (53,54), demonstration of these 2F5/4E10 targets as their *bona fide* self-ligands will require breeding of 2F5/4E10 KI mice to those with targeted disruptions in their putative self-reactive motifs. In terms of relevance to vaccine development, it will be important to determine the extent to which this kind of self-antigen mimicry limits BnAb generation, and the stage in B-cell development when BnAbs normally acquire tolerizing reactivity. Regarding this latter point, the data suggests that it can occur at any of several checkpoints: BnAbs like 2F5 may be tolerized in early BM B-cell development, since KI mice carrying reverted 2F5 BCRs undergo central deletion (Verkoczy L, Haynes BF, unpublished data), whereas others like CH103 and 4E10, whose reverted BCRs lack BnAb and self-specificity *in vitro* ((21••,55) and Haynes BF, unpublished data) may acquire tolerizing polyreactivity *de novo*, in the periphery.

Is there a correlation between self-reactivity of BnAbs and their unusual SHM levels?

Over 120 BnAbs have now been identified worldwide (10–15, reviewed in 16••,56•). Although these BnAbs often have greater breadth and/or potency than those initially isolated, many share predictive traits of negative selection: elongated HCDR3s, and, as examined by *in vitro* autoimmune assays, >1/2 exhibit poly- and/or autoreactivity (Fig. 1A). Furthermore, from this representative BnAb dataset emerges an additional feature common to all: an exceptionally high degree of somatic hypermutation (SHM)-mediated aa changes in rearranged immunoglobulins.

SHM, along with the linked mechanism of BCR affinity-dependent selection, comprise a general “fine-tuning” process that occurs in germinal centers (GC) known as affinity maturation (AM). AM is crucial for generating higher affinity BCRs/secreted Abs and formation of higher-affinity memory B-cells that confer long-term protection against future infections, the hallmark of secondary B-cell responses. During “typical” AM responses, SHM levels increase BCR affinity but can also inadvertently create *de novo* self-reactivity, and thus normally plateaus at ~5–6%. SHM in excess of that is believed to increase the probability of generating self-reactivity and decrease BCR affinity (62–64), resulting in decreased B-cell survival (57••,60,61). Thus, in addition to elongated CDRH3 regions, and poly-/autoreactivity, exceptionally high SHM levels in BnAb V(D)J rearrangements (15–48%) represents another trait associated with negative selection.

That all BnAbs identified thus far originate from subjects infected with HIV-1 for 2–4 years suggests such remarkable SHM frequencies are products of disfavored and/or highly-convoluted AM pathways (57••). The reasons for why such complex AM pathways are generated in chronic infection were unknown until recent examination of clonal BnAb lineages (21••,65) suggests a component is a co-evolutionary “arms race” between viral and host responses. These studies, and recent findings that most experimentally-reverted unmutated ancestors of BnAbs lack neutralizing specificity (57••,65–66) offer one explanation: naïve BCRs with extensive modification are required to achieve unusual structural requirements for dealing with extensive viral diversification (Fig. 2A). However, the observation that some *in vitro* poly/autoreactive BnAbs targeting the same general Env regions tend to be less mutated, relative to their less/non-polyreactive counterparts (eg: all MPER BnAbs, relative to 10E8) (Fig. 1B) raises an intriguing alternative hypothesis: negative selection may not only result from excess SHM, but also be its cause. At least two mechanisms address how tolerizing autoreactivity of BnAbs could drive unusually-high SHM (52••,57••,69••) (Figs. 2B, C). One possibility (52••,57••) (Fig. 2B) is that B-cells with germline BCRs that bind self-mimicking BnAb epitopes do not contribute to AM, because they are deleted in early development, necessitating recruitment of weakly cross-reactive, mutated B-cell clones to generate BnAbs via AM. We have proposed a second possibility (69••) (Fig. 2C), that peripheral tolerance drives additional SHM, when BnAb reactivity and self-reactivity are coupled. In this scenario, GC-mutated, self-reactive B-cells with BnAb specificity initially attempt to escape negative selection by acquiring additional mutations that remove the self-reactivity, but that also inadvertently remove BnAb specificity. Such a

