Exacerbated Autoimmunity in the Absence of TLR9 in MRL. Fas<sup>lpr</sup> Mice Depends on I<sub>ifnar1</sub>

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Exacerbated Autoimmunity in the Absence of TLR9 in MRL.Fas<sup>lpr</sup> Mice Depends on Ifnar1

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TLR9 suppresses TLR7-driven pathogenesis in the MRL.Fas<sup>lpr</sup> murine model of systemic lupus erythematosus, but the mechanisms by which TLR7 promotes and TLR9 prevents disease in this and other lupus models remain unclear. Type I IFNs (IFN-I) have also been implicated in the pathogenesis of lupus both in patients and in several murine models of disease, but their role in MRL.Fas<sup>lpr</sup> mice is controversial. Using MRL.Fas<sup>lpr</sup> mice genetically deficient in a subunit of the receptor for IFN-I, Ifnar1, we show that IFN-I contribute significantly to renal disease in this model. Ifnar1 had no effect on anti-nucleosome or anti-Sm autoantibody titers, but instead regulated anticytoplasmic and anti-RNA specificities. Moreover, Ifnar1 deficiency prevented the exacerbation of clinical disease observed in Tlr9-deficient animals in this lupus model. Thus, IFN-I signaling is an important mediator of lupus pathogenesis and anti-RNA Ab production that is dysregulated in the absence of Tlr9.

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Previously we observed an increase in the titer of serum IFN-α in MRL.Fas<sup>lpr</sup> mice lacking Tlr9 (5) and speculated that this cytokine could have contributed to the exacerbation of disease in those animals. IFN-α, a type I IFN (IFN-I), is elevated in some SLE patients with severe disease (11, 12) and has also been implicated in the pathogenesis of several murine lupus models (13–18). In contrast, a previous report suggested that IFN-I was dispensable for disease or even protective in the MRL.Fas<sup>lpr</sup> model, although only a small number of animals were examined in this study (19). Even if IFN-I were not required for MRL.Fas<sup>lpr</sup> disease, it remained possible that IFN-I could have contributed to the enhancement or acceleration of disease specifically in mice lacking Tlr9. We therefore backcrossed mice deficient in the receptor for IFN-I, Ifnar1, onto the MRL.Fas<sup>lpr</sup> model with or without Tlr9. In this article, we demonstrate that Ifnar1 indeed contributes to renal disease and production of anti-RNA, but not anti-nucleosome, autoantibodies in the MRL.Fas<sup>lpr</sup> model, in contrast with the previous report. Moreover, and most importantly, the exacerbation of disease seen in Tlr9<sup>−/−</sup> MRL.Fas<sup>lpr</sup> mice is substantially mitigated in Ifnar1<sup>−/−</sup> Tlr9<sup>−/−</sup> MRL.Fas<sup>lpr</sup> mice, suggesting that the proinflammatory effects of TLR9 deficiency in lupus are in large part mediated via increased IFN-I.

Materials and Methods

Mice

Ifnar1<sup>−/−</sup> mice were previously described (20) and were backcrossed to the MRL/MpJ-Fas<sup>+/+</sup>/J background (The Jackson Laboratory #006825) for eight generations before intercross. Tlr9<sup>−/−</sup> mice on the MRL/MpJ-Fas<sup>+/+</sup>/J background were previously described and were backcrossed an additional six generations to the MRL/MpJ-Fas<sup>+/+</sup>/J background before intercross. There were n = 11–32 animals per group in all assays except for 24 wk Ifnar<sup>−/−</sup>Tlr9<sup>−/−</sup> where n = 5 in all assays. All animal work was approved by the Yale Institutional Animal Care and Use Committee.

