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TLR9 in Peritoneal B-1b Cells Is Essential for Production of Protective Self-Reactive IgM To Control Th17 Cells and Severe Autoimmunity

Alexander D. Stoehr,*1 Carolin T. Schoen,*1 Maria M. M. Mertes,*1 Susanne Eoglmeier,* Vivien Holecska,* Alexandra K. Lorenz,* Tim Schommartz, † Anna-Lena Schoen, † Constanze Hess,* André Wünkler,* Hedda Wardemann,† and Marc Ehlers*†

The role of TLR9 in the development of the autoimmune disease systemic lupus erythematosus is controversial. In different mouse models of the disease, loss of TLR9 abolishes the generation of anti-nucleosome IgG autoantibodies but at the same time exacerbates lupus disease. However, the TLR9-dependent tolerance mechanism is unknown. In this study, we show that loss of TLR9 is associated with low peritoneal B-1b cell numbers and low levels of protective self-reactive IgM serum autoantibodies in lupus-prone FcγRIIB-deficient mice leading to the uncontrolled accumulation of proinflammatory CD4+ cells and exacerbated autoimmunity. TLR7 signaling was not able to compensate for the loss of TLR9 signaling in peritoneal B-1b cells to induce IgM Abs. Transfer of TLR9-expressing peritoneal B-1b cells from FcγRIIB-deficient mice or of recombinant monoclonal self-reactive IgM Abs was sufficient to reduce the frequency of proinflammatory Th17 cells and lupus disease in FcγRIIB/TLR9 double-deficient mice. Taken together, these data provide evidence for a TLR9-dependent tolerance mechanism of peritoneal B-1b cells generating protective self-reactive IgM in lupus-prone mice to control Th17 cell development and severe autoimmunity. The Journal of Immunology, 2011, 187: 2953–2965.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the generation of IgM and IgG autoantibodies against various nuclear Ags such as DNA, nucleosomes, and ribonuclear proteins. However, IgG autoantibodies are positively correlated and IgM autoantibodies are negatively correlated with SLE symptoms (1–4). In particular, the IgG/IgM ratio of self-reactive Abs can be used as prognostic marker for lupus nephritis during the course of the disease (3). Transfer of anti-dsDNA IgM Abs has further been shown to inhibit the development of nephritis in a murine model of SLE (5). These studies suggest that self-reactive IgM plays a key role in protecting against severe autoimmunity.

TLR7 and TLR9 stimulation and MyD88 signaling induced by RNA- and DNA-associated self-antigens, respectively, have been shown to play a major role in the activation of dendritic cells and self-reactive B cells (6–9). Accordingly, the TLR pathway is known to play an important role in the development of IgG autoantibodies and disease in lupus-prone mice (10–17). Although various TLR9-deficient lupus-prone mouse models do not develop anti-nucleosome IgG serum autoantibodies, they do show exacerbated disease progression, indicating that TLR9 also plays a role in self-tolerance (11, 15, 17–21). However, the tolerogenic function of TLR9 and the influence of TLR9 on the IgG/IgM ratio of self-reactive Abs in lupus-prone mice are unknown.

In this study, we used C57BL/6 mice deficient for the IgG inhibitory receptor FcγRIIB (FcγRIIB−/−) as a spontaneous model of SLE (12, 22) to determine the influence of TLR9 signaling on the onset of lupus nephritis. Lupus disease was exacerbated in TLR9-deficient FcγRIIB−/− (FcγRIIB−/−/TCRβ−/−) mice, with increased numbers of proinflammatory Th1 and Th17 cells and high levels of inflammatory IgG2c (IgG2a haplotype b) and IgG2b serum autoantibodies compared with those in FcγRIIB−/− mice. Loss of TLR9 was associated with low peritoneal B-1b cell numbers and low levels of self-reactive IgM serum autoantibodies in lupus-prone mice. Transfer of TLR9-deficient peritoneal B-1b cells from FcγRIIB-deficient mice or monoclonal self-reactive IgM Abs into FcγRIIB−/−/TLR9−/− mice inhibited the accumulation of proinflammatory Th17 cells. In summary, our data show a TLR9-dependent tolerance mechanism of peritoneal B-1b cells generating protective self-reactive IgM in lupus-prone mice to control the development of proinflammatory Th17 cells and severe autoimmunity.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories. FcγRIIB−/− and FcγRIIB−/−/MyD88−/− mice have been described previously (12, 22). TLR9−/− (23), JHT−/− (24), and μs (25) mice were crossed with FcγRIIB−/− mice to produce FcγRIIB−/−/TLR9−/−, FcγRIIB−/−/JHT−/−, and FcγRIIB−/− μs double-deficient mice. IL-17 receptor A-deficient mice

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Abbreviations used in this article: ANA, anti-nuclear Ab; BM, bone marrow; LN, lymph node; MZ, marginal zone; RF, rheumatoid factor; SLE, systemic lupus erythematosus.
(IL-17RA/−) (26) were crossed with FcγRIIB/−/TLR9−/− double-deficient mice to produce FcγRIIB−/TLR9−/−-IL-17RA−/− triple-deficient mice. All mice were on a C57BL/6 background. Mice were bred and maintained in accordance with institutional guidelines. Female mice were analyzed exclusively. Genotypes were determined by PCR on tail DNA or by FACS (12, 24).

**Bone marrow transfer**

Recipient 8- to 12-wk-old FcγRIIB−/− female mice were lethally irradiated with 7.5 Gy. Bone marrow (BM) was extracted from donor mice, and T cells were depleted by negative selection using anti-Thy1.2 (clone 30-H12) magnetic microbeads and a MACS magnetic column (Miltenyi Biotec). BM cells (5 × 10⁸) were transferred into the irradiated recipients. Mixed BM chimeric mice were produced as described previously (27).

