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Distinct Regulation of Murine Lupus Susceptibility Genes by the IRF5/Blimp-1 Axis

Ravichandran Panchanathan,*,† Hongzhu Liu,* Hongqi Liu,* Chee-Mun Fang,‡ Loren D. Erickson,§ Paula M. Pitha,‡ and Divaker Choubey*,†

Genome-wide association studies have identified lupus susceptibility genes such as *IRF5* and *PRDM1* (encoding for IFN regulatory factor 5 [IRF]5 and Blimp-1) in the human genome. Accordingly, the murine *Irf5* and *Prdm1* genes have been shown to play a role in lupus susceptibility. However, it remains unclear how IRF5 and Blimp-1 (a transcriptional target of IRF5) contribute to lupus susceptibility. Given that the murine lupus susceptibility locus *Nba2* includes the IFN-regulated genes *Ifi202* (encoding for the p202 protein), *Aim2* (encoding for the Aim2 protein), and *Fcgr2b* (encoding for the FcγRIIB receptor), we investigated whether the IRF5/Blimp-1 axis could regulate the expression of these genes. We found that an *Irf5* deficiency in mice decreased the expression of Blimp-1 and reduced the expression of the *Ifi202*. However, the deficiency increased the expression of *Aim2* and *Fcgr2b*. Correspondingly, increased expression of IRF5 in cells increased levels of Blimp-1 and p202 protein. Moreover, Blimp-1 expression increased the expression of *Ifi202*, whereas it reduced the expression of *Aim2*. Interestingly, an *Aim2* deficiency in female mice increased the expression of IRF5. Similarly, the *Fcgr2b*-deficient mice expressed increased levels of IRF5. Moreover, increased expression of IRF5 and Blimp-1 in lupus-prone C57BL/6.*Nba2*, New Zealand Black, and C57BL/6.*Sle123* female mice (as compared with age-matched C57BL/6 female mice) was associated with increased levels of the p202 protein. Taken together, our observations demonstrate that the IRF5/Blimp-1 axis differentially regulates the expression of *Nba2* lupus susceptibility genes, and they suggest an important role for the IRF5/Blimp-1/p202 axis in murine lupus susceptibility. ***The Journal of Immunology*, 2012, 188: 270–278.**

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Genetic studies involving systemic lupus erythematosus (SLE) patients have identified several lupus susceptibility genes, including *IRF5* and *PRDM1* (encoding for the IFN regulatory factor [IRF]5 and Blimp-1 transcriptional regulators) (1, 2). Correspondingly, the murine *Irf5* (3–7) and *Prdm1* (4) genes have been shown to play an important role in the development of lupus disease. However, it remains unclear how IRF5 and Blimp-1 transcription factors contribute to lupus susceptibility.

IRF5 is a member of the IRF family of transcription factors (8, 9). Murine IRF5 is primarily expressed as a full-length transcript in the $B220^+$ mature B cells, and levels of IRF5 decrease in $CD138^+$ plasma cells (10). Moreover, the female hormone estrogen upregulates the expression of the murine *Irf5* gene (11). IRF5 could be activated by both TBK1 and MyD88 to form homodimers, and activated IRF5 induces the transcription of type I IFN genes and the *Prdm1* gene (4, 8). The *Prdm1* gene encodes Blimp-1 protein, a master regulator of the B cell differentiation (12). The $Irf5^{-/-}$ mice, which were generated using embryonic stem cells from the 129sv strain on the mixed (C57BL/6 [B6] × 129) genetic background, show reduced serum levels of type I IFNs and develop an aging-dependent splenomegaly that is associated with an accumulation of $CD19^+B220^-$ B cells (4). Moreover, splenic cells from the $Irf5^{-/-}$ mice exhibit a decrease in the number of plasma cells and downregulation of Blimp-1 expression (4). Notably, murine IRF5 is required for the development of lupus-like disease in the $Fc\gamma RIIB^{-/-}$ Yaa and $Fc\gamma RIIB^{-/-}$ mouse models (3). Additionally, IRF5 is critical for the development of lupus in MRL/*lpr* mice (7). Interestingly, type I IFN receptor subunit 1-deficient $Fc\gamma RIIB^{-/-}$ Yaa mice maintained a substantial level of residual disease (3), thus raising the possibility of the IFN signaling-independent role for the IRF5 in the development of murine lupus disease. Accordingly, a recent study (5) noted that IRF5 contributes to murine SLE-like disease through its direct control of class switch recombination of the γ2a locus in B cells.

