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Expression of Natural Autoantibodies in MRL-lpr Mice Protects from Lupus Nephritis and Improves Survival

Kaiissar Mannoor,* Agata Matejuk,*+† Yang Xu, † Michael Beardali, † and Ching Chen†

Natural autoantibodies (NAA) and their associated B cells constitute a substantial proportion of the normal Ab and B cell repertoire. They often have weak reactivity toward a variety of self-Ags such as DNA, nucleoproteins, and phospholipids. It remains controversial whether NAA contribute to or protect from autoimmune diseases. Using site-directed transgenic (sd-tg) mice expressing a prototypic NAA, we investigated the effect of NAA and NAA-producing B cells in disease development in the autoimmune-prone MRL/MpJ-Fas+/− (MRL-lpr) mice. We found that the expression of NAA in MRL-lpr mice prevented proteinuria and reduced kidney immune complex formation. The mice had significantly improved survival. Administration of the IgM NAA to MRL-lpr mice also delayed the onset of nephritis. The sd-tg MRL-lpr mice had decreased levels of anti-dsDNA Abs, anti-Hep2 nuclear Abs, and anti-Sm/ribonucleoprotein Abs. There is a shift in the IgG subclass profile from IgG2a and IgG3 to IgG1 in the sd-tg MRL-lpr mice. The CD4+ T cells from the sd-tg MRL-lpr mice had increased expression of the negative costimulatory molecule CTLA-4 and increased production of IL-10 as compared with those from the wild-type mice. Furthermore, the NAA B cells produced large amounts of IL-10 upon TLR stimulation. These results indicate that NAA and NAA-producing B cells play an important role in protection from lupus nephritis and suggest that the NAA B cells may have an immune regulatory function via the provision of IL-10. The Journal of Immunology, 2012, 188: 000–000.

To study the cellular origin and physiologic function of NAA, we generated site-directed transgenic (sd-tg) mice in which the H chain gene of a prototypic NAA is inserted into the IgH locus. This NAA, named ppc1-5, is encoded by the V\textsubscript{i}, V\textsubscript{J}, and V\textsubscript{L} genes. It binds DNA, actin, p-nitrophenyl-phosphocholine (PC), as well as a variety of self- and foreign Ags (19). Despite their autoreactivity, B cells expressing ppc1-5H/1 NAA are not eliminated, but instead are positively selected during peripheral B cell maturation (20), underscoring the functional importance of NAA. The ppc1-5/1 NAA B cells are not restricted to the B1 cell compartment, but instead they are found in all B cell subsets, including follicular (FO) B cells, marginal zone (MZ) B cells, and peritoneal B1 cells (20). Using this sd-tg model, we have shown that the NAA B cells can participate in the early phase of T cell-dependent immune response and enter germinal centers (21). However, they are excluded from the IgG memory response, suggesting a checkpoint in the germinal center that prevents NAA B cells from developing to high-affinity IgG autoantibody production (21). This may in part explain why NAA B cells, though ubiquitously present in healthy individuals, do not generally cause autoimmune disease.

In autoimmune-prone individuals, immune regulation is defective, and therefore, NAA could serve as precursors for the production of pathologic autoantibodies. In support of this notion is the finding that patients with systemic autoimmune diseases have increased levels of polyreactive B cells (22, 23). In addition, examples exist in which NAA obtain increased autoreactivity via V gene mutation and isotype switching (24–26). In contrast, recent evidence has pointed to a role of natural IgM in maintaining self-tolerance. Mice deficient in secreted IgM, but retaining normal B cell numbers and IgG Abs, develop lupus-like autoimmune symptoms (27, 28). In contrast, the activation-induced cytidine deaminase-deficient MRL-lpr mice that lack IgG but have increased autoreactive IgM do not develop lupus nephritis (29). There are also reports that administration of IgM has beneficial effects in some experimental autoimmune models (30, 31). By analyzing a lupus patient’s sera against a multiplex autoantigen...
microarray, Mohan and colleagues (32) have found that the presence of IgM polyreactivity is correlated with reduced disease severity. Similarly, Witte et al. (33) discovered an inverse correlation between the level of IgM anti-dsDNA Abs and the severity of kidney disease. Taken together, the available information suggests complex function of natural poly-/autoreactive Abs in autoimmunity: on the one hand, they may occasionally promote autoimmunity by serving as a template for the high-affinity pathogenic autoantibodies, and on the other hand, they may generally suppress autoimmunity by regulating excessive autoimmune and inflammatory responses.