Evaluation of clinical disease

For skin disease, mice were scored for dorsal lesions on a scale of 0–5 based on affected area, with up to one additional point for presence of ear dermatitis and facial rash or loss of whiskers as described previously (5). Proteinuria was measured using a colorimetric dipstick assay (Albustix; Siemens, Tarrytown, NY). For kidney disease, formalin-fixed and paraffin-embedded tissue sections stained with H&E were scored for extent of interstitial and perivascular infiltrates on a 0–3 scale by an independent observer blinded to the genotype of the samples. Glomerulonephritis was scored on the same sections on a 0–6 scale as previously described (21).
Measurement of serum autoantibodies

HEp-2 immunofluorescence assays (Antibodies Inc., Davis, CA) were performed as previously described (5) with serum diluted at 1/200, and were scored for relative fluorescence intensity of cytoplasmic staining on a scale of 0–3 and for the presence or absence of mitotic chromatin by an observer blinded to the genotype of the samples. Antinucleosome and anti-Sm Ab ELISAs were performed as previously described (6). Anti-RNA Ab ELISAs were performed as described previously (22). Total serum IgG was determined by ELISA as previously described (6). Total serum IgM was determined by ELISA by coating polystyrene plates with goat anti-mouse IgM (clone B7-6). After blocking with 1% BSA in PBS, serial dilutions of serum from 1/50,000–1/1,350,000 were added. Specific Abs were detected with alkaline phosphatase–conjugated goat anti-mouse IgM (Southern Biotechnology Associates).

Results

To evaluate the role of IFN-I in the MRL. Fas<sup>lo</sup> model of SLE, we backcrossed mice genetically deficient in Ifnar1, a subunit of the heterodimeric receptor for IFN-I, onto the MRL. Fas<sup>lo</sup>/2J genetic background. Although a previous report found little effect of Ifnar1 deficiency on disease in the MRL. Fas<sup>lo</sup> model, this report evaluated a small number of animals and did not segregate them by sex (19). Because of the variable onset and severity of disease in this model, as well as the sex-dependent difference in disease kinetics (23), we considered that this report may not have had sufficient statistical power to accurately determine effects of Ifnar1 deficiency. In addition, we previously reported that several clinical parameters of disease were exacerbated in Tlr9-deficient MRL. Fas<sup>lo</sup> mice that also had elevated titers of serum IFN-α (5). Therefore, to test the hypothesis that an important mechanism by which TLR9 deficiency paradoxically promotes disease is via stimulation of excessive IFN-I secretion and signaling, we intercrossed Ifnar1<sup>−/−</sup> MRL. Fas<sup>lo</sup> animals with MRL. Fas<sup>lo</sup> genetically deficient in Tlr9. These crosses generated experimental cohorts lacking Ifnar1, Tlr9, or both Ifnar1 and Tlr9. Mice were evaluated at either 16 or 24 wk of age, and results were further segregated based on sex.

Renal function and glomerular disease are improved in Ifnar1-deficient MRL. Fas<sup>lo</sup> mice

We first evaluated parameters of renal disease. Proteinuria, a measure of kidney function, was reduced in Ifnar1-deficient female cohorts compared with Ifnar1-intact littermates at both 16 and 24 wk of age (Fig. 1A). In contrast, males had little proteinuria even by 24 wk in the Ifnar1-intact or -deficient groups. Importantly, proteinuria was elevated in 16-wk female and male, and 24-wk male mice lacking Tlr9 compared with Tlr9-intact animals, in agreement with our previous reports of exacerbated renal disease in the absence of Tlr9 (5, 6). In contrast, mice deficient in both Ifnar1 and Tlr9 had relatively little proteinuria, suggesting that the exacerbation of disease in Tlr9-deficient animals also requires the receptor for IFN-I.

