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Common Tolerance Mechanisms, but Distinct Cross-Reactivities Associated with gp41 and Lipids, Limit Production of HIV-1 Broad Neutralizing Antibodies 2F5 and 4E10

Yao Chen,*† Jinsong Zhang,*† Kwan-Ki Hwang,*† Hilary Bouton-Verville,*† Shi-Mao Xia,*† Amanda Newman,*† Ying-Bin Ouyang,‡,† Barton F. Haynes,*†,§ and Laurent Verkoczy*†,¶

Developing an HIV-1 vaccine has been hampered by the inability of immunogens to induce broadly neutralizing Abs (BnAbs) that protect against infection. Previously, we used knockin (KI) mice expressing a prototypical gp41-specific BnAb, 2F5, to demonstrate that immunological tolerance triggered by self-reactivity of the 2F5 H chain impedes BnAb induction. In this study, we generate KI models expressing H chains from two other HIV-1 Abs, 4E10 (another self-/polyreactive, anti-gp41 BnAb) and 48d (an anti-CD4 inducible, nonpolyreactive Ab), and find a similar developmental blockade consistent with central B cell deletion in 4E10, but not in 48d V H KI mice. Furthermore, in KI strains expressing the complete 2F5 and 4E10 Abs as BCRs, we find that residual splenic B cells arrest at distinct developmental stages, yet exhibit uniformly low BCR densities, elevated basal activation, and profoundly muted responses to BCR ligation and, when captured as hybridoma mAb lines, maintain their dual (gp41/lipid) affinities and capacities to neutralize HIV-1, establishing a key role for anergy in suppressing residual 2F5- or 4E10-expressing B cells. Importantly, serum IgGs from naive 2F5 and 4E10 KI strains selectively eliminate gp41 and lipid binding, respectively, suggesting B cells expressing 2F5 or 4E10 as BCRs exhibit specificity for a distinct spectrum of host Ags, including selective interactions by 2F5 BCR* B cells (i.e., and not 4E10 BCR* B cells) with those mimicked by its gp41 neutralization epitope. The Journal of Immunology, 2013, 191: 1260–1275.

Key to the development of an effective HIV-1 vaccine will be the ability to elicit broadly neutralizing Abs (BnAbs) (1). Experimental evidence supporting this notion comes from the robust protection conferred by BnAbs, either when passively transferred at physiological levels, preceding simian HIV challenge in nonhuman primate (2–5), or more recently, in humanized mice, prior to HIV-1 infection (6). Furthermore, passive infusion of BnAbs significa ntly mitigates viral rebound in HIV-1–infected subjects for whom highly active antiretroviral therapy has been disrupted (7). Unfortunately, BnAbs have not been elicited at significant levels by immunization and arise only in a minority of HIV-1–infected subjects, typically years after transmission (8, 9). The recent isolation of a number of new BnAbs from chronically infected subjects has expanded our understanding of vulnerable HIV-1 envelope (Env) regions as vaccine targets (10).

One important Env vaccine target is the membrane proximal external region (MPER), a conserved, linear, and well-characterized region in gp41 (11) containing several BnAb epitopes, which is critical for HIV-1 fusion with target cells (12). Explanations why BnAbs are so poorly elicited include direct effects of topological constraints in Env that limit epitope accessibility (13–18) and indirect effects such as preferential elicitation of nonneutralizing responses by immunodominant epitopes on nonnative Env conformations (19, 20). Additionally, unusual traits of BnAbs (i.e., extraordinary somatic mutation frequencies and HCDR3 lengths) may disfavor their generation (9, 21). However, an additional hypothesis has been proposed for the dearth of BnAbs in infected patients and vaccinees: the depletion, inactivation, and/or modification of BnAb-producing B cells via immunological tolerance (22). This hypothesis arose from the observation that two well-studied MPER-specific BnAbs, 2F5 (23) and 4E10 (24), exhibited self-/polyreactivity in vitro (25), a finding that now extends to several other recently isolated BnAb lineages (10, 21).

The tolerizing processes of clonal deletion, anergy, and receptor editing have been extensively studied in mice expressing autoreactive BCR (26–30), and we previously demonstrated that expression of the 2F5 H chain (HC) V(D)J rearrangement, either when paired to many endogenous L chains (LCs; 2F5 V H knockin [KI] mice) or with the 2F5 LC (2F5 complete KI mice), results in
profund deletion of BnAb-expressing immature B cells in the bone marrow (BM) (31, 32). Furthermore, residual 2F5 KI B cells express reduced levels of IgM on their surface, suggesting their ability to signal through BCR is compromised (33). These results are consistent with the 2F5 HC being sufficiently autoreactive to trigger profound B cell tolerance in vivo and are similar to other mice expressing highly self-polyreactive BCRs (26, 34, 35).

4E10 and 2F5 have specificity for adjacent, yet distinct, epitopes in the gp41 MPER (36, 37) and, in initial autoantigen-binding assays, exhibited a different spectrum of cross-reactivities (25); for example, 4E10 bound HIV-1 membrane lipids such as cardiolipin (CL) with higher affinity than 2F5, but the latter preferentially cross-reacted with centromere B. Furthermore, distinct conserved mammalian self-Ags that are avidly bound by the 2F5 and 4E10 mAbs have been identified: for 2F5, the XXX chemo-

FIGURE 1. Targeted replacement of the mouse IgH and Igk loci with the 4E10/48d VH(DJ)Ig and 4E10 VsJk rearrangements, respectively. (A and B) Site-di-
rected strategies used to knock in VH and Vl regions, respectively, showing the Ig targeting constructs, targeted Ig alleles (shown before and after homologous recombination), and the targeted alleles after Cre-mediated neo cassette deletion. Restriction fragment sizes are indicated for both WT and targeted loci. Genotyping primers are denoted by arrows. (A) Genomic structure of the targeted IgH allele, showing the endogenous mouse IgH cluster and Cre region, as well as the 4E10/48d VH expression cassette, comprised of a J558 H10 family promoter (p), the H10 split leader sequence (L), and the rearranged 4E10/48d VH(DJ)Ig (4E10/48d/VD10) coding segment. B) Geno-

Materials and Methods

Mice and flow cytometry

Female C57BL/6 Rag-1<−/−> and C57BL/6 IgH<sup>−/−</sup> mouse strains were purchased from The Jackson Laboratory. 4E10 VH<sub>10</sub>/<sup>−/−</sup> and 48d VH<sub>10</sub>/<sup>−/−</sup> KI mice were generated based on published methods for engineering the 2F5 VH<sub>10</sub>/<sup>−/−</sup> KI strain (31), whereas 4E10 VL<sub>10</sub>/<sup>−/−</sup> and complete KI strains were constructed as previously described to generate 2F5 VL<sub>10</sub>/<sup>−/−</sup>

Duke University Institutional Biosafety Committee–approved animal protocols. Flow cytometric analysis was performed as described previously (31). Brieﬂy, single-cell suspensions from BM, spleen, mesenteric lymph node, and peritoneal cavity lavage were isolated from 6–12-wk-old naive 4E10 and 2F5 KI mice and, for comparison, WT (C57BL/6) littermates. A total of 10<sup>6</sup> cells were suspended in FACS buffer containing 1× PBS (pH 7.2), 3% FBS (Sigma-Aldrich), and 0.01% sodium azide, and B cells were stained with premixed combinations of ﬂuorochrome-labeled mAbs at titration-determined optimal concentrations, and total B cells were gated as singlet, live, CD19<sup>+</sup>, and/or B220<sup>+</sup> lymphocytes. All Abs were from BD Biosciences unless otherwise stated. Primary labeled mAbs used were: Pacific Blue, allophycocyanin, or Texas Red–conjugated anti-B220 (clone RA3-6B2), PE-Cy7 anti-CD19, FITC–conjugated anti-IgD (clone 11-26), FITC–conjugated anti-IgM (clone 15F9), PE-conjugated anti-CD21, PE-Cy7–labeled anti-CD23 (eBioscience), allophycocyanin-conjugated anti-CD93 (eBioscience), FITC–conjugated anti-CD3, PE-conjugated anti-BP-1, allophycocyanin-labeled anti-human serum albumin, PE–conjugated anti-<xsub>κ</xsub>, and FITC–conjugated anti-<xsub>λ</xsub>–3. Depending on the experiment, either propidium iodide or v-amine live/dead violet dye (Invitrogen) was used to exclude dead cells and, for secondary staining, Texas Red–conjugated streptavidin. All FACS analysis was performed using a BD LSRII flow cytometer (BD Biosciences), and

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data were acquired and analyzed using FACSComp (BD Biosciences) and FlowJo (Tree Star) software, respectively.