**Transfer of peritoneal cells**

Peritoneal cells were obtained by injecting 3 ml PBS/BSA into the peritoneal cavities of 3-mo-old donor mice and washing with PBS/BSA. Then, 0.5 × 10⁸ to 1.5 × 10⁸ donor peritoneal cells or 1 × 10⁶ FACS-sorted peritoneal CD⁵⁺ MacIint IgMhigh B-1b cells were transferred into the recipients. Mixed BM chimeric mice were produced as described previously (27). Briefly, 4 × 10⁶ BM cells (80%) from FcγRIIB−/− JHT−/− mice together with 1 × 10⁶ BM cells (20%) from FcγRIIB−/−/TLR9−/−, or FcγRIIB−/− MyD88−/− mice were transferred into the irradiated recipients. Reconstituted mice were treated with antibiotics and analgesics in the drinking water for 2 wk.

**Production and purification of monoclonal IgM Abs**

HEK293T cells were cultured and transfected as previously described (29). The IgM Abs in the supernatant were purified with an anti-IgM Sepharose column (in-house preparation with anti-mouse IgM [clone M41]) and anti-IgB-activated Sepharose 4B (GE-Healthcare). The monoclonal IgM Abs were eluted with 0.1 M glycine, pH 2.7, collected in 1:10 vol of 1 M HCl, pH 9 and dialyzed against PBS. The concentration of the murine IgM Abs was determined by bicinecinonic acid assay (Pierce) and verified by IgM ELISA. Ab integrity was analyzed by SDS gel analysis, and self-reactivity was verified by ELISA.

**Evaluation of proteinuria**

Proteinuria was measured using Multistix 10 (Bayer) strips and scored as follows: 0; negative; 1, <75 mg/dl; 2, 125 mg/dl; 3, >125 mg/dl.

**Flow cytometry analysis and cell sorting**

The following fluorochrome-coupled anti-mouse Abs were used: anti-CD138 (clone N418), anti-IL-17 (TC11-18H10), anti-CD5 (5-7.3), anti-CD4 (RM4-5) (from BD), anti-CXCR3 (280083) (from R&D Systems), anti-CD43 (BioR2/60) (from ebiosiscience), anti-B220 (RA3-6B2), anti-CD4 (GK1.5 and YTS191), anti-CD44 (IM7), anti-CD62L (MEL14), anti-IFN-γ (AN18.17.24), anti-CD21 (7G6), anti-CD23 (B3/4), anti-Mac1 (M1/70.15.11), anti-IgM (M41), and anti-CD5 (19-3) (from house preparations). For intracellular cytokine analysis, cells were stimulated with PMA/ionomycin for 4 h. Brefeldin A was added after 1 h of stimulation. Cells were treated with cytochalasin D and sodium azide (BD Biosciences), according to the manufacturer’s instructions prior to Ab-mediated intracellular cytokine detection. Peritoneal MacIint IgMhigh B-1 cells, CD5⁺ MacIint IgMhigh B-1a cells, CD5⁺ MacIint IgMhigh B-1b cells, or MacI⁻ IgM⁻ B-2 cells were injected i.p. into recipient mice at the age of 5–6 mo. Blood Th17 cells or anti-natural Ab (ANA) serum IgG was determined by FACS or ELISA 9 d later.

**Cloning of monoclonal human/mouse chimeric and mouse IgM Abs**

The human IgG1 and κ constant regions in the H and L chain expression vectors were the monoclonal self-reactive and polyreactive human Ab ED38 and the control human Ab mg553 (29) and the self-reactive and polyreactive murine Ab 1R1Fg7 (30) were exchanged for the murine IgM H chain (Sall-BskiW) and murine κ chain (BskiW-HindIIIIII) constant regions, which were amplified by PCR from a generated C57BL/6 spleen cDNA, respectively (murine IgM H chain C region, forward primer 5'-CCTCCTGGGCTAGGTGAATCTATGCCATTAATTCGGATG-3', reverse primer 5'-TTATTCGGCTTCAAGCTTATTTTCTCTCCAGGTCGCTGTAATGGGCTCTGGTG-3', murine κ chain region forward primer 5'-CCTCTCCGGTTGGAACGGTTGATGAGTGGTTGATGCTGTTCT-3', reverse primer 5'-TTATTCGGGCTTCAAGCTTATTTTCTCTCCAGGTCGCTGTAATGGGCTCTGGTG-3'). The leader sequences of the original expression vectors were used (29).
Results
Exacerbated lupus disease in FcγRIIB−/−TLR9−/− mice is associated with increased levels of proinflammatory Th1 and Th17 cells and T cell-dependent IgG2c and IgG2b serum autoantibodies

In our mouse facility, ∼20% of lupus-prone FcγRIIB−/− mice developed nephritis and died of disease symptoms by the age of 9 mo (Fig. 1A, Supplemental Fig. 1). Mortality was significantly increased to 80% in FcγRIIB−/− mice lacking TLR9 (FcγRIIB−/−TLR9−/−), whereas a lack of MyD88 protected FcγRIIB−/− mice from developing lupus disease (Fig. 1A) (12). Loss of TLR9 was associated with low levels of anti-nucleosome (11, 17, 20) but high levels of anti-IgG RF and ANA serum IgG2c or IgG2b in 5- to 6-mo-old FcγRIIB−/−TLR9−/− mice compared with those in age-matched FcγRIIB−/− controls (Fig. 1B, Supplemental Fig. 1). Development of IgG autoantibodies required MyD88 signaling and T cell help in both FcγRIIB−/− and FcγRIIB−/−TLR9−/− mice (Fig. 1C, Supplemental Fig. 1). In addition, frequencies of

