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Human Ig knock-in mice to study the development and regulation of HIV-1 broadly neutralizing antibodies

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Summary

A major challenge for HIV-1 vaccine research is developing a successful immunization approach for inducing broadly neutralizing antibodies (bnAbs). A key shortcoming in meeting this challenge has been the lack of animal models capable of identifying impediments limiting bnAb induction and ranking vaccine strategies for their ability to promote bnAb development. Since 2010, immunoglobulin knock-in (KI) technology, involving inserting functional rearranged human variable exons into the mouse IgH and IgL loci has been used to express bnAbs in mice. This approach has allowed immune tolerance mechanisms limiting bnAb production to be elucidated and strategies to overcome such limitations to be evaluated. From these studies, along with the wealth of knowledge afforded by analyses of recombinant Ig-based bnAb structures, it became apparent that key functional features of bnAbs often are problematic for their elicitation in mice by classic vaccine paradigms, necessitating more iterative testing of new vaccine concepts. In this regard, bnAb KI models expressing deduced precursor V(D)J rearrangements of mature bnAbs or unrearranged germline V, D, J segments (that can be assembled into variable region exons that encode bnAb precursors), have been engineered to evaluate novel immunogens/regimens for effectiveness in driving bnAb responses. One promising approach emerging from such studies is the ability of sequentially administered, modified immunogens (designed to bind progressively more mature bnAb precursors) to initiate affinity maturation. Here, we review insights gained from bnAb KI studies regarding the regulation and induction of bnAbs, and discuss new Ig KI methodologies to manipulate the production and/or expression of bnAbs *in vivo*, to further facilitate vaccine-guided bnAb induction studies.

Keywords

broadly neutralizing antibodies (bnAbs); knock-in (KI) mice; B-cell tolerance controls; B-cell lineages; CD4-binding site (CD4bs); sequential immunization

Introduction

Neutralizing antibodies (nAbs) are critical contributors to protective responses against most viral infections (1, 2). However, HIV-1 differs from all other viruses for which successful nAb-eliciting vaccines have been made, in that it is both a rapidly integrating and mutating pathogen (3). These two features pose a unique and unprecedented challenge for developing an efficacious vaccine for HIV-1. The rapid establishment of a latently-infected CD4⁺ T-cell pool necessitates viral transmission be completely blocked, whereas at the population level, mutability of the HIV-1 envelope (Env) requires a humoral response to be adept in dealing with extreme viral diversity. Given these requirements, a fully protective HIV-1 vaccine will need to induce a rapid and robust memory antibody response capable of neutralizing a wide spectrum of HIV-1 strains, i.e. broadly neutralizing antibodies (bnAbs). Studies demonstrating absolute protection afforded by passive transfer (4, 5) or transduction (6) of monoclonal bnAbs, prior to viral challenge, lend support to this notion.

Although bnAbs have yet to be elicited by vaccination (7), recent findings that bnAbs do develop over years in a subset of HIV-infected subjects (8–12) provided renewed impetus for the HIV-1 vaccine field to devise new immunogens and strategies to induce them. High-throughput Ig cloning methodologies have enabled the discovery of many new monoclonal bnAbs over the past few years, providing two key insights for HIV vaccine design. First, the discovery of these new HIV-1 bnAbs has provided key structural information regarding five distinct, but conserved, regions in the Env trimer in which their epitopes cluster, thus providing a blueprint of the vulnerable regions a bnAb-eliciting vaccine can exploit. These five targets are the gp120 CD4-binding site (CD4bs), the gp41 membrane proximal external region (MPER), and three peptide/glycan epitope-rich regions, either found in gp120 V1/V2 or V3 variable loops (i.e the trimer apex and high-mannose patch, respectively), or in the gp120/gp41 bridging interface (13, 14). The second insight is that bnAbs have an intriguing set of shared characteristics, despite their independent origins. Thus, in addition to utilizing a restricted set of human V_H genes (3), all exhibit one or more of the following unusual traits: high levels of V(D)J somatic hypermutation (SHM), poly-/autoreactivity, and Ig heavy chain complementarity-determining regions (HCDR3s; critical antigen contact regions encoded by the V(D)J junction) that are exceptionally long. Indeed, it is the atypical nature of these features that likely confers the protective functions of bnAbs, by allowing them to overcome structural barriers in Env. For instance, elongated HCDR3s have a potential role in countering glycan occlusion of bnAb epitopes by penetrating the glycan shield (15), polyreactivity may allow B-cell receptor (BCR) “heteroligation” to deal with unusually low surface density of Env trimers on HIV virions (16, 17), and extensive SHM may provide structural flexibility to counter natural variation in otherwise relatively-conserved bnAb-targeting regions (3, 13, 18–20).

It has now become increasingly clear that these same unusual bnAb traits pose daunting immunologic obstacles to initiating and/or completing the maturation of bnAbs by vaccine strategies (reviewed in (21–23)). For example, long HCDR3s and *in vitro* polyreactivity are negatively correlated with function, specificity, and/or frequency of B-cells in the pre-immune repertoire, due to difficulties in their Immunoglobulin (Ig) heavy chains (HCs) pairing to surrogate or *bona fide* Ig light chains (LCs) and/or invoking central B-cell

tolerance controls (24–27). Furthermore, excessive SHM generated during bnAb development in the setting of infection, if also required during vaccination, would necessitate humoral responses involving highly disfavored, protracted affinity maturation (AM) pathways (28–31) that are likely impractical and/or unsafe to induce. Despite the daunting set of challenges such unusual traits pose, the good news is that bnAbs exhibit varying degrees of these features and/or combinations of them. For instance, while CD4bs “VRC01 class” bnAbs approach 50% SHM levels (3), they lack initial self-reactivity and have typical sized HCDR3s. On the other hand, bnAbs targeting the high-mannose patch (despite having long HCDR3s (13)) or those directed to the MPER (despite exhibiting considerable self-reactivity (32)), tend to accumulate fewer SHMs, relative to CD4bs-specific bnAbs (23, 33, 34).

Given the identification of these formidable, yet not insurmountable roadblocks, what are the steps most critical for developing a vaccine capable of inducing bnAbs? At least three major knowledge gaps need to be resolved. First, it is not yet known for individual bnAbs, and in the setting of vaccination, to what degree their unusual traits, or combinations of traits, are required for neutralization, and which are the most problematic to induce by vaccination. Thus, a deeper, more systematic understanding across *multiple* bnAb classes/lineages of what the minimum functional requirements for each feature is, relative to potential limitations they impart on vaccine-induced B-cell maturation, is key to create a *comprehensive* picture of whether certain bnAb target(s) are more tractable to elicit. For instance, it would be critical to know how much SHM is actually required by vaccination to develop neutralization breadth. This is currently an open question, since bnAbs originate from patients with prolonged HIV-1 infection, where bnAb-specific maturation issues, e.g. extensive host/virus co-evolution (35, 36) or potential purifying selection forces (23, 30, 31, 37) could generate high SHM, but other confounding issues, irrelevant to bnAb formation, e.g. inflammation (38) or B-cell dysregulation (39), may produce general “mutational noise”. Additionally, while one study has argued such extreme SHM levels (up to 48% VDJ aa mutation) confer FRW regions the necessary flexibility to deal with viral diversity (18), *in vitro* mutagenesis studies have shown only 25–30% of such high total SHM levels are functionally required (40). However, this latter report doesn’t take into account the non-linear acquisition of SHM accumulated during AM (29). Nevertheless, this issue is critical to resolve because if the minimal SHM needed for bnAb generation can be defined during vaccination and the level is considerably less than what occurs during infection, it would provide strong rationale to focus on devising vaccination strategies that “short-circuit” rather than recapitulate the evolutionary pathways deciphered during infection. Likewise, for the traits of long HCDR3 and *in vitro* poly/autoreactivity, understanding their *in vivo* tolerizing effects across bnAb lineages against distinct Env regions is critical for defining their tractability as vaccine targets, because immunization strategies can either be directed to lineages with no self-reactivity, or alternatively, focus on modulating tolerance, such as seeking to provide regimens with stronger or more specific BCR, Toll-like receptor (TLR), or helper T-cell signals (41–45).