ELISA and Luminex analysis of serum Abs

Serum samples were collected from naive 6–12-wk-old mice, and total serum Ab concentrations of all subclasses were determined by ELISA using a Milliplex Mouse Ig Isotyping kit (Millipore) and a Bio-Rad Luminex Bead Array Reader (Bio-Rad). Quantitative measurements of serum IgM- and IgG-specific binding to MPER epitope peptides SP62 (containing the 2F5 nominal MPER epitope) and MP6.03 (containing both 2F5 and 4E10 nominal MPER epitopes) were used in 2F5 and 4E10 KI strains, respectively, using 10-point serum dilution curves, based on previously described methods (31, 32). Plates were coated either directly with peptide (for 2F5-based assays), or with streptavidin followed by MP6.03-biotin (for 4E10-based assays). Binding Abs were detected using AP-conjugated goat anti-mouse IgG or -IgM reagents (both from Southern Biotechnology Associates), and plates were developed using TMB substrate solution (KPL) and stopped with 2N HCl. Ab concentrations were determined by comparison to a standard curve generated with serial dilutions of purified mouse Ig (H+L). Serial serum samples were tested on the same plate, and Ab concentrations were corrected for assay-to-assay variation, as previously described (31, 32).

Analysis of proximal and distal BCR signaling

Phosphoryosine protein immunoblotting assays were performed as previously described (39). Briefly, 5 × 10⁶ splenic B cells were isolated from WT or 4E10 complete KI mice by negative depletion of CD43+ cells using anti-mouse CD43 (Bio-Rad), followed by HRP-labeled goat anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories). Washed cells were resuspended in lysis buffer containing 1% MP-40, 1 mM EDTA, and 0.1% Triton X-100, and cell lysis was achieved by sonication. Cell extracts were fractionated by reducing SDS-PAGE gel (Invitrogen) and transferred to polyvinylidene difluoride membranes using Xcell II blot module (Invitrogen). Protein phosphoryosine was detected by Ab clone 4G10 (Millipore) and goat anti-rabbit IgG (Jackson ImmunoResearch). Blots were developed using the SuperSignal West Pico Substrate (Thermo Scientific Pierce). Calcium flux assays were performed using Fluo-4, following the manufacturer’s instructions (Invitrogen). Briefly, splenic cells were stained with Pacific Blue-labeled anti-B220 and allophycocyanin-labeled anti-CD43 (Bio-Rad), followed by staining buffer containing 5% FBS, 100 mM NaCl, and 2 mM MgCl₂, and resuspended in staining buffer at a concentration of 10⁶ cells/ml. Fluo-4 working solution was prepared by mixing Fluo-4 stock solution (1 mM) with Plutonic solution (10% v/v) at a ratio of 1/50. Cells were activated for 10 min, immediately quenched with 10 volumes RPMI 1640 + 10% FCS, and washed three times with RPMI 1640 + 10% FCS. Cells were then stimulated in vitro with 20, 5, or 1 µg/ml anti-IgM (Fab)₂ (Southern Biotechnology Associates) or 2 µg/ml ionomycin (Sigma-Aldrich) as a positive control for 5 min. Data were acquired on BD LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star).

BrdU labeling

Mice were injected i.p. with 200 µl 3 mg/ml BrdU (BD Pharmingen) in PBS every 12 h for 4 d. Splenic cells were then harvested and stained with PE–Texas Red–labeled anti-B220 (BD Biosciences) and allophycocyanin-labeled anti-CD43 (Bio-Rad), followed by fixation and incorporation of BrdU was detected with FITC-labeled anti-BrdU Ab, using the FITC BrdU Flow Kit (BD Pharmingen) all according to the manufacturer’s protocol. FACs analysis was performed using a BD LSRII flow cytometer (BD Biosciences); and data were acquired and analyzed using FACSComp (BD Biosciences) and FlowJo (Tree Star) software, respectively.

Hybridoma generation and analysis

Splenocytes from 4E10 or 2F5 complete KI mice were electrofused with NS0 Bcl-2 myeloma partner cells based on previously described methods (32). Briefly, after electrofusion, cells were seeded into 96-well plates at 1 × 10⁵ cells/well in DMEM medium containing 15% FCS and hypoxanthine-aminopterin-thymidine media supplement (Sigma-Aldrich). After incubation at 37°C, 5% CO₂ for 2 wk, plates were screened under a microscope for cell growth and culture supernatants from wells with cell colonies were assayed for Ab production, MPER/CL binding, as well as the ability to neutralize HIV-1 virus. Ab production was determined by ELISA to measure total IgM, IgG, or IgA using goat anti-mouse Ig (H+L) (Southern Biotechnology Associates) as capture Ab and HRP-conjugated goat anti-mouse IgM, IgG, and IgA (Bethyl Laboratories) as detection Ab, respectively. SureBlue TMB HRP substrate (KPL) was added for 5 min, after which the reaction was stopped by 1 N HCl and measured at 450 nm. MPER and CL binding were determined by ELISA using 2F5 nominal MPER epitope peptide SP62 or biotin-labeled 4E10 nominal MPER epitope MPR.03 binding to plated coated streptavidin and CL as target Ags and a mixture of HRP-labeled anti-mouse Ig M + IgG + IgA as detection Abs. HIV-1 neutralization ability was determined by the TZM-bl pseudovirus infectivity assay using BA-NAB-sensitive HIV-1 strain MN.3 as previously described (31, 32).

Certain randomly selected clones, including both MPER* and MPER clones, were subcloned at least one more time until cell lines were deemed to be monoclonal. For certain subclones, purified mAbs were purified from supernatants, and quantitative ELISAs to measure MPER, NIH-3T3 cytotoxicity, and mouse Ab, to demonstrate a profound developmental blockade in the BM, either when paired to many endogenous Lcs (2F5 VH KI mice) or with the 2F5 LC (2F5 VH × VH complete KI mice), consistent with 2F5 HC self-reactivity triggering profound deletion of immature B cells in the BM and thus impeding BnAb induction (31, 32). To explore if HC self-reactivity extends to other gp41-specific BnAbs and to formally exclude that may be a spurious effect of expressing chimeric HCs (i.e., human V + murine C regions) in vivo, we generated KI mice expressing the somatically mutated VH(V)3(D)14 rearrangements from two other well-characterized human HIV-1 mAbs: 4E10 (24), another self-/polyreactive MPER-specific BnAb with an unusually long HC CDR (HCDR3) and, for contrast, 48d, an mAb specific for the HIV-1 Env coreceptor CCR5-binding site (41), with an unremarkable HCDR3 (i.e., of average length and hydrophobicity) and no in vitro polyeactivity (M. Alam, unpublished observations). 4E10 and 48d VH KI mice were constructed using the same targeting constructs and strategies previously used to generate 2FS VH KI mice (31, 32) and, to mimic their expected locations as primary rearrangement events, involve their site-directed replacement of the murine Igh J H1–4 segments (Fig. 1A), thus allowing all primary rearrangement events, involve their site-directed replacements were confirmed by Southern blot (Fig. 1A, 1B), and homozygous offspring bearing germline trans- mission of 4E10 and 48d VH(D)14JH rearrangements were identified by PCR (Supplemental Fig. 1C, 1D).

