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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Abbreviations used: ALT, alanine transaminase; BAFF, B cell–activating factor belonging to the tumor necrosis factor family; HEL, hen egg lysozyme; iκ, intracellular κ; mRNA, messenger RNA; MZ, marginal zone; sIg, surface Ig.

B cells develop in the BM and fetal liver, generating antigen receptors with random combinations of heavy and light chains. As a consequence, many BCRs initially have affinity for self-tissues. Cells carrying autoreactive receptors are regulated by mechanisms including apoptosis, induction of anergy, or receptor editing. Receptor editing was first described as a process of secondary light chain gene rearrangement in immature autoreactive B cells of the BM resulting in the rescue of cells with reduced autoreactivity (Gay et al., 1993; Tiegs et al., 1993). Experiments in mice and humans showed that 20–50% of all developing B cells undergo editing (Harada and Yamagishi, 1991; Prak and Weigert, 1995; Retter and Nemazee, 1998; Bräuninger et al., 2001; Casellas et al., 2001; Oberdoerffer et al., 2003; Halverson et al., 2004; Wardemann et al., 2004). However, not all autoantigens are present in the BM and fetal liver. Many B cells confront self-antigens in the periphery and must be regulated by tolerance at later stages of development. B cell tolerance against specific tissue antigens was shown using several models, but the results varied from deletion to anergy to clonal ignorance (Russell et al., 1991; Akkaraju et al., 1997; Lang et al., 1997; Rojas et al., 2001). Even after these selections, a significant fraction (5–20%) of mature naive B cells are reported to retain self-reactivity (Dighiero et al., 1983; Rolink et al., 1987; Souroujon et al., 1988; Guigou et al., 1991; Hayakawa et al., 1999; Wardemann et al., 2003). Tolerance triggered by autoantigen has also been proposed to promote editing at even later

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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 $IgM^{lo}CD23^{+}CD93^{+}$ (Allman et al., 2001), and the recently defined $IgM^{lo}CD23^{lo}CD93^{+}$ 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/Vκ8 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^{b}, 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^{b} is only $\sim 3 \times 10^{5}\ M^{-1}$, whereas the HEL antibody affinity is ~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 (Aït-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 tissue-specific 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 (pUIiκ; Aït-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).

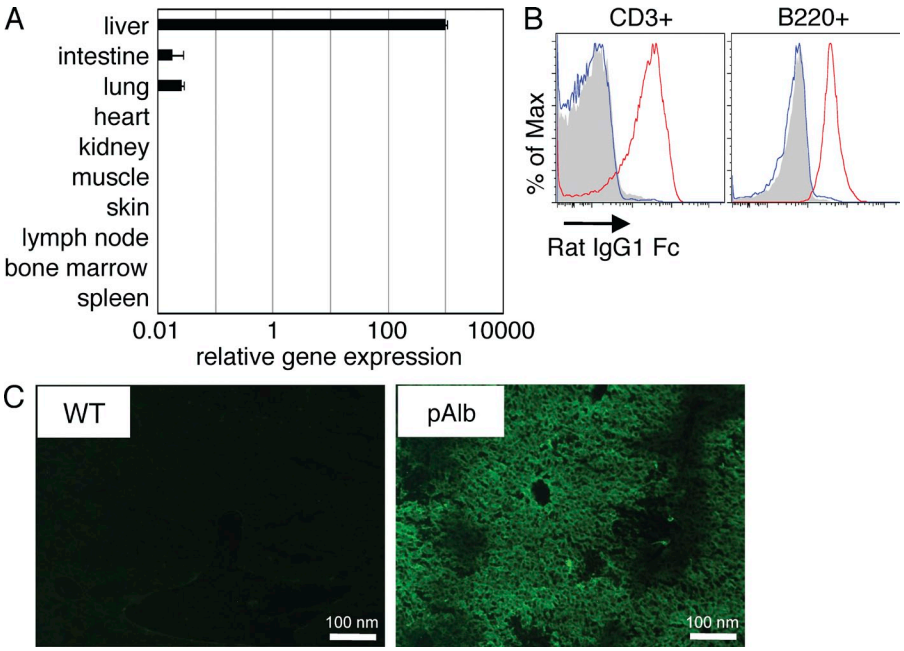

Figure 1. Liver-specific expression of pAlb κ superantigen transgene. RNA and protein expression analysis of κ superantigen gene expression in pAlb transgenic mouse line #26. (A) RNA was purified from the indicated tissues, and κ superantigen gene expression was determined by quantitative PCR. Shown 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 pUIiκ (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.