A second gap in knowledge towards developing a bnAb-eliciting vaccine is determining whether vaccination can evolve more than one class/target to a reasonable degree of breadth. Because viral escape mutants may develop in response to bnAbs targeting only a single

region (3, 35), and passively-infused combinations of two, less-potent/broad bnAbs to distinct targets can synergize in conferring near-complete breadth (3, 34, 46, 47), this suggests a bnAb-eliciting vaccine, based on an alternative strategy in which cumulative breadth is achieved by eliciting more modest breadth at two or more individual bnAb classes/targets, may not only be a more feasible approach, but may also reduce mutant escape frequency. In this regard, some bnAb intermediates already develop considerable neutralization breadth with more modest SHM levels; in one study, 40–80% breadth resulted from about 50% the total level of SHM observed in the V3/glycan-specific bnAb PGT121 (33), which already accumulates relatively less SHM (~20% VDJ aa mutation) than many other bnAbs. However, a potential caveat with this more modest estimated overall mutation level of ~10% for achieving modest breadth *in vitro* is that *at least* some additional SHM would likely be produced *in vivo* as intrinsic bystander mutation; although identifying new bnAbs accumulating even less SHM may help counter such concerns

The third knowledge gap required to inform bnAb-based vaccine design is to understand what types of HIV immunogens are best suited for priming and/or boosting humoral protective responses to HIV-1. On one end of the spectrum, long-standing proponents of polyvalent and/or consensus Env-based immunogens have argued that T-cell epitopes in Env are required to drive strong germinal center (GC) responses and thus inducing robust AM. Based on this notion, incorporating conserved epitopes from diverse Envs would provide global coverage of such responses at a population level (7). Furthermore, it also has been long been suggested that eliciting bnAb responses should require Envs be in native trimeric forms, which may more selectively display bnAb epitopes and occlude immunodominant non-nAb epitopes (48, 49). However, immunization with Env trimers on VLP surfaces (50, 51) or inactivated HIV-1 virions expressing fusion-active Env trimers (52) have thus far only elicited heterologous, tier 1 nAbs, (i.e. cross-reactive with multiple HIV-1 viral strains having high neutralization sensitivity). Furthermore, more recent studies using near-native soluble recombinant trimers have also resulted in induction of only predominantly tier 1 nAbs (53), although their elicitation of autologous, tier 2 nAbs (reactive to sequence-matched viral strains, having moderate neutralization sensitivity) suggest they may represent a useful starting point. On the other end of the spectrum, others argue for use of “minimal” “immunogens for targeting bnAb lineage/Env target-specific humoral responses in vaccine regimens, especially for priming responses, since traditional, native Env immunogens do not bind most precursors of bnAbs (54–58). However, such minimal immunogens are just beginning to be developed and evaluated, and peptide-based or partial protein subunit vaccine strategies in the past have been considerably less successful than protein subunit or whole virus vaccines (59, 60). In reality, it is also possible that hybrid approaches may be required: for example, amount or specificity of T-cell help, even in trimeric Env-based immunization regimens may be inadequate (due to tolerance controls) resulting in preferential activation of irrelevant non-bnAb responses. Additionally, the highly-glycosylated nature of trimeric Env may occlude precursor epitopes of matured bnAbs. Thus, minimal immunogens, combined with adding strong, exogenous (non-HIV) T-cell help may be well-suited for priming, while timing of multiple, sequentially-administered Env trimeric immunogens, with higher affinities and/or levels of glycosylation for binding progressively-matured intermediates of bnAbs, may be critical in boosting, as has been

proposed for newer B-cell lineage design-based approaches (55, 61, 62). Regardless of which types or combinations of immunogens are required in prime/boost strategies, this issue, as with the others mentioned above, would also benefit highly from more comprehensive testing in more practical *in vivo* platforms.

All three knowledge gaps noted above mandate methodical iterative testing in order to dissect the relative impacts of the roadblocks impeding development of a bnAb-based vaccine, which in turn requires more systematic and mechanistic evaluation of multiple, individual bnAb lineages (and their individual members/branchpoints) at distinct Env targets. A serious limitation in filling these knowledge gaps is the lack of appropriate *in vivo* platforms capable of addressing these questions. While no perfect immunization model exists in general, those best suited require a sufficient balance of physiological relevance and practicality, which can only be generated by mouse Ig locus-directed targeting of individual human bnAb precursor variable region exons or V, D, and J regions that can be developmentally assembled in mice. What individual criteria do such bnAb knock in (KI) models satisfy to achieve this unique balance? On the “practicality” end of the spectrum, bnAb KI mice meet two key criteria. First, they represent rapid, iterative testing platforms, since by expressing repertoires enriched for bnAb precursors of individual lineages as starting points, they enable studies to be focused on such lineages, where an unparalleled level of resolution and manageable numbers of maturation products from numerous trajectories can be examined and tracked in response to immunization. In contrast, such analysis is not feasible to achieve in human vaccine trials, primate studies, or other (unmanipulated) small animal models with normal polyclonal systems, since individual bnAb roadblocks manifest as a multifactorial set of limitations, resulting in eliciting subdominant bnAb responses, i.e. undetectable in plasma, and thus lack the resolution to measure incremental improvements in regimens. The second criteria that bnAb KI models meet for the required practicality is that they have considerable flexibility: only in such models can B-cell selection and SHM processes be genetically manipulated in the setting of focused bnAb lineages, thus allowing even further dissection of regulation and development of individual bnAbs. This can be done either by generating compound models via cross-breeding to other relevant, B-cell process-impacting KI/KO strains, or by genetically manipulating timing and/or stage of bnAb expression in the repertoire. The frequency and type of enriched repertoires can also be altered (as will be discussed further below); for instance, they can be derived either from HC only or HC/LC reverted bnAb rearrangements, or from preferentially-rearranging germline segments. All these maneuvers may be critical, especially since empirical studies have yet to yield an effective HIV vaccine, suggesting individual roadblocks in bnAb generation exist that require systematic identification.

On the “physiological relevance” side, since bnAb KI models need to be complementary to (and lead generators for) human trials or primate studies, they need only generate information that is sufficiently applicable to inform such studies. This minimal requirement is met because due to the way bnAb KI models are engineered, they retain all relevant mechanisms shared with humans relevant for bnAb activation and development, including those driving germinal center (GC) reactions, where high-affinity Ab formation is orchestrated. Specifically, affinity maturation in GCs is achieved through the combinations of SHM, positive selection for high affinity binding to foreign antigen, and purifying

selection (against self-antigen binding), all via nearly identical mechanisms in humans and mice. Thus, this approach differs from other types of humanized mice reconstituted with human immune systems such as the BLT (Bone marrow-Liver-Thymus) model, i.e. *Rag2*^{-/-}*γc*^{-/-}*CD47*^{-/-} mice reconstituted with human bone marrow, liver, and thymus), which have poor GC reactions. Furthermore, in regular outbred animal systems, not only is polyclonality an issue, but various Ig locus-related immunogenetic features may not be amenable to, or physiologically relevant for, human bnAb elicitation (23). By contrast, bnAb KI models are engineered to introduce specific human Ig elements functionally important for bnAbs, in the replace those at the wild-type (WT) mouse Ig loci that are limiting to bnAb development. For instance, WT mice have shorter D segments than humans, rendering them incapable of producing long (>20 aa) HCDR3s, a trait found in >2/3 of bnAbs (3, 23). Additionally, ~50% of bnAbs use diverse human λ LCs which cannot be recapitulated at the WT mouse Ig λ locus, which in relation to the human Ig λ locus, has restricted diversity and poor expression of V λ segments. Thus, by knocking-in longer human D segments (or human VDJ exons) into the mouse IgH locus, or human V λ J λ rearrangements into the mouse IgL locus, such issues can be overcome.

In this review, we focus on various types of human Ig KI mice available for HIV vaccine research, including bnAb KI strains developed by us and others in the field, with the goal of illustrating how their intended balance of physiological relevance and practicality allow more focused studies of how B-cell repertoires expressing human bnAbs are formed and selected, and to what extent broad and/or potent neutralizing responses to novel immunogens and/or vaccine strategies can develop. We begin with a historical background on the use of Ig KI technology to express V(D)J rearrangements from some of the original (1st generation) bnAbs, as well as more recent studies expressing more potent and/or broad 2nd generation and/or reverted bnAbs, in order to demonstrate the role of host tolerance in controlling their production, and to elucidate strategies to overcome these barriers. We then describe initial insights into vaccine-guided activation and initial development of bnAb lineages beginning to emerge from recent immunization studies done with newer human Ig KI models, made to either preferentially express reverted V(D)J rearrangements or fully germline (unrearranged) V gene segments of 2nd generation bnAbs. In the context of these latter studies, we will also review a number of technical advances developed for high-throughput production of, and conditional expression in, bnAb KI models, in order to accelerate basic studies of vaccine-guided bnAb development and regulation. Ultimately, we project how such methodological improvements will make systematic testing of individual bnAb lineages to distinct targets (and/or key intermediates within lineages) a more practical exercise, thus making feasible two of the key goals for HIV vaccine development: defining key parameters required for vaccine-guided bnAb induction and identifying which lineage-directed immunogens and prime/boost strategies can best do this.

Formal demonstration of bnAb tolerance controls using human Ig KI models

Ig KI models for studying B-cell tolerance and initial work in MPER bnAb KI mice

B-cell tolerance is not a single discrete process, but a complex series of phenomena, with *in vivo* tolerization effects manifest in a gradient of “severity”, driven by various factors during their interaction with B-cells, including the form self-antigens are presented (63), the affinity at which they bind (64, 65), and the location/site of their encounter (66, 67). Furthermore, tolerance occurs at several stages of B-cell development (24) by various distinct mechanisms that can impact B-cell frequency, function, and/or specificity (reviewed in (68)). In the past, conventional Ig transgenic or Ig KI models (i.e. those in which B-cells specific to defined self-antigens, were engineered with single pre-rearranged specificities (V_HDJ_H and/or V_LJ_L rearrangements) either randomly integrated into the genome or knocked-into the IgH and L loci, respectively) have been instrumental in providing the resolution required to systematically elucidate negative selection processes (reviewed in (69)). These Ig tg/KI-elucidated mechanisms include central deletion (63, 70–72), LC receptor editing (73, 74), V_H replacement (75), peripheral anergy (76, 77), follicular exclusion (78) and marginal zone sequestration (79).