FIGURE 1.  FcγRIIB−/−TLR9−/− mice exhibit exacerbated development of lupus disease and increased levels of anti-IgG RF IgG serum autoantibodies and proinflammatory Th1 and Th17 cells. A, Kaplan–Meier survival curves for wild-type C57BL/6 (n = 30), FcγRIIB−/− (RIIB−/−) (n = 21), RIIB−/−TLR9−/− (n = 21), and RIIB−/−MyD88−/− (n = 30) mice. All dead mice showed proteinuria before death. B, Anti-nucleosome (anti-nuc) and anti-IgG RF IgG2c and IgG2b serum autoantibody levels of 5- to 6-mo-old wild-type C57BL/6, RIIB−/−, and RIIB−/−TLR9−/− mice as analyzed by ELISA. Symbols represent data from individual mice. Horizontal lines show mean values with SEM. C, ANA serum IgG2c and IgG2b levels of 6-mo-old RIIB−/−TLR9−/−, RIIB−/−, and RIIB−/−MyD88−/− mice as analyzed by ELISA. One representative of at least two independent ELISAs is shown for each image. D, Frequencies of CD4+IFN-γ+ Th1 cells, CD4+IL-17+ Th17 cells, and CD4+CXCR3+ and CD4+CD44+CD62L− activated Th cells as determined by FACS in the blood or spleen of 5- to 6-mo-old wild-type C57BL/6, RIIB−/−, and RIIB−/−TLR9−/− mice as analyzed by ELISA. One representative of at least two independent ELISAs is shown for each image. E, IL-6 levels in 48-h supernatant of cultured splenocytes of 5- to 6-mo-old wild-type C57BL/6, RIIB−/−, RIIB−/−TLR9−/−, and RIIB−/−TLR9−/−TCRβ−/− mice as determined by ELISA. Bar graphs show the mean value with SEM for each group of mice. F, Kaplan–Meier survival curves for RIIB−/−TLR9−/− (n = 21; the same mice as in A) and RIIB−/−TLR9−/−IL-17RA−/− (n = 12) mice. All dead mice showed proteinuria before death.
CD138+ plasma cells were increased in the spleen and lymph nodes (LN) of FcyRIIB−/−TLR9−/− mice compared with those in FcyRIIB−/− mice (Supplemental Fig. 1).

Exacerbated lupus disease in FcyRIIB−/− mice lacking TLR9 was also reflected by increased frequencies of proinflammatory Th1 and Th17 cells in the blood, spleen, and LN and by increased frequencies of activated peripheral blood CXCR3+CD4+ and CD44+CD62L+CD4+ Th cells (Fig. 1D, Supplemental Fig. 1). The development of proinflammatory CD4+ T cells was inhibited if TLR signaling was abrogated in MyD88-deficient FcyRIIB−/− mice (Fig. 1D). Severe inflammation in FcyRIIB−/−TLR9−/− mice was further associated with increased production of the proinflammatory cytokine IL-6 and the frequent accumulation of macrophages and Th17 cells in the kidney, indicating the onset of lupus nephritis (Fig. 1E, Supplemental Fig. 2). The important role of IL-17 for the development of lupus nephritis in FcyRIIB−/−TLR9−/− mice was proved by the analysis of IL-17RA–deficient FcyRIIB−/−TLR9−/− mice. Mortality, proteinuria, and macrophage infiltration in the kidney were highly reduced in IL-17RA–deficient mice compared with those in IL-17RA–sufficient mice (Fig. 1F).

Thus, our data show that loss of MyD88 in lupus-prone FcyRIIB−/− mice inhibits inflammatory T and B cell responses and lupus disease. Loss of TLR9 in FcyRIIB−/− mice abolishes the induction of anti-nucleosome IgGs but leads to an uncontrolled accumulation of proinflammatory Th1 and Th17 cells and T cell–dependent IgG2c and IgG2b autoantibodies associated with exacerbated lupus disease.

**FIGURE 2.** Deletion of TLR9 in hematopoietic stem cells from FcyRIIB−/− mice exacerbates the accumulation of proinflammatory CD4+ T cells and lupus nephritis. A, Schematic illustration of the experimental setup used to reconstitute lethally irradiated FcyRIIB−/−mouse BM with BM from RIIB−/− (chim R) or RIIB−/−TLR9−/− (chim RT) mice. B, Frequency of CD4+IL-17+Th17 cells as determined by FACS in blood samples of chim R and chim RT mice 5 mo after BM chimerization. Symbols represent data from individual mice. Horizontal lines show mean values with SEM. C, Anti-nucleosome (anti-nuc) and ANA serum IgG2c and IgG2b levels of still-alive chim R and chim RT mice 6 mo after BM transfer as analyzed by ELISA. D, Kaplan–Meier survival curves of chim R (n = 9) and chim RT (n = 10) mice and control RIIB−/− (n = 21) and RIIB−/−TLR9−/− (n = 21) mice. All dead mice showed proteinuria before death. One representative of two independent experiments is shown.

