Identification of autoantigens recognized by the 2F5 and 4E10 broadly neutralizing HIV-1 antibodies

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Many human monoclonal antibodies that neutralize multiple clades of HIV-1 are polyreactive and bind avidly to mammalian autoantigens. Indeed, the generation of neutralizing antibodies to the 2F5 and 4E10 epitopes of HIV-1 gp41 in man may be proscribed by immune tolerance because mice expressing the V_H and V_L regions of 2F5 have a block in B cell development that is characteristic of central tolerance. This developmental blockade implies the presence of tolerizing autoantigens that are mimicked by the membrane-proximal external region of HIV-1 gp41. We identify human kynureninase (KYNU) and splicing factor 3b subunit 3 (SF3B3) as the primary conserved, vertebrate self-antigens recognized by the 2F5 and 4E10 antibodies, respectively. 2F5 binds the H4 domain of KYNU which contains the complete 2F5 linear epitope (ELDKWA). 4E10 recognizes an epitope of SF3B3 that is strongly dependent on hydrophobic interactions. Opossums carry a rare KYNU H4 domain that abolishes 2F5 binding, but they retain the SF3B3 4E10 epitope. Immunization of opossums with HIV-1 gp140 induced extraordinary titers of serum antibody to the 2F5 ELDKWA epitope but little or nothing to the 4E10 determinant. Identification of structural motifs shared by vertebrates and HIV-1 provides direct evidence that immunological tolerance can impair humoral responses to HIV-1.

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Abbreviations used: BCR, B cell receptor; BnAb, broadly neutralizing antibody; KYNU, kynureninase; MPER, membrane proximal external region; RUA, reverted unmutated ancestor; SF3B3, splicing factor 3B subunit 3.

Although uncommon, broadly reactive antibodies that neutralize multiple HIV-1 clades (broadly neutralizing antibodies [BnAbs]) and provide significant immune protection have been identified. BnAbs that block HIV infectivity contain viral spread under experimental conditions, preventing infection by HIV isolates in vitro (Mascola, 2003) and, at high concentrations, in vivo (Mascola et al., 1999, 2000; Balazs et al., 2012). Indeed, passive administration of BnAb 2F5, 2G12, b12, or 4E10 prevents simian HIV infection in monkeys (Mascola et al., 1999, 2000; Hessell et al., 2007, 2010). Likewise, humanized mice expressing transduced BnAb are protected from HIV infection (Balazs et al., 2012) and passive BnAb reduces the magnitude of viral rebounds after interruption of antiviral therapy in some patients (Trkola et al., 2005).

Several HIV-1 neutralizing epitopes are located along the membrane proximal external region (MPER) of gp41, a structure critical for viral fusion with target cells (Wyatt and Sodroski, 1998). The gp41-specific BnAbs 2F5, Z13, and 4E10 react with adjacent but distinct epitopes along the HIV-1 MPER (Muster et al., 1993; Zwick et al., 2001; Nelson et al., 2007), yet these Ab types are elicited in only a minority of HIV-1 patients and then only after years of infection (Yuste et al., 2006; Shen et al., 2009). These BnAbs carry high frequencies of mutations, suggestive of extraordinary selection

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of germinal center B cells (MacLennan, 1994) and, despite significant effort, no vaccine or immunization strategy induces robust MPER neutralizing Ab responses (Eckhart et al., 1996; Coëffier et al., 2000; Derby et al., 2006; Ofek et al., 2010a; Dennison et al., 2011).

Several explanations have been offered for the remarkable scarcity of gp41 HIV-1 BnAb after vaccination, including the complexity and genetic plasticity of HIV-1 epitopes, shielding of crucial antigenic determinants by glycosylation, competitive suppression by highly immunogenic, nonneutralizing envelope epitopes, and insufficient diversity in the primary Ab repertoire (Burton et al., 2004). Observations that the 2F5 and 4E10 BnAb recognize self-antigens (Haynes et al., 2005a; Verkoczy et al., 2010, 2011) offer an alternative explanation for the low frequencies of MPER-reactive BnAb in infected patients and vaccinees: immunological tolerance depletes most autoreactive B cells and consequently would impair Ab responses to HIV-1 epitopes that mimic self-antigens (Haynes et al., 2005b).

During development, self-reactive B cells are tolerized by apoptosis, anergy, or receptor editing (Goodnow, 1992), processes which have been intensively studied in mice expressing B cell receptors (BCRs) for authentic (Nemazee and Bürki, 1989; Erikson et al., 1991) or neo-self-antigens (Hartley et al., 1991). These experimental models have defined immature and transitional 1 B cells as targets of tolerizing apoptosis (Hartley et al., 1993) and identified anergy (Adams et al., 1990) and receptor editing (Gay et al., 1993; Tiegs et al., 1993) by characterizing B cell populations that escape apoptosis. Recently, these studies were extended to humans by expressing IgH and IgL rearrangements from single immature, transitional, or mature B cells and determining the frequencies at which these recombinant Abs reacted with self-antigens (Wardemann et al., 2003, 2004). In mice and humans, the frequency of autoreactive B cells declines with increasing developmental maturity (Wardemann et al., 2003, 2004), even when cells are recovered from peripheral sites (Meffre et al., 2004; Tsuiji et al., 2006).

The influence of tolerance on MPER-reactive B cell development has recently been investigated by the generation of 2F5 VDJ knockin (2F5 VDJ-KI) mice (Verkoczy et al., 2010, 2011). B cell development in 2F5 VDJ-KI mice is largely blocked at the transition of small pre–B to immature B cells (Verkoczy et al., 2010), a developmental blockade characteristically observed in mice expressing BCR for MHC (Nemazee and Bürki, 1989) or double-stranded DNA (Chen et al., 1995). However, unlike studies using BCRs with known autospecificity, the self-antigen that mediates selection against the development of B cells that recognize the 2F5 and 4E10 epitopes has not been identified.

Here, we identify kynureninase (KYNU) and splicing factor 3B subunit 3 (SF3B3) as the conserved self-antigens bound by the 2F5 or 4E10 BnAb, respectively. The KYNU H4 domain contains the complete 2F5 MPER epitope, and a single conservative mutation in the H4 motif abolishes 2F5 binding but not enzymatic activity. Remarkably, opossums naturally carry the same KYNU H4 mutation that destroys the 2F5 epitope, but they retain all 4E10 epitopes in SF3B3, and after immunization with HIV-1 envelope they develop unprecedented Ab responses against the 2F5 epitope but not the adjacent 4E10 determinant. Our findings implicate immunological tolerance as a natural mechanism by which HIV-1 evades robust immune responses.

RESULTS

Immunoprecipitation of human cell antigens by the 2F5 and 4E10 BnAb

To identify any cellular antigens recognized by 2F5 and 4E10, we immunoprecipitated whole-cell extracts of human HEp-2 cells with 2F5, 4E10, or an isotype-matched (IgG1/κ) human myeloma protein (151K). Specifically bound proteins were eluted from the BnAb and 151K, and resolved by SDS-PAGE (Fig. 1 A).

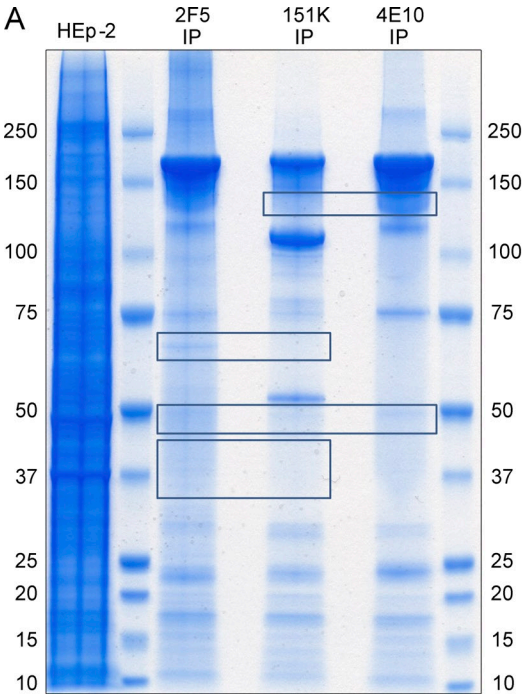
In this way, we identified discrete bands uniquely associated with 2F5, 4E10, or 151K eluates. Two discrete ligands of ∼70 and ∼50 kD were significantly enriched in all 2F5 eluates (Fig. 1 A); one notable band of ∼130 kD was found in 4E10 eluates. Additionally, 2F5 eluates were commonly enriched for a more diffuse band representing molecular mass of ∼40 kD, whereas 4E10 eluates contained a diffuse band of ∼70 kD. All other components of 2F5 and 4E10 immunoprecipitates were equivalently bound by the 151K myeloma control (Fig. 1 A).

Identification of cellular ligands bound by 2F5 and 4E10

We excised the protein bands preferentially immunoprecipitated by 2F5 and 4E10 BnAb and the corresponding areas of the 151K eluate (Fig. 1 A) for analysis by tandem mass spectrometry. A total of 15 proteins immunoprecipitated by 2F5 were identified in two or more of three independent experiments; all are typically cytoplasmic (Fig. 1 B). ATAD3A (ATPase family AAA domain-containing protein 3A), AIFM1 (apoptosis-inducing factor 1, mitochondrial precursor), HSP60 (60 kD heat shock protein, mitochondrial precursor), and SYNCRIP (heterogeneous nuclear ribonucleoprotein Q) were uniquely found in the 70-kD band of 2F5 eluate; TUBB4 (tubulin β-4A chain), TUFM (elongation factor Tu, mitochondrial precursor), KYNU, RPL4 (60S ribosomal protein L4), ALDH3A2 (fatty aldehyde dehydrogenase), and K-ALPHA-1 (tubulin α-1B chain) were identified in the 50-kD compartment; and ERLN2 (erlin-2), EMD (emerin), SLC25A11 (mitochondrial 2-oxoglutarate/malate carrier protein), RPL7 (60S ribosomal protein L7), and PHB (prohibitin) were recovered in the 40-kD band. Similarly, three proteins were found to associate preferentially with 4E10: SF3B3 from the 130-kD band, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and OST48 (dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kD subunit precursor) from the 50-kD band.

