Enhanced Antibody Responses to an HIV-1 Membrane-Proximal External Region Antigen in Mice Reconstituted with Cultured Lymphocytes

T. Matt Holl, Guang Yang, Masayuki Kuraoka, Laurent Verkoczy, S. Munir Alam, M. Anthony Moody, Barton F. Haynes and Garnett Kelsoe

*J Immunol* published online 3 March 2014
http://www.jimmunol.org/content/early/2014/03/01/jimmunol.1302829

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/03/01/jimmunol.1302829.DCSupplemental

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Enhanced Antibody Responses to an HIV-1 Membrane-Proximal External Region Antigen in Mice Reconstituted with Cultured Lymphocytes

T. Matt Holl,* Guang Yang,* Masayuki Kuraoka,* Laurent Verkoczy,†‡ S. Munir Alam,‡§ M. Anthony Moody,‡§ Barton F. Haynes,*†‡§ and Garnett Kelsoe*†

We have shown that the protective HIV-1 Ab, 2F5, avidly reacts with a conserved mammalian self-Ag, kynureninase, and that the development of B cells specific for the 2F5 epitope is constrained by immunological tolerance. These observations suggest that the capacity to mount Ab responses to the 2F5 epitope is mitigated by tolerance, but such capacity may be latent in the pretolerance and/or anergic B cell pools. In this study, we used B cell tetramer reagents to track the frequencies of B cells that recognize the HIV-1 2F5 epitope (SP62): in C57BL/6 mice, SP62-binding transitional B cells are readily identified in bone marrow but are lost during subsequent development. Unsurprisingly then, immunization with SP62 immunogen does not elicit significant humoral responses in normal C57BL/6 mice. Reconstitution of Rag2−/− mice with normal congeneric B cells that have matured in vitro restores the capacity to mount significant serum Ab and germinal center responses to this HIV-1 epitope. These B cell cultures are permissive for the development of autoantibody B cells and support the development of SP62-specific B cell compartments normally lost in 2F5 Ab knockin mice. The recovery of humoral responses to the 2F5/SP62 epitope of HIV-1 by reconstitution with B cells containing forbidden, autoreactive clones provides direct evidence that normal C57BL/6 mice latently possess the capacity to generate humoral responses to a conserved, neutralizing HIV-1 epitope. The Journal of Immunology, 2014, 192: 000–000.

Serum Ab responses to HIV-1 envelope Ags are elicited in infected individuals, but the initial Ab response is non-neutralizing and focused on epitopes that are poorly conserved among independent HIV-1 isolates (reviewed in Ref. 1). Neutralizing, strain-specific Ab responses to HIV-1 do emerge in a significant fraction of patients within months of infection (2, 3), and these select for resistant HIV-1 mutants (4). In contrast, serum Abs that effectively bind to neutralizing epitopes conserved on most or all HIV-1 clades are quite rare and arise in only a minority of infected individuals and after several years of chronic exposure to virus (5). These broadly neutralizing Abs (bnAbs) are protective in animal challenge experiments (6–9), but to date, no HIV-1 vaccine has been capable of inducing bnAb responses. A major goal of HIV-1 vaccine study is to understand how these rare bnAb responses are elicited.

Among the conserved, neutralizing epitopes of HIV-1 is the membrane-proximal external region (MPER) of gp41, a structure critical for viral fusion with target cell membrane (10). A series of neutralizing and broadly protective human Abs, 2F5, 4E10, Z13, and 10E8, react with linear epitopes of the HIV-1 MPER (11–14), and yet Abs specific for these MPER epitopes are rarely elicited (3). Indeed, despite significant effort, no vaccine or immunization strategy has been found to induce robust MPER bnAb responses (15–17).

A variety of hypotheses have been proposed to explain ineffective MPER Ab responses to HIV-1 infection and vaccines (reviewed in Ref. 4). We observed that the 2F5 and 4E10 bnAbs react with self-Ags (18), and we proposed that this structural mimicry would result in the loss of MPER-reactive B cells by immunological tolerance mechanisms (19); such mimicry would specifically impair Ab responses to HIV-1 epitopes that mimic host-Ags (19). This hypothesis has been supported by studies of 2F5 and 4E10 VDJ “knockin” mice that exhibit a potent blockade in B cell development at the transition of small pre-B to immature B cells (20–23); this developmental blockade is characteristic of transgenic/knockin mice expressing BCR for known autoantigens (24–26).

We subsequently demonstrated that the enzyme kynureninase (KNU) contains an epitope that is closely mimicked by the HIV-1 2F5 epitope and is avidly bound by the 2F5 bnAb and its unmutated ancestor (27). Because this KNU epitope is conserved in most mammalian species, the normal processes of immunological tolerance that remove KNU-specific B cells should purge equally B cells that are specific for the 2F5 MPER epitope of HIV-1. Likewise, the 4E10 bnAb was shown to react with many recombinant human proteins in microarrays and, under stringent conditions, to the RNA splice factor 3B subunit 3 (SF3B3) (27).
Both KYNU and SF3B3 are highly conserved, and the expected consequence of this epitopic mimicry is tolerization of 2F5 and 4E10-like B cells and impaired MPER Ab responses in most mammalian species, including mice and humans (19, 27). Indeed, laboratory opossums, which lack the cross-reactive KYNU epitope but retain SF3B3 mimicry, respond to immunization with HIV-1 envelope protein (gp140) with extraordinarily high titers of serum IgG to the 2F5 HIV-1 epitope but not to the adjacent 4E10 determinant (27). These observations strongly suggest that B cells specifically reactive with the KYNU/2F5 and SF3B3/4E10 HIV-1 epitopes are present before the first tolerance checkpoint but are then purged (28–30).

