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Germline Targeting Immunogens

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Summary

In 2009 D. Dimitrov's group reported that the inferred germline (iGL) forms of several HIV-1 broadly neutralizing antibodies (bNAbs), did not display measurable binding to a recombinant gp140 Env protein (derived from the dual-tropic 89.6 virus), which was efficiently recognized by the mature (somatically mutated) antibodies (1). At that time a small number of bNAbs were available, but in the following years, the implementation of high-throughput B cell-isolation and sequencing assays (2) and of screening methodologies (3) facilitated the isolation of greater numbers of bNAbs from infected subjects. Using these newest bNAbs, and a wide range of diverse recombinant Envs, we and others confirmed the observations made by Dimitrov's group. The results from these studies created a paradigm shift in our collective thinking as to why recombinant Envs are ineffective in eliciting bNAbs and has led to the 'germline-targeting' immunization approach. Here we discuss this approach in detail: what has been done so far, the advantages and limitations of the current germline-targeting immunogens and of the animal models used to test them, and we conclude with a few thoughts about future directions in this area of research.

Keywords

germline antibodies; B cell receptors; Env; immunogens; HIV

Broadly neutralizing antibodies and their targets on Env

Details on the identification and epitope specificities of HIV-1 bNAbs are discussed elsewhere in this issue. Briefly, bNAbs (which have been isolated from numerous infected subjects), target defined areas on the HIV-1 envelope glycoprotein (Env),: One in the gp41 subunit: the membrane proximal external region, MPER, and four in the gp120 subunit: the CD4-Binding Site (CD4-BS), the co-receptor binding site, a region at the base of the third hyper-variable region (V3) centered around the conserved N332 glycosylation site, and a glycopeptide epitope encompassing the N160 glycosylation site at the apex of the Env spike. bNAbs whose epitopes span elements of both gp120 and gp41 have also been identified.

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In some cases, bNAbs that target the same epitope are derived from identical, or very similar, VH and VL genes and despite extensive amino acid divergence, share similar structures (this is the case of certain anti-CD4-BS bNAbs discussed below), or have unusually long CDRH3 regions (this is the case of the anti-apex bNAbs). bNAbs that target the MPER region of gp41 often display autoreactivity (4–6). Here we focus our discussion to bNAbs that target the CD4-BS because of the wealth of information available on their ontogenies, their structures and the structures of their epitopes. In addition, immunogens that can bind the germline forms of these antibodies have been designed and are being characterized *in vivo*. Because structural and ontogeny-based information on apex-directed bNAbs are also available, we also highlight efforts to design immunogens capable of activating the germline forms of bNAbs that target the apex of the Env spike. Finally, we discuss germline-targeting immunogen-design efforts against N332-dependent bNAbs, for which promising immunogenicity studies have recently been conducted. Immunogens that aim to elicit anti-MPER antibodies and break through tolerance-related road blocks have also been evaluated in relevant animal models (7) and are addressed elsewhere in this issue. We first discuss the properties of the antibodies and their epitopes, then the design of 'germline-targeting' immunogens and finally the most recent immunization studies with these novel immunogens.

Anti-CD4-binding site bNAbs

The target of broadly- and non-neutralizing anti-CD4-BS antibodies

The CD4-BS on monomeric gp120 is formed by the interface of the outer and inner domains and of the bridging sheet of gp120 (8). The gp120 residues involved in direct CD4-binding are dispersed in the V1V2 stem, loop D, the β20-β21 hairpin, the β23 strand, the β15-α3 connection and the β24-α5 connection (8). Although areas of the CD4-BS are conserved in sequence and structure among diverse strains, a large part of the CD4-BS is variable in sequence and structure. The CD4-BS is immunogenic and anti-CD4-BS antibodies are readily generated during HIV-1 infection, but the majority of such antibodies display no (or very narrow) neutralizing activities (9). However, sera from infected subjects with broadly neutralizing antibody activities that target the CD4-BS have been identified (10, 11) and numerous broadly neutralizing CD4-BS monoclonal antibodies (MAbs) have been identified from infected subjects (12–17).

Non-neutralizing anti-CD4-BS antibodies can be elicited in small animals (mice, guinea pigs, rabbits) or non-human primates (18–27). Although broadly neutralizing anti-CD4-BS antibodies have not yet been elicited by recombinant Env immunization of humans, mice, rabbits, guinea pigs or non-human primates, cross-neutralizing anti-CD4-BS antibodies have been elicited in llamas immunized repeatedly with soluble recombinant gp140 Env proteins (28, 29). Because these llama antibodies lack light chains (LC) they may have easier access to the CD4-BS than human antibodies.

The relative accessibility of the CD4-BS within the trimeric, virion-associated Env spike is not only influenced by the relative positioning of the protomers forming the Env trimer (open vs. closed Env configurations (30)), but also by the length and orientation of the variable regions 1, 2 and 3 and by carbohydrate moieties present on specific N-linked

glycosylation sites (31–37). Anti-CD4-BS antibodies with broad neutralizing activities not only recognize the conserved elements of the CD4-BS, but they approach the site using angles that allow them to more easily bypass the above steric restrictions on the functional trimeric Env, while anti-CD4-BS antibodies with no, or narrow, neutralizing activities approach the CD4-BS in ways that are limited by the variable regions (30, 38–41).

Anti-CD4-BS broadly neutralizing antibodies

Based on their ontogenies and mode of recognition, CD4-BS bNAbs are grouped into two major types: CDRH3-dominated and VH-gene-restricted (16). Those that make contact with the CD4-BS primarily through their CDRH3 regions are further subdivided into four classes (so far): the CH103, HJ16, VRC13 and VRC16 classes. In contrast, VH-gene-restricted CD4-BS bNAbs make contact primarily through their CDRH2 domains. They are divided into two classes depending on their ontogenies: the VRC01-class antibodies which are derived from VH1-2 and the 8ANC131-class antibodies which are derived from VH1-46. Each antibody class is named by the first antibody isolated in that class. As we discuss below, a new anti-CD4-BS bNAb (IOMA) was recently isolated from an infected subject and its characterization revealed that although it is derived from VH1-2, it shares structural features of both VRC01-class and 8ANC131-class antibodies. So, the above classification is updated frequently.

Approximately twenty nine VRC01-class bNAbs have been isolated from nine chronically-infected subjects so far (12, 14–17, 42). Viruses circulating in subjects that develop VRC01-class antibodies can escape from such antibodies, but such escape has a toll on viral infectivity (43, 44). Nevertheless, VRC01-class antibodies are very efficient in preventing infection in animal studies (45–47), in reducing plasma viremia in the context of chronic HIV-1 infection (48–50), in contributing to the elimination of HIV-1 infected cells (51) and in delaying viral-rebound in the context of structured ART interruption treatments (52). The protective and therapeutic potentials of 8ANC131-class antibodies have not yet been described.

