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TLR9 Promotes Tolerance by Restricting Survival of Anergic Anti-DNA B Cells, Yet Is Also Required for Their Activation

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Nucleic acid-reactive B cells frequently arise in the bone marrow but are tolerated by mechanisms including receptor editing, functional anergy, and/or deletion. TLR9, a sensor of endosomal dsDNA, both promotes and regulates systemic autoimmunity in vivo, but the precise nature of its apparently contradictory roles in autoimmunity remained unclear. In this study, using the 3H9 anti-DNA BCR transgene in the autoimmune-prone MRL.Fas−/− mouse model of systemic lupus erythematosus, we identify the stages at which TLR9 contributes to establishing and breaking B cell tolerance. Although TLR9 is dispensable for L chain editing during B cell development in the bone marrow, TLR9 limits anti-DNA B cell life span in the periphery and is thus tolerogenic. In the absence of TLR9, anti-DNA B cells have much longer life spans and accumulate in the follicle, neither activated nor deleted. These cells retain some characteristics of anergic cells, in that they have elevated basal BCR signaling but impaired induced responses and downregulate their cell-surface BCR expression. In contrast, whereas TLR9-intact anergic B cells accumulate near the T/B border, TLR9-deficient anti-DNA B cells are somewhat more dispersed throughout the follicle. Nonetheless, in older autoimmune-prone animals, TLR9 expression specifically within the B cell compartment is required for spontaneous peripheral activation of anti-DNA B cells and their differentiation into Ab-forming cells via an extrafollicular pathway. Thus, TLR9 has paradoxical roles in regulating anti-DNA B cells: it helps purge the peripheral repertoire of autoreactive cells, yet is also required for their activation. The Journal of Immunology, 2013, 190: 1447–1456.

A utoreactive BCRs arise as a result of V(D)J recombination. As many as 55–75% of developing B cells display BCRs with measurable affinity for self-epitopes (1). Several self-tolerance mechanisms efficiently eliminate the majority of self-reactive BCR specificities before or shortly after entry into the mature B cell repertoire. These include editing of autoreactive BCRs through additional rounds of recombination at the L chain loci, deletion of autoreactive B cells, or the acquisition of a functionally unresponsive phenotype termed anergy (2, 3).

Recently, we and others have shown that TLR9, an endosomal innate immune sensor of dsDNA (4), is required for formation of spontaneous anti-DNA autoantibodies in several in vivo mouse models of systemic lupus erythematosus (SLE) (5–10). These findings are consistent with a model in which autoreactive B cells in SLE break tolerance due to the unique ability of nucleic acid–containing self-Ags to coengage the BCR and one or more innate immune sensors of nucleic acids, including TLR7 or TLR9, even in the absence of specific T cell help (11). In vitro evidence supports a role for TLR9 in this context (12). However, in vivo, the precise roles of TLR9 in autoimmunity may be more complex. Additional signals from both T cells and myeloid cells might substitute for TLR9 in B cells. Moreover, because TLR9 expression begins early in B cell development (13), TLR9 could play roles in B cell repertoire selection and the establishment of central tolerance, as has been suggested recently (14). To address the B cell–specific roles of TLR9 throughout autoreactive B cell development and activation, we examined the effect of TLR9 deficiency in the 3H9 anti-DNA BCR model (15, 16).

3H9 is an anti-DNA mAb, the H chain of which confers affinity for DNA via arginine residues in its CDRs (17). Depending on the Ig L chain with which the 3H9 VH pairs, the resulting Ab or BCR can bind to ssDNA or dsDNA (18). A subset of L chains (termed editors) significantly reduce the H chain’s affinity for DNA (19). When the 3H9 VH is expressed as a transgene (Tg) in the BALB/c strain, developing anti-dsDNA B cells are deleted, receptor edited, or anergized so that the peripheral B cell repertoire is enriched for editor L chains, and anti-dsDNA Abs are not detectable in the serum (15, 20–22). In contrast, when the Tg is expressed on the autoimmune-predisposed genetic background MRL.Fas−/−, a subpopulation of autoreactive cells at any given time has escaped tolerance and differentiated into short-lived plasmablasts that secrete anti-DNA autoantibodies (16).