Renal disease in SLE may be mediated by interstitial and perivascular lymphocyte infiltrates and/or by inflammation of the glomeruli (3). The extent of lymphocytic infiltrates in H&E-stained kidney sections was evaluated by a pathologist blinded to the genotypes of the animals and was not significantly different among any of the experimental groups, suggesting that neither Ifnar1 nor Tlr9 grossly affect recruitment and/or in situ expansion of lymphocytes in the kidneys (Fig. 1B). In contrast, the severity of glomerular disease was significantly diminished in 16-wk female and 24-wk female and male mice that lacked both Ifnar1 and Tlr9 compared with mice lacking only Tlr9, as reflected by differences in glomerular scores (Fig. 1C). Incidence of severe disease was also influenced by Ifnar1. The 24-wk female Tlr9<sup>−/−</sup> group had 13/22 severely affected (score ≥ 3), whereas the doubly-deficient group had 9/32 with severe glomerulonephritis (p = 0.0282, two-sided Fisher’s exact test); among the males, the Tlr9<sup>−/−</sup> group had 15/32 with severe disease, whereas only 3/31 Ifnar1<sup>−/−</sup>/Tlr9<sup>−/−</sup> mice were severely affected (p = 0.0017, two-sided Fisher’s exact test). Thus, Ifnar1 promotes progression of glomerular but not interstitial renal disease in the MRL. Fas<sup>lo</sup> model.

Onset of dermatitis is delayed in Ifnar1-deficient mice

We next evaluated other clinical parameters of disease. Aged MRL. Fas<sup>lo</sup> mice develop dorsal skin lesions, which are more frequently observed and more severe in females than in males, along with a loss of fur and whiskers on the muzzle and inflammation of the ears (24, 25). We did not observe significant skin lesions in Ifnar1-deficient animals at 16 wk of age regardless of Tlr9 genotype; in contrast, a fraction of female Ifnar1-intact animals had developed modest dermatitis by this age (Fig. 2A, 2B, left panels). By 24 wk of age, 30% of female Ifnar1-deficient mice had developed skin lesions that were as large in area as in affected Ifnar1-intact animals (Fig. 2A, 2B, right panels). Few males in this experimental cohort developed significant skin disease in either age group. Conclusions were fundamentally similar whether we considered skin score (Fig. 2A) or percentage of mice that were affected to a moderate or greater degree (Fig. 2B). Therefore, Ifnar1 deficiency modestly delays the onset of sex-dependent skin disease but does not have a significant effect on incidence or severity of dermatitis among older affected animals.
Lymphadenopathy is Ifnar1 dependent

MRL.Fas<sup>lpr</sup> mice develop splenomegaly and lymphadenopathy with age. In contrast, the MRL.Fas<sup>lpr</sup>/2J substrain, which arose via genetic drift in The Jackson Laboratory colony, has been reported to have less severe splenomegaly and lymphadenopathy than conventional MRL.Fas<sup>lpr</sup>/J, although the genetic alterations responsible have not yet been determined (http://jaxmice.jax.org/strain/006825.html). Consistent with these reports, we found that spleens in 16-wk animals in this experimental cohort, which had been backcrossed to the MRL.Fas<sup>lpr</sup>/2J strain, were relatively less expanded in size than in our previous reports (5, 6) (Fig. 3A, left) although moderate splenomegaly was observed in 24-wk animals (Fig. 3A, right). In the 24-wk cohort, splenomegaly was reduced when Ifnar1 was absent regardless of Tlr9 genotype. Similarly, in both 16- and 24-wk animals of either sex, Ifnar1 deficiency consistently led to smaller lymph nodes, independent of Tlr9 genotype (Fig. 3B). Thus, IFN-I contributes to the expansion of spleen and lymph nodes observed in the MRL.Fas<sup>lpr</sup>/2J strain.