VRC01-class antibodies are all derived from one of the possible five human VH1-2 alleles, the VH1-2*02 allele, and a few VLs (κ3-20, κ 3-15, κ 1-33 and λ 2-14) (14, 15, 42). The HCs of VRC01-class bNAbs have three germline-encoded amino acids ($Trp50_{HC}$, $Asn58_{HC}$ and$Arg71_{HC}$) in their CDRH2 domains that make key, conserved contacts with Env (42, 53–55). These three key amino acids are present in human VH1-2 alleles *02, *03 and *04, but not in the remaining two VH1-2 alleles, *01 and *05. 8ANC131-class antibody HCs lack $Trp50_{HC}$. $Trp50_{HC}$ hydrogen bonds with the conserved amino acid Asn280 in Loop D, $Asn58_{HC}$ hydrogen bonds (or interacts) with the conserved amino acid Arg456 in V5, while $Arg71_{HC}$ makes a key contact with amino acid Asp368 in β15 (53–55). Asn280 is also important in the binding of 8ANC131 bNAbs, but in these cases the interaction is mediated by elements of the LCs.

The CDRH3 domains of VRC01-class antibodies also participate in the recognition of the CD4-BS by interacting both with the outer and inner domains of gp120. The CDRH3 expands the overall binding surface areas between VRC01-class antibodies and Env (53, 55–57). The vast majority of known VRC01-class bNAbs have a Trp at position 100B

(Trp100B$_{HC}$) in their CDRH3, which is located precisely five amino acids prior to framework region 4 (FWR4). 8ANC131-class bNAbs do not express Trp100B$_{HC}$. Trp100B$_{HC}$ hydrogen bonds with Asn279 in Loop D of gp120 and mutation of this amino acid results in loss of neutralizing activity of VRC01-class antibodies (54). Because of its location in the CDRH3 domain it is unknown whether Trp100B$_{HC}$ is due to VDJ recombination, or if it is the product of somatic hypermutation. Next generation deep-sequencing of circulating human IgM+ cells from nine HIV-1 uninfected donors indicated that only a small fraction of VH1-2 *02, *03 and *04 sequences contained Trp100B$_{HC}$ (C. Yacoob, M. Pancera, et al unpublished data). Trp100B$_{HC}$ was preferentially found in the VH1-2 sequences derived from the *02 allele (9%) compared to the *03 (4.5%) or the *04 (2.8%) alleles, which may explain why all known VRC01-class bNAbs are derived from the *02 than the *03 or *04 alleles (54). Thus, it is likely that Trp100B$_{HC}$ already exists in the germline form of some VRC01-class antibodies and these antibodies may be preferentially selected by certain Envs to expand. Interestingly, although the VH/VL amino acid sequences of the presently known mutated VRC01-class MAbs can be up to 50% divergent, they adopt very similar structures which allow them to bind the same epitope in the CD4-BS in a nearly-identical way (14–16, 42, 58).

Interactions of ‘inferred’ germline forms of broadly neutralizing and non-neutralizing anti-CD4-BS antibodies with Env

Anti-CD4-BS bNAbs bind with high affinity to diverse recombinant Envs and structures of complexes of such antibodies with a few Envs have been characterized by crystallography and Cryo-EM (15, 16, 39, 42, 53, 55–59). When the VH and VL sequences of VRC01 were reverted to the predicted gene-encoded sequences and expressed as immunoglobulins, the iGL antibodies did not bind the recombinant gp120 protein that was used for crystallization purposes (93TH057) (55). Similarly, iGL forms of other VRC01-class antibodies, 12A21 and 3BNC60, did not recognize the ‘bait’ proteins used to isolate the B cells expressing the mature B cell receptor (BCR) forms of these antibodies (14). Binding screens with much larger sets of recombinant Envs derived from different clades confirmed and expanded upon these results (59–61). A similar lack of Env-recognition by the iGL 8ANC131-class antibodies has been reported (16). Also, diverse recombinant Envs readily activate B cell lines engineered to express the mutated, but not the iGL VRC01-class BCRs (59, 61). In contrast, the iGL forms of non-neutralizing (nNAbs) anti-CD4-BS antibodies bind diverse recombinant Envs (62–64) and diverse recombinant Envs activate B cells expressing the corresponding BCRs (63). Furthermore B cells expressing the iGL BCRs of nNAbs efficiently internalize and process Env, while B cells expressing iGL VRC01-class BCRs do not (63). The epitopes of nNAbs anti-CD4-BS antibodies are readily exposed on recombinant soluble Env proteins (65, 66), but are occluded on the trimeric Env spikes anchored in the viral membrane (38, 39, 67). The implication from the above studies is that diverse recombinant Env immunogens fail to activate VRC01-class precursor B cells during immunization, but instead activate and expand B cells expressing the germline BCRs of nNAbs. In sum, non-neutralizing B cell precursors have a major advantage over broadly neutralizing B cell precursors in the context of immunization and during infection. This is one reason why B cells that give rise to nNAbs dominate the anti-Env antibody responses during infection and immunization.

Structural comparison of the mutated and inferred germline VRC01-class antibodies

VRC01-class antibodies approach the CD4-BS at angles that are distinct from those of other anti-CD4-BS bNAbs, such as the 8ANC131 or the CDRH3-mediated bNAbs (16). The CDRH2 of VRC01-class antibodies resemble the interaction of the C″ β-strand of CD4 with the CD4-binding loop of gp120 (55, 56). However, despite similarities between VRC01-class antibodies and CD4, they do not interact with CD4 in an identical manner. For example, the CD4 molecule inserts a Phe (Phe43) in a hydrophobic 'pocket' between the gp120 bridging sheet and outer domain of gp120, something that does not occur with VRC01-class or 8ANC131 antibodies. All CDRH loops and parts of FWR2 and FWR3 participate in this interaction. The isolated VRC01-class antibodies display extensive somatic mutations in both the VH and VL genes (14, 15) and not only in the CDR domains, but also in the FWs (62). Many of these mutations are critical for the broad neutralizing activities of these antibodies (62, 68). Achieving levels of mutation found in potent CD4-BS bNAbs through vaccination will require innovative immunization schemes (69).

Despite the extensive amino acid changes, both in the CDRHs and FWR regions of the HCs, the three key amino acids in the HCs ($Trp50_{HC}$, $Asn58_{HC}$ and$Arg71_{HC}$) remain unaltered and make contacts with Env in the iGL versions of VRC01-class bNAbs (16, 53, 58). Thus, these amino acids anchor the germline BCR to Env and all subsequent amino acid alterations that occur during affinity maturation increase the affinity and selection of specific B cell clones. The structures of the mutated and iGL Abs have pre-formed antigen-binding conformations (although alterations in the orientation of CDRL1 between the bound and unbound forms of the iGL 3BNC60 antibody have been noted), which is different from most other antibodies (58). Furthermore, despite the fact that conserved amino acids remain unaltered during somatic mutation, the angle of approach (rotation and translation) of the mature and iGL HCs differ (58). Also, affinity maturation leads to the HCs of these antibodies to make extensive contacts with the inner gp120 domain, while this is not the case for the germline HC. Thus, affinity maturation increases the contact surface area between VRC01 antibodies and gp120 (53).

The LCs of VRC01-class antibodies also make important interactions with gp120. The unusually short, 5 amino acid long, CDRL3s of these antibodies interact with V5 and Loop D. The short CDRL3 length facilitates the CDRH2-dominated interaction with the CD4-BS and VRC01-class antibodies have evolved to avoid steric clashes with glycans attached to N276 in Loop D. This clash is avoided either by a shortening of CRL1 during affinity maturation, or by the introduction of glycines in the CDRL1 that increase its conformational flexibility (42).

