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Immune Tolerance Negatively Regulates B Cells in Knock-In Mice Expressing Broadly Neutralizing HIV Antibody 4E10

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A major goal of HIV research is to develop vaccines reproducibly eliciting broadly neutralizing Abs (bNAbs); however, this has proved to be challenging. One suggested explanation for this difficulty is that epitopes seen by bNAbs mimic self, leading to immune tolerance. We generated knock-in mice expressing bNAb 4E10, which recognizes the membrane proximal external region of gp41. Unlike b12 knock-in mice, described in the companion article (Ota et al. 2013. *J. Immunol.* 191: 3179–3185), 4E10HL mice were found to undergo profound negative selection of B cells, indicating that 4E10 is, to a physiologically significant extent, autoreactive. Negative selection occurred by various mechanisms, including receptor editing, clonal deletion, and receptor down-regulation. Despite significant deletion, small amounts of IgM and IgG anti-gp41 were found in the sera of 4E10HL mice. On a Rag1^{-/-} background, 4E10HL mice had virtually no serum Ig of any kind. These results are consistent with a model in which B cells with 4E10 specificity are counterselected, raising the question of how 4E10 was generated in the patient from whom it was isolated. This represents the second example of a membrane proximal external region-directed bNAb that is apparently autoreactive in a physiological setting. The relative conservation in HIV of the 4E10 epitope might reflect the fact that it is under less intense immunological selection as a result of B cell self-tolerance. The safety and desirability of targeting this epitope by a vaccine is discussed in light of the newly described bNAb 10E8. *The Journal of Immunology*, 2013, 191: 3186–3191.

Although no HIV vaccine exists, passive transfer of a number of broadly neutralizing Abs (bNAbs) can protect in animal models of disease (1–10). Hence, protection from HIV by vaccination is theoretically possible, but our lack of understanding of how to elicit bNAbs by immunization is a significant stumbling block. In this study, we focus on bNAbs 4E10, and, in the companion article (11), b12, which were, until recently, two of the most potent and broadly neutralizing HIV Abs known (12). Generation of mouse models expressing B cells of these specificities could aid in optimizing Ags capable of triggering such desirable B cells.

bNAb 4E10 was isolated by Katinger and colleagues (13). It neutralizes isolates from multiple clades with modest potency. Isolated from an HIV-infected patient as a hybridoma by fusion of peripheral blood cells with a heterohybridoma cell line (13), 4E10 Ab genes were recombinantly expressed, and the secreted IgG was tested for cross-neutralization (14). 4E10 recognizes a linear stretch

of amino acids in gp41, in the membrane proximal external region (MPER), centered on amino acids NWF(D/N)IT (15). In the co-crystal structure, the epitope is in helical conformation, forming a somewhat amphipathic structure with a hydrophobic face on one side, with W in the epitope involved in 36% of the contacts with 4E10 (16). The 4E10 combining site is also unusually hydrophobic in parts. Five of six CDRs are involved in epitope binding. But much of the long and hydrophobic H chain CDR3 does not directly contact the gp41 peptide. Cardoso et al. (16) speculated that 4E10's H chain CDR3 might contribute to viral binding by contacting the surface of the viral membrane through the tip of CDRH3, which is not involved in peptide binding but is predicted to be near the viral surface. Support for this notion was provided by enhanced binding of 4E10 seen in the presence of membranes (17) and in studies showing that viral neutralization, but not MPER peptide binding, was dependent upon CDRH3 residues (18, 19).

Surprisingly, in addition to their ability to bind to HIV Envelope, both 4E10 and b12 were suggested to be autoantibodies (20). This conclusion was based mainly on Ab-binding studies and was also extended to the Ab 2F5, which recognizes an epitope adjacent to that of 4E10 (20, 21). 2F5 has autoreactive properties when introduced as knock-in transgenes in mice (22). Recently, 4E10 was found to bind weakly to many human proteins present on protein microarrays, as well as to bind under "stringent" ELISA conditions to splicing factor 3B subunit 3 (21).