The effects of the targeted 4E10 and 48d VH rearrangements on B cell development were then evaluated by comparing BM,
splenic, and peritoneal B cell compartments in homozygous 4E10 and 48d V<sub>H</sub> KI mice to those in C57BL/6 littermates (Fig. 2). Indeed, 4E10 V<sub>H</sub> KI mice had a major blockade at the small pre-B to immature B transition (Fig. 2A), as well as residual splenic B cell populations with reduced surface Ig (sIg) densities (Fig. 2B), phenotypes previously reported in 2F5 V<sub>H</sub> KI mice (31). In contrast, 48d V<sub>H</sub> KI mice exhibited BM and splenic B cell subset frequencies as well as B cell sIg densities comparable to WT (C57BL/6) littermates (Fig. 2A, 2B), demonstrating that the chimeric 48d HC functions optimally in supporting B cell development. Interestingly, naive 4E10 and 48d V<sub>H</sub> KI mice both had significantly reduced peritoneal B-1a subsets (Fig. 2C), as well as reduced levels of serum isotypes predominantly associated with natural Ab production by innate B cells (i.e., IgM and IgG3) (Fig. 2D), results consistent with the notion that KI models bearing numerous HC V(D)J specificities, including (but not restricted) to those with self-reactivity, cannot support B-1 B cell development (42). Nevertheless, the profound blockade in early BM development selectively observed in 4E10 V<sub>H</sub> KI mice, is consistent with self-reactivity of the 4E10 HC, as we have previously seen with the 2F5 HC, being sufficient to trigger central deletion of B cells expressing it on their surface as BCR.

**Generation of 4E10 V<sub>H</sub> × V<sub>L</sub> KI mice and characterization of 4E10 and 2F5 KI strains**

To evaluate the effect of expressing the complete (V<sub>H</sub> × V<sub>L</sub>) 4E10 mAb as BCR and to more thoroughly compare B cell compartments and serum Ig subclasses between KI mice expressing targeted 4E10 or 2F5 rearrangements in all homozygous strains (i.e., V<sub>H</sub>, V<sub>L</sub>, and complete), we constructed 4E10 V<sub>L</sub> KI mice.

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**FIGURE 2.** Analysis of B cell development and serum Ig subclass levels in C57BL/6 (WT), 4E10 V<sub>H</sub><sup>+/+</sup>, and 48d V<sub>H</sub><sup>+/+</sup> KI strains. (A–C) Representative FACS contour histograms (representative of three experiments) comparing BM, splenic, and peritoneal B cell development in 4E10 V<sub>H</sub><sup>+/+</sup> and 48d V<sub>H</sub><sup>+/+</sup> KI mice (expressing the somatically mutated HCs of the polyreactive, MPER-specific BnAb 4E10, and the nonneutralizing HIV-1 mAb 48d, respectively). Also shown as controls are WT (C57BL/6) littermates; all mice were 6–12 wk old. (A) Analysis of BM B cell subsets, either less differentiated subpopulations (top panels), based on Hardy subfractionation, more differentiated subpopulations (middle panels), as revealed by sIgM and sIgD staining as previously described (32), or based on surface LC<sub>k</sub> and L<sub>1–3</sub> expression (bottom panels). Indicated B cell subsets were pre-gated as singlet, live CD19<sup>+</sup> B cells (top panels) or as singlet, live, total (CD19<sup>+</sup>B220<sup>+</sup>) B cells (middle and bottom panels), respectively. Numbers indicate percentages of B cells in each subset, with subsets defined as follows: pro/pre, pro-B/large pre-B (fractions A–C; B220<sup>lo</sup>CD43<sup>+</sup>); small pre-, small pre-B (fraction D; B220<sup>lo</sup>CD43<sup>+</sup>); imm/mat, mature+immature (fractions E and F; B220<sup>hi</sup>CD43<sup>+</sup>); imm, immature (IgM<sup>int</sup>/hiIgD<sup>+</sup>); T1/T2, transitional 1+2 (IgM<sup>lo</sup>IgD<sup>+</sup>/int); and mat, mature (IgM<sup>int</sup>IgD<sup>hi</sup>). (B) Analysis of splenic B cell subpopulations, as revealed either by surface CD93 and B220 expression of live lymphocytes (top panel) and by surface CD21 and CD23 or IgM and IgD expression of live, total (CD19<sup>+</sup>B220<sup>+</sup>) B cells (middle and bottom panels), respectively. Numbers indicate percentages of B cells in each subset, and B cell subsets indicated in top panels are defined as follows: T, transitional (B220<sup>+</sup>CD93<sup>+</sup>); Mat+MZ (B220<sup>+</sup>CD93<sup>+</sup>); NF, newly formed (i.e., transitional [CD21<sup>+</sup>CD23<sup>+</sup>]); MZ (CD23<sup>+</sup>CD21<sup>+</sup>); imm/mature, immature (fractions E and F; B220<sup>+</sup>CD43<sup>+</sup>); imm, immature (IgM<sup>hi</sup>IgD<sup>+</sup>) or T1/T2, transitional 1+2 (IgM<sup>lo</sup>IgD<sup>+</sup>/int); and mat, mature (IgM<sup>hi</sup>IgD<sup>+</sup>). (C) Analysis of peritoneal B cell subsets as revealed by surface staining of live lymphocytes with B220 and CD5<sup>+</sup> expression of live, total (CD19<sup>+</sup>B220<sup>+</sup>) B cells (middle and bottom panels), respectively. Numbers indicate percentages of B cells in each subset, and B cell subsets indicated in top panels are defined as follows: pro/pre, pro-B/large pre-B (fractions A–C; B220<sup>lo</sup>CD43<sup>+</sup>); small pre-, small pre-B (fraction D; B220<sup>+</sup>CD43<sup>+</sup>); imm/mat, immature (fractions E and F; B220<sup>+</sup>CD43<sup>+</sup>); imm/mature, immature (IgM<sup>hi</sup>IgD<sup>+</sup>/int); T1/T2, transitional 1+2 (IgM<sup>lo</sup>IgD<sup>+</sup>/int); and mat, mature (IgM<sup>hi</sup>IgD<sup>+</sup>). (D) Analysis of total Ig subclass levels in 4E10 V<sub>H</sub><sup>+/+</sup> and 48d V<sub>H</sub><sup>+/+</sup> KI mice or WT (C57BL/6) littermates. Serum samples were collected from 6–12-wk-old female naive mice, and serum concentrations for all Ig subclasses were determined by ELISA analysis. Each symbol represents an individual mouse, and horizontal lines represent averages for each group. Significance values were determined using a two-tailed student test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
using the identical targeting strategy and constructs used for generating 2F5 \( \text{V}_{\text{H}} \times \text{K}_{\text{I}} \) mice (31, 32). The prerearranged 4E10 \( \text{V}_{\text{H}} \times \text{K}_{\text{I}} \) rearrangement derived from the original (somatically mutated) 4E10 mAb was targeted into the murine Igk loci in place of the \( \text{J}_{\text{k}} \) 1–3 gene segments to mimic its expected location as the primary rearrangement event, thus allowing retention of kLC secondary rearrangements (Fig. 1B). 4E10 \( \text{V}_{\text{H}} \times \text{K}_{\text{I}} \) targeted embryonic stem cell clones and germline-transmitted homozygous mice were confirmed by Southern blot (Supplemental Fig. 1E) and genomic DNA PCR amplifications (Supplemental Fig. 1F), respectively. Finally, 4E10 complete \( (\text{V}_{\text{H}}^{+/+} \times \text{V}_{\text{L}}^{+/+}) \) KI mice were generated by cross-breeding 4E10 homozygous \( \text{V}_{\text{H}} \) and \( \text{V}_{\text{L}} \) lines.

Consistent with profound clonal deletion of B cells expressing either gp41-specific BnAb as a BCR, 4E10 and 2F5 complete KI mice, relative to C57BL/6 littermates, had a similar, major blockade at the pre-B to immature B transition, as measured both in terms of B cell subset frequencies (Fig. 3A) and absolute numbers (Table I). However, additional, minor developmental differences between 4E10 and 2F5 KI strains were also identified (Fig. 3B–E, Table I). In particular, 2F5 complete KI mice, relative to 4E10 complete KI mice, also had reduced frequencies and absolute numbers of total (CD19+\( \times \text{B220}^{+} \)) BM B cells (Fig. 3B, 3C, Table I), as well as an additional, modest blockade, at the pre/long pre-B to small pre-B transition (Fig. 3D, 3E, Table I), which we have reported previously (32). Interestingly, 4E10 \( \text{V}_{\text{H}} \) KI mice also exhibited this same, earlier blockade (Supplemental Fig. 2A, top panels), which may reflect suboptimal interactions between 4E10 HC s and mouse surrogate LCs, resulting in poor surface expression and inefficient allelic exclusion of endogenous HCs (Supplemental Fig. 3), even more than we reported for the 2F5 HC (31).