2F5 and 4E10 BnAb reactivity in human protein arrays

In an independent screen for 2F5 and 4E10 ligands, we used the ProtoArray microchip, which covers 9,400 human proteins,



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kD	2F5	4E10
130		SF3B3
70	ATAD3A, AIFM1, HSP60, SYNCRIP	
50	TUBB4, TUFM, KYNU, RPL4, ALDH3A2, K-ALPHA-1	GAPDH, OST48
35	ERLN2, EMD SLC25A11, RPL7, PHB	

Figure 1. Identification of 2F5 and 4E10 cellular antigens by immunoprecipitation. (A) HEp-2 cell lysate was immunoprecipitated with 2F5, 4E10, or human myeloma IgG (151K) and resolved under nonreducing conditions as described in Materials and methods. Boxes indicate bands that were differentially associated with 2F5, 4E10, or 151K eluates and were excised in pairs (BnAb versus 151K) for mass spectrometric analysis. HEp-2 lane contains whole-cell lysate of HEp-2; 2F5 IP, 151K IP, and 4E10 IP lanes include specifically bound proteins eluted from 2F5, 151K, and 4E10, respectively. Protein molecular masses (kD) are indicated on both sides of gel electrophoresis. The experiment was repeated on four independent occasions. (B) Excised protein bands were analyzed by tandem mass spectrometry; candidates were defined as proteins uniquely recovered in the BnAb, but not 151K control bands of the same molecular masses. To identify immunoprecipitates accurately, we required each protein candidate to be represented by three or more peptides with >95% peptide identification probability. Listed proteins were recovered in two or more out of three independent mass spectrometry tryptic digest analyses. Protein molecular mass indicates size of the band from which proteins were identified.

to compare the binding patterns of 2F5 and 4E10 to that of the human 151K. Anti–human IgG blotted on the array was used as an internal control for Ab loading, and human IgG on the array served as a loading control for the secondary detection reagent. Most proteins were bound equivalently by 2F5 and 151K (Fig. 2 A, diagonal binding axis). Intracellular high affinity Ig receptor TRM21 (Mallery et al., 2010) and all internal control proteins were equally recognized by both Abs (Fig. 2 A, top right square). However, 2F5 bound significantly better to KYNU and CKLF-like MARVEL transmembrane domain containing 3 (CMTM3) than did 151K (Fig. 2 A).

In contrast, 4E10 recognized 92.5% of the 9,400 microarray proteins better than the 151K control, and most proteins lay below the diagonal (Fig. 2 B). Internal controls of anti–human IgG were equally recognized by 4E10 and 151K, suggesting that the drastically skewed 4E10 binding pattern is not

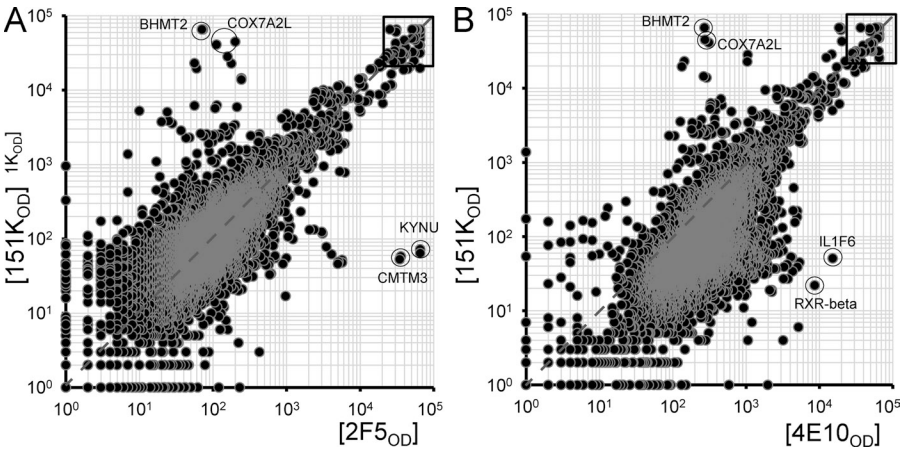

Figure 2. Identification of 2F5 and 4E10 self-antigens by protein array. Representative ProtoArray summary for protein arrays blotted with 2F5 (A), 4E10 (B), or 151K control. Axis values are relative fluorescence signal intensity in 151 array (y-axis) or BnAb array (x-axis). All proteins appear as duplicates on each array and each dot represents one replicate. Diagonal indicates equal binding by BnAb and 151K. Circles indicate repeatedly recovered positive hits by each Ab. BHMT2 (betaine-homocysteine methyltransferase 2) and COX7A2L (cytochrome *c* oxidase subunit VIIa polypeptide 2 like) were identified as the specific ligand for the human myeloma 151K in four or more independent experiments. As a control, we compared the binding pattern of a humanized therapeutic mAb against TNF, Infliximab, to that of the myeloma protein 151K (not depicted). Infliximab strongly associated with TNF, significantly better than the 151K, confirming that our system is capable of identifying the specific ligand of an Ab. IL1F6, IL-1 family member 6; Rxr-β, retinoid X receptor β. 2F5 and 151K arrays were repeated in three or more independent occasions; 4E10 protein array was performed twice.

a result of differences in Ab concentration but rather because of exceptional polyreactivity by 4E10. Unlike the 2F5 binding to KYNU and CMTM3, however, the majority of protein interactions by 4E10 are low avidity interactions, as indicated by the low fluorescence intensities. The three proteins that were identified to associate with 4E10 in immunoprecipitation, SF3B3, OST48, and GAPDH, also showed higher fluorescent binding to 4E10 than to 151K in the protein array. Additionally, we identified IL1F6 (interleukin-1 family member 6) and Rxr-β (retinoid X receptor β) as positive candidates in the 4E10 array, although the fluorescent intensities are fourfold lower than the positive hits in the 2F5 array.

Screening candidates for 2F5 and 4E10 self-ligands

To confirm that the proteins identified in mass spectrometry and protein array were bound by 2F5 or 4E10 with substantial affinity and high specificity, we obtained the full-length recombinant human proteins of all candidate 2F5 or 4E10 ligands and quantified their reactivity in ELISA. The initial ELISA, performed under permissive conditions (Kuraoka et al., 2011), identified six proteins, KYNU, CMTM3, TUBB4, RPL7, K-ALPHA-1, and SYNCRIP, to exhibit strong 2F5 binding (Fig. 3 A); ATAD3A, PHB, EMD, RPL4, ALDH3A2, and ERLN2 weakly associated with 2F5, and SLC25A11, HSP60, TUFM, and AIFM1 showed binding to 2F5 comparable to the negative control (BSA). The six strong binding proteins and ATAD3A were subjected to a second ELISA under more stringent binding conditions (Alam et al., 2008); KYNU and CMTM3 were the only proteins bound by 2F5 under these conditions (Fig. 3 B).

Similarly, the five candidate ligands of 4E10 were tested for 4E10 binding under permissive conditions. Two proteins, SF3B3 and OST48, reacted robustly with 4E10; the two low affinity 4E10 ligands identified in protein array (Fig. 2 B), IL1F6 and Rxr-β, exhibited detectable but weak binding to 4E10; and GAPDH had minimal interaction with 4E10 that is comparable to negative control. However, of the two strong binding proteins, under stringent conditions, only SF3B3 retained reactivity for 4E10 (Fig. 3 C). Thus, KYNU and CMTM3 are the main self-antigens that 2F5 specifically recognizes, and SF3B3 is the primary self-antigen of 4E10.

Confirmation of KYNU and SF3B3 as self-antigens of 2F5 and 4E10

Both KYNU and CMTM3 exhibited strong 2F5 binding under stringent ELISA conditions. 2F5 binds to KYNU, however, significantly more avidly than to CMTM3 because CMTM3 does not inhibit 2F5 binding to KYNU (Fig. 4 A) whereas KYNU inhibits 2F5 binding to CMTM3 even better than CMTM3 itself (Fig. 4 B). KYNU was discovered as a 2F5 ligand in both immunoprecipitation and protein array, whereas CMTM3 was only found in the protein array.

Interaction between 2F5 and cytoplasmic KYNU was confirmed in Western blots by the specific labeling of 2F5 50-kD immunoprecipitates with KYNU-specific Ab (Fig. 4 C). In addition, we stained HEp-2 epithelial cells with 2F5 using immunofluorescence. Under stringent binding conditions, the 2F5 BnAb labels the cytoplasmic and perinuclear regions of HEp-2 (Fig. 4 D). The 2F5 staining of HEp-2 cells colocalizes with KYNU-specific Ab staining (Fig. 4 D), suggesting that KYNU is the primary self-antigen of 2F5 in HEp-2 cells.

Similarly, the 4E10 BnAb labels perinuclear Golgi/ER regions of HEp-2 cells under stringent conditions, and colocalizes with a bound SF3B3-specific Ab (Das et al., 1999; Fig. 4 D). Collectively, these data indicate that KYNU and SF3B3 are the primary self-antigens of 2F5 and 4E10, respectively.

2F5 BnAb binds human KYNU via the H4 DKW motif

Soluble KYNU captured by plate-bound 2F5 mAb is recognized by anti-KYNU Ab (unpublished data), confirming that both plate-bound and soluble KYNU are recognized by 2F5. To compare 2F5 binding to KYNU and HIV-1 gp140, we inhibited 2F5 binding to gp140 JR-FL homologously with gp140 JR-FL and compared that to heterologous inhibition by KYNU in ELISA. The inhibition curves were superimposable (unpublished data), indicating that KYNU and HIV-1 gp140 are recognized by 2F5 at similar combining sites and with similar affinities.