These findings were interpreted to suggest that Envelope might have evolved to protect against the elicitation of neutralizing Ab by mimicking autoantigen. Among the assays in which 4E10 scored positive was in binding to HEp-2 cells, a clinical assay for autoantibodies, and in ELISA involving self constituents immobilized on microtiter plates, with 4E10 in solution. 4E10 bound to cardiolipin, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and the lupus autoantigen Ro (SSA). In addition, 4E10 had anticoagulant activity, a hallmark of antiphospholipid

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Abbreviations used in this article: BM, bone marrow; bNAb, broadly neutralizing Ab; MPER, membrane proximal external region.

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syndrome, although this activity was weak (23). Haynes et al. (20) suggested that tolerance to self explains the difficulty in generating Abs to the 4E10 determinant and the relative ineffectiveness of immunogens based on the MPER. 4E10, but not 2F5, reacted weakly in antiphospholipid assays and modestly prolonged activated partial thromboplastin time in vivo (23, 24). In the same study, b12 was found to bind to ribonucleoprotein, dsDNA, centromere protein, histones, and HEp-2 cells (20). The implication is that 4E10 and b12 cells are normally suppressed by immune tolerance, but they might respond under extraordinary circumstances. Patient responders might be resistant to AIDS but with a propensity to lupus-like autoimmunity. If these speculations are correct, they would have enormous implications for HIV vaccine design.

In this study, we generated knock-in mice in which the variable portions of 4E10 were introduced to the physiological mouse Ig H and L loci by gene targeting. The mice were then analyzed for B cell development and function. The results suggest that expression in mice can be a useful tool to establish the physiological significance of putative Ab autoreactivity and, in so doing, help us to prioritize vaccine epitopes.

Materials and Methods

Gene targeting

Targeting was carried out as essentially described in the companion article (11) but using 4E10 V region-coding sequences in place of b12 sequences.

Mice

All experiments were performed in accordance with relevant institutional and national guidelines and were approved by The Scripps Research Institute Animal Care and Use Committee. C57BL/6J, *Rag1*^{-/-}, *Ella-cre*, and IgH^a mice on the B6 background (B6.Cg-Igh^a Thy1^{tr} Gpi1^{tr}/J) were from The Jackson Laboratory. hCκ mice (25) were bred for >10 generations to C57BL/6J mice and then interbred to generate B6.IgH^{a/a}.Cκ^{h/h} mice for interbreeding with transgenic strains.

Flow cytometry and serum ELISA analyses

Analyses for surface markers and serum Abs were performed as described in the companion article (11).

HIV Ags

Peptide C1 sequence: QIQQEKNMYELLALDKWASLWNWFDITKWL-WYIKYGVYIVK-biotin. Design and preparation of MPER scaffold T93 were described (26).

ELISPOT

Polyvinylidene difluoride ELISPOT plates (Millipore, Billerica, MA) were coated with 10 μg/ml the MPER scaffold T93 in PBS and incubated overnight at 4°C. The rest of the assay was performed as previously described (27).

Statistical analysis

Statistical analyses were performed as described in the companion article (11).

Results

Generation of 4E10 H and L knock-in mice

As in the accompanying article (11), C57BL/6 ES cells were modified by gene targeting to introduce HIV Ab H and L chain variable exons, replacing the respective J clusters. Targetings were verified by PCR assay and Southern blotting (Supplemental Figs. 1, 2).

Analysis of 4E10 knock-in mice reveals evidence of physiological autoreactivity

Usage of targeted H chains was analyzed in B cells of 4E10H and 4E10HL mice carrying an IgH^a wild-type allele (the targeted allele

was IgH^b). Unlike b12 mice [see companion article (11)], 4E10HL mice and 4E10H mice failed to demonstrate efficient feedback suppression of endogenous Ig genes, as indicated by extensive expression on splenic B cells of IgM derived from the wild-type allele (IgM^a) (Fig. 1A). Analysis of κ allele usage in 4E10L and 4E10HL mice similarly showed frequent usage of the endogenous allele [marked using a human Cκ targeted replacement allele, Cκ^h (25)]; however, about half of the cells appeared to express only the targeted 4E10 L chain (Fig. 1B). Furthermore, 4E10H and 4E10HL mice had significantly reduced splenic B cell numbers (Fig. 1C). Analysis of bone marrow (BM) B cell fractions revealed a block in B cell development in 4E10H and 4E10HL mice, primarily at the preB to B cell transition [Hardy fraction D to E (28)]; however, there was also a reduction in the late proB cell fraction C (Fig. 1D).