To directly address the role of NAA and NAA-producing B cells in the development of autoimmune disease, we generated mice that expressed the prototypic NAA ppc1-5 on the autoimmune-prone MRL-lpr background. We show that the ppc1-5 H MRL-lpr mice developed little, if any, proteinuria and had markedly improved kidney pathology and decreased glomerular IC deposition; consequently, the mice had significantly prolonged survival. Injection of the purified ppc1-5 Ab to MRL-lpr mice also delayed onset of proteinuria. The wild-type (wt) and sd-tg MRL-lpr mice had very different IgG subclass profiles: whereas the former had high titers of IgG2a and IgG3, both of which are highly pathogenic in lupus nephritis, the latter had a predominance of the least pathogenic IgG1 subclass. The ppc1-5 H MRL-lpr mice had decreased levels of autoantibodies, including anti-dsDNA, anti-nuclear Abs (ANA), and anti-Sm/ribonucleoprotein (RNP). The overall lymphoproliferation as assessed by spleen weight and lymphocyte numbers was not significantly influenced by the expression of the ppc1-5 NAA. However, the CD4+ T cell compartment exhibited significant alterations with decreased CD69 expression and increased CTLA-4 expression, indicating an inhibition of T cell activation. In addition, there was an increase in the number of IL-10+CD4+ T cells in the ppc1-5 H MRL-lpr mice, suggesting an induction of the type 1 regulatory T cells (Tr1) (34). Importantly, the ppc1-5 B cells produced more IL-10 than the nontransgenic (tg) B cells upon CpG and LPS stimulation. Taken together, these findings demonstrate that NAA play an important role in protection from lupus nephritis, the latter having a predominance of the least pathogenic IgG1 subclass. The ppc1-5 H MRL-lpr mice had decreased levels of autoantibodies, including anti-dsDNA, anti-nuclear Abs (ANA), and anti-Sm/ribonucleoprotein (RNP). The overall lymphoproliferation as assessed by spleen weight and lymphocyte numbers was not significantly influenced by the expression of the ppc1-5 NAA. However, the CD4+ T cell compartment exhibited significant alterations with decreased CD69 expression and increased CTLA-4 expression, indicating an inhibition of T cell activation. In addition, there was an increase in the number of IL-10+CD4+ T cells in the ppc1-5 H MRL-lpr mice, suggesting an induction of the type 1 regulatory T cells (Tr1) (34). Importantly, the ppc1-5 B cells produced more IL-10 than the nontransgenic (tg) B cells upon CpG and LPS stimulation. Taken together, these findings demonstrate that NAA play an important role in protection from lupus nephritis, and suggest that NAA and/or NAA-producing B cells may be able to influence the function of CD4+ T cells.

Materials and Methods

Mice

The ppc1-5 H-chain sd-tg mice have been described previously (20). The ppc1-5 H MRL-lpr mice were crossed to the MRL-lpr background for at least 10 generations. The MRL/MpJ-Faslpr (MRL-lpr) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free conditions. In this study, heterozygous ppc1-5H MRL-lpr female mice were used for all of the experiments.

Renal function and histopathology

Proteinuria was measured semiquantitatively biweekly using Albustix assay strips (Siemens Healthcare Diagnostics), and the protein levels were scored as follows based on a previous publication (29): 0, negative; 1, trace; 2, 30 mg/dl; 3, 100 mg/dl; 4, 300 mg/dl; and 5, >2000 mg/dl. Histopathology was evaluated on formalin-fixed kidney sections (4 μm) stained with H&E or periodic acid-Schiff. The sections were examined in a blinded fashion for glomerular, tubular, and interstitial pathology. Disease was scored on a semiquantitative scale using the published criteria with modifications (35–37). For glomerulonephritis (GN): 0, normal; 1, mild to moderate increase in cellularity with mesangial proliferation; 2, moderate increase in cellularity with endocapillary and mesangial proliferation, increased matrix, and/or karyorrhexis; and 3, marked increase in cellularity with endocapillary proliferation, crescent formation, and/or necrosis, and/or sclerosis. For tubulointerstitial nephritis (TIN): 0, normal; 1, focal mild infiltrate of mononuclear cells (MNC); 2, multifocal, moderate MNC infiltrate and mild and focal tubular damage (dilatation, atrophy, necrosis); and 3, extensive MNC infiltrate, extensive and severe tubular damage, and/or interstitial fibrosis. Scores from 20 glomeruli were averaged to obtain a mean score for each kidney section.

Immunofluorescence

Mouse kidneys were embedded in OCT and flash frozen in dry ice-ethanol. Five-micron sections of frozen tissue were fixed in 2% paraformaldehyde, quenched in 0.3% H2O2, and blocked with 1% BSA. FITC-labeled anti-IgM, -IgG, -IgG1, -IgG2a, -IgG2b, -IgG3, or PE-labeled-C3 (Southern Biotechnology Associates) were added to the sections at 1:100 dilution and incubated for 60 min. The slides were then mounted with VECTASHIELD mounting medium (Vector Laboratories) and examined under a fluorescence microscope.

ELISA

Determination of Ig isotype and concentration was as described previously (19). Plates were coated with 2 μg/ml goat anti-mouse κ and/or λ, incubated with serum samples, and developed with alkaline phosphatase-labeled goat anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates). Ig concentration was calculated by comparing to titrations of purified mouse IgM or IgG standard (Southern Biotechnology Associates). Anti-dsDNA binding was carried out by a two-step solution phase ELISA as described previously (38). Mouse sera were diluted at 1:2000. A pooled serum sample from five 26-wk-old MRL-lpr mice was used as standard. The relative titer of anti-dsDNA Abs was calculated according to the pooled standard serum that was defined as a titer of 100. Anti-Sm and anti-Sm/RNP binding assays were performed using the anti-Sm and the anti-Sm/RNP ELISA kits (Diamedx, Miami, FL) following the manufacturer’s instruction. Briefly, Ag-coated wells were diluted with 100 μl assay buffer and incubated 1:100 for 30 min, washed three times, and incubated with 100 μl alkaline phosphatase-labeled anti-IgG or IgM for 30 min, followed by color development with the provided substrate solution for 30 min. The units of anti-Sm and anti-Sm/RNP Abs were calculated by comparing the absorbance of the serum samples to the absorbance of the provided calibrator using the manufacturer’s formula. The positive and negative controls showed appropriate values.