2F5 Ab recognizes a linear epitope specified by the Glu_{662}-Leu-Asp-Lys-Trp-Ala_{667} (ELDKWA) residues of the HIV-1 gp41 MPER ectodomain (Muster et al., 1993). Because of the similar behavior of KYNU and gp41 in terms of 2F5 binding, we looked into the sequence of KYNU. Remarkably, KYNU contained the complete ELDKWA sequence in its H4 domain α-helix, a domain which is accessible to Ab (Fig. 5 A). Interestingly, a BLAST search in Swiss-Prot database revealed KYNU as the only known protein besides HIV envelope to contain the complete 2F5 neutralizing epitope motif ELDKWA. Moreover, CMTM3 contains the DKW core of the ELDKWA motif, consistent with a lower affinity interaction to 2F5.

To confirm that KYNU is bound by 2F5 at the H4 ELDKWA motif shared with HIV-1 gp41, we generated a mutant human KYNU bearing the $D_{92}\rightarrow E$ replacement that destroys the 2F5 nominal epitope (Muster et al., 1993). Wild-type and mutant KYNU migrated similarly in SDS-PAGE gels (Fig. 5 B) and were equally recognized by KYNU Ab (Fig. 5 C). Importantly, wild-type and mutant KYNU exhibited identical enzymatic activity (Fig. 5 D), indicating little or no structural distortion. Nonetheless, 2F5 reactivity for mutant KYNU was completely abolished (Fig. 5 E), confirming that 2F5 binds KYNU at the single ELDKWA epitope shared by KYNU and the HIV-1 MPER.

4E10 BnAb binds human SF3B3 via novel lipophilic interactions

4E10 is also thought to recognize a linear epitope on HIV-1 MPER epitope specified by residues Asn_{671}-Trp-Phe-Asp/Asn-Ile-Thr_{683} (NWFD/NIT; Stiegler et al., 2001; Zwick et al., 2001). Unlike the DKW amino acid motif that

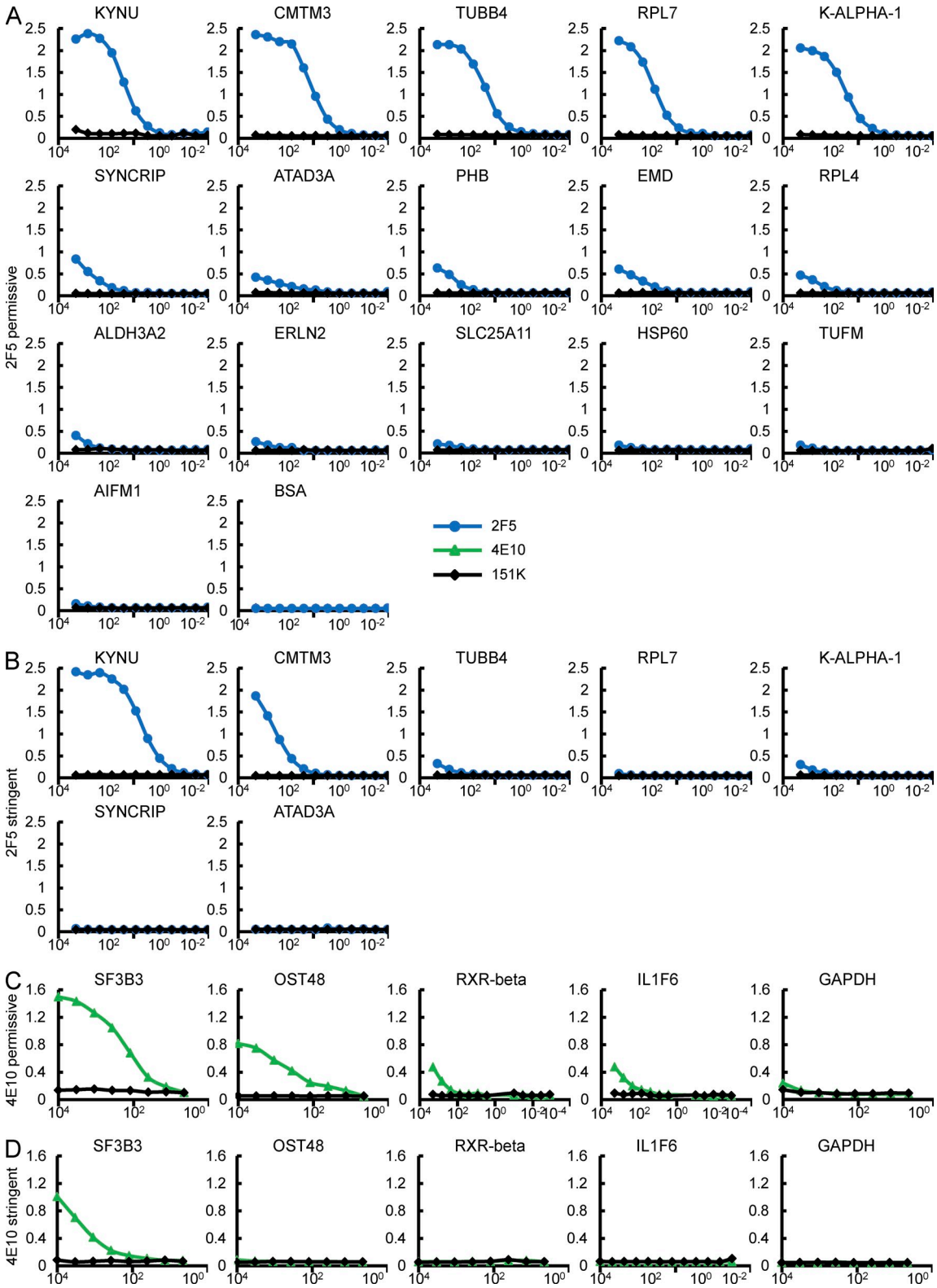

Figure 3. Screen of candidates for 2F5 and 4E10 self-ligands. Serial dilutions of 2F5 (A and B, blue), 4E10 (C and D, green), and 151K (black) in duplicates were incubated on ELISA plates preimmobilized with the recombinant candidate proteins identified in Figs. 1 and 2. BSA was used as negative control. The first round of screen was performed under permissive binding conditions as described in Materials and methods (A and C). Seven strongly recognized ligands for 2F5 and all 4E10 candidate ligands were then subject to a second round of screen under stringent binding conditions as described in Materials and methods (B and D). Axis values are OD (y-axis) or antibody concentrations (ng/ml; x-axis). All experiments were repeated in two or more independent experiments.

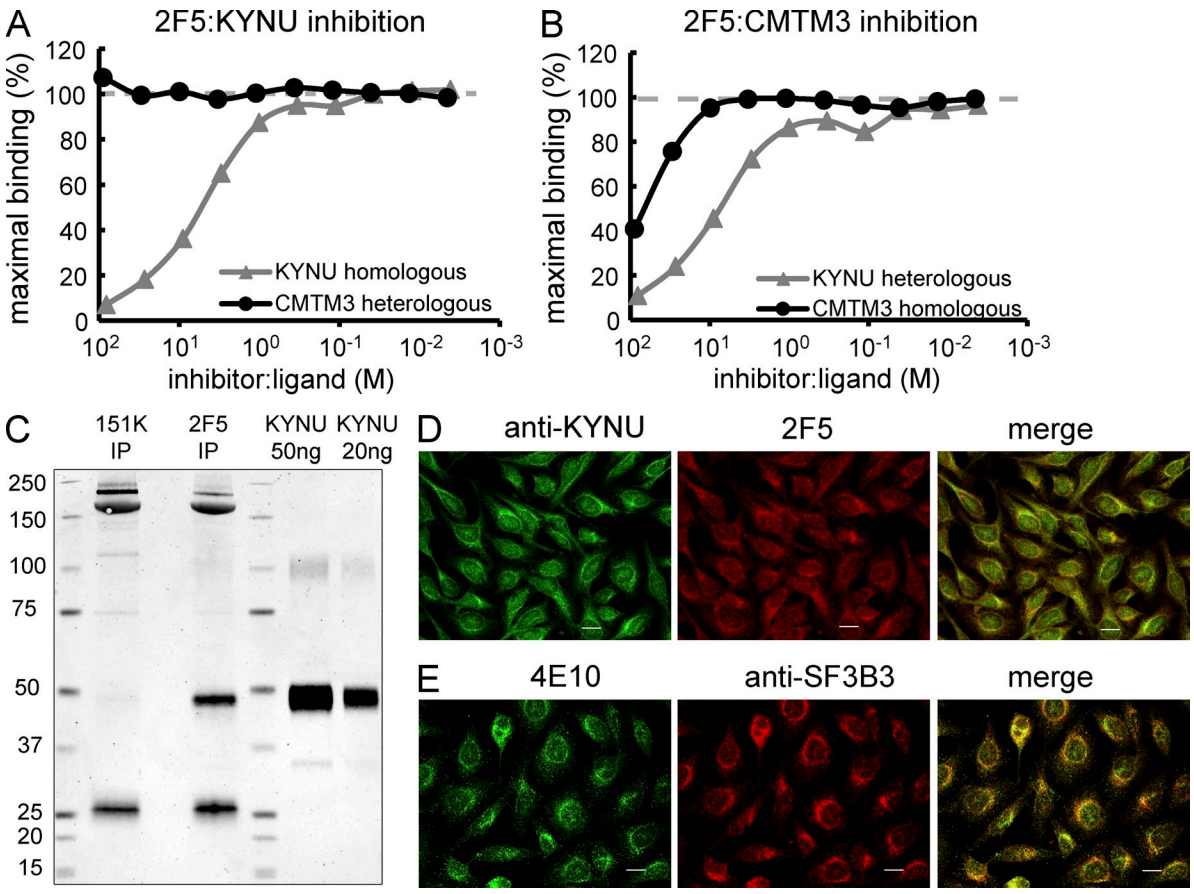

Figure 4. KYNU and SF3B3 are the main self-ligands of 2F5 and 4E10. Inhibition of 2F5 binding to immobilized recombinant KYNU (A) or CMTM3 (B) assessed in ELISA as described in Materials and methods. The y-axis indicates OD percentage of maximal binding, which is determined as the mean reading without inhibitors (100% binding is marked by gray dashes for comparison). The x-axis designates the molar ratio of inhibitor to plate immobilized ligand. (C) 2F5 and 151K immunoprecipitation eluates were stained with KYNU Ab in Western blot. Recombinant KYNU (rKYNU) was used as a positive control; molecular masses (kD) of protein markers are shown on the left of gel. (D and E) HEp-2 slides were indirectly labeled with anti-KYNU (FITC, green) and 2F5 (PE, red; D) or 4E10 (FITC, green) and anti-SF3B3 (PE, red; E). Negative controls were included to ensure specific staining and minimal cross-reactivity of all primary and secondary Abs. Bars, 15 µm. All data are representative of three or more independent experiments.