4E10 and 2F5 complete KI strains also had several notable differences in residual B cell compartments (Fig. 4A–F) and serum Ig subclass distributions (Fig. 4G). First, 2F5 complete KI strains had significantly reduced frequencies and absolute numbers of total (CD19+\( \times \text{B220}^{+} \)) splenic B cells than 4E10 complete KI strains (Fig. 4A, 4B, Table I), but residual splenic B cells in 4E10 complete KI mice preferentially accumulated as phenotypically less differentiated B-2 peripheral B cells (i.e., in addition to reduced B-1a subsets that were also seen in all \( \text{V}_{\text{H}} \) KI strains, including 48d; Fig. 2C), these subsets were most profound in 4E10 complete KI mice and also extended to the B-1b subset (Fig. 4F), coinciding with their near-complete elimination of serum IgM and IgG3 levels (Fig. 4G). Finally, 4E10 complete KI strains also had significant reductions in all other serum Ig isotypes (i.e., IgG1, IgG2b, IgA), relative to C57BL/6 littermates (Fig. 4G), consistent with their early blockade in peripheral B2 development. In contrast, 2F5 complete KI mice had lower levels of total serum IgG (Supplemental Fig. 4) and Ig subclass distributions most similar to 2F5 and 48d \( \text{V}_{\text{H}} \) KI mice, including selectively increased IgG2b (Figs. 2D, 4C, Supplemental Fig. 4). Overall, these findings therefore suggest that 2F5- and 4E10-expressing B cells undergo different degrees of clonal deletion in BM and peritoneal compartments and accumulate at distinct stages of splenic development.

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**FIGURE 3.** Flow cytometric comparison of BM B cell development in C57BL/6 (WT) and 4E10/2F5 complete \( \text{V}_{\text{H}}^{+/+} \times \text{V}_{\text{L}}^{+/+} \) KI strains. Shown are FACS contour plot histograms (representative of three experiments) indicating frequencies of: more differentiated BM B cell subsets (fractionated/annotated as indicated in Fig. 2A) and marginal zone (MZ) \( \text{CD23}^{\text{Int}} \text{CD21}^{\text{High}} \) B cell subsets (Fig. 4C, 4D, Table I). The transitional nature of residual 4E10 complete KI splenic B cells was confirmed by demonstrating their rapid in vivo turnover, as measured by incorporation of BrdU by the majority of CD93+ splenic B cells from continuously labeled 4E10 complete KI mice (Fig. 4E). In contrast, 2F5 complete KI strains had a more heterogeneous peripheral B cell subset distribution, with modest accumulation of newly formed/transitional B cells, but also substantial mature B2 (CD220+CD93–) and marginal zone (MZ) \( \text{CD23}^{\text{Int}} \text{CD21}^{\text{High}} \) B cell subsets (Fig. 4C, 4D, Table I). Additionally, although both 2F5 and 4E10 complete KI mice had significantly lower frequencies of B-2 peritoneal B cells (i.e., in addition to reduced B-1a subsets that were also seen in all \( \text{V}_{\text{H}} \) KI strains, including 48d; Fig. 2C), these subsets were most profound in 4E10 complete KI mice and also extended to the B-1b subset (Fig. 4F), coinciding with their near-complete elimination of serum IgM and IgG3 levels (Fig. 4G). Finally, 4E10 complete KI strains also had significant reductions in all other serum Ig isotypes (i.e., IgG1, IgG2b, IgA), relative to C57BL/6 littermates (Fig. 4G), consistent with their early blockade in peripheral B2 development. In contrast, 2F5 complete KI mice had lower levels of total serum IgG (Supplemental Fig. 4) and Ig subclass distributions most similar to 2F5 and 48d \( \text{V}_{\text{H}} \) KI mice, including selectively increased IgG2b (Figs. 2D, 4C, Supplemental Fig. 4). Overall, these findings therefore suggest that 2F5- and 4E10-expressing B cells undergo different degrees of clonal deletion in BM and peritoneal compartments and accumulate at distinct stages of splenic development.
Table I. Comparison of B cell subset absolute numbers in BM and spleen of WT, 2F5, and 4E10 complete KI mice

<table>
<thead>
<tr>
<th>Tissue/strain</th>
<th>n</th>
<th>Total B Cells</th>
<th>ProB/Large Pre-B</th>
<th>Small Pre-B</th>
<th>Immature B</th>
<th>T1/T2</th>
<th>Mature</th>
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<td>BM*</td>
<td></td>
<td>(B220&lt;sup&gt;+&lt;/sup&gt;CD19&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>(B220&lt;sup&gt;+&lt;/sup&gt;CD43&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>(B220&lt;sup&gt;+&lt;/sup&gt;CD43&lt;sup&gt;+&lt;/sup&gt;) (&lt;IgM&lt;sup&gt;+&lt;/sup&gt;IgD&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>(&lt;IgM&lt;sup&gt;+&lt;/sup&gt;IgD&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>(&lt;IgM&lt;sup&gt;-&lt;/sup&gt;IgD&lt;sup&gt;-&lt;/sup&gt;)</td>
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<td>WT</td>
<td></td>
<td>82.4 ± 16.0</td>
<td>157.7 ± 6.8</td>
<td>50.0 ± 16.8</td>
<td>9.7 ± 2.0</td>
<td>1.8 ± 0.6</td>
<td>5.1 ± 1.6</td>
</tr>
<tr>
<td>2F5VH&lt;sup&gt;+&lt;/sup&gt; × VH&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>61.7 ± 9.4</td>
<td>15.5 ± 4.8</td>
<td>24.7 ± 8.4</td>
<td>2.5 ± 1.2</td>
<td>0.013 ± 0.005</td>
<td>0.079 ± 0.021</td>
</tr>
<tr>
<td>4E10VH&lt;sup&gt;+&lt;/sup&gt; × VL&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>56.8 ± 9.5</td>
<td>12.5 ± 4.5</td>
<td>54.5 ± 7.1</td>
<td>1.7 ± 1.0</td>
<td>0.016 ± 0.007</td>
<td>0.094 ± 0.022</td>
</tr>
</tbody>
</table>

Values are absolute cell numbers (× 10<sup>6</sup> ± SEM).

*BM cells were taken from both femurs for FACS analysis.

*Cells were gated on singlet/live/lymphocytes. B cell subset annotations are based on those defined in Fig. 2A and 2B.

However, despite the above differences, residual sIg<sup>+</sup> B cells from 2F5/4E10 VH or complete KI strains, relative to those in control (C57BL/6 and 48d VH KI) mice, expressed uniformly reduced BCR densities (i.e., lower sIgM and sIgD levels) and reduced BCR densities not only restricted to T3 B cells, but also included more differentiated (mature + MZ) splenic subsets (Figs. 2F5/4E10 VH or complete KI strains, relative to those in control (C57BL/6 and 48d VH KI) mice, expressed uniformly reduced BCR densities not only restricted to T3 B cells, but also included more differentiated (mature + MZ) splenic subsets (Figs. 2A, 2B, bottom panels), as well as BM B cells (Supplemental Fig. 3). Interestingly, the degree of sIg reductions in total splenic B cells in all 4E10 and 2F5 KI strains was quantitated either by surface κ or IgM median fluorescence intensities (Fig. 4H) were greater in complete KI strains than VH<sup>+</sup> KI strains (Supplemental Fig. 2E), with 4E10 complete KI mice having the greatest reductions (Fig. 4H, bottom panel). Because the degree of sIg reduction across these strains also inversely correlates with the differentiation stage at which their residual peripheral B cells accumulate, it is possible that these quantitative differences could be a manifestation of progressively higher levels of self-reactivity retained by residual B cells across these KI species, as has been seen in comparisons of anti-DNA KI models having varying degrees of self-Ag affinity (35, 43). Nevertheless, these phenotypic data collectively suggest that anergy is the common mechanism suppressing most residual 4E10 or 2F5 HC–expressing B cells, regardless of the peripheral developmental stage at which they are arrested.