Natural Envs that bind germline CD4-BS and anti-apex bNAbs

The unavailability of samples from the early stages of infection (or at the time when the production of VRC01- antibodies begun) from subjects that developed VRC01-class bNAb responses has prevented the identification of natural Env clones that bound the germline BCR forms of such antibodies and initiated their production. However, in three cases where the gradual development of bNAbs and of the concomitant viral evolution have been well-documented, it was shown that the initiation of bNAb-production required the emergence of

viral clones expressing Env variants with particular features that allowed them to engage the pre-immune BCRs of those particular bNAbs. In one case, the UCA of the CAP256-VRC26 bNAb lineage, targeting the apex of the Env spike, neutralized a superinfecting virus in the CAP 256 donor, which presumably expressed an Env capable of engaging that UCA (70). In the second and third cases, two inter-dependent CD4-BS lineages (CH103 and CH235) evolved in the same subject (13, 71, 72). The CH505 transmitted/founder Env engaged both the CH103 UCA and CH235 UCA lineages. In all three cases, a cascade of viral Env evolutionary and BCR maturation steps ensued leading to the production of specifically mutated antibodies capable of neutralizing not just the autologous virus, but heterologous viruses as well.

These studies, coupled with the lack of diverse recombinant Env recognition by iGL forms of VRC01- and 8ANC131 class antibodies, strongly suggest that specific viral Envs with rare features emerged in a subset of infected individuals and initiate the production of VRC01-class antibodies. In the absence of natural Envs that bind germline VRC01- or 8ANC131-class antibodies, the design of specific recombinant Env-derived proteins for their engagement is required (see below).

Development of recombinant Env-derived proteins that bind inferred germline VRC01-class antibodies

Knowledge of key structural elements, many of which are gene-encoded, of VRC01-class antibodies and of gp120 helped us and others design recombinant proteins that can engage iGL VRC01-class antibodies (57, 59, 61, 63, 73). Our efforts to design Envs that would recognize iGL VRC01-class antibodies begun by identifying recombinant Envs that were recognized with high affinity by the mutated antibodies and then introducing targeted alterations at key positions, based on available structural information.

The available crystal structures of A/E 93TH057 gp120 bound to a chimeric version of the VRC01-class NIH45-46 antibody (glHCmLC) (53, 54) and of the Cryo-EM structure of BG505 SOSIP.664 bound to PGV04 (39) suggested that a conserved Trp in the LC (W67LC) interacted with glycans on the conserved N276 on Loop D gp120. That information and the fact that the germline CDRL1 of several VRC01-class antibodies is shorter by two amino acids than the mature form, suggested that the LC of germline Abs will clash with elements at position N276 (53, 55, 59). Indeed, when we eliminated the glycosylation site N276 by mutagenesis (replacing N by a D) on the clade C Env 426c, the iGL NIH-45-46 and its clonal relative VRC01 (61), as well as the iGL PGV19 and PGV20 antibodies bound (73). By eliminating two additional NLGS in V5 (N460 and N463) we improved the binding affinities of these iGL antibodies. So the 426c-derived modified Env (gp140 form) lacking these three NLGS (triple mutant, or 426c TM) bound not only the mutated VRC01-class antibodies but some iGL antibodies as well. Despite the conserved nature of the above-mentioned NLGS in Loop D and V5, their elimination from other Envs did not result the binding of iGL VRC01-class antibodies (73). The reasons for the unusual properties of the 426c Env are not fully known. Subsequently, introducing additional optimizing modifications on the 426c TM, which included the elimination of variable regions 1, 2 and 3 and other mutations (59), an optimized 426c-derived construct was designed that was

recognized by most known iGL VRC01-class antibodies (73). For simplicity we refer to this optimized construct as '426c core' (this construct has also been termed TM4ΔV1-3 (58, 69, 73)).

The group of W. Schief at Scripps utilizing a combination of computational design and yeast-based evolutionary approaches engineered a highly mutated version of the outer domain of the HxB2 gp120 (termed eOD-GT6) (that included the elimination of N276) that bound diverse iGL VRC01-class antibodies (especially its 60meric nanoparticle form) (59). eOD-GT6 was further optimized for iGL VRC01-class antibody binding (eOD-GT8) which was recently used as a bait to isolate B cells that express *bona fide* germline VRC01-class BCRs from HIV-1- subjects (57). Thus, in eOD-GT6/8 and 426c TM/426c core cases, the central role that carbohydrate moieties at the conserved NLGS in Loop D and in V5 play in preventing the binding of iGL VRC01-class antibodies became evident. In sum, eOD is a highly redesigned outer domain of gp120 derived from the clade B lab-adapted HxB2 virus, while the 426c core is a modified and truncated gp120 that expresses both the inner and outer domains of the primary clade C 426 virus.

We note that: a) 426c derived 'germline-targeting' proteins do not bind iGL 8ANC131 antibodies (16). b) Additional constructs, based on different Env backgrounds and designs are being developed to engage not only the germline forms of VRC01-class antibodies, but also antibodies that bind neutralization epitopes outside the CD4-BS (74) (see also review by Moore and Sanders). c) eOD-GT8 binds to CD4-BS B cells expressing germline VH1-2*02 HC paired with germline LCs with longer than 5 amino acid long CDRL3 domains as well. In fact, the majority of eOD-GT8-isolated naïve human B cells express germline VH1-2*02 HCs that are paired with LCs do not expressing 5 amino acid long CDRL3 (57). Therefore these 'germline-targeting' constructs will not exclusively stimulate precursor VRC01-class antibodies, but also other types of Env-binding antibodies that may, or may not be associated with HIV-1 neutralizing antibodies. The immunogenicity studies in knock-in mice, which we discuss below (69, 75) confirm this. d) A new broadly neutralizing anti-CD4-BS antibody, IOMA, was recently isolated and characterized by the Nussenzweig and Bjorkman groups (Gristick. H. B, et al.,2016 NSMB in press). IOMA is derived from a VH1-2 HC paired with a LC VL2-23 with 8 amino acid long CDRL3 and shares structural features with both VRC01-class and 8ANC131-class antibodies). IOMA is less mutated (22 HC and 15 LC) than VRC01-class antibodies and neutralizes ~50% strains tested (with a mean IC50 of 2.3 μg/ml). IOMA-like bNAbs may thus be elicited more easily than VRC01-class antibodies through immunization, since LCs with 8 AA long CDRL3s are more frequently found in the human repertoire (54, 76, 77), but first, immunogens capable of binding their germline forms must be engineered. e) Although N276-linked carbohydrates are hindering the binding of germline VRC01-class antibodies, they are critically important for the binding of other CD4-BS bNAbs (78, 79). Overall, different immunogens will most likely be required to stimulate anti-CD4-BS broadly neutralizing antibody responses that are as broad as possible by immunization.

