International Immunology, Vol. 20, No. 7, pp. 881–891
doi:10.1093/intimm/dxn049

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

Toshihiko Kobayashi[1,2], Koichiro Takahashi[1], Yoshinori Nagai[1,6], Takuma Shibata[1], Masako Otani[3], Shozo Izui[3], Shizuo Akira[4], Yoshiyuki Gotoh[5], Hiroshi Kiyono[5] and **Kensuke Miyake[1]**

[1]Division of Infectious Genetics, Institute of Medical Science, University of Tokyo, Tokyo, Japan
[2]Japan Society for the Promotion of Science, Tokyo, Japan
[3]Department of Pathology and Immunology, University of Geneva, Geneva, Switzerland
[4]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan
[5]Division of Mucosal Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

[6]Present address: Department of Immunobiology and Pharmacological Genetics, Graduate School of Medicine and Pharmaceutical Science for Research, University of Toyama, Toyama, Japan

Keywords: autoimmunity, B cells, innate immunity

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).

Correspondence to: K. Miyake; E-mail: kmiyake@ims.u-tokyo.ac.jp

Received 4 February 2008, *accepted* 22 April 2008

Transmitting editor: K. Inaba

Advance Access publication 19 May 2008

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$^{-/y}$ mice were provided by S. Akira (Osaka University). RP105$^{-/-}$ autoimmune mice were generated by intercrossing C57BL/6 RP105$^{-/-}$ mice with MRL/MpJ$^{lpr/lpr}$ 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: TCCATGACGTTCCTGATGCT) 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-CD3ε 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, gatatatggctgaaagcttgctaa and μ anti-sense, tgtgtgtctatatgtgtgcctg a; for γ3 sense, gactgtaccctccagcacct and γ3 anti-sense, actgggcttgggtattctagg; for γ2b sense, ggccaagtcagaccgtca and γ2b anti-sense, aggacaggggttgattgttg. Each sample was normalized by mouse hypoxanthine guanine phosphoribosyl transferase (HPRT) expression (Mm 00446968_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 exudates 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 polynuclear 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 T_h1 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

