Tonic B cell activation by Radioprotective105/MD-1 promotes disease progression in MRL/lpr mice

Toshihiko Kobayashi1,2, Koichiro Takahashi1, Yoshinori Nagai1,6, Takuma Shibata1, Masako Otani3, Shozo Izui3, Shizuo Akira4, Yoshiyuki Gotoh5, Hiroshi Kiyono5 and Kensuke Miyake1

1Division of Infectious Genetics, Institute of Medical Science, University of Tokyo, Tokyo, Japan
2Japan Society for the Promotion of Science, Tokyo, Japan
3Department of Pathology and Immunology, University of Geneva, Geneva, Switzerland
4Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan
5Division of Mucosal Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan
6Present address: Department of Immunobiology and Pharmacological Genetics, Graduate School of Medicine and Pharmaceutical Science for Research, University of Toyama, Toyama, Japan

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Abstract
Toll-like receptors (TLRs) have a crucial role in sensing microbial products and triggering immune responses. Recent reports have indicated that TLR7 and TLR9 have an important role in activating autoreactive B cells. In addition to TLR7 and TLR9, mouse B cells express TLR2, TLR4 and structurally related Radioprotective105 (RP105). We have previously shown that RP105 works in concert with TLR2/4 in antibody response to TLR2/4 ligands. We here report that B cells are constitutively activated by TLR2/4 and RP105. Such B cell activation was revealed by the γ3 germ line transcript and serum IgG3 production, both of which were impaired by the lack of RP105 or TLR2/4. Serum IgG3 was not altered in germ-free or antibiotics-treated mice, suggesting that the microbial flora hardly contributes to the continuous activation of B cells. The lack of RP105-dependent B cell activation ameliorated disease progression in lupus-prone MRL/lpr mice. RP105−/− MRL/lpr mice showed less lymphoadenopathy/splenomegaly and longer survival than MRL/lpr mice. Whereas glomerulonephritis and auto-antibody production were not altered, improvement in blood urea nitrogen and lower incidence of renal arteritis indicated that renal function was ameliorated in the absence of RP105. Our results suggest that RP105-dependent tonic B cell activation has a pathogenic role in MRL/lpr mice.

Introduction
Systemic lupus erythematosus (SLE) is multisystem, autoimmune, connective tissue disorder with a broad range of clinical features. Pathogenic mechanisms underlying SLE are largely unknown. Mouse models of SLE have provided significant insights into the identification of critical molecular pathways that mediate the generation of this autoimmune disease. Two mouse strains have been extensively studied. The NZB/NZW F1 (BWF1) lupus nephritis model shares pathogenic features with human SLE (1). The other strain, MRL/lpr, is also a well-established murine model of SLE, in which the disease is characterized by glomerulonephritis with immune complex deposition, vasculitis, splenomegaly, lymphadenopathy and auto-antibody production (2). Auto-antibody is a general feature of lupus, and the mechanisms that result in the selection and expansion of auto-antibody-producing B cells have been suggested to involve the Toll-like receptors (TLRs) (3). TLRs are known to recognize microbial molecules ranging from nucleic acids to membrane lipids. These receptors can activate diverse cell populations of the immune system to initiate or enhance protective immune responses. On a susceptible genetic background, however, TLR signaling also induces autoimmunity. In particular, TLR7 and TLR9 can provide a co-stimulatory signal for auto-antibody production (4, 5). The lack of TLR7 or TLR9 down-regulated auto-antibody production in autoimmune-prone mice (5).
Tonic B cell activation in autoimmunity