To investigate the role of TLR9 in establishing and breaking tolerance in autoimmune MRL.Fas−/− mice, we studied mixed bone marrow (BM) chimeras lacking TLR9 in B cells and crossed the 3H9 anti-DNA Tg onto the Tlr9−/− MRL.Fas−/− genetic background. In this article, we show that the absence of TLR9 expression in B cells prevents the spontaneous production of anti-DNA autoantibodies via an extrafollicular (EF) pathway. Surprisingly, we found that TLR9 was not just required for activation, but also...
controlled self-tolerance. DNA-reactive 3H9/λ1 B cells in TLR9-deficient MRL.Fas<sup>−/−</sup> mice were neither activated nor deleted. Rather, they entered the B cell follicle (FO) and accumulated as long-lived resting cells despite evidence of Ag exposure and anergy. These results identify a novel protective role for TLR9 in regulating autoreactive B cell life span and localization.

**Materials and Methods**

**Mouse**

Mixed BM chimeras were prepared by whole-body x-irradiation of J<sub>2</sub>−/−/MRL.Fas<sup>−/−</sup> recipients with 750–800 rad. After 1–2 h, animals were given ~4 × 10<sup>8</sup> BM cells from donors mixed at indicated ratios by i.v. injection. Anti-CD45R-B220<sup>−</sup> splenocytes were taken directly from C57BL/6J mice at 8 weeks of age. Anti–BrdU-Alexa 647 (clone RO11-153), anti–F4/80-Alexa 647 and CD4-Pacific Blue (GK1.5) in blocking buffer for 1 h, then washed 3× in PBS/BSA/Tween followed by immersion in PBS. Slides were then stained with SA-Alexa 555 in blocking buffer. Sections were covered with ProLong Anti-Fade Gold (Life Technologies) and a cover slip.

**Microscopy**

Seven-micrometer spleen sections were prepared from OCT-frozen tissues and fixed in cold acetone for 10 min. Slides were blocked in PBS with 1% BSA (w/v), 0.1% Tween, and 10% normal rat serum for 20 min. Slides were stained with anti-CD19-Alexa 488 (ID3.2), anti–lambdal-biotin (R11-153), anti–F4/80-Alexa 647 and CD4-Pacific Blue (GK1.5) in blocking buffer for 1 h, then washed 3× in PBS/BSA/Tween followed by immersion in PBS. Slides were then stained with SA-Alexa 555 in blocking buffer.

**Flow Cytometry**

For BrdU labeling, mice were given an i.p. injection of 0.5 mg BrdU in PBS before the addition of anti–BrdU-Alexa 647 (clone RO11-153) and 5 min of additional data were collected.

**Immunofluorescence**

For analysis of α<sub>1</sub> cell distribution in the FO, images of individual FOs were cropped in Photoshop and analyzed with CellProfiler version 2.0 (r10997) software (27). In brief, the algorithm identified α<sub>1</sub> cells and measured their total area within two regions. One region was the portion of the CD19<sup>+</sup> B cell FO within 50 μm of the CD4<sup>+</sup> T cell zone; CD19<sup>+</sup> and CD4<sup>+</sup> regions were manually drawn on their respective single-color images, and the border region was computed by expanding the CD4<sup>+</sup> region by 50 μm in every direction and masking on the intersection of this expanded mask with the CD19<sup>+</sup> region. The other region was the whole CD19<sup>+</sup> FO. The ratio of α<sub>1</sub> pixels in these two regions, normalized for the relative areas of the regions, was defined as the distribution index. Images in which the area of the T/B border was <20% or >80% of the area of the B cell FO were considered to be atypical cross sections and were excluded from analysis.

**Statistical Analysis**

Unless otherwise stated, statistical comparisons were by two-tailed Mann–Whitney U test. Significance values are p < 0.05, p < 0.01, and p < 0.001, as calculated by GraphPad Prism software.