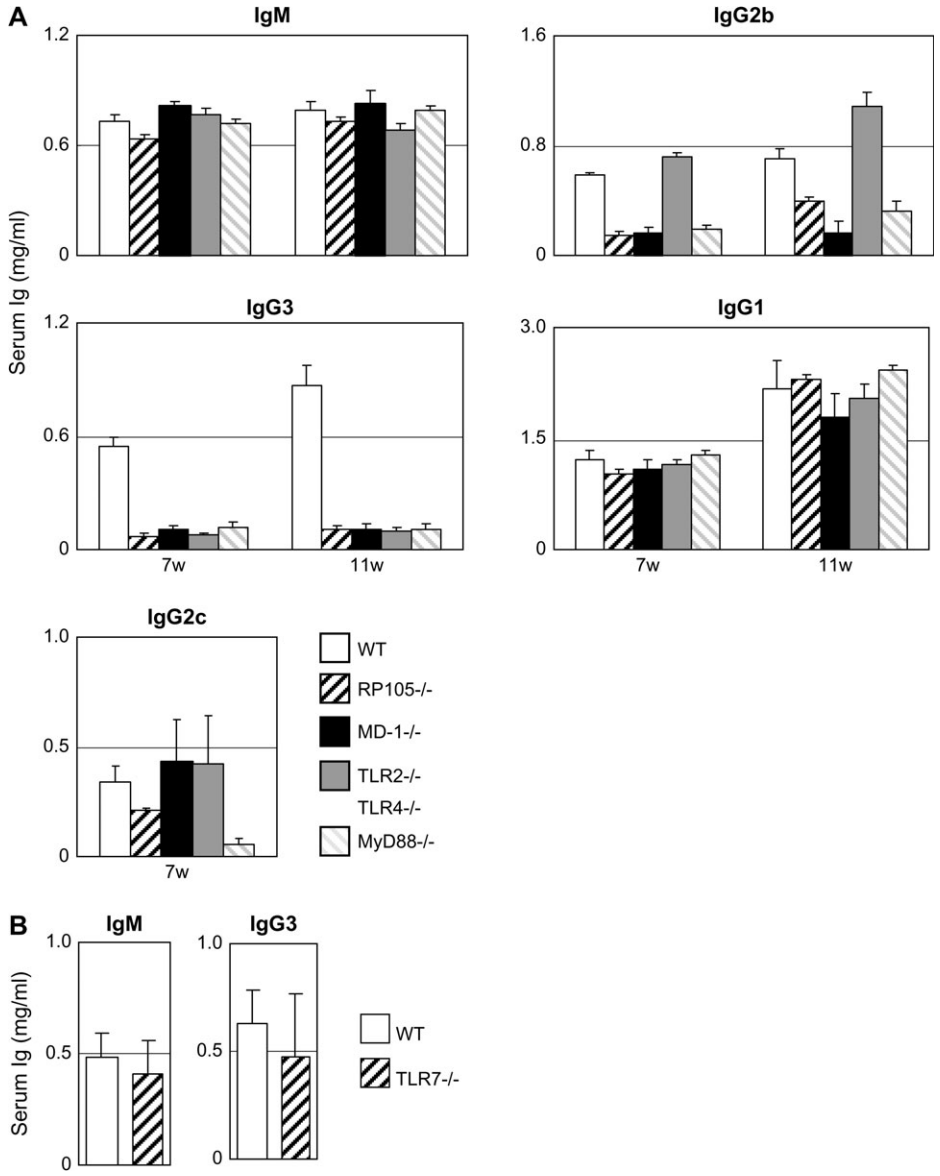

Fig. 1. Serum IgG3 is dependent on TLR2, TLR4 or RP105/MD-1. (A) Levels of serum IgM, IgG1, IgG2b, IgG2c and IgG3 in indicated mice (wild-type, RP105$^{-/-}$, MD-1$^{-/-}$, TLR2$^{-/-}$TLR4$^{-/-}$, MyD88$^{-/-}$ mice) were determined by ELISA. The results are represented by mean values with standard deviation (SD) from four to five mice at 7 and 11 weeks of age (except for IgG2c; measured only at 7 weeks of age). (B) The levels of serum IgM and IgG3 in wild-type and TLR7$^{-/-}$ mice were determined by ELISA as in (A). The results are represented by mean values with SD from six mice at 8 weeks of age.

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.

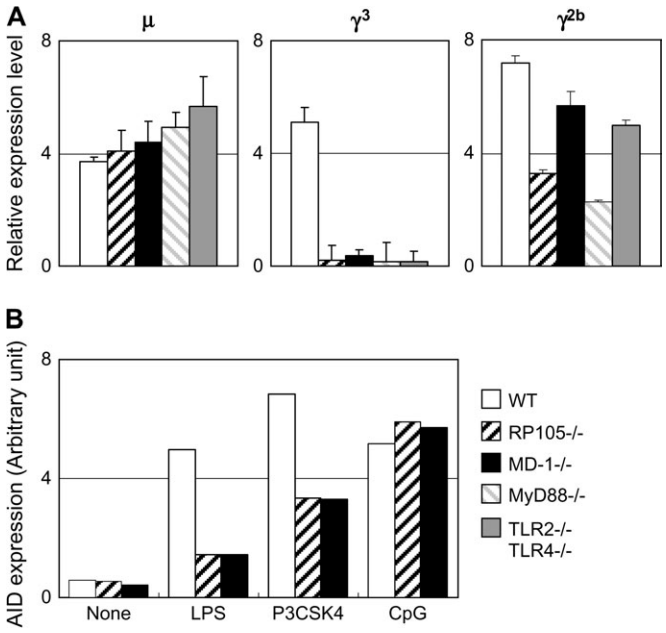

Fig. 2. γ3 germ line transcript is dependent on TLR2, TLR4 and RP105/MD-1. (A) Expression of mRNA encoding μ (left), γ3 (middle) and γ2b (right) chain in enriched splenic B cells was measured by real-time reverse transcription (RT)–PCR. Enriched splenic B cells were obtained from wild-type, RP105$^{-/-}$, MD-1$^{-/-}$, MyD88$^{-/-}$ or TLR2$^{-/-}$TLR4$^{-/-}$ mice as indicated in the figure. RT–PCR was conducted as described in Methods. The results are represented as mean value with standard deviation from triplicate samples. (B) Splenic B cells from indicated mice were stimulated with LPS (1 μg ml^{-1}), P3CSK4 (1 μg ml^{-1}) or CpG (1.0 μM) for 2 days. Quantitative RT–PCR was conducted for measuring AID mRNA expression. Each expression level was normalized by mouse HPRT expression. Results are representatives of more than three independent experiments.

To address a role of microbial flora, we studied germ-free mice (Fig. 3A). Serum IgA in germ-free mice was reported to be reduced (28). In keeping with this, serum IgA was significantly down-regulated in the germ-free mice used in this study, demonstrating the effectiveness of germ-free condition. Nonetheless, the rest of isotypes including IgG3 and IgG2b was not reduced. Compared with control mice, IgM and IgG3 were rather up-regulated. We next employed antibiotics treatment to deplete microbial flora. Mice were fed with antibiotics-containing water during 4–8 weeks of age. These mice were similar to germ-free mice in that serum IgA was significantly reduced, confirming the reduction of microbial flora (Fig. 3B). Despite the effective treatment with antibiotics, serum IgG3 was not decreased at all. Instead, IgG2b was significantly down-regulated by antibiotics treatment (Fig. 3B). The exact reason for IgG2b reduction is currently unknown. These results did not necessarily negate roles for microbial flora, but strongly suggested that microbial flora is dispensable for tonic B cell activation driven by TLR2, TLR4 and RP105/MD-1.

