Liver-expressed Igκ superantigen induces tolerance of polyclonal B cells by clonal deletion not κ to λ receptor editing

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Little is know about the nature of peripheral B cell tolerance or how it may vary in distinct lineages. Although autoantibody transgenic studies indicate that anergy and apoptosis are involved, some studies claim that receptor editing occurs. To model peripheral B cell tolerance in a normal, polyclonal immune system, we generated transgenic mice expressing an Igκ–light chain–reactive superantigen targeted to the plasma membrane of hepatocytes (pAlb mice). In contrast to mice expressing κ superantigen ubiquitously, in which κ cells edit efficiently to λ, in pAlb mice, κ B cells underwent clonal deletion. Their κ cells failed to populate lymph nodes, and the remaining splenic κ cells were anergic, arrested at a semi-mature stage without undergoing receptor editing. In the liver, κ cells recognized superantigen, down-regulated surface Ig, and expressed active caspase 3, suggesting ongoing apoptosis at the site of B cell receptor ligand expression. Some, apparently mature, κ B1 and follicular B cells persisted in the peritoneum. BAFF (B cell–activating factor belonging to the tumor necrosis factor family) overexpression rescued splenic κ B cell maturation and allowed κ cells to populate lymph nodes. Our model facilitates analysis of tissue–specific autoimmunity, tolerance, and apoptosis in a polyclonal B cell population. The results suggest that deletion, not editing, is the major irreversible pathway of tolerance induction among peripheral B cells.

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Developmental stages (Sandel and Monroe, 1999; Hippen et al., 2005; Rice et al., 2005), although the extent to which tolerance is responsible for RAG-mediated recombination in the peripheral B cells and the state of maturity of these cells are controversial (Nemazee and Weigert, 2000).

B cells released from the BM complete their maturation through several transitional stages that have been best characterized in the spleen. T1, T2, and T3 transitional B cells are defined by cell surface phenotype and functional characteristics (Allman et al., 2001; Chung et al., 2002; Su and Rawlings, 2002). In the mouse, all transitional B cell subsets express CD93, and BrdU labeling studies indicate that they turn over relatively rapidly (Allman et al., 1993; Rolink et al., 1998). It has been suggested that clonal deletion of autoreactive cells might take place among transitional cells (Carsetti et al., 1995; Allman et al., 2001; Merrell et al., 2006; Duong et al., 2010). T1 cells are the least mature; upon BCR stimulation in vitro, T1 cells fail to proliferate and are induced to apoptosis. T2 cells appear to be more responsive to stimuli, including BCR ligands and the cytokine BAFF (B cell–activating factor belonging to the tumor necrosis factor family), and they can mature into B2 and marginal zone (MZ) subsets. Other subsets of immature B cells include T3 cells, which are IgM<sup>hi</sup>CD23<sup>−</sup>CD93<sup>−</sup> (Allman et al., 2001), and the recently defined IgM<sup>hi</sup>CD23<sup>−</sup>CD93<sup>hi</sup> T3’ (T3-like) population. Detailed analyses using BCR transgenic mouse models suggested that the T3 subset contains many anergic cells whose phenotype is maintained by continuous antigen stimulation through the BCR (Merrell et al., 2006). T3’ phenotype cells are few in number in WT mice, but are abundant in the 3H9-56R/V<sub>k</sub>8 double-stranded DNA–specific BCR transgenic model (Kiefer et al., 2008). T3 and T3’ populations have been shown to undergo secondary light chain rearrangement (Kiefer et al., 2008); however, the data were obtained from BCR transgenic rather than WT mice.

BAFF (TNFSF13B) is essential for follicular and MZ B cell survival and development (Mackay and Schneider, 2009). The soluble form binds to three receptors, BAFF-R, TACI, and BCMA. BAFF-R is most highly expressed on mature B cells. Null mutants of BAFF or BAFF-R show a block of B cell development at the T2 stage. In contrast, the overexpression of BAFF causes increased B cell number and elevated serum Ig and induces a systemic lupus erythematosus–like disease. The serum level of BAFF is elevated in systemic lupus erythematosus and Sjögren’s syndrome patients (Mackay et al., 1999; Cheema et al., 2001; Zhang et al., 2001; Groom et al., 2002). Excess BAFF can promote the survival of autoreactive B cells (Lesley et al., 2004; Thien et al., 2004; Ota et al., 2010), but it is not known how BAFF affects tissue-specific autoreactive B cells.