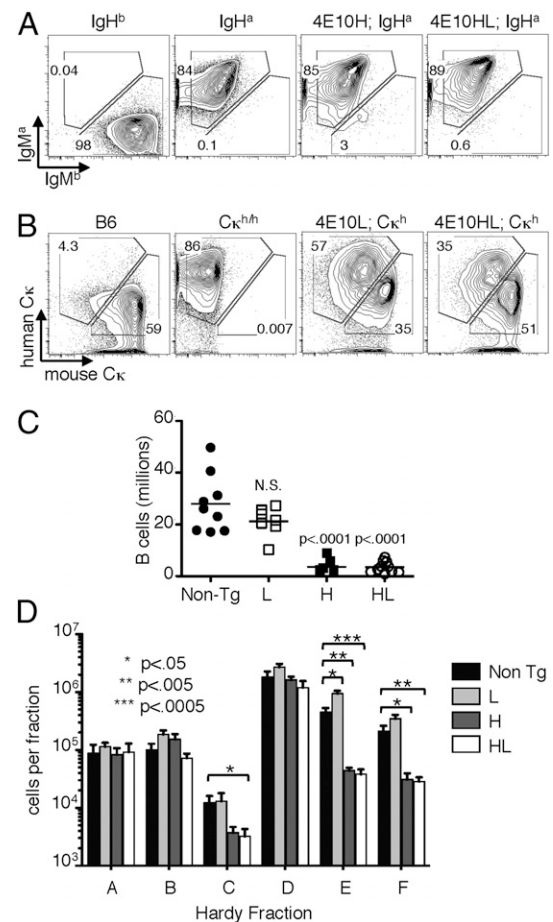


FIGURE 1. B cell development and allelic inclusion in 4E10 mice. (**A** and **B**) Knock-in mice carrying serologically distinguishable endogenous H and L chain constant regions were generated by breeding homozygous knock-in mice with IgH^{a/a}.Cκ^{h/h} mice. Splenic B cells of the indicated genotypes were assessed by flow cytometry using Abs against IgM^b and mouse Cκ, to detect the transgene H and L alleles, respectively, and Abs against IgM^a and human Cκ were used to identify chains derived from endogenous genes. Data are representative of results obtained with 4–10 mice/group. (**C**) Reduced splenic B cell numbers in 4E10 knock-in mice. B220⁺CD19⁺ splenic B cells were enumerated from mice of the indicated genotypes. Each symbol represents an individual mouse. Horizontal lines show means. Data were from at least three independent experiments. (**D**) Analysis of BM B cell developmental fractions according to the staining scheme of Hardy et al. (28). Fractions represent developmental stages as follows: A–C, proB; D, small preB; E, immature B; F, recirculating B. Six mice were analyzed for 4E10HL, and four mice were analyzed for the other genotypes, carried out in one experiment.