**Loss of TLR9 in BM-derived B cells promotes development of proinflammatory CD4+ T cells and lupus disease in FcyRIIB−/− mice**

To ascertain whether the loss of TLR9 expression in hematopoietic cells is associated with exacerbated lupus disease in FcyRIIB−/−TLR9−/− mice, we reconstituted lethally irradiated FcyRIIB−/− (RIIB−/−) mice with BM from RIIB−/− (chim R) or RIIB−/−TLR9−/− (chim RT) mice (Fig. 2A). The irradiation dose was thereby sufficient to inhibit host recovery of the immune system (Supplemental Fig. 4). Chim RT mice resembled FcyRIIB−/−TLR9−/− mice in that they showed high numbers of activated proinflammatory CD4+ T cells, high levels of IgG2c ANA IgG2c and IgG2b in the absence of anti-nucleosome serum autoantibodies, and increased nephritis-induced mortality in comparison with those of chim R mice (Fig. 2B–D). Reconstitution of lethally irradiated RIIB−/−TLR9−/− mice with BM from RIIB−/− (chim TR) led to a comparable mortality rate compared with those of chim R and chim RT−/− mice (Supplemental Fig. 5). Although it cannot be excluded that the transferred BM contained mature B cells, these data suggest a TLR9 tolerance effect of BM-derived hematopoietic cells in lupus-prone FcyRIIB−/− mice.

To determine further whether TLR9 signaling in BM-derived B cells induces the development of anti-nucleosome IgG2c and IgG2b autoantibodies but at the same time controls the development of proinflammatory Th1 and Th17 cells in FcyRIIB−/− mice, we generated mixed BM chimeric FcyRIIB−/− mice with either TLR9−/− or MyD88-deficient B cells: lethally irradiated FcyRIIB−/− mice were reconstituted with 80% BM from B cell-
deficient FcγRIIB−/−JHT−/− mice plus 20% BM from FcγRIIB−/−TLR9−/− mice to generate FcγRIIB−/− mice, in which all B cells lack TLR9 (chim RJ+RT); or with 20% BM from FcγRIIB−/−MyD88−/− mice to generate FcγRIIB−/− mice, in which all B cells lack MyD88 (chim RJ+RM); or with 20% BM from FcγRIIB−/− as a control group (chim RJ-R) (Fig. 3A). Reconstitution of the B cell and non-B cell pool by mixed donor BM cells functioned as intended (Supplemental Fig. 6). Chim RJ+RT mice showed similar reductions in the levels of anti-nucleosome IgG2c and IgG2b serum autoantibodies as FcγRIIB−/−TLR9−/− mice, indicating that TLR9 stimulation in B cells is involved in the induction of anti-nucleosome IgG autoantibodies in FcγRIIB−/− mice (Fig. 3C). At the same time, however, levels of Th1, Th17, and activated CXCR3+ CD4+ and CD44+CD62L−CD4+ Th cell numbers and ANA IgG2c as well as nephritis-induced mortality were enhanced in comparison with those of control chim RJ+R mice (Fig. 3B–D). By contrast, chim RJ+RM mice with MyD88-deficient B cells lacked inflammatory T cell responses and IgG2c and IgG2b serum autoantibodies and showed no mortality (Fig. 3B–D).

We therefore conclude that B cell-intrinsic MyD88 signaling is important for the induction of inflammatory immune responses. However, B cell-intrinsic TLR9 signaling is not only crucial for the generation of anti-nucleosome IgG2c and IgG2b autoantibodies but also for controlling the accumulation of proinflammatory T cells and inflammatory autoimmune reactions associated with end-organ damage and lupus disease.

**B cell-intrinsic TLR9 stimulation regulates self-reactive IgM levels**

TLR-dependent tolerance mechanisms in B cells have been associated with production of the anti-inflammatory cytokine IL-10, protecting from autoimmunity (31). However, IL-10 production by total splenocytes or by peritoneal cells of TLR9-deficient FcγRIIB−/− mice was not reduced compared with that by TLR9-sufficient FcγRIIB−/− mice (Fig. 4A).

Self-reactive IgM autoantibodies have also been described to play a role in protecting against the development of lupus disease symptoms in mice and humans (3–5, 32). To establish whether exacerbated lupus disease in TLR9-deficient FcγRIIB−/− mice is associated with changes in self-reactive IgM levels, we measured self-reactive serum IgM Ab levels by ELISA (Fig. 4B). Five- to six-month-old FcγRIIB−/− mice showed increased levels of self-reactive ANA and anti-IgG RF and total serum IgM levels in comparison with those of age-matched C57BL/6 wild-type control mice. (Fig. 3).
mice (Fig. 4B). Surprisingly, self-reactive and total serum IgM Ab levels were significantly reduced in 5- to 6-mo-old MyD88- and TLR9-deficient FcγRIIB−/− mice and were thus comparable with the levels measured in age-matched C57BL/6 wild-type controls (Fig. 4B). These differences in IgM levels were already seen in young 3-mo-old animals (Fig. 4C and data not shown). Self-reactive ANA or anti-IgG RF IgM levels were also reduced in chimeric RT mice and chimeric RJ+RT and chimeric RJ+RM mice with TLR9- or MyD88-deficient B cells, respectively, compared with those in control chim R and chim RJ+R mice (Fig. 4D, 4E).

We therefore conclude that the development of self-reactive IgM serum Abs in autoimmune FcγRIIB−/− mice depends on B cell-intrinsic MyD88-mediated TLR9 signaling.