As a positive control, we show the superantigen expression on pUIiκ 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−, $B220^{+}CD23^{lo}CD21^{hi}$). 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 $B220^{hi}κ^{+}$ recirculating cell population (Fig. 2, A and H), but other compartments were normal, including all compartments of λ cells (Fig. 2, B, G, and H), indicating that the perturbations caused by superantigen expression were restricted to late stages of κ B cell maturation. For comparison, we show the analysis of κ and λ cells in pUIiκ mice, in which B cells encounter superantigen in the BM and undergo central tolerance with receptor editing (Fig. 2, A and B). In contrast to pUIiκ mice, the BM of pAlb mice lacked a significant increase in newly formed, $B220^{int}$ λ BM cells or the massive (approximately sevenfold) increase in recirculating λ cells characteristic of pUIiκ mice (Fig. 2, B, G, and H; Aït-Azzouzene et al., 2005). In peritoneum, κ B cell frequencies in pAlb mice were reduced by ∼90% (Fig. 2 I). As B1 cells are positively selected by some autoantigens (Hayakawa et al., 1999) and are generally 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 ($CD19^{hi}B220^{lo}$) and B2 ($CD19^{+}B220^{hi}$) 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 $CD23^{lo}IgM^{lo}IgD^{+}CD93^{hi}$ 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