B cell peripheral tolerance to tissue-restricted membrane antigen was previously studied mainly with two BCR transgenic mouse models. In the 3–83 model, liver-specific expression of a weakly bound autoantigen, MHC class I K<sub>β</sub>, led to significant reductions in B cell numbers in the spleen and their almost total elimination in lymph nodes (Russell et al., 1991; Lang et al., 1997; Kench et al., 1998; Kouskoff et al., 2000). However, in a high affinity anti–hen egg lysozyme (HEL) transgenic mouse crossed to a thyroid-specific membrane HEL strain, autoreactive B cells were functional, and Ig deposition was observed in the thyroid (Akkaraju et al., 1997). The reasons for the differences in the fates of the autoreactive B cells in the two models has not been completely defined but is presumably explained by differences in antigen accessibility to B cells, rather than BCR affinity for autoantigen, because the affinity of the 3–83 BCR for K<sub>β</sub> is only \( \sim 3 \times 10^9 \text{ M}^{-1} \), whereas the HEL antibody affinity is \( \sim 1,000\)-fold tighter. An additional complication in these studies was that they used conventional Ig transgenic mice, which cannot undergo receptor editing efficiently.

To clarify the fate of autoreactive B cells in an independent way that is less likely to bias results than those based on BCR transgenic mice, we developed a mouse expressing a κ superantigen restricted to liver tissue. This approach takes advantage of a custom superantigen technology in which a membrane–tethered single-chain antibody reactive to Igκ (called κ-macroself antigen) is expressed on host cells (Ait-Azzouzene et al., 2005). The strategy avoids manipulation of B cells in any way but permits analysis of their responses to tolerogen in a polyclonal immune system. The goal was to assess the ability of an Igκ–reactive macroself antigen expressed specifically on liver cells to promote B cell tolerance, with a view to establishing a general model for tolerance to tissuespecific antigen that is useful for screens of mutations affecting this process. As a proof of principle, we show that BAFF overexpression can abrogate peripheral tolerance in this system and that T cells do not facilitate tolerance.

RESULTS

Development of liver-specific κ-macroself transgenic mice

A liver-directed κ-macroself transgene (pAlb) was generated by expressing the κ superantigen protein–coding elements, described previously for a ubiquitously expressed version (pULκ; Ait-Azzouzene et al., 2005), under the control of the albumin promoter and both albumin and α-fetoprotein enhancers. This design has been successfully used to make liver-specific cre-expressing mice (Postic et al., 1999). pAlb transgenic mice were generated by microinjection of C57BL/6 (B6) zygotes. We generated 26 founder mice carrying the transgene. Four founders that had to varying extents reduced serum κ concentration were initially selected for more detailed study. The data presented in this study come from one typical line of mice derived from founder #26. First, we evaluated the tissue specificity of gene expression by quantitative RT-PCR. As shown in Fig. 1 A, κ-macroself messenger RNA (mRNA) expression was extremely specific to the liver, although we detected weak gene expression in the lung and intestine (\(<0.1\% \) as much). Next, superantigen protein expression was measured using antibody to the κ-macroself protein. As predicted based on RNA expression, pAlb splenocytes lacked detectable staining above the background defined in splenocytes from WT mice (Fig. 1 B, blue lines).
As a positive control, we show the superantigen expression on pULik splenocytes (Fig. 1 B, red lines). We confirmed that there was a high level of protein expression in liver by staining frozen sections (Fig. 1 C). The results thus show liver-specific superantigen expression in pAlb mice.

Because Igκ was absent from the sera of pAlb mice (<0.1 μg/ml; see Fig. 4 C, NL), we assessed using flow cytometry the changes in phenotypes and numbers of κ B cells in lymphoid tissues. As shown in Fig. 2, in pAlb mice, the numbers of B cells expressing κ were reduced by 80–90% in spleen (Fig. 2, A and C) and by >99% in lymph nodes (Fig. 2, A and D). In contrast, those expressing λ were barely affected, with a 50% numerical increase compared with WT in spleen (Fig. 2, B and C) and no significant difference in lymph nodes (Fig. 2 D). The increase of λ cells specifically in the spleens of pAlb mice could be explained by homeostasis of the MZ B cell compartment, as its size was normal in pAlb mice but now made up exclusively of λ cells, representing a ninefold increase of λ MZ B cells over WT (Fig. 2, E and F; note λ MZ identified here as κ−, B220+CD23+CD21hi). The remaining κ B cells found in pAlb spleens had a distinctly lower level of B220 compared with κ-negative B cells (Fig. 2 A), whereas λ cells were similar in phenotype to those of WT mice. In BM, pAlb mice lacked the B220+κ+ recirculating cell population that was resistant to cell death (Otero et al., 2006), we expected most of the remaining κ B cells in pAlb peritonea to be B1 B cells. Surprisingly however, they were comprised of similar proportions of B1 (CD19hiB220hi) and B2 (CD19+B220hi) B cells (Fig. 2 J). Serum IgG and IgM levels in pAlb mice were reduced compared with WT by 58% and 67%, respectively (IgG, 1,628 ± 296 μg/ml vs. 3,919 ± 1,169 μg/ml; IgM, 11.1 ± 1.10 μg/ml vs. 33.7 ± 9.3 μg/ml; pAlb, n = 14; WT, n = 11). Overall, the phenotype of pAlb mice was consistent with deletion of κ B cells at a late stage in B cell development outside of the BM, with a residual peritoneal κ population that was resistant to, or had possibly failed to encounter, autoantigen.

