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Ifih1 Gene Dose Effect Reveals MDA5-Mediated Chronic Type I IFN Gene Signature, Viral Resistance, and Accelerated Autoimmunity

Steve P. Crampton,* Jonathan A. Deane,† Lionel Feigenbaum,‡ and Silvia Bolland*

Type I IFNs (IFN-I) are normally produced during antiviral responses, yet high levels of chronic IFN-I expression correlate with autoimmune disease. A variety of viral sensors generate IFN-I in their response, but other than TLRs, it is not fully known which pathways are directly involved in the development of spontaneous immune pathologies. To further explore the link between IFN-I induced by viral pathways and autoimmunity, we generated a new transgenic mouse line containing multiple copies of Ifih1, a gene encoding the cytoplasmic dsRNA sensor MDA5 with proven linkage to diabetes and lupus. We show that MDA5 overexpression led to a chronic IFN-I state characterized by resistance to a lethal viral infection through rapid clearance of virus in the absence of a CD8+ or Ab response. Spontaneous MDA5 activation was not sufficient to initiate autoimmune or inflammatory pathology by itself, even though every immune cell population had signs of IFN activation. When combined with the lupus-susceptible background of the FcγR2B deficiency, MDA5 overexpression did accelerate the production of switched autoantibodies, the incidence of glomerulonephritis, and early lethality. Thus, MDA5 transgenic mice provide evidence that chronic elevated levels of IFN-I are not sufficient to initiate autoimmune or inflammation although they might exacerbate an ongoing autoimmune pathology. The Journal of Immunology, 2012, 188: 1451–1459.

Several families of germline-encoded innate immune sensors, including TLRs and RIG-I–like and NOD-like receptors, recognize pathogens within the endosome, cytosol, and extracellular regions of the cell (1). TLRs located in endosomes primarily activate phagocytic cells and APCs, whereas cytoplasmic nucleic acid sensors are designed to recognize RNA from viral replication intermediates in infected cells (2). The RIG-I–like receptor family, consisting of RIG-I, MDA5, and LGP2, are localized in the cytosol of cells throughout the body and recognize small RNA products derived from picorna-, flavi-, and paramyxoviruses (2). Activation of MDA5 and RIG-I leads to induction of the type I IFNs, which have a wide range of effects such as inducing the expression of antiviral genes, upregulating Ag presentation, and shaping the lymphocyte response against the pathogen (3, 4).

Because of the range of effects IFN-I can have on the immune system, it is not surprising that it has been implicated in systemic autoimmune (5, 6). For instance, patients with systemic lupus erythematosus (SLE) display elevated levels of transcripts for IFN-I target genes in their circulation (7). IFN-I–containing serum from lupus patients was shown to induce dendritic cell (DC) maturation of human monocytes (8). Furthermore, high levels of IFN-I in patients are associated with increased IgG antibody titers, whereas lower IFN-I levels are associated with the IgM isotype, suggesting type I IFN may play a role in class switching in SLE (9). A variety of mouse models have demonstrated a positive role for IFN-I in lupus (10–12). However, IFN-I has also been shown to be protective in another model of SLE (13, 14).

The link between IFN-I and spontaneous autoimmune disease in human and mouse experimental settings implies that there are IFN-I–producing pathways capable of spontaneous and chronic activation, which may be active even in the absence of pathogenic triggers. TLR7 is an example of an IFN-I–producing pathway that contributes to autoimmune disease through spontaneous activation (15–17). TLR7 was shown to be necessary and sufficient for the development of autoimmune inflammatory disease in mice. Although it is assumed that the pathogenic effect of TLR7 activation is mainly due to increased production of IFN-I, this claim has not been formally proven. Furthermore, IFN-α/βR deficiency in the lupus-prone FcγR2B yaa mice, which harbor an extra copy of Tlr7, show reduced but not eliminated pathology (11). Thus, it is uncertain whether IFN-I alone can initiate autoimmune inflammatory disease. It is also unclear whether pathways other than TLRs play a role in spontaneous immune activation and whether this activation can lead to the chronic elevated IFN-I gene expression that is detected in autoimmune pathologies.