The majority of residual 2F5- and 4E10-expressing B cells are functionally anergic

The uniform reduced sIgM and sIgD densities in most splenic B cells from 2F5/4E10 KI complete KI mice (Figs. 2–4) is unexpected in that it does not resemble the classic anergic phenotype (IgM<sup>+</sup>–IgD<sup>+</sup>) originally reported in the HEL transgenic system (44, 45), believed to occur predominantly at the splenic T3 checkpoint (46). However, it is now becoming apparent that anergy is more complex than previously thought, both in terms of the stages it may occur and the phenotypic heterogeneity of functionally anergic B cells (reviewed in Ref. 33). For example, functionally anergic B cells have been reported in later splenic B2 differentiation in some autoreactive BCR models (27, 47–53) and B cells functionally analogous to anergic cells, with low densities of both sIgM and sIgD observed at varying degrees in anti-DNA KI models having differing affinities for self-Ag (43).

To test if the splenic B cells expressing these uniformly low sIg densities in our MPER BnAb complete KI models represented bona fide anergic B cells, we first examined total (B220<sup>+</sup>CD19<sup>+</sup>) splenic B cells from complete KI strains by flow cytometry for basal activation of surface activation markers. We found the total peripheral B cells in naive 4E10 and 2F5 complete KI B cells, relative to WT (C57BL/6) littermate B cells had uniformly upregulated surface expression of MHC class II and CD95, two phenotypic hallmarks of anergic peripheral B cells (33) (Fig. 5A), whereas CD25, CD40, CD69, CD80, and CD86 were unchanged (data not shown). That basal expression of these activation markers was increased in the majority of total B cells from both KI strains suggests that anergy is not restricted to the T3 transitional subset, because they only make up a minority of splenic B cell compartments in the 2F5 complete KI strains, which has a predominant B220<sup>+</sup>CD93<sup>+</sup> (mature and MZ) B cell subset. To confirm this uniformly anergic phenotype of MPER BnAb-expressing B cells functionally, we examined calcium mobilization in response to BCR ligation using Flu-4 flow cytometric staining, which allowed comparison of proximal signaling in splenic transitional (B220<sup>+</sup>CD93<sup>+</sup>) and mature B2 (B220<sup>+</sup>CD93<sup>+</sup>) subsets of 4E10 and 2F5 complete KI strains, relative to comparable subsets from control (i.e., WT BM/6 and 48d VH KI) mice. Indeed, both transitional and mature B2 B cell subsets in either 2F5 or 4E10 complete KI mice exhibited significantly muted calcium induction in response to BCR cross-linking (Fig. 5B), demonstrating B cell anergy, regardless of maturational stage.

Finally, to further confirm the anergic status of MPER BnAb complete KI B cells functionally, we monitored several other independent proximal and distal signaling responses to BCR ligation in total splenic 4E10 and 2F5 complete KI B cells. Indeed, protein tyrosine phosphorylation immunoblotting of total naive 4E10 complete KI splenic B cells revealed their higher basal levels of tyrosine phosphorylation, relative to those from WT mice, and their failure to induce additional tyrosine phosphorylation upon BCR aggregation by anti-IgM F(ab')<sub>2</sub> (Fig. 5C). Additionally, upon in vitro BCR cross-linking of total splenic naive B cells from WT, 4E10, or 2F5 complete KI mice, the majority of total 4E10 and 2F5 complete KI B cells failed to upregulate basal expression of early/intermediate activation markers CD25 and CD69 (Fig. 5D, bottom panels and data not shown) or the late activation marker CD86 (Fig. 5D, top panels) and exhibited abrogated proliferation (Fig. 5E). As with B cells from 4E10 complete KI mice, total splenic B cells from 4E10 VH KI mice, the majority of which are mature (B220<sup>+</sup>CD93<sup>+</sup>) B cells, also had uniformly increased basal surface expression of MHC class II and CD95 and, in response to BCR ligation, also exhibited muted proliferation, CD25, CD69, and CD86 upregulation, and calcium induction, relative to total splenic 48d VH KI B cells (data not shown). Collectively, these data confirm that functional silencing is the predominant mechanism controlling most residual 2F5- and 4E10-specific B cells in vivo.
FIGURE 4. Distribution of splenic and peritoneal B cell subsets in C57BL/6 (WT) and 4E10/2F5 complete (VH+/+ VL+/+) KI strains. (A) FACS contour plot histograms (representative of three experiments) showing frequencies of total (singlet/live/lymphocyte, B220+CD19+) B cells in spleens of 6–12-wk-old naive mice. (B) Statistical analysis of total splenic B cell percentages (based on flow cytometric analysis shown in (A), with (Figure legend continues)
Characterization of splenic hybridomas from complete 2F5 and 4E10 KI mice

In addition to their low sIg levels, it is interesting that 2F5/4E10 V_H and complete KI strains exhibit elevated surface LC κ/λ ratios, relative to WT and 48d V_H KI mice (Supplemental Fig. 3F), indicating biased pairing to κLCs by both BnAb HCs. In this regard, it is also intriguing that central deletion triggered by 2F5/4E10 HC self-reactivity occurs independently of endogenous LC pairing (Fig. 2A, Supplemental Fig. 3A) (32) and that most BM 2F5 complete KI B cells, when cultured in vitro (under conditions that permit rescue from clonal deletion) undergo extensive κLC editing, but still retain an anergic phenotype (32). This suggests that: 1) pairing constraints imparted by 2F5/4E10 HCs prevent λ editing; and 2) κ-editing only partially removes self-reactivity. We therefore asked if, in vivo, residual splenic 4E10/2F5 KI B cells, with their uniformly lower BCR expression, attempt LC editing (or any other V region modifications) prior to being routed into an anergic pathway.

To examine this, hybridomas from naive 4E10 and 2F5 complete KI splenic B cells were generated, and the percentage of clones retaining MPER epitope specificity and neutralization ability were determined. Plates were first screened for cell growth, with 6.7% (320 out of 4800) and 16.8% (510 out of 3040) of wells from 4E10 and 2F5 fusions exhibited growth, respectively, and subcloned lines were screened for Ab secretion, with 150 4E10 and 225 2F5 hybridomas identified, all of the IgM isotype (Table II). The majority, 95.3% (143 out of 150), of 4E10 Ab-producing hybridoma lines bound the MPER.03 peptide (containing the 4E10 neutralization epitope), and 99.6% (224 out of 225) of Ab-producing 2F5 lines bound the SP62 peptide (containing the 2F5 neutralizing epitope) (Fig. 6A, Table II). CL ELISA assays also revealed that 100% of MPER-reactive hybridomas maintained CL reactivities from both KI strains (Data not shown). Additionally, all hybridoma culture supernatants that bound the MPER also neutralized HIV MN.3 pseudovirus in standard TZM-bl assays (Fig. 6A).

To determine IgH/IgL V region sequences of 4E10 and 2F5 KI strain hybridomas, we selected 51 4E10 KI lines and 30 2F5 KI lines from our primary screens for an additional round of subcloning to confirm monoclonality (Table III). All 30 cloned 2F5 KI hybridoma lines retained their original knocked-in 2F5 V_H and V_L rearrangements, which had no, limited, or silent somatic mutations (Table III). Forty-nine out of 51 of the 4E10 KI hybridoma lines also retained their original 4E10 V_H and V_L regions/sequences, but interestingly, the two exceptions secreted unusually high amounts of Ab, and both underwent V_H replacement of their 4E10 HCs, as well as completely lost their lipid/MPER reactivities and neutralization abilities (Tables III, IV, Fig. 6B–D). Finally, to fully verify reactivity and neutralization profiles, we also generated purified mAbs from 11 MPER+ representative cloned 4E10 lines, 2 MPER– representative cloned 4E10 lines, and, for comparison, 6 MPER+ representative cloned 2F5 lines. All purified MPER+ 2F5s and 4E10 mAbs maintained comparable reactivity to NIH-3T3 cell and their respective nominal MPER epitopes (Fig. 6B, 6C). Furthermore, all 2F5 and 4E10 MPER+ mAbs maintained CL reactivities, but as anticipated, 4E10 KI hybridomas bound CL at generally higher affinities than 2F5 KI hybridomas (Fig. 6D), consistent with differential CL affinities reported for 4E10 and 2F5 BnAbs (25, 54, 55).