During their development, self-reactive, immature B cells are tolerized by apoptosis, receptor editing, or anergy (24, 25, 31–35). Self-reactive B cells that are not purged in the bone marrow (BM) remain susceptible to mechanisms of peripheral tolerance that limit their capacity to respond productively to Ag ligands (36). These activities rely in part on the cellular environment, and we have demonstrated that immunological tolerance can be bypassed in a stromal cell–independent culture system that supports B lymphopoiesis (37). The culture-derived (CD) B-lymphocyte cells that develop in vitro are phenotypically and functionally similar to their in vivo counterparts (37). In the absence of the normal BM environment (38, 39), however, CD B cells are enriched for autoreactivity, including high-affinity, autoreactive VDJ rearrangements that are normally deleted at the first tolerance checkpoint; this biased repertoire is retained even after transfer to RAG1-deficient hosts (37).

The generation of mature, functional CD B cells that mature in the absence of central B cell tolerance allows us to test directly whether the weak immunogenicity of the conserved, neutralizing 2F5 epitope of the HIV-1 MPER is intrinsic or the consequence of immune tolerance. The answer to this question is crucial to HIV vaccine design: do HIV-1 vaccines fail to elicit bnAbs because the weak immunogenicity of the conserved, neutralizing 2F5 epitope remains stable through in vitro development, and RAG1-deficient C57BL/6 (BL/6) mice reconstituted with CD B and T cells rescue germinal center (GC) and serum IgG responses to a MPER HIV-1 peptide immunogen containing the 2F5 epitope. Indeed, reconstituted mice mount GC and serum IgG responses to the 2F5 immunogen that are 20- to 40-fold greater than BL/6 controls despite their significantly reduced ability to respond to 4-hydroxy-3-nitrophenylacetyl hapten (NP)–chicken gamma globulin (CGG). The provision of mature, 2F5 epitope-reactive B cells rescues the virtual unresponsiveness of BL/6 mice to immunization with a simple HIV-1 MPER immunogen, further strengthening the hypothesis that at least some of the conserved neutralizing epitopes of HIV-1 mimic self-Ags and thereby evade effective immune control.

Materials and Methods

Mice

BL/6 and congenic RAG-1–/– (B6.129S7-Rag1tm1Mom/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). 2F5 VH-KI mice (BL/6 genetic background) were provided by L. Verkoczy. RAG-deficient mice reconstituted with culture-derived B cells (CD-RAG) and RAG-deficient mice reconstituted with lymph node (LN) cells (LN-RAG) were generated by tail vein injection of 2 × 10^7 CD or LN cells into Rag1–/– mice as described (37). CD-RAG and LN-RAG mice were used no earlier than 4 wk after reconstitution and paired with age-matched BL/6 controls; in all other experiments, mice were used at 6–8 wk of age. All mice were housed in a pathogen-free barrier facility in sterile bedding and fed ad libitum. These studies were approved by the Duke University Animal Care and Use Committee.

Abs and flow cytometry

To identify, characterize, and isolate lymphocytes, mAbs included: B220-Pacific Blue (RA3-6B2), CD23-biotin (B3B4), CD93-allophycocyanin (APC) (AA4.1), GL7-FITC (GL7) and APC-Alexa Fluor 750–conjugated streptavidin were purchased from BD Pharmingen (San Diego, CA); and anti-mouse IgM-PE-Cy7 (eB121-15F9), anti-mouse IgD-FITC (11-26), CD21-PE (eBioSD9), and TCR-APC (H57-597) were purchased from eBioscience (San Diego, CA). Single-cell suspensions of dissociated tissues and cultured cells were counted on a hemacytometer using trypan blue exclusion to determine total cell numbers. Cells (10^6) were suspended in FACS buffer and labeled with mAbs described above. FACS buffer contained 1× PBS (pH 7.2) with 3% FBS (Sigma-Aldrich) and 0.01% sodium azide. Propidium iodide was used to exclude dead cells from our samples. All FACS analyses were performed using a BD LSR II or FACsCanto cytometer and presented with FlowJo software. Cell sorting was performed on a BD FACSVantage cytometer.

Pepitides and tetrators

All peptides were synthesized by SynPep (Dublin, CA). All tetratomers were created as previously described (40). Peptides used for immunization, ELISA, tetramer, and ELISPOT analysis include: DP178-224 (B220 IgM-IgD CD21-CD23-CD93), immature B cells (B220 IgM-IgD CD21-CD23-CD93); T1 B cells (B220 IgM-IgD CD21-CD23-CD93); transitional-1 (T2) B cells (B220 IgM-IgD CD21-CD23-CD93); marginal zone (MZ) B cells (B220 IgM-IgD CD21-CD23-CD93); and mature B cells (B220 IgM-IgD CD21-CD23-CD93 ). Characteristic flow cytometry gatings are illustrated in Supplemental Fig. 2. The B cell tetramers used to identify Ag-specific B cells in different B cell compartments replaced CD93; we therefore relied on expression patterns of B220, IgM, IgD, CD21, and CD23 to characterize the developmental state of MPER-binding B cells. CD cells were analyzed as described (37).

Definition of B cell subsets

We have defined developmental B cell subsets by their surface phenotypes following the definitions of R.R. Hardy (41) and our prior work (42). Briefly, B cell subsets were defined as: pro/pre-B cells (B220 IgM-IgD CD21-CD23-CD93); immature B cells (B220 IgM-IgD CD21-CD23-CD93); T1 B cells (B220 IgM-IgD CD21-CD23-CD93); transitional-1 (T2) B cells (B220 IgM-IgD CD21-CD23-CD93); marginal zone (MZ) B cells (B220 IgM-IgD CD21-CD23-CD93); and mature B cells (B220 IgM-IgD CD21-CD23-CD93). Characteristic flow cytometry gatings are illustrated in Supplemental Fig. 2. The B cell tetramers used to identify Ag-specific B cells in different B cell compartments replaced CD93; we therefore relied on expression patterns of B220, IgM, IgD, CD21, and CD23 to characterize the developmental state of MPER-binding B cells. CD cells were analyzed as described (37).