Roles for RP105 in serum Ig titers of lupus-prone mice

TLRs have been implicated in autoimmune diseases. Especially, in MRL/lpr mice, TLR7 was reported to deteriorate the autoimmune disease (5). MRL/lpr mice reared in a germ-free state were similar to those in conventional conditions with regard to lymphoproliferation and B cell autoimmunity (29), indicating that autoimmunity in MRL/lpr is independent of live, non-pathogenic microbes. This report suggests that B cells are excessively activated by non-microbial, environmental stimuli in MRL/lpr mice. It is possible that such excessive B cell activation is driven not only by TLR7 but also by TLR2, TLR4 and RP105. To address this possibility, we established RP105$^{-/-}$ MRL/lpr mice. We considered that RP105$^{-/-}$ mutation is more appropriate than TLR2$^{-/-}$TLR4$^{-/-}$ double mutation. The lack of RP105 impairs TLR2/4 responses only in B cells (16), whereas the lack of TLR2/4 blunts all the TLR2/4 responses not only in B cells but also in dendritic cells, macrophages, endothelial cells or fibroblasts. It is difficult to discriminate the effect of TLR2/4 mutation in B cells from that in non-B cells. We also introduced RP105 deficiency into another type of lupus-prone mice, BWF1 mice. We studied the levels of serum Igs at 20 weeks of age and found that RP105$^{-/-}$ MRL/lpr mice were significantly lower than RP105$^{+/+}$ MRL/lpr mice in serum IgG3 and IgG2a. RP105$^{-/-}$ BWF1 mice were lower than RP105$^{+/-}$ BWF1 mice in serum IgG3 and IgG2b (Fig. 4).

Roles for RP105 in autoimmunity in MRL/lpr

RP105$^{-/-}$ mutation ameliorated mortality in the MRL/lpr 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/lpr mice, whereas no significant difference was detected between RP105$^{+/-}$ and RP105$^{-/-}$ BWF1 mice (Fig. 5B). We further examined glomerulonephritis in MRL/lpr 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/lpr mice spontaneously develop systemic-necrotizing arteritis of small- and medium-sized muscular arteries, which is characterized by the infiltration of mononuclear and/or polynuclear leukocytes and by the destruction of the arterial wall (30). Notably, necrotizing arteritis in kidneys was observed less frequently in RP105$^{-/-}$ MRL/lpr than in RP105$^{+/+}$ MRL/lpr (Fig. 5D). Impaired renal perfusion may be responsible for deterioration of renal function revealed by elevated BUN.

MRL/lpr 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/lpr mice (Fig. 6A and B). Due to unknown reason, axillary lymph nodes were most apparent in difference between RP105$^{-/-}$ MRL/lpr and RP105$^{+/+}$ MRL/lpr mice (Fig. 6B). We next examined lymphocyte subpopulation in the spleen. Decreases 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).

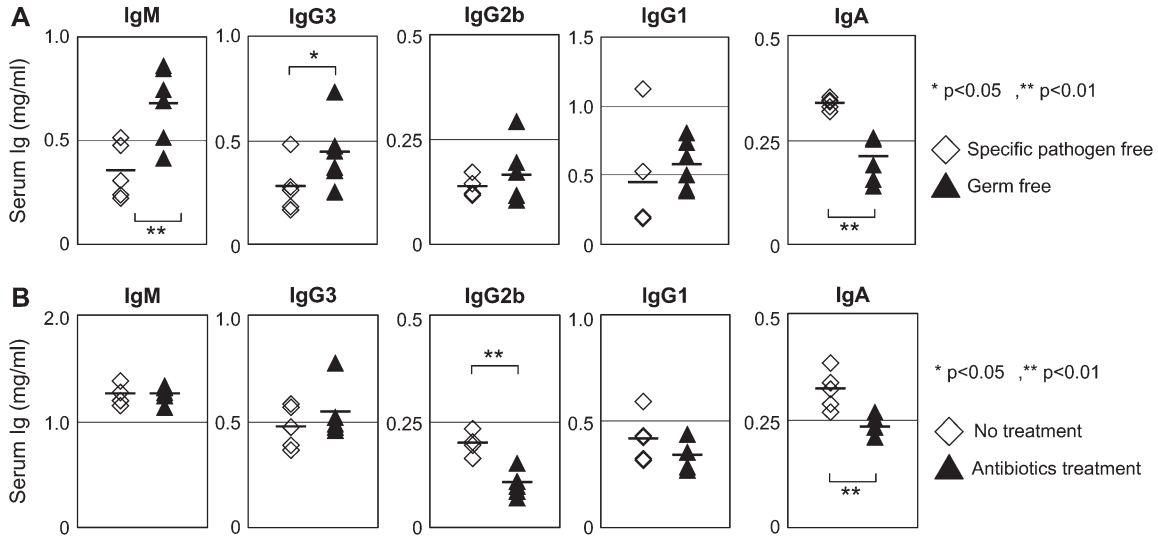

Fig. 3. Serum IgG3 is not influenced by microbial flora depletion. Sera were collected from 7-week-old ICR mice grown under specific pathogen-free (open diamond) or germ-free condition (closed triangle) as indicated in the figure (A) or from 8-week-old BALB/c mice fed with water with (closed triangle) or without antibiotics (open diamond) during 4–8 weeks of age (B). The levels of serum IgM, IgG3, IgG2b, IgG1 and IgA were determined by ELISA. The results were represented as individual values and mean values shown by horizontal bars from six (A) or five (B) mice. The experiment using germ-free mice was conducted once, whereas antibiotic treatments were independently performed three times and gave similar results. $*P < 0.05$, $**P < 0.01$.