appears to mediate 2F5 binding to CMTM3 (Fig. 3 B), no amino acid triplet in human SF3B3 matches to this canonical 4E10 epitope. Consequently, we also searched the SF3B3 amino acid sequence for the presence of two additional epitopes that have been suggested to mediate 4E10 binding to HIV-1 envelope, LWVITVYYGVPVWK (gp120) and AV/L/MFLGFLGAA (gp41; Hager-Braun et al., 2006). Nine amino acid triplets present in these alternative epitopes were present in human SF3B3. To determine whether this overlap of 4E10 epitope triplets was merely a chance outcome, we randomly selected 150 protein sequences from the set of 9,400 human proteins in the protein array (comprising 77,283 amino acid residues; Fig. 2) and determined the frequencies of amino acid triplets in these proteins, both individually and pooled, that were also present in the 2F5 and 4E10 linear epitopes. By this comparison, the occurrence of 2F5 epitope triplets in human KYNU was found to be significantly enriched compared with the randomly selected control proteins ($P = 2.8 \times 10^{-6}$; Student's *t* test). In contrast, the frequencies of 4E10 epitope amino acid triplets in the control proteins were not significantly different from that in SF3B3 ($P = 0.25$; Student's *t* test). We conclude that the avid binding of 4E10 to SF3B3 (Fig. 3 C) is not mediated by any of the known 4E10 linear epitopes but rather by unknown linear or conformational motifs.

To compare the nature of 4E10 binding to SF3B3 and gp41, we inhibited the SF3B3 binding to 4E10 in ELISA using either recombinant HIV-1 gp41 or liposomes decorated with cardiolipin (Alam et al., 2007). 2F5 binding to KYNU is readily inhibited by gp41, but not by liposomes, even at high concentrations (Fig. 6 A). In contrast, 4E10 binding to SF3B3 was strongly reduced by the liposome/cardiolipin inhibitor but not by gp41 (Fig. 6 B). Although there is controversy regarding the cardiolipin reactivity of 2F5, it is accepted that 4E10 binds anionic phospholipids, including cardiolipin, with high affinity (Alam et al., 2007; Scherer et al., 2007; Vcelar et al., 2007). This phospholipid interaction is mediated by the long hydrophobic HCDR3 of

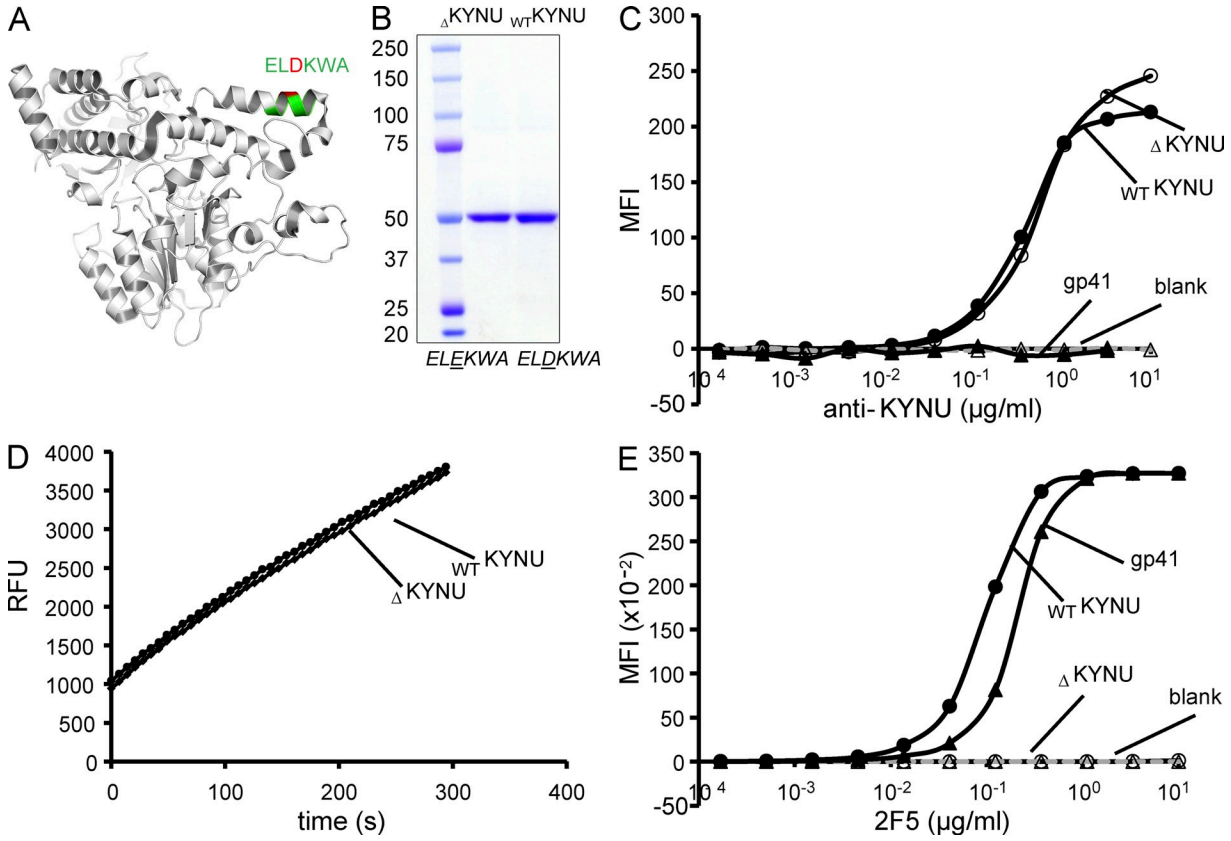

Figure 5. 2F5 recognizes KYNU through the H4 DKW shared with HIV-1 gp41. (A) Structure of monomeric KYNU as it appears in the dimer complex. Green highlights 2F5 epitope within the H4 domain of KYNU; red indicates D amino acid in the core of 2F5 epitope (Lima et al., 2007). (B) Recombinant wild-type and mutant KYNU were resolved in nonreducing SDS-PAGE and stained with coomassie blue with molecular mass standards (KD) on the left. (C and E) gp41, recombinant KYNU, and mutant KYNU binding to anti-KYNU (C) or 2F5 (E) in Luminex assays. Uncoupled beads (blank) were used as negative control. MFI, mean fluorescence intensity. (D) Enzymatic activities of wild-type and mutant KYNU were measured as described in Materials and methods. RFU, relative fluorescence unit. Enzymatic measurements were repeated in three or more independent experiments. All other data were collected twice in duplicates.

4E10, whereas the hydrophobic properties of this extended HCDR3 are not required for interaction with gp41 protein (Alam et al., 2009). With these points in mind, we propose that the 4E10 BnAb may not bind to SF3B3 in the way that it does to the HIV-1 gp41 MPER (Fig. 6 B) but does use the lipophilic HCDR3 in a way that confers substantial specificity (Fig. 2 B). Significantly, the lipophilic HCDR3 of both 2F5 and 4E10 are crucial for BnAb activity (Alam et al., 2009).

Germline precursors of 2F5 and 4E10 bind human KYNU and SF3B3

The avid binding of 2F5 and 4E10 to KYNU and SF3B3 suggested that endogenous KYNU and SF3B3 might tolerize developing B cells bearing BCR like 2F5 or 4E10. Because this tolerance would necessarily act on unmutated B cells, we generated the reverted unmutated ancestor (RUA) Ab for 2F5 and 4E10 (Alam et al., 2011). Two RUAs for 2F5 with identical posterior probabilities were identified (RUA variant 1 and RUA variant 2). These germline Abs exhibit lower, but significant, affinities for the 2F5 epitope (variant 1, K_d = 0.11 μM; variant 2, K_d = 4.8 μM) than the 2F5 Ab (K_d = 4.5 nM; Alam et al., 2011); RUA variant 1 bound KYNU strongly in both ELISA and Western blots, although less strongly than 2F5 (Fig. 7 A). Like 2F5, the 2F5 RUA variant 1 binds to KYNU via the ELDKWA motif because the interaction is completely abolished upon D_{92} substitution (Fig. 7 B). 2F5 RUA variant 2 Ab was not positive in our assays, but more sensitive plasmon resonance studies have demonstrated its capacity to bind the ELDKWA motif (Alam et al., 2011).

Similarly, the RUA for 4E10 was generated (4E10 GL1). In serological assays, 4E10 GL1 binds SF3B3, albeit with ~10-fold lower avidity than 4E10 and control SF3B3 Ab (Fig. 7 C). Therefore, the immature B cells expressing 4E10 germline Ab may well be subject to tolerance deletion by endogenous SF3B3.