Before 2009, only a handful of monoclonal bnAbs (2G12, 2F5, 4E10, b12 and Z13e) had been identified. Of these “1st generation” bnAbs, only three (2G12, 2F5, and 4E10) expressed natural HC/LC pairs by virtue of being directly isolated from chronically HIV-1 infected subject PBMCs using traditional hybridoma technology, while the other two were obtained using random combinatorial phage display libraries. Of these three “1st generation” bnAbs obtained by hybridoma cloning, both 2F5 and 4E10, directed to linear adjacent epitopes in the MPER, were reported to exhibit traits that represent predictors of negative B-cell selection: elongated, hydrophobic HCDR3s, and *in vitro* polyreactivity and/or autoreactivity, as determined by HEp-2 immunohistochemical staining and a standard clinical autoantigen panels, respectively (80). This led to the hypothesis that bnAb responses, at least in part, are impaired by B-cell tolerance mechanisms (81). Analogous to the approaches previously used to test *in vivo* tolerizing effects on KI B-cells reactive to DNA (64, 71) erythrocytes (67, 72), or to artificially-introduced self-antigens in dual KI/tg systems such as the α -MHC Ig tg/KI+MHC 3–83 tg (70, 82) or α -HEL Ig tg/KI+HEL tg models (63), this hypothesis was tested by three independent groups who expressed the original (mutated) V_HDJ_H and $V_HDJ_H+V_LJ_L$ rearrangements of 2F5 and/or 4E10 (83–87). Strikingly, all 2F5/4E10 KI models had profound blockades at the pre-B to immature B-cell transition in the bone marrow, characteristic of clonal deletion at the first tolerance checkpoint, and similar to those seen in Ig KI/tg mice expressing high affinity and/or membrane-bound self-antigens (63, 64, 70, 71). Interestingly, the 1st generation CD4bs⁺ bnAb b12, originally reported to be poly/autoreactive (80), lacked obvious *in vivo* tolerizing self-reactivity in KI mice (88), although the significance of this finding remains ambiguous, since b12’s origin from a phage library makes it unclear if its HC/LC pairing existed *in vivo*, and detailed analysis of peripheral B-cell subsets in b12 KI mice was not reported.

Central deletion and other B-cell tolerance mechanisms observed in bnAb KI models

Notably, although central deletion is profound in 2F5 and 4E10 VDJ+VJ KI models, it is not complete, as small populations of residual KI B-cells are observed in the periphery of both, which could serve as targets for immunization. These residual KI B-cells, however, were found to be limited by additional, 2^o tolerance mechanisms, including having poor BCR expression and signaling, characteristic of being anergic (functionally-silenced) (89), and/or undergoing extensive LC receptor editing events that mitigated self-reactivity and promoted rescue from clonal deletion (84–86) (Fig 1). Despite having undergone extensive LC editing, many peripheral clones remained anergic (84, 85), which along with the finding that a version of this KI model in which V_HDJ_H rearrangements expressed alone could invoke profound tolerance controls (83–87), suggested that the 2F5/4E10 HC self-reactivities are dominant and cannot be fully vetoed by LC editing.

In terms of tolerance mechanisms controlling bnAb induction, the 2F5 VDJ+VJ strain, as the prototype bnAb KI tolerance model, has been most extensively evaluated to date (Fig 1), and in addition to the processes of deletion, anergy, and receptor editing noted above, further studies have also led to identification of a novel process termed Affinity Reversion/Ab redemption that represents a “last resort” mechanism for anergic 2F5⁺ B-cells that persist into mature B-cell subsets (including the aforementioned “uneditable” anergic population) for escaping apoptosis via purifying selection (37). The same phenomenon has independently been uncovered and elegantly described in a well-controlled set of HEL KI models by Goodnow and colleagues (30). Recently, evidence for this process in limiting completion of bnAb maturation has been observed in the development of 2F5-like lineages in vaccinated primates (90). Based on these emerging data in support of this process, one hypothesis to account for the high degree of SHM in bnAbs has been proposed, wherein the incomplete overlap of bnAb epitopes with self-antigens creates a selection “tug-of-war” during AM: for HIV neutralization affinity, but against self (23, 31, 91). Since bnAbs appear to require protracted AM pathways during HIV co-evolution in chronically-infected subjects (3), it will now be of interest to explore the relevance of Affinity Reversion during vaccine approaches aimed at eliciting other bnAb lineages, since such a mechanism, if generalizable, would have obvious importance in guiding novel immunogen design approaches, in which “escape clones” that have de-coupled Env and self-reactivities, i.e. with neutralizing potential, but lacking self-binding, could be identified and used to map Env mutants retaining only the residues critical for function.

From initial studies in 4E10/2F5 KI models, three key, related questions have emerged: 1) whether tolerance controls limit induction of all MPER⁺ bnAbs, 2) to what extent do they control responses of bnAbs targeting other Env regions, and 3) are they also operational in bnAb precursors, and as such, at least partially contribute to high SHM levels. Understanding tolerance prevalence across bnAb lineages to distinct Env regions now becomes a critical question for the field, because understanding their relative tractability as vaccine targets will inform as to whether vaccine strategies should either be directed to lineages with no self-reactivity, or alternatively, be focused on modulating tolerance. Based on the studies done in classic self-Ig KI models and 2F5/4E10/b12 bnAb KI models, it is clear these systems now represent the “gold standard” to definitively test if and how *in vitro*

self-reactivity physiologically impacts development of “2nd generation” bnAbs (i.e. more potent and/or broad bnAbs identified 2009 or later), and their precursors (before or during immunization), for two reasons. First, the presence or absence of self-reactivity *in vitro* is often, but not always a predictor of invoking host tolerance (reviewed in (23)). For instance, false negatives can arise due to self-antigens having restricted expression or affinities sufficient to trigger deletion *in vivo* (92) but below detection *in vitro*. Conversely, false positives may occur when *in vitro* reactivities to self-antigens don’t reflect those capable of invoking tolerance at physiologically relevant sites of B-cell encounter. Secondly, because host tolerance occurs as a continuum of developmental checkpoints and processes (22, 91) only through analysis in Ig KI models can precise mechanisms responsible and most critically, ways to manipulate them, be identified, as has been previously done to examine various auto-Ab specificities.

Although potential *in vivo* tolerization has not yet been evaluated for most 2nd generation bnAbs or their precursors, several initial insights have begun to emerge from studies in newer KI models expressing reverted/unmutated versions of these bnAbs. One such example is a study addressing whether high SHM levels in bnAbs can accumulate at least in part due to elimination of self-reactivity, which would predict a more stringent degree of tolerization in precursors (57). In this study, mice expressing 2F5’s unmutated ancestor (UA) V_HDJ_H + V_κJ_κ rearrangements were generated, and found to be under more profound deletion and anergy controls relative to the original (mutated) 2F5 dKI mice (90), demonstrating this is the case, at least in certain instances. Another example is a recent report in which a gl-3BNC60 VDJ+VJ KI model, co-expressing V germline-reverted V_HDJ_H and V_LJ_L rearrangements of 3BNC60, a VRC01-class bnAb, were found to exhibit extensive LC editing and peripheral anergy (93), thus providing an initial clue that at least some CD4bs specific bnAbs may also be controlled by tolerance. Interestingly, several other recent studies in settings other than KI models also support findings of considerable tolerance prevalence across bnAb lineages, thus complementing the above-mentioned initial studies in newer (non-MPER bnAb) KI models. In one, a protein array of 9500 host proteins was used to screen a large array of HIV Abs (including many 2nd generation bnAbs), and found >2/3 were poly- and/or autoreactive (94), including two of the most potent and broad ones, the MPER⁺ bnAb 10E8 and the CD4bs specific bnAb VRC01, both for which self-reactivity was not originally detected in standard *in vitro* assays (95–97). Further in support of host controls limiting many (or most) bnAbs is a new study supporting the corollary that bnAbs should be more easily generated in autoimmune patients (due to defective tolerance), which has been supported by anecdotal observations of disproportionately lower frequencies of SLE⁺ patients with HIV infection (98–102). In this recent study, two HIV-infected cohorts differing in their abilities to produce bnAbs were compared, and relative to subjects that failed to generate bnAb responses, those producing bnAbs had higher frequencies of circulating auto-Abs and CD4⁺ T follicular helper cells and lower levels of T regulatory cells (103).

KI mice as models to identify strategies to break bnAb anergy and test pathogenicity

Two key related goals for learning how to induce bnAbs, especially if tolerizing self-reactivity of bnAbs is a prevalent issue (i.e. present in bnAbs against multiple Env targets,

and/or at key branchpoints in common maturation pathways of many lineages), are whether bnAb self-reactivity can be overcome by vaccine strategies and if so, whether inducing them by vaccination can be done safely? With respect to the first goal, a common theme emerging from studies in 2F5 UA and gl-3BCN60 HC/LC KI mice is that although KI B-cells are already tolerized prior to vaccine-driven maturation (90, 93), it is nevertheless encouraging that not all KI-B cells are eliminated early in development by clonal deletion, resulting in residual anergic KI⁺ B-cell populations that persist in peripheral tissues at various developmental stages, and thus are available for vaccine regimens to target/re-activate. However, this is not trivial to do, since anergic B-cells are preferentially selected against by immunization-mediated exclusion (78). Furthermore, it is now becoming better appreciated that B-cell anergy is not a discrete phenotype, both in terms of stages at which it can occur and the phenotypic and functional heterogeneity of anergic B-cells (89); thus, severity or reversibility of BCR unresponsiveness to immunization exhibited by anergic B-cells may vary.