Germline CDRH3

The CDRH3 sequences of mutated antibodies cannot be reverted with certainty to their germline sequences, and as a consequence the iGL antibodies express CDRH3 sequences present in the mutated antibodies. Does this limit our ability to design better immunogens? We believe it does, especially for antibodies that recognize their target primarily through their CDRH3 domains. Such as those that bind the apex of the Env spike (see below), those that recognize elements of the V3 loop in a N332-dependent fashion (80–83), but also the CDRH3-dependent anti-CD4-BS antibodies, as well as antibodies that bind the membrane proximal region of the gp41 subunit (MPER) (84–87).

The role of CDRH3 in the interaction of germline VRC01-class or germline 8ANC131-class antibodies with Env is not well understood. Although these antibodies make contact with the CD4-BS primarily though their CDRH2 domains, their CDRH3s contribute to that interaction (see above). Germline VRC01-class antibodies isolated recently using eOD-GT8 display a wide range of binding affinities, despite of having germline VH1-2*02 sequences (57) and one potential reason for that could be due to the distinct CDRH3 sequences expressed by those antibodies.

Presently it is unknown how diverse the CDRH3 repertoire is in naturally circulating B cells that express germline VH1-2*02 HCs and whether particular types of CDRH3s are linked with LCs expressing 5 amino acid long CDRL3s; whether different CDRH3s are associated with the *02, *03 and *04 alleles; and whether the currently available germline-binding constructs (eOD and 426c) bind all possible putative germline VRC01-class antibodies, or only a fraction of such antibodies because of differences in their CDRH3 regions.

Antibodies that target the ‘apex’ of Env

The ‘apex’ region of Env

The V1/V2 domains of three interacting protomers form a canopy at the top of the viral Env spike (38, 40, 88, 89). The V1V2, assumes a four or five stranded anti-parallel greek key motif from which the V1 and V2 emanates and partially covers the third hypervariable (V3). Upon binding to CD4, the trimer undergoes extensive conformational changes that include the partial dissociation of the apex, such that the V3, which encompasses the co-receptor biding site, protrudes towards and interacts with the target cell (89).

The Env apex is highly immunogenic and is the target of both non-neutralizing and broadly neutralizing antibodies. Some of the broadest bNAbs identified to date target the V1V2 domain (90). The PG9 and PG16 antibodies were the first ‘apex’ MAbs isolated that displayed very potent and broad neutralizing activities (3). PG9 and PG16 potently neutralize ~80% and ~70% (respectively) of strains tested. They were isolated from a Clade A infected donor from the IAVI protocol G cohort (donor 24) by screening single well B cell culture supernatants in a micro-neutralization assay. They primarily (PG9) or exclusively (PG16) recognize the membrane-anchored form of Env (rather than soluble recombinant Env constructs, with a few exceptions which we will discuss below), and their epitope includes carbohydrate moieties and is quaternary in nature. Their binding depends on *N*-linked glycosylation sites in the V2 region of Env: at position N160, and to a lesser extent

N156 or N173 (3). In addition to N160, a lysine rich region of the V1/V2 surrounding K169 is a critical determinant of for the neutralizing activity of these (70, 91, 92).

Several PG9/PG16-like Abs were isolated since from different HIV-1-infected subjects: Antibodies PGT141-145 from IAVI donor 84 (93), and antibodies CH01-CH04 from CHAVI donor 0219 (94). These antibodies neutralize between 40–80% (PGT141-145) and 40–50% (CH01-CH04) of strains tested against. More recently, the VRC26-CAP256 antibody lineage has been isolated from donor 256 of the CAPRISA cohort. Unlike the other V1/V2 apex antibodies which were isolated from a single time point during chronic infection, longitudinal PBMC samples were obtained from the CAP256 donor, which permitted tracking the evolution of the bNAb lineage and of viral variants expressing Envs that stimulated the corresponding B cells (70). As mentioned above the V1V2 also elicits nNAbs and several antibodies that recognize quaternary epitopes in V1V2 have also been isolated from infected individuals (95). One key difference between these two types of ‘apex’ antibodies is that the bNAbs neutralize viruses with glycans on N160 (common feature of primary HIV-1 strains) while the nNAbs cannot neutralize such viruses (95–97). The V2 also elicits antibodies that recognize linear epitopes, including those encompassing K169. Such antibodies were elicited in the RV144 clinical trial and although they did not display broad neutralizing activities they were associated with reduced risk of infection in that trial (98, 99).

Common structural features of ‘apex’ bnAbs

A common feature of the V1/V2 apex bNAbs is their unusually long, anionic CDRH3 regions which often contain sulfated tyrosines (3, 70, 88, 93, 94, 100–103): 24 AA for CH01-04 Abs, 28 AA for PG9 and PG16, 31-32AA for PGT 141-145 Abs, 35-37AA for the VRC26-CAP256 lineage Abs. The CDRH3 regions of all ‘apex’ bNAbs share a similar β-hairpin conformation and protrude from the face of the binding face of the Fab. In the PGT145 case, the long B-hairpin extends vertically along the long axis of the Fab (88), while in other Apex bNAbs the β-hairpin is kinked to assume a more perpendicular orientation towards to the long axis of the Fab. The CDRH3s of PG9 and PG16 assume a “hammerhead” configuration, while those of CAP256 and CH01, 03 and 04 antibodies assume an “axe head” like configuration.

Stoichiometry of ‘apex’ bNAbs-Env interactions

Defining the precise epitope targeted by these antibodies at atomic resolution has proven difficult due to the quaternary nature of the epitope. Co-crystal structures of PG9 and PG16 with protein scaffolds expressing the V1/V2 domain are available and revealed that the long CDRH3s of PG9 and PG16 are essential for making contact with, but also for reaching through, the carbohydrate moieties on N160 and N156. This allows the sulfated tyrosines to interact with the positively-charged lysine rich region surrounding residue K169 in strand C at the base of V1V2 (82, 88). The liganded structures of CH03 and CH04 display similar glycan and strand C interactions as PG9 and PG16 do. However, the liganded structure of CH03 was solved with a trimeric V1/V2 scaffold and revealed that this Ab (and probably all other ‘apex’ bNAbs) makes extensive contact with glycans from an adjacent protomer. Superimposition models of the structure of V1/V2 on protein scaffolds with that of the

V1/V2 present on soluble trimeric, stabilized full-length Env (BG505 SOSIP) suggests that a single Fab can simultaneously interact with glycans at N160 from two adjacent protomers (100), something that was predicted when the structure of trimeric Env was first characterized (38). These observations are in agreement with negative-stain EM studies that indicated that a single 'apex' Fab binds each Env trimer (1:1 stoichiometry) (67, 70, 90, 104). These results are also in agreement with the preference of these antibodies for quaternary epitopes on the 'apex' of Env and their lack of binding to monomeric gp120 and or conventional gp140 recombinant Env proteins, on which the protomers more frequently adopt an 'open' configuration (105). In contrast, up to three anti-CD4-BS antibodies can engage an Env spike.