Anti-nuclear Ab detection

Mouse sera diluted in buffer (1% BS A, 0.02%NaNO3) at 1:200 were tested for ANA using a Hep-2 ANA test kit (Antibodies Incorporated). The Hep-2 slides were incubated with the sera for 45 min followed by washing with PBS. Subsequently, the slides were stained with FITC-labeled anti-IgG,-IgG1,-IgG2a,-IgG2b, or -IgG3 (Southern Biotechnolgy Associates) and visualized using a fluorescence microscope.

Flow cytometric analysis

Cell preparation for flow cytometry was as described previously (20). Cells (1 x 10^7) were stained with combinations of the following anti-mouse Abs labeled with FITC, PE, biotin, or alkaline phosphatase: anti-CD19 (BD Biosciences), and intracellular cytokines were detected by fluorochrome-conjugated anti-cytokine Ab (anti–IL-10, –IFN-γ, –IL-2, –IL-4, –IL-17, –IL-12, –IFN-γ, –IL-2, –IL-4, –IL-12, –IFN-γ, and –TNF-α; BD Biosciences). For measurement of cytokine production, the secreted IL-10, purified CD19+ B cells were cultured as above (no monensin was added), and the supernatants were collected. IL-10 concentration was measured by using the IL-10 ELISA kit (BD Biosciences).
Results

Proteinuria, kidney histopathology, and IC deposition

To study the role of NAA in autoimmune disease development, we crossed the ppc1-5H sd-tg to the MRL-lpr mice for at least 10 generations to assure a near complete MRL background. The ppc1-5H MRL-lpr mice had ∼15–20% of the ppc1-5H A1 NAA B cells in the peripheral B cell repertoire, whereas the wt MRL-lpr mice had very few λ1+ B cells (Fig. 1), a finding similar to that described previously in the ppc1-5H B6 mice (20). The significant enrichment of NAA B cells in the ppc1-5H MRL-lpr mice allows us to assess the effect of NAA expression on disease development. Groups of female ppc1-5H MRL-lpr mice and their wt littermates were monitored for the development of proteinuria. As expected, the wt MRL-lpr mice had significant proteinuria by 16 wk of age, and the protein level reached peak by 22 wk of age (Fig. 2A). On the contrary, the urine protein level of the ppc1-5H MRL-lpr mice remained very low (<2.5 score or <100 mg/dl) over the 6-mo period of observation. The mice were euthanized at 6 mo of age, and their kidneys were examined for histopathologic changes and for IC deposition. In accordance with the proteinuria data, the ppc1-5H MRL-lpr mice had much milder GN and TIN as compared with the wt MRL-lpr mice (Fig. 2B, 2C). Glomerular deposition of IgG and C3 was also significantly less in ppc1-5H than in wt mice (Fig. 2D, 2E). The ppc1-5H MRL-lpr mice had a predominantly mesangial pattern of cellular proliferation and IC deposition (World Health Organization class I and II lupus nephritis), whereas most of the wt mice had a combined mesangiocapillary or membranoproliferative pattern (World Health Organization class IV) (35).

It has been shown that some IgG subclasses are more pathogenic than others (39–42). We therefore examined glomerular deposition of IgG1, IgG2a, IgG2b, and IgG3 (Fig. 2D, 2E). Although all IgG subclasses formed IC deposits in wt MRL-lpr mice, the IgG3 deposits appeared to be particularly strong and often had a membranous pattern in addition to a mesangiocapillary pattern, suggesting their important contribution to the proteinuria (35). The ppc1-5H MRL-lpr mice had much less deposition in all four IgG subclasses as compared with the wt littermates.

To examine the role of the secreted NAA per se in protection from lupus nephritis, purified ppc1-5 and control IgM Abs were administered to wt MRL-lpr. As shown in Fig. 3, the level of proteinuria was substantially decreased in mice injected with ppc1-5 IgM as compared with the mice that received either PBS or control IgM Abs. These data indicate that the secreted form of the ppc1-5 IgM is able to protect, at least to some extent, from lupus nephritis.

Skin lesions

Skin lesions, mainly in the neck and head areas, are one of the major disease manifestations in MRL-lpr mice. The lesions are thought to be caused by vasculitis secondary to IC deposition (43). The ppc1-5H and the wt MRL-lpr mice were monitored for the development of skin lesions for 36 wk. We observed a significant decrease in the incidence of skin lesions in the ppc1-5H mice (Fig. 4): whereas >90% of the wt MRL-lpr mice developed skin lesions by 26 wk of age, only 50% of the ppc1-5H MRL-lpr mice did. The severity of the skin lesions among those mice that had dermatitis was also significantly reduced in ppc1-5 MRL-lpr mice (data not depicted).

Survival

The female ppc1-5H MRL-lpr mice had significantly longer survival than their wt littermates (Fig. 5). The mortality rate at 50 wk was 28% for the ppc1-5H MRL/lpr mice, but was 80% for the wt mice. To rule out a nonspecific transgene-related effect on survival, we compared the survival data of the ppc1-5H mice to those of the 76RH MRL-lpr mice. The latter is an anti-dsDNA sd-tg mouse model we generated previously with a targeting construct similar to that of the ppc1-5H (44). We had shown that the 76RH MRL-lpr mice had accelerated onset of autoantibody production and developed severe kidney disease (44). In this study, we demonstrate that the 76R MRL-lpr mice had a shortened lifespan with 100% mortality rate at 40 wk of age (Fig. 5). Therefore, the protective role of ppc1-5H is not due to a gene insertion effect, but rather it is associated with the specificity of the inserted IgH.