Previous studies examining signals required to overcome B-cell unresponsiveness in the prototypical anergy HEL transgenic model have shown that providing stronger T-cell help (41, 43) and/or BCR signals (42, 104) to enhance B-cell survival (in situations where such signals are normally limiting), can re-activate anergic B-cells to secrete class-switched Abs, and other studies have similarly shown that BCR/TLR co-ligation can trigger residual anti-DNA⁺ self-reactive peripheral B-cells (44, 45). Interestingly, recent immunization studies in WT mice, in the presence of cytokines providing strong costimulatory signals and/or enhanced peripheral B-cell survival, exhibit enhanced anti-HIV Ab responses (105, 106). In one, pre-administration with BAFF/BlyS resulted in increased frequencies of self-reactive B-cells that was accompanied by increased potencies of Tier 1 serum nAb responses to Env gp120 immunization (105), whereas in another, DNA vectors expressing multimerized BAFF or APRIL, when added to an Env gp140 + IL-12 immunization regimen, induced more robust GCs and modestly enhanced autologous Tier 1 & 2 serum nAb titers (106). However, due to limitations of WT mice we discussed related to murine immunogenetics and system polyclonality, and the fact Env specificities of Ab responses in these studies were cumulative and variable, it could not be ascertained how individual bnAb lineages were initiated by such modified vaccine regimens, if at all.

In bnAb KI studies, where it is possible to evaluate specific effects of anergy-targeting vaccine regimens on development of individual bnAb lineages, results have begun to provide proof-of-principle that self-reactivity of bnAbs (or their precursors) can indeed be overcome by immunization. In gl-3BCN60 VDJ+VJ KI mice, McGuire *et al.* found that anergic KI⁺ cells could be re-activated, but only when 426c.TM4 V1-3, a soluble GL-targeting gp140 immunogen, was highly multimerized (93), although even then, the gl-3BNC60 transgene underwent no or minimal SHM. Likewise, in 2F5 UA VDJ+VJ KI mice, Zhang *et al.* demonstrated that a residual anergic KI⁺ B-cell subset could be targeted for expansion/activation by a vaccine regimen comprised of MPER peptides when conjugated to liposomes and formulated with TLR agonists, but underwent minimal class switching and SHM (90), raising the possibility that additional signal(s) may be required to activate a T-dependent response. Interestingly, Verkoczy *et al.* previously found this same MPER peptide/liposome/TLR regimen was also capable of inducing residual anergic dKI⁺ clones in original

(matured) 2F5 VDJ+VJ mice to produce high (mg/ml) titers of serum bnAb IgGs (Fig 1) (37). However, unlike 2F5 UA KI⁺ cells, they were able to undergo class switching (possibly due to being under less profound tolerance controls (90) and/or having higher affinity for the MPER epitope (57)), but vaccine-mediated re-activation of 2F5 mature KI⁺ anergic B-cells still occurred in a T-independent manner, suggesting T cell help and/or BCR signals were insufficient for activating 2F5 mature dKI⁺ B-cells, and to an even greater degree, 2F5 UA dKI⁺ B-cells. Notably, while this earlier study also found that both presentation of the MPER 2F5 bnAb epitope in liposomes and TLR adjuvanting were critical to break anergy (37), it did not ascertain if this was due to antigen multimerization in combination with TLR co-ligation (thus working as a classic Type 2 T-independent immunogen), or if more specific components in the regimen were involved. One clue supporting the latter possibility has come from follow-up studies that further dissected the regimen, in which specific presentation of the MPER peptide in liposomes i.e. in an orientation similar to that in the HIV-1 virion (in contrast to general multimeric display), and only with MPLA (versus other TLR agonists tested), were both key to induce optimal serum bnAb IgG titers in 2F5 and 4E10 VDJ+VJ mice ((107); H. Verville, L. Verkoczy et al, manuscript in preparation), suggesting a key role for lipid components in overcoming anergy of MPER⁺ bnAbs. An intriguing remaining question regarding overcoming MPER bnAb anergy is whether strength of T_H priming and degree of tolerization are related (i.e. is stronger T cell help required to fully re-activate and drive maturation of more profoundly anergic bnAb precursors?), an issue that can be rigorously examined via parallel evaluation of priming strategies in 2F5 mature and UA KI models. More generally, it will also be of interest to understand if signals for triggering anergic KI cells in gl-3BCN60 and 2F5 UA models are similar to those potentially needed to re-activate bnAb precursors targeting other Env regions.

Finally, most deletion or anergy-inducing poly-/autoreactive Abs are not pathogenic. Thus, bnAb KI mice can serve as platforms to not only test if and how to feasibly re-activate tolerizing bnAbs, but also to evaluate if any potential adverse pathogenic effects occur in doing so. An example of their utility for this purpose is a series of studies done in the original (affinity matured) 2F5 and 4E10 dKI models. Previously, it had been found that 2F5 and 4E10 exhibit vastly different *in vitro* self-reactivity and *in vivo* tolerization profiles, which also manifest distinctly in terms of pathogenicity. Specifically, 4E10 exhibits considerably higher lipid reactivity than 2F5 (80). Furthermore, while 4E10 exhibits a high degree of polyreactivity in protein arrays, 2F5 has none, and instead, has high affinity for Kynureninase, an auto-antigen involved in tryptophan metabolism that mimics the 2F5 minimal epitope ELDKWA (108). Interestingly, the selective *in vivo* LC editing-mediated removal of 2F5 MPER epitope and lipid binding, in peripheral follicular B-cells of mature 2F5 and 4E10 mature dKI mice, respectively, mirror these *in vitro* reactivities (85). Furthermore, while significant anti-coagulant activity has been reported for 4E10 *in vitro* (likely resulting from its cardiolipin reactivity), none was reported for 2F5 (109), and when passively administered to humans, 4E10 was biologically active and prolonged partial thromboplastin time, whereas no clinically adverse effects were reported in passive infusions with 2F5 (110). Importantly, remaining concerns about eliciting 2F5-like bnAbs as a safe vaccine target have now been alleviated in a recent pathogenicity study done in 2F5 mature

KI mice and macaques immunized with the MPER-liposome regimen, where, despite the high circulating plasma IgG 2F5 or 2F5-like Kynureninase cross-reacting Ab titers elicited, respectively, no associated autoimmune disease were seen in either model, nor were any disruptions in Kynureninase activity or tissue tryptophan metabolism observed (T. Bradley, B Haynes *et al*, ms submitted).

Germline reverted bnAb KI models for testing new HIV vaccine strategies

Induction of VRC01-like Abs in mouse models

VRC01-class antibodies (Abs) target the CD4bs of gp120 (96, 97, 111). All VRC01-class Abs utilize the IGHV1-2 HC, which acts as a structural mimic of CD4. The LCs of VRC01 Abs are variable, but all contain a short 5-amino acid (aa) CDR L3 to avoid steric clash with gp120 (97, 112, 113). To elicit VRC01-class Abs, immunization needs to mobilize B-cells that express a IGHV1-2 HC and a LC with the 5-aa CDR L3. Given the rarity of LCs with this 5-aa CDR L3, the frequency of VRC01 precursors in each individual may be quite limited. Another daunting aspect of VRC01-class Abs is their high levels of SHM. Not all the mutations are critical for neutralization activity; VRC01 still retained substantial neutralization activity when a substantial fraction of its mutations were removed (40, 114). Nevertheless, SHM is a largely random process, and it is not technically feasible to direct the mutation machinery to a specific position. In order to hit the desired spot, unwanted mutations may be unavoidable. Thus, to elicit a matured VRC01-like Ab, immunization may need to induce more mutations than the minimal set. Lastly, the strong reactivity of the matured VRC01 toward UBE3A may limit the induction of this type of Ab (94). Since the VRC01 paratope for UBE3A and gp120 appear to overlap, VRC01 affinity for the two antigens may go hand in hand. During immunization, when VRC01 intermediates have gained sufficient affinity for UBE3A, further maturation may run into roadblocks of tolerance control.

Since conventional vaccination strategies have so far failed to elicit bnAbs, a sequential immunization scheme has been proposed to overcome the impasse (55, 56, 61, 115, 116). In this scheme, bnAb development is initiated by a priming antigen that activates B-cells expressing the bnAb precursor. The priming reaction would generate memory B-cells that have undergone certain extent of SHM. The boost immunization employs an immunogen that resembles the native Env more than the priming antigen. Such boosting would preferentially reactivate the memory B-cells expressing Abs that have matured toward recognizing more native forms of Env. The process would be reiterated with more native-like Env antigens. Eventually, native Env would be used to select out B-cells expressing matured bnAb. The central concept of this immunization scheme is stepwise Ab maturation, which puts realistic demands on the SHM levels achieved at each immunization step.