In addition to nucleic acid-sensing TLRs, mouse B cells express TLR2 and TLR4/MD-2, both of which were reported to respond to microbial membrane components and non-microbial products derived from tissue damages (6–11). B cells also express Radioprotective105 (RP105)/MD-1, which is structurally and functionally related to the LPS sensor TLR4/MD-2 (12–15). RP105 forms a complex with MD-1 and can transmit strong proliferation signals into B cells when ligated by a specific mAb. Although RP105/MD-1 is expressed on dendritic cells and macrophages as well, TLR2/4 responses were impaired only in B cells (16). RP105/MD-1 was reported to be associated with TLR4/MD-2 (17), suggesting that RP105/MD-1 is activated simultaneously with TLR4 by co-clustering. RP105−/− mice were as low as myeloid differentiation gene 88 (MyD88−/−) mice in serum IgG3 production. Little is known about roles for RP105/MD-1 in autoimmune diseases. We here studied roles for RP105 in B cell activation in the steady state and in autoimmune diseases in MRL/lpr and BWF1 mice. The present study demonstrated that RP105/MD-1 works in concert with TLR2/TLR4 in constitutive B cell activation in the steady state and has a pathogenic role in autoimmune diseases in MRL/lpr mice but not in BWF1 mice. Taken together, tonic B cell activation by the cell surface complex consisting of TLR2, TLR4 and RP105/MD-1 may have a pathogenic role in MRL/lpr mice.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC co. (Shizuoka, Japan) and used for analyses at 7–12 weeks of age. Mutant mice were maintained in the animal facility at the Institute of Medical Science, the University of Tokyo. C57BL/6 RP105−/−, MD-1−/− mice have been described previously (14, 18). MyD88−/−, TRIF−/−, TIRAP−/− and TLR7−/− or TLR7−/− mice were provided by S. Akira (Osaka University). RP105−/− autoimmune mice were generated by intercrossing C57BL/6 RP105−/− mice with MRL/MPJ/JmJ mice. After ninth backcross generation, mice were analyzed at 19–20 weeks of age. RP105−/− New Zealand Black (NZB) and RP105−/− New Zealand White (NZW) mice were generated by intercrossing C57BL/6 RP105−/− mice with NZB or NZW mice. After backcrossing 10 generations, RP105−/− or RP105−/− NZB/W F1 (BWF1) mice were generated by breeding NZB RP105−/− and NZW RP105−/− mice.

Germ-free mice at 7 weeks of age (ICR mice) and their control-specific pathogen-free mice were purchased from CLEA Japan. Germ-free mice were maintained in the sterile condition from their birth and fed with sterilized chow. Germ-free mice were confirmed by the absence of bacterial colony in feces. For antibiotics treatment, mice were fed with drinking water containing ampicillin 1 g l−1, neomycin 1 g l−1, vancomycin 0.5 g l−1 and metronidazole 0.5 g l−1 during 4–8 weeks of age. Unless otherwise noted, female mice were used for all experiments in this study. All the animal experiments were conducted with the approval of the Animal Research Committee at the Institute of Medical Science in the University of Tokyo.

Reagents

LPS from Escherichia coli (serotype O55:B5) was purchased from Sigma–Aldrich (St Louis, MO, USA). N-palmitoyl-S-(2,3-bis (palmitoyloxy)-(2R,S)-propyl)-(R)-cysteinyl-seryl-(lysyl)3-lysine (P3CSK4) was purchased from EMC microcollections (Tubingen, Germany). CpG (CpG1668: TCCATGACGTTCTGATGCT) was purchased from Hokkaido System Science (Sapporo, Japan). Loxoribine (7-allyl-8-oxo-guanosine) and poly(I:C) were purchased from InvivoGen (San Diego, CA, USA). Lymphocytes from 19-week-old mice were stained with the following antibodies: FITC-anti-CD4, PE-anti-CD8 and PE-Cy7-anti-CD3e for T cell separation; PE-Cy7-anti-CD19 for B cell separation. FITC-anti-CD44 and biotinylated anti-CD69 followed by allophycocyanin-streptavidin for the analysis of lymphocyte activation status, respectively. Stained cells were analyzed by flow cytometry (BD FACSCalibur; BD Biosciences).

Measurement of serum Ig titer

The levels of serum Igs were measured by ELISA as described previously (16). Briefly, goat anti-mouse Ig (H+L) antibody (SouthernBiotech, Birmingham, AL, USA) was coated onto the ELISA plate and blocked with 0.5% BSA, and serial serum dilutions were applied. Bound antibodies were detected by peroxidase-conjugated goat antibodies to IgG1, IgG2a, IgG2b, IgG2c (for C57BL/6 mice), IgG3, IgM or IgA.