The transcriptional activation of the *Prdm1* gene by murine IRF5 is associated with the terminal differentiation of B cells to $CD138^+$ plasma cells (12). Blimp-1–mediated transcriptional repression of certain target genes, such as Pax5 and c-Myc, is required for the terminal differentiation of B cells. However, Blimp-1 induces the expression of XBP-1 in plasma cells (12). The optimal core DNA consensus sequence (GAAAG) that is bound by human and murine Blimp-1 is essentially identical to that bound by the IRF family members (13). Interestingly, Blimp-1 represses the transcription of the *Aim2* gene (14).

IRF5 transcription factor participates in cell type-dependent key signal transduction pathways, such as TLR signaling and type I IFN production (8, 9). These signaling pathways are implicated in the development of SLE (9, 15). The IFN family of cytokines includes type I (IFN-α and IFN-β) and type II (IFN-γ) IFNs (16). The IFNs

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Abbreviations used in this article: B6, C57BL/6; ER, estrogen receptor; IRF, IFN regulatory factor; NZB, New Zealand Black; NZW, New Zealand White; SLE, systemic lupus erythematosus.

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exert multiple biological effects on the immune system by affecting differentiation, proliferation, and survival of immune cells (17, 18). The IFN-inducible genes encode "effector" proteins that mediate the immunomodulatory functions of IFNs (19). Increased serum levels of IFN-α and the "IFN signature" have been reported in SLE patients (20, 21). Accordingly, lupus-prone New Zealand Black (NZB) mice that are deficient in the type I receptor do not develop disease (22). Interestingly, the SLE-associated variant of IRF5 has been linked to higher IFN-α levels in sera of human SLE patients (23).

The telomeric chromosome 1 in the mouse (and its syntenic equivalent 1q21–q44 region in humans) has shown a strong linkage to systemic autoimmunity (24). In the mouse, three loci have been identified in the autoimmunity susceptibility region: New Zealand White (NZW)-derived *Sle1* in the NZM2410 strain (25), and NZB-derived *Lbw7* (26) and *Nba2* (24, 27–32) in (NZB × NZW)F_1 mice. Interestingly, both *Sle1* and *Nba2* intervals contain candidate lupus susceptibility genes, which include members of the *Fcgr* family, members of the *SLAM* family, and members of the *Ifi200* family (28, 30). The *Nba2* locus (~90-97 cM) has been shown to be a major genetic contributor from the NZB strain to lupus susceptibility in the (NZB × NZW)F_1 spontaneous mouse model of SLE (28–32). Accordingly, B6 mice congenic for the *Nba2* interval (congenic mice indicated as B6.*Nba2*-ABC) produce detectable levels of anti-nuclear Abs at ~7 mo age, but they do not develop a kidney disease (28), thus suggesting interactions of the *Nba2* locus with other loci for the development of the kidney disease. Based on the sequence polymorphisms that are identified in the *Nba2* interval genes, it has been proposed that the interval may contain several candidate lupus susceptibility genes (28–32). The candidate genes include the *Slam* family genes (31, 32), the *Fcgr2b* gene (encoding for the inhibitory FcγRIIB receptor) (29), and the IFN-inducible *Ifi202* gene (28, 33, 34). Interestingly, the B6.*Nba2* congenic mice that are deficient in the type I receptor do not develop a lupus-like disease and express reduced levels of the *Ifi202* mRNA (35).

Generation of B6.*Nba2*-ABC subcongenic lines (B6.*Nba2*-A, -A′B, -B, and -C) and their characterization reveal that the B6.*Nba2*-C subcongenic mice, which harbor the *Ifi200* gene cluster, do not develop anti-nuclear Abs and do not produce type I IFN (32). Consistent with these observations, we were able to detect the expression of the p202 protein in the B6.*Nba2*-ABC female mice (age, ~4 mo), but not the parental B6 or B6.*Nba2*-C congenic mice (36). Moreover, genes within the subinterval C (comprising the *Ifi200* family genes) negatively regulate the expression of the *Fcgr2b* gene and inhibit the FcγRIIB-induced apoptosis (32).