Hypergammaglobulinemia in Tlr9-deficient mice requires Ifnar1

MRL.Fas<sup>lpr</sup> mice have high concentrations of serum IgGs that are even further elevated in Tlr9-deficient animals (5, 6). Therefore, we measured total serum IgM and IgG concentrations by ELISA. In 16-wk Ifnar1-deficient animals, we observed a modest reduction in serum IgM compared with Ifnar1-intact animals regardless of Tlr9 genotype, although this effect was not observed in older mice (Fig. 4A). In contrast with our previous reports (5, 6), and likely because of differences in disease kinetics between MRL.Fas<sup>lpr</sup>/J and MRL.Fas<sup>lpr</sup>/2J strains, we did not see a significant increase in amounts of serum IgG among 16-wk animals when Tlr9 was absent (Fig. 4B, left). Nonetheless, the absence of Ifnar1 resulted in a reduction in total IgG concentrations among 16-wk males (Fig. 4B, left). In contrast, among 24-wk animals, Tlr9 deficiency did lead to the increase in IgG concentrations in both female and male animals, as expected from our prior studies (5, 6).
Ifnar1 (Fig. 5A). As previously reported (4–6), Tlr9-deficient mice did not make antimitotic chromatin autoantibodies; this was also unaffected by Ifnar1 genotype. Not did Ifnar1 deficiency affect the titers of anti-nucleosome autoantibodies in 16- or 24-wk-old MRL.Fas<sup>+/+</sup>/2J mice of either sex (Fig. 5B). In agreement with our previous reports and the HEp-2 ANA, Tlr9-deficient animals had approximately a two-log reduction in anti-nucleosome titer; this was not affected by Ifnar1 genotype. Therefore, Ifnar1 does not affect production of Tlr9-dependent anti-nucleosome autoantibodies.

Ifnar1 promotes production of anti-RNA autoantibodies

We previously showed that the exacerbation of disease in Tlr9-deficient mice required intact Tlr7 and was associated with an increase in titers of some autoantibodies to RNA-containing autoantigens (6). To evaluate whether Ifnar1 contributed to Tlr7-dependent anti-RNA autoantibodies either in the presence or absence of Tlr9, we first scored HEp-2 slides for the intensity of staining of cytoplasmic components. Such cytoplasmic staining is Tlr7 dependent and increased in Tlr9-deficient animals (6). In agreement with our previous findings, Tlr9-deficient animals had greater cytoplasmic HEp-2 staining than Tlr9-intact mice (Fig. 6A). However, cytoplasmic HEp-2 staining was reduced when Ifnar1 was also absent, suggesting that this staining pattern includes IFN-I–dependent specificities.

To further define this effect, we examined the Tlr7-dependent autoantibodies anti-Sm and anti-RNA by ELISA. Few mice were making anti-Sm at 16 wk of age (Fig. 6B, left). By 24 wk of age, a greater proportion of female and male animals were making anti-Sm (Fig. 6B, right). Among 24-wk-old males, Ifnar1 deficiency led to reduced titer and incidence of anti-Sm; however, Ifnar1 did not affect anti-Sm production in Tlr9-deficient mice, with a substantial fraction of Ifnar1<sup>−/−</sup>/Tlr9<sup>−/−</sup> animals being anti-Sm producers. Thus, we conclude that Ifnar1 is not necessary for the production of anti-Sm autoantibodies. In contrast with anti-Sm, we observed a significant decrease in anti-RNA titers when Ifnar1 was absent, with a 2-fold reduction in titer between 24-wk Ifnar-intact and -deficient males (Fig. 6C). Moreover, although anti-RNA was elevated by 2.2-fold among 24-wk females and 2.6-fold among 24-wk males when Tlr9 only was absent, this elevation was not observed in mice lacking both Ifnar1 and Tlr9. Thus, Ifnar1 contributes to the production of anti-RNA autoantibodies and cytoplasmic HEp-2 staining, but not anti-nucleosome or anti-Sm autoantibodies.