**Results**

**B cell expression of TLR9 is required for anti-DNA generation in lupus mouse**

Anti-nucleosome autoantibodies were reduced in MRL.Fas<sup>−/−</sup> lupus-prone mice genetically deficient in TLR9 (5). However, these animals had markedly exacerbated disease, suggesting complex roles for TLR9 in both promoting and regulating autoimmunity (5, 6). TLR9 is expressed in B cells from at least the pro-B stage on immunofluorescence, and in intrinsic, we prepared mixed BM chimeras in which TLR9 deficiency to only the hematopoietic compartment (13), but is also expressed in other hematopoietic lineages including macrophages and DCs. To determine whether the requirement for TLR9 in anti-nucleosome Ab production was B cell intrinsic, we prepared mixed BM chimeras in which TLR9 deficiency was restricted primarily to the B cell compartment. In this experimental system (Fig. 1A), B cell–deficient (J<sub>2</sub>−/−) MRL.Fas<sup>−/−</sup> recipients were irradiated and reconstituted with a mix of 80% J<sub>2</sub>−/−/3H9<sup>−/−</sup>/Faslpr<sup>−/−</sup>/IgM<sup>−/−</sup> BM and either 20% J<sub>2</sub>−/−/3H9<sup>−/−</sup>/Faslpr<sup>−/−</sup>/IgM<sup>−/−</sup>/CD4<sup>+</sup> BM or 20% J<sub>2</sub>−/−/3H9<sup>−/−</sup>/Faslpr<sup>−/−</sup>/IgM<sup>−/−</sup>/CD4<sup>+</sup>CD<sup>+</sup> BM (B<sub>2</sub>/B<sub>2</sub>) group. Only J<sub>2</sub>−/−/3H9<sup>−/−</sup>/Faslpr<sup>−/−</sup>/IgM<sup>−/−</sup>/CD4<sup>+</sup> BM is capable of generating B cells, whereas cells of other hematopoietic lineages are derived from both donors. Control recipients were provided with BM from only J<sub>2</sub>−/−/3H9<sup>−/−</sup>/Faslpr<sup>−/−</sup>/IgM<sup>−/−</sup>/CD4<sup>+</sup> (All.9/All.9/All.9/All.9) or only J<sub>2</sub>−/−/3H9<sup>−/−</sup>/Faslpr<sup>−/−</sup>/IgM<sup>−/−</sup>/CD4<sup>+</sup>CD<sup>+</sup> (B<sub>2</sub>/B<sub>2</sub>) donors. Extent of chimerism was confirmed by quantitative real-time PCR (data not shown).

Restriction of TLR9 deficiency to only the hematopoietic compartment resulted in a shift in the dominant ANA pattern from a DNA-associated homogenous nuclear staining pattern (Fig. 1B, All.9/All.9/All.9/All.9 group) to a speckled nuclear or cytoplasmic staining pattern (Fig. 1B, All.9/All.9/All.9/All.9 group), similar to intact MRL.Fas<sup>−/−</sup> lacking TLR9 in all tissues (5, 6). This shift was also seen when TLR9 was absent specifically in B cells but present in most non-B cells (Fig. 1B, B<sub>2</sub>/B<sub>2</sub>/B<sub>2</sub>/B<sub>2</sub> group). Positive mitotic chromatin staining in the ANA (Fig. 1C) and anti-nucleosome IgG autoantibodies (Fig. 1D) also required TLR9 expression specifically in the B cell compartment, indicating that B cell intrinsic
expression of TLR9 is necessary for anti-nucleosome autoantibody in lupus-prone mice.

**TLR9 is not required for L chain editing**

Because TLR9 is expressed from early in B cell development (13) and components of the TLR9 signaling pathway have been implicated in central tolerance (14), we examined the requirements for TLR9 in L chain editing and development of anti-DNA B cells using the 3H9 Tg MRL.Faslpr system, in which L chain usage is a surrogate marker for autoreactivity amid a restricted but polyclonal repertoire. We sorted developing B cell fractions (Fr.s) by a surrogate marker for autoreactivity amid a restricted but polyclonal repertoire. We found no difference in V\_l sequence (RS), a direct measure of attempted L chain editing (28), that should be undergoing receptor editing (28), among mature A–C B cells (Fig. 2B, Fr. F). Intriguingly, we observed an increase in the frequency of \( l^+ \) cells among recirculating mature 3H9+ Fr. F B cells when TLR9 was absent, as will be further investigated later.