κ B cells in pAlb mice are immature and anergic

We further characterized the maturation state and functionality of κ B cells in the spleens of pAlb mice to determine whether they might be anergic or simply immature. Gating on κ+ cells revealed a lack of MZ and follicular cells and few CD93− B cells, whereas gating on splenic λ+ B cells showed a typical B cell profile, although with a higher proportion of MZ B cells (Fig. 2, E and F). In pAlb spleens, κ+ cells had significantly lower IgD expression and slightly lower IgM expression than controls (Fig. 3, A–C). This reduced BCR density was also seen in comparison with CD93− B cells of WT mice (Fig. 3 B). Moreover, most κ+ B cells in pAlb spleens had the CD23hiIgMhiIgD+CD93hi T3+ phenotype (Fig. 3 C). These cells also had high levels of CD24 (heat-stable antigen), which is characteristic of newly formed B cells (Fig. 3 B). The reduced surface Ig (sIg) levels and up-regulated MHCII levels were consistent with, and confirm, the immature status of these cells.

Figure 1. Liver-specific expression of pAlb κ superantigen transgene. RNA and protein expression analysis of κ superantigen gene expression in pAlb transgenic mouse line #26. (A) RNA was purified from the indicated tissues, and κ superantigen gene expression was determined by quantitative PCR. Shown are mean and SEM from three different mice. (B) Superantigen expression on splenocytes identified with anti–rat IgG and gated for expression of CD3 or B220. WT (gray histogram), pAlb (blue line), and pULik (red line; positive control) are shown. Similar results were obtained in five independent experiments. (C) Liver frozen sections from WT or pAlb mice were stained with FITC anti–Rat IgG1 and enhanced with Alexa Fluor 488 anti–FITC, and results were similar in three independent pairs of WT and pAlb mice.
Figure 2. Reduced numbers of κ B cells in pAlb spleen, lymph node, and peritoneum. The indicated tissues from WT, pAlb, or pUliκ mice were analyzed by flow cytometry using the indicated antibody combinations. Plots shown were gated on CD4−CD8− lymphocytes. (A and B) Flow cytometric analysis of κ (A) and λ (B) B cells in BM, spleen, and lymph nodes of pAlb mice. Top panels indicate with bottom and top boxes B220int Ig+ immature B cells and B220hi Ig+ recirculating B cells, respectively. (C and D) Statistical analysis of absolute κ and λ B cell numbers in spleen (C) and lymph nodes (D) from five WT and seven pAlb mice. (E and F) MZ B cell analysis. E shows analysis scheme defining λ MZ B cells among B220+κ− cells along with summary data in F. (G and H) Analysis of BM immature and recirculating B cell numbers in gates defined in A and B from three WT, three pAlb, and three pUliκ mice. (I and J) κ+ B cells in peritoneum (I) were further analyzed for CD19 and B220 expression levels (J). Boxes indicating CD19−B220hi (left) and CD19−B220lo (right) identify B1 and B2 B cells, respectively. Results were derived from at least three independent experiments except for the data from pUliκ mice, which were obtained in one experiment. Shown are means and SEM. *, P < 0.05; **, P < 0.005; ***, P < 0.0005; ns, not significant (two-tailed t test).
Anergy and apoptosis but no receptor editing in pAlb κ B cells. (A–J) Analysis in pAlb and WT mice of sIg levels, maturation markers, Ca^{2+} response, and RAG expression, among spleen cells (A–F) and analysis of sIg L-chains and apoptosis in liver B cells (G–J). (A) IgD and IgM expression level in B220+ B cells. (B) Cell surface markers of B220+ gated splenocytes. pAlb (red line), WT (blue line), CD93+ (black line), and negative control (gray area) are shown. HSA, heat-stable antigen. (C) Transitional cells, identified as B220+CD93+, are defined as T1-T3 and T3′ subfractions in the quadrants of the rightmost plots as follows: T1, IgM+CD23--; T2, IgM+CD23++; T3, IgMloCD23++; and T3′, IgMloCD23-. (D) Spleen cells of the indicated genotypes were loaded with Indo-1, gated on CD4−CD8−Gr-1− lymphocytes, and stimulated with anti-κ at a final concentration of 2 µg/ml. Indo-1 405/485-nm emission ratio fluorescence data were acquired at room temperature for 200 s. (E) Analysis of κ and λ expression on B220-gated pAlb or WT spleen cells. (F) Analysis of Rag1 and Rag2 expression by quantitative PCR. WT BM fraction was sorted with B220 microbeads (MACS), and κ splenocytes of the indicated mice were purified using biotinylated anti-κ followed by antibiotin microbeads. Data in A–C and E are representative of five mice/group; data in D are representative of three mice/group. F shows means and SEM of three mice/group. (G–J) Lymphocytes from mice of the indicated genotypes were isolated over density gradients from perfused livers and stained with the indicated antibodies. The gray area in J shows the level of staining on T cells. Data are representative of four mice/group.