We have explored the possibility of spontaneous activation of MDA5 as a trigger of inflammatory disease because Ifih1, the gene that codes for MDA5, has been linked to type I diabetes and lupus in humans (18–22). Specifically, a loss-of-function allele is associated with resistance to disease, and susceptibility genotypes are associated with elevated expression, raising the possibility that increased expression or activity of MDA5 could contribute to autoimmune development (18, 20). Levels of Ifih1 are often highly elevated during viral infections and inflammatory diseases (23–

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Abbreviations used in this article: BAC, bacterial artificial chromosome; BM, bone marrow; cDC, conventional dendritic cell; DC, dendritic cell; IFN-I, type I IFN; ISG, IFN-I–stimulated gene; KO, knockout; pDC, plasmacytoid dendritic cell; SLE, systemic lupus erythematosus; Tg, transgenic; VSV, vesicular stomatitis virus; WT, wild-type.

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induces chronic Type I IFN

Materials and Methods

Mice

MDA5 transgenic (Tg) mice were made using a bacterial artificial chromosom(e) (BAC) Tag approach. The BAC clone RP23-3408 containing MDA5 genomic DNA (565 bp) was modified using a BAC recombineering kit (Gene Bridges, Denvers, Germany). The upstream genes gca and Kcnq7 were replaced with a neomycin resistance cassette so that only 565 bp genomic DNA remained in the BAC. The resulting BAC was used to inject C57BL/6 embryos in our Tg facility (National Cancer Institute, Frederick, MD). Founders were screened for the presence of the transgene using the following PCR primers: 5'-AGTTTACAAGTGCAAGCTAAGCG-3' (forward) and 5'-GCCATACCTGAGAAGACCTG-3' (reverse). Other genotyping for the transgene was conducted with these primers or real-time PCR primers (see below). All experiments were conducted using a single founder. Type 1 IFN-α/β receptor 1 knockout (KO) (IFNrb1−/−) and FcRyR2−− mice were obtained from the Taconic National Institute of Allergic and Infectious Diseases colony. All animal experiments were approved by the National Animal Care and Use Committee.

Abs/Flow cytometry

Abs against the following Ags were used for flow cytometric analysis of splenocytes: B220, GL7, FAS, IgG2a, CD13, CD48, CD69, CD45r, CD44, CD11b, Ly6G, Ly6C, GR1, Ly6a/E, ICOS, CD3e, TNF, and CXCR5 from BD Biosciences (San Jose, CA) and CD62L, CD8α, CD11c, CD122, IFN-γ, IL2, KLRL1, PD1, and Fc block (CD16/32) from eBioscience (San Diego, CA). Flow cytometry was conducted using a FACSCalibur or LSR2 (BD Biosciences) and CD62L, CD8ε, IL2, KLRG1, PD1, and Fc block (CD16/32) from eBioscience (San Diego, CA). Flow cytometry was conducted using a FACSCalibur or LSR2 (BD Biosciences). Flow data were analyzed using FlowJo (Tree Star, Ashland, OR). For detection of vesicular stomatitis virus (VSV) Ag-specific CD8+ T cells, splenocytes were stained with a H-2Kb pentamer with the peptide epitope from the nucleoprotein (n52-59) (Proimmune, Oxford, U.K.) (26).

Genotyping and real-time PCR primers

The following primers were used for real-time PCR analysis: IFN regulatory factor 7, 5'-CAGCGGAGTGCTTGTGAGGAC-3' (forward) and 5'-AAATGGTGCTCACCCATGC-3' (reverse); Mx1, 5'-GATCCGACTCTTCCTCAGATGG-3' (forward) and 5'-CATCTGAGTGTGTCTCACCC-3' (reverse); Isg55, 5'-AGCAAGCACGAGACACTC-3' (forward) and 5'-GGAAAGCCGACACCAATC-3' (reverse); Oas2, 5'-CCGGCCCGTGCCAAGTGGAGG-3' (forward) and 5'-CATGAGGACACCCAGGAC-3' (reverse); MDA5, 5'-GTGATGAGGACGCAGCTGTGC-3' (forward) and 5'-ATTCATCGCTGTGCTCAGAGTC-3' (reverse); Tlr7 (TaqMan), 5'-ATGAAGAGCCAGCGTGCAGAAGAG-3' (forward) and 5'-TTCATAAGGTCACATGTGAGG-3' (reverse); and 5'-GGGTCCGTTGTTCTCAACAAAC-3' (reverse). For genotyping IFNrb1−/− mice, four primers were used: IFNRb1 upper (5'-CATCAGTCTGATGCTCGAGTC-3') and IFNRb1 lower (5'-TCTTCTCCCTCTGCTGACAGGA-3') generate a 487-bp fragment from the wild-type allele, and MDA5 upper (5'-AAATGCAGTGCTCCTGAGTTTCTGCG-3') and MDA5 lower (5'-ATTATATACAAAGGGAAGGCGAAGTCG-3') generate a 150-bp fragment from the wild-type (WT) allele and a 1300-bp fragment from the KO allele. For genotyping FcγR2−− mice, three primers were used: FcRε1 forward (5'-AAGCGTTGCTCAGAACGCC-3') and OL 4080 (5'-TTCTCTGCTGCTGCTGACAGGA-3'), and OL 1413 (5'-TCTGCTGCTGCTGCTGACAGGA-3'), which gave approximately 190-bp WT fragment and a ~300-bp KO fragment. For measurement of VSV real-time PCR, see below. For measurement of IFN signature genes by real-time PCR, all whole splenocytes, purified B cells, or CD4-depleted splenocytes were used from multiple mice.