The IFN-inducible *Ifi200* genes family includes structurally related mouse genes, including *Ifi202a*, *Ifi202b*, and *Aim2* (33, 34). The 200-gene family region of the mouse chromosome 1 (~6000 kb) is syntenic to a region at the 1q23 on human chromosome 1, which contains four genes (*IFI16*, *IFIX*, *MNDA*, and *AIM2*) (33, 34). Proteins in the p200 family share at least one HIN200 domain. The domain participates in protein–protein interactions as well as detection of cytosolic DNA (37). The Aim2 protein, which contains a pyrin domain, recruits ASC adapter protein to form an Aim2 inflammasome (38, 39). Generation of the *Aim2*-deficient mice revealed that the Aim2 protein negatively regulates type I IFN responses and the expression of the *Ifi202* (possibly both *Ifi202a* and *Ifi202b* genes) lupus susceptibility gene (36, 40). Given that the expression of the *Ifi202* gene is regulated by the female sex hormone estrogen (41) and that p202 protein suppresses the expression of both *Aim2* and *Fcgr2b* genes located within the *Nba2* interval (42), we investigated whether the IRF5/Blimp-1 axis could regulate the expression of the *Nba2* lupus susceptibility genes. We report that the axis differentially regulates the expression of *Nba2* lupus susceptibility genes. These observations suggest that the IRF5/Blimp-1/p202 axis contributes to lupus susceptibility in mice.

Materials and Methods

Mice

Generations of *Irf5*- (4) and *Aim2*-deficient mice (40) on the mixed (129 × B6) genetic background have been described. Wild-type and *Irf5*-deficient mice either 76% (KO-P) or 98% (KO-C) on the B6 genetic background were housed in specific pathogen-free animal facilities of The Johns Hopkins University (Baltimore, MD). The B6.*Nba2* (or B6.*Nba2*-ABC) and B6.*Nba2*-C mice were housed in specific pathogen-free animal facilities at the University of Virginia (Charlottesville, VA). The *Fcgr2b*-deficient mice on the B6 genetic background and the corresponding wild-type mice were purchased from Taconic Farms (Germantown, NY). B6 and NZB male and female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The B6.*Sle123* (B6.NZM*Sle1*/*Sle2*/*Sle3*) female mice (43, 44) (age, ~10 wk) were purchased from The Jackson Laboratory. Mice were housed in specific pathogen-free animal facilities at the University of Cincinnati (Cincinnati, OH). The Institutional Animal Care and Use Committees at the institutions where the mice were housed approved the protocols for mice used in these studies.

Splenocyte isolation, cell culture, and treatments

Total splenocytes were prepared from age- and strain-matched male or female mice as described previously (36). Cells were resuspended in RPMI 1640 medium supplemented with 10% FBS. When indicated, splenic B cells ($B220^+$) or plasma cells ($CD138^+$) were purified using magnetic beads (purification kit purchased from Miltenyi Biotec) by the positive selection of cells. The purified (90–95% pure) cells were used immediately for the experiments. In most cases, unless indicated otherwise, cells from two or more age- and gender-matched mice were pooled to prepare total RNA or protein extracts.

The RAW264.7 murine macrophage cell line was purchased from the American Type Culture Collection, and cells were maintained as suggested by the supplier.

Plasmids and nucleofections

Plasmids to express murine IRF5 (pCMV-mIRF5) (10), Blimp-1 (pCMV-mPrdm1) (13), and p202 (pCMV-202) (41) have been described. RAW264.7 cells (2×10^6) from subconfluent cultures were nucleofected with highly purified (endotoxin-free) plasmid DNA encoding for the indicated protein. As a control, we used pCMV plasmid. For nucleofections, we used the Nucleofector II device (Amaxa Biosystems, Köln, Germany) with kit V and the program D-032. After nucleofections, cells were incubated for 24–30 h before harvesting for total RNA or proteins. Nucleofections resulted in ~60% cell survival after 24 h.

Reporter assays

Promoter reporter assays were performed essentially as described previously (41). In brief, subconfluent cultures of RAW264.7 cells (in a six-well plate) were transfected with the reporter plasmids 202-Luc (1.0 μg) and pRL-TK (0.2 μg) along with either an empty vector pCMV (0.8 μg) or equal amounts of a plasmid that allowed the expression of murine IRF5 (pCMV-mIRF5) or Blimp-1 (pCMV-mBlimp1) using the FuGENE 6 (Roche Applied Science, Indianapolis, IN) transfection reagent as suggested by the supplier. Cells were harvested between 40 and 45 h after transfections, and the firefly and *Renilla* dual luciferase activities were determined.