Auto-antibody measurement

Serum levels of IgG auto-antibodies against chromatin and double-stranded DNA (dsDNA) were determined by ELISA as described previously (19, 20). The assay was developed with alkaline phosphatase-conjugated anti-mouse IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies (SouthernBiotech). For the measurement of anti-IgG2a rheumatoid factor (RF), ELISA plates were coated with 2,4-dinitrophenyl (DNP)–BSA and then incubated with IgG2a anti-DNP (Hy1.2) mAb as a competitor against anti-DNA antibodies (21). These plates were then incubated with two different dilutions of sera (1:200 and 1:1000), and the assay was developed with alkaline phosphatase-conjugated anti-mouse IgG3.

Blood urea nitrogen

Amount of blood urea nitrogen (BUN) in mice sera was measured by using BUN-kainos kit (Kainos Inc., Tokyo, Japan), according to the manufacturer’s instruction.

Quantitative reverse transcription–PCR

Splenic B cells from non-immune mice were separated by CD43 negative selection using AutoMACS system (Miltenyi Biotec). B cell purity was >95%, as judged by flow cytometry analyses. Total RNA was extracted from splenic B cells using RNeasy kit (QIAGEN) and cDNA was synthesized with SuperScript III First-Strand cDNA Synthesis System for reverse transcription–PCR (Invitrogen) according to the manufacturer’s instruction. Quantification of cDNA was performed by using the 7300 Real Time PCR System (Applied Biosystems). Activation-induced cytidine deaminase (AID) expression was quantified by using TaqMan Gene expression
assays for AID (Mm 00507774_m1) (Applied Biosystems), while μ, γ3 and γ2b expression were quantified by using the sets of specific primers in Universal Probe Library (Roche Diagnostics). Primer sequences were as follows: for μ sense, gatatgtgtaagctgtgcta and μ anti-sense, tgttgctatatgtggtctg; for γ3 sense, gactgtacctcagacct and γ3 anti-sense, actgggtcttgattcgcg; for γ2b sense, ggccagtcagcagct and γ2b anti-sense, aggacaggttgatgttg. Each sample was normalized by mouse hypoxanthine guanine phosphoribosyl transferase (HPRT) expression (Mm 00469668_m1).

**Histopathology**

Kidneys were obtained from 18- to 19-week-old MRL mice, and histological sections were stained with either periodic acid-Schiff or hematoxylin–eosin (H&E). Glomerulonephritis was scored on a 0–4 scale based on the intensity and extent of histological changes. A grade 0 was given to kidneys without glomerular lesions. The 1+ lesions corresponded to minimal thickening of the mesangium, 2+ lesions contained noticeable increases in both mesangial and glomerular cellularity, 3+ lesions were characterized by the preceding conditions with superimposed inflammatory exudes and capsular adhesions and in 4+ lesions the glomerular architecture was obliterated in >70% of glomeruli and tubular cast formation was extensive. Grades 3 and 4 glomerulonephritis were considered to be significant contributors to clinical disease and/or death. Incidence of necrotizing arteritis of small- to medium-sized muscular arteries in kidneys was assessed by analyzing two independent sections of each animal. The infiltration of mononuclear and/or polymuclear inflammatory cells in association with the destruction of the arterial wall was considered positive for necrotizing arteritis. All histological examination was controlled by a renal pathologist in a blinded manner.

**Statistical analysis**

Paired data were evaluated by Student’s t-test. A value of P < 0.05 was considered statistically significant. Mortality assessments of MRL/lpr mice and BWF1 mice were evaluated by log-rank test. Incidence of renal vasculitis was evaluated by chi-square test.