Shared Genetic signatures of 'apex' bNAbs

The CDRH3 domains of antibodies are the product of V, D, and J recombination. Thus, long CDRH3s can be the product of such recombination with particular D and J genes (i.e, they can be present at the pre-immune level of the BCR). But, they could also be the result of affinity maturation. Analysis of sequentially isolated CAP256 lineage Abs, revealed that the long CDRH3 was the product of V,D,J recombination since it was present in the unmutated common ancestor (UCA) (70). Although the germline forms of CH01-04 antibodies are unknown, next generation sequencing and phylogenetic analysis of antibody heavy chain sequences isolated soon after the initiation of this lineage, allowed for the identification of the 'earliest maximum likelihood intermediates' of CH01-04 lineage antibodies (100, 106). These early intermediates had long CDRH3 regions, suggesting that germline antibody forms also had it.

The PG9, CAP256, and CH01-lineage antibodies are derived from the VH3 family: PG9 from 3-33*05, CAP256 from 3-30*18 and CH01-04 from 3-20*01. The PG9 and CAP256 VH regions differ by only one amino acid which is present at the C-terminus of the CDRH3. The PG9, PG16 and CAP256 antibodies also share an YYD motif in their CDRH3s which is encoded by the D3-3*01 gene. The YYD motif contains the sulfated tyrosines mentioned above which are critical for the strong binding of these antibodies with strand C on Env (88). This D3-3*01 gene encoded motif is also present in the CAP 256 UCA and is found in all members of the CAP256 lineage (70). Although the true germline progenitor of PG9 is not known, because PG9 and CAP256 share the same D gene usage it is plausible that the YYD motif was present in the true PG9 precursor as well, and thus it is not the product of somatic mutation. In contrast, the VH of CH01 is more divergent from the VHs of PG9 and CAP256, with 19 and 20 amino acid differences, respectively. The PGT145 antibody is more distant still, and utilizes VH1-8 rather than a VH1-3 gene. Overall, despite their derivation from distinct VH genes, 'apex' bNAbs share remarkable structural similarities in their unusually long CDRH3 domains, which are the primary means by which they interact with Env, i.e, those features are selected by Env during infection.

Envs that engage the germline forms of 'apex' antibodies

V1/V2 apex bNAbs arise earlier and more frequently during infection (70, 107–111) than other bNAb specificities, and are less mutated than other bNAbs (such as the CD4-BS bNAbs discussed above) and thus are an attractive vaccine target.

In contrast to the lack of Env-recognition and virus neutralization by the above-mentioned iGL CD4-BS antibodies, a few Envs have been identified that are recognized by the iGL/UCA forms of some ‘apex’ antibodies and some iGL apex antibodies neutralize some viruses (70, 92, 112). In those rare cases, the Env does not interact with all iGL ‘apex’ antibodies. The identification of soluble Envs capable of binding many iGL ‘apex’ antibodies is exacerbated by the quaternary nature of their epitopes, which are not recapitulated by most recombinant Envs (with a few exceptions, discussed below). However, because of the shared structural features of their CDRH3 domains, which appear to be present at the level of the germline antibody forms, it has been proposed that immunogens can be specifically designed to engage germline ‘apex’ antibodies (92, 100).

Here, we highlight two promising, but distinct, approaches currently employed to design immunogens that target multiple V1/V2 apex bNAb progenitors and initiate the production of such antibodies. The first approach, takes advantage of the development of technologies that have facilitated the production of soluble trimeric Env spikes, that better mimic the virion-associated Env spikes (SOSIP constructs) (67). The second approach is based on the design of small scaffold peptides on which the V1V2 are expressed in biologically-relevant conformations (113).

SOSIP-based approaches—Early studies revealed that the iGL forms of some ‘apex’ antibodies display neutralizing activity towards a limited number of viruses. This contrasts with observations made with the iGL CD4-binding site antibodies (discussed above). For example, iGL PG9 neutralizes the ZM233M virus (101), iGL CH01-04 not only neutralizes ZM233M, but also Q23.15, WITO and A244 (94), while the UCA of CAP256-VRC26 neutralizes a superinfecting virus isolated from the CAP256 donor (70, 114). However, with the exception of the CH01-CH04 UCA which binds weakly to the A244 gp120 (94), the iGL of apex V1V2 bNAbs do not bind to conventional soluble gp120 or gp140 constructs (70, 115). More recently, large panels of viruses were screened for susceptibility to neutralization by iGL and/or UCA versions of ‘apex’ antibodies to identify several Envs that could serve as templates for the design of appropriate ‘germline-targeting’ immunogens (92, 100). In this manner, Andrabi and colleagues identified five viral isolates that were neutralized by at least 2 iGL ‘apex’ antibodies each (92). Three were neutralized by iGL versions of ‘apex’ antibodies from different donors (PG9, CH01 and CAP256.09). Similarly, Gorman et al independently identified a number of viral strains that were neutralized by multiple iGL ‘apex’ antibodies (100).

Because ‘apex’ bNAbs typically do not bind monomeric gp120 or commonly used gp140 recombinant Envs, Andrabi and colleagues produced a well-ordered and stable soluble trimeric Env derived from CRF02_AG_250 virus which is neutralized by the iGL forms of PG9, CH01, and CAP256.09. Gorman et al took a different approach to produce soluble native-like Envs. By swapping the V1/V2 region from 9 strains sensitive to neutralization by iGL ‘apex’ bNAbs onto the stabilized BG505.664 protein (116), soluble recombinant Envs were produced that were bound by two or more V1/V2 apex antibodies. Three of these Envs: ZM233M, BB201.B432 and KER2018 SOSIP were able to bind iGL versions of PG9, CH01-04 and the CAP256-VRC26 iGL antibodies. None of the trimeric Envs were able to

bind the iGL version of PGT145 which may be a reflection of its unique VH gene usage or of a more divergent CDRH3 structure (88).

A number of native-like Envs are thus now available that bind to one or more iGL bNAbs with a range of affinities. Even though there are many commonalties between ‘apex’ bNAbs, the differential binding of iGL bNAbs to these Envs implies that one or more Env immunogens may be required to efficiently target all potential apex bNAb progenitors. Thus, evaluating the ability of these immunogens to elicit, or at least initiate the expansion of ‘apex’ antibody progenitors alone, or in combination, in appropriate animal models, should help determine which of these Envs might be the most successful in targeting the appropriate B cell progenitors in humans.

V1/V2 epitope scaffold approaches—An alternative to the native-like SOSIP engineering approach, is based on the synthesis of dimeric peptides corresponding to the conserved lysine rich region on strand C of V2, which as discussed above is the major region of contact of the CDRH3 of ‘apex’ antibodies. Dimeric peptides corresponding to Env amino acids 148–184 from the A244 strain were synthesized with either Man5 or Man3 glycans at positions 160 and 156. iGL PG9 and CH01 antibodies bound to peptides containing Man5 glycans with nanomolar apparent affinities. These antibodies also bound to dimeric peptides with Man3 glycans, albeit with lower avidity. The UCA of the non-neutralizing CH58 monoclonal antibody displayed micromolar avidity for these peptides with Man3 or Man5 glycans. Thus, it has been proposed that these small peptides that encompass the critical neutralization determinant on strand C of the HIV-1 Env may be a way to selectively prime progenitors of anti-V1/V2 apex bNAbs (113).