Isolation of RNA from splenocytes and RT-PCR

Splenocytes ($5–8 \times 10^6$ cells) were used to prepare total RNA using the TRIzol (Invitrogen, Carlsbad, CA) method (36). RNA (0.5–2 μg) was used for RT-PCR reaction using the SuperScript one-step RT-PCR system (from Invitrogen). Semiquantitative regular PCR reaction was performed using a pair of primers specific to the *Irf5* (forward, 5′-AATACCCCACCACCTTTTGA-3′; reverse, 5′-TTGAGATCCGGGTTTGAGAT-3′), *Prdm1* (forward, 5′-TTCTTGTGTGGTATTGTCGGGACTT-3′; reverse, 5′-TTGGGGACACTCTTTGGGTAGAGTT-3′), *Ifi*202 (forward, 5′-GGTCATCTACCAACT CAGAAT-3′; reverse, 5′-CTCTAGGATG CCACTGCTGTTG-3′), *Aim2* (forward, 5′-ACAGTGG CCACGGAGA-3′; reverse, 5′-AGGTGACTTCACTCCACA-3′), *Fcgr2b* (forward, 5′-AAGTCTAGGA AGG-

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ACACTGC-3′; reverse, 5′-ATCCTGGCCTTTCTGGCTTGC-3′), or the murine *Ifnb* (forward, 5′-CTGCGTTCCTGCTGTGCTTCTCCA-3′; reverse, 5′-TTCTCCGTCATCTCCATAGGGATC-3′) genes. The conditions for the regular PCR have been described (36).

To perform quantitative real-time TaqMan PCRs, we used the 7300 real-time PCR system (from Applied Biosystems, Foster City, CA) and the commercially available real-time TaqMan gene expression assays. The PCR cycling program has been described previously (36). The TaqMan assays for the murine *Irf5* (assay Mm00496478_g1), *Prdm1* (assay Mm0118 7285_ml), *Ifi202* (assay Mm0304 8198_m1; the assay allows the detection of both the *Ifi202a* and *Ifi202b* mRNA levels), *Aim2* (assay Mm01295719_m1), *Ifnb* (assay Mm00439552_s1), *Fcgr2b* (assay Mm00438875_m1; the assay allows the detection of mRNA encoding both the B1 and B2 isoforms of the FcγRIIB receptor), the endogenous *Actb* control (catalog no. 4352933E), and β_2-*microglobulin* (assay Mm00437762_m1) were purchased from Applied Biosystems and used as suggested by the supplier.

Immunoblotting

Total cell extracts containing equal amounts of proteins were subjected to immunoblotting as described previously (36). The p202 antiserum, which allows the detection of both p202a and p202b proteins in immunoblotting, has been described (45). Additionally, we also used the polyclonal (catalog no. IMG-6670A from Imgenex, San Diego, CA) or monoclonal (sc-166253; from Santa Cruz Biotechnology, Santa Cruz, CA) anti-p202 Abs to detect the p202 protein. We have described polyclonal Abs against the murine Aim2 protein (36). Abs to murine IRF5 (catalog no. 4950), Blimp-1 (catalog no. 9115), STAT1 (catalog no. 9172), and β-actin (catalog no. 4967) were purchased from Cell Signaling Technology (Danvers, MA). Abs to detect the murine FcγRIIB receptor (sc-28842) were purchased from Santa Cruz Biotechnology.

Statistical analyses

The statistical significance of differences in the measured mean frequencies between the two groups of observations was calculated using the Student two-tailed *t* test. A p value <0.05 was considered significant.

Results

An Irf5 *deficiency in mice decreases expression of Blimp-1 and p202*

Expression of IRF5 is critical for the development of lupus in certain models, including in FcγRIIB$^{-/-}$ Yaa and FcγRIIB$^{-/-}$ mice (3), as well as in MRL/*lpr* mice (7). Because development of lupus-like disease in B6.*Nba2* congenic female mice depends on type I IFN signaling (35), and alterations in the expression of *Ifi202*, *Aim2*, and *Fcgr2b* genes that are located within the *Nba2* autoimmunity locus are associated with the development of lupus-like disease in mice (28, 32, 36, 42), we first investigated whether an *Irf5* deficiency in nonautoimmune mice could regulate the expression of these genes. As shown in Fig. 1, an *Irf5* deficiency in splenic cells isolated from female mice (age, ~12 wk) on the B6 genetic background decreased levels of both Blimp-1 (a transcriptional target of IRF5) and p202 proteins as compared with age-matched B6 females. Given that the increased levels of the p202 protein in immune cells negatively regulates the expression of *Aim2* and *Fcgr2b* genes (36, 42), we also compared the steady-state levels of Aim2 and FcγRIIB proteins. Notably, levels of the Aim2 and FcγRIIB proteins were higher in IRF5-null cells than in the wild-type cells. Consistent with our above observations (Fig. 1*A*), we also noted decreases in steady-state levels of *Prdm1* and *Ifi202* mRNA levels in *Irf5*-deficient splenocytes from female mice as compared with age-matched B6 females in regular PCR (Fig. 1*B*) and quantitative real-time PCR (Fig. 1*C*). Moreover, levels of the *Aim2* and *Fcgr2b* mRNA were higher in *Irf5*-deficient cells than in wild-type cells. Interestingly, we also detected reduced levels of *Ifnb* mRNA in *Irf5*-deficient cells than in wild-type cells. Taken together, these observations indicated that an *Irf*5 gene deficiency in mice decreases the expression of Blimp-1, IFN-β, and p202 proteins, whereas the deficiency increases the levels of Aim2 and FcγRIIB proteins.