**TLR9 is required for anti-nucleosome production in 3H9 Tg MRL.Faslpr mice**

Because TLR9 did not affect primary repertoire development in 3H9+ mice, we examined whether DNA-reactive B cells were dependent on TLR9 for activation and differentiation into Ab-forming cells (AFCs). Sera from 3H9 \( Tlr9^{+/+} \) MRL.Faslpr or 3H9 \( Tlr9^{-/-} \) MRL.Faslpr at 13–20 wk of age were assessed for anti-nucleosome Abs by ELISA (Fig. 3A). \( Tlr9 \) was indeed required for production of anti-nucleosome IgM Abs in the 3H9 model, whereas total serum IgM titers were similar in \( Tlr9^{-/-} \) and -deficient animals (Fig. 3B).

Cells expressing \( \lambda 1 \) are anergic anti-DNA B cells in 3H9 Tg animals (16, 29). We first asked whether \( \lambda 1^+ \) anti-DNA B cells could become AFCs in the absence of TLR9. \( \lambda 1^+ \) IgM ELISPOT-forming cells, which were present at high frequency in 3H9 \( Tlr9^{+/+} \) MRL.Faslpr spleens, were undetectable in the spleens of 3H9 \( Tlr9^{-/-} \) MRL.Faslpr mice (Fig. 3C). Similarly, \( \lambda 1^+ \) cells with an AFC phenotype were not observed by FACS in 3H9 \( Tlr9^{-/-} \) MRL.Faslpr mice (Fig. 3D). TLR9-deficient 3H9 \( MRL.Faslpr \) mice also had reduced cell-surface expression of CD44 (Fig. 3E) and CD86 (Fig. 3F) compared with non-Tg animals (Fig. 2D), suggesting that TLR9 does not affect receptor editing of anti-DNA B cells during development. TLR9 deficiency also had no impact on the frequency of cells using VA1, an L chain that permits dsDNA binding when paired with 3H9 IgH (16, 29), among the developing Fr. E B cells (Fig. 2C). Intriguingly, we observed an increase in the frequency of \( \lambda 1^+ \) cells among recirculating mature 3H9+ Fr. F B cells when TLR9 was absent, as will be further investigated later.

**Autoreactive B cells accumulate in the FO in Tlr9-deficient mice**

The absence of spontaneous anti-DNA AFCs in TLR9-deficient animals could have been because of differences in precursor population frequency and/or establishment and maintenance of
anergy. Because we observed no differences in the primary repertoire or development of anti-DNA λ1+ B cells in the BM (Fig. 2), we next enumerated λ1+ B cells in the spleens and lymph nodes (LNs) of 3H9 + Tlr9+/+ or 3H9 + Tlr9−/− MRL.Faslpr as well as 3H9− mice by FACS. Surprisingly, not only were λ1+ anti-DNA cells present in the spleens of TLR9-deficient mice, but the percentage of FO and LN B cells that were λ1+ was actually greater in 3H9 + Tlr9−/− mice than in 3H9 + Tlr9+/+ mice (Fig. 4A, 4D). In contrast, the percentage of marginal zone (MZ) B cells using λ1 was unaffected by TLR9 expression (Fig. 4B). The frequencies of nonautoreactive λx+ B cells in the LN and splenic FO, MZ, or transitional populations were not different in the presence or absence of TLR9 (Supplemental Fig. 3). Importantly, the proportion of CD93+ transitional B cells that were λ1+ was also not affected by Tlr9 genotype (Fig. 4C), indicating that TLR9 does not affect DNA-reactive B cell accumulation before completion of maturation. This conclusion is also consistent with the lack of effect of TLR9 expression on λ1 or λx usage in Fr. E in the BM and the