with previous antigen encounter (Cambier et al., 2007), as was the reduction in CD19 expression (Fig. 3 B). In response to BCR ligation, these cells mobilized Ca^{2+} poorly upon stimulation with anti-κ (Fig. 3 D) or anti-IgM (Fig. S1 A); however, we were unable to detect any elevation in basal Ca^{2+} levels in Fig. S1 B). Moreover, BrdU labeling experiments indicated that these cells turned over rapidly (Fig. S1 C). These data indicated that κ B cells in spleens of pAlb mice were mainly recently formed and autoantigen–experienced anergic cells.
No obvious editing of κ B cells to λ

As the extent to which peripheral B cells might undergo receptor editing in response to an encounter with autoantigen is unclear, we assessed this by measuring RAG mRNA levels and by looking for evidence of editing at the level of light chain protein expression. We considered the possibility that, in the pAlb model, κ B cell survival might require editing in the periphery to λ or perhaps the coexpression of λ chain along with κ. However, we obtained little evidence supporting this idea. Compared with WT mice, the absolute number of λ B cells in pAlb mice was only slightly increased in spleens and not at all in lymph nodes (Fig. 2, C and D), and we failed to detect any increase in the proportion of κ/λ–double positive cells (Fig. 3 E). (As noted previously, in mice expressing the κ-macroself antigen ubiquitously, which induces significant editing, λ B cell numbers are elevated by sevenfold [Ait-Azzouzene et al., 2005].) In sorted pAlb κ+ B cells, Rag1 mRNA expression levels were equivalent to those of WT splenocytes, whereas Rag2 mRNA was slightly elevated, and both were far lower than that found in BM B cells (Fig. 3 F; note log scale). We conclude that receptor editing in pAlb spleen cells was insignificant or absent and that κ autoreactive B cells were mainly or exclusively tolerized by clonal elimination.

sIg down-regulation and apoptosis in the liver

To assess the phenotype of B cells at the site of superantigen expression, tissue-associated lymphocytes were isolated from livers of pAlb or control mice after perfusion to remove blood cells. In pAlb mice, liver B cells had no detectable sIgλ (Fig. 3 H), but many scored positive for intracellular λ (iλ; Fig. 3 I), indicating that they had down-regulated their receptors. These cells had a distinctly reduced level of B220 and CD19 compared with λ cells present in the same tissue, reminiscent of the pattern in spleen (Fig. 3, G, I, and J). Interestingly, a significant subset of CD19+ B cells in pAlb livers was positive for active caspase 3 (Fig. 3 G; 1.6 ± 0.2% in pAlb vs. 0.08 ± 0.01% in WT; P < 0.0001), suggesting that at any given time ~3–4% of κ B cells in livers of pAlb mice had initiated an apoptotic pathway after sIg engagement by superantigen. We were unable to detect evidence of λ B cell survival requiring λ chain expression in the pAlb liver, and we observed no increase in the proportion of κ/λ–double positive cells (Fig. 3 E).

Figure 4. Maternal Ig affects peripheral tolerance in pAlb mice. pAlb male mice were crossed to WT female mice, and pups were analyzed. Based on the frequency of κ+ B cells in the peripheral blood, mice were separated into two groups, nonleaky (NL) and leaky (L). (A) Representative κ and λ staining is shown for spleen and lymph nodes. (B) κ and λ B cell numbers in spleen. Shown are means and SEM of three nonleaky and five leaky mice. (C) Serum Igκ concentration (conc) from mice of the indicated types was measured by ELISA. Each point represents the value obtained from an individual mouse (NL, n = 6; L, n = 9; WT, n = 10). Horizontal lines represent means of the individual values. (D) Comparison of the transgene mRNA expression levels in the liver of the indicated mice as measured by quantitative PCR. Shown are the mean and SEM of three nonleaky and five leaky mice. **, P < 0.005; ***, P < 0.0005 (two-tailed t test).

Figure 5. No T cell requirement for peripheral B cell tolerance in pAlb mice. pAlb male mice were bred to TCR-β−/−Δ−/− mice to generate TCR-β−/−Δ−/− pAlb mice (TCR−/− pAlb mice), and B cell deletion was analyzed by flow cytometry. (A) Representative analysis of κ B cells in spleen and lymph nodes. (B) CD93 expression in κ+ B cells in spleen. TCR−/− pAlb (black line) and TCR−/− (gray area) are shown. (C) Shown is the statistical analysis of the total κ and λ cell numbers in TCR−/− and TCR−/− pAlb mice. Means and SEM of three TCR−/− pAlb and four TCR−/− pAlb mice are shown. *, P < 0.05 (two-tailed t test).
We conclude that maternal IgGs probably masked the binding site of superantigen and impaired tolerance induction in a subset of mice and that this effect did not occur in transgenic offspring of pAlb mothers because of their low serum Ig \( \kappa \) levels.