Serum Ab levels

Total serum Ig. To examine the effect of ppc1-5 NAA expression on overall Ab production, we measured the concentrations of serum IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 in ppc1-5H and wt MRL-lpr mice at 10 and 20 wk of age. The IgM level was higher in ppc1-5H than in wt MRL-lpr mice (Fig. 6A), likely due to the constitutive activation of the self-reactive NAA-producing B cells (20). The total serum IgG levels were similar between the two groups of mice (Fig. 6A), indicating that NAA expression does not affect the overall B cell activation and plasma cell differentiation. Further evaluation of the IgG subclasses, however, revealed important differences. At 20 wk of age, the ppc1-5H mice had significantly lower levels of IgG2a and IgG3 and a higher level of IgG1 as compared with the wt mice. Notably, the reduction of IgG3 was already evident at 10 wk of age. This indicates that the expression of ppc1-5 NAA can influence Ig isotype switching with a preferential suppression of IgG2a and IgG3, isotypes that play a major role in SLE pathogenesis (41, 42).

Anti-dsDNA Abs. Given that the ppc1-5 NAA can bind DNA and other self-Ags, it is conceivable that the expression of ppc1-5H in MRL-lpr mice may increase the level of anti-DNA Abs. Indeed, at 10 wk of age, the ppc1-5H mice had a higher level of IgM anti-dsDNA Abs than the wt mice (Fig. 6B). Such a difference was not apparent at 20 wk of age when anti-dsDNA Abs reached high levels in both ppc1-5H and wt MRL-lpr mice. In contrast, the IgG anti-dsDNA Ab level was lower in ppc1-5H than in wt mice. In particular, a significant reduction was seen in IgG2a and IgG3 anti-dsDNAAs at both 10 and 20 wk of age. The IgG1 anti-dsDNA Ab levels were similar in the two groups despite that the ppc1-5H mice had a higher level of total IgG1 (Fig. 6A).

Anti-nuclear Abs. Additionally, we examined the ANA in ppc1-5H and wt MRL-lpr mice using the Hep-2 ANA assay. The intensity of Hep-2 nuclear staining was significantly lower in ppc1-5H than in wt MRL-lpr mice in all four IgG subclasses (Fig. 7A, 7B). Three

![FIGURE 1](image-url)
major nuclear staining patterns were previously described by Shlomchik and colleagues (45): the homogeneous pattern, the speckled pattern, and the mixed homogeneous and speckled pattern. The homogeneous pattern is thought to correlate with anti-dsDNA and antichromatin reactivity, whereas the speckled pattern correlates with binding to RNA-containing Ags such as Sm and U1RNP. Evaluation of the staining patterns of individual serum samples revealed that most of the samples from the wt MRL-lpr mice exhibit a mixed homogeneous and speckled nuclear staining pattern, indicating the presence of Abs to dsDNA and RNA–protein complexes in these animals (Fig. 7A, 7C). In contrast, sera from the ppc1-5H mice showed mainly a homogeneous pattern, suggesting a significant loss of reactivity toward RNA-containing Ags.

Anti-Sm and anti-Sm/RNP Abs. To further evaluate the Ab response against RNA-containing Ags in ppc1-5H MRL-lpr mice, we examined the serum levels of anti-Sm and anti-Sm/RNP Abs by ELISA. Both anti-Sm and anti-RNP Abs are directed against RNA-containing small nuclear RNP particles. These Abs are of great clinical significance (46). In MRL-lpr mice, anti-Sm and anti-RNP represent major autoantibody responses (47–49). As shown in Fig. 7D and 7E, the wt MRL-lpr mice had high levels of IgG anti-Sm and anti-Sm/RNP Abs at 20 wk of age. In contrast, the age-matched ppc1-5H MRL-lpr mice had only modest levels of IgG anti-Sm and anti-Sm/RNP Abs, although the titers were slightly higher than those of the control B6 mice. The IgM anti-Sm and anti-Sm/RNP Abs were very low in ppc1-5H MRL-lpr mice, comparable to those of the B6 mice. The wt MRL-lpr mice also had low levels of IgM anti-Sm and anti-Sm/RNP Abs, but the values were significantly higher than those of the ppc1-5H mice (Fig. 7D, 7E). Taken together, these results and the ANA data indicate a reduced Ab response to RNA-containing Ags in ppc1-5H MRL-lpr mice, which may contribute to the lessened disease manifestation because there is evidence that Abs to RNA-containing Ags play an important role in lupus pathogenesis (45, 50).

Lymphoid hyperplasia and lymphocyte populations. Lymphoid hyperplasia, mainly ascribed to the accumulation of CD4+ CD8– double-negative (DN) T cells, is a constant feature of MRL-lpr mice. To determine whether the expression of ppc1-5H would affect lymphoid hyperplasia, we first measured the weight of the spleens from the ppc1-5H and wt MRL-lpr mice. We found no significant difference in the mean spleen weight between the two groups of mice (0.33 versus 0.31 g, Table I); both groups had significantly larger spleens than the age-matched B6 mice (0.08 ±
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MRL-lpr mice, as were the CD8+ T cells (Table I). The CD4 class II (I-A/E), and CD44.

The spleen architecture was also more preserved in ppc1-5H mice. The numbers of MZ B cells were comparable between the two groups of mice. The spleen architecture was also more preserved in ppc1-5H mice (data not depicted). No differences were detected in the expression of activation-associated markers on splenic B cells between the ppc1-5H and wt MRL-lpr mice; these included CD69, CD80 (B7-1), CD86 (B7-2), MHC class II (I-A/E), and CD44.