FIGURE 2. L chain editing is not impaired in TLR9-deficient MRL.Faslpr mice. (A) Sorting strategy for isolation of BM B cell Hardy Frs. in 6- to 9-wk-old mice. Representative staining from a 3H9 + Tlr9+/+ MRL.Faslpr animal is shown. (B) Quantitative real-time PCR of genomic Vκ-RS rearrangements from Frs. sorted in (A), expressed relative to a total B6 splenocyte κ+ B cell control. n = 3 per group from 3 independent sorts of n = 1 per each genotype. (C) Proportion of live BM cells that are λ1+ B220− CD93− (Fr. E, left) or λ1+ B220− CD93+ (Fr. F, right). (D) Proportion of live BM cells that are λx+ B220+ CD93+ (Fr. E, left) or λx+ B220+ CD93− (Fr. F, right). Data in (C) and (D) are pooled from five cohorts of 13- to 20-wk-old mice. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 3. TLR9 is required for anti-DNA Abs in 3H9 MRL.Faslpr mice. (A) Serum anti-nucleosome IgM measured by ELISA from 17- to 20-wk-old mice. (B) Total serum IgM measured by ELISA. Data in (A) and (B) are pooled from three independent cohorts. (C) Splenic AFCs secreting λ1 IgM Abs detected by ELISPOT. Data are pooled from five independent cohorts. (D) TCRβ− CD44+ CD138− surface-λ1int intracellular-λ1/2high splenic AFCs enumerated by FACS. Data are pooled from four independent cohorts. (E) CD44 and (F) CD86 mean fluorescence intensity (MFI) was measured on CD19+ splenocytes. Data in (E) and (F) are representative of three independent experiments. (G and H) Immunofluorescent staining of spleen sections from 19-wk-old (G) 3H9 + Tlr9+/+ and (H) 3H9 + Tlr9−/− MRL.Faslpr with CD19 (red), λ1 (green), and CD4 (blue). Scale bar, 1 mm. **p < 0.01, ***p < 0.001.
increased frequency of λ1 cells in BM Fr. F (Fig. 2C, 2D). Thus, TLR9 deficiency led to the accumulation of a population of FO anti-DNA B cells, which nonetheless do not progress to AFCs.

**Autoreactive B cells in Tlr9-deficient mice are Ag exposed and functionally anergic**

We next asked whether TLR9-deficient and -sufficient anti-DNA B cells were similarly anergic. We first compared surface IgL expression as a function of Tlr9 genotype in 3H9 Tg MRL.Faslpr mice. Similar to anergic B cells in several models including 3H9 (29, 31, 32), BCR downregulation was observed in both TLR9-deficient and -sufficient λ1 splenic follicular B cells (Fig. 5A–C). 3H9/Vα1 BCR expression was slightly lower in TLR9-deficient compared with TLR9-intact animals (Fig. 5C). In contrast, the nonautoreactive 3H9/Vαx BCs in the same animals were not downregulated compared with non-Tg mice, either with or without Tlr9 (Fig. 5D–F).

Although BCR downregulation indicated Ag exposure, this observation alone did not establish functional anergy. Anergic B cells have high basal BCR signaling but poor responses to in vitro IgM cross-linking (2). Therefore, we asked whether TLR9 influenced BCR signaling. Basal Syk phosphorylation was indeed increased in the autoreactive 3H9/Vα1 B cell population compared with non-Tg MRL.Faslpr mice, but this increase did not depend on Tlr9 genotype, suggesting that the autoreactive population is anergized without or without TLR9 (Fig. 5G, 5H). Importantly, there were no differences in basal Syk phosphorylation in the Vαx nonautoreactive population with or without either the 3H9 Tg or Tlr9 (Fig. 5I). In addition, FO B cells from both 3H9 and 3H9 Tlr9+/– MRL.Faslpr mice had similarly high basal intracellular Ca²⁺ concentrations compared with Tg animals, which did not increase as much in 3H9 cells compared with non-Tg cells after IgM cross-linking, irrespective of Tlr9 genotype (Fig. 5J–O). Thus, with respect to both BCR downregulation and signaling, anti-DNA B cells in 3H9 Tlr9+/– MRL.Faslpr mice are anergized to a similar extent as their TLR9-intact counterparts.