"tug-of-war" process would likely invoke multiple rounds of mutation/selection to de-couple self-reactivity from affinity, yielding high SHM levels. Evidence for this, previously proposed as a model for the GC reaction (70), is observed in 2F5 KI mice. In these mice self-reactivity encoded by the original 2F5 V(D)J rearrangement involves a high degree of neutralization epitope mimicry (45••,52••) that is difficult to fully eliminate by receptor editing (43,44), resulting in an anergic, mature B-cell population (45••). Importantly, an $MPER^{-}$ subset of this peripheral anergic population undergoes additional targeted SHM at 2F5 V_H CDR residues (69••, Verkoczy L, Diaz M, Haynes BF, et al. unpublished data) presumably in an attempt to remove BnAb epitope-associated self-reactivity. A similar "affinity reversion/de-maturation" process has also been documented in the HEL KI model (Sabouri Z, Goodnow CC, personal communication).

Examining the extent to which BnAb lineages are under host tolerance controls

Given that all BnAbs identified thus far have at least one of the three above-mentioned traits associated with negative selection (Fig. 1), another emerging consideration for vaccine strategies is understanding how often host tolerance limits BnAb production: namely, do they limit all $MPER^{+}$ BnAb lineages, to what extent are BnAb lineages targeting other Env regions predisposed to such mechanisms, and what percentage of those target Env epitopes mimicking host structures, and finally, can such host controls be safely overcome by immunization? Thus, understanding how reliably the two BnAb traits most-associated with tolerance controls, *in vitro* autoreactivity and polyreactivity, i.e. as arbitrarily measured by common clinical assays (35,58) can predict *in vivo* tolerizing effects, is thus critical. Reassuringly, such *in vitro* poly-/autoreactivities exhibit reasonable concordance with immune tolerance, as suggested by the fact that *in vitro* poly-/autoreactivity of the normal human repertoire progressively wanes at developmental stages coinciding with previously-defined tolerance checkpoints in mice (58).

However, it is important to be mindful that i*n vitro* poly-/autoreactivity assays can yield both "false positives" and "false negatives". The former can occur because BCR/Ab affinities to host antigens *in vivo* are too low to effect tolerance (reviewed in 53). Given that ~15–20% of the mature T-dependent B2 population exhibit such "acceptable" *in vitro* poly/autoreactivity in healthy individuals' repertoires, it may be fairly common (58). In addition, a large proportion of other B-cell subsets (i.e. MZ, B-1) (71,72), recently shown to harbor specificities resembling those of some BnAbs (73) also exhibit *in vitro* poly-/autoreactivity. "False negatives" can result from technical inter or intra-assay variation between laboratories. For example, the BnAb 10E8, initially reported to lack poly/autoreactivity measured by common assays (59) has strong affinity for a ubiquitous human protein, when detected in protein microarrays (Liu Y, Yang G, Kelsoe G, *et al*. unpublished data), and has also been reported to exhibit functionally-relevant lipid reactivity (74•). Furthermore, some relevant autoantigens could escape detection by existing technologies if they are tissue/cell-restricted, like insulin B-islets in the pancreas. Finally, "false negatives" could also result from the atypical biology of BnAbs with exceptionally high SHM, which, as products of

BnAb lineages that otherwise exhibit prevalent self-reactivity, could represent atypical "snapshots".