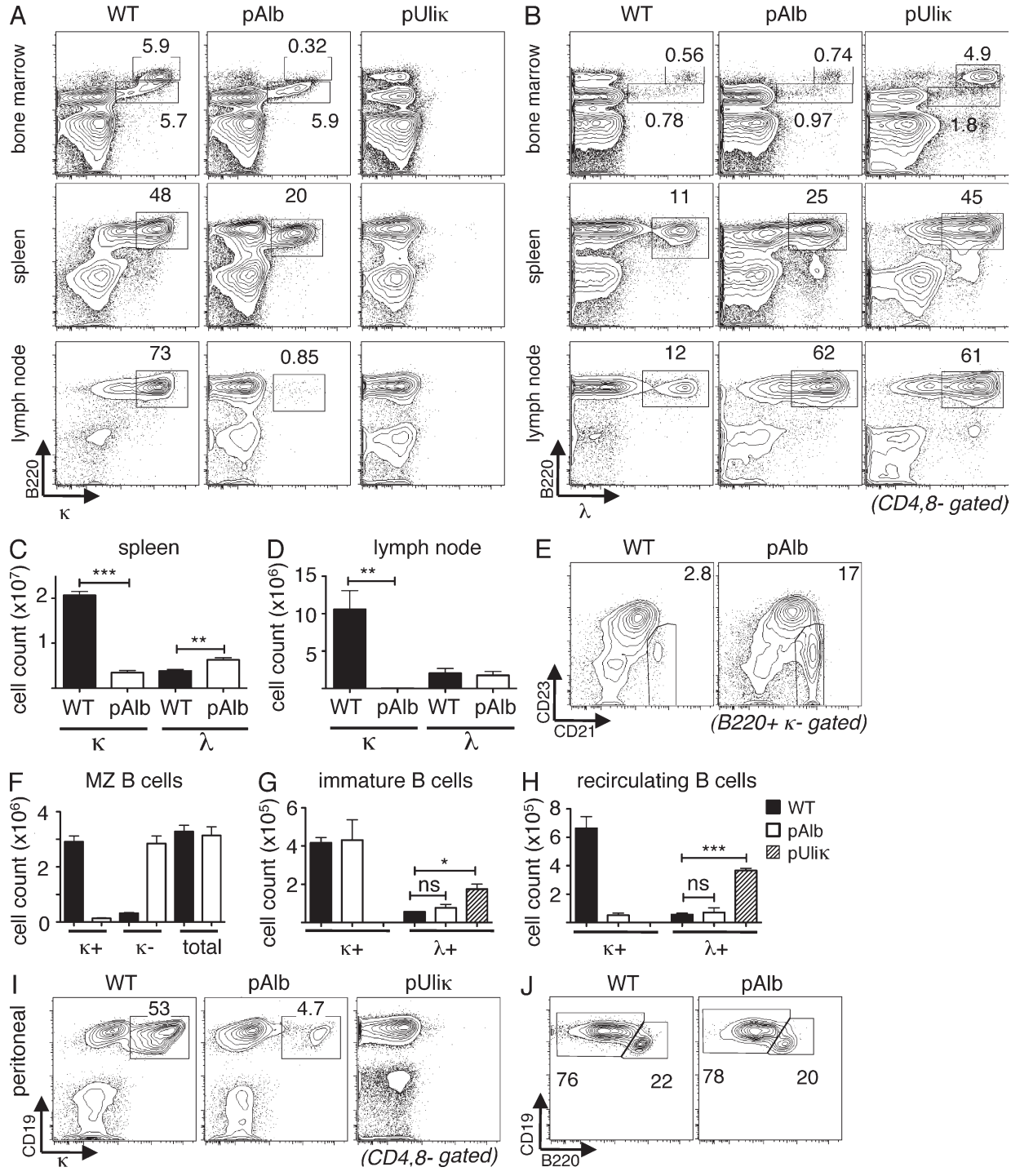

Figure 2. Reduced numbers of κ B cells in pAlb spleen, lymph node, and peritoneum. The indicated tissues from WT, pAlb, or pUliκ mice were analyzed by flow cytometry using the indicated antibody combinations. Plots shown were gated on CD4⁻CD8⁻ lymphocytes. (A and B) Flow cytometric analysis of κ (A) and λ (B) B cells in BM, spleen, and lymph nodes of pAlb mice. Top panels indicate with bottom and top boxes B220^{int}sIg^{+} immature B cells and B220^{hi}sIg^{+} 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^{+}B220^{int} (left) and CD19^{+}B220^{hi} (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).