Low levels of self-reactive IgM Abs in FcγRIIB−/−TLR9−/− mice correlate with low numbers of BM-derived peritoneal B-1b cells

Marginal zone (MZ) B cells and B-1 cells have been associated with the generation of self-reactive IgM Abs (33–36). To ascertain whether differences in self-reactive serum IgM levels in 5- to 6-mo-old FcγRIIB−/− and FcγRIIB−/−TLR9−/− mice are associated with abnormalities in these B cell compartments, we measured the frequency of splenic and peritoneal B cell populations by FACS (Fig. 5, Supplemental Figs. 7, 8). No significant differences in the frequency of MZ B cells were observed between FcγRIIB−/− and FcγRIIB−/−TLR9−/− mice (Supplemental Fig. 7). Furthermore, reduction of MZ B cells via injection of an anti-CD21 Ab tend to reduce total serum IgM but not ANA serum IgM levels in FcγRIIB−/− mice (Supplemental Fig. 7) (28).

However, numbers of peritoneal B-1b cells but not of B-1a or B-2 cells were significantly reduced in 5- to 6-mo-old FcγRIIB−/−TLR9−/− mice in comparison with FcγRIIB−/− mice; peritoneal B-1b cell numbers were also reduced in FcγRIIB−/−MyD88−/− mice (Fig. 5A–D, Supplemental Fig. 8). B-1b cell frequencies were already reduced in FcγRIIB−/−TLR9−/− mice as young as 1.8 mo (Supplemental Fig. 8), suggesting a role of TLR9 in the maintenance of peritoneal B-1b cell numbers. Indeed, peritoneal B-1b cells but not B-1a cells were already reduced in TLR9-deficient C57BL/6 mice compared with those in wild-type C57BL/6 mice (Fig. 5B–D).

Loss of FcγRIIB might activate peritoneal B-1b cells in a TLR9-dependent way leading to the production and accumulation of self-reactive serum IgM in FcγRIIB−/− mice. Because activated B-1b cells have been described to migrate to the spleen to produce additional self-reactive IgM, we investigated CD4+ CD43+ IgMhigh CD23low B-1a cells and CD5+ CD43+ IgMhigh CD23+ B-1b cells in the spleen (36). Indeed, spleen B-1b but not B-1a cell numbers were increased in FcγRIIB−/− mice compared with those in wild-type C57BL/6 mice (Fig. 5E–H).

To investigate whether differences in self-reactive IgM serum Ab levels in FcγRIIB−/− and FcγRIIB−/−TLR9−/− mice correlated with the low number of peritoneal B-1b cells in FcγRIIB−/−TLR9−/− mice, we measured the levels of self-reactive IgM Abs...
FIGURE 5. Peritoneal B-1b cell numbers and peritoneal self-reactive IgM levels are reduced in FcγRIIB−/−TLR9−/− mice. A, Representative FACS analysis of peritoneal cells and gated Mac1int IgMhigh B-1 cells further analyzed by CD5 expression of FcγRIIB−/− (RIIB−/−) mice. B, Frequencies of peritoneal (perit.) CD5+ Mac1int IgMhigh B-1a cells, CD5− Mac1int IgMhigh B-1b cells, and Mac1+ IgMlow B-2 cells of 5- to 6-mo-old wild-type C57BL/6, RIIB−/−, RIIB−/−TLR9−/−, TLR9−/−, and TLR9−/−MyD88−/− mice. Symbols represent data from individual mice. Horizontal lines show mean values with SEM. C, Total peritoneal cell numbers. D, Peritoneal B-1a, B-1b, or B-2 cell numbers calculated from frequencies and total peritoneal cell numbers as analyzed in B and C. E, Representative FACS analysis of splenocytes and gated IgMhigh CD23int cells further analyzed by CD43 and CD5 expression of

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in the supernatant of equal numbers of cultured total splenocytes or peritoneal cells after 96 h (Fig. 5I). Total splenocytes and also peritoneal cells from FcγRIIB−/− mice produced more self-reactive IgM compared with wild-type C57BL/6 mice. Total splenocytes from FcγRIIB−/−TLR9−/− mice also produced more self-reactive IgM compared with wild-type C57BL/6 mice, but total peritoneal cells produced even less self-reactive IgM compared with wild-type C57BL/6 mice (Fig. 5J).

Thus, low levels of self-reactive IgM serum Abs in FcγRIIB−/−TLR9−/− and FcγRIIB−/−MyD88−/− mice correlate with low numbers of peritoneal B-1b cells (Supplemental Fig. 8). Peritoneal B-1b cell numbers were already reduced in TLR9-deficient C57BL/6 mice suggesting a general role of TLR9 in the maintenance of peritoneal B-1b cell numbers.

**TLR7 signaling does not compensate loss of TLR9 signaling in B-1b cells to induce proliferation and IgM production**

TLR signaling in B-1 cells has been associated with the generation of self-reactive IgM (37, 38). To investigate the role of TLR9 and the lupus-relevant TLR7, we sorted peritoneal B-1a, B-1b, and B-2 cells from FcγRIIB−/− and FcγRIIB−/−MyD88−/− mice and stimulated them in vitro with either the TLR9 ligand CpG ODN 1826 or the TLR7 ligand imiquimod or a combination of both for 96 h. CpG induced comparable and MyD88-dependent proliferation of all three sorted peritoneal B cell subsets. However, MyD88-dependent total and self-reactive IgM production by B-2 cells was significantly reduced compared with that by B-1a and B-1b cells as has been shown recently (Fig. 6A, 6B, Supplemental Fig. 9, and data not shown) (37). As described recently, TLR7 signaling counteracts TLR9 signaling in B cells (39). Indeed, the activation of TLR9 and TLR7 together reduced B cell proliferation and IgM production. TLR7 signaling alone induced neither B cell proliferation nor IgM production (Fig. 6A–D). Any induction of self-reactive IgG and IgA Abs was below the detection limit (Supplemental Fig. 9). It is worth mentioning that proliferation of B-1a cells was reduced compared with that of B-1b cells when total peritoneal B-1 cells were sorted and stimulated in vitro with CpG (Fig. 6C, 6D).