KYNU and SF3B3 are phylogenetically conserved

Immunization of various animals with peptides bearing the 2F5 and 4E10 epitopes has demonstrated its poor immunogenicity across species (Montero et al., 2008), indicating that

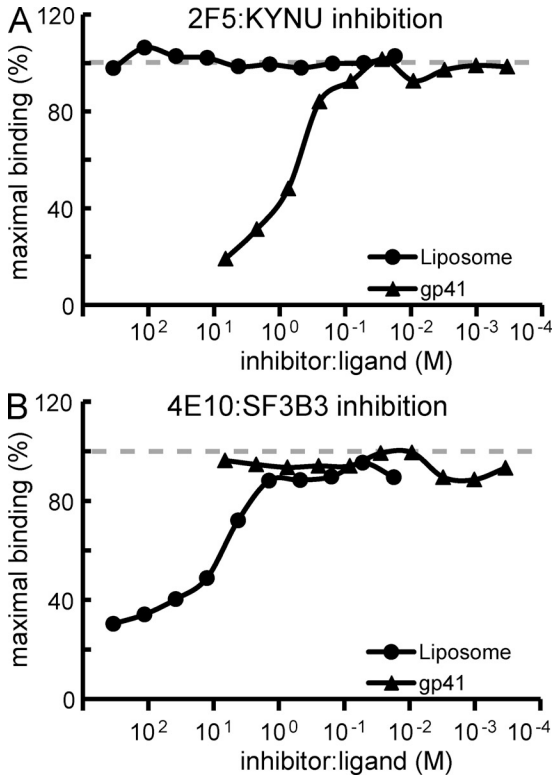

Figure 6. 4E10 binds SF3B3 via hydrophobic interactions. Competitive inhibition of 2F5 binding to KYNU (A), and 4E10 binding to SF3B3 (B) by gp41 (triangle) or liposome containing 75% cardiolipin and 25% phosphatidylcholine (circle). Recombinant KYNU and SF3B3 were immobilized, and threefold serial dilutions of the indicated inhibitor were added, followed by 200 ng/ml 2F5 (A) or 4E10 (B). BnAb binding was detected as described in Materials and methods. The y-axis indicates OD percentage of maximal binding, which is determined as the mean reading without inhibitors (100% binding is marked by gray dashes for comparison). The x-axis designates the molar ratio of inhibitor to plate immobilized ligand. All experiments were performed in duplicates.

the suppression of potential Ab responses by tolerance mechanisms is conserved among mammals. Indeed, we observed similar staining patterns to human HEp-2 cells when 2F5 and 4E10 were used to label mouse fibroblasts (Verkoczy et al., 2010), suggesting that the self-ligands of 2F5 and 4E10 are present in both species. Therefore, we sought to compare the sequence similarity of mammalian KYNU and SF3B3.

The ELDKWA sequence present in human KYNU, an enzyme which catalyzes the cleavage of L-kynurenine and 3-hydroxykynurenine into anthranilic acid and 3-hydroxyanthranilic acid, respectively (Saito et al., 1957), is located in a surface-exposed H4 domain that mediates homodimerization (Lima et al., 2007). Such sequence within the H4 domain of KYNU is remarkably conserved. All mammals for which genomes have been sequenced carry the ELDKWA H4 domain except gray short-tailed opossum, where glutamic acid (E_{92}) replaces aspartic acid (D_{92}; ELEKWA). This substitution also occurs in zebrafish and conserves enzymatic function (Fig. 5 C) but completely abrogates 2F5 binding (Fig. 5 A). Therefore, KYNU from all mammalian species but opossum should exert tolerizing pressure for B cells expressing 2F5-like BCR. In addition, the DKW sequence of CMTM3 is also conserved among humans, chimpanzees, and mice, but not opossums (Fig. 8).

SF3B3 is also highly conserved in mammalian species (Fig. 8); the amino acid sequences of human, nonhuman primates, mouse, rat, and wolf SF3B3 are ≥99.7% identical. Even in opossum, a marsupial mammal, SF3B3, shares 94.9% amino acid identity with humans.

Robust Ab responses to the 2F5, but not the 4E10 epitope, by *Monodelphis domestica*

Opossums naturally carry the D_{92}→E exchange at the site of 2F5 binding to KYNU (Fig. 8) that abolishes 2F5 reactivity but not enzyme activity (Fig. 5; Muster et al., 1993). In contrast, opossum SF3B3 from liver cell lysates is strongly bound

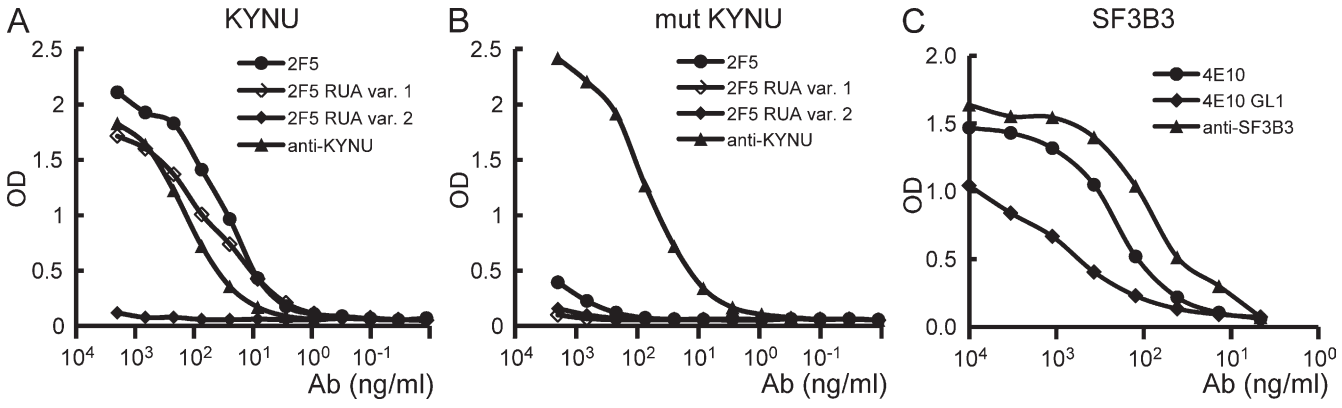

Figure 7. KYNU and SF3B3 are recognized by germline precursors of 2F5 and 4E10. (A and B) Recombinant KYNU (A) or mutant KYNU (B) were immobilized on ELISA plates and detected by duplicate threefold serial dilutions of 2F5, 2F5 RUA variant 1, 2F5 RUA variant 2, and anti-KYNU. (C) Recombinant SF3B3 was immobilized, duplicate serial dilutions of 4E10, 4E10 GL1, and anti-SF3B3 were added, and binding was detected as described in Materials and methods. All experiments include negative controls with BSA plates and isotype control Ab (not depicted). All data are representative of at least three independent experiments.

A

gp41 HIV-1

	2F5 epitope	
...ELL	ELDKWA	SLW NWFDIT...

B

KYNU

Homo sapiens	LEE	ELDKWA	KIA
Pan troglodytes	LEE	ELDKWA	KIA
Mus musculus	LEE	ELDKWA	KMG
Monodelphis domestica	LEE	EL**E**KWA	KMG

C

CMTM3

Homo sapiens	QLN	DKW	QGL
Pan troglodytes	QLN	DKW	QGL
Mus musculus	QLN	DKW	QGL
Monodelphis domestica	KIN	**E**K**I**	PGI

D

SF3B3

Species	Identity (%)
Homo sapiens	-
Pan troglodytes	100.0
Macata mulatta	99.7
Canis lupus	99.9
Bos taurus	100.0
Mus musculus	99.9
Rattus norvegicus	99.8
Monodelphis domestica	94.9

Figure 8. KYNU, CMTM3, and SF3B3 are phylogenetically conserved. (A) Amino acid sequence flanking the 2F5 epitope within the MPER of HIV-1 gp41. (B) The amino acid sequences of the H4 domain of KYNU in human, chimpanzee, mice, and opossums are listed. (C) CMTM3 sequence flanking DKW in human, chimpanzee, mice, and opossums. (D) Overall amino acid sequence identity of mammalian SF3B3 compared with human SF3B3. Sequences of all mammalian species for which the genome has been sequenced are also conserved in the 2F5 epitope in KYNU (not depicted).

by the 4E10 Ab (not depicted); 4E10 is also highly polyreactive (Fig. 2 B). We predict, therefore, that 4E10-like B cells are tolerized in opossums. The absence of the 2F5 epitope in opossum KYNU (and CMTM3) but retention of 4E10 self-reactivity imply that immunization of opossums with HIV-1 antigens containing both determinants will induce Ab to the 2F5 epitope but not to the adjacent 4E10 determinant.

A cohort of 12 opossums was immunized with HIV-1 gp140 JR-FL and boosted at 21-d intervals with liposomes bearing an MPER peptide and TLR ligands (Dennison et al., 2011). The MPER peptide immunogen carries both 2F5 and 4E10 epitopes. Sera were collected 2 wk after each immunization and analyzed for Ab to gp41 and peptides containing the 2F5 or 4E10 epitopes (Muster et al., 1993; Zwick et al., 2001). For comparison, C57BL/6 mice were similarly immunized with the same protein and liposome immunogens.

In opossums, the initial immunization elicited significant levels of gp41 Ab with ELDKWA-specific Ab present after the second immunization; by the third immunization, serum Abs specific for gp41 and 2F5 peptide were equivalent (Fig. 9 A). Ab responses to the 2F5 epitope continued to increase on boosting, reaching a mean end-point titer (week 14) of $10^{-4.7}$ (geometric mean, $n = 12$, 1:47,622). By the fourth immunization, the three highest responders generated a mean Ab titer of $10^{-5.2}$ (1:163,897) with one opossum achieving a titer of $10^{-5.5}$ (1:340,920). These high levels of serum Ab were maintained with no significant change after a fifth immunization (Fig. 9 A). Despite the remarkable speed and scale of these ELDKWA Ab responses, reactivity against the 4E10 epitope in all immunized opossums was virtually absent (geometric mean, $n = 12$, 1:2.6; Fig. 9 A).