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.

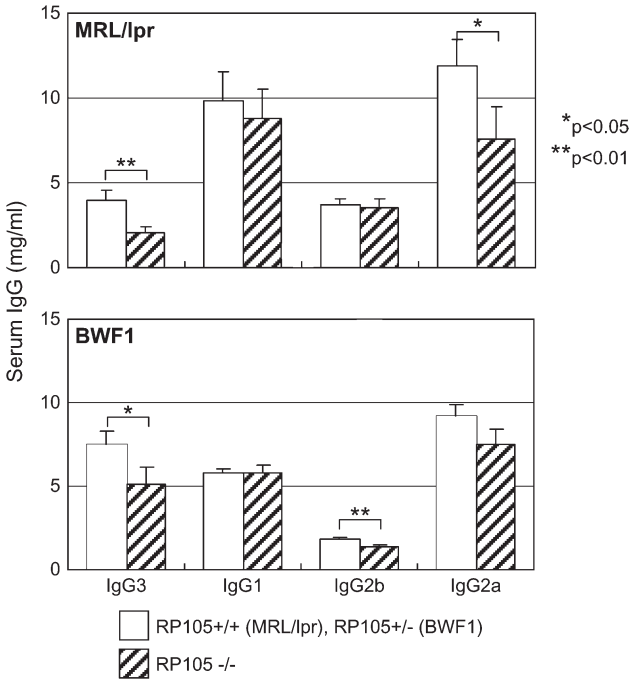

Fig. 4. $RP105^{-/-}$ mutation decreased serum IgG3 in lupus-prone mice. Sera were collected from $RP105^{+/+}$ MRL/lpr (20 week old, $n = 9$), $RP105^{-/-}$MRL/lpr (20 week old, $n = 10$), $RP105^{+/-}$ BWF1 (20 week old, $n = 17$) or $RP105^{-/-}$ BWF1 (20 week old, $n = 17$) as indicated in the figure. The levels of serum IgG3, IgG1, IgG2b and IgG2a were determined by ELISA. The results were represented as mean values with standard errors.