**Results**

**Serum IgG3 is dependent on TLR2, TLR4 and RP105/MD-1**

We previously reported that RP105−/− B cells were impaired in responses to TLR2/TLR4 ligands (16). MD-1−/− B cells showed hyporesponsiveness to LPS and a TLR2/TLR1 ligand P3CSK4, which were indistinguishable from RP105−/− B cells. The analysis of serum Igs revealed that MD-1−/− mice were similar to RP105−/− mice in reduced serum IgG3, demonstrating a requirement for the RP105−/−MD-1 complex in serum IgG3 production (Fig. 1A). RP105 does not have a TLR-like signaling domain in the cytoplasmic region (13), and RP105-mediated B cell activation was not altered in MyD88−/− mice (16, 22). Considering that serum IgG3 was severely reduced in MyD88−/− mice as well (Fig. 1A), MyD88-dependent TLRs are likely to work in concert with RP105/MD-1 for IgG3 production (16, 23). Given the functional relationship between RP105 and TLR2/4 (16), TLR2/4 are most likely to contribute to serum IgG3 production. Mice lacking TLR2 or TLR4 were, however, not as low as MyD88−/− or RP105−/− mice (16). We, therefore, suspected that TLR2 and TLR4 were redundant for IgG3 production and established mice lacking both TLR2 and TLR4. TLR2−/−TLR4−/− mice were as low as MyD88−/− mice in serum IgG3 (Fig. 1A). Along this line, mice lacking TIRAP, an adaptor molecule required for signaling via TLR2 or TLR4 (24), were similar to TLR2−/−TLR4−/− mice in reduced serum IgG3 (T. Kobayashi, S. Akira, K. Miyake, unpublished data). No change was observed in a T-dependent isotype IgG1, whereas IgG2b was low in mice lacking RP105 or MD-1 but not in TLR2−/−TLR4−/− mice. IgG2c production was considerably impaired in MyD88−/− mice due to their defect in Tα1 development (25).

These results clearly demonstrated that TLR2, TLR4 and RP105/MD-1 were critically involved in basal production of serum IgG3. Because serum IgG3 was reported to be low in TLR7−/− MRL/lpr mice (5), we also studied TLR7−/− C57BL/6 mice, which showed, however, no significant alteration in serum IgG3 (Fig. 1B). We also found that serum IgG2b production was dependent on RP105/MD-1 (Fig. 1A).

**Impaired expression of the γ3 germ line transcript in RP105−/− and MD-1−/− mice**

Specific reduction in serum IgG3 and IgG2b suggested that RP105−/− or MD-1−/− B cells have a defect in class switching from μ to γ3 or to γ2b. Class switch recombination to γ3 or γ2b requires the germ line transcript for γ3 or γ2b and AID, a master regulator of class switch recombination (26, 27). To gain insight into a mechanism regulating serum IgG3 and IgG2b, we first examined expression of the germ line transcript for γ3 and γ2b. The germ line transcripts for γ3 or γ2b, but not for γ1 or γ2a, were reported to be detectable in splenic B cells without any activation (27). It has not been clarified whether these germ line transcripts are induced autonomously or non-autonomously. The latter possibility suggests that splenic B cells continuously respond to environmental stimulation including microbial flora. Interestingly, the γ3 germ line transcript, which is constitutively expressed in wild-type mice, was almost completely abolished in mice lacking RP105, MD-1, MyD88 and TLR2−/−TLR4−/− without any significant change in mRNA for μ chain (Fig. 2A), demonstrating that the γ3 transcript expression is dependent on TLR2, TLR4 and RP105/MD-1. On the other hand, moderate down-regulation of the γ2b transcript was observed in RP105−/− or MyD88−/− B cells. The changes of the γ2b germ line transcript in MD-1−/− or TLR2−/−TLR4−/− mice were much less pronounced. The γ2b germ line transcript seemed to be differently regulated in comparison to the γ3 germ line transcript.

Constitutive TLR signaling was able to induce the γ3 germ line transcript but apparently not sufficient for AID induction, since AID was not constitutively expressed in splenic B cells (27). We studied AID induction in response to TLR ligands. Enriched B cells were stimulated with LPS, P3CSK4 or CpG for 2 days. AID mRNA was up-regulated by all the TLR ligands examined (Fig. 2B). AID induction in B cells lacking RP105 or MD-1 was partially impaired in response to LPS or P3CSK4 but not at all in response to CpG. Because the γ3
germ line transcript is constitutively expressed in B cells, its induction by LPS stimulation was much less than that of AID (data not shown). Only ~30% increase in the γ3 germ line transcript was seen with LPS stimulation at 1 μg ml⁻¹. Similar results were reported by Muramatsu et al. (27). Constitutive expression of the γ3 germ line transcript is likely to be sufficient for the class switching from μ to γ3.