Discussion

The importance of the TLR7 and TLR9 pathways in SLE has been clearly demonstrated in several in vivo murine models of lupus pathogenesis (4–10). Autoreactive B cells in lupus break tolerance because of the ability of their nucleic acid–containing ligands to coligate both the BCR and either TLR7 or TLR9 (26–28). Mice lacking TLR7 or the adaptor MyD88 have reduced anti-RNA titers, as well as reduced renal disease (5, 6). Paradoxically, however, mice lacking TLR9 instead have exacerbated disease, despite lacking anti-nucleosome autoantibodies (6–10). The reasons for the divergent effects of TLR7 and TLR9 deficiency on disease remain incompletely understood, and resolving these issues will have important implications for our understanding of lupus pathogenesis.

In this study, we have connected the IFN-I signaling pathway to exacerbated disease in the Tlr9-deficient MRL.Fas<sup>+/+</sup> model. As
was the case in mice lacking both Tlr7 and Tlr9 (6), mice lacking both Ifnar1 and Tlr9 had reduced anti-RNA autoantibodies and reduced renal disease compared with mice lacking Tlr9 only. Thus, disease that is normally suppressed by Tlr9 requires both Tlr7 and Ifnar1. This represents an important advance in understanding the puzzle of why Tlr9 deficiency paradoxically exacerbates lupus disease. In fact, most, but not all, of the Tlr7-dependent disease exacerbation phenotypes are also mitigated by the loss of Ifnar1. Hence we propose that TLR7 and IFN-I work in series or alternatively in a positive feedback loop: either TLR7 signals are enhanced in the absence of TLR9, leading to increased production of IFN-I that ultimately promotes disease; or lack of TLR9 directly leads to increased IFN-I secretion, which upregulates expression of TLR7 in one or more target cell types. If both effects were in play, then they would be self-reinforcing; genetic inactivation of either Tlr7 or Ifnar1 breaks this proposed feedback cycle and ameliorates disease. Nonetheless, Ifnar1 deficiency did not absolutely prevent disease in all Tlr9-deficient animals; a fraction of older females in the Ifnar1+/−/Tlr9+/− group did progress to develop glomerulonephritis, dermatitis, or both. It is likely that the delay in disease progression in Ifnar1+/−/ mice can be overcome by alternative pathways, including, for example, pathways downstream of IFN-γ or IL-6 (29, 30).

In keeping with the effects of Ifnar1 in modulating disease exacerbation in the absence of Tlr9, but in contrast with a previous report (19), we found that Ifnar1 by itself did indeed contribute to full expression of renal disease in the MRL.Fas+/− model. In our experience, and in agreement with other reports (23), the age of spontaneous disease onset and the kinetics of disease progression can vary significantly from animal to animal even among genetically identical mice. As a result, the previous report may have examined too few animals to reveal the effects of Ifnar1 deficiency on disease. Moreover, lupus disease expression and autoantibody titers are highly sex dependent in the MRL.Fas+/− model (23), as is also the case in human patients (31); the previous study did not report the sex of the animals examined. Our analysis, which was robust with respect to numbers of animals and considered sex, indeed did show that Ifnar1 is important for mediating renal disease progression in MRL.Fas+/−, consistent with reports of the role of Ifnar1 in other mouse models of SLE (13-16). This is of significance because heretofore the MRL.Fas+/− model has been frequently cited as differing from other murine lupus models with respect to a protective rather than pathogenic role for IFN-I (32, 33); we show in this study that this is not the case.

An unfortunate confounding factor in this study is the existence of two commercially available substrains of MRL.Fas+/− with significantly different disease kinetics. Over time, The Jackson Laboratory’s MRL.Fas+/− colony experienced genetic drift, acquiring as yet unidentified mutation(s) that reduced the severity and increased the age of onset of clinical disease, most likely because of selective pressures in a breeding colony toward animals with reduced disease, and hence increased fertility. All of the genetic alleles examined in this study were backcrossed to the MRL.Fas+/− substrain with slower disease kinetics, MRL.Fas+/−/2J, for at least six generations before intercross. As a result, the kinet-1ics of disease differed substantially from our previous reports on Tlr7-deficient MRL.Fas+/− (5, 6). Most strikingly, although we previously found that Tlr9−/− MRL.Fas+/−/2J mice reached 50% morbidity or mortality by 16.4 wk of age (5), in the experimental cohorts described in this article, we observed few deaths before 24 wk of age in any experimental group. Total Ab and autoantibody titers, spleen and lymph node weights, and extent of renal disease were all reduced in the Ifnar1+/+Tlr9+/+ and Ifnar1+/−/Tlr9−/− groups compared with similar animals in previous experimental cohorts at the same age. Nonetheless, Tlr9−/− MRL.Fas+/−/2J did exhibit more severe renal disease and elevated IgG titers than their Tlr9+/+ littermates, in broad agreement with our previous reports. It is therefore important to emphasize that all comparisons in this article were generated via a “littermate-control” design from progeny of a similar genetic background.