FIGURE 3. Decreased proteinuria in MRL-lpr mice after receiving ppc1-5 IgM injection. Female MRL-lpr mice were injected with purified ppc1-5 IgM, s.c. 100 μg three times a week starting at 8 wk of age. The control groups received either a control IgM Ab (an IgM hybridoma isolated from B6 mouse in our laboratory) or PBS injection. There were eight mice in each group. The mice receiving ppc1-5 IgM had significantly reduced proteinuria as compared with the control groups at all time points from 12 wk on. The top asterisk signs represent the differences between the ppc1-5 IgM and PBS groups, and the lower asterisk signs represent the differences between the ppc1-5 IgM and the control IgM groups. *p < 0.05, **p < 0.01, ***p < 0.001, Student t test.

0.02 g). Next, we examined splenic lymphocyte populations by flow cytometry. The ppc1-5H MRL-lpr mice consistently had more CD19+ B cells than the wt mice, although the difference did not reach statistical significance (Table I). It is known that the MRL-lpr mouse have relatively decreased CD21+CD23+ FO B cells and relatively increased CD21+CD23- MZ B cells as compared with the non-autoimmune mouse. The expression of ppc1-5H resulted in a more normalized FO B cell population (Fig. 8A, Table I), which is likely responsible for the increased B cells in ppc1-5H mice. The numbers of MZ B cells were comparable between the two groups of mice. The spleen architecture was also more preserved in ppc1-5H mice (data not depicted). No differences were detected in the expression of activation-associated markers on splenic B cells between the ppc1-5H and wt MRL-lpr mice; these included CD69, CD80 (B7-1), CD86 (B7-2), MHC class II (I-A/E), and CD44.

The total T cell numbers were comparable in the ppc1-5H and wt MRL-lpr mice, as were the CD8+ T cells (Table I). The CD4+CD8- DN T cells were decreased in ppc1-5H mice, although the difference did not reach statistical significance (p = 0.057). The number of CD4+ T cells was higher in ppc1-5H than in wt MRL-lpr mice. We further divided the CD4+ T cells into three subsets based on the expression of CD44 and CD62L: the naive T cells (CD44lowCD62Lhigh), activated T cells (CD44highCD62Llow), and memory/activated T cells (CD44highCD62Lhigh). The ppc1-5H MRL-lpr mice had higher number of naive T cells, but comparable numbers of activated and memory T cells as compared with the wt mice (Fig. 8B, Table I). It therefore appears that the increased CD4+ T cells in the ppc1-5 H mice can be attributed to the more abundant naive T cells in these mice. The expression of the early activation marker CD69 was decreased in ppc1-5H mice (Table I). In contrast, the expression of CTLA-4, a potent negative regulator of T cell activation (51), was significantly increased in the ppc1-5H MRL-lpr mice (Fig. 8C, Table I). No differences were found in the number of CD4+CD25+ or CD4+Foxp3+ regulatory T cells between the two groups of mice (Table I).

Taken together, these results show that the expression of ppc1-5 NAA in MRL-lpr mice did not significantly affect the overall lymphoid hyperplasia or the total B and T cell numbers. It did have, however, a number of impacts on T cell compartment: there was a decrease in the number of DN T cells, an increase in naive T cells, and a suppression of CD4+ T cell activation as indicated by the decreased CD69 expression and increased CTLA-4 expression.

Cytokine production. To determine whether the expression of ppc1-5 NAA might affect cytokine production, we initially measured the serum cytokine levels in ppc1-5H and wt MRL-lpr mice. No significant differences were detected between the two groups of mice in the serum concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-23, IFN-γ, and TNF-α (data not depicted). Because serum cytokine concentrations are the resultant of cytokine production and consumption by various cell types, they do not necessarily reflect the amounts of cytokines produced by the lymphocytes. We therefore quantified cytokine-producing T cells by intracellular staining following a short period of in vitro anti-CD3 stimulation. The ppc1-5H MRL-lpr mice had more IL-10–producing CD4+ T cells than the wt mice (Fig. 9A). The number of IFN-γ–producing CD8+ T cells was decreased in ppc1-5H MRL-lpr mice, whereas the number of IFN-γ–producing CD4+ T cells was not significantly different between the two groups of mice (Fig. 9B). No differences were detected in the numbers of IL-2, IL-4, IL-10, IL-17, or TNF-α–producing T cells (data not depicted).
Additionally, we measured cytokine production by B cells. Upon CpG (TLR9 ligand) or LPS (TLR4 ligand) stimulation, there were significantly more IL-10–producing B cells in the ppc1-5H than in the wt MRL-lpr mice (Fig. 9C, 9D). Quantification of the secreted IL-10 in the culture supernatants of the purified B cells also demonstrated a significantly higher amount of IL-10 in the ppc1-5H than in the wt B cell cultures (Fig. 9E). No significant differences were observed in the production of IL-2, IL-4, TGF-β, or TNF-α between the two groups of mice (data not depicted). Stimulation with anti-IgM, anti-CD40, or a combination of both had little effect on B cell cytokine production (data not depicted).

FIGURE 6. Total serum Ig and anti-dsDNA Abs. Total serum Ig subclasses (A) and anti-dsDNA Abs (B) in ppc1-5H and wt MRL-lpr mice at 10 and 20 wk of age. Data are for groups of eight female mice. The relative titers of anti-dsDNA Abs were calculated based on the values of a pooled serum sample from five 26-wk-old MRL-lpr mice. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, determined by Student t test.