Measurement of blood cell populations

Differential blood counts and blood chemistry were measured at the National Institutes of Health's Department of Laboratory Medicine using standard procedures.

VSV infections

The Indiana strain of VSV was provided by J. Yewdell (National Institute of Allergy and Infectious Diseases/National Institutes of Health). VSV was injected i.v. at 2 × 106 PFU/mouse in 200 µl HBSS. For lethal dose experiments, 2 × 106 PFU/mouse was used.

VSV neutralization assay

To determine the level of VSV neutralizing Abs in VSV-infected mouse serum, a Vero cell neutralization assay was performed. Vero cells were plated the night before in 96-well flat-bottom dishes at 3.3 × 103 cells/well. The next day, 160 PFU VSV (in 100 µl volume) was incubated 1:1 with various dilutions of serum from infected animals (serum dilutions 1:1,280, 1:2,560, 1:5,120, 1:10,240, and 1:50,960) for 1 h at 37°C. This neutralized VSV was then plated on the Vero cells at 100 µl/well and left for 1 h at 37°C. Supernatants were then replaced with 1% methylenediamine diluted in MEM and left overnight at 37°C. The next morning, the cells were fixed in 50% ethanol, 5% parafomaldehyde, and 4.25% NaCl for 30 min at room temperature and stained with 0.5% crystal violet for 1 h at room temperature. Plaques were then counted from dried plates, and anti-VSV serum titers were determined by the plaque dilution required to inhibit 50% of plaque formation compared with VSV without Abs.

ELISAs/Cytokine bead arrays

Total IgG, IgG1, and IgG2a were measured in the sera of mice using an ELISA (Southern Biotechnology Associates, Birmingham, AL). Anti-nuclear Abs were detected in sera using an ELISA (Alpha Diagnostic International, San Antonio, TX). IgG anti-dsDNA Abs were detected using a commercially available anti-dsDNA ELISA kit (Calbiochem, San Diego, CA) and secondary goat anti-mouse IgG-Alkaline Phosphatase Ab (Southern Biotechnology Associates). Circulating IFN-γ, TNF-α, IL-12p70, and MCP-1 were detected using the Mouse Inflammation Kit cyto metric bead array (BD Biosciences).

Measurement of VSV by real-time PCR

The Indiana strain of VSV mRNA was measured in the spleen and liver of infected mice using fluorescent probe-based real-time PCR (27). The following PCR primers used were as follows: 5'-TGAATACAGTACAATTTTGGGAC-3' (forward), 5'-GAGACTTCTCTGTAATGGAG-3' (reverse), and 5'-GTGATGAGGACGCCAGCTGTGC-3' (probe), and 5'-TTCATCCAAAGGTCAGAAGATG-3' (forward) and 5'-GGGTTGCTGTTCTCAACAAAC-3' (reverse). For genotyping IFNrb1−/− mice, four primers were used: IFNRb1 upper (5'-CATCAGTCTGATGCTCGAGTC-3') and IFNRb1 lower (5'-TCTTCTCCCTCTGCTGACAGGA-3') generate a 487-bp fragment from the wild-type allele, and MDA5 upper (5'-AAATGCAGTGCTCCTGAGTTTCTGCG-3') and MDA5 lower (5'-ATTATATACAAAGGGAAGGCGAAGTCG-3') generate a 150-bp fragment from the wild-type (WT) allele and a 1300-bp fragment from the KO allele. For genotyping FcγR2−− mice, three primers were used: FcRε1 forward (5'-AAGCGTTGCTCAGAACGCC-3') and OL 4080 (5'-TTCTCTGCTGCTGCTGACAGGA-3'), and OL 1413 (5'-TCTGCTGCTGCTGCTGACAGGA-3'), which gave approximately 190-bp WT fragment and a ~300-bp KO fragment. For measurement of VSV by real-time PCR, see below. For measurement of IFN signature genes by real-time PCR, all whole splenocytes, purified B cells, or CD4-depleted splenocytes were used from multiple mice.