A major obstacle in realizing this immunization scheme is that germline reverted VRC01 (gl-VRC01) Abs interact poorly with Env proteins (54–56, 97). To initiate VRC01 Ab development, Env protein must be engineered into a form that can bind VRC01 precursor Abs with sufficient affinity. A major impediment for gl-VRC01-Env interaction are certain N-linked glycans near the CD4bs. Removal of these glycans, by mutating the glycosylation site at N276 in loop D, N460 and N463 in V5 domain of a clade C virus (426c), significantly

enhanced the binding affinity between the mutant Env and gl-VRC01 Abs (55). However, the triple glycosylation mutations do not provide a universal solution to the problem of Env/glVRC01 interaction; the triple mutant 426c Env binds to some gl-VRC01-class Abs, but not others. In a more drastic modification of the Env protein, an engineered minimal gp120 outer domain (eOD) was used as a starting point for immunogen development (56, 117). The original eOD base interacts poorly with gl-VRC01 Abs. Through a series of structure-based modifications, the eOD-base was eventually evolved into a broad gl-VRC01 binder, eOD-GT8 (117). However, gl-VRC01 Abs contain CDR3 from mature VRC01 Abs and may not be equivalent to *bona fide* VRC01 precursors. To evaluate the potential of eOD-GT8 to engage genuine VRC01 precursors and to assess their frequency in humans, eOD-GT8 binding B-cells was isolated from human peripheral blood (117). From 61.6 million naïve B-cells, 26 isolated B-cells (a frequency of 1 in 2.4 million) express Abs that were composed of IGHV1-2 HC and LC with 5-aa CDR L3. Moreover, based on structural studies, the isolated VRC01-like Ab binds to the CD4bs in essentially the same manner as VRC01-class Abs. These experiments demonstrated that eOD-GT8 is a promising candidate for priming the immune response for VRC01-class Abs.

Immunization studies in the VRC01gH KI model—The efficacy of eOD-GT8 as a priming immunogen was evaluated in a VRC01 KI model (Fig. 2; A) (118). In this model, a rearranged gl-VRC01 HC (VRC01gH) was integrated into the mouse J_H locus, and 85% of B-cells express the VRC01gH. VRC01 LC was not introduced into this model. The hypothesis was that mouse LCs with 5-aa CDR L3 could function in association with IGHV1-2 HC as VRC01 precursors. For immunization purposes, eOD-GT8 was formulated as 60-mer nanoparticles, with the goal of binding cognitive BCRs with higher avidity than monomeric antigens and activating B-cells more effectively through receptor crosslinking. Immunization of VRC01gH mice with eOD-GT8 60mer elicited memory B-cells that expressed VRC01-like Abs. Specifically, nearly 90% of CD4bs-specific IgG⁺ B-cells expressed Abs composed of VRC01gH and mouse LCs with 5-aa CDR L3; moreover, the CDR L3 was enriched for a partial VRC01 consensus motif QQYXX. Functionally, these Abs bound specifically to the CD4bs, but exhibited no neutralization activity, owing to limited SHM. These results indicate that eOD-GT8 60mer is an effective priming antigen to activate B-cells expressing VRC01 precursor Abs. By contrast, immunization with native trimeric Env protein, BG505 SOSIP, elicited no VRC01-like Abs, likely due to poor interaction between native Env proteins and VRC01 precursors.

Given low SHM levels after the priming step, VRC01-like Abs at this stage may not be able to effectively recognize native Env proteins. This consideration prompted the development of bridging immunogens for initial boosting (119). One such immunogen, BG505coreGT3, contains the complete gp120 core of BG505 Env, including inner domain and bridging sheet, which were absent in the streamlined eOD-GT8. In addition, BG505coreGT3 contains fewer mutations than eOD-GT8 so that its CD4bs is more native-like. An important property of BG505coreGT3 is that it binds mature VRC01-class Abs with >1000-fold higher affinity than the germline reverted counterparts. Thus, boost immunization with BG505coreGT3 would promote the maturation of VRC01-like precursor Abs. As an alternative boosting immunogen, BG505GT3 was also produced as native Env trimers, i.e. BG505 GT3 SOSIPs.

To test the efficacies of these bridging immunogens, VRC01gH mice were immunized with two immunization protocols (119). In one scheme, the mice were primed with eOD-GT8 60mer, then boosted once with BG505 GT3 nanoparticles, and finally boosted twice with BG505 SOSIP N276D. In the second scheme, the mice were primed with eOD-GT6 60mer, then boosted once with BG505 GT3 SOSIP, and finally boosted twice with BG505 SOSIP N276D. Both immunization protocols elicited CD4bs-specific Abs that were composed of VRC01gH and mouse LCs with 5-aa CDR L3. Additionally, substantial levels of SHM were induced, and some of the mutations corresponded to those found in VRC01-class bnAbs. Particularly remarkable was the strong enrichment for the conserved E residue within the 5-aa CDR L3; in fact, some CDR L3s have acquired the complete consensus QQYEF motif of VRC01-class bnAbs. Along with induction of SHM, these immunizations also elicited Abs that were able to neutralize multiple tier 2 HIV-1 isolates with the N276 mutation. Two of these could neutralize a tier 2 virus N276 mutant with comparable potency as the mature VRC01 Ab, and were able to weakly neutralize the native form of this viral isolate that has the intact N276 glycosylation site. Other viral isolates with intact N276 glycosylation site remained refractory to neutralization by these and other cloned Abs. Thus, these boosting regimens matured VRC01-like Abs to an intermediate stage, validating the strategy of sequential immunization as an effective approach to mature VRC01-like Abs.

Immunization studies in the 3BNC60 KI model—Immunization studies have also been carried out in KI models for another VRC01 family member, 3BNC60. Similar to the VRC01gH KI model, one 3BNC60 KI model (GLV_H) expresses the gl-3BNC60 V_HDJ_H rearrangement (Fig. 2; B). B cell development in this model appeared largely normal, with essentially all naive B-cells expressing the KI gl-3BNC60 HC (120). The mice were immunized with two kinds of germline VRC01 binding antigens: eOD-GT8 60mer and multimerized 426c.TM4 V1-3 (93). The 426c.TM4 V1-3 immunogen contains mutations in three N-linked glycosylation sites near the CD4bs as well as truncations of variable loops 1-3 (V1-3); these modifications improved the binding affinity of 426c Env toward gl-VRC01 Abs. Immunization of GLV_H mice with these antigens induced robust Ab responses; in the case of eOD-GT8 60mer immunization, the serum Abs showed detectable preference for CD4bs. However, from sorted CD4bs-specific B-cells, 5-aa CDR L3 was found only in one out of four immunized mice. The less dramatic induction of VRC01-like Abs in GLV_H model relative to VRC01gH model could potentially be attributed to differences in the HC transgenes. Although both transgenic HCs utilize germline IGHV1-2*02, VRC01gH contains the HCDR3 from the mature VRC01 Ab, whereas the gl-3BNC60 HC contains HCDR3 from the mature 3BNC60 Ab. In this regard, eOD-GT8 binds to gl-VRC01 Ab with about 10-fold higher affinity than gl-3BNC60.

Immunization studies have also been carried out in a mouse model (MuV_H) that expressed mature 3BNC60 V_HDJ_H rearrangements (Fig. 2; C) (120). An Ab composed of mature 3BNC60 HC and a LC with 5-aa CDR L3 was considered a synthetic 3BNC60 intermediate, and the model was designed to test the ability of immunogens to promote further maturation of this intermediate, primarily through optimization of the LC. The introduction of a mature 3BNC60 HC into this model dramatically improved its immune response to eOD-GT8 60mer and native Env trimer, BG505 SOSIP. In both cases, immunization led to marked

induction of CD4bs-specific Abs and neutralization activities in sera. Correspondingly, the frequency of CD4bs-specific IgG⁺ B-cells in MuV_H mice increased dramatically in response to immunization with either eOD-GT8 or BG505 SOSIP, and most of these B-cells expressed mouse LCs with 5-aa CDR L3s, which contains an E residue at the 4th position, a signature of VRC01-class Abs. Moreover, these cloned Abs were CD4bs-specific and recapitulated the serum neutralization activities. Overall, BG505 SOSIP elicited broader neutralization activities than eOD-GT8 60mer. These results reinforce the notion that maturation of intermediate Abs requires native-like Env immunogens.

Expression of germline reverted VRC01 or 3BNC60 KI HCs alone did not cause any major problems for B cell development. However, as described earlier, co-expression of gl-3BNC60 V_HDJ_H and V_LJ_L rearrangements in the third 3BNC60 model severely impeded B cell development (Fig. 2; D) (93). In these mice, the mature B cell population was severely depleted, presumably a reflection of autoreactivity or polyreactivity of gl-3BNC60 Ab. The depletion of gl-3BNC60 B cells was not complete, and some did populate peripheral lymphoid tissues. Presumably because the gl-3BNC60 expressing B-cells were anergic, the mice responded poorly to c426.TM4 V1-3 immunization. Multimerization of c426.TM4DV1-3 antigen significantly improved the Ab response, which appeared to be partially specific for CD4bs. Most of the elicited Abs were unmutated and exhibited no neutralization activity, a result that was not surprising, given that these mice have received only one immunization. Another issue is that multimeric antigen could activate B-cells through T-independent pathways, which do not induce SHM. Alternatively, the preliminary activation observed may have broken the anergic state, thus enabling B-cells to participate in normal T-dependent responses in subsequent rounds of immunization.