FIGURE 7. ANA patterns and the levels of anti-Sm and anti-Sm/RNP autoantibodies. (A) Examples of three major ANA patterns: homogeneous (left panel), speckled (middle panel), and mixed (right panel). Arrows indicate mitotic figures. Fluorescence stain. Original magnification ×400. (B) Fluorescence intensity scores of ANA. (C) Percentages of serum samples stained with each pattern in ppc1-5H and wt MRL-lpr mice. There were 12 female mice (16 wk old) in each group. Anti-Sm (D) and anti-Sm/RNP Abs (E) in ppc1-5H and wt MRL-lpr mice. Data are for groups of seven to eight female mice at 20 wk of age. The units of anti-Sm and anti-Sm/RNP Abs were calculated based on the values of a calibrator provided by the manufacturer (see Materials and Methods). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, determined by Student t test.
B cell proliferation and Ab production upon TLR stimulation. The significant effects of CpG and LPS on IL-10 production by B cells from ppc1-5H MRL-lpr mice suggest that the ppc1-5 NAA B cells may be particularly responsive to TLR stimulation. To further test this notion, we cultured the B cells in the presence or absence of LPS or CpG for 48 h and quantitated the numbers of total B cells and $\lambda^+$ B cells; the latter represent cells expressing the ppc1-5/\lambda^+ NAA. As expected, there was a marked increase in B cell numbers in both the ppc1-5H and wt cell cultures after LPS or CpG stimulation (Fig. 10A, 10B). However, the ppc1-5H B cells exhibited a significantly greater increase than did the wt B cells. Remarkably, the difference was almost entirely due to an increase in $\lambda^+$ B cells. We have noticed an increased staining with anti-$\lambda$ reagent in the ppc1-5H B cells, in particular, after LPS and CpG stimulation (Fig. 10A). To further characterize these B cells, hybridomas were made from the stimulated ppc1-5H B cells. Our preliminary analysis showed 17 and 25% of $\lambda^+$ clones and 83 and 75% of $\kappa^+$ clones from LPS and CpG cultures, respectively (K. Mannoor and C. Chen, unpublished observations). These findings indicate that the B cells stained weakly with $\lambda$ in the LPS and CpG cultures are not true $\lambda$-producing B cells but rather $\kappa^+$ B cells with a high background staining. This high background is likely due to the high levels of ppc1-5/$\lambda^+$ NAA in the culture supernatants (see below).

Next, we determined the Ab concentrations in the culture supernatants. As shown in Fig. 10C, the ppc1-5H B cells produced more IgM/\lambda Abs than the wt B cells did after LPS and CpG stimulation. There were very few, if any, IgG/\lambda Abs in both the ppc1-5H and wt B cell cultures. No significant differences were observed in the concentrations of IgM/\kappa and IgG/\kappa between the ppc1-5H and wt B cell cultures (data not depicted). Of note, there were more $\kappa^+$ than $\lambda^+$ Abs in both the ppc1-5H and wt B cell cultures. Taken together, these results indicate that the ppc1-5/\lambda NAA B cells have a heightened response to TLR4 and TLR9 stimulation with vigorous cell proliferation, IgM Ab secretion, and IL-10 cytokine production.

**Discussion**

To critically examine the role of natural autoantibodies and their associated B cells in the development of autoimmune diseases, we generated IgH sd-tg mice, in which a prototypic NAA, ppc1-5, is expressed by a substantial proportion of the peripheral B cells. We showed that the expression of ppc1-5 NAA in the lupus-prone

<table>
<thead>
<tr>
<th>B cell proliferation and Ab production upon TLR stimulation.</th>
<th>pp1-5 MRL-lpr</th>
<th>wt MRL-lpr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen weight (g)</td>
<td>0.33 ± 0.07</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>B cells (absolute no. $\times 10^6$/spleen)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total B (CD19+)</td>
<td>52.0 ± 6.4</td>
<td>39.8 ± 6.6</td>
</tr>
<tr>
<td>$\lambda^+$</td>
<td>11.2 ± 1.2</td>
<td>2.9 ± 0.6***</td>
</tr>
<tr>
<td>FO (CD21$^{hi}$CD23$^{lo}$)</td>
<td>31.1 ± 3.2</td>
<td>16.7 ± 3.4**</td>
</tr>
<tr>
<td>MZ (CD21$^{lo}$CD23$^{hi}$)</td>
<td>19.1 ± 4.6</td>
<td>17.5 ± 3.7</td>
</tr>
<tr>
<td>T cells (absolute no. $\times 10^6$/spleen)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total T (CD3$^+$)</td>
<td>63.5 ± 8.1</td>
<td>60.5 ± 9.0</td>
</tr>
<tr>
<td>CD4$^+$</td>
<td>33.5 ± 3.7</td>
<td>23.6 ± 2.7*</td>
</tr>
<tr>
<td>CD8$^+$</td>
<td>16.4 ± 2.9</td>
<td>17.5 ± 3.3</td>
</tr>
<tr>
<td>CD4$^+$CD8$^-$</td>
<td>11.6 ± 2.8</td>
<td>18.0 ± 2.3</td>
</tr>
<tr>
<td>Subsets of CD4$^+$ T cells (% CD4$^+$ T cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4$^{lo}$CD62L$^{hi}$</td>
<td>10.3 ± 1.6</td>
<td>5.2 ± 1.2*</td>
</tr>
<tr>
<td>CD4$^{lo}$CD62L$^{hi}$</td>
<td>31.2 ± 3.3</td>
<td>25.8 ± 3.0</td>
</tr>
<tr>
<td>CD4$^{hi}$CD62L$^{lo}$</td>
<td>53.3 ± 3.9</td>
<td>61.3 ± 2.1</td>
</tr>
<tr>
<td>CD69$^+$</td>
<td>38.2 ± 2.8</td>
<td>52.3 ± 2.8**</td>
</tr>
<tr>
<td>CTLA-4$^+$</td>
<td>39.9 ± 7.4</td>
<td>24.2 ± 2.1*</td>
</tr>
<tr>
<td>CD25$^+$</td>
<td>17.0 ± 4.9</td>
<td>11.3 ± 3.4</td>
</tr>
<tr>
<td>Foxp3$^+$</td>
<td>12.4 ± 4.6</td>
<td>8.3 ± 2.2</td>
</tr>
</tbody>
</table>