Comparable results were seen after in vivo i.p. stimulation of FcγRIIB−/− or FcγRIIB−/−MyD88−/− mice with CpG, imiquimod, or a combination of both (Fig. 6E–H, Supplemental Fig. 10). CpG highly induced MyD88-dependent peritoneal B-1b and B-2 cell proliferation and IgM but hardly IgG or IgA levels. However, proliferation of peritoneal B-1a cells was highly reduced.

Thus, MyD88-dependent TLR9 but not TLR7 signaling induces peritoneal B-1b cell proliferation and IgM production, TLR9 signaling does not induce B-1a cell proliferation in vivo, and B-2 cell proliferation by TLR9 stimulation is accompanied by only low IgM production.

**Transfer of peritoneal B-1b cells from FcγRIIB−/− mice into FcγRIIB−/−TLR9−/− mice inhibits Th17 cell accumulation and lupus nephritis**

To determine further the tolerogenic role of TLR9, we repeatedly transferred FcγRIIB-deficient total peritoneal cells into FcγRIIB−/−TLR9−/− mice starting at the age of 4 mo. Peritoneal cell transfer inhibited the uncontrolled accumulation of proinflammatory Th17 cells and the nephritis-induced mortality rate in FcγRIIB−/−TLR9−/− mice (Fig. 7A, 7B). Instead, repeated transfer of peritoneal cells from FcγRIIB−/−µs mice producing no soluble IgM into FcγRIIB−/−TLR9−/− mice failed to reduce Th17 cell accumulation and lupus disease (Fig. 7A, 7B) indicating that IgM produced by peritoneal B cells plays an essential role in inhibiting Th17 cell accumulation. Accordingly, repeated transfer of peritoneal B-1b cells from FcγRIIB−/− mice into FcγRIIB−/−TLR9−/− mice reduced Th17 cell accumulation, ANA serum IgG, and lupus disease (Fig. 7A–C).

Furthermore, transfer of peritoneal cells from FcγRIIB−/− but not from FcγRIIB−/−TLR9−/− mice into 5- to 6-mo-old FcγRIIB−/−TLR9−/− mice was sufficient to reduce the frequency of Th17 cells already after 9 d (Fig. 7D, 7E). Accordingly, transfer of peritoneal B-1b cells from FcγRIIB−/− mice temporarily tended to enhance self-reactive IgM levels and reduced Th17 cell frequencies (Fig. 7F), whereas transfer of peritoneal B-1a or B-2 cells from FcγRIIB−/− mice failed to reduce blood Th17 cells in FcγRIIB−/−TLR9−/− mice (Fig. 7G).

Thus, transfer of TLR9-expressing peritoneal B-1b but not B-1a or B-2 cells from FcγRIIB-deficient mice into FcγRIIB−/−TLR9−/− mice inhibits the accumulation of proinflammatory Th17 cells.

**Monoclonal self-reactive IgM Abs reduce Th17 cell accumulation and nephritis-induced mortality in FcγRIIB−/−TLR9−/− mice**

To ascertain whether self-reactive IgM can control the accumulation of proinflammatory CD4+ T cells and lupus disease in FcγRIIB−/−TLR9−/− mice, we i.p. treated FcγRIIB−/−TLR9−/− mice with a recombinant human/mouse chimeric monoclonal self- and polyreactive IgM Ab (ED38; 200 µg) twice a week starting at the age of 3.5 mo (Fig. 8A–D, Supplemental Figs. 11, 12). ED38 IgM-treated FcγRIIB−/−TLR9−/− mice showed lower nephritis-induced mortality rates than those of untreated or human/mouse chimeric control IgM (mgo53)-treated FcγRIIB−/−TLR9−/− mice (Fig. 8A). The development of mouse IgG1 Abs against chimeric human/mouse IgM had obviously no effect (Supplemental Fig. 12). ED38 IgM-treated FcγRIIB−/−TLR9−/− mice further showed lower levels of proinflammatory splenic or blood CD4+ T cells and CD138+ plasma cells than those of untreated or control IgM (mgo53)-treated FcγRIIB−/−TLR9−/− mice (Fig. 8B, 8C).

IgG2c and IgG2b serum autoantibody levels are higher in 5- to 6-mo-old FcγRIIB−/−TLR9−/− mice, which develop proteinuria, compared with those in mice that do not develop proteinuria until the age of 8 mo (Fig. 8D). Accordingly, ED38 IgM-treated mice tend to have lower self-reactive IgG serum Ab levels compared with those of control IgM (mgo53)-treated mice (Fig. 8D). Furthermore, at the age of 9 mo, ED38 IgM-treated mice showed IgG2c immune complexes but no macrophage infiltration in the kidney in comparison with untreated FcγRIIB−/−TLR9−/− mice with proteinuria (Supplemental Fig. 12). Notably, one i.p. transfer (200 µg) of the monoclonal self-reactive and polyreactive ED38 IgM Ab (1RIIgc7) but not of the control IgM Ab (mgo53) into 5- to 6-mo-old FcγRIIB−/−TLR9−/− mice was sufficient to reduce the frequency of proinflammatory Th17 cells already after 9 d (Fig. 8E).