Ultimately, the "gold standard" for confirming the physiological relevance, i.e *in vivo* tolerizing effects of a given BnAb (or its lineage members') *in vitro* poly-/autoreactivity measurements, is gene targeting of its/their V(D)J specificities. The power of the KI approach is evident in *the vivo* evaluation of 4E10, 2F5, and b12's negative selection-associated traits (45••,46••,75•). While all these BnAbs have elongated HCDR3 regions and exhibit poly-/autoreactivity *in vitro*, b12 exhibits normal B-cell development when knocked into the mouse Ig locus (75•), unlike 4E10 and 2F5 (45••,46••). However, since b12 was generated via phage display, it remains to be seen if any BnAbs from naturally-derived H+L chain pairs represent "false positives" like notable exceptions in the general B-cell literature (76,77), and conversely, if candidate "false negative" BnAb lineages, like 10E8 or VRCO1, in fact do effect host tolerance controls *in vivo*. Higher-throughput KI approaches, based on RAG blastocyst-complementation (78) in which BnAb V(D)J rearrangements could be rapidly inserted, may facilitate this type of analysis. Finally, another independent, potentially fruitful avenue to exploring the prevalence of tolerance controls on BnAb production is examining frequencies and spectra of BnAbs produced by autoimmune patients. The first example of such an analysis is recent characterization of CH98, a CD4bs-specific BnAb isolated from an SLE patient (79).

Functional and practical aspects of BnAb traits associated with negative selection

Each of the above-mentioned traits of BnAbs are paradoxical because while they may predispose BnAbs to host tolerance controls, they may also be critical in conferring their function. Below, we discuss the potential practical relevance of each trait for vaccine development.

First, regarding ***BnAb polyeractivity***, their degenerate recognition (and generally lower-affinity binding) to multiple distinct antigens, may have both general and specific relevance. General polyreactivity of Env-specific Abs may increase their overall affinity via heteroligation (68), a process proposed for dealing with the low density of Env trimer spikes found on HIV-1 (80,81). An elegant example of this is the structural analysis of bound 21c, a polyeractive Env Ab, which suggests interaction with not only its expected CD4i epitope, but also an adjacent CD4 receptor (82). Regarding BnAb function specifically, the lipid polyreactivities of 2F5, 4E10, 10E8, and m66 are believed to be an essential part of a general neutralization mechanism for all MPER-specific BnAbs, in which initial low-affinity contacts with the viral lipid membrane may extract lipid-immersed critical residues, thus allowing subsequent high-affinity interactions with exposed MPER epitopes (74•,83–86). There is growing consensus that for MPER-based vaccine strategies, appropriate presentation of 2F5/4E10 minimal peptide epitope in lipid membranes is required (87–90). Indeed, in 2F5 and 4E10 KI mice, only immunization regimens in which 4E10/2F5's neutralization epitopes are presented in virosomes, and in the presence of the lipid component/TLR agonist MPLA, can elicit robust serum BnAb responses (91). With respect to ***BnAb autoreactivity***, this trait merits practical considerations only if a BnAb's relevant

(tolerizing) self-antigen(s) exhibits considerable structural overlap with its neutralization epitope, as is likely for BnAbs targeting the 2F5 epitope (44,45••,52••). In such instances, a key question is whether vaccines can trigger either residual anergic B-cells (that retain both BnAb specificity and self-reactivity), or more ideally, rare peripheral "escape" variants (which have de-coupled self-reactivity and BnAb specificity). Although it remains to be determined if such latter clones exist, evidence for the former is suggested by the coincident generation of 2F5-like BnAbs and auto-Abs reported in sera from an HIV-1 infected subject (92). Importantly, immunization studies in 2F5 KI mice demonstrate that such residual anergic B-cells can be appropriately activated, thus providing proof-of-principle that their autoreactivity can be overcome by vaccination (69••). Furthermore, concerns about the pathogenicity of eliciting 2F5-like BnAbs are alleviated by passive infusion studies of 4E10, 2F5, or 2G12, which have not shown clinically adverse effects (93–95), except anticoagulant-activity for 4E10 (96), which is consistent with its higher lipid affinity, relative to 2F5 (35).