IFN α, β response PCR Gene Expression Array

Type 1 IFN response genes were measured using a real-time quantitative PCR array (PAMM-016) from SABiosciences (Frederick, MD). RNA was isolated from CD43 spleen B cells from WT, MDA5 Tg, and B6.1 Ha mice. cDNA was synthesized and subjected to QPCR as described in the kit. Raw Ct values were analyzed using the RT2 Profiler PCR Array Data Analysis (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php?target=upload).

Glomerulonephritis scoring

For glomerulonephritis scoring, kidney sections from WT (n = 3) FcγR2−− (n = 6), and MDA5 Tg FcγR2−− (n = 8) mice were stained with H&E. The diameter was measured for multiple Glomeruli, and a threshold for “affected” was determined to be ≥78 µm (this diameter was the greatest diameter for a healthy WT mouse). A score was then generated for each kidney based on the percentage affected glomeruli (0, 0–19% affected; 1, 20–30% affected; 2, 31–50% affected; 3, 51–70% affected; and 4, 100% affected).
Statistics

In all experiments, unless otherwise specified in the figure legend, a one-way ANOVA with Bonferroni post hoc test was performed for multiple groups.

Results

MDA5 Tg mice display a chronic IFN-I signature with minor pathological consequences

We first characterized Tg mice with increased expression of MDA5 (MDA5 Tg), which we generated by insertion of 12 copies of a BAC clone encoding the endogenous murine Ifih1 gene (Fig. 1A). Ifih1 mRNA levels in MDA5 Tg splenocytes were ~25-fold increased over WT controls (Fig. 1A).

Because activation of MDA5 leads to production of IFN-I (2), we next tested for spontaneous IFN-I-induction in MDA5 Tg mice. Indeed, expression of the IFN-I–stimulated genes (ISGs) Ifih1, Mx-1, Oas2, and Isg15 was 5- to 15-fold increased compared with controls. ISG expression in MDA5 Tgs was comparable or even greater than in the highly inflammatory and pathogenic environment of mice with a similar extra copy number of Tlr7 (~8–16 copies) (Fig. 1B) (16).

Despite elevated levels of IFN-I target genes in MDA5 Tg mice, there were surprisingly no pathological abnormalities in the liver, kidney, gut, lymph nodes, pancreas, and thymus as determined by histological analysis. Circulating inflammatory cytokine levels were mostly comparable between MDA5 Tg and controls; only MCP-1 was significantly elevated in MDA5 Tgs (Supplemental Fig. 1A). There was also a small but significant increase in spleen size and blood analysis revealed mild anemia in the form of reduced mean corpuscular volume of erythrocytes in MDA5 Tgs (Supplemental Fig. 1B, data not shown). In contrast to TLR7 overexpressing mice, which die starting at 2 mo of age (16), MDA5 Tg mice exhibited identical morbidity to Tg-negative littermate controls, up to 10 mo of age, despite having a spontaneous IFN signature (data described below).

Evidence of cellular IFN-I activation, yet normal immune cell homeostasis in MDA5 Tgs

Given that IFN-I produced by antiviral pathways has been associated with a number of changes in immune cells (28, 29), we next analyzed the ex vivo phenotype of splenic cell populations in MDA5 Tgs using flow cytometry. The cell surface markers Ly6C and Ly6A/E (or Sca-1) are commonly upregulated in response to IFN-I (30). Indeed, both markers were highly upregulated on most splenocyte populations, including B cells, CD8+ T cells and DCs, whereas only Ly6A/E was enriched on CD4+ T cells (Fig. 2A).

Populations in the bone marrow (BM) were comparable in numbers but also showed signs of IFN-I stimulation in MDA5 Tgs. Ly6C and Ly6A/E were enriched on DCs and Ly6A/E on CD11b+ cells in the BM (Fig. 2A, Supplemental Fig. 1C, left panel). The IFN-I–inducible plasmacytoid dendritic cell (pDC) marker PDCA-1 was also upregulated in multiple myeloid and lymphoid populations in the spleen and BM, albeit at intermediate levels compared with the normally high expression on pDCs (Fig. 2B) (31).