AID induction by TLR2/4 ligands was not so severely impaired as compared with the γ3 germ line transcript in the steady state (Fig. 2A and B), suggesting that the impaired induction of the γ3 germ line transcript in the steady state is principally responsible for reduced serum IgG3. These results suggested that the constitutively expressed γ3 germ line transcript was induced by TLR2/4 and RP105/MD-1.

Given that MyD88⁻/⁻ and TIRAP⁻/⁻ mice are similar to TLR2⁻/⁻-TLR4⁻/⁻ double-deficient mice in impaired serum IgG3 production, the TLR2/4-dependent signaling is required for serum IgG3 production. By contrast, we do not have direct evidence for requirement of RP105 signaling in induction of the γ3 germ line transcript. It remains clarified how RP105/MD-1 is involved in serum IgG3 production.

Roles for microbial flora in serum IgG3 production
Considering that the expression of the γ3 germ line transcript depends on TLR2, TLR4 and RP105 in resting B cells, B cells are likely to respond to TLR ligands without any infection or immunization. Microbial flora may stimulate splenic B cells, although spleen is not exposed to microbial flora.
Roles for RP105 in autoimmunity in MRL/prp

RP105−/− mutation ameliorated mortality in the MRL/prp strain (Fig. 5A). In contrast, the lack of RP105 did not influence mortality in BWF1 mice despite IgG3 reduction (Fig. 5A). Elevation of BUN, which correlated with renal dysfunction, was significantly improved in RP105−/− MRL/prp mice, whereas no significant difference was detected between RP105−/− and RP105−/− BWF1 mice (Fig. 5B). We further examined glomerulonephritis in MRL/prp mice. Despite the improvement in BUN accumulation (Fig. 5B), any significant change was not found in the intensity and extent of glomerular alterations when assessed at 18–19 weeks of age (Fig. 5C). MRL/prp mice spontaneously develop systemic-necrotizing arteritis of small- and medium-sized muscular arteries, which is characterized by the infiltration of mononuclear and/or polymuclear leukocytes and by the destruction of the arterial wall (30). Notably, necrotizing arteritis in kidneys was observed less frequently in RP105−/− MRL/prp mice than in RP105−/− MRL/prp (Fig. 5D). Impaired renal perfusion may be responsible for deterioration of renal function revealed by elevated BUN.

MRL/prp mice develop severe splenomegaly and lymphadenopathy due to accumulation of lymphocytes expressing an activated phenotype in these organs (31). The weight of lymphoid organs including spleen, mesenteric lymph nodes, and axillary lymph nodes was examined at 19 weeks of age. We found that splenomegaly and lymphadenopathy were both significantly reduced in the absence of RP105 in MRL/prp mice (Fig. 6A and B). Due to unknown reason, axillary lymph nodes were most apparent in difference between RP105−/− MRL/prp and RP105−/− MRL/prp mice (Fig. 6B). We next examined lymphocyte subpopulation in the spleen. In the percentages of CD4+ T cells and CD8+ T cells were significantly improved, whereas no significant difference was observed in the percentages of CD4+/CD8− double-negative T cells (DNTCs) and B cells (Fig. 6C).
RP105 deficiency seemed to decrease the total number of DNTC without influencing the percentage of DNTC in spleen, as revealed by ameliorated splenomegaly (Fig. 6A and B). Activation status of splenic B cells was analyzed by cell surface staining of CD44 and CD69, which did not reveal any difference in the absence or presence of RP105 (Fig. 6D). In contrast, CD44+/CD4+ activated T cells in mesenteric lymph nodes were significantly decreased in the absence of RP105 in MRL/lpr mice (Fig. 6E).