Finally, eight purified 4E10 and five purified 2F5 mAbs were selected for assay against a panel of viral isolates in TZM-bl neutralization assays. Interestingly, the 2F5 mAbs (all containing unmodified V regions) were found to neutralize with similar potency and breadth as the control (human rIgG) 2F5 Ab, whereas the six 4E10 mAbs with unmodified V regions neutralized with a modestly lower potency than the control (human rIgG) 4E10 Ab (Table IV). One potential explanation for these differences may be related to our previous finding that mouse 2F5 IgM mAbs in unreduced form (containing monomeric/panmertic mixtures) unexpectedly neutralize more poorly than reduced (monomeric) mouse 2F5 IgM or IgG (32), thus counteracting the lower potency of 2F5 Abs containing mouse, rather than human C regions (31). In this regard, given 4E10’s neutralization epitope in native HIV-1 viruses may not be as conformationally accessible as 2F5’s (56), 4E10 pentameric IgM mAbs may thus not be capable of enhancing neutralization potency by whatever mechanism 2F5 pentameric IgM mAbs achieves this, due to structural (size) constraints.

Nevertheless, our above data collectively indicate that a significant fraction of anergic peripheral B cells from naive 2F5 and 4E10 KI mice retain their original, functional specificity in vivo and have not undergone V_H or V_L modification prior to (or after) acquiring their anergic phenotypes.

Serum Ab specificities of naive 4E10 and 2F5 KI strains reveal distinct routes of positive selection

Despite the predominant, uniformly anergic phenotype of residual 2F5 and 4E10 complete KI B cells, their distinct numbers/peripheral subset distributions, and the recent demonstration that 4E10 and 2F5 BnAbs exhibit markedly different polyreactivity profiles and bind distinct autoantigens in vitro (38), nevertheless predict that distinct positive selection routes are used in vivo by each circle, square, and triangle representing an individual 6–12 wk-old C57BL/6 (WT), 2F5 complete KI, and 4E10 complete KI mouse, respectively. Horizontal lines represent averages for each group. Significance values were determined using a two-tailed Student t test. (C) Representative FACS contour plot histograms showing analysis of splenic B cell subsets derived from 6–12 wk-old naive mice, fractionated and annotated as indicated in Fig. 2B. (D) Statistical analysis of splenic B cell subsets from individual WT or 2F5/4E10 complete KI mice, represented as frequencies of newly formed/transitional (NF/T; CD21 CD23); MZ (CD23 CD21); and Mat (CD23 CD21) subsets (top panel) or transitional (B220 CD93)+Mat-MZ (B220 CD93 ) ratios (bottom panel). (E) In vivo turnover of the predominant, transitional splenic B cell subset in 4E10 complete KI mice. Eight-week-old naive 4E10 complete KI or control C57BL/6 (WT) mice (bottom and middle panels, respectively) were continuously labeled with BrdU for 4 d, prior to flow cytometric enumeration of the percent of BrdU+ B cells within singlet/live, lymphocyte-gated transitional (B220 CD93+) and mature/MZ (B220 CD93 ) splenic B cell subsets, as indicated in the shaded histograms. Also shown as negative controls are C57BL/6 (WT) mice without BrdU treatment (top panel). Data are representative of two mice/group. (F) Representative FACS contour plot histograms showing analysis of peritoneal B cell subsets as revealed by surface staining of live lymphocytes with CD5 and either B220 (top panel) or IgM (bottom panel), with numbers in each gate indicated, and B cell subsets indicated in the top panel are defined as for Fig. 2C. (G) Analysis of total serum Ig subclass levels in C57BL/6 (WT) mice and 2F5/4E10 complete KI strains. Serum samples were collected from 6–12 wk-old naive mice, and Ig subclass concentrations were determined by Luminex analysis. Each symbol represents an individual mouse, and horizontal lines represent averages for each group. (H) Flow cytometric analysis of sIg densities in total (singlet/live) lymphocytes, B220 CD19+ splenic B cells from 6–12 wk naive mice, as measured by median fluorescence intensity (MFI) quantifications of surface sLC (bottom panel) or surface IgM (top panel) expression. **p ≤ 0.01, ***p ≤ 0.001.
FIGURE 5. Phenotypic analysis of basal activation levels and functional analysis of signaling responses to in vitro BCR cross-linking in MPER BnAb KI splenic B cells. (A) Flow cytometric analysis of surface MHC class II and CD95 expression in naive WT and 4E10 or 2F5 complete KI B cells, represented as gray shadow and blue or red line histograms, respectively. (B) Calcium flux analysis of control WT C57BL/6 or 48d V_{H}^{+} KI and 2F5 or 4E10 complete KI B cells (shown in red, orange, blue, and green lines, respectively). Prestained splenocytes from naive mice were loaded with ionomycin or anti-Ig F(ab')_{2} (Figure legend continues)
Table II. Summary of isotypic distribution and MPER reactivities in primary screen of hybridoma clones derived from 2F5 and 4E10 complete KI spleens

<table>
<thead>
<tr>
<th>Fusion Identification no.</th>
<th>Mice</th>
<th>Total Seeded Wells</th>
<th>Wells with Cell Growth*</th>
<th>IgM#</th>
<th>IgG*</th>
<th>IgA*</th>
<th>MPER**</th>
</tr>
</thead>
<tbody>
<tr>
<td>2F5-V101</td>
<td>2F5V1a/4a × VLa/4a</td>
<td>3040</td>
<td>510</td>
<td>225</td>
<td>0</td>
<td>0</td>
<td>224</td>
</tr>
<tr>
<td>4E10-V2</td>
<td>4E10V1a/4a × VLa/4a</td>
<td>3200</td>
<td>146</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>4E10-V3</td>
<td>4E10V1a/4a × VLa/4a</td>
<td>1600</td>
<td>174</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

All fusions were performed using NS0-bk12 myeloma fusion partner lines and represent a different individual mouse.

C Cultured plates were screened under microscope for cell growth.

IA levels/isotypes of all cloned hybridoma lines were determined by ELISA as described in Materials and Methods. Lines were considered positive if >3× above background OD binding was detected in 1:40 diluted supernatants.

4E10 and 2F5 complete KI mice for their reactivity to two components required for their neutralization abilities: their peptide-neutralizing epitopes and membrane lipids, either or both of which could also be predicted to be removed due to self-polyreactivity.

We found that MPER and CL-specific serum IgM levels were comparable to background levels in the WT (C57BL/6) strain (data not shown), but total serum IgM production in both complete KI strains relative to WT mice was profoundly reduced (Fig. 4G) and thus was not a fair comparison. For all strains, we therefore normalized MPER and CL-specific IgM to total IgM levels and, as expected, found that the low amounts of serum IgM secreted in 4E10 and 2F5 complete KI mice retained substantial MPER- and CL-specific reactivity (Fig. 5A, 7B, left panels), consistent with serum IgM originating predominantly from residual self-reactive B cell pools, either from the profoundly depleted peritoneal fractions and/or anergic immature/transitional self-reactive B cells.

In contrast, serum IgG-specific MPER and CL titers in 4E10 and 2F5 KI strains, when normalized to total serum IgG levels, revealed strikingly distinct binding profiles (Fig. 7). In particular, 2F5 KI strains had serum IgG-specific CL titers significantly higher than background (WT/B6) levels, whereas 4E10 KI strains completely lacked CL reactivity (Fig. 7A, right panel), mirroring the higher CL affinities, relative to 2F5, of the original (IgG) human 4E10 mAb (25) and IgM* 4E10 KI hybridomas in this study (Fig. 6D) and suggesting more stringent elimination of CL reactivity in 4E10-expressing IgG* mature B-2 cells. Conversely, 2F5 complete KI mice exhibited minimal binding to their MPER epitope, whereas a large fraction of 4E10 complete KI mice maintained binding to theirs (Fig. 7B, right panel), suggesting more stringent selection against MPER binding in 2F5-expressing B cells. Collectively, these data are consistent with distinct self-Ags driving the tolerization of 2F5- and 4E10 BCR-expressing B cells in vivo, with positive selection of residual 2F5 KI cells involving the elimination of self-reactive residues associated with binding the 2F5 neutralizing epitope, whereas that of 4E10 KI cells involved elimination of binding to membrane lipids (and/or other correlated self-components comprising 4E10’s general polyreactivity, but not associated with binding to its neutralizing epitope).