MDA5 Tg spleens had normal numbers of B220+ B cells, CD4+ T cells, and CD8+ T cells. In the B cell compartment, there was a slight, yet nonsignificant increase in IgG1 class-switched MDA5 Tg -B cells (Fig. 2C). There was no additional evidence of spontaneous B cell activation: numbers of germinal center B cells and plasma cells were comparable in WT and MDA5 Tg animals. Likewise, CD4+ T cells did not display signs of an activated phenotype in MDA5 Tgs, with the exception of increased CD69 expression (Fig. 2D). In addition, FOXP3+ regulatory T cell frequency was similar between WT and MDA5 Tg mice (data not shown). Numbers of conventional DCs (cDCs) and granulocytes were comparable between MDA5 Tg and WT (Fig. 2E). Despite the obvious signs of IFN-I production, which is thought to promote cDC maturation, we did not find evidence of cDC activation, as measured by CD40, MHCI-II and CD86 expression on CD11c+CD11b+ cells from MDA5 Tg mice (Supplemental Fig. 1C, right panel). In addition, we did not observe an increase in pDC frequency, defined by CD11c+PDCA1+ cells (Fig. 2E). One splenic cell population that expanded significantly in MDA5 Tg mice was that of monocytes expressing high levels of Ly6C (Fig. 2E). This expansion was observed in the spleen and blood but not in the BM of MDA5 Tgs (Fig. 2F). Overall, this type of cellular activation is characterized by IFN-I–inducible surface markers but is fundamentally different from what we previously observed in the highly inflammatory TLR7 Tg environment (16).

FIGURE 1. MDA5 Tg mice display a chronic IFN-I signature with minor pathological consequences. A, Ifih1 genomic and mRNA levels were measured by real-time PCR on ear DNA or splenic RNA from mice of the indicated genotype. β-Actin was used as standard. B, IFN-I–target gene expression was measured by real-time PCR on RNA from spleens (CD4-depleted or CD43-negative) from mice of the indicated genotype. TLR7 Tg mice contain between 8 and 16 copies of TLR7 genomic DNA (16). Each dot represents an individual mouse. Data are pooled from multiple experiments. For B, a statistical t test was performed. *p < 0.05, **p < 0.01.
To test whether the phenotype observed in MDA5 Tg was driven by chronic IFN-I, we crossed them to mice deficient for the type I IFN receptor (IFNARβ1), which eliminates all IFN-I signaling (32, 33). As expected, the upregulation of Irf7, Mx1, Oas2, and Isg15 mRNA in MDA5 Tg splenocytes was completely reversed to WT levels in MDA5 Tg mice deficient for IFNARβ−/− (Fig. 3A). In

**FIGURE 2.** Evidence of cellular IFN-I activation, yet normal lymphocyte homeostasis in MDA5 Tgs. A, Ly6C and Ly6A/E surface expression was evaluated by flow cytometry on CD8+, B220− CD4+, and CD11c+ cells from WT and MDA5 Tg spleens or BM. B, PDCA-1 surface expression levels were evaluated on B cells and cDCs in the spleen as well as cDCs and monocytes in the BM of WT and MDA5 Tg mice. C–E, Splenocytes from MDA5 Tg and WT littermate controls were gated by flow cytometry using the indicated markers. Each dot represents absolute numbers of cells from an individual mouse. Mice were between 2 and 6 mo of age with little age-dependent differences observed. A statistical t test was performed comparing WT to MDA5 Tg. **p < 0.01. F, Representative dot plots of Ly6C staining on monocytes with a gate showing the frequency of CD11b+Ly6CHi cells. In A, B, and F, representative cytometric plots from at least three mice in each group are shown.

Cellular phenotype in MDA5 Tgs is dependent on type I IFN and is initiated in radio-resistant cells

To test whether the phenotype observed in MDA5 Tg was driven by chronic IFN-I, we crossed them to mice deficient for the type I IFN
congenically marked BM (WT CD45.1+) was transferred into lethally irradiated mice below each graph. And Ly6A/E measured by flow cytometry on splenic cells gated as indicated were measured by real-time PCR. Values are plotted as fold average WT.