With respect to ***elongated and/or hydrophobic BnAb HCDR3s***, hydrophobic residues appear critical for neutralization-conferring lipid reactivity of all MPER-specific BnAbs examined thus far, including 2F5/4E10 (83,84,97,98), 10E8 (74•) and M66 (86), but for BnAbs targeting other Env regions, it is unknown if their hydrophobicities (36) have functional relevance. However, for BnAbs with exceptionally elongated (>25aa) HCDR3s, such as the glycan/V1V2-specific BnAbs PG9/PG16, this unusual length is required to form the "hammerhead" structures thought critical for accessing occluded residues found in complex, glycan-masked epitopes (99). Encouragingly, while the frequencies of B-cells bearing exceptionally long CDRH3 regions are severely limited either by pre-antigenic constraints (HC/LC pairing and N-addition/VDJ recombination events) and/or various tolerance checkpoints (reviewed in 36), they nonetheless are present in repertoires of most healthy individuals (100).

High SHM levels in BnAbs, represent a potentially more formidable roadblock for vaccination because conventional vaccines cannot recapitulate AM pathways during infection that generated such mutational levels; existing HIV-1 immunization regimens produce SHM levels (4–5%) more in-line with autologous nAbs (56•). However, the degree to which observed mutations in BnAbs are required for neutralization breadth/potency is unknown, although several recent studies have begun assessing this (67•,101•,102). In one, selective reversion of V(D)J framework regions (FRW) from a panel of BnAbs impacted breadth/potency in most (67•) leading to the proposal that exceptionally high SHM levels in BnAb FRWs provide structural flexibility. However, two recent *in vitro* mutagenesis-scanning studies found that only ~10–25% of reverted mutations impacted neutralization function for 10E8 and VRCO1 (two of the most mutated BnAbs) (101•,102). One explanation for the disparate results is that highly-mutated BnAbs may result from the stochastic nature of SHM driving numerous rounds of AM to create the desired combination of mutations that enhance structural flexibility (FRW's), and epitope binding affinity. Alternatively, SHM above the minimum required for neutralization, may be result of conflicted selection pressure in the GC: negative (for tolerance) and positive (for affinity) (Fig. 2C). It is also possible that a component of "excess" SHM found in BnAbs, may be

related to chronic HIV-1 infection (34•) and its potential effects on immune function (see 103, 104), since high mutation levels (although not as dramatic as in BnAbs) is also produced in other chronic infection settings, like influenza (57••,105). Ultimately, proof-of-concept immunization studies, including those in KI mice expressing reverted ancestors/intermediates of individual BnAb clonal lineages, cross-bred to strains with altered SHM levels (106) may provide some insight on this issue.

Assuming a considerable degree of the SHM generated in BnAbs during infection is required during immunization, new vaccine strategies will have to re-create disfavored and/or convoluted AM pathways that generated such extensive SHM. B-cell lineage design has been recently proposed for this purpose (21••,57••), an approach aimed to use immunogens that optimally bind a given BnAb lineage's ancestors and AM intermediates, administered either sequentially (to help "guide" AM pathways) or in combination (to mimic HIV-1 diversity). Perhaps the most straightforward and physiological use of this approach involves using the actual Env proteins identified from sampling serial isolates of BnAb lineages generated during infection, to re-create similar pathways by vaccination (21••). However, other variations have also been proposed, involving use of computational and *in vitro* selection methodologies to engineer "super-immunogens" that can bind BCRs of multiple clonal members from BnAb lineages with common epitopes (107•,108•).