N332-dependent broadly neutralizing antibodies

A number of potent bNAbs recognize the base of the V3 loop in a N332-dependent manner (80–83, 117–120). N332-dependent bNAbs are frequently detected in sera from HIV-1-infected subjects that developed broad neutralizing antibody responses (93). Because these antibodies are derived from different germline VH/VL genes and because the precise way they recognize their epitopes vary, the N332-directed epitope was defined as the N332-supersite on gp120 (120). Briefly, while all antibodies interact with N322 glycan, PGT128-like antibodies also contact glycans at position N301 and the base of the V3 loop (83), PGT135-like antibodies interact with additional glycans at N386, N392 and protein region on the outer domain of gp120 (120) and PGT121-like antibodies contact glycans at N301, N137 and N156 as well as regions of the V1 and V3 loops (81, 82, 119).

PGT121-like antibodies were shown to protect non-human primates from experimental SHIV-infection in passive immunization studies (47, 121) and transiently reduce plasma viremia to undetectable levels in the context of chronic SHIV infection (122). PGT128-like antibodies potently, but transiently, reduce viral load in humanized mice infected with HIV-1 (123). Thus, the elicitation by vaccination of such antibodies is highly desirable. Indeed, immunogens designed to target the germline precursors of the PGT121-derived antibodies have been developed and evaluated in animal immunization studies very recently (see below) (Steichen et al., 2016 Immunity, in press and Escolano et al., 2016 Cell, in press).

‘Germline-targeting’ immunogen-design efforts to elicit N332-like bNAbs

As mentioned above bNAbs that target the N332 supersite, display diverse gene usage and employ distinct structural solutions to attack this site. Thus, structure based ontogeny approaches that afford design of immunogens capable of binding to the Env ‘apex’ or the CD4-BS, are not readily applicable for N332 supersite-targeting bNAbs.

Like other bNAbs discussed above, iGL PGT121 does not display recombinant Env recognition (81, 124). To identify Env variants that bind to iGL PGT121, mutagenized Env (based of BG505 T332N) were expressed on the surface of mammalian cells and evaluated for iGL PGT121-binding (Steichen et al., 2016 Immunity, in press). Because it is difficult to predict the germline CDRH3 that give rise to PGT121, 5 putative iGL variants of PGT121 were employed, but none displayed Env-binding. Thus the Env library was screened against putative PGT121 intermediates with 3 (GL+3) or 9 (GL+9) light chain mutations. GL+3 recognized an Env variant with 5 mutations in the V1 (5MUT) and GL+9 selected for different mutations in V1 that disrupt NLGS sites a 133 and 137 (3MUT). An Env variant that combined the mutations in 5MUT and 3MUT, called 7MUT showed detectable binding to one of the 5 putative iGL PGT121 variants. Subsequently MUT 7 was randomly mutagenized in V1 and V3 and screened against iGL PGT121 variants. Selection of mutations that were enriched during this screen, were used to produce an Env variant termed, 10MUT which showed weak but detectable binding to 5/5 iGL PGT121 variants A follow-up targeted mutagenesis a 10MUT, lead to the selection of a variant ($11MUT_B$), with higher affinity for 3/5 iGL PGT121 precursors. The $11MUT_B$, 10MUT, 7MUT, 5MUT and 3MUT were all subsequently produced as stabilized SOSIP trimers and as liposome-bound constructs. $11MUT_B$ but not 10MUT SOSIP variants presented on liposomes were able to activate one variant of iGL PGT121 expressing B cells *ex vivo* ($GLC_{DR3rev4}$). Yet despite being unable to activate iGL PGT121 B cells when highly multimerized, immunization of iGL PGT121 knock in mice with 10MUT induced a detectable serological antibody response (Steichen et al., 2016 Immunity, in press and Escolano et al., 2016 Cell, in press). Consistent with its ability to activate B cells *ex vivo*, immunization of iGL PGT121 knock-in mice with $11MUT_B$ induced B cell responses in these mice which were stronger than those elicited by 10MUT. This study highlights the utility of the directed evolution approach to generate ‘germline-targeting’ immunogens where a shared pattern of ontogeny and structural recognition is not available.

Immunogenicity studies

The main purpose of ‘germline-targeting’ immunogens is to activate naïve B cells that express specific germline BCRs. It is not expected that repeated immunizations with ‘germline-targeting’ immunogens will induce the complete set of somatic mutations that are associated with broad neutralizing antibody activities (62, 64, 69). During natural infection, viral diversity drives the maturation of antibodies towards their broad neutralizing activities (13). To accomplish this feat by vaccination, immunization schemes involving boosts with Envs that are modified in specific way will be necessary.

a) 'Germline-binding' immunogens for the elicitation of CD4-BS bNAbs

'The priming phase': Small animals, such as mice, rats, or rabbits (54), as well as non-human primates (NHPs) (77) do not express VH1-2*02 orthologs. In addition, LCs with 5 amino acid long CDRL3s may even be rarer in NHPs (rhesus macaques of Indian origin) than in humans (77). Thus, specifically engineered animals are required to evaluate VRC01 antibody-targeting immunogens, before such constructs are evaluated in humans.

Knock-in transgenic mice have been engineered to express the human iGL HC variable domains of two VRC01-class antibodies, VRC01 (75) or 3BNC60 (69). Knock-in mice expressing both the iGL heavy chain and iGL light chain variable regions of 3BNC60 have also been engineered (73). In these three mouse systems the entire iGL variable domain of the HC (V, D and J) was knocked-in and thus, the CDRH3 sequences are those of the mutated VRC01 and 3BNC60 antibodies.

Immunizations of iGL HC 3BNC60 mice were conducted with eOD-GT8 (60meric nanoparticle form) and '426c core' (a dextramer-based multimeric form) (69). Proteins (10ug) were administered in Alum. B cells isolated prior to immunization expressed the exogenous iGL HC 3BNC60, but none expressed mouse LCs with 5 amino acid long CDRL3. Thus, the frequency of B cells expressing iGL VRC01 BCRs is very low in these mice. One or two immunizations with these two 'germline-targeting' immunogens elicited serum antibodies, a significant fraction of which displayed CD4-BS specificity. In contrast, multiple immunizations with recombinant native-like Env failed to elicit a significant serum antibody response, consistent with a lack of iGLVRC01-binding to this Env. Individual CD4-BS specific B cells were isolated from eOD-GT8-immunized animals and their VH/VL sequenced. Eight clonal B cell lineages were identified (with 2 to 13 clonal members each). Only one of the expanded clones displayed the classical 5 amino acid long CDRL3 signature. Neither the serum nor MAbs from the immunized animals displayed neutralizing activity (as expected).

eOD-GT8 was also evaluated in the iGL HC VRC01 mice (75). Next generation sequencing of LC B cell transcripts indicated that 5 amino acid long CDRL3s were present at ~0.2%, thus the frequency of VRC01-like naïve B cells in these mice, is also very low. Here eOD-GT8 was administered either as a 60meric nanoparticle form, or as a trimer, adjuvanted with Alum, Iscomatrix or Ribi. Individual B cells specific for the CD4-BS were isolated and their VH/VL genes sequenced. Remarkably, 92% of those cells expressed LCs with 5 amino acid long CDRL3s. Although very few somatic mutations were recorded overall, Ribi tended to induce more somatic mutations than the other adjuvants. As was observed in the iGL 3BNC60 knock in mice (69), eOD-GT8 elicited antibodies from the iGL VRC01 knock-in mice did not display neutralizing activities. The reasons for the difference in the efficiency of eOD-GT8 to select VRC01 precursors versus iGL VRC01 iGL3BNC60 knock-in mice are not currently known.