Anergic B cells have a short half life, although the reasons for this are unclear (2, 33, 34). The increased number of λ1 FO B cells in 3H9 Tlr9+/– MRL.Faslpr mice compared with 3H9 Tlr9+/- MRL.Faslpr mice (Fig. 4A) suggested that TLR9 might regulate the survival of anergic anti-DNA B cells. We therefore measured the kinetics of BrdU uptake in 3H9/Vα1 cells from TLR9-intact or -deficient MRL.Faslpr mice. Provided that cells are not proliferating within a particular compartment itself, the frequency of BrdU+ B cells in peripheral compartments after long-term labeling is proportional to the rate of entry of cells into that population from the BM as the unlabeled cells resident in that population turn over, as specifically shown for the B cell lineage (35). BrdU was provided by i.p. injection twice daily for 2 or 4 d. Under these conditions, transitional B cells were labeled with BrdU at a similar rate regardless of Tlr9 genotype, with approximately half of the 3H9/Vα1 (Fig. 6A) or 3H9/Vα1– (Fig. 6D) transitional B cells in the spleen having incorporated BrdU by 4 d of labeling, suggesting that rates of immature B cell entry into the spleen from the BM are unaffected by TLR9. In contrast, the frequency of BrdU+ cells among 3H9/Vα1 FO B cells was 2-fold greater in TLR9-intact MRL.Faslpr mice compared with Tlr9+/– animals (Fig. 6B), consistent with the greater turnover rate of FO B cells in the TLR9-intact animals. This TLR9-dependent difference in labeling kinetics was also observed for λ1 B cells in the LNs (Fig. 6C). Labeling of the λ1 FO B cells was also greater in TLR9-sufficient mice (although to a lesser extent than among the λ1 population; Fig. 6E), most likely reflecting the mixture of both anergic anti-DNA B cells and nonautoreactive edited B cells in the λ1 population (22). Finally, labeling of LN λ1 cells was not significantly affected by TLR9 (Fig. 6F).

The TLR9-dependent difference in the rate of BrdU incorporation among 3H9/Vα1 FO B cells (Fig. 6B) suggested that this population was turning over more rapidly in TLR9-intact than -deficient 3H9+ MRL.Faslpr mice. Alternatively, the difference could have reflected proliferation of TLR9-stimulated activated B cells directly within the compartment. To address this possibility, we measured BrdU incorporation among CD19+ CD21+ CD23+ FO B cells, and found a TLR9-dependent difference in label in the λ1 population (Supplemental Fig. 4A), but not among total naive B cells (Supplemental Fig. 4B). To further evaluate whether TLR9 affected proliferation of FO B cells directly, we pulsed 3H9 MRL.Faslpr mice with BrdU for 2 h via a single i.p. injection; given the short half life of BrdU, this labels only those cells in S phase at the time of injection. Under these conditions, ~10% of B220+ BM cells were labeled with BrdU (Supplemental Fig. 4C), but BrdU incorporation in the λ1+ FO population was not significantly above background (Supplemental Fig. 4D–F), suggesting that the vast majority of FO B cells are indeed nonproliferative. In particular, the implied rates of proliferation of FO B cells are much too low to account for proliferation causing the extent of labeling seen over a 2- or 4-d period; rather, such labeling must have come from maturation over that period of a more immature proliferating precursor, as originally proposed by Allman and colleagues (35). Taken together, these results suggest that follicular 3H9/Vα1 cells are turning over more rapidly in TLR9-intact than -deficient MRL.Faslpr mice, consistent with and resulting in the observed increase in this population’s frequency when TLR9 is absent (Fig. 4A).