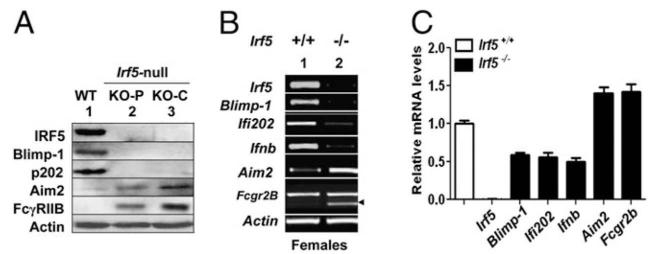

FIGURE 1. An *Irf5* deficiency in mice decreases expression of Blimp-1 and p202 proteins. *A*, Total cell lysates prepared from splenic cells isolated from wild-type (*lane 1*) or *Irf5*-deficient female mice (age, ~12 wk) either 76% (*lane 2*) or 98% (*lane 3*) on the B6 background were analyzed by immunoblotting using Abs specific to the indicated proteins. *B*, Total RNA prepared from splenic cells isolated from wild-type (*lane 1*) or an *Irf5*-deficient female mice (98% on B6 background; *lane* 2) was analyzed by semiquantitative PCR using a pair of primers specific to the indicated genes. *C*, The RNA samples in *B* were also subjected to quantitative real-time PCR using TaqMan assays specific to the indicated genes. The ratio of the mRNA levels for a test gene to β_2-*microglobulin* mRNA was calculated in units (one unit being the ratio of the test gene to β_2-*microglobulin* mRNA). The ratio of mRNA levels in the wild-type mice is indicated as 1. The error bars represent the SD.

IRF5 upregulates the expression of Blimp-1, IFN-β, and p202

To investigate further whether IRF5 stimulates the expression of the *Prdm1*, *Ifnb*, and *Ifi202* genes, we chose to overexpress murine IRF5 in murine macrophage RAW264.7 cells. We chose these cells because they do not express adaptor protein ASC (46), thus allowing expression of a desired gene after nucleofection of a plasmid DNA into cells without the activation of the Aim2 inflammasomes, which can induce cell death upon sensing cytosolic DNA (38, 39). Additionally, these cells express low levels of TLR9, which allows the activation of IRF5 upon sensing DNA that is taken up by cells that survive nucleofection. As shown in Fig. 2*A* and 2*B*, nucleofection of the pCMV-mIRF5 plasmid allowing the expression of IRF5 in cells, but not an empty vector, increased steady-state levels of IRF5 and Blimp-1 mRNA.

Expression of IRF5 and its activation induce the expression of type I IFNs (8, 9). Therefore, our above observation that nucleofection of the pCMV-mIRF5 plasmid into RAW264.7 cells induced the expression of Blimp-1 expression prompted us to test whether IRF5 expression could induce IFN-β expression and the IFN-inducible *Ifi202* gene. As shown in Fig. 2*C* and 2*D*, nucleofection of the plasmid DNA also increased steady-state levels of *Ifnb* and *Ifi202* mRNAs. Moreover, consistent with our earlier observation (36), levels of *Aim2* mRNA were inversely correlated with *Ifi202* mRNA (Fig. 2*E*). Correspondingly, levels of STAT1, p202, and Blimp-1 proteins were higher in cells that were nucleofected with the pCMV-mIRF5 plasmid as compared with an empty vector (Fig. 2*F*). Furthermore, increased expression of IRF5 in promoter reporter assays using RAW264.7 cells stimulated the activity of the 202-luc-reporter plasmid ~6-fold (Fig. 2*G*). Taken together, these observations indicated that overexpression of the murine IRF5 in RAW264.7 cells after nucleofection of plasmid DNA stimulates the expression of Blimp-1, IFN-β, and p202.