The 426c core immunogen was tested in mice engineered to express both the iGL HC and iGL LC of 3BNC60. Several 426c core multimeric forms were evaluated: gp120 or gp140 dextrameric-forms, trimeric gp140 form, C4b-based 7meric form, or Ferritin-based 24meric form (73). Although knock-in mice expressing the iGL HC of 3BNC60 display

physiological B cell phenotypes (69), B cells expressing both the iGL HC and iGL LC of 3BNC60 display an autoreactive phenotype. Despite the anergic phenotype of B cells, a single immunization with multimerized forms of 426c core was sufficient to elicit and antibody response that was partially CD4-BS directed in the majority of animals. In contrast, immunization with a multimeric wild type 426c immunogen (non-germline-binding) did not elicit a serum response. Interestingly, although only 20% of naive B cells present in the spleen of these mice express the exogenous human gl3BNC60 κLC (presumably because they are selected against), the majority of vaccine-specific B cells isolated expressed the exogenous human κLC. Thus, in that mouse model, the 426c core immunogen selected for B cells that express the exogenous gl3BNC60 LC with a 5 amino acid long CDRL3s.

More recently, mice were engineered that predominately express the human VH1-2*02 V_H gene found in VRC01-class antibodies (Tian, M et al 2016 Cell, in press). In this case, the human V_H gene recombines with the endogenous mouse D and J genes and the expressed HCs present a diverse repertoire of CDRH3s which can pair with a wildtype murine LC repertoire. eOD-GT8 was evaluated as a prime in these mice. VH1-2*02 knock-in mice (3 animals per group) were immunized with 15, 30, or 60 μg of eOD-GT8 with poly I:C as adjuvant. B cells were isolated after a single immunization and their VH/VL genes were sequenced. None of the 49 cells clones expressed a LC with 5 amino acid long CDRL3 in the 15μg dose group. 6 of 140 cells expressed such LCs in the 30μg dose group, while 7 out of 188 cells expressed such LCs in the 60μg dose group. As with the immunization studies discussed above, a substantial fraction of the immune response was not specific for the CD4-BS. So, eOD-GT8 selected for infrequent B cells that expressed VRC01 B cell progenitors, but as in the case of the iGL 3BNC60 HC knock-in mice, the majority of B cells isolated and sequenced expressed LCs with longer than 5 amino acid long CDRL3s. As in the studies discussed above, immunizations with regular recombinant Envs did not elicit a significant antibody response.

The results from these studies prove that ‘germline-targeting’ immunogens behave *in vivo* as hoped. They support the proposal that they are better suited than regular recombinant Envs to initiate the process of VRC01-class antibody production *in vivo*. But, they also reveal that B cells with off-target specificities will also be stimulated by these immunogens, as predicted from *in vitro* experiments (63).

‘The boosting phase’: Two recent immunization studies aimed to induce the maturation of germline VRC01-like antibodies elicited by ‘germline-targeting’ immunogens towards their neutralizing forms. One study was conducted by groups at the Harvard Medical School, the Vaccine Research Center and the Duke University School of Medicine (Tian, M et al 2016 Cell, in press). It was conducted in mice expressing the V_H domain of VH1-2*02 with diverse CDRH3 regions (described above) but with the restricted the fully rearranged iGL VRC01 LC. Animals were ‘primed’ with eOD-GT6 60mer (an earlier version of eOD-GT8), and subsequently boosted with sequentially with ‘426c core’ and several derivatives. The immunization protocol was devised to prime the antibody response with the most minimalist ‘germline-binding’ construct, which expresses only the outer domain of gp120 (eOD-GT6), then boost with a heterologous ‘germline-targeting’ construct that expresses both the inner and outer gp120 domains (426c core), followed by 426c core derivatives onto which the

NLGS in V5 and Loop D were gradually restored. The final boost was with a full-length, native-like 426c SOSIP Env. In total, six different immunogens were used. Only the first four constructs bind iGL VRC01. In this 'sequential immunization' scheme, one animal from the group (6 animals) was sacrificed after each immunization. As a result, only one animal from the group received all 6 immunogens. The 'sequential immunization' scheme was effective in generating CD4-BS antibodies, although a fraction of the elicited antibodies were not specific for the CD4-BS. During the course of immunization, the mutation frequencies of the iGL heavy and light chains steadily increased. Furthermore, in many instances the immunization scheme induced mutations that are found in the mutated VRC01-class antibodies. Somatic mutations in the LCs appeared primarily focused on CDRL1. As mentioned above, mutations in this region are critical for avoiding clashes with carbohydrate moieties in the highly conserved N276 position in Loop D. Importantly, serum from stepwise immunized mice exhibited neutralizing activities against 426c viruses that lack all three NLGS (N276D, N460D, N463D) or only one (N276D) sites, but also against a heterologous wild type HIV-1 Env 45_01dG5 naturally lacking N276. CD4-BS directed MAbs cloned from these animals displayed neutralizing activities similar to that observed in sera and one antibody displayed weak neutralizing activity against the fully glycosylated, tier 2 426c virus.

A different sequential immunization scheme was recently tested in iGL VRC01 HC knock-in mice by several groups at The Scripps Research Institute (Briney, B et al., 2106 Cell, in press). In this study, murine B cells expressing the iGL variable region of the VRC01 HC (with the CDRH3 sequence found in the mutated antibody) pair with a diverse murine LC repertoire described above (75). The immunogen used during the 'prime' was eOD-GT8 60mer. Two different proteins were evaluated as a subsequent first boost: a) a gp120 core version derived from the BG505 strain (BG505 core GT3). This construct lacks the 3 key NLGS that are important for germline VRC01-class antibody-binding and the variable regions 1, 2 and 3, and expresses both the inner and outer domains of gp120 (a similar concept to the above discussed '426c core'(73)) and was expressed on the same nanoparticle platform (NP) as eOD-GT8. b) A modified SOSIP BG505 trimer lacking the 3 NLGS mentioned above (BG505 GT3 SOSIP). In both cases a final boost was administered with a modified SOSIP BG505 trimer lacking only N276 (ie., the NLGS in V5 were restored to their native form). Ribi was used during the prime, while the boosts were performed with either Ribi or PBS.