Auto-antibody production is not altered by the lack of RP105
We finally studied auto-antibody production against DNA and chromatin. Unexpectedly, we could not find any significant difference between RP105−/− and RP105+/+ MRL/lpr mice (Fig. 7). Kikuchi et al. (32) reported that the transgenic mice expressing IgG3 against IgG2a RF developed necrotizing arteritis in small- and medium-sized arteries of the kidney. We therefore determined the serum levels of IgG3 anti-IgG2a RF by ELISA (Fig. 8). We could not find any reduction in the IgG3 anti-IgG2a RF activity regardless of improvement in necrotizing arteritis in RP105+/+ MRL/lpr mice. Although RP105 was likely to have a pathogenic role in necrotizing arteritis formation in the kidneys of MRL/lpr mice, IgG3 anti-IgG2a RF did not seem to be involved in the RP105-dependent arteritis.

Discussion
The present study demonstrated that B cells are constitutively activated by pathogen sensors including TLR2, TLR4 and RP105/MD-1. Continuous B cell activation leads to the induction of the γ3 germ line transcript and production of serum IgG3. In our previous study (16), we showed that antigen-specific, T-independent Ig production driven by TLR2/4 ligands is all dependent on RP105. A role for RP105/MD-1 in serum Ig production seems, however, to be more specific for IgG3 than that in T-independent B cell
responses. TLR-independent mechanisms, either T dependent or independent, would have an important role in serum Ig production. In this regard, serum IgG3 production seems to be most dependent on TLRs and least dependent on TLR-independent mechanisms. By contrast, serum IgM and IgG2b are likely to be influenced by TLR-independent mechanisms as well as by TLR-dependent mechanisms.

Although the requirement for these pathogen sensors suggested a role for microbial flora in inducing serum IgG3, we could not detect any alteration in serum IgG3 in germ-free or antibiotics-treated mice (Fig. 3). With regard to IgG2b production, MD-1−/− mice are impaired only in serum IgG2b, but not in the γ2b germ line transcript (Figs 1A and 2A). In this regard, MD-1−/− mice were distinct from RP105−/− mice, which are impaired in both serum IgG2b and the γ2b germ line transcript (Figs 1A and 2A). The discrepancy between MD-1−/− and RP105−/− mice might suggest a pleiotropic role for soluble MD-1. IgG3 is predominantly produced in T-independent responses, whereas IgG2b is produced in T-dependent responses as well. In this regard, it is of note that MD-1 was reported to have a regulatory role in T cell responses (33). Further study is required for clarifying this issue.

The results in Figs 1–3 together with our previous study (16) demonstrated that TLR2, TLR4 and RP105/MD-1 have a role in tonic B cell activation. We next asked a pathogenic role for the RP105-dependent tonic B cell activation in autoimmune diseases by introducing RP105−/− mutation into
autoimmune-prone mice. RP105<sup>−/−</sup> mutation ameliorated lymphoproliferation in MRL/lpr mice (Fig. 6A and B). Considering that deficiency of TLR2, TLR4, RP105 or MD-1 does not influence the B cell differentiation (14, 18, 34, 35), the effect of RP105<sup>−/−</sup> mutation on autoimmune diseases is likely to be due to defective B cell activation in the steady state. MRL/lpr mice reared in a germ-free condition were similar to those in conventional conditions with regard to lymphoproliferation and B cell autoimmunity (29), indicating that autoimmunity in these mice is independent of live, non-pathogenic microbes. Taken together with our results from wild-type mice (Figs 1–3), B cells are likely to be activated by non-microbial, environmental stimuli in both healthy and autoimmune-prone mice. Although RP105/MD-1 is expressed on dendritic cells and macrophages as well as on B cells, RP105<sup>−/−</sup> mice showed impaired response to LPS or lipopeptides only in B cells but not in dendritic cells or macrophages (16). These results suggest that TLR2, TLR4 and RP105/MD-1 on splenic B cells are continuously activated in a microbial flora-independent manner not only in wild-type mice but also in MRL/lpr mice.

What activates B cells in the steady state in MRL/lpr mice? In MRL/lpr mice, abnormal T cells accumulate as a consequence of defect in Fas-dependent apoptosis and become a major source of necrotic cells (2). Resultant necrotic cells were suggested to activate TLR7/9 to produce auto-antibody (3, 5). Necrotic cells might also activate TLR2/4 and RP105/MD-1 on B cells in healthy and autoimmune-prone mice. Alternatively, dietary exposure may have a role for stimulating TLR2/TLR4/RP105 on B cells, since the previous report suggested a possible role for dietary exposure in inducing lymphoproliferation in MRL/lpr mice (29).