Immunization studies in KI models with diverse VRC01 precursor repertoires

—Both VRC01gH and 3BNC60 KI models express rearranged IGHV1-2*02 HCs with fixed HCDR3 from mature Abs. By contrast, in the human Ab repertoire, IGHV1-2 is linked to diverse HCDR3s, a subset of which may be compatible with the development of VRC01-like Abs. Thus, a key issue is to assess immunogens in a setting where the human IGHV1-2 is expressed in association with diverse HCDR3s. One way to achieve this is by generating a KI model where the human IGHV1-2 segment substitutes for one of the mouse V_H segments. During V(D)J recombination, IGHV1-2 recombines with mouse D and J_H segments to create a diverse range of HCDR3s. However, the low frequency of B-cells expressing potential VRC01 precursors in this kind of model may pose practical difficulties for immunization studies. It would be desirable to have a KI model that has a diverse repertoire of IGHV1-2 Abs, but at high enough frequencies to generate a robust immunization readout. These considerations prompted development of the V_H1-2 KI model, where IGHV1-2*02 substituted for the mouse V_H81X gene segment at the endogenous IgH locus (Fig. 2; E) (121). V_H81X is the most proximal V_H segments relative to D segments. Owing at least in part to its proximity to D segments, V_H81X is the most frequent utilized V_H segments during HC rearrangement (121). The bias for V_H81X rearrangement becomes even more pronounced when a regulatory element at the intergenic region between V_H and D segments, IGCRI, is deleted. Similarly, when IGHV1-2*02 replaced V_H81X in the context of IGCRI deletion, it was utilized in approximately 45% of peripheral B-cells (Fig. 2; E)

(122). In this setting, IGHV1-2*02 recombines with the whole set of mouse D and J_H segments to create a diverse range of HCDR3s. Therefore, VH1-2 KI mice can serve as an assay to evaluate the efficacy of immunization strategies to selectively engage the VRC01 precursor from a diverse pool of Abs expressing IGHV1-2*02 HC. Immunization of VH1-2 mouse model with eOD-GT8 60mer elicited noticeable serum Ab response targeting CD4bs, and the frequencies of CD4bs-specific memory B-cells increased correspondingly. Among the LCs expressed by CD4bs-specific memory B-cells, the frequency of 5-aa CDR L3 is significantly higher than that in the pre-immune repertoire, and CDR L3 is enriched for the partial consensus VRC01 motif of QQYXX. In binding assays, the elicited VRC01-like Abs were specific for CD4bs, but have not matured enough to attain neutralization activities. These results showed that, even in the challenging environment of a complex Ab repertoire, it is feasible to selectively activate B-cells expressing VRC01-like precursors with a high affinity germline VRC01 binder.

Due to diverse Ab repertoires, the number of VRC01-like precursors in the VH1-2 model is limited. To facilitate studies on AM, a model with higher precursor frequencies was generated. In this model (VH1-2/LC), a rearranged gl-VRC01 LC was integrated into the J_κ locus of the VH1-2 model (Fig. 2; F). Following a sequential immunization strategy, the VH1-2/LC model was first primed with eOD-GT6 60mer to selectively activate B-cells expressing VRC01-like precursor Abs. eOD-GT6 is related to eOD-GT8, but has lower binding affinities to gl-VRC01 Abs. The first set of boosting immunogens was based on the Env protein of 426c virus; the use of Env antigens from different viral strains for the prime and boost protocol was intended to help focus immune response to CD4bs. The 426c gp120 core with mutations in three glycosylation sites (N276, N60, N463) was used for the first boost immunization. At this early stage of immunization, the precursor Ab is unlikely to have progressed far in AM; the triple glycosylation site mutations would facilitate the interaction of the immunogen with early intermediates. On the other hand, the 426c core immunogen contains both the outer and inner domains of gp120, whereas eOD-GT6 consists primarily of a minimal outer domain. So, the use of 426c core immunogen served as the first step to guide Abs toward recognizing CD4bs in a more native context. In subsequent immunizations, 426c core with two, one or no glycosylation site mutations were used sequentially to gradually select for B-cells that express Abs capable of accommodating these glycans near CD4bs. The final boost employed the native trimeric form of 426c Env protein. This stepwise protocol elicited CD4bs-specific VRC01-like Abs, which were composed of IGHV1-2*02 HCs and the transgenic gl-VRC01 LC. These Abs accumulated substantial levels of SHM, some of which mimic those in VRC01-class Abs. Some of the VRC01-like Abs bound the autologous WT Env trimer, 426c SOSIP, but only 1/15 Abs bound weakly to the heterologous BG505 Env trimer. In neutralization assays, 12/15 Abs isolated 22 weeks post immunization exhibited neutralization activities against the autologous 426c virus with N276D mutation, and the potencies of some of these Abs were comparable to that of the mature VRC01 Ab; 4/15 Abs neutralized a heterologous virus that lacks N276 glycosylation. One Ab showed weak neutralization activity toward the WT 426c virus. By contrast, immunization with the native trimeric BG505 SOSIP did not elicit any detectable nAbs.

Immunization studies in Kymab mice—Although the VH1-2 mouse model contains a diverse repertoire of IGHV1-2*02 HCs, the frequency of B cells express IGHV1-2*02 HC (45%) is much higher than that in human Ab repertoire (~3%) (123–125). To evaluate the efficacies of eOD-GT8 as a priming immunogen in a fully polyclonal environment, immunization studies have also been done in Kymab mouse, in which the complete human IgH, Igκ and Igλ variable region repertoire were incorporated into the corresponding mouse loci (126). In Kymab mice, the human IGHV1-2 HC represents about 1% of the Ab repertoire, lower than, but close to, that of human Ab repertoire (~3%) (123–125). However, the frequency of 5-aa CDRL3s in Kymab mouse is 0.018%, which is lower than that in humans (~0.9%) by 50-fold (123). Taking into account that mice have fewer B-cells than humans, VRC01-like precursors are expected to be more rare in Kymab mice than in humans. Consistent with this prediction, no VRC01-class B-cells were identified by eOD-GT8 sorting among 300 million B cells from naïve Kymab mice (123); using the same technique, VRC01-class B cells were detected at a frequency of 1 in 2.4 million human B-cells (117). Remarkably, in spite of the extreme paucity of VRC01-class B cells in Kymab mice, immunization with eOD-GT8 60mer elicited CD4bs-specific Abs, about 1% of which were composed of IGHV1-2 HC and various LCs with 5-aa CDRL3s (123). The CDRL3 is enriched for the partial VRC01 consensus, QQYXX, and some of the LCs correspond to those in VRC01-class Abs. Altogether, VRC01-like Abs were detected in 29% of immunized Kymab mice (123). Because the mice were immunized only once, none of the elicited VRC01-like Abs matured enough to attain neutralization activities. Similar to immunization results in other mice models, immunization with WT Env trimer, BG505 SOSIP, failed to elicit VRC01-like Abs in the Kymab model. These experiments represent the most stringent test so far for eOD-GT8, and the result further confirmed the ability of this antigen to serve as an effective priming immunogen for VRC01-class Abs.

In summary, various immunization studies, now done in four types of mouse models, have thus far provided several important insights and pointed directions for future studies. First, priming with high affinity germline-binding immunogens, such as the eOD-GT8 60mer, is necessary to initiate the development of VRC01-like Abs. Second, sequential immunization with progressively more native-like Env immunogens is an effective way to mature precursor VRC01-like Abs toward nAbs. Third, the accommodation of N276 glycosylation appears to be a bottleneck for VRC01-like Ab development. In mature VRC01 Abs, CDRL1 truncation or mutation helps to accommodate the N276 glycan. Some of the elicited Abs from the immunized VRC01 mouse models utilized mouse LCs with short CDRL1s (118–120, 122), and these Abs may eventually overcome the N276 glycan obstruction with additional AM. Fourth, neutralization activities were induced in three types of VRC01 models (VRC01gH, 3BNC60, and VH1-2). However, in these mouse models, VRC01 HCs and/or LCs were expressed at much higher frequencies than those in humans. On the other end of the spectrum, Kymab mice offer a potentially even harder condition than that in humans to elicit VRC01-class Abs. Although the eOD-GT8 priming has successfully elicited VRC01-class precursor Abs in a fraction of immunized Kymab mice, it would be challenging to mature these precursor Abs into nAbs. In a complex Ab repertoire, boost immunization with more native forms of Env antigens, as those employed in the other three VRC01 mouse models,

would likely induce irrelevant Ab responses, which may dominate over the development of VRC01-class Abs.