Cell lines

P3 and 13H11 cell lines were grown and maintained in DMEM media (Life Technologies) containing 10% FCS, 1% nonessential amino acids, 2 M 2-ME, and penicillin/streptomycin (P/S) antibiotics. The RA4 cell line was grown and maintained in DMEM media (Life Technologies) containing 20% FCS, 1% MEM nonessential amino acids, 2 M 2-ME, and P/S antibiotics as described (44).

B cell culture system

BM cells were cultured to generate CD B cells as described (37). Briefly, mice were killed by cervical dislocation and BM was collected from tibiae and femurs by flushing with cold, serum-containing media. BM was plated for 5–10 min in a humidified CO2 incubator at 37°C to remove adherent cell populations. Nonadherent cells were collected and centrifuged at ~400 g at 4°C for 5 min. RBCs were lysed using 1× ACK buffer. Cells were washed and the number of live cells was determined by trypan blue exclusion. BM cells were plated at 7.5 × 10^5 cells/ml (25 ml) in T-75 flasks for 4 d in IMDM (Life Technologies) containing 10^{-4} M 2-ME, 10% HyClone serum (verified), and P/S antibiotics. Recombinant cytokines were added at 10 ng/ml IL-7 or 20 ng/ml BAFF from R&D Systems (Minneapolis, MN).

ELISA

ELISA plates (BD Falcon) were coated overnight, 4°C with 2–5 μg/ml (50 μl/well) capture reagent (NIP-BSA or DP178-Q16L) in carbonate buffer (0.1 M; pH 9.5). Coated plates were washed with 1× PBS (pH 7.4)
containing 0.1% Tween 20 and 0.5% BSA (USB). Wells were incubated (2 h at 25˚C) with blocking buffer (PBS [pH 7.4], 0.1% Tween 20, and 0.5% BSA [USB]). For Ag-specific Ab-forming cells (AFCs), activated B cells (5 µg/ml LPS and 20 ng/ml BAFF; 72 h) were washed and plated at 1.5–2 × 10^5 wells in triplicate on ELISPOT plates coated with goat anti-mouse Ig(H+L) (2 h at 25˚C) with blocking buffer (PBS [pH 7.4], 0.1% Tween 20, and 0.5% BSA [USB]), and then washed and subblocked for 1–2 h using washing buffer. To identify MPER-specific AFCs, membranes were subsequently incubated with 20 µg/ml biotin-DP178-Q16L or biotin-R4A peptide for 2 h at room temperature. Streptavidin-AP (SouthernBiotech) and SigmaFast BCIP/NBT (Sigma-Aldrich) were then used to enumerate MPER- or R4A-specific AFCs. This method identifies all MPER AFCs regardless of H or L chain type. ELISPOTs were photographed using a Canon EOS 20D digital camera with an EFS 60-mm lens.

### Immunizations

For NP-CGG immunizations, 6- to 8-week-old BL/6 mice were immunized (i.p.) with 50 µg NP-CGG (5 µg) precipitated in aluminum sulfate and suspended in 200 µl PBS. CD-RAG mice were immunized with equivalent amounts of Ag 3.5 wk after CD B cell transfer. Mice were bled before and 12 d after immunizations to determine Ag-specific serum Ab levels. For MPER immunizations, 6- to 8-week-old BL/6 mice were immunized (i.p.) once or twice with DP178-Q16L peptide (10 µg) precipitated in aluminum sulfate and suspended in 200 µl PBS. CD-RAG mice were immunized (i.p.) once or twice with DP178-Q16L peptide (10 µg) precipitated in aluminum sulfate and suspended in 200 µl PBS 3.5–4 wk after CD B cell transfer. Secondary immunizations came 28 d after the primary immunization. Mice were bled 16 d after each immunization as indicated to determine Ag-specific serum Ab levels. Spleen and mesenteric LNs (MLNs) were harvested 16 d after immunization and analyzed via FACS and immunofluorescent labeling of tissue sections.

### Immunofluorescence assay histology

A portion of the spleen and individual MLNs from naive and immunized mice were embedded in OCT compound, snap frozen using N2-chilled 2-methylbutane, and stored at −80˚C. Five-micrometer sections were prepared using a cryostat and polylysine-coated slides. Slides were fixed with 1:1 acetone/methanol for 10 min at −20˚C and labeled with 2B20-biotin, TCRβ-PE (red), and GL-7-FTC (green) mAb. FITC signal was acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope. Slides bearing fixed tissues were incubated with goat-anti-mouse IgG-AP and IgM-AP detection Ab. SigmaFast BCIP/NBT (Sigma-Aldrich) was used to develop spots.

### Results

**MPER tetramer binding to mouse B cells is specific**

The 2F5 epitope is present in the Ag KNYU of both humans and mice (27). We hypothesized that the 2F5 epitope-binding B cell population in BL/6 mice is subject to regulation by tolerance mechanisms. To identify Ag-specific B cells, we prepared B cell tetramer reagents consisting of linear HIV-1 envelope peptides synthesized with biotin and subsequently tetramerized with streptavidin-APCy (40). Similar tetramer reagents have been used to identify and isolate B lymphocytes for HIV-1 envelope Ags, including the V3 loop of gp120 and the immunodominant region of gp41 (45, 46); their specificity has been demonstrated by surface plasmon resonance and competitive inhibition (40, 47, 48). Biotinylated polypeptides encompassing the 2F5 epitope were tetramerized with streptavidin-APCy. This MPER tetramer was bound only by human and mouse mAbs specific for the 2F5 epitope of gp41 as determined by surface plasmon resonance and reactivity to mAb-coated beads (Supplemental Fig. 1) (40). Matched tetramers bearing a scrambled MPER peptide were not bound by the same mAb, and irrelevant mAbs did not bind to the MPER tetramer (Supplemental Fig. 1) (40).