The lack of RP105 extended the survival of MRL/lpr mice probably due to amelioration of overall renal function, as documented by improvement of BUN elevation. Although we were unable to detect any alteration in glomerulonephritis, the amelioration of renal function in RP105<sup>−/−</sup> MRL/lpr would be due to the significantly lower incidence of renal-necrotizing arteritis than in MRL/lpr mice. Systemic-necrotizing arteritis was reported to be observed in MRL/lpr mice but not in BWF1 mice (2), which may in part explain a reason why renal function was ameliorated in MRL/lpr but not in B/WF1 by the lack of RP105 (Fig. 5B). Necrotizing arteritis was shown to be induced by IgG3 anti-IgG2a RF in MRL/lpr (32). We, however, could not detect any difference in IgG3 anti-IgG2a RF in RP105<sup>−/−</sup> MRL/lpr mice (Fig. 8). Moreover, production of all IgG subtypes of antibodies against dsDNA or chromatin was not changed in RP105<sup>−/−</sup> MRL/lpr mice (Fig. 7). Given that serum IgG3 and IgG2a were down-regulated by RP105<sup>−/−</sup> mutation in MRL/lpr mice, these antibodies to at least uncharacterized auto-antigens could be responsible for renal arteritis. TLR2 and TLR4 react with

Fig. 6. The effect of RP105 deficiency in the lymphoid organs of MRL/lpr mice. RP105<sup>+/+</sup> (white bars, n = 12, female), RP105<sup>−/−</sup> (slashed bars, n = 9, female) and healthy MRL (black bars, n = 5, female) mice were assayed for various parameters of disease activity and immune activation. (A) Weight of mesenteric lymph nodes or spleen from mice used in (Fig. 6B) was shown. (B) Weight of axillary lymph nodes from mice used in (Fig. 6B) was shown (graph). The results were represented as mean value with standard errors. Representative photograph of the axillary lymph nodes from RP105<sup>+/+</sup> or RP105<sup>−/−</sup> mice is also shown. Black bar indicates 1 cm. *P < 0.05, **P < 0.01. (C–E) Lymphocyte subpopulations and their activation status were analyzed by flow cytometry. Shown were proportions of splenic CD4<sup>+</sup> T cells, CD8<sup>+</sup> cells, CD4<sup>+</sup>/CD8<sup>−</sup> DNTE, B cells (C), CD44<sup>+</sup> and CD69<sup>+</sup> splenic B cells (D) and CD44<sup>+</sup>/CD4<sup>+</sup> T cells in mesenteric lymph node (E).
mice may be explained by a B cell-dependent but auto-
antibody-independent mechanism. When rendered B
cell deficient, MRL/lpr mice developed substantially less
lymphadenopathy or splenomegaly and lacked glomerulonephritis,
as well as skin disease (36, 37). Ab-independent roles
for B cells in autoimmune diseases were revealed by com-
paring B cell-deficient MRL/lpr mice and those lacking only
 serum Igs. Survival of serum Ig-deficient MRL/lpr was be-
 tween MRL/lpr and B cell-deficient MRL/lpr (38). MRL/lpr-
lacking serum Igs were ameliorated in glomerulonephritis as
much as MRL/lpr-lacking B cells, whereas no improvement
was seen in renal vasculitis, suggesting a role for B cells
per se but not for auto-antibodies in renal vasculitis. In
humans, rituximab, a humanized anti-CD20, is broadly used
for autoimmune disease therapies to deplete autoreac-
tive B cells. Rituximab treatment in humans (39), or in trans-
genic mice expressing human CD20 (40), reduced the
disease severity but not altered auto-antibody levels, indicat-
ing that B cells play a role in autoimmune disease via their
role as antigen-presenting cells. With regard to RP105+/−-
MRL/lpr mice, lymphadenopathy/splenomegaly and CD44+
activated T cells in mesenteric lymph nodes were apparently
decreased (Fig. 6A, B and E). Such differences in T cells
can reflect reduced activity of B cells as antigen-presenting
cells in RP105+/− MRL/lpr mice.