Blimp-1 induces expression of Ifi202

Blimp-1 suppresses expression of c-Myc and E2F1 transcription factors (12). Interestingly, both c-Myc and E2F1 negatively regulate the *Ifi202* expression (47, 48). Moreover, Blimp-1 can bind to the GA sequence (bound by the IRFs) that is present in the 5′ regulatory region of the *Ifi202* gene (13, 49). Therefore, our above observations that IRF5 expression can stimulate the expression of

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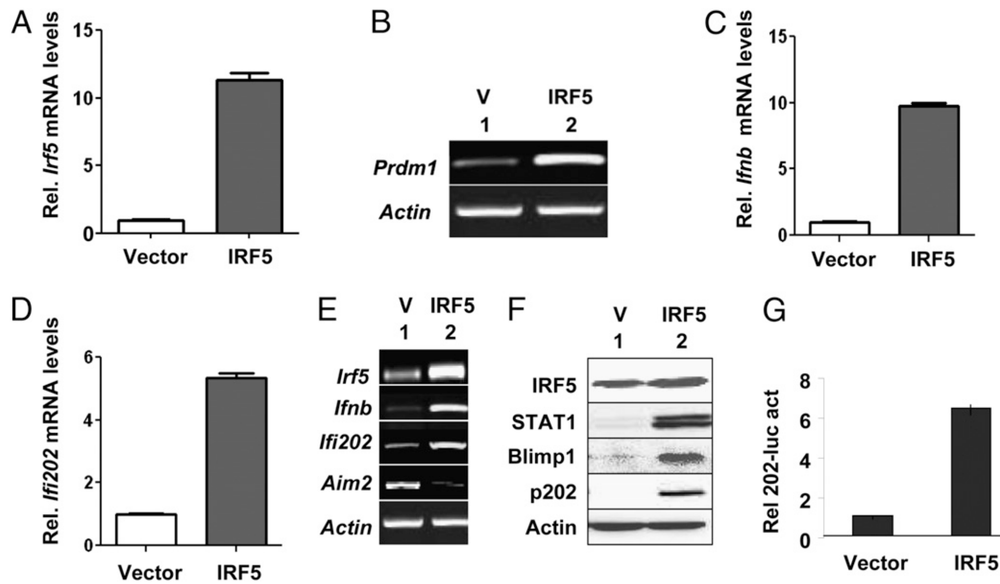

FIGURE 2. IRF5 upregulates the expression of Blimp-1, IFN-β, and p202 proteins. *A*, RAW264.7 murine macrophage cells (2×10^6) were either nucleofected with an empty vector pCMV (2 μg) or equal amounts of pCMV-mIRF5 plasmid. Twenty-four hours after nucleofections, cells were harvested and total RNA was analyzed by quantitative real-time PCR to assess the steady-state levels of IRF5 mRNA. The ratio of the IRF5 mRNA levels to actin mRNA was calculated in units (one unit being the ratio of the test gene to actin mRNA). The ratio of mRNA levels in the vector nucleofected cells is indicated as 1. The error bars represent the SD. *B*, The RNA samples described in *A* were analyzed by regular PCR using a pair of primers specific to the *Prdm1* gene or actin. *C* and *D*, The RNA samples described in *A* were analyzed by quantitative real-time PCR to assess steady-state levels of *Ifnb* (*C*) or *Ifi202* (*D*) mRNA levels. The ratio of mRNA levels in the vector nucleofected cells is indicated as 1. The error bars represent the SD. *E*, The RNA samples described in *A* were also analyzed by regular PCR using a pair of primers specific to the indicated genes. *F*, Actively proliferating RAW264.7 murine macrophage cells (2×10^6) were either nucleofected with an empty vector pCMV (2 μg) or equal amounts of pCMV-mIRF5 plasmid. Twenty-four hours after nucleofections, cells were harvested and total cell lysates were analyzed by immunoblotting using Abs specific to the indicated proteins. *G*, Subconfluent cultures of RAW264.7 cells in a six-well plate were transfected with the reporter plasmids 202-Luc (1.0 μg) and pRL-TK (0.2 μg) along with either an empty vector pCMV (0.8 μg) or equal amounts of a plasmid that allowed the expression of murine IRF5 (pCMV-mIRF5). Cells were harvested between 40 and 45 h after transfections, and the firefly and *Renilla* dual luciferase activities were determined. The normalized relative luciferase activity in the vector transfected cells is indicated as 1. V, empty vector.