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

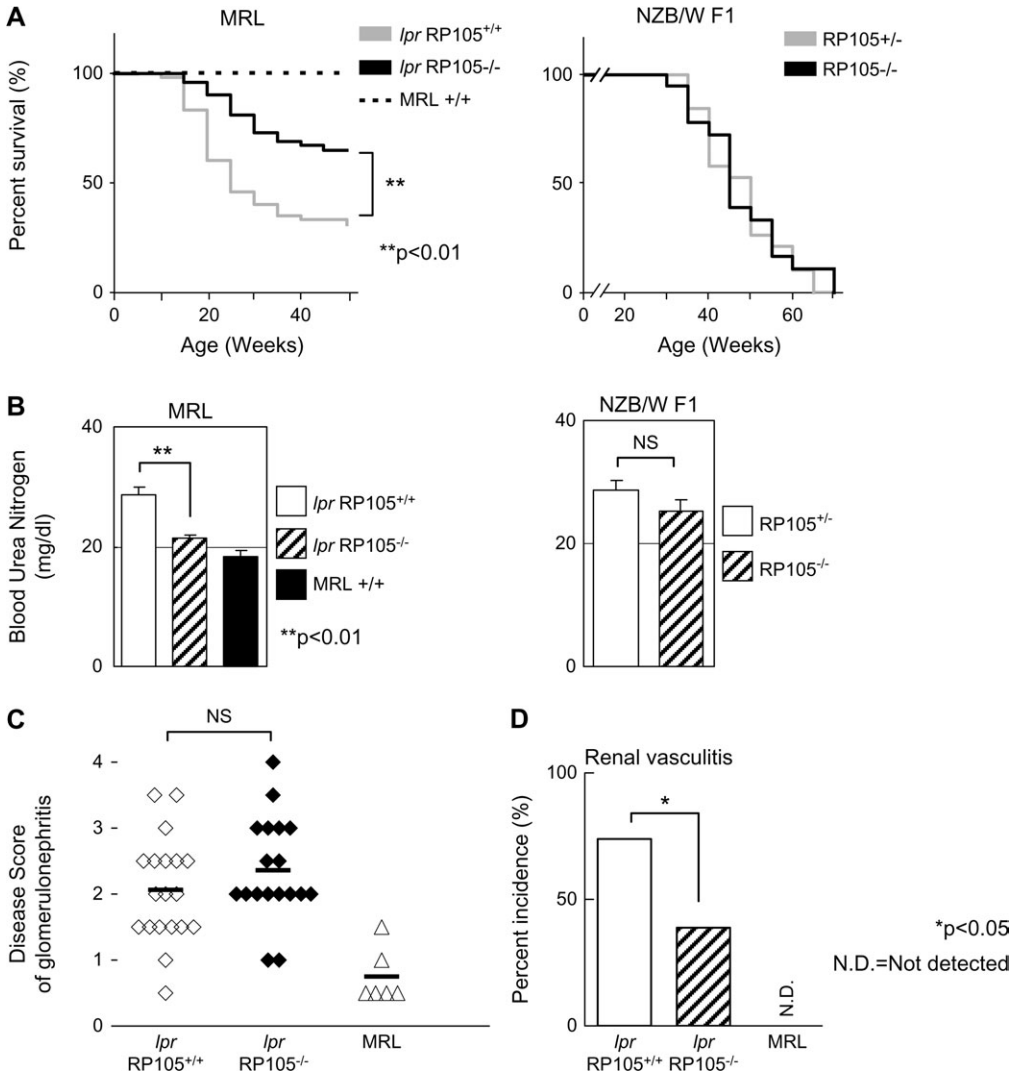

Fig. 5. Lack of RP105 ameliorated autoimmune disease in MRL/lpr mice but not in BWF1 mice. (A) Survival of MRL (n = 5, female), RP105$^{+/+}$ MRL/lpr (n = 52, female), RP105$^{-/-}$ MRL/lpr (n = 53, female), RP105$^{+/-}$ BWF1 (n = 19, female) or RP105$^{-/-}$ BWF1 (n = 18, female) mice were shown until 50 weeks of age. *P < 0.01 by log-rank test. (B) Sera from MRL/lpr (n = 12, 19 week old), MRL/lpr RP105$^{-/-}$ (n = 9, 19 week old), MRL$^{+/+}$ (n = 5, 19 week old), RP105$^{+/-}$ BWF1 (n = 17, 20 week old) or RP105$^{-/-}$ BWF1 (n = 17, 20 week old) mice were used for measuring BUN. The results were represented as a mean value with standard deviation. (C) The severity of glomerulonephritis in MRL/lpr (n = 19, female), RP105$^{-/-}$ MRL/lpr (n = 18, female) or MRL (n = 6, female) mice at 18–19 weeks of age was scored on a 0–4 scale (see Methods). Horizontal bars denote mean values. NS, not significant. (D) The percentages of mice suffering from necrotizing arteritis in kidneys were shown in the same mice as used in (C). ND, not detected. *P < 0.05 by chi-square test.