In mice given the same immunogens, development of serum Ab was significantly delayed and reduced. Serum Ab to both the 2F5 and 4E10 epitopes was not observed until 4 wk

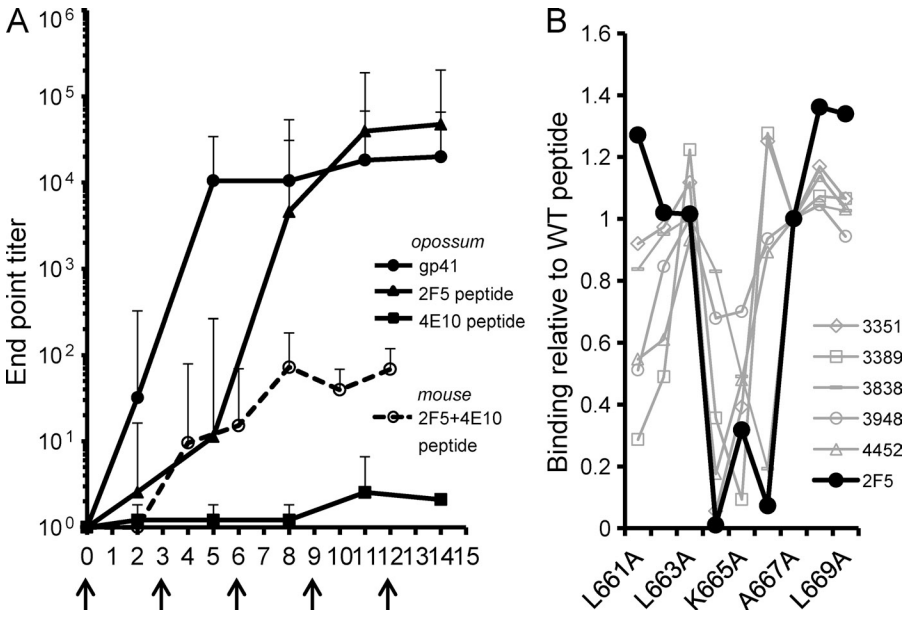

Figure 9. Opossums mount robust 2F5-like Ab responses after immunization with HIV-1 envelope protein. (A) 12 laboratory opossums were immunized with JR-FL gp140 and boosted with liposomes containing MPER peptide and TLR ligands. MPER peptide contains both 2F5 and 4E10 determinants. Sera were assayed by ELISA for opossum IgG against gp41, biotinylated 2F5-epitope peptide, and biotinylated 4E10 epitope peptide. All ELISA plates contained positive controls of 2F5 or 4E10 and negative controls of human IgG (not depicted). End-point titers ($n = 12$; geometric means ± SD) were determined as described in Materials and methods. For comparison, BL/6 mice were primed and boosted i.p. with the same JR-FL gp140 and liposome immunogens given to opossums at 2-wk intervals for 12 wk. Mouse sera (pre and postimmunization) were assayed by ELISA for IgG Ab using the MPER656 peptide that includes both the 2F5 and 4E10 epitopes. End-point titers ($n = 5$; geometric means ± SD) were determined as for opossums. (B) Single alanine mutations were introduced into a collection of 2F5 epitope peptides spanning residues 661–669. The effects of these mutations were evaluated by SPR and compared with binding of opossum serum to wild-type 2F5 peptide. The five sera with highest SP62 responses in SPR are shown.

after the primary immunization and serum titers never climbed (week 12) above $10^{-1.9}$ (geometric mean, $n = 5$, 1:72; Fig. 9 A). Serum Ab responses to the 2F5 and 4E10 MPER epitopes in mice were <0.5% of the 2F5 and gp41 responses observed in opossums.

To map the fine specificity of the serum 2F5-like Ab generated by opossums, we determined the reactivity of each serum against a library of alanine-mutated 2F5 peptides by SPR. Remarkably, the 2F5 epitope binding activity of the opossum sera mapped most strongly to the single aspartic acid (D_{92}) residue absent in opossum KYNU (Fig. 9 B). This focused binding is consistent with our hypothesis that robust 2F5-like Ab responses to HIV-1 gp41 in immunized opossums was the consequence of removing tolerizing selection by endogenous KYNU. Despite the similar binding properties of opossum ELDKWA serum Ab with 2F5 (Fig. 9 B), there was no neutralizing activity in the plasma of immunized opossums, consistent with the additional requirement of lipid reactivity by induced Ab for virus neutralization (Ofek et al., 2004; Alam et al., 2009).

DISCUSSION

HIV-1 exhibits exceptional antigenic plasticity and diversity; consequently, vaccines that elicit neutralizing serum Ab to conserved epitopes of HIV-1 envelope proteins are a key research focus (Haynes and Montefiori, 2006; Burton et al., 2012). The gp41 MPER located at the Env stem contains the fusion machinery necessary for viral cell entry (Wyatt and Sodroski, 1998) and is the target of several potent HIV-1 BnAbs, including 2F5 and 4E10 (Muster et al., 1993; Zwick et al., 2001). However, despite its genetic and structural conservation among clades (Sun et al., 2008), HIV-1 gp41 MPER epitopes are poorly immunogenic even when administered in a variety of contexts and in several animal models (Montero et al., 2008). Similarly, only a minority of HIV-1–infected subjects mount robust MPER BnAb responses and even then only after years of infection (Burton et al., 2004). To explain this remarkable lack of immunogenicity, it has been proposed that the physiological processes of immunological tolerance may limit the production of MPER BnAb against epitopes that mimic host structures (Haynes et al., 2005b). This hypothesis offers a mechanistic explanation for the rarity of MPER-reactive Ab responses after vaccination and infection, is testable (Verkoczy et al., 2010, 2011), and predicts that neutralizing HIV-1 MPER epitopes have been selected to mimic host antigens (Haynes et al., 2005a,b).

Host mimicry by microbial pathogens is well known. For example, Ab elicited by bacterial adhesin FimH of fimbriated pathogens cross-reacts with LAMP-2 (lysosomal membrane protein 2) and causes pauci-immune FNGN (focal necrotizing glomerulonephritis; Kain et al., 2008). Similarly, Ab to *Campylobacter jejuni* lipooligosaccharide (LOS) frequently reacts with mammalian gangliosides present on neural cells (Yu et al., 2011), a mimicry associated with only modest serum Ab responses in a minority of *Campylobacter* infections (Bowes et al., 2002). Immunization of normal mice with *C. jejuni* LOS elicits weak, T cell–dependent Ab responses, but these are greatly enhanced in knockout mice that cannot generate complex gangliosides (Bowes et al., 2002). This observation identifies microbial mimicry of host antigens as an effective strategy to mitigate humoral immunity in the infected host. We submit that this evolutionary strategy is widespread and a principal component of the poor BnAb responses to HIV-1 MPER epitopes.

We used two approaches to identify candidate self-ligands mimicked by 2F5 and 4E10 epitopes. HEp-2 human cell lysates were incubated with 2F5 or 4E10 and immunoprecipitated, and Ab–antigen complexes were separated by gel electrophoresis and analyzed by tandem mass spectrometry (Fig. 1). In an independent screen, the reactivity of 2F5 and 4E10 was determined by protein arrays containing some 9,400 human recombinant proteins (Fig. 2). By these methods, 15 proteins preferentially bound by 2F5 were detected. An ELISA screen under permissive (low affinity) binding conditions confirmed 2F5 reactivity to these 15 proteins, but under stringent conditions that reduced weak, unspecific protein–protein interactions, only KYNU and CMTM3 could be demonstrated as authentic 2F5 ligands (Fig. 3). The specificity of this binding maps unambiguously to the ELDKWA and DKW motifs in KYNU and CMTM3, respectively (Fig. 5). Structural analyses of the 2F5 F_{ab} complexed with MPER peptides have defined ELDKWA as the 2F5 epitope (Ofek et al., 2004); notably, KYNU is the only known protein that carries the complete ELDKWA 2F5 epitope and this H4 domain motif is conserved across widely divergent mammalian species (Fig. 8). A single replacement mutation (ELDKWA→ELEKWA) within the human KYNU H4 domain abrogates 2F5 binding (Fig. 5). The 2F5 BnAb binds KYNU with high affinity and exquisite specificity (Figs. 3 and 4) for a rare structural motif that matches exactly the 2F5 MPER epitope (Figs. 5 and 8); we conclude that the host antigen mimicked by the 2F5 MPER epitope is the H4 domain of KYNU, a ubiquitously expressed enzyme of tryptophan metabolism (Soda and Tanizawa, 1979).

Of the five candidate ligands for the 4E10 BnAb (Figs. 1 and 2), only SF3B3 was bound by 4E10 in ELISA under stringent binding conditions (Fig. 3). In contrast to the single linear 2F5 epitope, at least three different antigenic determinants through which 4E10 binds the HIV-1 envelope have been proposed (Stiegler et al., 2001; Zwick et al., 2001; Hager-Braun et al., 2006). However, none of these linear determinants are present in human SF3B3; instead, our results indicate that the 4E10 BnAb recognizes this ubiquitously expressed splicing factor through novel linear or conformational motifs that can be disrupted by cardiolipin but not gp41 (Fig. 6). In contrast to the obvious structural mimicry of the 2F5 epitope, the 4E10 MPER may mimic host structures more subtly by imitating conformation-dependent patterns of surface-accessible hydrophobic regions.