An interesting implication of our findings is that they further emphasize that anti-nucleosome autoantibodies, despite being the clinical diagnostic hallmark of SLE, are actually entirely dispensable for renal disease. Tlr9−/− animals lack anti-nucleosome autoantibodies, but have more severe renal disease, likely because of the presence of other pathogenic specificities including anti-RNA, as well as an increase in class switch to complement-fixing IgG2a and IgG3 isotypes (6). Conversely, Ifnar1−/− mice have comparable titers of anti-nucleosome autoantibodies with the Ifnar1+/+ group, but have relatively little clinical disease. This could be because of the reduction in anti-RNA titers, and/or Ifnar1 may mediate inflammation in situ downstream of or independent of Ab deposition, for example, by promoting T cell activation.

One of the most notable effects of Ifnar1 deficiency was the shift in autoantibody specificities. Whether Ifnar1 acts directly in autoreactive B cells to exert its effects on repertoire or indirectly through other cell types has not yet been determined. One group described a model in which IFN-I affected reactivity to TLR7-dependent autoantigens by upregulating TLR7 itself in B cells, thereby increasing the likelihood that RNA-containing self-Ags that cross-link the autoreactive BCR would also deliver a TLR7-mediated “second signal” to break tolerance (34). Such an effect could explain the differences in anti-RNA titer and cytoplasmic HEp-2 staining that we observed between Ifnar1+/+ and Ifnar1−/− animals, particularly when Tlr9 was absent. However, anti-Sm, which is also a Tlr7-dependent specificity (5, 6), was not affected by the absence of Ifnar1. One explanation for this discrepancy is that these specificities may differ in their ability to receive T cell help; Sm is an RNA-associated protein Ag, whereas anti-RNA may lack a specific protein component and, therefore, could be more strongly dependent on Ifnar1 and Tlr7-dependent signals. Nonetheless, with regard to the interaction between Tlr7 and Ifnar1, these data indicate that not all effects are strictly in series.

In conclusion, in addition to clarifying that, in fact, some components of MRL.Fas+/− lupus disease do have a dependency on IFN-I, our studies add a significant piece to the puzzle in understanding how TLR9 exacerbates lupus in murine models. Lack of TLR9 causes increased IFN-I secretion (5) and in this article we demonstrate its relevance, as loss of IFNAR-I signaling negates many of the disease-enhancing effects of TLR9 deficiency. In this regard, the phenotype of Tlr9−/−/Ifnar1−/− MRL.Fas+/− mice resembles that of Tlr7−/− MRL.Fas+/− mice (6), although (as discussed above with regard to autoantibody repertoire) they are not complete phenocopies. The connections between TLR7 and IFNAR1 signaling in vivo remain to be worked out, issues that will require a combination of tissue-specific deletion, as well as defined signaling experiments. Furthermore, the effects of Tlr7 deletion on exacerbated disease in Tlr9−/− MRL.Fas+/− mice that are nonoverlapping with and therefore independent of Ifnar1 also are still unresolved. Thus, the ongoing work to unravel these highly interrelated pathways, of which this report is but one significant component, continues to be of great interest, with clear implications for understanding disease and therapeutic targeting.

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