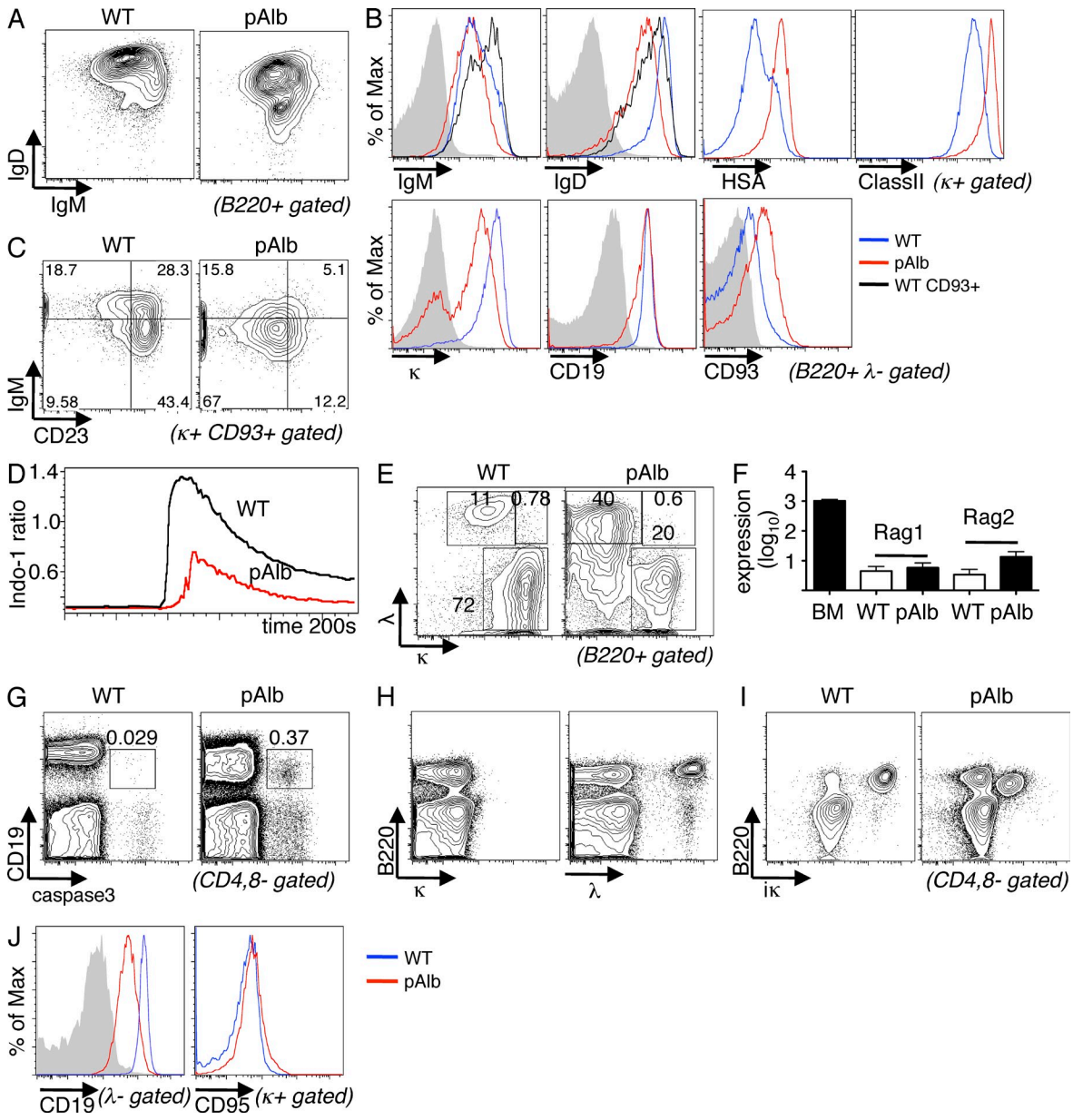

Figure 3. Anergy and apoptosis but no receptor editing in pAlb κ B cells. (A–J) Analysis in pAlb and WT mice of sIg levels, maturation markers, Ca^{2+} response, and RAG expression, among spleen cells (A–F) and analysis of sIg L-chains and apoptosis in liver B cells (G–J). (A) IgD and IgM expression level in $B220^+$ B cells. (B) Cell surface markers of $B220^+\lambda^-$ 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, $IgM^{lo}CD23^+$; and T3′, $IgM^{lo}CD23^-$. (D) Spleen cells of the indicated genotypes were loaded with Indo-1, gated on $CD4^-CD8^-Gr\text{-}1^-\lambda^-$ 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.

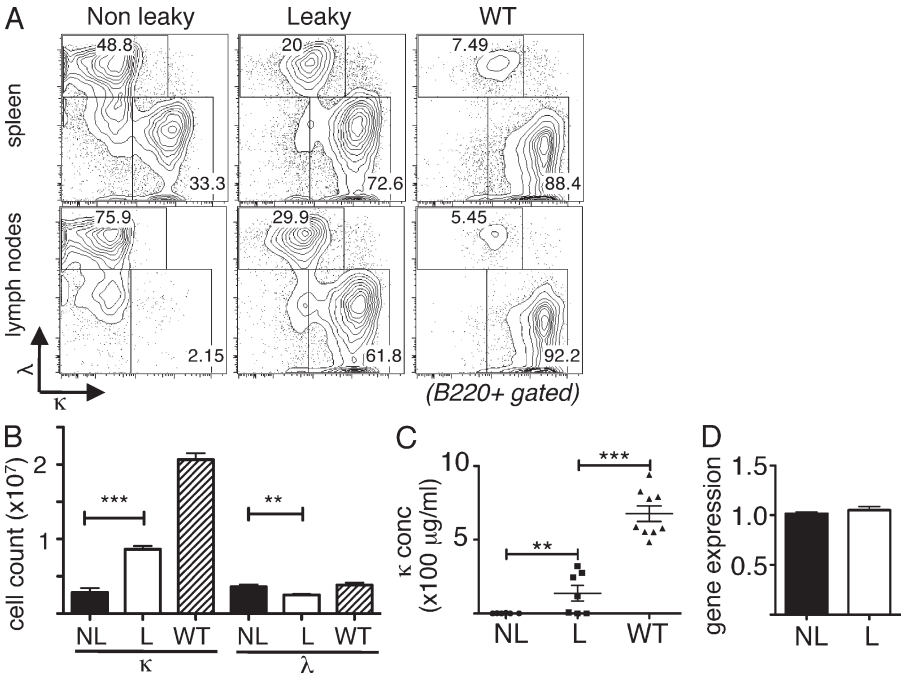

Figure 4. Maternal Ig affects peripheral tolerance in pAlb mice. pAlb male mice were crossed to WT female mice, and pups were analyzed. Based on the frequency of κ+ B cells in the peripheral blood, mice were separated into two groups, nonleaky (NL) and leaky (L). (A) Representative κ and λ staining is 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).

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 [Aït-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 sIgκ (Fig. 3 H), but many scored positive for intracellular κ (iκ; 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

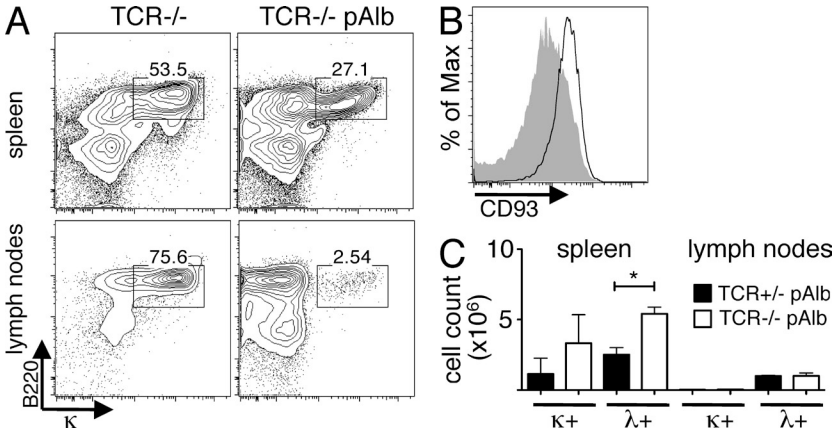

Figure 5. No T cell requirement for peripheral B cell tolerance in pAlb mice. pAlb male mice were bred to TCR-$\beta^{-/-}\delta^{-/-}$ mice to generate TCR-$\beta^{-/-}\delta^{-/-}$ pAlb mice ($TCR^{-/-}$ pAlb mice), and B cell deletion was analyzed by flow cytometry. (A) Representative analysis of κ B cells in spleen and lymph nodes. (B) CD93 expression in κ+ 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).