Discussion

This study provides key mechanistic insight into why gp41-targeted BnAbs like 2F5 and 4E10, for which epitopes are both located in the gp41 MPER HR2 region, are difficult to elicit. We demonstrate that site-directing targeting of either 4E10 or 2F5 VH/Vl pre-rearrangements results in a common, profound blockade in early BM B cell ontogeny, coinciding the pre-B to immature B cell transition, the developmental stage at which such BnAbs would normally be expressed by B cells as surface Ag receptors. These results are similar to other transgenic models expressing autoreactive BCRs with high affinity for self-Ags (26, 34, 35) and are thus consistent with Ag-mediated clonal deletion being the predominant, common mechanism suppressing B cells expressing gp41+ BnAbs as BCRs. Moreover, KI mice expressing 2F5 or 4E10 VH rearrangements alone (i.e., paired to multiple endogenous mouse LCs, instead of to those expressing 2F5 or 4E10 Vl rearrangements, respectively) also exhibit a profound developmental blockade, suggesting that 2F5/4E10 HC self-reactivities are sufficient to trigger central deletion, as seen in other KI mice with site-directed targeting of autoreactive HCs containing long and/or positively charged HCDR3 regions like the anti-DNA HC 3H9-76R (35, 57). Furthermore, that we observe an additional, modest blockade in both 2F5 and 4E10 VH KI mice at the pro/pre-B stage suggests that 2F5/4E10 HCs may also partially impact B cell development even prior to BCR expression. This finding is also consistent with previous studies demonstrating selection against pre-BCR+ B cells bearing HCDR3s that are elongated and/or enriched in positively charged/
Aromatic residues (58–61) and could either be due to inefficient HC/surrogate LC pairing and/or pre-BCR–mediated self-reactivity (reviewed in Refs. 42, 62–64). Finally and importantly, the fact that normal BM and splenic B2 B cell development is supported in KI mice expressing the \( V_H \) of 48d, a human HIV-1 mAb bearing a HCDR3 of average charge and length (and constructed in identical fashion as those expressing 2F5/4E10 \( V_H \) regions) argues against the possibility that expression of chimeric 2F5/4E10 Abs (i.e., bearing human \( V_H \) /mouse C regions) non-somatically limits B cell development in our KI models.

An intriguing question our results raise is whether clonal deletion represents a global mechanism for limiting all B cells expressing MPER epitope-specific BnAbs. Of relevance to this issue is recent identification of 10E8, a potent MPER-specific BnAb lacking in vitro polyreactivity, but having an extraordinarily high degree of somatic mutations (65). Three explanations could account for the reported differences observed between 4E10/2F5 and 10E8 with respect to in vitro self-/-polyreactivity: 1) not all MPER-specific BnAb-expressing B cells may indeed be clonally deleted in vivo; 2) BnAbs like 10E8 have no polyreactivity, yet bind specific autoantigen(s) and thus would only be detectable in vitro in protein array scans (and not general HEP-2 or a predefined autoantigen panel screens), akin to the minimally polyreactive BnAb 2F5, which avidly and selectively binds KYNU and CMTM3 (38) and for which physiologically relevant autoreactivity has been confirmed via the in vivo measurement of negative B cell selection (31, 32); and 3) BnAbs like 10E8 may originate from a rare subset of B cell clones isolated from HIV-1–infected patients who acquired additional somatic mutations at some point during affinity maturation in germinal centers (GCs), which eliminated their poly/self-reactivity, yet maintained their neutralization specificity. Future studies involving generation and characterization of KI mice expressing 10E8 will allow these possibilities to be distinguished.

Another key related question raised by this study is whether precursors in the naive B cell repertoire expressing germline (i.e., unmutated common ancestor [UCA]) forms of original, (i.e., somatically mutated) BnAbs derived from chronically infected HIV-1 subjects, like 2F5 and 4E10, undergo central clonal deletion or alternatively are normally deleted later in peripheral differentiation, during or after the GC reaction. With respect to the 2F5 BnAb lineage specifically, UCA-expressing B cell precursors are also likely profoundly deleted early in BM development, based on several initial observations. First, it has recently been demonstrated in vitro that the 2F5 UCA binds lipids with higher affinity, exhibits stronger HEP-2 reactivity than the original 2F5 Ab, and, depending on the allelic variant, retains M PER epitope reactivity at varying degrees (66). Additionally, the deduced somatic mutations acquired by the original 2F5 HC do not include HCDR3 residues involved in MPER reactivity (37), lipid reactivity (56), and/or positively charged amino acids believed to be critical for general polyreactivity (57). Importantly, and consistent with this notion, we have recently demonstrated in KI mice expressing the 2F5 UCA \( V_H \) rearrangement, a similar blockade in BM development (i.e., at the pre-B to immature B cell stage) relative to those expressing

![FIGURE 6. Antigenic reactivity and neutralization profiles of splenic hybridoma lines isolated from naive 2F5 or 4E10 complete KI mice. (A) MPER reactivity and HIV-1 neutralization profiles of B cell hybridoma subcloned lines derived from primary screens of fusions using naive 2F5 or 4E10 complete KI spleens. Data are represented as scatter plots, with MPER nominal epitope reactivities (left panels) or percent MN-3 neutralization inhibition scores (right panels) plotted against supernatant IgM concentrations in 2F5 and 4E10 complete KI hybridoma lines (top and bottom panels, respectively). (B-D) Antigenic reactivity profiles of purified mAbs selected from primary screens. Shown are representative ELISA binding curves of purified 4E10 and 2F5 complete KI-derived mAbs (solid and dashed lines, respectively) to their MPER neutralizing epitopes (B) (as specified by MPB.03 peptides), NIH-3T3 cytoplasmic/nuclear components (C), and CL (D), all carried out as previously described (32). V3-1.4 (a 2F5 IgM mAb) (32) or 4E10-V2-6 CL4 (a representative 4E10 IgM mAb identified in this study, bearing 4E10 \( V_H /V_L \) sequences) and AID 3G11 (a nonneutralizing IgM mAb lacking self-polyreactivity) (69) were used as positive and negative controls for binding self-/-polyreactive components, respectively. Criteria for positivity were arbitrarily set at saturating concentrations and relative to control mAbs as follows: (+++), ≥80% of (4E10-V2-6 CL4 or V3-1.4) binding; (+), 25–50% of (4E10-V2-6 CL4 or V3-1.4) binding, but more than AID 3G11 binding.](http://www.jimmunol.org/Downloadedfrom)
<table>
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<tr>
<th>Clone Identification no.</th>
<th>MPER</th>
<th>CL</th>
<th>Neutralization Ability</th>
<th>VH Rearrangement Used</th>
<th>VL Rearrangement Used</th>
<th>VH Mutation Type/Location</th>
<th>VL Mutation Type/Location</th>
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<tr>
<td>4E10-V2-2-3 CL1</td>
<td>+++</td>
<td>N/A</td>
<td>+++</td>
<td>KI 4E10V H VDJ</td>
<td>No mutation</td>
<td>KI 4E10V H VDJ</td>
<td>KI 4E10V L VJ No mutation</td>
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<tr>
<td>4E10-V2-2-5 CL1</td>
<td>+++</td>
<td>N/A</td>
<td>+++</td>
<td>KI 4E10V H VDJ</td>
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<td>KI 4E10V H VDJ</td>
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<td>4E10-V2-2-6 CL4</td>
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<td>+++</td>
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<td>No mutation</td>
<td>KI 4E10V H VDJ</td>
<td>KI 4E10V L VJ No mutation</td>
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(Table continues)
the original 2F5 V_H rearrangement (L. Verkoczy, unpublished observations), suggesting HC self-reactivity of mAb 2F5 is already encoded in unmutated B cell precursors of the 2F5 lineage. In contrast, the 4E10 UCA is expressed normally as BCR when transfected in vitro in B cells (67) and in HEp-2 ANA and general autoreactivity assays, exhibits low affinity for various self-Ags (including lipids), and lacks MPER specificity (B. Haynes, unpublished observations), suggesting in vivo, 4E10-expressing B cells may be deleted only after acquiring somatic mutations during affinity maturation. Generation and direct comparison of KI mice expressing complete (V_H × V_L) UCA s of 4E10, 2F5, and 10E8 will be critical to address where in concentrations and relative to control mAbs as follows: ++++, >80% of (4E10-V2-6 CL4 or V3-1.4) binding; ++, 50–80% of (4E10-V2-6 CL4 or V3-1.4) binding; and +, 25–50% of (4E10-V2-6 CL4 or V3-1.4) binding, but more than 13H11 (or AID 3G11) binding.