BG505 core GT3 NP in Ribi elicited the highest frequency of CD4-BS specific memory IgG^+ B cells. An extensive analysis of HC and LC sequences from sorted CD4-BS specific B cells was performed. VRC01-like antibodies (i.e., the exogenous HC was paired with mouse light chains with 5 amino acid long CDRL3s) were amplified in all animals at different rates. Importantly, the complete heterologous boosting scheme resulted in significantly mutated sequences. Up to 17% mutation rates in HCs were observed and many of the mutations were similar to those found in the mutated VRC01 antibody. Equally important is the observation that there was evidence for sequence convergence of vaccine-elicited LCs with a critical glutamate at position 96 in CDRL3 which is found in most VRC01-class antibodies and makes critical contacts with gp120, as discussed above. Purified serum IgG after the final immunization did not display neutralizing activities

against a panel of 8 WT viruses. When N276 was eliminated by mutagenesis from these viruses, some became susceptible to neutralization by some of serum IgG. These results suggest that the vaccine-elicited antibodies could not bypass the carbohydrate moieties present on that critical site. Based on these promising results a second immunization study was conducted, following the above general 'prime-boost' scheme but one group of mice was boosted at the final stage with a cocktail of 3 SOSIP constructs (one each from clade A, B and C), all lacking N276. Serum IgG did not display neutralizing activity. MAbs from the immunized mice were produced and several displayed neutralizing activities against viruses with a mutagenized NLGS at position N276. Interestingly two MAbs also neutralized a WT tier 2 virus, 191084 B7-19. Thus, there is preliminary evidence that these MAbs bypass the carbohydrates on N276. Notably, a smaller number of constructs was used to drive an incomplete maturation of germline VRC01-like antibodies towards their neutralizing forms than in the above-mentioned study by Tian el al.

b) 'Germline-binding' immunogens for the elicitation of N332 supersite bNAbs—Escolano et al recently generated a knock in mouse that carried an iGL version of the N332-supersite specific PGT121 antibody (A. Escolano et al, 2016 Cell, in press). Naïve sera from these mice do not recognize recombinant Env, and immunization with native-like Envs failed to initate an antibody response, consistent with previous observations that iGL PGT121 does not display neutralizing activity or binding to any recombinant Env protein (62, 81, 90). Despite being unable to activate iGL PGT121 knock in cells *ex vivo*, a single immunization with 10MUT initiated a detectable serum antibody response, which was not neutralizing. To test whether sequential immunization schemes with the above-mentioned selected/modified Envs would guide the maturation of the 10MUT-elcited antibodies towards their neutralizing forms, sera from the 10MUT primed animals were screened for reactivity against less mutated native-like Env variants representing intermediates between WT BG505 T332N and 10MUT (7MUT, 5MUT, 3MUT, see above). The least modified Env variants that displayed serum reactivity after each immunization were selected as a boost. This iterative prime-boost strategy elicited serum responses that could bind to the WT BG505 T332N Env protein and to a cocktail of "heterologous" WT Envs with differing variable loops. This 'cocktail' was used as an immunogen in two final immunizations.

Importantly, serum IgG from 5/7 iGL PGT121 knock-in mice at the end of this iterative immunization regimen, displayed impressive cross-clade neutralizing activity on a 12 virus panel. The neutralizing activity of serum IgG from one mouse was shown to neutralize 12/50 Tier 2 and Tier 1B viruses that are susceptible to neutralization by the mutated PGT121. To better characterize the antibodies elicited by this regimen, 40 monoclonal antibodies were isolated, 15 of which displayed cross-clade tier 2 neutralizing activity on a 12 virus panel. The neutralizing activity of the cloned MAbs correlated with the number of somatic mutations. Interestingly, two of these MAbs were isolated from animals that did not display serum neutralizing activity.

iGL PGT121 knock-in mice repeatedly immunized with the germline targeting Env (10MUT), or 10MUT immunization followed by immunization with the WT BG505 N332T SOSIP immunogen failed to elicit serum neutralizing antibodies. Similarly, monoclonal antibodies isolated from mice immunized repeatedly with 10mut were non-neutralizing,

validating the ‘sequential immunization’ platform for the elicitation of bNAbs against HIV-1.

Despite the limitations of the animal model used here (discussed below) this is the first study that demonstrates that HIV-1 broadly neutralizing antibody responses can be elicited by vaccination.

Limitations of current ‘germline-targeting immunogens and immunization studies

The number of available ‘germline-targeting’ immunogens is presently limited. The search for additional ‘germline-targeting’ immunogens is very active and different strategies are being pursued by several groups. We anticipate that the repertoire of available ‘germline-targeting’ immunogens will increase rapidly fairly soon. Ideally, more than one immunogen targeting a specific class of bNAb-precursors (i.e., a particular epitope) will become available and that a side-by-side evaluation of such constructs will inform on their advantages and limitations. Such comparative studies will improve our ability to activate the desired naïve B cells in human vaccinees.

Immunogens capable of simultaneously activating germline BCRs that target distinct epitopes will also become available. For example, BG505.SOSIP-based constructs have been engineered that can activate, *in vitro* and *in vivo*, B cells expressing germline BCRs targeting the ‘apex’ and the CD4-BS (74) (J.P. Moore and R. Sanders, personal communications). Thus, two separate B cell linages targeting two distinct neutralization epitopes could expand simultaneously in response to a single immunogen. There is an obvious advantage in eliciting more than one type of bNAbs by vaccination, since HIV-1 can easily escape from individual bNAbs.

The immunogenicity studies discussed above represent the first step in assessing ‘germline-targeting’ immunogens and evaluating different boosting schemes. These animal systems provide the most direct evidence about whether or not a particular BCR is activated by a particular immunogen. However, they do have limitations, which should not be overlooked. With one exception, the knock-in HC genes express a predetermined (optimal) V,D,J recombination. The CDRH3 is thus pre-fixed and has the amino acid sequences found in the mutated antibodies. This can be a problem especially in the case of ‘apex’ antibody-targeting immunogens, because these antibodies interact with their epitopes primarily through their CDRH3 regions and the lack of information on the germline CDRH3 for all but the CAP256 lineage limits the optimal design of immunogens.

A second limitation is that the majority of the B cells in these mice express the desired HC and or LCs. In humans, there will be competition between the B cells that express the desired BCR and a majority of B cells that bind outside the desired epitope. The observation that even in these artificial animal models ‘germline-targeting’ immunogens elicit substantial off-epitope B cell responses should serve a warning, but also as guide to further improve the design of ‘germline-targeting’ immunogens. Immunization studies in animals expressing the full set of human VH/VLs (125–127) are being pursued and they may provide information on how these germline-targeting immunogens fair in a polyclonal B cell landscape. But even

such animals may not tell the whole story, as the expression frequencies of the desired B cells may differ from that found in humans.

What is consistent in all the immunization studies discussed here is that repeated immunizations with the 'germline-targeting' immunogen will not lead to extensive somatic hypermutation in HC and LCs and will not lead to the development of bNAbs. Boosting with additional Env variants will be required (69) (A. Escolano et al, 2016 Cell, in press). Although the immunogens employed during the boost will amplify the desired B cell clones stimulated during the prime, they will also prime undesirable B cell lineages *de novo*.

Concluding remarks

The broadly neutralizing antibody field has undergone a renaissance in the past 5–6 years, with: i) the availability of large, well characterized large cohort of HIV-1-infected subjects; ii) the isolation and characterization of many broadly neutralizing MAbs from infected subjects; iii) improvements in technologies that allow their structural characterization; and iv) the generation of new animal models systems. The tools we now have at our disposal allow us to interrogate the immune system before, during and after immunization in exquisite detail that was impossible a few years ago. We are thus optimistic that the development of broadly neutralizing antibodies by vaccination, once considered by some as an impossible task, will soon become a reality.

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