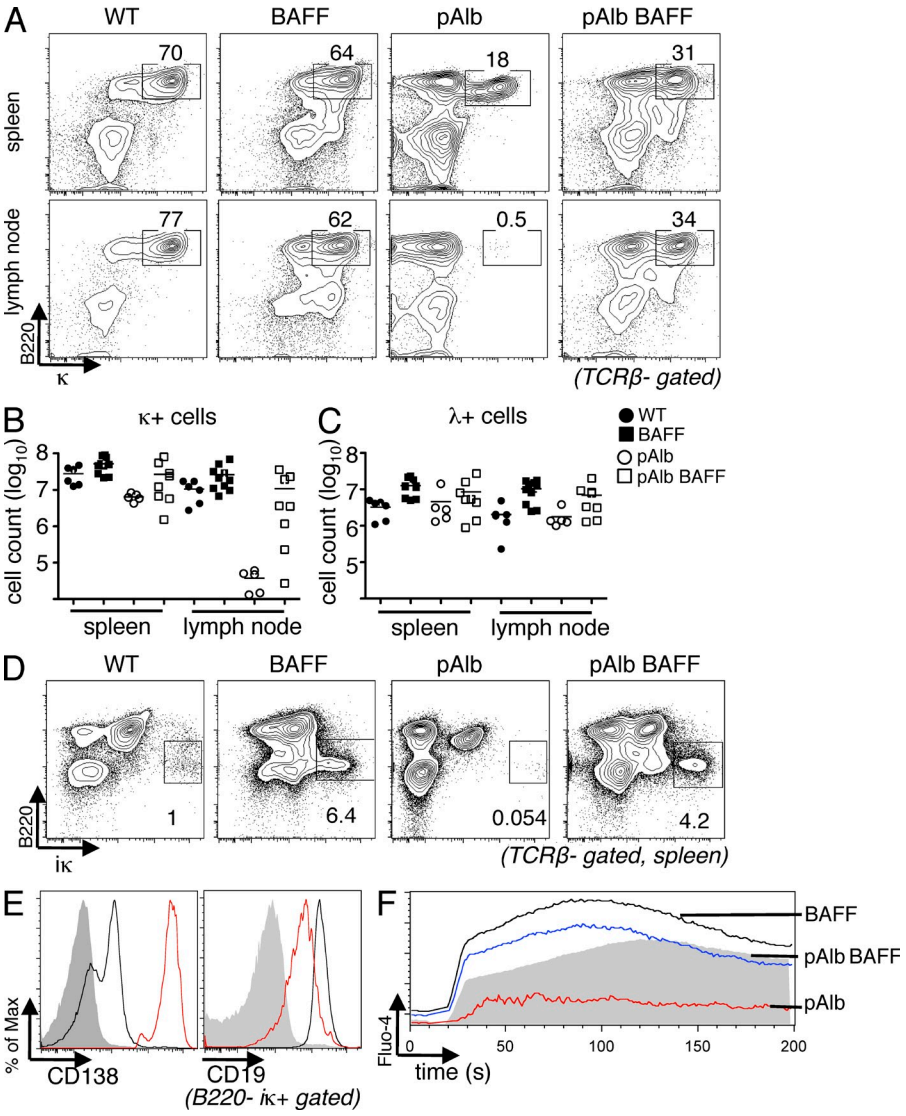

Figure 6. Overexpression of BAFF allows κ B cell escape from tolerance. Analysis of peripheral B cell tolerance in pAlb/BAFF double transgenic mice. (A–D) Flow cytometry analysis of κ B cell numbers in spleen and lymph node cells. For iκ staining (D), surface κ was blocked by unlabeled antibody (187.1) before fixation and permeabilization. (B and C) Horizontal lines represent means of the individual values. (E) CD138 and CD19 expression on $B220^{lo}i\kappa^{+}$ gated cells (red line, $B220^{lo}i\kappa^{+}$; black line, $B220^{+}$; gray area, TCR-β^{+}). (F) Ca response in κ^{+} B cells from mice of the indicated genotypes. Cells were gated to CD4, CD8, Gr-1, λ-negative population (gray area, WT). Data were obtained from the following numbers of mice/group: $n = 6$ WT, $n = 5$ pAlb, $n = 10$ BAFF, and $n = 8$ pAlb/BAFF.

to detect a difference in active caspase 3 in the spleens of pAlb mice, presumably because such cells were rapidly phagocytosed or because the rate of apoptosis was lower.