**Tlr9 influences distribution of anti-DNA B cells in the FO**

Previous studies demonstrated that anergic cells, including 3H9/Vα1 B cells, are excluded from the FO and accumulate at or near the T/B border (33, 36). In Fasl-deficient animals, 3H9/Vα1 cells were shown to enter the FO to a much greater extent than in Fas-
FIGURE 5. λ1+ B cells in TLR9-deficient mice are Ag experienced and anergic. (A) Representative overlays of surface λ1 staining among CD22+ CD21/35int CD23+ splenic FO B cells from 6- to 8-wk-old 3H9+ Tlr9+/+ MRL.Fas−/− (gray shaded), 3H9+ Tlr9+/+ MRL.Fas+/+ (solid line), and 3H9+ Tlr9−/− MRL.Fas−/− (dashed line). (B) Overlays of surface λ1+ staining in (A), gated on the λ1+ population. (C) MFI of surface λ1+ staining among the λ1+ population. (D–F) Overlays and MFI of surface λx staining among FO B cells from the same animals in (A)–(C). Data are representative of at least four (A) or three (λx) independent experimental cohorts. (G) Representative overlay of anti-phosphorylated Syk staining in CD22+ λ1+ B cells from 8- to 9-wk-old 3H9− MRL.Fas−/− mice either fixed immediately upon spleen disaggregation (solid line) or fixed and treated with alkaline phosphatase (gray shaded). (H) Change in p-Syk MFI between untreated or alkaline phosphatase–treated CD22+ λ1+ B cells. (I) Change in p-Syk MFI between untreated or alkaline phosphatase–treated CD22+ λx+ B cells. (J) Representative overlay of indo-1 (violet)/indo-1 (blue) ratio over time among CD19+ CD35int CD23+ FO B cells in 8- to 9-wk-old 3H9− MRL.Fas−/− (red), 3H9+ Tlr9+/+ MRL.Fas+/+ (blue), or 3H9+ Tlr9−/− MRL.Fas−/− (green). Anti-IgM was added at t = 60 s. (K) Basal indo-1 (violet)/indo-1 (blue) ratio was determined as the mean ratio for t = 14 s to t = 45 s. (L) (Figure legend continues)
sufficient animals (37). Nonetheless, we noted that in a significant Fr. of pre-autoimmune 3H9 MRL. Faslpr FOs, the distribution of $\lambda_1^+$ cells did not appear to be as uniform throughout the FO as was observed in nontransgenic animals (Fig. 7B, 7C). We therefore developed an image analysis algorithm to assess the distribution of $\lambda_1^+$ cells in the FO more rigorously than can be accomplished by visual inspection of representative images. Using CellProfiler image analysis software, we identified a portion of the B cell FO that was immediately adjacent to the T cell zone and enumerated the $\lambda_1^+$ pixels in that region. This number was expressed as a ratio to the number of $\lambda_1^+$ pixels in the FO as a whole and normalized to the relative areas of the two regions, to define a “distribution index.” A distribution index of 1 would therefore indicate that, per unit area, there are as many $\lambda_1^+$ cells on the edge of the FO closest to the T zone as there are in the FO as a whole, whereas a distribution index >1 indicates a relative enrichment of $\lambda_1^+$ cells on the edge of the FO nearest the T zone.

Using this approach, we found that the FOs of nontransgenic MRL. Faslpr mice had a median distribution index of 0.93 for $\lambda_1^+$ cells (mean ± SEM: 0.919 ± 0.020). In contrast, the median distribution index of 3H9 MRL. Faslpr FOs was 1.27 (mean ± SEM: 1.309 ± 0.030), indicating a relative enrichment of 3H9 V$\lambda_1$ cells on the edge of the FO closest to the T zone as there are in the FO as a whole, whereas a distribution index >1 indicates a relative enrichment of $\lambda_1^+$ cells on the edge of the FO nearest the T zone.

Discussion

The involvement of TLR9 in SLE has been evident from a number of in vivo and in vitro experimental systems, but its precise roles remain puzzling and apparently paradoxical. In particular, although TLR9 is required for anti-DNA Ab generation in lupus-prone mice, its absence promotes disease in at least five different murine lupus models (6–10). Why is this?

In this article, we have provided several significant pieces to the puzzle of TLR9 function in systemic autoimmunity. Initially, we showed, using mixed BM chimeras, that TLR9 must be expressed in the B cell compartment for the generation of anti-DNA in the MRL. Faslpr model of SLE. Thus, although TLR9 is expressed in many myeloid cells and is linked to the secretion of type I IFNs and immune cell activation that may also promote autoantibodies, its expression in B cells per se is absolutely required for anti-DNA production. We then used the 3H9 BCR Tg model to track the effects of TLR9 expression on the fate of a defined population of autoreactive anti-DNA B cells through development in comparison with nonautoreactive B cells within the same animals. We found that anti-DNA APCs are produced via an EF plasmablast response rather than a germinal center, in an entirely TLR9-dependent manner, analogous to the situation for rheumatoid factor B cells (30).

Most unexpectedly, we found that TLR9 also governed peripheral anti-DNA B cell homeostasis, promoting tolerance rather than autoreactivity. In contrast, TLR9 deficiency had no detectable effect on the development, accumulation, labeling kinetics, or receptor editing of anti-DNA B cells in the BM or among immature splenic B cells, nor did TLR9 affect BCR-proximal establishment

**p < 0.01, ***p < 0.001.

Inducible Ca$^{2+}$ flux expressed as the difference between peak indo-1 (violet)/indo-1 (blue) ratio (mean from t = 118 s to t = 159 s) and basal indo-1 ratio. (M–O) Similar plots to (J–L) with the addition of an anti-$\lambda_1$/2 $F_{(ab)}$ gate. Data are representative of three independent experiments, n = 3–7 per group. **p < 0.01, ***p < 0.001.
of anergy. Taken together, these results provide a comprehensive analysis of TLR9’s influence on anti-DNA B cells throughout B cell development and maturation, revealing novel and seemingly paradoxical roles for TLR9 in the establishment and maintenance of B cell tolerance.