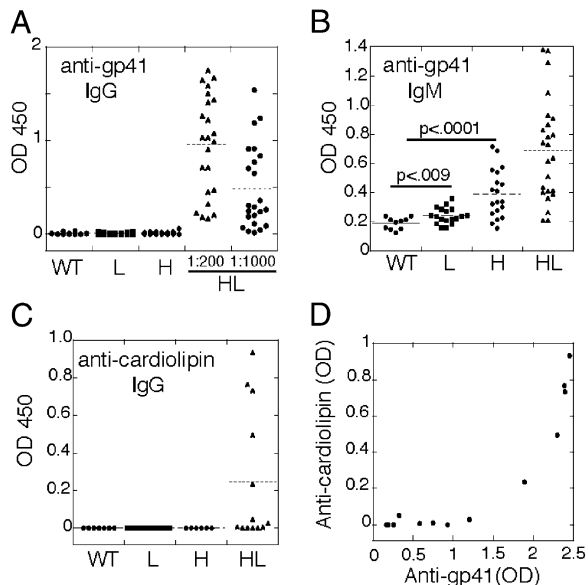


FIGURE 2. Analysis of spontaneous anti-gp41 activity in the sera of 4E10 HL mice. Sera from 6–10-wk-old mice of the indicated genotypes were tested for reactivity to gp41. (A and B) Total IgG and IgM anti-gp41 activity of normal sera from individual adult mice of the indicated genotypes. Readings were from ELISA using serum at 1:200 dilution unless otherwise indicated. (C) IgG anti-cardiolipin assay. (D) Correlation between anti-gp41 and anti-cardiolipin IgG activity. Horizontal lines in (B) and (C) show group means.

Weak, but reproducible, spontaneous anti-gp41 secretion in 4E10HL mice

Despite the poor retention of 4E10 BCR expression on most B cells, analysis of serum Abs revealed that 4E10HL mice, but not mice carrying only the H or L transgene, expressed detectable serum IgG anti-gp41 activity (Fig. 2A). We presume that this indicates that there was some secretion of 4E10HL-like Ab. Serum IgM anti-gp41 activity was also seen, although this correlated less well with coexpression of 4E10 H and L (Fig. 2B). Moreover, a subset of 4E10HL mice produced IgG reactive with cardiolipin (Fig. 2C). Consistent with the notion that 4E10 is an autoantibody, the 4E10HL sera expressing the highest levels of IgG anti-gp41 activity also contained IgG reactive with cardiolipin ($r = 0.9$, Fig.

2D), whereas sera from 4E10H and 4E10L mice lacked both activities (Fig. 2A, 2C). Total Ig levels in 4E10HL mice were slightly subnormal, although this was only statistically significant for IgG1 (Fig. 3). Therefore, we conclude that, in 4E10HL mice, some 4E10-like Ab was produced despite the obvious negative regulation of B cells carrying this specificity.

Enforced expression of 4E10 leads to B cell deletion

In an effort to enforce 4E10 BCR expression, we carried out genetic strategies to increase the dosage of transgenic receptors or to reduce editing by competing endogenous receptors. We generated mice homozygous for the 4E10 H chain knock-in (HH mice) or L chain knock-in (LL mice) or compound homozygotes (HHLL mice). In addition, we bred 4E10HL mice to the *Rag1*^{-/-} background or to mice with deletions of J_H and J_κ clusters, yielding *Rag1*^{-/-}; HL and J_H^{+/-}J_κ^{+/-};HL mice, respectively. If the 4E10 BCR were innocuous and reduced BCR expression was the cause of the subnormal B cell development, it should be rescued, to some extent, in HHLL mice. In contrast, if 4E10 were autoreactive, the increased barriers to editing and increased BCR expression in the 4E10 BCR in HHLL mice would lead to stronger negative selection and a further decrease in B cells. B cell numbers in the periphery were indeed reduced further, rather than rescued, by increasing knock-in gene copy number or suppressing endogenous Ig gene rearrangements (Fig. 4A). Total B cell numbers in mice with enforced 4E10 BCR expression amounted to $<10^6$ /spleen. In addition, those B cells that populated the spleen were made up of a much higher proportion of CD93⁺ cells (Fig. 4B). These few remaining cells had reduced BCR density (Fig. 5A, 5B), yet some carried low levels of 4E10HL receptors, as indicated by their ability to bind to MPER peptide tetramerized on streptavidin (Fig. 5C, 5D). HH or LL mice, which carry the same transgenes without their partner, had higher BCR surface densities than did HHLL mice, indicating that each individual chain was well expressed and suggesting that B cells expressing both chains had downregulated their BCRs. Consistent with this interpretation, BM B cells in *Rag1*^{-/-};HL mice included a major subset with downregulated IgM and CD19 levels (Fig. 6A, right panels). The B220⁺CD19^{low} population (left middle panel) is diagnostic of cells undergoing prolonged attempts at receptor editing (29). As a positive control in this experiment, we used BM cells from IgM^b-macroself transgenic mice, which defined this phenotype (29) (middle col-