The pp1-5 and wt MRL-lpr mice were all female, 16–20 wk old. There were 5–10 mice in each group. Values are the mean ± SEM.

*p < 0.05, **p < 0.01, ***p < 0.001, pp1-5 versus wt mice (Student $t$ test).

**FIGURE 8.** Flow cytometric analysis of the splenic cells from pp1-5H and wt MRL-lpr mice. The Abs are indicated. (A) is gated on CD19$^+$ B cells; (B) and (C) are gated on CD4$^+$ T cells. Data are representative of six to eight female mice in each group. The mice were 14–16 wk old.
MRL-lpr mice prevented proteinuria, reduced renal histopathology and IC deposition, and improved skin disease. Consequently, the mice had significantly prolonged survival. Furthermore, injection of the purified ppc1-5 IgM to MRL-lpr mice also significantly delayed the onset of proteinuria. These findings indicate that the ppc1-5 NAA and their associated B cells play an important role in protection from systemic autoimmune disease.

A concern about the IgH transgenic model is the relatively restricted Ab repertoire, which was thought to prevent the development of autoimmune disease in a conventional anti-DNA tg mouse model (52). Although we cannot completely rule out the influence of IgH restriction in our sd-tg model, we do not think it is the major cause of the alleviated disease observed in the ppc1-5H MRL-lpr mice for several reasons. First, the ppc1-5H B cells are able to undergo isotype switching, somatic mutation, and receptor editing, all of which would lead to a more diverse Ab repertoire. This is supported by our preliminary hybridoma analysis in which 20 out of 30 (67%) IgG hybridomas from the ppc1-5H MRL-lpr mice expressed a non–ppc1-5 VH due to receptor editing, and a variety of κ L chains were used (K. Mannoor and C. Chen, unpublished observations). Second, a major consequence of a very restricted B cell repertoire in autoimmune mice is a reduction in activated/memory T cells (52). This was supported by our preliminary hybridoma analysis in which 20 out of 30 (67%) IgG hybridomas from the ppc1-5H MRL-lpr mice expressed a non–ppc1-5 VH due to receptor editing, and a variety of κ L chains were used (K. Mannoor and C. Chen, unpublished observations). Second, a major consequence of a very restricted B cell repertoire in autoimmune mice is a reduction in activated/memory T cells (52). This was not observed in ppc1-5H MRL-lpr mice, indicating a functionally diverse B cell repertoire. Third, the 76R anti-dsDNA IgH sd-tg mice, which were created in the same fashion as the ppc1-5H mice and had a limited B cell repertoire (44), had accelerated disease and shortened survival. Fourth, when Kench et al. (53) crossed the anti–H-2K H/L tg to the MRL-lpr background, the anti-H2K/MRL-lpr mice had very restricted B cell repertoire, yet they did not show improved survival as compared with the non-tg MRL-lpr mice. Lastly, administration of the ppc1-5 IgM NAA into the wt MRL-lpr mice (which have a full range of B cell repertoire) significantly delayed the onset of proteinuria. Our preliminary adoptive transfer experiments also showed that infusion of the ppc1-5H B cells but not the wt B cells into the MRL-lpr mice prevented clinical nephritis (K. Mannoor and C. Chen, unpublished observations). These facts strongly argue against the restricted B cell repertoire or other nonspecific transgene effect being responsible for the alleviated disease observed in ppc1-5H sd-tg mice. Instead, they support a specific protective effect of the ppc1-5 encoded Ab and the associated B cells.

The delayed onset of proteinuria in mice infused with purified ppc1-5 IgM demonstrates that the secreted ppc1-5 NAA per se have a protective effect. This finding is consistent with a previous report that administration of an anti-dsDNA IgM to NZB/W F1 mice reduced renal disease (30). The mechanisms by which NAA protect from autoimmunity are not well understood. It has been postulated that the NAA can promote clearance of apoptotic cells and therefore suppress autoimmune reactions because apoptotic...
cells represent a major source of lupus-associated autoantigens (54–57), and deficiencies associated with delayed clearance of apoptotic cells lead to autoimmune manifestations (58–61). When cells are undergoing apoptosis, cellular constituents are fragmented and reorganized to form a variety of neo–self-Ags (62). It is not hard to envision that the broadly autoreactive NAA such as ppc1-5 could bind to these self-Ags and promote clearance of apoptotic cells. Silverman and colleagues (63–65) have demonstrated that the T15 anti-PC natural Ab can bind to the PC-containing neo–self-Ag on the apoptotic cells and subsequently recruit C1q and MBL; this complex interacts with the scavenger receptors on dendritic cells and macrophages, thereby enhancing clearance of apoptotic cells on one hand and suppressing dendritic cell activation/maturation on the other. Through these mechanisms, the T15 Ab functions as an immune regulator and suppresses autoimmunity. Our preliminary studies have shown that ppc1-5 IgM can bind to apoptotic cells (K. Mannoor and C. Chen, unpublished observations). Experiments are underway to examine the mechanisms of action of ppc1-5 NAA.