Induction of PGT121-like Abs in KI models

The PGT121 lineage of bnAbs target the V3 and V1 regions near the apex of the Env trimer (127, 128). The epitopes of this class of bnAbs are complex, involving a combination of glycans and peptides (127–130). Induction of PGT121-class Abs faces some of the same obstacle as those for other bnAbs, namely, low precursor frequency and high SHM. PGT121-class bnAbs employ a long 26-residue HCDR3 to penetrate through the glycan layer to interact with the underlying peptides; HCDR3s of such length are rare in the human Ab repertoire. SHM frequencies for PGT121-class bnAbs, although lower than those in VRC01-class Abs, are still far above average, and their LCs also contain indels. Tolerance control may not be a major concern for PGT121-class Abs, as PGT121 exhibits no overt poly-/autoreactivity at least *in vitro* (94).

The sequential immunization strategy is equally applicable to the induction of PGT121-class of Abs. Similar to other reverted bnAbs, gl-PGT121 Abs exhibited no appreciable binding affinity toward WT Env proteins (131). Through multiple rounds of mutagenesis and selection, BG505 Env proteins was engineered into gl-PGT121 binder (10MUT and 11MUTB) (131). Additionally, some of the optimization intermediates (5MUT and 7MUT), i.e. Env proteins with fewer mutations and lower binding affinities for gl-PGT121, served as effective immunogens for AM intermediates and were instrumental to the induction of neutralization Abs. The efficacies of the PGT121 immunogens were tested in two mouse models that express either the gl-PGT121 Ab, which is composed of germline V and J segments and the HCDR3 of the least mutated member in this lineage, or a synthetic intermediate composed of a mature PGT121 HC and gl-PGT121 LC (132). The rearranged V(D)J exons of these HC and LC were integrated into the mouse J_H and J_κ loci, respectively, to generate the mouse models for gl-PGT121 Ab (GL_HL121) (Fig. 3; A) and intermediate PGT121 Ab (Mut_HGL_L121) (Fig. 3; B). Consistent with the lack of noticeable poly-/autoreactivity of PGT121 Ab, expression of these Abs did not cause obvious problems for B cell development, and essentially all B-cells express the transgenic HC and LC. Repeated immunization of GL_HL121 and Mut_HGL_L mice with native trimeric Env proteins, YU2 SOSIP or BG505 SOSIP, failed to elicit any detectable Ab response. The outcome came as no surprise, as GL_HL121 and Mut_HGL_L Abs do not interact appreciably with native trimeric Env. In contrast, a single immunization with the germline-binding immunogen 10MUT was sufficient to elicit readily detectable autologous antigen binding Abs in serum. The elicited Abs were PGT121-like, as interaction with 10MUT was abolished by mutations within the PGT121 epitope. After priming, the serum gained binding affinity for an engineered version of BG505 Env (7MUT) with fewer mutations, thus closer to native Env, than 10MUT. Accordingly, trimeric 7MUT SOSIP was used for the second immunization to steer AM toward recognition of more native form of Env proteins. After second immunizations, serum Abs gained detectable affinity toward even less mutated forms of BG505 Env, 5MUT, which was then chosen as the third boost. After the third immunization, binding activity for native Env trimers was detectable in serum, leading to the logical step of using native BG505 trimer as the fourth boost. Moreover, to broaden the

immune response, a cocktail of native-like BG505 Env trimers with diverse variable loop sequences (VLC) was used as the final boosts. After this series of immunization, cross-clade viral neutralization activities were detectable in 5/7 GL_{HL}121 mice and 7/7 Mut_HGL_L121 mice. The neutralization breadth of the elicited Abs was assessed on a panel of 54 tier 2 viruses and 2 tier 1B viruses and compared side by side with the mature PGT121 Ab. Abs from GL_{HL}121 and Mut_HGL_L121 mice were able to neutralize 12 and 23 viruses in the test panel, respectively; for comparison, mature PGT121 Ab could neutralize 50 viral isolates. Thus, immunization induced nAbs with substantial breadth. In parallel with the development of neutralization activities, the elicited Abs accumulated substantial levels of SHM, some of which mimic those in PGT121-class Abs.

In conjunction with immunization studies in VRC01-class KI models, these experiments in PGT121 KI models further highlight both the value of Env engineering for developing efficacious immunogens and testing their potential in human Ig KI mouse models. The successful induction of neutralizing activities, the closest to bnAbs in animal models so far, also further validates the concept of sequential immunization. However, going forward, the induction of PGT121-like Abs faces a similar challenge as that for VRC01: the immunization strategy must be able to elicit bnAbs in a complex Ab repertoire, where the precursor Ab is present at physiological frequencies. The problem is likely to be more acute for PGT121-class Abs, and other long HCDR3-expressing bnAbs, than for VRC01. HCDR3 constitutes a major part of the interface in the complex of PGT121-class Abs and Env; for this reason, PGT-121 class Abs have stringent requirements for both the sequence and length of HCDR3. By comparison, VRC01-class Abs are more flexible with respect to HCDR3 sequence, and their more typical HCDR3 length are usually not associated with counter selection that long HCDR3-expressing B-cells potentially face during B cell development. Thus, the precursor frequency for PGT121-like Abs is likely to be even lower than that for VRC01-class Abs. Immunization in a complex Ab repertoire poses yet another difficulty: Ab responses that target irrelevant epitopes. In the case of VRC01, the problem can be ameliorated at least in part by using a minimal outer domain where the antigenic variable loops are trimmed away. This solution may not apply to PGT121 immunogens, since V1 and V3 are part of the bnAb epitope. Furthermore, V1-V3 loops mediate protomer association (133, 134), and as shown by immunization studies in the PGT121 KI model, trimeric but not monomeric immunogens are necessary to elicit adequate immune response (132). Thus, V1-V3 loops need to be preserved in PGT121 immunogens, but the presence of these variable and potentially antigenic loops may increase the chance of diverting immune responses.

Conclusions and future perspectives

Tremendous progress has been made in this current “golden era” of bnAb discovery, where information emerging from cloning and characterization of new bnAbs that began ~5 years ago, is now starting to be more effectively applied to immunogen design and for formulating new vaccine strategies. Yet, much still remains to be learnt in the pursuit of achieving vaccine-guided bnAb induction. Given these advances, we must now ask what are the most critical questions in a path to developing a bnAb-based HIV vaccine, and how the most relevant issues can best be aided by further improvements in existing human Ig KI

methodologies, in order to allow even more iterative and practical testing of new vaccine candidates in driving efficacious bnAb induction.

One such critical question moving forward is: what will the most effective stimulating antigens be to initiate development of bnAb lineages, and then a key related issue would be: what types of human Ig KI models will be best suited to study this issue? A major consideration in properly designing such studies will be for candidate immunogens to be capable of binding precursor naïve B-cells bearing *bona fide* germline (gl) rearrangements i.e. direct products of V(D)J recombination originating from unmutated human variable region exons, for any new bnAb of interest. Thus, of all human Ig KI models potentially available to test this question (Fig. 4), only those capable of generating such true gl rearrangements, can be physiologically relevant with certainty. In particular, of the gl-reverted models discussed in this review, those which are “conventional KI” mice, i.e. VRC01gH, gl-3CN60, and gl-PGT121 have pre-rearranged V(D)Js that use gl V segments but retain mature HCDR3s (118, 120) (Fig. 4; **Models “A1”**). Thus, while potentially informative to develop boosting regimens and/or prime early potential intermediates of maturation pathways (assuming vaccine-induced pathways will reproducibly form similar mature HCDR3 junctions) they can’t reliably inform truly initial priming phases of complete maturation pathways. Thus, which other KI models are best suited to address this key issue?

This largely depends both on the molecular characteristics a bnAb has acquired, as well as the information that is (or will be) available regarding its evolutionary pathway. For example, for bnAbs on which information regarding its molecular evolution is not available (or limited), the best-suited, existing KI model is one having unrearranged V_H and/or J_H segments knocked-in (Fig. 4; **Models “B”**), made like the VH1-2 KI model (122), since due to its ability to preferentially rearrange V_H1-2, can express a repertoire enriched for VRC01-like lineages, in contrast to “fully” Ig-humanized models expressing completely polyclonal, human V(D)J-derived repertoires, i.e. Kymab® (123, 126) or Regeneron VelocImmune® mice (135, 136) (Fig. 4; **Model “E”**), which rarely express such bnAb rearrangements, thus making iterative study of bnAb development impractical. On the other hand, if complete virus and Ab co-evolutionary pathways from which a bnAb develops have been elucidated via comprehensive longitudinal sampling, including having a high-probability inference of this bnAb’s unmutated common ancestor (UCA), the goal is to employ the “B-cell lineage immunogen design” vaccine strategy (61), in which priming is done with the lineage’s original transmitted founder virus, and sequential boosting is done using Env immunogens generated from the viral isolates that co-evolved with its progressively-matured Ab intermediates. In such instances, then the study focus becomes using such a regime as a roadmap to recapitulate (and potentially, short-circuit maturation of this UCA seen during infection), during immunization, and using alternative versions of “conventional” bnAb KI models, in which pre-rearranged V(D)J’s of such bnAbs’s UCAs have been knocked-in (Fig. 4; **Models “A2”**) is warranted. As an example, bnAb KI mice bearing the CH103 lineage UCA rearrangements would hold particular promise for evaluating the B-cell lineage design vaccine sequential approach, since the entire CH103 lineage’s co-evolutionary trajectory has been molecularly elucidated (35), its bnAbs have acquired less SHM (relative to other CD4bs bnAbs), and its viral co-evolution can be re-capitulated in near identical fashion in