**FIGURE 3.** Cellular phenotype in MDA5 Tgs is dependent on type I IFN and is initiated in radioreistant cells. A, mRNA levels of the gene indicated were measured by real-time PCR. Values are plotted as fold average WT. n = 3–5 mice in each group. B, Surface expression of Ly6C and Ly6A/E measured by flow cytometry on splenic cells gated as indicated below each graph. n = 3–5 mice in each group. C, T cell-depleted congenically marked BM (WT CD45.1+) was transferred into lethally irradiated WT or MDA5 Tg CD45.2+ recipients (see schematic). Spleen cells from reconstituted mice were analyzed 3 mo postransfer, gated CD45.1+ (donor-derived cells), and stained with the indicated markers for flow cytometry. Absolute numbers of cells per gate for individual mice are displayed. A statistical t test was performed. BM transfer experiment was conducted twice with similar results. One experiment is depicted. *p < 0.05, **p < 0.01, ***p < 0.001.

addition, mRNA levels for Ifih1 were reduced by 60% in MDA5 Tg IFNRαβ−/− mice, suggesting that an IFN-I–dependent feed-forward pathway drives higher expression of Ifih1. We next looked at the cellular phenotype of MDA5 Tg mice deficient in the IFNRαβ−/−. High expression of Ly6C and Ly6A/E on CD8+ and B220+ cells in MDA5 Tg mice, as well as the expansion of Ly6Chigh monocytes, were completely dependent on the IFN-I response and is initiated in radioreistant cells. Furthermore, Ly6Chigh monocytes derived from WT BM were expanded when they developed in the MDA5 Tg environment. In mixed BM chimera studies, where MDA5 Tg BM was allowed to develop with WT BM, neither WT nor MDA5 Tg CD8+ T cells acquired the Ly6C+Ly6A/E+ phenotype, and CD11b+Ly6C+ cells did not expand (Supplemental Fig. 2). Thus, we conclude that the major cellular source for IFN-I in MDA5 Tg mice is a radiation-resistant population.

**MDA5 Tg mice are resistant to lethal VSV infection**

Because the chronic levels of IFN-I in the MDA5 Tg mice had surprisingly little effect on B cell activation, T cell activation and spontaneous disease development, we questioned whether the IFN-I levels induced by the transgene were physiologically important. Hence, we tested the MDA5 Tgs ability to respond to a virus that is sensitive to the IFN-I response (32). We chose VSV because of its ability to induce IFN-I, a B cell response, and a cytotoxic T cell response (34).

We measured virus titers in the spleens and livers of mice infected with VSV early postinfection before there is an appreciable adaptive immune response (34). MDA5 Tgs had significantly lower levels of VSV mRNA, measured at 32 or 72 h in the spleen or at 72 h postinfection in the liver (Fig. 4A). Thus, the MDA5 Tg environment is not permissive to VSV survival early during the course of infection.

Because MDA5 Tg mice rapidly cleared VSV compared with WT mice, we next asked whether they were resistant to the LD50 of VSV. When we challenged the mice with 2 × 10⁶ PFU of VSV, a dose high enough to kill 50% of WT mice, only 1 of 16 MDA5 Tg mice succumbed to the infection (Fig. 4B).

The possibility still remained that MDA5 Tgs were mounting a stronger VSV-specific CD8 or B cell response against the virus and this accounted for the increased survival. Therefore, we measured the Ag-specific CD8 T cell response against the virus using a pentamer specific for a VSV immunodominant epitope (H-2Kb VSV N52–59 peptide). Although there was a robust expansion of Ag-specific CD8 T cells from WT mice at day 8 postinfection, the MDA5 Tg response was significantly muted (Fig. 4C). To rule out the possibility of a stronger humoral response against the virus in MDA5 Tgs, we measured neutralizing Ab titers. MDA5 Tgs had a significantly lower titer of VSV neutralizing Abs 16 d postinfection compared with WT controls (Fig. 4D). Importantly, although there was still detectable viral neutralizing activity in WT sera 69 d postinfection, there was no detectable neutralizing activity in MDA5 Tg sera, indicating that the B cell response against the virus was not delayed in these animals. Therefore, MDA5 transgene overexpression appears to poise animals to be protected from viral infection, in the absence of priming of B and T lymphocytes normally associated with long-term memory.

**The MDA5 transgene enhances B cell responses in FcγR2B−/− mice**

Because type I IFN has been associated with SLE and single nucleotide polymorphisms in Ifih1 are associated with SLE, we next tested whether Tg expression of MDA5 could exacerbate spontaneous disease in a lupus-prone mouse strain (5, 19). We crossed the MDA5 Tgs to FcγR2B-deficient mice, known to develop spontaneous disease characterized by the presence of anti-nuclear Abs, splenomegaly and glomerulonephritis (35). Because FcγR2B-deficient mice develop lethal disease between 6 and 9 mo of age, we analyzed mice between the ages of 3 and 6 mo old for signs of accelerated disease.