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

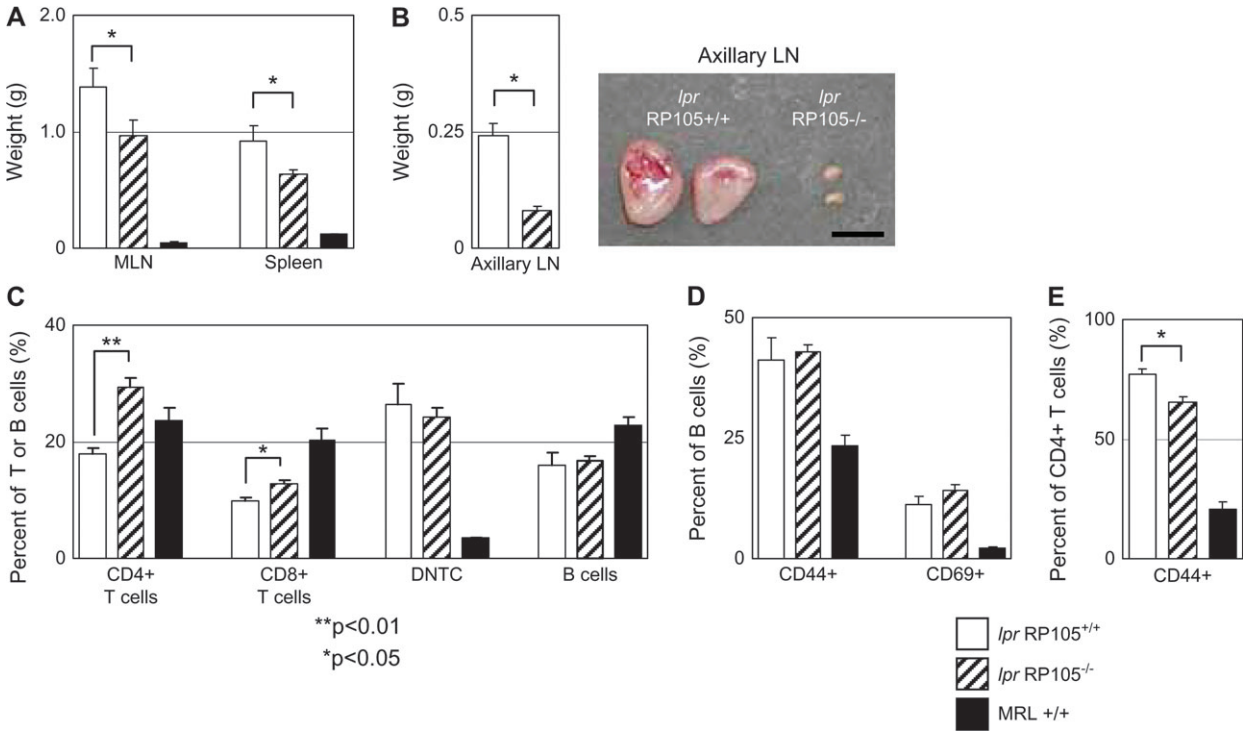

Fig. 6. The effect of RP105 deficiency in the lymphoid organs of MRL/lpr mice. RP105$^{+/+}$ (white bars, n = 12, female), RP105$^{-/-}$ (slashed bars, n = 9, female) and healthy MRL (black bars, n = 5, female) mice were assayed for various parameters of disease activity and immune activation. (A) Weight 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$^{+/+}$ or RP105$^{-/-}$ 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$^+$ T cells, CD8$^+$ cells, CD4$^-$/CD8$^-$ DNTC, B cells (C), CD44$^+$ and CD69$^+$ splenic B cells (D) and CD44$^+$/CD4$^+$ T cells in mesenteric lymph node (E).

autoimmune-prone mice. RP105$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ MRL/lpr mice (Fig. 8). Moreover, production of all IgG subtypes of antibodies against dsDNA or chromatin was not changed in RP105$^{-/-}$ MRL/lpr mice (Fig. 7). Given that serum IgG3 and IgG2a were down-regulated by RP105$^{-/-}$ mutation in MRL/lpr mice, these antibodies to as yet uncharacterized auto-antigens could be responsible for renal arteritis. TLR2 and TLR4 react with

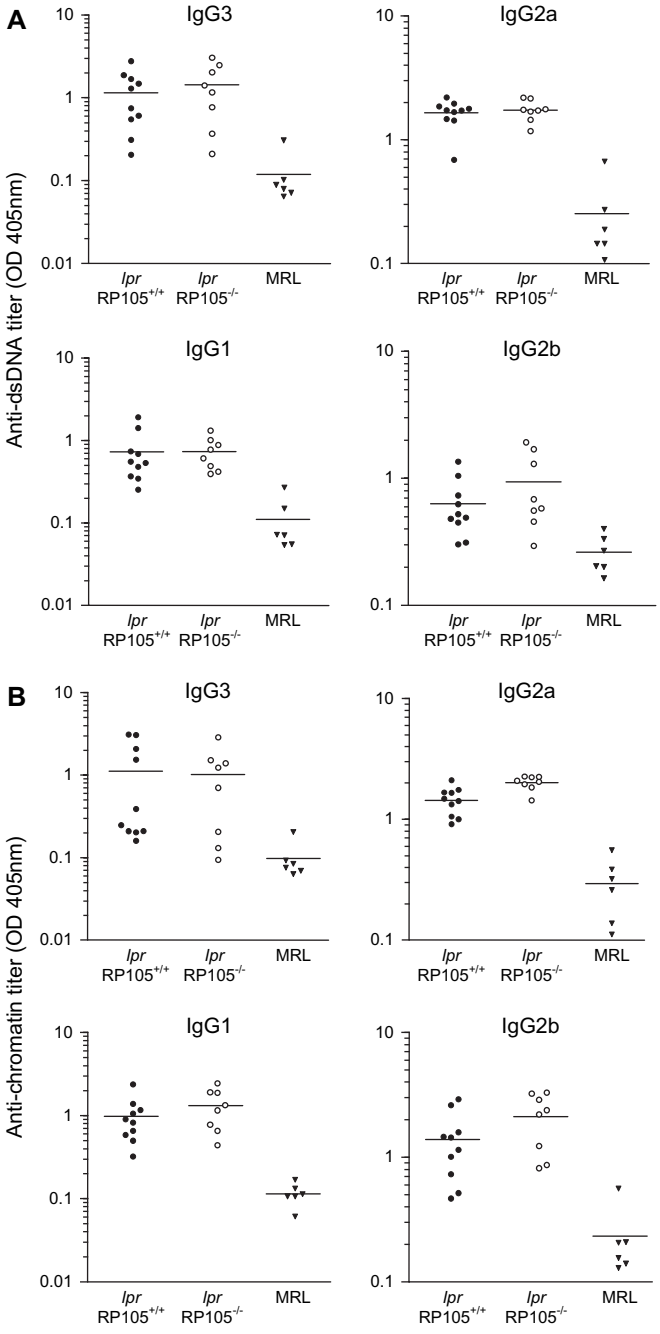

Fig. 7. Anti-dsDNA or anti-chromatin auto-antibodies were not altered in RP105$^{-/-}$ MRL/lpr mice. Sera from MRL/lpr (n = 10, 18 week old, female), MRL/lpr RP105$^{-/-}$ (n = 9, 18 week old, female), MRL$^{+/+}$ (n = 6, 18 week old, female) mice were used for ELISA measuring the titers of anti-dsDNA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