Although 2F5 weakly interacts with nonprotein self-antigens including cardiolipin (Haynes et al., 2005a; Mouquet et al., 2010), the binding of 4E10 to cardiolipin is significantly more avid (Fig. 6; Alam et al., 2007). The efficient inhibition of

4E10 binding to human SF3B3 by cardiolipin, but not HIV-1 gp41 (Fig. 6), suggests to us that the major component of 4E10, but not 2F5, autoreactivity involves the extended, hydrophobic HCDR3 of 4E10 (Alam et al., 2007). The differential capacities of gp41 and cardiolipin to inhibit 2F5 or 4E10 binding to KYNU and SF3B3, respectively, suggests that tolerance to the H4 domain of KYNU, rather than a more general lipid reactivity, is responsible for the rarity of B cells that express 2F5-like Ab. Consistent with this hypothesis, B cell escape mutants from 2F5 VDJ-KI mice exhibit no MPER binding but retain substantial lipid reactivity (Verkoczy et al., 2010, 2011). We predict that a knockin of a mutant 2F5 VDJ that exhibits diminished binding to lipid, but not gp41 (Alam et al., 2009), will effect a B cell developmental blockade similar to that of 2F5 VDJ-KI mice (Verkoczy et al., 2010, 2011). In contrast, if the autoreactivity of 4E10 depends on its hydrophobic, lipid-interacting HCDR3 [Fig. 6 and (Alam et al., 2007)], we predict that any B cells that escape tolerance in 4E10 VDJ-KI mice will be characterized by reductions in both lipid reactivity and gp41 binding.

Many HIV-1 BnAbs are considered polyreactive (Haynes et al., 2012), a feature which has been proposed to promote HIV-1 neutralization by heteroligation and a consequential increase in apparent affinity for Env epitopes (Mouquet et al., 2010). There has been, however, some controversy regarding the polyreactivity of the 2F5 and 4E10 BnAb (Scherer et al., 2007; Vcelar et al., 2007). Haynes et al. (2005a, b) first reported that 2F5 and 4E10 bind several autoantigens, including cardiolipin, and suggested that their polyreactivity might lead to down-regulation by immunological tolerance. 2F5 and 4E10 polyreactivity was confirmed and shown to increase the apparent affinity (Mouquet et al., 2010) and neutralizing capacity (Mouquet et al., 2012) of these HIV-1 BnAbs. Here, to quantify polyreactivity, we used human protein arrays and compared binding patterns of 2F5 and 4E10 in a pairwise fashion to the isotype/type-matched myeloma protein 151K and therapeutic mAb Infliximab. Indeed, when compared with both 151K and Infliximab, 4E10 is broadly polyreactive, binding >90% of microarray proteins with low affinity (Fig. 2). Such stickiness is likely a result of hydrophobic interactions, as we and others have noted that 4E10 binds phospholipids with much higher affinity than 2F5 (Matyas et al., 2009). Significantly, SF3B3 binding by 4E10 is inhibited by cardiolipin-decorated liposomes (Fig. 6), supporting the notion that 4E10 promiscuity is mediated by its hydrophobic HCDR3 (Alam et al., 2009). In contrast, among all 9,400 proteins tested, 2F5 reacted only with KYNU and CMTM3 under stringent binding conditions (Fig. 2 A). Such specific cross-reactivity is presumably mediated by a shared antigenic epitope.

In contrast to our results, an earlier analysis of 2F5 and 4E10 binding in protein arrays concluded that neither BnAb was polyreactive (Scherer et al., 2007). This study used a small (400 protein) array, did not identify specific ligands for 2F5 or 4E10 (KYNU, CMTM3, and SF3B3 were not included), and concluded that none of the tested HIV-1 BnAbs were "unusually cross-reactive" because the "cross-reactivity profiles of the different mAbs cluster together" (Scherer et al., 2007). These cross-reactivity profiles represented the rank ordered binding of each BnAb to each protein ligand. Remarkably, the authors did not emphasize the fact that the binding distributions for different BnAbs were made similar by changing the order of ligand binding. In fact, the rank order of BnAb binding to each antigen differed for each tested mAb (Scherer et al., 2007); reordering the bound ligands made these differences difficult to detect and effectively reduced any differences in cross-reactivity among the tested mAb. Indeed, when our protein array data are replotted in the same way, the cross-reactivity profiles of 4E10, 2F5, 151K, and Infliximab cluster together indistinguishably (unpublished data). Nonetheless, 4E10 unequivocally exhibits heightened cross-reactivity for virtually all of the protein ligands in the array (Fig. 2 B) and is substantially more polyreactive than 2F5, 151K, and Infliximab.

Our results suggest that newly generated B cells bearing 2F5- or 4E10-like antigen receptors that cross react with host KYNU (and CMTM3) or SF3B3 undergo physiological tolerization by deletion, editing, or inactivation. We put this hypothesis to the test by immunizing opossums. Opossums naturally lack the endogenous KYNU and CMTM3 epitope mimicked by the HIV-1 gp41 MPER (Fig. 8). In contrast, opossum and human SF3B3 are comparably bound by the 4E10 mAb (unpublished data); moreover, the strong polyreactivity of 4E10 would also promote tolerization in opossum (Wardemann et al., 2003). We demonstrate that immunization of opossums results in rapid and robust Ab responses to the 2F5 gp41 epitope but not to the adjacent 4E10 determinant that is also present on the immunogen (Fig. 9). As a matter of fact, the 2F5 epitope titer is equivalent to the gp41 titer; MPER Ab response of immunized opossums is, therefore, specifically focused on the 2F5 epitope that is absent from its own KYNU and CMTM3 but not generally enhanced anywhere else.

The substantial binding of KYNU and SF3B3, respectively, by the unmutated RUA forms of the 2F5 and 4E10 BnAbs strongly suggests that newly formed human B cells with 2F5- or 4E10-like BCR are subject to immunological tolerance. The 2F5 RUA variant 1 represents a V_H2-5 gene segment allele that contains an aspartate residue (D_{54}) in HCDR2 that forms a salt bridge with the lysine residue (K_{665}) in the 2F5 epitope of gp41 (Alam et al., 2011). The RUA variant 2 represents the alternative V_H2-5 allele with a D_{54}→N replacement; it is unable to form the salt bridge with gp41. Plasmon resonance studies show that these structural differences produce different affinities for the ELDKWA epitope, with RUA variant 1 binding with a K_d of 0.11 μM and variant 2 with a K_d of 4.8 μM (Alam et al., 2011). This 44-fold difference in affinity likely accounts for robust binding of 2F5 RUA variant 1 to KYNU in ELISA, whereas RUA variant 2 binding was undetectable (Fig. 7). We conclude that similar binding forces control 2F5 binding to both the H4 domain of human KYNU and to the HIV-1 MPER. It is certainly

plausible that the immature B cell that gave rise to the 2F5 BnAb recognized endogenous KYNU but fortuitously escaped immunological tolerance. Despite its inability to bind KYNU in ELISA (Fig. 7), the demonstrated affinity of the 2F5 RUA variant 2 for the ELDKWA epitope (Alam et al., 2011) falls within the range of central tolerization in mice (Lang et al., 1996) and is likely susceptible to tolerization by the identical epitope on KYNU. Alternatively, this tolerization might occur later in the periphery and perhaps even during the germinal center reaction (Han et al., 1995; Pulendran et al., 1995; Shokat and Goodnow, 1995). The generation of 2F5 RUA KI mice will address this issue.

2F5 RUA variant 1 binds KYNU better than HIV-1 gp140 (unpublished data), suggesting that naive B cells bearing 2F5-like RUA 1 BCR may not constitute a significant component of early humoral responses to HIV-1 Env after infection or immunization. The later appearance of MPER BnAb in infected patients may be the result of the relaxation of tolerance by chronic infection/inflammation (Moir et al., 2011). Alternatively, late appearing BnAb may arise from nonautoreactive B cells that acquire V(D)J mutations in response to environmental antigens (Liao et al., 2011) and acquire autoreactivity (Tiller et al., 2007; Mietzner et al., 2008) that predisposes for responses to MPER epitopes that mimic self-antigens (Haynes et al., 2012). The high frequencies of V(D)J mutations that characterize many BnAb are consistent with these possibilities.

Despite great ingenuity and effort, most attempts to elicit MPER Ab by vaccination have either failed or generated only low quantities of serum Ab (Eckhart et al., 1996; Coëffier et al., 2000; Derby et al., 2006). Intense immunization regimens with MPER antigens containing the 2F5 epitope can induce in mice and nonhuman primates serum Ab responses directed to the DKW core of the 2F5 epitope, but these are only minor components of the elicited Ab (Ofek et al., 2010a; Dennison et al., 2011; Guenaga et al., 2011). Analyses of KI mice (Verkoczy et al., 2010, 2011) and our demonstration of discrete autoantigens bound by the 2F5 and 4E10 BnAbs suggest immunological tolerance as a major roadblock in the generation of vaccines that elicit MPER BnAb. An obvious test of our hypothesis is the immunization of animals that lack these tolerizing ligands with standard MPER vaccines. Fortuitously, KYNU and CMTM3 in the laboratory opossum lack the 2F5 epitopes present in most mammalian species, whereas opossum SF3B3 maintains 4E10 reactivity. The absence of endogenous 2F5 epitopes resulted in unprecedented serum Ab responses to the 2F5 epitope of HIV-1 MPER, but not the nearby 4E10 determinant also present in the immunogen. We conclude that 2F5 BnAb production in humans and most other mammalian species is limited by the ELDKWA sequence of endogenous KYNU, and/or DKW of CMTM3. We hypothesize that KYNU is the major tolerizing self-antigen because it binds 2F5 with higher affinity (Fig. 4), and we are currently generating KI mice bearing a D92E mutation in KYNU to extend our studies of the role of KYNU-induced tolerance in the induction of 2F5-like BnAb.

Even though MPER immunization elicited high titers of serum Ab specific for the 2F5 epitope, opossum immune sera did not neutralize HIV in vitro. It has been shown that both peptide epitope and lipid reactivity are essential for 2F5 to neutralize HIV (Alam et al., 2009; Ofek et al., 2010b; Scherer et al., 2010), and we suspect that although opossums are permissive for the generation of ELDKWA-reactive Ab, our immunization scheme did not select for the long and hydrophobic 2F5 HCDR3 that is crucial for virus neutralization (Ofek et al., 2004; Alam et al., 2009).