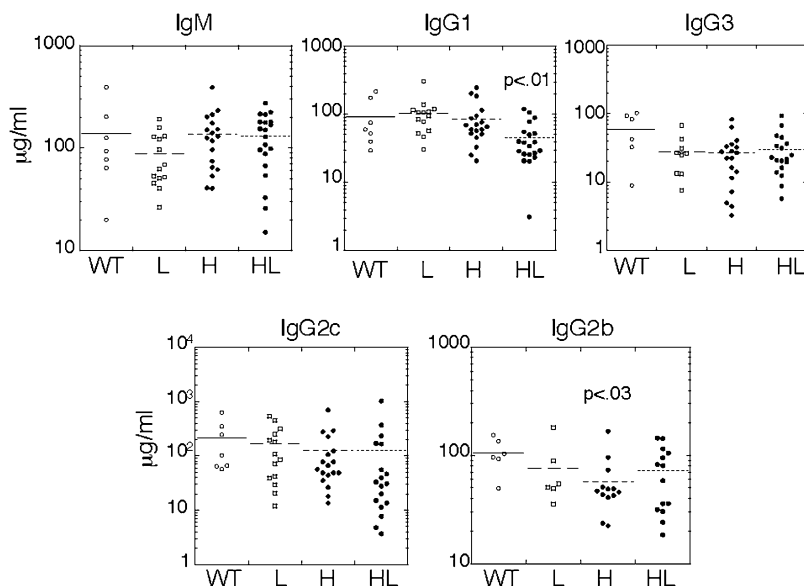


FIGURE 3. Normal serum Ig levels in mice of the indicated genotypes carrying one or no copies of the knock-in H and L alleles. Each symbol represents an individual mouse. Horizontal lines show arithmetic means of the indicated groups.

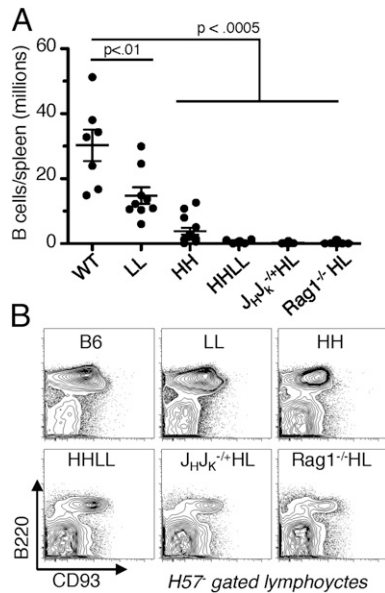


FIGURE 4. Effect of enforcing 4E10 expression on B cell numbers and maturation. Spleen cells of mice homozygous for 4E10 H- or L-targeted loci or double homozygotes (LL, HH, and HHLL, respectively), were compared with 4E10HL hemizygous mice deficient in the second allele ($J_H J_k^{+/+}HL$) or lacking *Rag1* ($Rag1^{-/-}HL$). **(A)** Total B cell numbers ($CD19^+B220^+$) in spleens. **(B)** Analysis of CD93 and B220 coexpression in splenic lymphocytes gated to exclude $TCR\beta^+$ cells using MAb H57.

umn of panels). The rare splenic B cells of $Rag1^{-/-}HL$ mice were $CD93^+$, showed downregulated IgM, and had relatively low levels of CD23 (Fig. 6B), placing the cells in the T3' category of highly autoreactive B cells. Overall, we conclude that the 4E10 BCR is autoreactive, to a physiologically significant extent, and is counterselected by various mechanisms, including receptor editing, clonal deletion, and receptor downregulation.