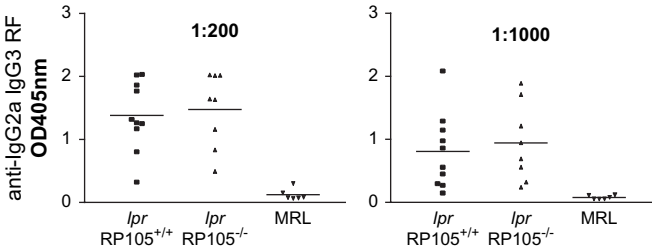

Fig. 8. RP105 deficiency did not influence the level of anti-IgG2a RF in MRL/lpr mice. Sera from MRL/lpr (n = 10, 18 week old, female), MRL/lpr RP105$^{-/-}$ (n = 9, 18 week old, female), MRL$^{+/+}$ (n = 6, 18 week old, female) mice were used for ELISA measuring the titers of IgG3 anti-IgG2a RF. Results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

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 production. Ameliorated renal function in RP105$^{-/-}$ MRL/lpr 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, vasculitis and skin disease (36, 37). Ab-independent roles for B cells in autoimmune diseases were revealed by comparing B cell-deficient MRL/lpr mice and those lacking only serum Igs. Survival of serum Ig-deficient MRL/lpr was between 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 the autoimmune disease therapies to deplete autoreactive B cells. Rituximab treatment in humans (39), or in transgenic mice expressing human CD20 (40), reduced the disease severity but not altered auto-antibody levels, indicating 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 may 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 FcRγ chains (41). Direct activation of circulating 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 FcRγ chain did not ameliorate lupus nephritis (43), implying 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. Moreover, 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

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.

Funding

Ministry of Education, Culture, Sports, Science and Technology for Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases; Strategic cooperation to control emerging and reemerging infections funded by The Special Coordination Funds for Promoting Science and Technology of Ministry of Education, Culture, Sports, Science and Technology;Swiss National Foundation for Scientific Research.

Acknowledgements

We thank N. Tanimura for excellent technical assistance and helpful discussions and Y. Tsukamoto for provision of sera from TLR7-deficient mice.

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-seryl-(lysyl)3-lysine
RF	rheumatoid factor
RP105	Radioprotective105
SLE	systemic lupus erythematosus
TLR	Toll-like receptor

References

1 Theofilopoulos, A. N. and Dixon, F. J. 1985. Murine models of systemic lupus erythematosus. *Adv. Immunol.* 37:269.
2 Andrews, B. S., Eisenberg, R. A., Theofilopoulos, A. N. *et al.* 1978. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *J. Exp. Med.* 148:1198.
3 Marshak-Rothstein, A. and Rifkin, I. R. 2007. Immunologically active autoantigens: the role of toll-like receptors in the development of chronic inflammatory disease. *Annu. Rev. Immunol.* 25:419.
4 Lau, C. M., Broughton, C., Tabor, A. S. *et al.* 2005. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J. Exp. Med.* 202:1171.
5 Christensen, S. R., Shupe, J., Nickerson, K., Kashgarian, M., Flavell, R. A. and Shlomchik, M. J. 2006. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* 25:417.
6 Schaefer, L., Babelova, A., Kiss, E. *et al.* 2005. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *J. Clin. Invest.* 115:2223.
7 Paterson, H. M., Murphy, T. J., Purcell, E. J. *et al.* 2003. Injury primes the innate immune system for enhanced Toll-like receptor reactivity. *J. Immunol.* 171:1473.
8 Wu, H., Chen, G., Wyburn, K. R. *et al.* 2007. TLR4 activation mediates kidney ischemia/reperfusion injury. *J. Clin. Invest.* 117:2847.
9 Apetoh, L., Ghiringhelli, F., Tesniere, A. *et al.* 2007. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat. Med.* 13:1050.
10 Vogl, T., Tenbrock, K., Ludwig, S. *et al.* 2007. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat. Med.* 13:1042.
11 Miyake, K. 2007. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin. Immunol.* 19:3.
12 Miyake, K., Shimazu, R., Kondo, J. *et al.* 1998. Mouse MD-1, a molecule that is physically associated with RP105 and positively regulates its expression. *J. Immunol.* 161:1348.
13 Miyake, K., Yamashita, Y., Ogata, M., Sudo, T. and Kimoto, M. 1995. RP105, a novel B cell surface molecule implicated in B cell activation, is a member of the leucine-rich repeat protein family. *J. Immunol.* 154:3333.
14 Ogata, H., Su, I., Miyake, K. *et al.* 2000. The toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. *J. Exp. Med.* 192:23.
15 Miyake, K. 2003. Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2. *Int. Immunopharmacol.* 3:119.
16 Nagai, Y., Kobayashi, T., Motoi, Y. *et al.* 2005. The radioprotective 105/MD-1 complex links TLR2 and TLR4/MD-2 in antibody response to microbial membranes. *J. Immunol.* 174:7043.
17 Divanovic, S., Trompette, A., Atabani, S. F. *et al.* 2005. Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. *Nat. Immunol.* 6:571.
18 Nagai, Y., Shimazu, R., Ogata, H. *et al.* 2002. Requirement for MD-1 in cell surface expression of RP105/CD180 and B-cell responsiveness to lipopolysaccharide. *Blood* 99:1699.
19 Luzuy, S., Merino, J., Engers, H., Izui, S. and Lambert, P. H. 1986. Autoimmunity after induction of neonatal tolerance to alloantigens: role of B cell chimerism and F1 donor B cell activation. *J. Immunol.* 136:4420.
20 Vyse, T. J., Rozzo, S. J., Drake, C. G., Izui, S. and Kotzin, B. L. 1997. Control of multiple autoantibodies linked with a lupus nephritis susceptibility locus in New Zealand black mice. *J. Immunol.* 158:5566.
21 Kuroki, A., Moll, T., Lopez-Hoyos, M. *et al.* 2004. Enforced Bcl-2 expression in B lymphocytes induces rheumatoid factor and anti-DNA production, but the Yaa mutation promotes only anti-DNA production. *Eur. J. Immunol.* 34:1077.
22 Yazawa, N., Fujimoto, M., Sato, S. *et al.* 2003. CD19 regulates innate immunity by the toll-like receptor RP105 signaling in B lymphocytes. *Blood* 102:1374.
23 Pasare, C. and Medzhitov, R. 2005. Control of B-cell responses by Toll-like receptors. *Nature* 438:364.
24 Yamamoto, M., Sato, S., Hemmi, H. *et al.* 2002. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* 420:324.
25 Schnare, M., Barton, G. M., Holt, A. C., Takeda, K., Akira, S. and Medzhitov, R. 2001. Toll-like receptors control activation of adaptive immune responses. *Nat. Immunol.* 2:947.