Blimp-1 and p202 proteins raised the possibility that Blimp-1 could regulate the expression of *Ifi202* gene. As shown in Fig. 3, nucleofection of pCMV-Prdm1 plasmid (allowing the expression of the murine Blimp-1 protein), but not an empty vector, into RAW264.7 cells increased levels of p202 mRNA (Fig. 3*A*) and protein (Fig. 3*B*). Furthermore, increased expression of Blimp-1 in promoter reporter assays using RAW264.7 cells stimulated the activity of the 202-luc-reporter plasmid ~14-fold (Fig. 3*C*). Given that the p202 protein suppresses the expression of the *Aim2* gene (33, 39) and that Blimp-1 also suppresses the expression of the *Aim2* gene (14), we also explored whether p202 protein could regulate Blimp-1 expression. Interestingly, p202 overexpression in RAW264.7 cells increased levels of Blimp-1 protein (Fig. 3*D*). Taken together, these observations revealed that Blimp-1 and p202 proteins are part of a positive feedback loop.

Gender-dependent upregulation of IRF5 levels in Aim2-*deficient mice*

Expression levels of IRF5 and its nuclear localization are regulated by the female sex hormone estrogen (11). Given that an *Irf5* deficiency in mice increased expression levels of Aim2 protein (Fig. 1), we sought to investigate whether an *Aim2* deficiency could

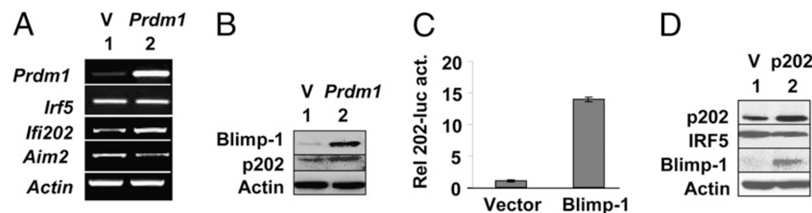

FIGURE 3. Blimp-1 induces expression of the *Ifi202* gene. *A*, RAW264.7 cells (2×10^6) that were proliferating actively were nucleofected with an empty pCMV plasmid (2 μg) or a plasmid (pCMV-mPrdm1) allowing expression of murine Blimp-1 protein. Nucleofected cells were harvested after 24 h, and total RNA was prepared. Samples were analyzed for steady-state levels of mRNA for the indicated genes by regular PCR. *B*, Cells were nucleofected as described in *A*, and 24 h after nucleofections total cells lysates were prepared. Lysates containing equal amounts of proteins were analyzed for levels of indicated protein by immunoblotting. *C*, Subconfluent cultures of RAW264.7 cells in a six-well plate were transfected with the reporter plasmids 202-Luc (1.0 μg) and pRL-TK (0.2 μg) along with either an empty vector pCMV (0.8 μg) or equal amounts of a plasmid that allowed the expression of murine Blimp-1 (pCMV-mBlimp-1). Cells were harvested between 40 and 45 h after transfections, and the firefly and *Renilla* dual luciferase activities were determined. The normalized relative luciferase activity in the vector-transfected cells is indicated as 1. *D*, RAW264.7 cells were nucleofected as described in *A* with either an empty vector or a plasmid (pCMV-202) allowing the expression of p202 protein. Twenty-four hours after nucleofections cells lysates were analyzed by immunoblotting. V, empty vector.

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regulate levels of IRF5 protein. As shown in Fig. 4*A*, levels of IRF5 protein were higher in splenic B220$^+$ B cells from wild-type female mice as compared with age-matched males. Interestingly, levels of the IRF5 protein were higher in the *Aim2* deficient females than the age-matched wild-type females (compare *lane 4* with *lane 3*), and IRF5 expression was not detectable in *Aim2*-deficient males. Consistent with these observations, we detected increased levels of IRF5 mRNA in splenic B220$^+$ B cells from *Aim2*-deficient females as compared with age- and gender-matched wild-type mice (Fig. 4*B*). Correspondingly, we also detected increased levels of mRNA encoding for Blimp-1, IFN-β, and p202 proteins in *Aim2*-deficient B cells (B220$^+$) than in wild-type cells. Taken together, these observations indicated that expression of Aim2 and IRF5 proteins is inversely correlated and that gender-dependent factors regulate the expression of IRF5 in *Aim2*-deficient immune cells.