Conclusions

Recent studies using KI models demonstrate immune tolerance can profoundly limit BnAb induction, and traits associated with negative selection in many recently discovered BnAbs suggest this has relevance for BnAbs at distinct Env targets. Some aspects of tolerance can be overcome, for example immunization of 2F5 KI mice demonstrates overcoming deletion/anergy is possible, provided a structurally-compatible immunogen is used, and passive infusion studies suggest pathogenicity would not result from their elicitation. Other aspects, like overlap of self-reactivity with BnAb specificity, which may result in inadvertent selection against the latter, may be more problematic. Further examination of the prevalence of this issue across BnAb lineages will benefit from autoantigen discovery/modeling and high-throughput KI approaches. Finally, immunoregulatory factors other than immune tolerance, that may contribute to subdominance of BnAb responses, including shaping of the initial B-cell repertoire by incidental exogenous antigen exposure (28), or host genetic determinants like allelic variation of V_H segments utilized by BnAb lineages (109) and MHC class II-restriction of overlapping $CD4^+$ T_H/BnAb epitopes (110) may also have to be considered for vaccine strategies.

A key for vaccine development will be determining the feasibility of immunization to elicit AM pathways that can generate breath/neutralization in various BnAb lineages. The development of novel immunization strategies like B-cell lineage design, more powerful KI-based immunization models (expressing limited repertoires of unrearranged germline segments from representative BnAb lineages) to more efficiently probe AM pathways *in vivo*, and isolation of new clonal lineages from both infection and immunization settings will be key. Finally, it should be noted that even when only considering the minimally-required mutations for neutralization in highly-mutated BnAbs like VRC01, vaccination schemes

would still have to generate much higher SHM levels than existing regimens can elicit. We therefore submit that BnAbs with relatively less SHM may be more desirable/tractable targets.

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involving selected removal of an N-glycosylation site, was used instead, to select for immunogens that could bind/activate B-cells expressing germline reverted “VRCO1-class” CD4bs-specific BnAbs as BCRs. [PubMed: 23530120]

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Key Points

- Numerous broadly neutralizing antibodies (BnAbs) have recently been identified, many of which have unusually long, hydrophobic HCDR3 regions and/or *in vitro* poly-/autoreactivity, traits normally associated with negative B-cell selection.
- The exceptional degree of somatic mutation found in all BnAbs isolated thus far suggest their generation via convoluted affinity maturation pathways, which directly or indirectly, may result from mimicry of self-antigens by BnAb epitopes.
- Knock-in mice of BnAbs targeting the gp41 MPER region have provided evidence that BnAb poly-/autoreactivity can invoke host tolerance controls that profoundly impacts their production.
- The unusual HCDR3 regions, poly-/autoreactivity, and excess somatic mutation of BnAbs may all be functionally relevant, thus necessitating novel vaccine strategies capable of triggering and/or generating B-cells having such features.