As expected, MPER tetramer binding to 13H11 cells, a 2F5 epitope–reactive hybridoma line (49), was highly specific (Fig. 1A, 1B). Approximately 60% of 13H11 cells were labeled by APCy conjugated MPER tetramer whereas APCy conjugated empty (no peptide) or irrelevant (R4A) tetramers labeled ≤20% of 13H11 cells (Fig. 1A, 1B). Labeling of the parental fusion line, P3, by MPER or control tetramers was even lower (<5%) (Fig. 1A). To further ensure the specificity of MPER tetramer binding, 13H11 cells were incubated with either unlabeled homologous or irrelevant tetramer or peptide (0.6- to 20-fold molar excess) and subsequently exposed to APCy conjugated MPER tetramer (representative histograms in Supplemental Fig. 1). Homologous peptide and unlabeled tetramer comparably reduced the frequency and intensity of labeled 13H11 cells to background levels in a dose-dependent manner (Fig. 1A, 1B). In contrast, preincubation with heterologous peptide or tetramer resulted in little or no reduction of MPER tetramer labeling (Fig. 1A, 1B).

To determine whether MPER tetramer binding to mouse lymphocytes was equally specific, we incubated BL/6 BM cells (>2 × 10^6) in ice-cold medium or medium containing a 10-fold molar excess of unlabeled MPER peptide, washed the cells, and exposed them to APCy conjugated MPER tetramer (125 ng/10^6 cells). Subsequently, the BM cells were reacted with B220 mAb to identify B-lineage cells. Whole BM cell populations contained a small (≤0.2%), but reproducible, population of MPER tetramer[+] B220[+] cells; in those BM samples preincubated with soluble, homologous peptide, the frequency of MPER tetramer[+] cells was reduced by ≥80% (Fig. 1C). We conclude that the substantial majority of B cells labeled by MPER tetramer specifically bound the MPER peptide, and that the MPER tetramer identifies Ag-specific B cell populations (Fig. 1C).

**MPER-reactive B cells are lost after transitional B cells leave the BM**

B cell development is strikingly impaired in 2F5 V_{H}KI mice by a developmental blockade consistent with the loss of self-reactive immature and transitional B cells by tolerance mechanisms (20). To determine whether MPER-reactive B cells expressing endogenous Ig rearrangements might also be lost during development, we determined the frequencies of MPER- and control tetramer–binding B cells in distinct developmental compartments of the BM and spleen (Supplemental Fig. 2) (41, 43).
Empty tetramer controls were used to determine background of tetramer labeling in B cell subsets. Because MPER tetramer+ B cells were rare (∼0.3%), a stringent cut-off was defined: 4 SDs of the frequency of B cells binding empty tetramers. Pro/pre-B cells (B220loIgM2IgD2CD212CD232) and immature B cells (B220loIgM+IgD2CD212CD232) did not exhibit significant MPER tetramer binding, whereas low but significant (p, 0.05) frequencies (∼0.3%) of T1 (B220loIgMhiIgDloCD212CD23lo) and T2 (B220hiIgMhiIgDhiCD21loCD23hi) BM B cells were labeled by the MPER tetramer (Fig. 2). In contrast, frequencies of splenic T1 and T2 B cells that bound MPER tetramer were significantly lower (p, 0.05), with splenic T1 binding falling below background (Fig. 2).

Reactivity with the MPER tetramer was not a general property of BM B cells, as mature, recirculating, B cells (B220hiIgMintIgDhiCD21loCD23hi) in the BM exhibited only background frequencies of MPER binding (p, 0.05 compared with BM T1 B cells; Fig. 2). Although splenic T2 and mature follicular (MF; B220hiIgMintIgDhiCD21hiCD23hi) B cells were labeled by MPER tetramer above background levels, tetramer binding in these compartments was about half that of BM T1 and T2 B cells (p < 0.05). Similar to splenic T1 B cells, MZ (B220hiIgMhiIgDhiCD21hiCD23hi) B cells were not labeled by MPER tetramer above background levels (Fig. 2). Despite MPER tetramer binding frequencies that fell above (T2 and MF) or below (T1 and MZ) background, the frequencies of MPER-binding splenic B cells were not significantly different from each other (T1 to T2, p = 0.051; T2 to MF, p = 0.999; MF to MZ, p = 0.497; T1 to MF, p = 0.052; T1 to MZ, p = 0.115; T2 to MZ, p = 0.493).

The R4A peptide is a DNA mimotope and labels autoreactive B cells subject to tolerance control (44, 50). We observed a developmental kinetics for R4A-reactive B cells that was similar to that for the MPER tetramer, with peak binding frequencies in the BM T1 and T2 compartments followed by substantial declines in all splenic compartments (Fig. 2, dashed and solid line). R4A-binding cells were less frequent, however, than those binding the MPER tetramer, and most of these values fell below background. Indeed, only BM T1 B cells exhibited a mean R4A-binding frequency above background (p > 0.05) (Fig. 2). Nonetheless, the similar patterns of MPER and R4A tetramer binding suggest that similar processes control the development of MPER- and DNA-reactive B cells.

Taken together, these data are consistent with the generation of HIV-1 MPER-reactive B cells and their subsequent loss following the T1 and T2 stages of B cell development in the BM. This period is known to be a major checkpoint of central B cell tolerance (51), and our experiments constitute to our knowledge the first demonstration of developmentally regulated reductions in the numbers of MPER-specific B cells in normal mice.
In vitro B cell culture supports the development of 2F5 VDJ-KI immature and transitional B cells

B cell development in 2F5 VDJ-KI mice is blocked, resulting in significantly reduced numbers of immature, transitional, and mature B cells (20). Earlier, we developed a stromal cell–independent, B cell culture method that generates substantial numbers of IgM+ B cells from pro-B and large pre-B cell precursors, including those normally lost to immunological tolerance (e.g., DNA-specific 3H9 HC-KI); autoreactive, CD B cells persist after adoptive transfer into congenic, RAG1 knockout recipients (37).