26 Honjo, T., Nagaoka, H., Shinkura, R. and Muramatsu, M. 2005. AID to overcome the limitations of genomic information. *Nat. Immunol.* 6:655.
27 Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y. and Honjo, T. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102:553.
28 Macpherson, A. J., Gatto, D., Sainsbury, E., Harriman, G. R., Hengartner, H. and Zinkernagel, R. M. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288:2222.
29 Maldonado, M. A., Kakkanaiah, V., MacDonald, G. C. *et al.* 1999. The role of environmental antigens in the spontaneous development of autoimmunity in MRL-lpr mice. *J. Immunol.* 162:6322.
30 Alexander, E. L., Moyer, C., Travlos, G. S., Roths, J. B. and Murphy, E. D. 1985. Two histopathologic types of inflammatory vascular disease in MRL/Mp autoimmune mice. Model for human vasculitis in connective tissue disease. *Arthritis Rheum.* 28:1146.
31 Cohen, P. L. and Eisenberg, R. A. 1991. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243.
32 Kikuchi, S., Pastore, Y., Fossati-Jimack, L. *et al.* 2002. A transgenic mouse model of autoimmune glomerulonephritis and necrotizing arteritis associated with cryoglobulinemia. *J. Immunol.* 169:4644.
33 Gorczynski, R. M., Kai, Y. and Miyake, K. 2006. MD1 expression regulates development of regulatory T cells. *J. Immunol.* 177:1078.
34 Takeuchi, O., Hoshino, K., Kawai, T. *et al.* 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11:443.
35 Hoshino, K., Takeuchi, O., Kawai, T. *et al.* 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162:3749.
36 Chan, O. and Shlomchik, M. J. 1998. A new role for B cells in systemic autoimmunity: B cells promote spontaneous T cell activation in MRL-lpr/lpr mice. *J. Immunol.* 160:51.
37 Shlomchik, M. J., Madaio, M. P., Ni, D., Trounstein, M. and Huszar, D. 1994. The role of B cells in lpr/lpr-induced autoimmunity. *J. Exp. Med.* 180:1295.
38 Chan, O. T., Hannum, L. G., Haberman, A. M., Madaio, M. P. and Shlomchik, M. J. 1999. A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *J. Exp. Med.* 189:1639.
39 Anolik, J. H., Barnard, J., Cappione, A. *et al.* 2004. Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. *Arthritis Rheum.* 50:3580.
40 Hu, C. Y., Rodriguez-Pinto, D., Du, W. *et al.* 2007. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. *J. Clin. Invest.* 117:3857.
41 Clynes, R., Dumitru, C. and Ravetch, J. V. 1998. Uncoupling of immune complex formation and kidney damage in autoimmune glomerulonephritis. *Science* 279:1052.
42 Bergtold, A., Gavhane, A., D'Agati, V., Madaio, M. and Clynes, R. 2006. FcR-bearing myeloid cells are responsible for triggering murine lupus nephritis. *J. Immunol.* 177:7287.
43 Matsumoto, K., Watanabe, N., Akikusa, B. *et al.* 2003. Fc receptor-independent development of autoimmune glomerulonephritis in lupus-prone MRL/lpr mice. *Arthritis Rheum.* 48:486.
44 Santiago-Raber, M. L., Baccala, R., Haraldsson, K. M. *et al.* 2003. Type-I interferon receptor deficiency reduces lupus-like disease in NZB mice. *J. Exp. Med.* 197:777.
45 Hron, J. D. and Peng, S. L. 2004. Type I IFN protects against murine lupus. *J. Immunol.* 173:2134.
46 Ehlers, M., Fukuyama, H., McGaha, T. L., Aderem, A. and Ravetch, J. V. 2006. TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. *J. Exp. Med.* 203:553.