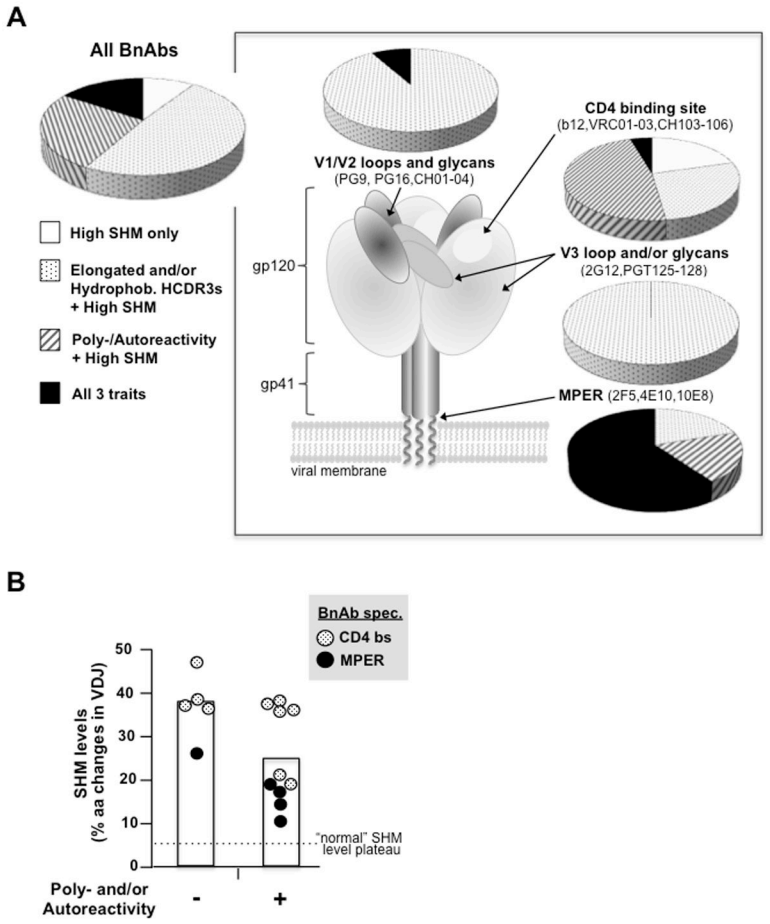

Figure 1. Distribution of the three BnAb traits associated with negative selection

A. The relative distribution of BnAbs with only high SHM (≥10% aa changes in V(D)J rearrangements), or in addition, *in vitro* poly-/autoreactivity or elongated and/or hydrophobic HCDR3 regions [see (16••) for individual BnAb refs] are shown for either all BnAbs, or broken down into the four general Env regions they target (inset)(16••,29,30); schematic diagram of trimeric Env based on (57••), with representative BnAbs also listed. HCDR3 lengths are reported in Kabat nomenclature and BnAbs with elongated HCDR3s are defined as those ≥2 standard deviations (i.e.≥18aa) above the median length (13.5 aa) in the normal mature human repertoire (58), and unusual hydrophobicity was calculated as previously described (36,58). For all calculations, independently-discovered individual BnAbs and BnAb clonal lineages are equally weighted (and clonal members with different characteristics as fractions therein) and only BnAbs for which all traits have been assessed, including poly-/autoreactivities measured *in vitro* in common clinical autoimmune screens (14,35,59), are included. **B.** Correlation between poly-/autoreactivity and SHM levels in BnAbs specific to either the MPER or CD4 binding site. Note the cutoff line labeled "normal SHM level plateau" is defined as the theoretical maximum during typical secondary Ab responses (57••,60,61) and also approximate levels generated in autologous nAb responses. SHM data shown for BnAb lineages are averages of their clonally related BnAb members.