Maternal IgG affects the efficiency of peripheral tolerance

Because maternally derived IgG/κ Igs transported to the fetus might mask the κ superantigen expressed in the liver, diminishing tolerance induction, in most experiments, we avoided this by crossing the female pAlb transgenics to male B6 WT mice. However, to assess the potential effect of maternal IgGs, we tested offspring of male pAlb bred to B6 females. In these (WT × pAlb)F1 pups, 7 out of 13 (54%) had more κ B cells in the periphery (Fig. 4, A and B) than did (pAlb × WT)F1 mice. In the subset with higher levels of κ cells, κ B cells were detected in lymph nodes, and serum Igκ was detected, albeit at significantly lower levels than in WT mice (Fig. 4 C; indicated in the figure as "leaky" [L], as opposed to "nonleaky" [NL], i.e., mice with low κ B cell levels). Leaky and nonleaky mice did not differ in their superantigen mRNA expression (Fig. 4 D). 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 Igκ levels.

No requirement for T cells in peripheral deletion of κ 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 ($TCR^{-/-}$ pAlb) generated by introducing null mutations of TCR-β and TCR-δ (TCR-$\beta^{-/-}\delta^{-/-}$; Mombaerts et al., 1992, 1994). As in TCR-sufficient pAlb mice, κ cells in $TCR^{-/-}$ pAlb mice were efficiently deleted (Fig. 5 A), and the remaining κ splenocytes in these mice expressed higher levels of CD93 when compared with $TCR^{-/-}$ control mice lacking superantigen (Fig. 5 B). The absolute numbers of κ B cells in $TCR^{-/-}$ pAlb spleens were almost the same as in $TCR^{+/-}$ pAlb mice, but λ cells were increased by 30% (Fig. 5 C). No other obvious differences between T cell–sufficient and –deficient pAlb mice were seen. In $TCR^{-/-}$ pAlb mice, interestingly, pups from female TCR-$\beta^{-/-}\delta^{-/-}$ mice crossed to male pAlb TCR-$\beta^{-/-}\delta^{-/-}$ mice lacked κ 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 κ B cell escape in the periphery

We next investigated the effects of BAFF overexpression in the pAlb model because of the correlations between elevated