Anti-gp41 secretion in 4E10 mice with enforced transgene expression

These data raised the question of how the 4E10 B cell arose in the patient from whom it was isolated. As mentioned above, 4E10HL

mice produced anti-gp41 serum Abs. Therefore, we asked whether MPER-reactive Abs were still made in the mice with enforced 4E10 expression. Interestingly, low, but reproducible, levels of IgG2a and IgG1 anti-MPER activity were found in most HHLL and $J_H^{+/+}J_K^{+/+};4E10HL$ sera, but little to none was detected in $Rag1^{-/-};4E10HL$ sera (Fig. 7A, 7B). IgM anti-MPER levels were uniformly low (Fig. 7C). Consistent with these findings, $Rag1^{-/-};4E10HL$ mice lacked any detectable serum IgM (Fig. 7D). Because $Rag1^{-/-};4E10HL$ mice are unable to express any endogenous Ig chains and lack T cells, we infer that anti-MPER production might require T cells, B cells expressing a mixture of both endogenous and 4E10 Ig molecules, or both. In any case, 4E10-like Ab levels were low and maintained by a surprisingly small number of plasma cells, as determined by ELISPOT analysis of HHLL mice (Fig. 7E). Most anti-MPER plasma cells were found in the spleen and mesenteric lymph nodes, but combined analysis of several lymphoid organs enumerated <100 Ab-secreting cells/per mouse (Fig. 7E). Overall, although tolerance is evidently not complete, these data establish that 4E10HL B cells are negatively selected, even when B cell competition is lacking and editing is inhibited.

Discussion

B cells in knock-in mice expressing 4E10 are negatively regulated by immune-tolerance processes. In contrast to b12 mice [discussed in the companion article (11), 4E10HL mice clearly have suppressed B cell production, mainly by central tolerance and clonal deletion, leading to B cell lymphopenia. The remaining B cells that appear in the periphery of these mice have lost 4E10 H chain expression and a substantial fraction also failed to express 4E10 L chain. However, analysis of normal sera indicated that at least some B cells in this model make 4E10 Ab. It is likely that the phenotype of 4E10 knock-in mice is a consequence of 4E10's autoreactivity, as might have been predicted by Haynes et al. (20) and subsequent studies (21). However, the claim that b12 is autoreactive (20) was not supported by our studies presented in the companion article (11). Our findings, along with the study by Verkoczy et al. (22) on 2F5 knock-in mice, underscore the value of biological measures of autoreactivity. In this regard, it is of interest that B cell line transfectants expressing 4E10HL appeared to be difficult to propagate and appeared to have desensitized receptors, whereas cells carrying

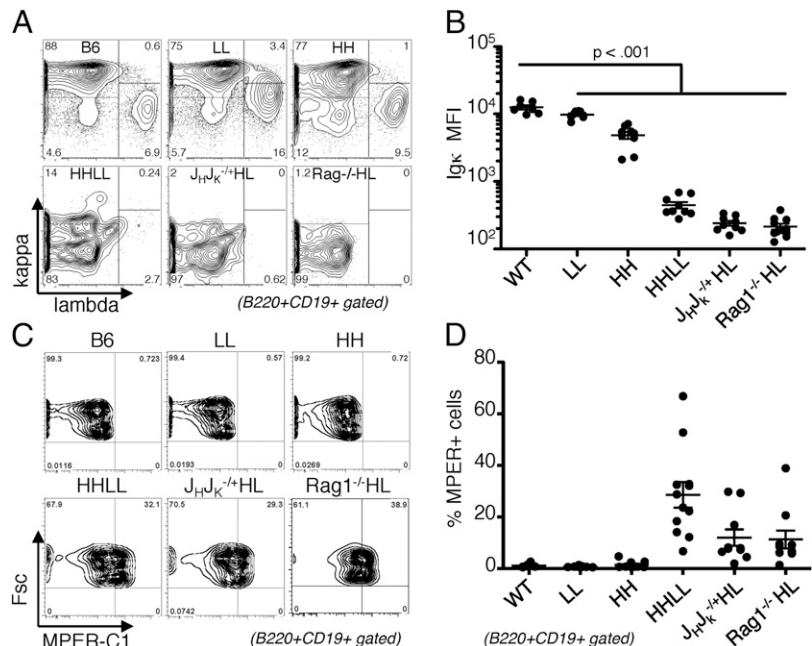


FIGURE 5. Analysis of BCR surface density and MPER binding among splenic B cells from mice with enforced 4E10 expression. **(A and B)** Analysis of surface Ig L chain density on splenic B cells of mice of the indicated genotypes. **(C and D)** Analysis of the frequency of MPER-binding splenic B cells using the biotinylated C1 peptide. Symbols represent data from individual mice obtained over at least two experiments. Horizontal lines in (B) and (D) represent mean group values. Error bars indicate SEM.