No requirement for T cells in peripheral deletion of \( \kappa \) B cells

Because some autoreactive B cells have been shown to be killed by CD4 T cells through Fas signaling (Rathmell et al., 1995), we assessed B cell deletion in pAlb mice lacking all T cells (TCR-\( \beta \)-/pAlb) generated by introducing null mutations of TCR-\( \beta \) and TCR-\( \delta \) (TCR-\( \beta \)-/\( \delta \)-/; Mombaerts et al., 1992, 1994). As in TCR-sufficient pAlb mice, \( \kappa \) cells in TCR-\( \beta \)-/pAlb mice were efficiently deleted (Fig. 5 A), and the remaining \( \kappa \) splenocytes in these mice expressed higher levels of CD93 when compared with TCR-\( \beta \)/control mice lacking superantigen (Fig. 5 B). The absolute numbers of \( \kappa \) B cells in TCR-\( \beta \)-/pAlb spleens were almost the same as in TCR-\( \beta \)/pAlb mice, but \( \kappa \) cells were increased by 30% (Fig. 5 C). No other obvious differences between T cell–sufficient and –deficient pAlb mice were seen. In TCR-\( \beta \)/pAlb mice, interestingly, pups from female TCR-\( \beta \)-/\( \delta \)-/ mice crossed to male pAlb TCR-\( \beta \)-/\( \delta \)-/ mice lacked \( \kappa \) B cell escape as described in Fig. 4 for T cell–sufficient mice, probably because of their lower maternal IgG levels. In any case, these data exclude a role for T cells in the B cell deletion in pAlb mice.

BAFF overexpression allows \( \kappa \) B cell escape in the periphery

We next investigated the effects of BAFF overexpression in the pAlb model because of the correlations between elevated
BAFF levels, autoimmune disease, and the abrogation of B cell tolerance (Lesley et al., 2004; Thien et al., 2004; Mackay and Schneider, 2009; Ota et al., 2010). Accordingly, we bred female pAlb mice with male pCD68-BAFF transgenics (Gavin et al., 2005) and analyzed λ cells in these pAlb/BAFF double transgenic mice (Fig. 6 A, right). κ B cell deletion was impaired in seven of eight analyzed pAlb/BAFF mice, as revealed by increased numbers of κ+ B cells in lymph nodes (Fig. 6, A–C). However, one pAlb/BAFF mouse had almost no κ+ B cells in the lymph nodes, indicating that in this mouse, tolerance induction was intact and that BAFF overexpression failed to promote B cell escape (Fig. 6 B).

Several other parameters indicated that κ splenic B cells of pAlb/BAFF mice had restored function. Most cells appeared more mature as they had reduced expression of CD93 (Fig. S2 C). BCR cross-linking induced a more normal Ca2+ flux in pAlb/BAFF κ+ splenocytes compared with pAlb κ+ cells (Fig. 6 F), and κ staining of splenocytes revealed a massively expanded κ+ plasma cell population (Fig. 6 D, compare boxes in the two panels on the right). These B220κhi λ B cells were confirmed to be plasma cells by their CD138hi CD19lo cell surface phenotype (Fig. 6 E).

**Periportal lymphocytic infiltrates in pAlb and pAlb/BAFF mice**

Hematoxylin and eosin (H&E) stain histological examination of the liver was performed to assess possible inflammation and hepatocyte damage at the site where the tolerogenic ligand was expressed. We detected some lymphocyte infiltration in the periportal vein areas in pAlb liver and, more frequently, in pAlb/BAFF livers (Fig. 7 A). In WT and BAFF mice, no infiltrate was observed (Fig. 7 A). We also examined mouse liver sections with anti-IgM and anti-IgG1 staining, revealing Ig deposition in pAlb/BAFF but also in BAFF samples (Fig. 7 A, bottom). Interestingly, we failed to see an increase of alanine transaminase (ALT) in the sera of either strain (Fig. 7 B), suggesting minor tissue damage and inflammation despite lymphocytic infiltration and autoantibody production.

**DISCUSSION**

Our findings support the notion that self-ligands expressed on hepatocytes promote peripheral clonal deletion of autoreactive B cells with little or no induction of receptor editing. κ B cells of pAlb mice were reduced in number in the spleen and in lymph nodes were deleted almost completely. Moreover, remaining splenic κ B cells were arrested in development at a transitional stage. At the site of superantigen expression, the liver, κ B cells were present but had down-regulated sIg and expressed a reduced density of CD19, obviously suggesting that they had encountered autoantigen. A fraction of κ liver B cells contained active caspase 3, indicating that they were in the process of apoptosis. Although active apoptosis has been seen in models of peripheral B cell deletion in response to a bolus of tolerogen of exogenous origin (Murakami et al., 1992; Tsubata et al., 1994; Finkelman et al., 1995; Goodyear and Silverman, 2004), the present model is, to our knowledge, the first to demonstrate ongoing peripheral B cell apoptosis in the steady-state.