To determine whether this in vitro recovery might also rescue the development of immature and transitional B cells specific for the 2F5 epitope, we cultured nonadherent BM cells from 2F5 VDJ-KI knobin mice (20) and characterized the B cells that developed in vitro. Although the BM of 2F5 VDJ-KI mice contains significantly lower numbers (<10% of BL/6; p < 0.01) of immature and T1/T2 B cells (Fig. 3), cultures of 2F5 VDJ-KI BM (37) contained substantially higher numbers (~40% of BL/6 controls; p = 0.34) of immature (B220+IgM+IgDCD93+3P) and T1/T2 (B220+IgM+IgDCD93+) 2F5 VDJ-KI B cells (Fig. 3). The increased frequencies of immature and transitional 2F5 VDJ-KI CD B cells suggests cultures are permissive for the development of 2F5-like B cells that are normally tolerized in vivo (20, 21, 27).

Culture of BL/6 BM supports development of 2F5-like B cells

Mouse B lymphocytes that generate Abs specific for the 2F5 MPER epitope are rare (Fig. 2) (40). Because CD B cell populations contain clones that do not normally mature in vivo (38, 39), we tested whether these cultures support the development of 2F5-like B cells from normal BL/6 BM. After expansion and differentiation (37), CD cells were incubated with control (empty), R4A tetramer or MPER tetramer (Fig. 1A, 1B) along with mAb reagents specific for B220, IgM, and IgD (37) to identify R4A and MPER tetramer–binding B cells (Fig. 4A).

Whereas pro/pre-B (B220hiIgM+IgD−) CD B cells did not exhibit (empty, R4A, or MPER) tetramer binding, MPER tetramer labeled significantly higher frequencies (p < 0.05, compared with matched, empty tetramer controls) of immature (B220+IgMloIgD−) and T1 (B220+IgMhiIgDlo) CD B cells (Fig. 4A). Increased frequencies of R4A tetramer–binding cells were observed in the CD T1 compartment whereas empty tetramer labeled no or few immature or T1 CD B cells (<0.1%) (Fig. 4A). For T2 (B220hiIgM+IgDhi) CD B cells, the frequencies of MPER-binding cells remained elevated and similar to that observed in vivo (p = 0.40; Fig. 2) but were not significantly (p > 0.05) higher than the frequencies of cells labeled with empty or R4A tetramers (Fig. 4A).

To confirm the presence of MPER-specific CD B cells, we induced CD B cells to differentiate into AFCs in the presence of BAFF and LPS (37) and enumerated R4A- and MPER-specific AFCs using reverse ELISPOT assays (40). The frequencies of R4A- and MPER-specific AFCs (all isotypes/types) (~0.1–0.4%) in LPS/BAFF-activated CD B cells (Fig. 4B, unselected) were congruent with the frequencies of R4A- or MPER-specific B cells determined by tetramer labeling (Fig. 4A); prior enrichment of MPER tetramer+ CD B cells by flow cytometry dramatically and specifically increased (~12-fold) the frequency of MPER-reactive AFCs (Fig. 4B, selected). In comparison, selection of MPER tetramer+ CD B cells showed no significant change (p > 0.05) in the frequency of R4A-reactive AFCs (Fig. 4B). We conclude that in vivo, MPER-reactive CD B cells are generated from BL/6 BM and that tetramer labeling of B cells can be used to enumerate and enrich for Ag-specific cells (52).

Reconstitution with CD, but not LN cells, results in serum autoantibody

To generate CD-RAG and LN-RAG mice, congenic RAG-1−/− mice were reconstituted with lymphocytes by i.v. transfer of (2 × 106) CD or LN cells (37). Both CD and LN cells effectively reconstituted RAG-1−/− mice, as evidenced by comparable populations of B220+IgM+IgDhi cells present in spleen and LN 4 wk.
after transfer (Fig. 5A). Additionally, we observed substantial populations of B220hiIgMhiIgDlo B cells in the spleen and LNs of both CD-RAG and LN-RAG mice, a phenotype consistent with B cells that have undergone class-switch recombination (Fig. 5A). Interestingly, in both CD-RAG and LN-RAG animals, the frequency of splenic B cells with a transitional/MZ-like phenotype (B220hiIgMhiIgDlo) was elevated (33 and 46%, respectively) compared with BL/6 controls (∼20%; Fig. 5A). CD-RAG mice contained comparable frequencies of mature follicular (B220hiIgMhiIgDlo) and transitional/MZ-like B cells to those of LN-RAG mice; however, LN-RAG mice contained elevated (∼3- to 5-fold) frequencies of transitional/MZ-like B cells in the lymph node compared with either BL/6 or CD-RAG mice (Fig. 5A). These data indicated that LN or CD cells were equivalent in their capacity to reconstitute RAG-1−/− mice.

We detected DNA autoantibody in the serum (1:160 dilution) of BL/6, CD-RAG, and LN-RAG mice using C. luciliae direct immunofluorescence assay (53) by rating the observed binding as either strong (+), weak (+/−), or no (−) reactivity (representative images, Fig. 5B). BL/6 sera samples contained only weak (1 of 5) or no (4 of 5) reactivity to DNA (Fig. 5C). In contrast, CD-RAG sera samples contained mostly (4 of 5) strong reactivity to DNA (Fig. 5C), whereas most LN-RAG samples (3 of 5) showed no reactivity to DNA (Fig. 5C). To ensure that differences in serum DNA Ab were not the result of unequal IgG reconstitution, we directly measured serum IgG levels in BL/6, CD-RAG, and LN-RAG mice.
using ELISA (Fig. 5C). We observed that both CD-RAG and LNRAG mouse sera contained similar (∼1.5 mg/ml) amounts of IgG to that of BL/6 controls (Fig. 5C). These data support our conclusion that the B cell repertoire formed in vitro is qualitatively different from the mature, peripheral B cell repertoire of BL/6 mice, indicating that the CD-RAG animal model can be used to study B cell populations that are normally excluded from the mature repertoire.