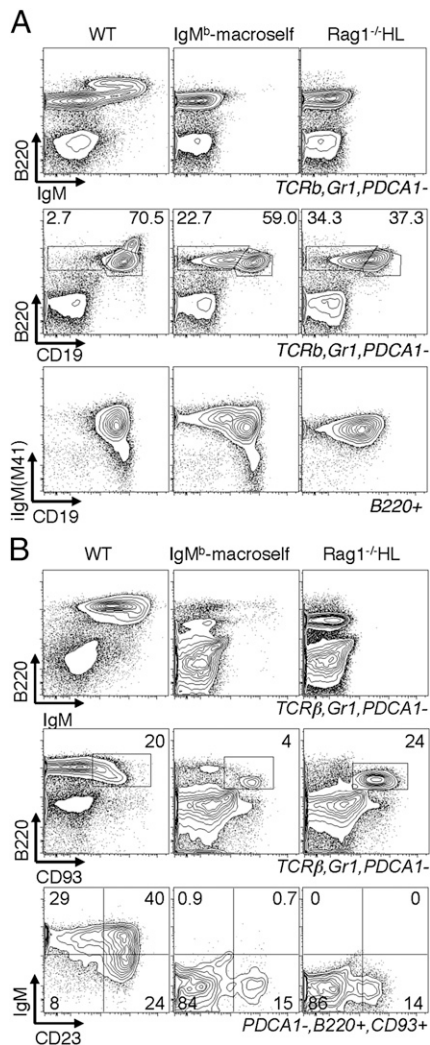


FIGURE 6. BCR internalization and developmental block of B cells in *Rag1*^{-/-}HL mice. **(A)** Analysis of BM B cells for surface markers B220, IgM, CD19, and intracellular IgM (iIgM). BM cells were gated to exclude most myeloid and T cells (*top* and *middle* panels). For iIgM analysis, cells were gated on B220⁺ (*bottom* panels). *IgM*^b-macroself Tg mice express a superantigen reactive to IgM^b (28). **(B)** Analysis of splenic B cells from mice of the indicated genotype. The *middle* panels show the gating used for the analysis shown in the *bottom* panels.

b12HL were propagated more easily and could signal Ca²⁺ mobilization upon stimulation with soluble trimers (30).

Splenic B cells of 4E10 HHLL mice had downregulated BCRs compared with 4E10 HH or LL mice. Because 4E10 HH and LL splenic B cells individually had surface BCR expression > 10-fold higher than B cells coexpressing 4E10 H and L chains, the 4E10 transgenes appear to be well expressed. Similar transgenic expression is predicted for b12 [see companion article (11)] and 4E10 mice because we used identical targeting vectors, including promoter elements. We infer that the low B cell numbers and expression of endogenous H chains among escaped B cells in 4E10HL mice were the result of inefficient H chain editing, followed by the preferential survival of cells that eliminated the 4E10 specificity through expression of endogenous Ig chains. 4E10H mice also had reduced B cell numbers. The 4E10 H chain CDR3 is quite hydrophobic and might yield an autoreactive Ab when combined with a multitude of endogenous L chains. The 4E10 H chain locus lacks unrearranged J_H elements, but it might be silenced by recombinase-mediated destruction by D-to-V_{DJ} rearrangements