The expression of ppc1-5H did not affect the total amount of serum IgG but skewed the IgG subclasses: there was a significant decrease in IgG2a and IgG3 and a concurrent increase in IgG1. The specific suppression of IgG3 is important. It has been shown that the production of IgG3 is responsible for the development of lupus nephritis in MRL-lpr mice (41). Our data have also shown that IgG3 forms particularly strong membrane IC deposits in the kidney (Fig. 2). The greater ability of IgG3 to form IC than other Ig isotypes is thought to be related to their greater cationicity and cryoglobulin properties (41). The regulatory mechanisms for IgG3 production are unclear. Because IgG3 is generally considered to be the Ab subclass in response to TI-2 Ags, it is possible that there is a dysregulation in TI-2 immune response in MRL-lpr mice and that this dysregulation can be corrected by the expression of ppc1-5 NAA. There are also reports that IgG3 production is IFN-γ dependent (66, 67). It is therefore possible that the expression of ppc1-5 NAA in MRL-lpr mice can down-regulate IFN-γ production (possibly by CD8+ T cells as shown in Fig. 9B or by non-T cells) and thereby suppresses isotype switching to IgG3.

Aside from Ab production, the NAA B cells may regulate autoimmunity via Ab-independent functions. B cells are important APC. There is clear evidence that B cells as APC can induce T cell tolerance (68–71). In some reports, it has been shown that tolerance induction was mediated by upregulation of negative co-stimulatory molecules such as CTLA-4 and programmed cell death-1 on T cells (70, 71). Our finding that the ppc1-5H MRL-lpr mice have increased numbers of CTLA-4+ T cells suggest that the ppc1-5H NAA B cells may be able to deliver a tolerogenic signal to CD4+ T cells. Notkins and colleagues (72) have reported that B cells expressing polyclonal BCRs could present Ag to T cells but failed to cause T cell proliferation.

B cells may also regulate T cell function via cytokine secretion. B cells can secrete a variety of cytokines (73, 74), among which IL-10 is of particular interest because it is closely associated with the regulatory function of B cells. There is convincing evidence in animal models that IL-10–producing B cells can suppress autoimmune and inflammatory processes (75–77). Our finding that the NAA B cells produced large amounts of IL-10 upon TLR stimulation points to their regulatory function and suggests that they may be part of the growing family of regulatory B cells (77–79). The NAA-expressing B cells may be especially responsive to TLR stimulation because their polyreactive BCRs often bind TLR ligands such as DNA, RNA, and microbial Ags, which in turn facilitates TLR activation by dual engagement of BCR and TLR (80–82). Our data have shown a vigorous response of ppc1-5 NAA B cells to TLR-9 and, to a lesser extent, to TLR-4 stimulation. The activated NAA B cells produce more NAA and more IL-10, which in turn inhibit autoimmunity. It has been shown that ablation of TLR9 exacerbates lupus-like disease in MRL-lpr mice despite decreased anti-dsDNA Abs in such mice (45, 83). The role of TLR9 in the enhancement of NAA B cell regulatory function may in part account for this effect.

One of the intriguing findings of our current study is that in the presence of ppc1-5 NAA B cells, anti-CD3 stimulation of T cells led to a significant increase in IL-10+ CD4+ T cells. This is in agreement with a previous report that coculture of IL-10–producing B cells with CD4+ T cells induced differentiation of CD4+ IL-10+ Tr1 cells (84). This was thought to be a mechanism by

FIGURE 10. B cell proliferation and Ab production upon LPS and CpG stimulation. Splenic cells from ppc1-5 and wt MRL-lpr mice were cultured for 48 h in the presence of LPS, CpG, or with medium alone. The numbers of total B cells and the A1+ B cells were measured by cell counting with trypan blue exclusion and flow cytometry. The Ab concentrations in the supernatants were measured by ELISA. (A) Flow cytometric analysis of A1+ B cells. (B) Numbers of total B cells and A1+ B cells. (C) IgM/A1 Ab concentrations after LPS or CpG stimulation. There were five to six female mice (14–16 wk old) in each group. Data are presented as mean ± SEM. *p < 0.05, ***p < 0.001, determined by Student t test.
which regulatory B cells might control autoimmunity. In addition to induction of Tr1, there are also reports that IL-10 and regulatory B cells can suppress Th1 response and influence Th1/Th2 polar-
ization (84, 85). Our finding of the decreased Th1 type IgG2a Ab and the increased Th2 type IgG1 Ab in pp1-5H MRL-lpr mice supports this notion.

In summary, our data show strong evidence that NAA and NAA B cells can suppress autoimmune disease and suggest several poten-
tial mechanisms. First, the secreted IgM NAA per se can suppress lupus nephritis, presumably by promoting removal of self-Ags and apototic cells. Second, NAA B cells can function as tolerogenic APCs and induce T cell expression of negative regulators such as CTLA-4. Third, NAA B cells upon activation by TLR ligands can differentiate to IL-10–producing regulatory B cells, which can induce Tr1 differentiation and restore Th1/Th2 balance. We expect future experiments in this model system to provide further insight into the mechanisms by which NAA and NAA B cells suppress autoimmune response and protect from lupus nephritis.

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Disclosures
The authors have no financial conflicts of interest.

References