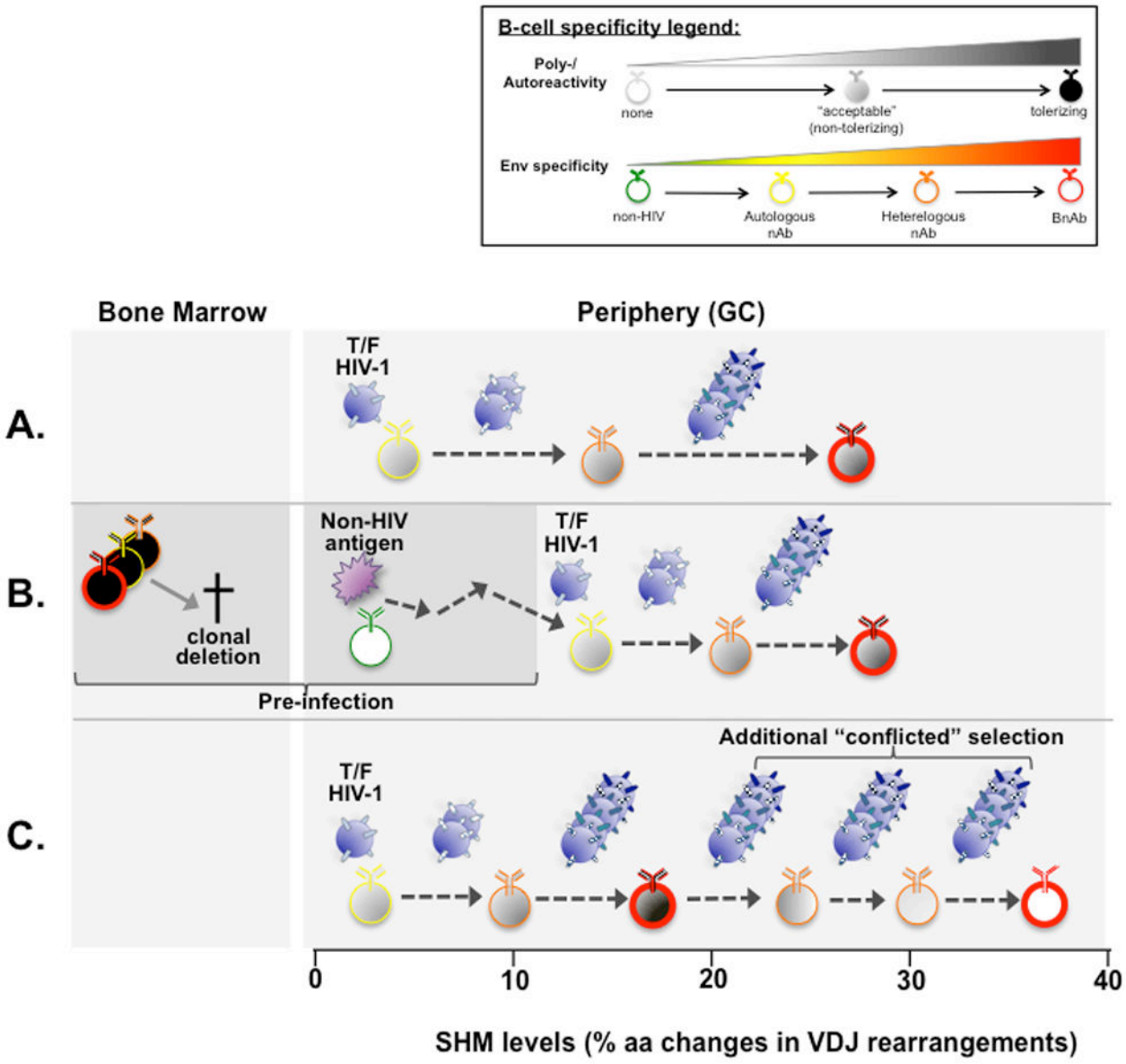

Figure 2. Hypotheses for why BnAb$^+$ B-cells acquire unusually high levels of VDJ mutations during chronic HIV-1 infection

In a standard (non-tolerance) model **(A)**, GC B cells with no/minimal reactivity to mature Envs (57••,65,66) interact with founder/transmitted Envs and undergo extensive AM, driven by viral escape/Env diversification pressure (21••), which demands difficult structural modifications to achieve both FRW flexibility and Env affinity (67•). Note that as shown, B-cells with "acceptable" (non-tolerizing) polyreactivity prior to entering the GC reaction may be selected for their ability to heteroligate Env (68), but poly-/self-reactivity may also be acquired during AM, as in the CH103-CH106 BnAb lineage (21••). In a model where self-tolerance indirectly impacts excess SHM (52••,57••) **(B)**, B-cells with BnAb reactivity that mimic host antigens are clonally deleted in BM development, creating "holes in the repertoire". In the absence of competition, weakly cross-reactive clones that already underwent non-Env driven AM can then participate in additional, Env-driven AM. Finally,

in a model where self-tolerance directly drives excess SHM (69••)**(C)**, B-cells that acquire both self and BnAb specificity via AM use additional SHM/selection to retain BnAb specificity, while removing tolerizing self-reactivity. Such a process would assume close, but not complete BnAb mimicry of self-epitopes. Thus, the degree of additional selection/SHM would be proportional to how closely a particular BnAb epitope mimics self-antigen(s). Note that these hypotheses are not mutually exclusive: for example, the "tolerance-based" models shown in (**B–C**) are shown in combination with the "standard" model, depicted in (**A**).