The robust 2F5-like Ab responses of immunized opossums suggests the possibility of human KYNU polymorphisms that abolish this endogenous 2F5 epitope. Such natural variants could contribute to the probability of 2F5-like BnAb in HIV-1 patients. This hypothesis remains to be tested, as the patient from whom 2F5 was originally isolated is unidentifiable (Buchacher et al., 1994). Perhaps more usefully, we can imagine HIV-1 vaccination strategies based upon the selection of naive B cells that do not react with self- or conserved MPER neutralizing epitopes but can acquire these reactivities by V(D)J hypermutation (Haynes et al., 2012). Our identification of HIV-1 mimicry of conserved host antigens may prove useful in selecting HIV-1 vaccine antigens that minimize the induction of autoreactivity while compelling responses to HIV-1 neutralizing epitopes (Haynes et al., 2012).

MATERIALS AND METHODS

Immunoprecipitation. HEp-2 cells were cultured to 90% confluence. Total protein was harvested using IP Lysis buffer (Thermo Fisher Scientific) containing protease inhibitors (Sigma-Aldrich) according to the manufacturer's instructions. The cell lysate was centrifuged and then the supernatant was collected.

For IP reactions, Dynabeads Protein G (Invitrogen) was cross-linked with 2.5 mM BS^3 (Thermo Fisher Scientific). Cell lysates were precleared with human myeloma protein 151K (SouthernBiotech) before incubation with 2F5, 4E10 (Polymun Scientific), or 151K Dynabeads Protein G for immunoprecipitation. Ab–Ag complexes were eluted and then boiled in sample buffer (Invitrogen).

Eluates were resolved under nonreducing conditions and stained with colloidal blue (Invitrogen). Individual bands were excised and subsequently analyzed by tandem mass spectrometry at the Duke proteomics core facility.

Protein array microchip. 2F5- and 4E10-reactive antigens were identified according to the instructions of the microchip manufacturer. In brief, ProtoArray 5 (Invitrogen) was blocked and blotted with 2 µg/ml 2F5, 4E10, Infliximab (Remicade, Centocor Ortho Biotech Inc.), or human myeloma 151K for 90 min. Protein–Ab interactions were detected using 1 µg/ml anti–human IgG conjugated with Alexa Fluor 647. The arrays were scanned at 635 nm with 10-µm resolution using 100% power and 600 gain (GenePix 4000B scanner; Molecular Devices). Fluorescence intensities were quantified using GenePix Pro 5.0 (Molecular Devices). Lot-specific protein spot definitions were provided by the microarray manufacturer and aligned to the image data. The protein array data were deposited in NCBI GEO (accession no., GSE42673).

Immunoblotting. Proteins were separated on NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes (iBlot; Invitrogen). The membranes were blocked with 1% casein protein in PBS (Bio-Rad Laboratories). Membranes were then probed with 2 µg/ml of the following Abs in blocking buffer: anti–human KYNU (R&D Systems), 2F5, and anti-His (EMD

Millipore). Appropriate secondary Abs (Sigma-Aldrich or Invitrogen) were used and blots were visualized by Alkaline Phosphatase substrate (Promega) or Odyssey infrared imaging (LI-COR Biosciences), respectively.

Immunofluorescence. HEp-2 slides (MBL Bion) were blocked with PBS containing powdered milk (1%), normal goat serum (5%), and Tween-20 (0.05%). Slides were labeled with 50 µg/ml 2F5 or 4E10, and anti-KYNU or anti-SF3B3 (Abcam), extensively washed, and then stained with 20 µg/ml FITC or PE-labeled species-specific secondary Ab. 151K, goat serum IgG (R&D Systems), and a mouse IgG1κ mAb specific for chicken IgG (CGG-8) were used as negative controls and had minimal binding under the same image acquiring settings. Secondary Abs were tested to be species specific and had minimal cross-reactivity in co-stain experiments. Images were acquired with a confocal microscope (TCS SP5; Leica) using a 40× objective with an NA of 1.25 (HCX PL APO lambda blue) using oil medium at room temperature. Images were acquired by PMT using Application Suite Advanced Fluorescence (Leica). The lite version of the same software was used to adjust color balance after image acquisition.

Luminex assay. Luminex assays were performed as reported (Tomaras et al., 2011). In brief, 5×10^6 microspheres (Luminex Corp.) were covalently linked to 25 µg of recombinant protein and incubated with serially diluted Ab. Bound Abs were detected with 4 µg/ml biotinylated mouse anti–human IgG (SouthernBiotech) or donkey anti–goat IgG (SouthernBiotech), followed by incubation with 5 µg/ml streptavidin-PE (BD). Fluorescence was measured on a Bio-Plex instrument (Bio-Rad Laboratories).

Production of recombinant human KYNU. The amino acid sequence of human KYNU was obtained from GenBank (accession: NM_003937). The gene construct was codon-optimized using highly expressed human housekeeping gene codons (André et al., 1998). KYNU construct with a 3′ sequence encoding a C-terminal His-6 tag was de novo synthesized (GenScript) and cloned into pcDNA3.1(+) plasmid (Invitrogen). To produce mutant KYNU, a single nucleotide substitution from C to G at position 276 in the coding region of human KYNU plasmid was introduced by site-directed mutagenesis, resulting in a change from aspartic acid to glutamic acid at amino acid position 92 (D92E). Both wild-type and D92E mutant KYNU were produced in 293T cells by transient transfection using polyethylenimine (Smith et al., 2009) and purified over a nickel column (Thornburg et al., 2007).

KYNU enzymatic assay. 2 ng/µl of recombinant KYNU was added to 50 µl of 200 µM 3-hydroxykynurenine substrate. The reaction product 3-hydroxyanthranilinic acid was read on a fluorescent plate reader at excitation and emission wavelengths of 315 and 415 nm, respectively. The conversion factor for 3-hydroxyanthranilinic acid concentration was determined from a 3-hydroxyanthranilinic acid standard (Sigma-Aldrich). Enzyme activity (pmol/min/µg) was calculated and adjusted to substrate only controls.

Immunizations. Opossums were housed at the Texas Biomedical Research Institute. Priming immunizations involved intramuscular, intranasal, and sublingual routes with a total of 100 µg JR-FL in Emulsigen (Mvp Laboratories, Inc.) with oCpG distributed in a 2:1:1 ratio, respectively. Opossums were immunized with gp140 JR-FL at weeks 0 and 3, and then boosted with liposomes every 3 wk. Boosting liposomes contained the MPER656 peptide (NEQELLELDKWASLWNWFNITNWLW) and TLR7/8 and TLR4 ligands as previously described (Dennison et al., 2011), and were given via intramuscular, intranasal, and sublingual routes at 66, 7, and 3.5 µg each. Sera were sampled before immunization and 2 wk after each immunization and stored at −80°C until use.

C57BL/6 mice were primed with the same JR-FL gp140 in Emulsigen with 25 µg oCpG administered to opossums at week 0 and then boosted i.p. with the 25 µg of the same liposomes given to opossums every 2 wk. Sera were sampled before immunization and 10 d after each immunization and stored at −80°C until use.

ELISA. ELISA was performed as previously described (Kuraoka et al., 2011). In brief, 96-well microplates (BD) were coated with capture reagent (0.5–2 µg/ml). Plates were then blocked with PBS/0.5% BSA/0.1% Tween-20 under permissive binding conditions, or PBS/4% whey protein/15%.normal goat serum/0.5% Tween-20 under stringent binding conditions. Ab samples were diluted to 10 µg/ml in blocking buffer and then threefold serially diluted in duplicates. HRP-conjugated secondary Ab (SouthernBiotech) was used to detect bound Ab.

For competitive inhibition assays, plates were directly coated with ligands (KYNU, CMTM3, or human SF3B3). After blocking, 200 ng/ml 2F5 or 4E10 was added together with threefold gradients of inhibitors. After extensive washing, plate-bound Ab was detected by anti–human Ig-HRP (SouthernBiotech).

To determine end-point titers after opossum immunization, sera were serially diluted from 1:10 to 1:196,830, and then reacted with plate-bound gp41, biotinylated 2F5 epitope peptide (SP62, QQEKNEQELLELDKWASLW), and biotinylated 4E10 epitope peptide (SLWNWFNITNWLWYIK). Positive (4E10 or 2F5) and negative controls were included in each plate. HRP-conjugated rabbit anti–opossum IgG (Bethyl Laboratories, Inc.) was used to detect bound IgG. Mouse serum IgG response to MPER epitopes was determined in the same way but using the MPER656 peptide that contains both the 2F5 and 4E10 epitopes. ELISA OD values were adjusted by subtracting matched, prebleed serum readings. End-point titers were defined as the highest dilution yielding an adjusted OD reading two-fold or greater above background. For statistical analyses, sera that did not exhibit specific binding in ELISA were assigned an end-point titer of 1. Geometric means and SD of end-point titers were calculated for graphing and analysis.

Surface plasmon resonance. SPR studies were performed as previously described (Dennison et al., 2011). For measurement of KYNU affinity to 2F5 family Ab, recombinant KYNU was immobilized on a Biacore CM5 chip. The binding was measured using 2F5 mAb (at 1.0, 0.8, 0.6, 0.4, 0.2, and 0.1 µg/ml) and 2F5 RUA variant 2 mAb (at 100, 80, 60, 40, 20, 10, and 5 µg/ml). 2F5 mAb and 2F5 RUA variant 2 mAb binding curves were fitted globally to a Bivalent analyte model. KYNU mutant (D92E) was also immobilized on an adjacent flow cell of the same sensor chip as a control and no binding was observed with 2F5 mAb, 2F5 Fab, or 2F5 RUA variant 2.

Structural analysis. Figure was generated using the PyMOL Molecular Graphics System (version 1.3; Schrödinger, LLC). Previously reported protein structures were used for analysis (Ofek et al., 2004; Lima et al., 2007; Julien et al., 2008).

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