DNA from cloned hybridoma lines were amplified using leader-specific and κ-specific primers, and PCR products were cloned and sequenced in both orientations as described in the Materials and Methods. KI VH and VL regions were analyzed for mutations using Lasergene software (DNASTAR, Inc., Madison, WI).

HCs that cannot align to 4E10 VH were analyzed using the Ig BLAST algorithm. These two clones keep 3′ end partial 4E10 VH sequence but replace their 5′ end with endogenous VH segments in a region of the 4E10 VH that coincides with a cryptic recombination signal sequence containing a perfect consensus-embedded nonamer and heptamer (70). N/A, Not available.

Table IV. Neutralization profiles of purified IgM mAbs selected from cloned hybridoma lines

<table>
<thead>
<tr>
<th>Clone Identification no.</th>
<th>MPER</th>
<th>Neutralization Ability</th>
<th>VH Rearrangement Used</th>
<th>VH Mutation Type/Location</th>
<th>VL Rearrangement Used</th>
<th>VL Mutation Type/Location</th>
</tr>
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<tr>
<td>2F5-V101-160 CL1</td>
<td>+++</td>
<td>++</td>
<td>KI 2F5V_H VDJ</td>
<td>No mutation</td>
<td>KI 2F5V_L VJ</td>
<td>No mutation</td>
</tr>
<tr>
<td>2F5-V101-165 CL1</td>
<td>+++</td>
<td>++</td>
<td>KI 2F5V_H VDJ</td>
<td>No mutation</td>
<td>KI 2F5V_L VJ</td>
<td>No mutation</td>
</tr>
<tr>
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<td>No mutation</td>
</tr>
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<td>++</td>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<tr>
<td>2F5-V101-305 CL1</td>
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<td>No mutation</td>
<td>KI 2F5V_L VJ</td>
<td>No mutation</td>
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*Shown are IC50s of affinity-purified mAbs required to neutralize each HIV-1 isolate listed in the TZM-bl cell assay.

**SVA, SVA-MLV (simian virus amphotropic murine leukemia virus), a murine retrovirus used as a background control for nonspecific activity.

†Recombinant human 2F5, 4E10, and mouse mAb V3-1.4 were used as positive control Abs.

‡Mouse mAbs AID 3G11 and 13H11 were used as negative control.

§Clones underwent VH replacement.

The development of somatic mutations in a process called V(D)J recombination is critical in shaping the immune repertoire by introducing diversity into the Ig genes in B cells. This process occurs at two distinct stages: V(D)J recombination and SHM. V(D)J recombination is a complex and error-prone process that generates a diverse repertoire of antigen receptors. SHM, on the other hand, is a process of somatic hypermutation, which occurs in mature B cells and enhances the affinity of antibody binding by introducing point mutations into the variable regions of the Ig genes.

The table shows the IC50 values for different mAbs, indicating their neutralizing activity against various HIV-1 isolates. The table also includes the VH and VL rearrangement types, mutations, and location types for each clone. This information is crucial for understanding the genetic basis of antibody diversity and for developing new therapeutic strategies against HIV-1.
residues [many also associated with binding the 2F5 MPER for somatic mutations that eliminate multiple self-reactive HCDR on demonstration in 2F5 KI strains of "affinity reversion" (i.e., selection lacks a consensus cryptic RSS (32), as well as our recent dem-

Consistent with this notion is the fact that the 2F5 V\text{H} sequence able (i.e., with self-reactive residues for which V\text{H} replacement is 

resort safeguard during affinity maturation to temporarily deal the purpose of lowering affinity to self-Ags could represent a last-

FIGURE 7. MPER epitope and lipid reactivities of serum Abs from naive 2F5 and 4E10 KI strains. Shown are reciprocal endpoint titers of serum IgM and IgG-specific binding in 4E10 or 2F5 complete KI strains (open and closed triangles, respectively) and control WT (C57BL/6) littermates or RAG1-deficient mice to MPER 4E10/2F5 neutralization epitope-containing peptide MPR.03 (A) or to CL (B), with each rectangle representing an individual mouse and horizontal lines representing mean titers for each group. 4E10 complete KI mice having reciprocal anti-MPER IgG >1000 were grouped as having high MPER neutralization epitope reactivity (framed in black rectangle) and compared with WT as well as 2F5 complete KI mice. For all indicated groups, serum samples were collected from 6–12-wk-old naive mice and subjected to quantitative ELISA based on previous methods (32) and as described in Materials and Methods, with endpoint titers calculated as the reciprocal of the highest serum dilution used in which more than three background binding fluorescence were still observed. Data shown are normalized to the total IgM or IgG levels (mg/ml) shown in Fig. 4D and Supplemental Fig. 4A, respectively. Significance values were determined using a two-tailed student test. *p ≤ 0.05.

mechanism proposed for irreversibly stopping BCR expression (68). In the context of the 2F5 KI model specifically, in which we previously demonstrated that most B cells expressing 2F5 HCs paired with endogenous mouse LCs (either in 2F5 V\text{H}\text{+/+} KI mice or via LC editing in cultured 2F5 complete KI BM B cells) lose MPER binding, yet remain anergic, the general lowering of slg for the purpose of lowering affinity to self-Ags could represent a last-resort safeguard during affinity maturation to temporarily deal with residual self-reactivity of the 2F5 HC as one that is unedit-

4E10 and 2F5 bind neighboring epitopes in the MPER HR2 region (37) and use a similar mode of HIV-1 neutralization, re-

quiring sequential engagement of Env membrane lipids, followed by interaction with their respective MPER epitopes (56). Our comparative studies of 2F5 and 4E10 KI strains in this study additionally demonstrate that these two BnAbs originate from B cells profoundly limited in vivo by the same fundamental, overriding immune tolerance mechanisms: deletion and anergy. However, the underlying biology responsible for tolerization of 4E10- and 2F5-expressing B cells have different features, with general polyreactivity (including, or correlating with, lipid reactiv-

ity) and neutralization epitope-associated self-reactivity appearing to play disparate roles, as revealed by their selective losses in serum IgG fractions from these two strains. In particular, that 2F5 complete KI serum IgG and cultured B cells from sorted mature B2 compartments lose their MPER neutralization epitope specificity (but retain most of their basal lipid reactivity) suggests positive selection of 2F5-expressing B cells involves eliminating self-antigenic residues associated with its neutralization epitope, whereas the stringent loss of the significant initial lipid reactivity of serum IgG from 4E10 complete KI mice (but most retain 4E10 neutralization epitope specificity) implies that positive-selection of peripheral 4E10 KI B cells instead occurs via selectively eliminating lipid binding (and/or other correlating reactivities comprising 4E10’s polyreactivity). The distinct residual specific-

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ities of 2F5 and 4E10 KI serum IgG for these two neutralization components are intriguing in light of the recent findings of Yang et al. (38), in which KYNU and CMTM3, the conserved mam-

malian self-Ag targets identified for 2F5, are perfectly mimicked by its complete and core neutralization epitopes, respectively, whereas SFB3B, the primary Ag target of 4E10, has no homology to its epitope. Furthermore, in this study, 4E10 was found to have considerable polyreactivity, in addition to its higher lipid affinity, than 2F5 (25, 54).

Collectively, our results therefore provide the first in vivo evidence, to our knowledge, supporting the notion that 4E10 and 2F5 receptor-expressing B cells exhibit specificity for markedly distinct host Ags and suggests this involves 2F5-expressing B cells (but not 4E10-expressing B cells) having cross-reactivity for self-Ag(s) mimicked by its neutralization epitope. This in turn may have important implications for HIV-1 vaccine research, in that immunization strategies aimed at circumventing tolerance/anergy of BnAbs directed to distinct neutralization MPER epitopes may need to consider designing minimal immunogens that mimic components in distinct host Ags. More generally, beyond the field of HIV vaccinology, this study’s identification of KI strains expressing two, related, but distinct antiviral Igs (i.e., one with high polyreactivity versus one with selective reactivity for host Ags mimicked by an infectious agent), together provides an ele-

gent, comparative series of B cell tolerance models to examine how protective Ab responses are regulated by infectious pathogens that use molecular mimicry for immune evasion.

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