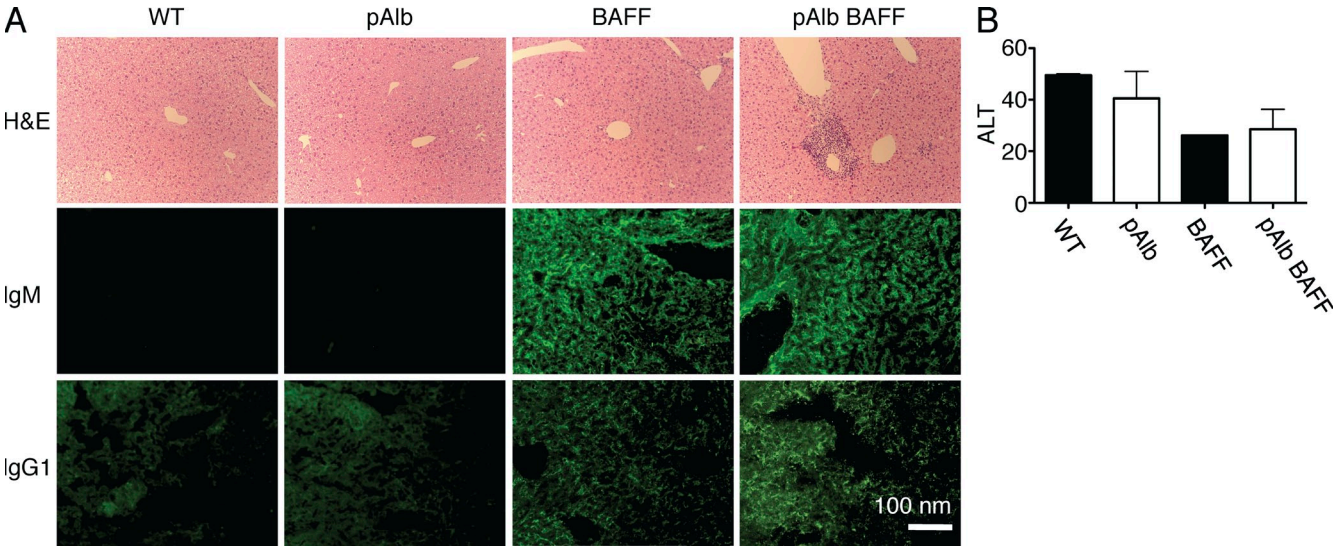

Figure 7. Periportal vein lymphocytic infiltration in pAlb/BAFF double transgenic mice. pAlb, BAFF, pAlb/BAFF, and WT mice were compared for inflammation and Ig deposition in liver. (A) Perfused livers were either fixed and analyzed by H&E staining (top) or frozen with Tissue-Tek and stained with Alexa Fluor 488–rat anti–mouse IgM or with FITC–rat anti–mouse IgG1. (B) ALT levels in sera of mice of the indicated genotypes. Three mice of each genotype were analyzed in these experiments. Error bars indicate SEM.

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 κ 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 Ca^{2+} flux in pAlb/BAFF κ^+ splenocytes compared with pAlb κ^+ cells (Fig. 6 F), and iκ staining of splenocytes revealed a massively expanded κ^+ plasma cell population (Fig. 6 D, compare boxes in the two panels on the right). These $B220^{lo}i\kappa^{hi}$ B cells were confirmed to be plasma cells by their $CD138^{hi}$ $CD19^{lo}$ 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 recombinase 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 Ca^{2+} responses. Furthermore, among the clearly antigen-experienced, sIgκ 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 sIg 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 Igκ 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^{hi}IgM^{lo}CD93^{hi} T3 cells (Merrell et al., 2006). In BCR transgenic mice, anergic B cells were characterized as having reduced sIgM 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 sIg, rapid turnover, and blunted Ca^{2+} 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, CD21$^-$CD23$^-$CD93$^+$IgM^{lo}IgD^{int} (Kiefer et al., 2008). In addition, we were unable to detect an elevated basal level of Ca^{2+} (Fig. S1 B), 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/Vκ8 and 3H9-56R/Vκ8 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 transgenes 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-K^b models (Russell et al., 1991; Kouskoff et al., 2000) suggests that there was no fundamental difference between κ superantigen and native self-antigen (K^b) 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 K^b 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 transfusion 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 lymph 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 in a way that similarly diminishes B cell tolerance to their targets and ultimately increases the risk of autoimmunity.

The phenotype of κ B cells in the peritoneal cavity of pAlb mice defied our predictions in that they included not only B1 cells but also B2 cells. B1 cells are well known to be enriched in cells with weak self-reactivity, likely owing to active positive selection by self-ligands and a resistance to apoptosis (Hayakawa et al., 1999; Otero et al., 2006). However, in pAlb mice, we saw a reduced frequency of κ B cells with little change in the B1/B2 ratio. The surface phenotype of these remaining κ cells was not indicative of antigen encounter. As the κ superantigen, when accessible, engages the BCRs of these cells similarly, we conclude that the cells probably escaped by clonal ignorance owing to their distinct migratory properties rather than any intrinsic BCR signaling differences.

We found that in pAlb mice, the overexpression of BAFF could rescue κ B cells from anergy and deletion and promote their differentiation to plasma cells. It is interesting to compare this result with the inability of the same BAFF transgene to overcome tolerance to an IgD-macroself antigen (Duong et al., 2010). In that case, tolerogen was ubiquitously expressed and encountered at the T1/T2 transitional stage, whereas here the B cells encountering autoantigen were clearly more mature and BAFF responsive. However, we cannot yet definitively exclude the possibility that in the pAlb/BAFF mice the frequency of competing, nonautoreactive B cells was too low compared with the IgD-macroself model to allow normal competition-dependent homeostasis. In pAlb/BAFF double transgenic mice, direct immunofluorescence analysis revealed binding of IgM and IgG1 to hepatocytes. However, similar binding was seen even in the absence of superantigen in BAFF singly transgenic mice. Although other BAFF transgenic models have been seen to have immune complexes and Ig deposition in kidney (Mackay et al., 1999), to our knowledge, the liver has not been described as an autoantibody target. A key difference between pAlb/BAFF and BAFF mice was the lymphocyte infiltrations observed in the periportal vein area of the former mice, which bear some similarities to autoimmune hepatitis. Ig deposition was observed, but serum ALT levels were not elevated, and histological analysis of H&E-stained sections revealed no obvious cell necrosis. Presumably, hepatocytes are able to limit further inflammation caused by Ig deposition. Autoimmune hepatitis is characterized by autoantibodies to nuclear antigens, smooth muscle, and liver/kidney microsomes and is accompanied by chronic inflammatory cell infiltration to the liver. Therefore, BAFF transgenic mice may be a good model for the predisease stage of this disease, whereas the pAlb/BAFF double transgenic mice could be an interesting model for the more advanced disease stage.