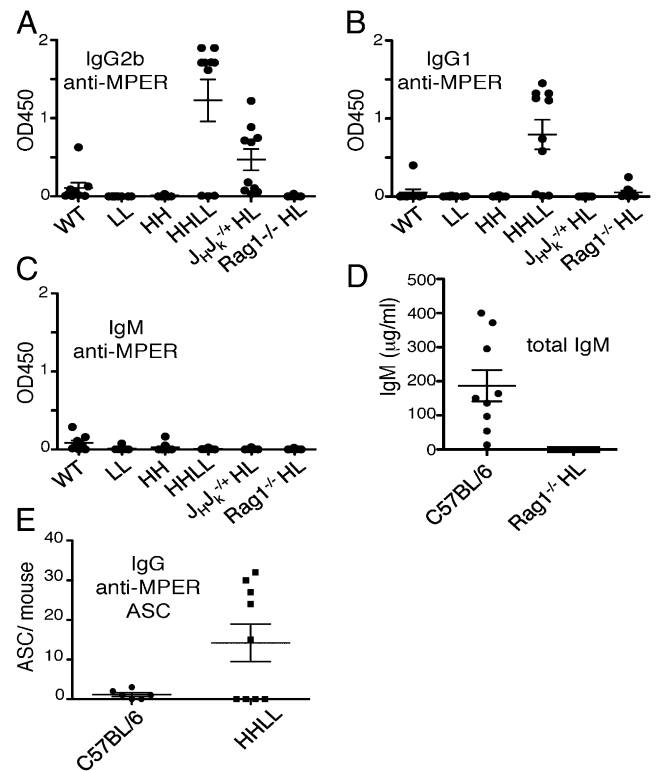


FIGURE 7. Analysis of spontaneous anti-MPER activity in the sera of mice with limited ability to edit because of 4E10 gene homozygosity or gene knockout. **(A and B)** Analysis of IgG2b and IgG1 anti-MPER titers in mice of the indicated genotypes. **(C)** IgM anti-MPER levels in the samples shown in (A) and (B). **(D)** Total IgM levels in *Rag1*^{-/-};HL mice compared with WT controls. **(E)** Assessment of IgG anti-MPER Ab-secreting cell (ASC) numbers in HHLL mice. BM, spleen, and selected lymph nodes were assessed for anti-gp41 activity using an ELISPOT assay. Serological assays were carried out in a single experiment for optimal comparison, but they had similar results to assays done with the same sera at different dilutions or on different days. Each symbol represents the mean value from an individual mouse. Horizontal lines show mean values and error bars show SEM.

at cryptic heptamer sites (31) or be replaced by V_H-to-V_{DJ} rearrangements, which can be functional (32). When endogenous Ig rearrangements were blocked in *Rag1*^{-/-};4E10HL mice, B cell development was aborted at the preB/B cell transition, with the few remaining B cells exhibiting BCR downmodulation.

The autoreactivity and negative selection of 4E10 B cells established in this study, along with similar data in the 2F5 system (22) and an independently generated 4E10 mouse (33) (published while this article was in revision), raise the questions of how 4E10 was initially produced and whether it would be effective or desirable to target the MPER for human vaccination. A larger epitope overlapping with those of 2F5 and 4E10 is seen by the bNAbs 10E8 (34). 10E8 is both more potent than 4E10 and 2F5, and unlike 4E10, it appears not to be polyreactive. It would be of interest to confirm this in a 10E8 knock-in model and to assess in both knock-in models the immunization conditions that promote or prevent Ab formation to defined MPER epitopes. Short of barring a B cell response, a tolerance barrier could slow the evolution of B cell maturation in the germinal center toward desirable epitopes, particularly if the autoantigen in question is present in the germinal center environment (35, 36). One possible explanation for the autoreactivity of both 2F5 and 4E10 is that both recognize relatively short, linear peptide determinants, which, by chance, might have many cross-reactive homologs in the body. A high-affinity

autoantigen recognized by 2F5 has been identified (20), but even low-affinity interactions can efficiently promote B cell tolerance when the autoantigen is sufficiently accessible (37, 38). It is tempting to speculate, but premature to conclude, that conformational epitopes might be more specific and, hence, safer vaccine targets than are linear ones.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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