TLR9 deficiency uncovered a previously unsuspected tolerogenic role of TLR9 in the peripheral B cell compartment: there was a striking accumulation of anergic anti-DNA B cells within the FO and in the LN. Interestingly, this increase was not seen in CD93+ immature B cell compartments (Fr. E) in either the BM or spleen. Thus, the effects of TLR9 in preventing autoreactive cell accumulation occur either at the E-to-F transition or take place within Fr. F. This is an important distinction to make because it may relate to the observation that polyreactive B cells in human patients who lack either IRAK-4, MyD88, or Unc93b were not filtered from the repertoire (14). Curiously, elimination of cells reacting in an HEp2-extract ELISA assay, which may relate more directly to the anti-DNA specificity we studied, was faulty only in the IRAK-4– and MyD88-deficient patients, but not in patients deficient in UNC93b, a discrepancy that has not yet been resolved. Regardless, the explanation for how the lack of these genes in humans affects autoreactivity in the B cell repertoire, whether negative selection, positive selection, or receptor editing, could not be addressed in human studies. However, based on differences seen in “new emigrant” circulating B cells, these genes were thought to affect the repertoire in the immature rather than mature compartments. In contrast, we found the effects of TLR9 were instead seen on accumulation and turnover rate of mature FO B cells in the periphery, whereas in developing anti-DNA B cells, neither receptor editing nor cellular homeostasis of immature B cells were affected.

There are a number of possible reasons for the apparent differences in our findings and those of Isnardi et al. (14). First, the specificities under study were different. In the human studies, polyclonal and HEp-2 reactivity were measured, which comprise multiple specificities that may differ in TLR dependence. In this report, we focused on a single, well-defined, chromatin-reactive clonotype, allowing for a more precise interpretation of the relationship between BCR specificity and cell fate. Second, although both mice and humans express functional TLR9 during B cell development, there could still be species-specific differences, with mice potentially filtering their B cell compartment at a later stage but in an analogous way. Third, we looked specifically at TLR9, whereas the human studies used less targeted deficiencies based on the availability of patients with rare mutations. These mutations affect multiple signaling pathways that include, but are not limited to, TLR9. Finally, humans who lack these signaling pathways in all cells from birth are immunodeficient, and an indirect effect of this contributing to the different phenotype cannot be excluded.
compartments at steady state. This tolerogenic role for TLR9 in regulating autoreactive cell life span is most likely B cell intrinsic rather than an indirect effect of differences in production of survival factors such as BAFF by non-B cells. This notion is supported by the difference in labeling between the VÀ1+ cells, which are uniformly anti-DNA, and the λ1 population, which includes both edited nonreactive and anti-DNA cells. In contrast, a B cell–extrinsic effect should have affected all types of B cell. Although TLR9 did not affect expression of CD44 or CD86 in nonautoreactive cells, it supported the expression of these markers in DNA-reactive B cells, further illustrating how TLR9 plays an ongoing role in shaping the peripheral self-reactive B cell repertoire.

In concert with the effects of TLR9 on anti-DNA B cell turnover and cell-surface marker expression in the mature compartment, TLR9 expression also modestly affected the positioning of these self-reactive B cells. In TLR9-sufficient mice, anti-DNA B cells tended to be located closer to the T/B border in contrast with a somewhat less asymmetric FO distribution in TLR9-deficient mice. Localization to the T/B border has been shown in some models of autoreactivity to be linked to Fas-independent cell death (38), and thus localization might contribute, in part, to the differences we observed in B cell turnover between TLR9-intact and -deficient mice.

Despite differences in activation, differentiation, and other cell-intrinsic effects mediated by TLR9 expression, anergy per se (defined as high basal BCR-proximal signaling and poor induced intrinsic effects mediated by TLR9 expression, anergy per se) was not altered expression of the BCR and the coactivation marker CD86, as to be directly controlled by the BCR only. Notably, however, other (defined as high basal BCR-proximal signaling and poor induced intrinsic effects mediated by TLR9 expression, anergy per se) mice.

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Disclosures

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