One i.p. transfer of the monoclonal self-reactive and polyreactive mouse IgM Ab (1RIIgc7; 200 µg) into 5- to 6-mo-old FcγRIIB−/− mice had no effect after 9 d (Fig. 8F).
FIGURE 6. TLR9 stimulation in vitro and in vivo induces proliferation of peritoneal B-1b cells and accumulation of IgM Abs. A and B. Sorted peritoneal (perit.) CD5<sup>+</sup> MacI<sup>int</sup> IgM<sup>high</sup> B-1a cells, CD5<sup>−</sup> MacI<sup>int</sup> IgM<sup>high</sup> B-1b cells, or MacI<sup>−</sup> IgM<sup>int</sup> B-2 cells of 2- to 3-mo-old FcγRIIB<sup>−/−</sup> (RIIB<sup>−/−</sup>) or RIIB<sup>−/−</sup> MyD88<sup>−/−</sup> mice were stimulated in vitro with either CpG ODN 1826 or imiquimod or a combination of both and analyzed 96 h later (n = 3). A. Total cell numbers 96 h after stimulation. Bar graphs show the mean value with SEM for each group of mice. B. Total and ANA IgM levels in the supernatant 96 h after stimulation as analyzed by ELISA. One representative of two independent experiments is shown.

C and D. Sorted peritoneal MacI<sup>int</sup> IgM<sup>high</sup> B-1 cells of 2- to 3-mo-old RIIB<sup>−/−</sup> mice were stimulated with either CpG (n = 5) or imiquimod (n = 6) or a combination of both (n = 6) and analyzed 96 h later. C. Total numbers of CD5<sup>−</sup> MacI<sup>int</sup> IgM<sup>high</sup> B-1a and CD5<sup>−</sup> MacI<sup>int</sup> IgM<sup>high</sup> B-1b cells 96 h after stimulation calculated by considering total cell numbers and FACS analysis (data not shown). D. Total IgM Ab levels in the supernatant 96 h after stimulation as analyzed by ELISA. One representative of five independent experiments is shown.

E–H. Eight-week-old RIIB<sup>−/−</sup> mice were i.p. stimulated in vivo with either CpG (n = 5) or imiquimod (n = 6) or a combination of both (n = 6) and analyzed at day 7. E. Representative FACS analyses of peritoneal cells and gated MacI<sup>int</sup> IgM<sup>high</sup> B-1 cells further
Thus, injection of monoclonal self-reactive IgM Abs prevents the uncontrolled development of proinflammatory Th17 cells, accumulation of IgG serum autoantibodies, and lupus disease in FcγRIIB<sup>-/-</sup>TLR9<sup>-/-</sup> mice.

**Discussion**

TLR as well as MyD88 signaling are known to play an initial role in the activation of APCs leading to the induction of inflammatory T and B cell responses. Our data further clarified that also B cell intrinsic MyD88 signaling is required for the development of pathogenic IgG autoantibodies and lupus disease in FcγRIIB<sup>-/-</sup> mice. However, MyD88 signaling is not only involved in the TLR9-dependent development of anti-nucleosome IgG2c and IgG2b autoantibodies but also in cell maturation via other TLRs (e.g., TLR7) (7–12, 14, 15, 17, 40, 41).

Loss of TLR9 in FcγRIIB<sup>-/-</sup> mice abrogates the development of anti-nucleosome IgG2c and IgG2b autoantibodies but not the activation of the immune system via other TLRs, which is even enhanced by loss of TLR9 tolerance. In contrast, loss of MyD88 in FcγRIIB<sup>-/-</sup> mice inhibits signaling via different TLRs and thereby abolishes the uncontrolled accumulation of Th1, Th17, and B-2 cells, pathogenic IgG2c and IgG2b autoantibodies, nephritis, and mortality. However, MyD88 signaling is also involved in the TLR9-dependent tolerance mechanism, which however does not phenotypically become obvious at first sight in healthy MyD88<sup>-/-</sup> FcγRIIB<sup>-/-</sup> double-deficient mice compared with lupus-prone TLR9<sup>-/-</sup>FcγRIIB<sup>-/-</sup> mice.

A tolerogenic function of TLR stimulation in B cells has previously been linked to the production of the anti-inflammatory cytokine IL-10 (31). We obtained no evidence of an IL-10 decline in TLR9<sup>-/-</sup>FcγRIIB<sup>-/-</sup> mice compared with FcγRIIB<sup>-/-</sup> mice. However, alterations in IL-10 have not been completely ruled out. Previous work has suggested also that self-reactive IgM Abs play a protective role in autoimmunity (3–5, 32, 42, 43) and...
that B-1b cells have a tolerogenic function, but underlying mechanisms have been unknown (44, 45). Our findings now show that loss of MyD88-dependent TLR9 signaling is associated with reduced peritoneal B-1b cell numbers and reduced protective self-reactive IgM serum levels in lupus-prone FcγRIIB−/− mice leading to the uncontrolled accumulation of proinflammatory CD4+ T cells and severe autoimmunity.

TLR9-deficient but not TLR9-sufficient FcγRIIB−/− mice showed a significant reduction of peritoneal B-1b cells compared with C57BL/6 wild-type mice even at young age suggesting that TLR9 plays a key role in regulating peritoneal B-1b cells. This observation was confirmed by the fact that already TLR9−/− mice showed reduced B-1b cell numbers and that B-1b cells, but hardly B-1a cells, are highly responsive to CpG in vivo. Thus, TLR9 seems to be essential for peritoneal B-1b cell number maintenance.