Robust germinal center responses in CD-RAG mice immunized with MPER Ag

CD B cells contained MPER-specific populations (Figs. 3, 4) and were able to reconstitute lymphocyte-deficient mice with a unique repertoire of B cells (37) (Fig. 5); we therefore immunized BL/6 and CD-RAG mice with MPER peptide precipitated in aluminum sulfate. The spleen and MLNs of naive control and immunized mice (day 16 after immunization) were harvested and the frequency of germinal center (GC) B cells (B220hiGL-7hi) within the total B220+ population (Fig. 6A, 6B) was determined for each tissue. Additionally, the presence of GC structures was confirmed by histological analysis of spleen and MLN samples (representative examples in Fig. 6C, 6D). Reconstitution of RAG-1−/− mice with either CD or LN cells did not lead to hypertrophy of the spleen or MLNs, nor did we observe increased morbidity or mortality in reconstituted animals (not shown).

In BL/6 mice, immunization with MPER Ag did not increase (1′ p = 0.80; 2′ p = 0.52) the frequency of MLN GC B cells when compared with naive animals (Fig. 6A). Histological analysis (Fig. 6C) confirmed that BL/6 mice did not form robust splenic GC responses after MPER Ag immunization, consistent with minimal increases (1′ p = 0.04; 2′ p = 0.10) in the frequency of GC B cells determined by FACS (Fig. 6B). The lack of robust GC responses in BL/6 mice after MPER peptide immunization is consistent with the loss of MPER-specific mature B cells (Fig. 2).

In contrast, immunization of CD-RAG mice with MPER Ag significantly increased (1′ p = 0.01; 2′ p = 0.05) the frequency of MLN and splenic GC B cells (Fig. 6A, 6B). Histological analysis of spleen samples from these immunized CD-RAG mice confirmed that GL-7hi B cells were organized into GC structures (Fig. 6C, 6D). Compared to BL/6 controls, CD-RAG mice harbored significantly elevated (MLN, 1′ p < 0.01, 2′ p = 0.01; Spl, 1′ p = 0.01, 2′ p = 0.05) frequencies of GC B cells after each immunization with MPER Ag (Fig. 6A, 6B). These data demonstrate that CD-RAG mice mount robust GC responses to MPER Ag immunization and we correlated these enhanced GC responses

![Graph](https://example.com/graph.png)

**FIGURE 6.** CD-RAG mice form robust GC responses after immunization with MPER peptide. BL/6 (○) and CD-RAG (■) mice were immunized (i.p.) with 10 μg DP178-Q16L (MPER) peptide in aluminum sulfate. (A) MLN and (B) spleen cells were harvested at day 16 after one to two immunizations. Cells were labeled with mAb to B220, IgM, IgD, TCRβ, and GL-7. The percentage of B220hiGL-7hi B cells in B220hi cells was determined by flow cytometry. Each group contained multiple mice (n = 6–12) compiled over multiple (n = 2–4) independent experiments. Significant differences (*p ≤ 0.05, **p ≤ 0.01) between groups were determined by a two-tailed Student t test. Five-micrometer sections of spleen from BL/6 and CD-RAG mice at day 16 after (C) primary or (D) secondary immunizations were labeled with mAbs to B220-AF350 (blue), TCRβ-PE (red), and GL-7-FITC (green). FITC signal was amplified using anti–FITC-AF488 Ab. Scale bars, 50 μm for all images. Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope at ≈200 original magnification.
to MPER with the recovery of MPER-reactive B cells in the in vitro culture system.

**Enhanced serum IgG responses to MPER in CD-RAG mice**

Historically, 2F5-like gp41 MPER-specific serum Ab is poorly elicited in mouse models after immunization with HIV-1 Ag (15–17, 54). CD B cells reconstituted peripheral lymphoid tissues, organized into follicles, and formed robust GCs upon MPER Ag immunization (Figs. 5, 6). We therefore collected serum Ab of naive and immunized CD-RAG mice and quantified Ag-specific Abs by ELISA. As a positive control, we immunized BL/6 and CD-RAG mice with NP-CGG and compared NIP-specific IgG response to the generation of gp111 MPER-reactive Abs.

Immunization of BL/6 and CD-RAG mice with NP-CGG/aluminum sulfate elicited a large increase (~100- and 30-fold, respectively) in NIP-specific serum IgG Ab compared with naive animals (Fig. 7A). NIP-specific serum IgG of CD-RAG mice was ~3-fold less than that elicited in BL/6 mice (Fig. 7A), indicating that CD-RAG animals are capable of mounting a B cell response to Ag immunization that is proportional to their level of cellular reconstitution.

B cells modify their BCR in the GC reaction, resulting in the preferential expansion of high-affinity clones whereas low-affinity clones are eliminated, a process termed affinity maturation (55–57). To compare the extent of affinity maturation, we measured high-affinity serum IgG reactivity to NIP using NIP$_{25}$-BSA–coated ELISA. Whereas in immunized BL/6 mice ~30% of NIP$_{25}$-BSA-reactive serum IgG binds NIP$_{25}$-BSA, NIP$_{3}$-reactive IgG is essentially nonexistent in the serum of immunized CD-RAG mice (Fig. 7A).

Prior to immunization, sera from most naive animals did not contain detectable amounts of MPER-reactive IgG Ab. However, 4 of 13 naive BL/6 mice and 11 of 18 CD-RAG mice contained detectable amounts of MPER serum IgG (~0.5 µg/ml) that was near the limit of detection by ELISA (Fig. 7B). If such low levels of MPER Ab represent authentic binding, the enriched MPER reactivity in naive CD-RAG mice is consistent with higher levels of serum autoantibody (37). After primary immunization of BL/6 mice with MPER peptide, we detected a significant increase ($p < 0.01$) in MPER-specific serum IgG; however, the average amount of Ab was low (~0.7 µg/ml) (Fig. 7B). After secondary challenge of BL/6 mice with MPER peptide, the level of Ag-specific IgG did not significantly increase (~2-fold) over primary challenge (Fig. 7B), indicating that the humoral memory to this MPER peptide was not formed.