The liver may be an especially efficient organ for the induction of B cell tolerance because of its size and accessibility to B cells in the blood. The slow blood flow and fenestrated hepatic endothelia directly expose blood-borne cells to the surfaces of hepatocytes, and the liver is known to be particularly tolerogenic 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 autoreactive 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-$\beta^{-/-}\delta^{-/-}$ mice (B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/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′-AAGGAGGATCCTTAATTAACTAGCTTCCTTAGCATGACGTTCCA-3′) and A63 AlbR (5′-TTTCGGACTAGTCAAGCTGGAGAACGAGTTCAAGC-3′) and A64 AfpF (5′-AAGGGACTCGAGCCTGAAAGAGTGAAAGTCATTTTCTGC-3′) and A65 AFpR (5′-TTTCTTGGCATATGTTAATTAAATCCCATAATCAGCTTCCAAACGCTG-3′), respectively. All cloned products were confirmed by sequencing. The albumin fragment was digested with BamH1 and SpeI and cloned into BglII–BamH1-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. SalI–SacI-digested κ superantigen fragment including the poly A site was then introduced to this vector to yield pLive3-187.1. This plasmid was digested with PacI 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/Cytoperm kit (BD) according to manufacturer's recommendations. The following mAbs were used at 1:200 in all experiments described: B220 (RA3-6B2; FITC; BioLegend), CD19 (1D3; PE/Cy7; eBioscience), CD21 (7E9; FITC; eBioscience), CD23 (B3B4; PE; eBioscience), CD86 (GL1; PE; BD), CD93 (AA4.1; PE or allophycocyanin; eBioscience), IgD (11-26; PE; eBioscience), Igκ (187.1; 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 (53-6.7; BioLegend). All samples were read on an LSR-II instrument (BD) and analyzed using the FlowJo program (Tree Star, Inc.).

Ca^{2+} flux assays. Total spleen cells isolated from pAlb mice and their nontransgenic littermates were pooled and resuspended at 2×10^7 cells/ml in RPMI. A total of 10^7 spleen cells was then preincubated with 1.5 μM Indo-1 (Invitrogen), 5 μg Fc Block (2.4G2; BD), and 1 μg each of PerCP/Cy5.5-labeled CD4, CD8, and APC–anti-λ for 30 min at 37°C, followed by another 30 min at room temperature. Aliquots of 4×10^6 cells (0.5 ml) were then stimulated at room temperature with 2 or 10 μg/ml anti–mouse IgM (M41) or anti–mouse κ (187.1) in IgG form. Ca^{2+} signals were recorded for 200 s, gating on CD4/CD8/Gr-1/λ-negative cells and measuring the 405/485-nm emission ratio of Indo-1 fluorescence upon UV excitation.

Quantitative RT-PCR. $κ^+$ and $B220^+$ B cells were purified from spleen or BM using biotinylated anti-κ plus antibiotin microbeads or with B220 microbeads (Miltenyi Biotec), respectively, and their total RNA was isolated using RNeasy Plus (QIAGEN) per kit instructions. RT was performed with a QuantiTect RT kit (QIAGEN) per the manufacturer's protocol. PCR reactions were performed using GoTaq quantitative PCR (Promega) and analyzed on the 7900HT Fast Real-Time PCR system (Applied Biosystems). Annealing temperature per cycle was set to 60°C. Quantifications of *RAG1* and *RAG2* mRNA were normalized with concomitant amplifications of *β-actin* message in each triplicate sample. The following primers were used: RAG1 F, 5′-GGAGGCCTGTGGAGCAAGGTAG-3′; RAG1 R, 5′-GTTCTGACCACCAGGCTTCTCT-3′; RAG2 F, 5′-CCCAGTGCATGGATTTGGAAGAAC-3′; RAG2 R, 5′-GGAGTTTGCAATGCTCTTGCTATCTG-3′; β-actin F, 5′-CAACCGTGAAAAGATGACCCAGA-3′; and β-actin R, 5′-CACAGCCTGGATGGCTACGTAC-3′.

Serum ELISA. Maxisorp plates (Thermo Fisher Scientific) were coated with 2 μg/ml rat IgG–adsorbed donkey anti–mouse IgG (H+L; Jackson ImmunoResearch Laboratories, Inc.) overnight at room temperature, blocked with 1% BSA in buffered saline (ELISA buffer) for 1 h, and incubated for 1 h with mouse sera diluted in ELISA buffer. Purified 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 Ca^{2+} mobilization and BrdU uptake in $κ^+$ B cells of pAlb spleen, documenting their hyporesponsiveness and rapid turnover. 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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