In contrast to B-1a cells, secretion of IgM Abs by B-1b cells requires Ag-mediated activation (46–52). Loss of FcγRIIB-dependent negative feedback mechanisms in self-reactive peritoneal B-1b cells may promote their activation by TLR9 ligand

**FIGURE 8.** Reconstitution of self-reactive IgM levels by transfer of monoclonal self-reactive IgM Abs in FcγRIIB−/−TLR9−/− mice inhibits accumulation of proinflammatory Th17 cells and prevents lupus disease. A–E. Self-reactive ED38 IgM protects from lupus disease. A, Kaplan–Meier survival curves for untreated FcγRIIB−/−TLR9−/− mice (RIIB−/−TLR9−/−; n = 21) and ED38 IgM-treated (n = 15) and control IgM-treated (ctrl.; mgo53; n = 5) RIIB−/−TLR9−/− mice (200 μg twice a week starting at the age of 3.5 mo). B. Frequencies of spleen or blood CD4+IFN-γ+ Th1 cells or CD4+IL-17+ Th17 cells as analyzed by FACS of 5- to 6-mo-old untreated wild-type C57BL/6 and RIIB−/−TLR9−/− mice or 9-mo-old RIIB−/−TLR9−/− mice treated with ED38 IgM or control (mgo53) IgM (Th1 and Th17 cells in spleen; C57BL/6 mice, n = 3; untreated RIIB−/−TLR9−/− mice, n = 3-4; ED38 IgM-treated RIIB−/−TLR9−/− mice, n = 5). Bar graphs show the mean value with SEM for each group of mice. Symbols represent data from individual mice. Horizontal lines show mean values with SEM. C. Frequencies of splenic CD138+ plasma cells (PC) in 5- to 6-mo-old untreated RIIB−/−TLR9−/− mice treated with ED38 IgM or control (mgo53) IgM (Th1 and Th17 cells in spleen; C57BL/6 mice, n = 7) and 9-mo-old RIIB−/−TLR9−/− mice treated with ED38 IgM (n = 5) as determined by FACS. D, ANA and anti-IgG RF serum IgG2c and IgG2b levels as analyzed by ELISA of 5- to 6-mo-old untreated wild-type C57BL/6 and RIIB−/−TLR9−/− mice or 9-mo-old RIIB−/−TLR9−/− mice treated with ED38 IgM or control (mgo53) IgM (Th1 and Th17 cells in spleen; C57BL/6 mice, n = 3; untreated RIIB−/−TLR9−/− mice, n = 3-4; ED38 IgM-treated RIIB−/−TLR9−/− mice, n = 5). Bar graphs show the mean value with SEM for each group of mice. Symbols represent data from individual mice. Horizontal lines show mean values with SEM. One representative experiment is shown. E and F, Frequencies of blood CD4+IL-17+ Th17 cells in 5- to 6-mo-old (E) RIIB−/−TLR9−/− or (F) RIIB−/− mice before (d0) and 9 d after (d9) i.p. transfer of 200 μg monoclonal control (mgo53), self-reactive ED38, or self-reactive 1RIlg7 IgM Ab. One representative of two independent experiments is shown for each approach.
containing Ags via the BCR and TLR9 and MyD88 costimulation (7, 34, 48, 53) and thus the secretion of self-reactive IgM serum Abs (54).

Thus, two mechanisms might be responsible for the reduced levels of self-reactive IgM serum autoantibodies in TLR9- and MyD88-deficient FcγRIIB−/− mice: 1) less B-1b cell numbers and/or 2) reduced B-1b cell activation.

We demonstrate that the transfer of TLR9/MyD88-sufficient peritoneal B-1b cells from FcγRIIB−/− mice or monochlocal self-reactive IgM Abs controls the development of Th17 cells and reduces lupus disease in FcγRIIB−/−/TLR9−/− mice, thus supporting the idea that the tolerogenic function of B-1b cells may rely on the TLR9-dependent generation of self-reactive serum IgM Abs. The importance of IgM serum Abs in the maintenance of self-tolerance has previously been demonstrated in mice with aggravated lupus disease due to lack of soluble IgM Abs and by the finding that transfer of a monoclonal anti-DNA IgM Ab delays the onset of nephritis in NZB/W mice (5, 32). Our findings now show that transfer of monoclonal self-reactive IgM Abs is sufficient to reduce Th17 cell numbers already after 9 d suggesting that Th17 cells are short-lived. Self-reactive IgM might contribute to self-antigen clearance to avoid Th17 cell priming by APCs (4, 5, 32, 35). Additionally, recent data have shown that clearance of apoptotic cells by self-reactive IgM induces tolerance in dendritic cells via an active mechanism inhibiting inflammatory TLR responses and avoids Th17 cell development (42, 43). Deletion of the IL-17RA in FcγRIIB−/−/TLR9−/− mice strongly reduced the infiltration of effector immune cells in the kidney, proteinuria, and mortality, further demonstrating the important role of Th17 cells in the induction of lupus disease.

Taken together, our data suggest that the tolerogenic function of MyD88-dependent TLR9 signaling is mediated by the production of self-reactive serum IgM Abs by peritoneal B-1b cells, which protects from uncontrolled accumulation of Th17 cells and severe autoimmunity. These findings provide new insight in the tolerogenic function of TLR9, B-1b cells, and self-reactive IgM to control autoimmunity. These findings provide new insight in the tolerogenic function of TLR9, B-1b cells, and self-reactive IgM to control autoimmunity. These findings provide new insight in the tolerogenic function of TLR9, B-1b cells, and self-reactive IgM to control autoimmunity. These findings provide new insight in the tolerogenic function of TLR9, B-1b cells, and self-reactive IgM to control autoimmunity.