Previous studies revealed differences between two types
of lupus-prone mice, MRL/lpr and BWF1. FcγRs have been
implicated in autoimmune glomerulonephritis in BWF1 mice,
as documented by the protection of lupus nephritis in these
strains deficient in FcγR chains (41). Direct activation of cir-
culating FcγR-bearing myeloid cells, including monocytes/
macrophages, by glomerular immune complex deposits is
likely to be sufficient to initiate inflammatory responses (42).
MRL/lpr mice are distinct from BWF1 mice in which the lack
of FcγR chain did not ameliorate lupus nephritis (43), imply-
ing heterogeneity of pathophysiologic basis of autoimmune
nephritis. Another difference was revealed by type I IFN. In
NZB or BWF1 mice, lupus-like disease was accelerated and
enhanced by type I IFNs themselves or their inducer. More-
over, disease was ameliorated in NZB mice lacking the type
I IFN receptor (IFNAR) (44). In contrast, the absence of
IFNAR exacerbated lupus disease of MRL/lpr mice (45).

The present study revealed another difference between

microbial membrane components, raising a possibility that
RP105-dependent antibodies may react with self-membrane
components such as phospholipids.

It is also possible that the pathogenic role for RP105 in
MRL/lpr mice is not directly linked with auto-antibody pro-
duction. Ameliorated renal function in RP105+/− MRL/lpr

Fig. 7. Anti-dsDNA or anti-chromatin auto-antibodies were not altered
in RP105+/− MRL/lpr mice. Sera from MRL/lpr (n = 10, 18 week old,
female), MRL/lpr RP105+/− (n = 9, 18 week old, female), MRL+/− (n = 6, 18
week old, female) mice were used for ELISA measuring the titers of
IgG3, IgG2a, IgG1 and IgG2b (top) or anti-
chromatin IgG3, IgG2a, IgG1 and IgG2b (bottom). Horizontal bars
denote mean values.

Fig. 8. RP105 deficiency did not influence the level of anti-IgG2a RF
in MRL/lpr mice. Sera from MRL/lpr (n = 10, 18 week old, female),
MRL/lpr RP105+/− (n = 9, 18 week old, female), MRL+/− (n = 6, 18
week old, female) mice were used for ELISA measuring the titers of
IgG3 anti-IgG2a RF. Results from different dilution of sera were shown
(1:200, left; 1:1000, right). Horizontal bars denote mean values.

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MRL/lpr and BWF1 in which the lack of RP105 ameliorated disease progression in MRL/lpr mice but not in BWF1 mice. These two strains may be distinct from each other in roles for TLRs in induction of lupus nephritis. Along this line, a recent report is noteworthy that the lack of TLR9 ameliorated lupus nephritis in an autoimmune-prone mouse strain (46). Although the autoimmune-prone mice used in this study were distinct from BWF1, both are similar in that FcγR-dependent inflammation has a pathogenic role in lupus nephritis. By contrast, TLR9 had an opposing role in MRL/lpr in which the lack of TLR9 did not ameliorate but rather exacerbated lupus nephritis (5). These results suggested two distinct types of lupus nephritis, which were discriminated by roles for FcγR, IgG3 auto-antibody, RP105/MD-1, TLR2, TLR4, TLR9 and type I IFNs.

The present study stressed that TLR2, TLR4 and RP105 continuously transduce the signal without any apparent infection, indicating that these innate pathogen sensors may continuously respond to environmental stimuli. The discrimination between infectious and steady state by TLRs is still an important and unresolved issue in the innate immune system.

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Abbreviations

AID
activation-induced cytidine deaminase
BUN
blood urea nitrogen
DNP
2,4-dinitrophenyl
DNTC
double-negative T cell
HPRT
hypoxanthine guanine phosphoribosyl transferase
MyD88
myeloid differentiation gene 88
P3CSK4
N-palmitoyl-S-(2,3-bis (palmitoyloxy)-(2R,S)-propyl)-(R)-cysteinyl-serglycyllysine
RF
rheumatoid factor
RP105
Radioprotective105
SLE
systemic lupus erythematosus
TLR
toll-like receptor

References