We analyzed B cell subsets in FcγR2B-deficient mice with or without the MDA5 transgene. As shown earlier, the MDA5 Tg alone was not sufficient to drive B cell activation or isotype...
switching (Figs. 2D, 5A). In mice homozygous for the FcγR2B deletion, however, MDA5 Tg expression significantly enhances the number of class-switched B cells (B220 + IgG+) and plasma-blasts (B220 intCD138+) (Fig. 5A). Germinal center B cell and follicular helper CD4+ T cell numbers were comparable in MDA5 Tg FcγR2B2/2 compared with FcγR2B2/2 mice (Fig. 5A, 5B).

We did, however, detect significantly higher ICOS expression on CD4+ cells from these mice (Fig. 5B). In addition, we observed higher levels of Ly6C and Ly6/E on B cells in MDA5 Tg FcγR2B2/2 mice (data not shown). The observed higher number of Ab producing and Th cells correlated with elevated concentration of serum IgG2a in MDA5 Tg FcγR2B2/2 mice (Fig. 5C).

The MDA5 transgene accelerates autoimmunity in FcγR2B−/− mice

We tracked the survival of a cohort of mice heterozygous or homozygous for the FcR mutation that either lacked or contained the MDA5 transgene.

In a 10-mo period, 40% of FcγR2B−/− mice survived, whereas 100% of MDA5 Tg FcγR2B−/− mice succumbed to autoimmunity by 9 mo of age (Fig. 6A, left panel). We did not observe any difference in survival of MDA5 Tg mice that were heterozygous for FcγR2B. A higher fraction of MDA5 Tg FcγR2B−/− mice contained detectable amounts of protein in their urine, indicative of kidney disease (Fig. 6A, right panel). In addition, the MDA5 Tg
accelerated the splenomegaly seen in FcγR2B-deficient mice (Fig. 6B). Serum antinuclear Abs in MDA5 Tg FcγR2B−/− were significantly elevated compared with WT, FcγR2B−/−, or MDA5 Tg littermates (Fig. 6C). The same trend was observed in Abs specific for dsDNA (Supplemental Fig. 3). Glomerulonephritis was apparent in MDA5 Tg FcγR2B−/− mice at an age when FcγR2B−/− mice show no signs of inflammation (Fig. 6D). These data indicate that MDA5 overexpression does not drive autoimmune phenotypes but can serve as a modifier of autoimmunity.

Discussion

MDA5 is a well-established ubiquitous viral dsRNA sensor that induces IFN-I through activation of IPS-1 and IFN regulatory factor 3 (2). The gene that encodes MDA5, Ifih1, has been linked to autoimmune diseases such as lupus and diabetes (18–22), and it seems plausible that aberrant expression or function of the MDA5 pathway could have pathological consequences. Our characterization of mice bearing multiple copies of the endogenous murine Ifih1 gene shows that augmented expression of MDA5 can lead to spontaneous generation of IFN-I with concomitant systemic upregulation of ISGs, but it does not induce detectable inflammatory cytokines or overt pathology. This result contrasts with the prevailing view that high levels of IFN-I are the cause of pathology both in patients and in a number of mouse models of disease (6, 10–12). Instead, our data imply that high levels of IFN-I could aggravate an ongoing inflammatory condition but that factors other than IFN-I are necessary for disease initiation. Given the difference in terms of which IFN signature genes are modulated between the TLR7 and MDA5 Tg animals, it is also possible that the qualitative differences in IFN signature genes determine the disease outcome.

The spontaneous production of IFN-I in MDA5 Tg mice is likely an amplification of a normally occurring low-level activation of this pathway, possibly in a minor cell population that is habitually exposed to small RNAs that could act as MDA5 ligands. The presence of IFN-I in the MDA5 Tg is readily detectable through the upregulation of ISGs in every immune cell population that we have tested. In fact, ISG upregulation must be widely spread in MDA5 Tg mice, because it is effective in preventing VSV replication when infected with lethal levels of the virus. The observed high expression of the Mx1 and Isg15 genes spontaneously induced by MDA5 seems sufficient to explain this preventive effect because
VSV has been reported to be particularly sensitive to high expression of these genes (36, 37). If high levels of ISGs induced by MDA5 overexpression are protective against a lethal viral infection, there must be a downside to explain why high expression of MDA5 and its antiviral target genes are not naturally selected. One negative effect of chronic high levels of IFN-I seems to be the inhibition of RBC production (38). Indeed, we observe a mild anemia in MDA5 Tg that fits the pattern of what has been described as anemia of chronic disease and that somewhat mimics the anemia detected in patients that receive repeated IFN-β treatments (39, 40). Another issue that appears to result from this high level of ISGs is the fact that adaptive immune priming in response to a virus is impaired in MDA5 Tg animals. Because of this, long-term memory from this infection is likewise impaired.