In contrast, primary immunization of CD-RAG mice resulted in significantly ($p < 0.01$) increased MPER-specific IgG serum Ab than in immunized BL/6 mice (Fig. 7B). Indeed, the actual capacity to elicit MPER-specific IgG responses is likely underestimated in CD-RAG mice because the mature B cell compartments were not fully reconstituted (Fig. 5A) and humoral responses to a model Ag, NP-CGG, were only 30% of that mounted by BL/6 controls (Fig. 7A). The actual enhancement of MPER-specific IgG responses in CD-RAG mice may be ~12-fold greater than in BL/6 mice. CD-RAG mice that received secondary immunization significantly ($p < 0.01$) increased (~10-fold) the amount of MPER-specific IgG (~25 µg/ml) over primary challenge (Fig. 7B). This robust expansion of MPER-specific IgG suggests that CD-RAG mice had formed humoral memory to the MPER peptide during the initial immunization. These data demonstrate that the humoral immune response to this HIV-1 gp41 MPER peptide Ag can be restored in mice when the constraints of B cell tolerance have been relaxed.

**Discussion**

The inability to mount robust B cell responses to several conserved, neutralizing MPER epitopes appears to be phylogenetically conserved from rodents to humans (58, 59). Recent work has characterized the Ab response to HIV gp41 in patients whose serum contains moderate to high virus-neutralizing activity (60). Interestingly, these mAbs were not able to compete away the binding of the 2F5 and 4E10 bnAbs for their respective MPER epitopes (60), illustrating the rarity of humoral responses to the MPER region of the gp41 envelope Ag. We previously confirmed that the 2F5 epitope of the MPER mimics the self-Ag KNYU, and that the abundance of B cells reactive to such epitopes may be limited by tolerance mechanisms (20, 21, 23, 27).

An alternative explanation for the late onset and infrequency of MPER bnAb responses to HIV-1 infection and vaccines is that these Ags are normally evanescent or imperfectly represented in vaccine immunogens. The possibility of imperfect Ags has been rigorously addressed by Schief and colleagues (61, 62) who have generated immunogenic Ag “scaffolds” that stabilize and present the 2F5...
reactivity to the MPER peptide mediates the tolerizing deletion of for the MPER peptide during B cell development, verifying that MPER determinant and not generalized lipid reactivity that is binding to self-Ag KYNU is only blocked by MPER peptide and affinity for cardiolipin and phosphatidylserine (18), the 2F5 BL/6 animals (75–78), supporting the possibility that the abundance of MPER-reactive B cells before and after tolerance checkpoints should reveal stage- and Ag-specific B cell losses (28, 72, 73).

Our tetramer studies demonstrate the loss of MPER tetramer-reactive cells in the splenic T1/T2 B cell compartments, suggesting their removal by central tolerance mechanisms (e.g., deletion or receptor editing). A critical question is whether other B cell compartments that are typically enriched for self-reactivity contain elevated frequencies of HIV-1 MPER-reactive cells. In mice, the MZ B cell compartment is a natural reservoir of auto-reactive B cells (reviewed in Ref. 74). Our data demonstrate that in normal mice, the MZ B cell compartment was not enriched for MPER tetramer B cells (Fig. 2), indicating that MPER-reactive B cells are efficiently deleted or edited as demonstrated in knockout animals (20, 21). In contrast, our data indicate that B cells reactive to this MPER epitope can be recovered in mice that have been reconstituted with B cells that have matured in the absence of the first tolerance checkpoint. CD B cells are enriched for autoreactive specificities (37) (Fig. 5C) and substantially increase humoral responses to the 2F5 epitope shared by the HIV-1 MPER and KYNU (27) (Figs. 6, 7B). We submit that the enhanced GC and serum Ab responses of CD-RAG mice are analogous to our immunizations of laboratory opossums: opossums mount robust humoral response to the 2F5 MPER epitope because their KYNU lacks the shared determinant and does not tolerate (27), whereas responses are enhanced in CD-RAG mice because their B cells have not been tolerated by self-Ags.

We have reported that BL/6 mice mount poor humoral responses to the MPER peptide compared with BALB/c mice and that BALB/c mice harbor significantly higher frequencies of MPER-reactive splenic B cells than do BL/6 mice and that this difference maps to the Igh locus (40). These observations indicate that genetics may play a crucial role in the efficacy of tolerization for MPER/self-reactive B cells. Interestingly, BALB/c mice are known to be relatively more susceptible to autoimmune syndromes than are BL/6 animals (75–78), supporting the possibility that the abundance of MPER-specific B cells may be enriched when tolerance controls are reduced (79).

Although 2F5 contains a long, hydrophobic CDR3 and exhibits affinity for cardiolipin and phosphatidylserine (18), the 2F5 binding to self-Ag KYNU is only blocked by MPER peptide and not by cardiolipin (27). We conclude that it is the KYNU/2F5 MPER determinant and not generalized lipid reactivity that is critical for the induction of tolerance control. Indeed, using MPER tetramers we directly demonstrated reduction of B cells specific for the MPER peptide during B cell development, verifying that reactivity to the MPER peptide mediates the tolerizing deletion of 2F5-like B cells. This notion is supported by observations that many residual peripheral B cells that escape central clonal deletion in 2F5 V_{H1}-KI mice lose MPER reactivity but retain their capacity to bind lipids (21).

Normally, the GC reaction must balance affinity maturation with the elimination of self-reactive B cells that arise as a consequence of somatic hypermutation (80). Therefore, it is somewhat surprising that both 2F5 and 4E10 exhibit the hallmarks of affinity maturation in the GC, yet retain high affinity for phylogenetically conserved self-Ags (27). Although it is known that post-GC B cells may acquire increased autoreactivity (81), we propose that for B cells to acquire HIV-neutralizing capacity, a tortuous path of mutation and Ag-mediated selection is required to balance tolerizing clonal with increased affinity for viral epitopes that mimic host determinants (82). In mice, the mature peripheral B cell pool is purged of MPER-reactive cells that would be normally recruited to the GC reaction upon infection or immunization (Fig. 2). Our B cell transfer model may enhance Ab and GC responses to the 2F5 and 4E10 epitopes simply by increasing the initial frequencies of B cells that can initiate this difficult path to Ab production.