Liver-expressed superantigen failed to promote significant receptor editing. Although in spleen Rag2 gene expression was slightly elevated, Rag1 levels were normal. Given the enrichment in pAlb spleen of immature B cells, which are known to have some residual Rag1 and Rag2 message (Yu et al., 1999), the requirement for both Rag-1 and -2 for functional recombine activity, and the fact that most of the population was effectively autoreactive, the slight difference in Rag2 mRNA was not consistent with extensive ongoing editing.
Analysis of λ B cell numbers as a surrogate marker for receptor editing gave a similar picture: although λ cell number was slightly increased in the spleens of pAlb and TCR-/- pAlb mice, no such increase was seen in lymph nodes. We ascribe the modest increase in λ cells in the spleens not to editing but rather to expansion of λ MZ B cells to fill that compartment. However, it is difficult to completely exclude the possibility that editing occurs in a small subset of B cells. Nor can we formally exclude the possibility that extra BM editing might occur in B cells encountering autoantigens in peripheral tissue other than liver or because of contact with antigens in special forms, although we suspect that this is unlikely. We conclude that receptor editing in response to the superantigen was negligible, instead κ B cells predominantly underwent clonal deletion, likely preceded by a short-lived anergic phase.

Our data suggest that deletion in pAlb mice might require multiple antigenic hits over time. Despite the absence of superantigen expression in the spleen, splenic κ B cells showed signs of antigen experience, including BCR down-modulation, MHCII up-regulation, and BCR desensitization as manifested by reduced BCR-triggered Ca²⁺ responses. Furthermore, among the clearly antigen-experienced, Sgk down-modulated B cells of liver, only a small subset expressed caspase 3. To explain these features, we imagine that B cells might leave the liver after a first pass, returning through the blood to the spleen in an anergic state, possibly later reentering the circulation whence they again encounter liver antigens. (Anergic B cells are known to have altered migration patterns, including improved chemotaxis to CCR7 ligands [Cyster et al., 1994; Fulcher et al., 1996; Reif et al., 2002].) Eventually, a certain proportion of κ B cells undergo apoptosis. This scenario would explain why the splenic κ population is largely made up of anergic cells with high MHCII levels and only a small subset of liver κ B cells were actively apoptotic despite their uniformly profound Sgk down-regulation. It is likely that most B cells encounter liver antigens before their migration to the spleen because of the liver's more significant blood flow and volume, including massive inputs from both the hepatic artery and portal vein. It is unlikely that soluble, shed superantigen is responsible for the effects on splenic κ B cells because (a) it is present at too low a level to explain these results and (b) significant levels should have affected BM development and triggered editing. (Using a reagent that sees the rat IgG1Fc tag portion of the superantigen, we measured serum superantigen at 93 ± 44 ng/ml, but this material lacked detectable Igk binding activity [<1 ng/ml].) In a previous study in 3-83 BCR transgenic mice, we found that an anergic phenotype was induced when the frequency of cells carrying cognate membrane autoantigen was low, but when this frequency was high, it led to deletion and receptor editing (Lang and Nemazee, 2000). Because changing the frequency of antigen–carrying cells should not greatly affect the strength of signal caused by any particular encounter between B cells and antigen–carrying cells, we proposed that the frequency of encounter of antigen by B cells was important. Also consistent with this model was the finding that in pAlb mice, a small subset of κ⁺ cells in the peritoneal cavity appeared to be resistant to deletion and included both B-1 and conventional B cells. We suggest that these cells escaped deletion because their trafficking history resulted in less frequent exposure to the liver, possibly because of preferential trafficking to the gut tissues, where the superantigen is not expressed.

The deletion of κ B cells was clearly T cell independent. Previous studies in the MD4/soluble HEL model suggested that cognate helper T cells might be toxic to anergic B cells (Rathmell et al., 1995, 1996), whereas a more recent study in the same system suggested that bystander T cells promote improved survival of these same anergic cells (Lesley et al., 2006). Our results fit better with the latter study, although we have not specifically explored the effects of cognate helper T cells.