Another downside of augmented MDA5 function and IFN-I levels is that they aggravate an ongoing autoimmune disease. Although autoreactivity is not apparent in MDA5 Tg mice, this same transgene increases severity of disease in the lupus prone FcγR2B-deficient mouse. The type of autoimmune acceleration observed in the MDA5 Tg crossed to FcγR2B−/− is reminiscent of the Y-chromosome linked autoimmune accelerator (Yaa) mouse (41), which does not succumb to autoimmunity on its own. We demonstrated earlier that the presence of an additional copy of the Tlr7 gene was responsible for the autoimmune accelerator effect in Yaa (17). Whereas the health of TLR7 overexpressing animals is compromised when expressed 2-fold, MDA5 Tg mice remain healthy even when levels are 10-fold higher than WT animals. Furthermore, TLR7 activation in pDCs induces IFN-I production, and TLR7 expression itself is IFN inducible (42, 43). We thought it possible that upregulation of TLR7 could explain why the presence of the MDA5 Tg was also accelerating lupus. However, we did not observe a significant upregulation of Tlr7 message in MDA5 Tgs, and the phenotype was unaltered when crossed to Tlr7−null mice (data not shown). In addition, when comparing IFN-I target gene expression in B cells, the MDA5 Tg had a broader and higher magnitude of induced ISGs than Yaa B cells (Supplemental Table I). These data suggest that Tlr7 gene duplication and the MDA5 Tg do not have a similar IFN-I–target gene profile and could be accelerating lupus in FcγR2B−/− mice in distinct ways. It is possible that the TLR7 Tg has B cell-intrinsic activation effects, whereas in the MDA5 Tg, B cells are mostly affected by expression of stromal cell-derived IFN-I. It would be interesting to investigate the qualitatively different contributions...
of other innate immune receptors to type I IFN gene induction and disease acceleration. The main effect of MDA5 Tg in autoimmune prone mice seems to be the increase in isotype-switched Ab-producing cells at early stages of disease. This is consistent with the known effects of IFN-1 on B cell IgG class-switching during a normal ongoing immune response as well as an autoimmune response (44, 45). These data together demonstrate the importance of regulating MDA5 expression for generating an antiviral response, and in the development of autoimmunity, in particular to prevent the IFN enhancement of B cell activation that seems to be at the core of inflammatory disease. The MDA5 Tg represents an excellent system to study the effect of chronic IFN-I in the absence of inflammation.

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Disclosures

The authors have no financial conflicts of interest.

References

SUPPLEMENTAL FIGURE 1
Characterization of the immune phenotype of MDA5 Tg mice. (A) MCP-1 levels elevated in MDA5 Tg mice. Serum cytokines measured using the cytokine bead array from BD Biosciences. (B) Enhanced spleen size in MDA5 Tgs. Spleens were weighed or enumerated for cellularity. (C) Signs of IFN-I exposure in bone marrow CD11b^+ cells and no evidence of DC maturation in MDA5 Tg mice. Ly6A/E levels were measured on bone marrow CD11b^+ cells in WT or MDA5 Tg mice (left). CD11c^+CD11b^+ splenocytes from WT or MDA5 Tg mice were stained for CD40, MHC class II or CD86 and analyzed using flow cytometry (right). For (A) and (B), a statistical T test was performed. ***P<0.001.
Supplementary Data

**SUPPLEMENTAL FIGURE 2**
IFN-I phenotype is not transferred through bone marrow-derived cells. Congenically marked CD45.1+/CD45.2+ bone marrow was mixed with either WT or MDA5 Tg CD45.2+/CD45.2+ bone marrow and transferred into lethally irradiated CD45.2+/CD45.2+ hosts. 2 months later spleens were harvested and CD45.1+ cells were analyzed for the MDA5 Tg phenotype.

**SUPPLEMENTAL FIGURE 3**
Anti-dsDNA antibody titer enhanced in MDA5 Tg FcγR2b−/− mice. anti-dsDNA antibody titers were measured in the sera isolated from 3-5 month old WT, MDA5 Tg, FcγR2b−/− and MDA5 Tg FcγR2b−/− mice. Absorbance plotted has been background-subtracted. Each dot represents an individual mouse.
Supplementary Data

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Expression of IFN-I target genes in purified CD43<sup>+</sup> splenic B cells from MDA5 Tg or B6.Yaa mice. Values are expressed as fold change compared to WT controls.