That immune tolerance may be a major factor in the weak immunogenicity of neutralizing MPER epitopes has been challenged by the discovery of the 10E8 bnAb (14). This bnAb recognizes an epitope that overlaps the conserved 4E10 MPER determinant and exhibits great neutralizing breadth (98% [178 of 181] of HIV-1 isolates tested) in vitro. The 10E8 bnAb was reported to have no affinity for phospholipids, including phosphatidylcholine-cardiolipin, did not label fixed HEp-2 human epithelial cells, nor did it bind several human autoantigens diagnostic for various autoimmune diseases (14). Nonetheless, the 10E8 bnAb is encoded by highly mutated V(D)J rearrangements that are typical of virtually all potent bnAbs and indicative of complex maturation pathways (82). The extraordinary frequency of V(D)J mutations in the 10E8 bnAb is consistent with the recruitment of mutated, cross-reactive B cells into the empty niche created by the tolerization of B cells specific for an MPER epitope that mimics a self-Ag. We predict, therefore, that 10E8 may recognize some host Ags that were absent in the initial screenings for autoreactivity. Indeed, in a protein microarray (27), 10E8 exhibited no polyreactivity but strong affinity for a human protein that is expressed in testis, brain, pituitary gland, and several fetal tissues (M. Liu, G. Yang, B.F. Haynes, and G. Kelsoe, manuscript in preparation). Whether this autoreactivity is physiologically significant remains to be determined by the generation of 10E8 knockout mice (20, 21, 23).

Our experiments are likely relevant to HIV-1 vaccine design and development. MPER-specific Ab responses in normal BL/6 mice are virtually absent (Figs. 6, 7) (40), but this deficit is substantially ameliorated by reducing B cell tolerance (Fig. 7). CD-RAG mice are genetically identical to their intact controls and differ only in the way the B cell repertoire develops. Significantly, CD-RAG mice do not exhibit any evidence of systemic autoimmunity; changes in the repertoire of CD B cells do not lead to evident autoimmunity but are permissive for MPER responses. We conclude, therefore, that even small differences in the repertoire of naïve B cells can result in significant differences in the intensity and scope of HIV-1 serum Ab responses. It would be interesting to determine whether differences in the repertoire of naïve human B cells might be associated with different probabilities of mounting bnAb responses. Could a modest relaxation of B cell tolerance increase the chances for bnAb induction in response to infection or immunization? If so, one might expect bnAb activity to be present in HIV-1 patients with disordered B cell tolerance.
Indeed, an autoreactive mAb, CH98, recently recovered from an SL/E/HIV-1 patient has been found to possess strong bnAb activity, suggesting that bnAb and SEL autoantibodies may arise from the similar pools of B cells (83).

More speculatively, if the likelihood of mounting bnAb responses is increased by broadening the primary B cell repertoire, pharmacologic agents (e.g., BAFF; see Refs. 84, 85) that transiently suppress B cell tolerance may be useful in conjunction with HIV-1 vaccines. We imagine that HIV-1 vaccines that normally activate few or no naive B cells (Figs. 6, 7) (61, 62) might induce enhanced Ab responses in the context of a more diverse B cell repertoire.

Acknowledgments

We thank D. Liao, Y. Li, and L.C. Armand for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


10. Ab RESPONSES TO HIV MPER Ags


Supplemental Figure 1. Specific labeling of B-cell lines by MPER tetramer. 13H11 cells (1-1.3 x 10^6) were incubated in PBS + 3% FCS alone or buffer containing equivalent molar excess amounts of either i) unlabeled MPER-tetramer, ii) MPER peptide, iii) unlabeled R4A tetramer or iv) R4A peptide for 30 min. at 0° C. Unlabeled peptide and tetramer concentrations were established to represent 0.6, 1.3, 2.5 and 5.0 M excess of tetramer-associated peptide epitope. Subsequently, cells were labeled with 125 ng of APC-conjugated MPER-tetramer for 30 min. at 0° C. Independent aliquots of 13H11 cells were labeled with APC-conjugated Empty-tetramer as a negative control for peptide-independent binding. 13H11 cells were subsequently analyzed by flow cytometry and the fraction of tetramer binding cells (inset no.) and the M.F.I. of tetramer^+ cells were determined compared to unlabeled controls. Each histogram is representative of at least 3 independent measurements (n≥3) compiled over 2 independent experiments. All data was acquired using a BD LSRII cytometer and histograms were created using FlowJo software.
Supplemental Figure 2. Cell surface marker expressions on BM and splenic B cell populations. BM and spleen cells from BL/6 mice were labeled with mAb to B220, IgM, IgD, CD23, CD21 and CD93. Flow diagrams in panel A were pre-gated on live, single B220+ cells. Specific B cell compartments in the BM and/or spleen were defined as: pro/pre B cells (B220hi IgM- IgD- CD21 CD23 CD93hi); immature B cells (B220lo IgM+ IgD+ CD21 CD23 CD93hi); T1 B cells (B220lo IgMhi IgDlo CD21 CD23lo CD93hi); T2 B cells (B220lo IgMhi IgDhi CD21lo CD23hi CD93int); MZ B cells (B220hi IgMhi IgDlo CD21hi CD23lo CD93hi), and mature B cells (B220lo IgMlo IgDhi CD21lo CD23hi CD93hi). When B cell tetramers were used to identify Ag-specific B cells, CD93 was replaced by tetramers, and only B220, IgM, IgD, CD21, and CD23 were used to characterize B cell development. Data were acquired using a BD LSRII flow cytometer and analyzed using FlowJo software.