The κ B cells in the spleens of pAlb mice were, like sullen teenagers, semi-mature and anergic. Previously described anergic cells are thought to have a short half-life, require continuous presence of autoantigens to maintain the tolerant state (Goodnow et al., 1991; Fulcher and Basten, 1994; Gauld et al., 2005), and are identified as CD23⁻IgM⁺CD93⁻ T3 cells (Merrell et al., 2006). In BCR transgenic mice, anergic B cells were characterized as having reduced SgkM expression, near normal IgD, elevated MHCII expression, and impaired Ca responses to BCR ligation (Goodnow et al., 1988; Healy et al., 1997; Cornall et al., 1998; Benschop et al., 2001; Seo et al., 2003). The κ B cells of pAlb spleens had elevated MHCII levels, reduced Sgk, rapid turnover, and blunted Ca²⁺ responses, but their surface phenotype differed from anergic B cells described in other models (Cambier et al., 2007), in that they had a T3’ phenotype, CD23⁻CD23⁻CD93⁻IgM⁺IgD⁻ (Kiefer et al., 2008). In addition, we were unable to detect an elevated basal level of Ca²⁺ (Fig. S1B), which has been described in some anergic B cells continually exposed to soluble antigen (Healy et al., 1997). Cells with the T3’ phenotype represent a small subset in WT mice and were defined in 3H9/Vk8 and 3H9-56R/Vk8 anti-DNA site-directed transgenic mice, in which they are more numerous (Kiefer et al., 2008; Ota et al., 2010). The differences in models might be the result of differences in the nature of the BCR transgenics studied, as B cells had already encountered cognate antigen in the BM in those studies and had continuous exposure to tolerogen in the spleen.

As this study demonstrates, superantigens facilitate the study of lymphocyte responses by allowing the stimulation of a significant subset of cells in a polyclonal repertoire. One advantage of their use over alternative techniques, such as BCR transgenic models, is that superantigens do not perturb B cell development before their encounter. Custom designed superantigen transgenics have the additional advantage of facilitating expression in a controlled and tissue-specific way. Previous studies of peripheral B cell tolerance of tissue-restricted membrane self-antigens made use of conventional Ig transgenic mice (Russell et al., 1991; Akkaraju et al., 1997), which are nonideal systems in terms of permitting efficient receptor editing. Moreover, they usually skew B cell lineages in favor of particular subsets even in the absence of cognate autoantigen.
and often lead to premature BCR expression and accelerated B cell development. In pAlb mice, liver tissue antigens, rather than B cells, were modified, which should have permitted normal B cell development up to the point that B cells encountered the superantigen, including providing Ig genes in a normal genomic context that should facilitate receptor editing. In any case, the fact that the B cell tolerance phenotype of the pAlb mice was very similar to previously investigated 3-83/liver-Kb models (Russell et al., 1991; Kouskoff et al., 2000) suggests that there was no fundamental difference between κ superantigen and native self-antigen (Kb) in the ability to promote tolerance.

Using targeted Ig genes that changed BCR specificity upon interferon-induced cre/lox-mediated inversion, Lam and Rajewsky (1998) assessed the fate of mature B cells that acquired the 3-83 receptor on a Kb background. Consistent with our findings, these authors concluded that the autoreactive B cells underwent deletion. Similar results were obtained in a model in which B cells in normal adult mice were challenged by transfection with cells carrying an IgD-macroself antigen (Duong et al., 2010). These studies were potentially capable of detecting elevated editing in mature peripheral B cells but failed to do so.

Maternal IgGs affected tolerance of κ B cells in pAlb mice, presumably by masking liver-expressed superantigen. As a consequence, in the offspring of WT females, κ B cells appeared to significantly escape deletion and to populate lymphoid nodes, an effect which was retained long after weaning and thus was likely perpetuated by host κ Ig and/or κ B cell production (Fig. S2, A and B). However, the effect was incomplete. Compared with WT, these mice had a lower frequency of κ B cells and a higher frequency of λ cells. In contrast, offspring of female pAlb mice were fully tolerant, with minimal escape from deletion and little or no serum Igκ. In any case, these findings support the suggestion (Wang and Shlomchik, 1998) that maternal IgG autoantibodies might imprint offspring to B cells in the blood. The slow blood flow and fenestrated surfaces of hepatocytes, and the liver is known to be particularly tolerant to T cells (Thomson and Knolle, 2010). Moreover, this tissue is usually quite resilient, with the ability to regenerate after damage. As this study shows, the liver appears to be quite resistant to the negative effects of autoantibodies. The liver is also a significant potential host cell target of gene therapies. Adenovirus vectors and even naked plasmid DNA can be taken up and expressed by hepatocytes (Liu et al., 1999; Zhang et al., 1999; Ota et al., 2009; Somanathan et al., 2010). Our study suggests that expression of autoantigens or macroself antigens of interest in the liver might be an interesting way to treat diseases caused by tissue-specific autoimmune B cells and their antibodies.