humans and primates (137), suggesting its co-evolved bnAb lineage maturation trajectories are reproducible. Finally, for bnAbs with long HCDR3 regions (~2/3 of all bnAbs), even if their full maturation trajectories have been deciphered by longitudinal sampling, it may still be challenging to correctly deducing their UCA rearrangements with a high likelihood, given that assigning nucleotides originating from SHM rather than N-addition at V(D)J junctions becomes increasingly difficult, the longer the HCDR (138). Thus, given the ambiguity of inferring *bona fide* UCA variable exon sequences of lineages producing bnAbs with long HCDR3s, determining whether the transmitted/founder virus, or an earlier stimulating antigen primes development of such lineages may not be feasible. In such cases, the current “2nd generation” Ig bnAb KI model V_H1-2, expressing unrearranged V_H and J_H (Fig. 4; **Models “B”**) would need further alterations to accommodate the longer human D and/or J regions (relative to those in WT mouse IgH loci), required to produce elongated HCDR3s (139). Such additional modifications could involve either replacing the murine D cluster with a relevant long human D (Fig. 4; **Model “C”**), replacing the murine J_H cluster with a human DJ_H containing a long DJ_H junction, or generating a “limited germline repertoire” KI model that preferentially expresses rearrangements derived from a limited set of V, D, and J segments, common to bnAbs with long HCDR3s (Fig. 4; **Model “D”**),.

As we discussed, two other critical, related questions for HIV vaccinology will be whether certain Env targets are more tractable for eliciting by vaccination (either due to bnAbs targeting them requiring less SHM and/or not being under tolerance controls) and assuming more than one is tractable to elicit, if achieving more modest breadth to such targets can work as apart of a “cumulative breath” strategy. Both of these questions require generating a larger volume of bnAb KI models, to allow for systematic identification of impediments common or unique at distinct targets, and then, devising strategies with modified adjuvants, T cell primes, and/or immunogens to test how to overcome them. Thus, determining relative tractability of such targets for vaccine candidates will require comprehensive testing of tolerance prevalence and minimal SHM requirements of bnAbs across all Env targets, which in turn will necessitate use of higher-throughput KI-producing methodologies. The technology employed in generating the V_H1-2 mouse models, termed RDBC (Rag2 deficient blastocyst complementation) (Fig. 5) (140) would help in this endeavor. Using this methodology, the multiple genetic modifications in the V_H1-2 models were introduced into the genomes of ES cells, which were injected into Rag2 deficient blastocysts. As Rag2 is critical for V(D)J recombination, all the B and T cells in the resultant chimeric mice are derived from the injected ES cells. For this reason, the chimeric mice can be used directly for immunization experiments. The RDBC method obviates the need for lengthy and costly mouse breeding during germline transmission, and is particularly advantageous for mouse models containing multiple genetic modifications on different chromosomes, which will segregate during mouse breeding. Construction of mouse models can also be further aided by the application of CRISPR technology that can greatly expedite genetic manipulations in ES cells. With these technological advances, it will now be feasible to accelerate the production bnAb KI models so that a large array can be made, thus making it more feasible to analyze bnAb development across multiple lineages, including those targeting Env regions other than the CD4bs. Furthermore, with the combination of these two technologies, it will not only enable study of multiple individual bnAb lineages, but

intermediate members of key lineages, thus making it possible to study critical maturation bottlenecks unique to particular lineages. This may be critical, since extensive SHM can either remove or introduce self-reactive residues (30, 31, 37, 141–143), and matured bnAbs could have less tolerizing self-reactivity than their precursors (90), or more. Thus, critical lineage-specific selection bottlenecks can occur at distinct stages of maturation, and KI models expressing intermediates can allow testing of lineage-specific vaccine regimens to overcome unique bottlenecks at unparalleled resolution.

Finally, in addition to the advent of higher-throughput techniques to produce KI models, other novel approaches that may further facilitate study of bnAb development will include those in which stage-specific expression is manipulated. An instance where this could be particularly useful to study responses of bnAb⁺ B-cells that normally are not permitted by host tolerance to progress into mature B-cell compartments, a potentially significant technical hurdle, given the large number of 2nd generation bnAbs that also exhibit features predicted to predispose them to near-complete deletion in bone marrow, for example bnAbs like VRC26, whose unusually long HCDR3s (36) normally undergo negative selection in early B-cell development (25). One specific example of such an approach would be employing a Cre-loxP inversional recombination system to conditionally express a “passenger” bnAb specificity in the periphery, analogous to strategies previously used to modify B-cell specificities in memory B-cells (144). Another example of further refinements to bnAb KI models revolves around mechanistic issues of SHM in bnAb maturation, including specifically, whether high SHM levels are inherent to and/or generally required (or perhaps even detrimental) for bnAb development, and if so, whether inducing such high levels or unusual, insertion/deletion events associated with such high SHM rates, but key for function of many bnAbs (145), is both feasible and safe for eliciting via vaccines. In this regard, examples of KI models that could be generated to facilitate such questions are those co-expressing passenger KI alleles, in order to better dissect the intrinsic mutability component of bnAb responses (146), as well as those bred to other genetically modified KI/KO lines with altered SHM and/or GC B-cell selection. One example of such a “compound” bnAb KI line is one generated via crossing to a dominantly active form of polymerase- ζ , i.e. rev3 KI mice (147), in which SHM rates are accelerated, and thus SHM levels during immunization-guided bnAb lineage development would be forced to reach those seen during chronic infection.

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Fig 1. B-cell tolerance controls in the 2F5 bnAb KI mouse model

Shown is a pictorial summary of all bnAb regulation and induction studies done in the setting of 2F5 $V_HDJ_H^{+/+}V_KJ_K^{+/+}$ KI mice. The original (affinity matured) version of 2F5, upon knocking its VDJ/VJ rearrangements into B-cells, fails to pass multiple tolerance checkpoints (83–85). However, while nearly all 2F5⁺ B-cells are clonally deleted at the 1st checkpoint (the pre-→imm. B transition), 5% populate the periphery, but most have been functionally silenced. Amongst these residual anergic B-cells, a minority remove all self-reactivity & escape tolerance altogether, but due to the inability of 2F5 receptor editing to remove dominant 2F5 HC self-reactivity, purifying selection occurs in later development, by a T-dependent, SHM-driven process, “Affinity Reversion/Ab Redemption” (23, 30, 31, 37). Finally, serum bnAb IgG responses can be elicited in 2F5 KI mice immunized with an MPER peptide-lipid-TLR agonist vaccine regime through re-activation of unedited (MPER⁺) anergic KI B cells not yet subjected to purifying selection (84, 85).

Fig 2. Mouse KI/chimeric models for testing VRC01 class-targeting vaccine strategies
Schematic illustration of VRC01gH, 3BNC60 GLV_H, 3BNC60 MuV_H, or gl-3BNC60 HC/LC mouse KI and V_H1-2 or V_H1-2/LC chimeric model alleles generated to evaluate the ability of CD4bs-targeting sequential immunization strategies to induce VRC01-like Abs.

Fig 3. PGT121-class bnAb KI models

Schematic illustration of PGT121 GL_{HL} and PGT121 Mut_HGL_L KI alleles generated to evaluate the ability of V3-targeting sequential immunization strategies to induce PGT121-like Abs.

Fig 4. Types of Ig-humanized mouse models to study vaccine-guided bnAb development

Pictorial representations of the structural organization of targeted alleles from various kinds of current or planned Ig-humanized models are shown, relative to the WT murine allele. For simplicity, only HC loci are depicted, and only at one allele. Models that have been published (with corresponding references to the right), or models which are being generated, are denoted in black text (Models A, B, and E), while theoretical germline/unrearranged models (that could be made to further bridge the current gap between physiological relevance and practicality to study “difficult to infer, long HCDR3” bnAb specificities; Models C and D), are annotated blue text. Mouse and human germline-derived sequences/segments are depicted in red and blue, respectively. Denoted as a green segment in the A1 type of models are “mutated” HCDR3s from the human bnAbs VRC01 and PGT121, i.e. from either the original/mature VRC01 bnAb, but with a single mutation to remove an unpaired cysteine (118), or for PGT121, from the least mutated lineage member (132). The intergenic control region 1 (IGCR1) normally found in WT HC loci is denoted by a large circle, which has been removed in models B,C, D, to further facilitate rearrangement of D-proximal (V_H 81X-positioned) V_H segment(s) of interest. p=promoter region; L=leader sequence.

Fig. 5. High-throughput *in vivo* expression of bnAbs in chimeric mice using RDBC

Shown are the two major steps involved in generating bnAb KI chimeric mice using RAG-dependent blastocyst complementation: generation of blastocyst chimeras by microinjection of bnAb KI ES cells (A), and implantation of blastocysts in pseudopregnant RAG2^{-/-} foster mothers, resulting in chimeric offspring (B) whose lymphocytes all express the knocked-in bnAb specificity and can be used for immunization. Shown as an optional step is germline transmission of bnAb KI allele to generate a standard KI model (C), which can be pursued if more in-depth studies with a particular model are desired.