**Materials and Methods**

**Mice.** 8–12 wk-old mice were used in most experiments. C57BL/6J mice were purchased from the Scripps Research Institute breeding colony. pAlb mice were generated on the C57BL/6J background (see next section). All other transgenic and mutant strains used were backcrossed at least 10 generations onto C57BL/6J. BAFF transgenic mouse line MB21 (Gavin et al., 2005) was bred and maintained in the Scripps Research Institute Animal Resources facility according to The Scripps Research Institute Institutional Animal Care and Use Committee guidelines. TCR-β−/−δ−/− mice (B6.129P2-Tcrb−/−δ−/−Tkr118bw/J) were obtained from the Jackson Laboratory.
Transgenic mouse generation. The albumin enhancer and α fetoprotein enhancer II region were amplified from B6 genomic DNA using primer pairs A62 AlbF (5′-AAAGGACTCTTAATTAACCTA-GCTTCTTACAGTGGACCTTCCA-3′) and A63 AlbR (5′-TTTGGCGACTGATTCGAGGAAGATCTTTCAAG-3′) and A64 AfpF (5′-TTTTCTTGCCATGATTTAATACGTTTACATAATCAGCTTCCACACTTG-3′), respectively. All cloned products were confirmed by sequencing. The albumin fragment was digested with BamHI and Spel and cloned into BglII–BamHI-digested pLIVE plasmid (Mirus Bio LLC). Subsequently, the α fetoprotein fragment was digested with XhoI and NdeI and cloned into XhoI–NdeI-digested plasmid carrying the albumin enhancer. Sall–SacI-digested κ superantigen fragment including the poly A site was then introduced to this vector to yield pLIVE3-187.1. This fragment was digested with Pael and used to generate pALb mice by microinjection of B6 zygotes. 24 founders were obtained. Data presented here were from a single representative line.

Flow cytometry analyses. Flow cytometric analyses for surface markers were performed using standard protocols as previously described (Duong et al., 2010). To visualize intracellular proteins, cells were first permeabilized using the Cytofix/Cytperm kit (BD) according to manufacturer’s recommendations. The following mAbs were used: CD4 (RM4-5; BioLegend), CD21 (7E9; FITC; eBioscience), CD23 (B3B4; PE; eBioscience), CD86 (GL1; PE; BD), CD93 (AA4.1; PE or allophycocyanin; eBioscience), IgD (11-26; PE; eBioscience), Igk (181.7; FITC or Pacific blue; BD), and Igλ (RLM-42; APC; BioLegend). mAbs against mouse IgM (M41; Alexa Fluor 488) and B220 (Pacific blue) were labeled in-house. To gate out most T cells, in some analyses we included a dump antibody mixture consisting of PerCP/Cy5.5-labeled mAbs against CD4 (RM4-5; BioLegend) and CD8 (53-67; BioLegend). All samples were read on an LSR-II instrument (BD) and analyzed using the FlowJo program (Tree Star, Inc.).

Ca2+ flux assays. Total spleen cells isolated from pALb mice and their nontransgenic littermates were pooled and resuspended at 2 × 107 cells/ml in RPMI. A total of 107 spleen cells was then preincubated with 1.5 µM Indo-1 labeled CD4, CD8, and APC–anti-mouse IgG1 κ (MG1-45; BioLegend) was used as a standard. Horseradish peroxidase–conjugated anti-κ (187.1; BD) diluted in ELISA buffer was used to report signals using 1-Step Ultra-TMB substrate (Thermo Fisher Scientific) per the manufacturer’s instructions. Signals were recorded at 450 nm using a microplate reader (VersaMax; MDS Analytical Technologies).

Isolation of liver lymphocytes. Isolation of intrahepatic lymphocytes was performed as previously described (Isogawa et al., 2005). In brief, livers were perfused with 10 ml of FACS buffer via the portal vein to remove circulating lymphocytes, and the liver cell suspension was pressed through a 70-µm cell strainer and digested with 10 ml of RPMI 1640 medium (Invitrogen), containing 0.02% (wt/vol) collagenase IV (Sigma-Aldrich) and 0.002% (wt/vol) DNAse I (Sigma-Aldrich), for 40 min at 37°C. Cells were washed with FACS buffer and then overlaid on lympholyte-M (Cedarlane). After centrifugation for 20 min at 1,500 g, the intrahepatic lymphocytes were isolated at the interface, washed twice with FACS buffer, and used for further analysis.

Histology. Perfused liver was fixed (Z-FIX; Anatech) and H&E sections were processed at the Scripps Research Institute Histology Core. Direct immunofluorescence was performed to examine the in vivo deposition of IgG/M on the hepatocytes. The perfused livers were embedded in OCT compound (Sakura). Each sample was incubated with a 100-fold dilution of Alexa Fluor 488–anti-IgM (M41), FITC IgG1 (A85-1; BD), or FITC rat–IgG1 (RG11/39.4; BD). The specimens were examined using a fluorescence microscope (Eclipse E8000; Nikon).

Online supplemental material. Fig. S1 shows analysis of Ca2+ mobilization and BrdU uptake in κ† B cells of pALb spleen, documenting their hyporesponsive and rapid turn-over. Fig. S2 shows reanalysis of leaky mice at 5 mo of age, documenting the stability of their phenotype after maternal antibody should be absent. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20102265/DC1.

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