Fcgr2b *deficiency increases IRF5 levels*

Deficiency of the inhibitory FcγRIIB receptor in immune cells induces type I IFN response and also induces expression of the *Ifi202* gene (42). Therefore, increased levels of FcγRIIB receptor in IRF5-deficient cells (Fig. 1) prompted us to test whether the deficiency of the FcγRIIB receptor could regulate expression of IRF5. As shown in Fig. 5*A*, levels of IRF5 protein were higher in *Fcgr2b*-deficient cells from male and female mice as compared with age- and gender-matched B6 mice. Correspondingly, we also detected higher steady-state levels of IRF5 mRNA in *Fcgr2b*-deficient cells as compared with age- and gender-matched B6 mice (Fig. 5*B*). Taken together, these observations indicated that the lack of FcγRIIB receptor expression in immune cells increases levels of IRF5 mRNA and protein.

Increased expression of IRF5 and Blimp-1 in lupus-susceptible mice is associated with increased expression levels of p202 protein

Lupus-prone preautoimmune (much before the detection of autoantibodies; age, ∼4 mo) B6.*Nba2*-ABC (same as B6.*Nba2*) female mice express increased levels of IFN-β, exhibit activation of IFN signaling, and express increased levels of p202 protein as compared with age-matched B6.*Nba2*-C and B6 non–lupus-prone female mice (36). Therefore, our above observations that both IRF5 and Blimp-1 stimulated the expression of *Ifi202* gene in immune cells and an *Irf5* deficiency decreased p202 protein levels (Fig. 1) encouraged us to investigate whether levels of IRF5 and/or Blimp-1 proteins are higher in the lupus-prone B6.*Nba2*-ABC mice as compared with non–lupus-prone B6.*Nba2*-C and B6 mice.

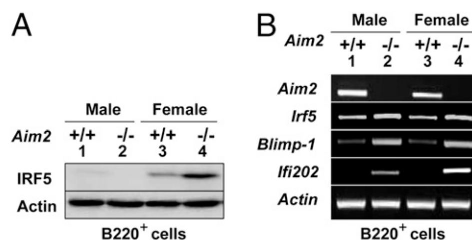

FIGURE 4. Gender-dependent upregulation of IRF5 levels in Aim2-deficient mice. *A*, Total cell lysates prepared from purified splenic B cells (B220$^+$) that were isolated from wild-type (*lanes 1* and *3*) and age-matched *Aim2*-deficient (*lanes 2* and *4*) male or female mice (age, 6–8 wk) were analyzed by immunoblotting using Abs specific to the indicated proteins. *B*, Total RNA prepared from purified splenic B cells (B220$^+$), which were isolated from wild-type (*lanes 1* and *3*) and age-matched *Aim2*-deficient (*lanes 2* and *4*) male or female mice (age, 6–8 wk), were analyzed by regular PCR for mRNA levels.

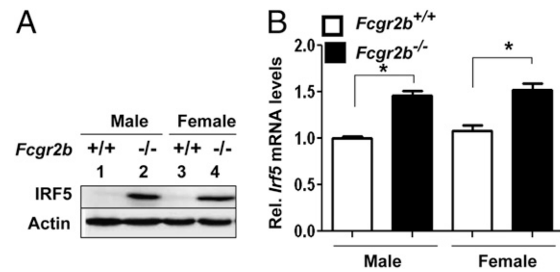

FIGURE 5. *Fcgr2b* deficiency increases IRF5 levels. *A*, Total cell lysates prepared from splenic cells that were isolated from wild-type (*lanes 1* and *3*) and age-matched *Fcgr2b*-deficient (*lanes 2* and *4*) male or female mice (age, ∼9 wk) were analyzed by immunoblotting using Abs specific to the indicated proteins. *B*, Total RNA prepared from splenic cells, which were isolated from wild-type (*lanes 1* and *3*) and age-matched *Fcgr2b*-deficient (*lanes 2* and *4*) male or female mice (age, 6–8 wk), were analyzed by quantitative real-time PCR for steady-state levels of IRF5 mRNA. The ratio of the IRF5 mRNA levels to actin mRNA was calculated in units (one unit being the ratio of the test gene to actin mRNA). The ratio of the mRNA levels in the wild-type male splenic cells is indicated as 1. The error bars represent the SD. $^*p < 0.01$.

As shown in Fig. 6*A* and 6*B*, we detected higher levels of IRF5 and Blimp-1 mRNA in splenic cells from B6.*Nba2*-ABC female mice as compared with the age-matched B6.*Nba2*-C or B6 female mice. Accordingly, we also detected the increased levels of both IRF5 and Blimp-1 proteins in splenic cells from B6.*Nba2*-ABC female mice as compared with the age-matched B6.*Nba2*-C and B6 mice (Fig. 6*C*). Interestingly, B6.*Nba2*-C splenic cells isolated from the female mice had higher levels of IRF5 but not Blimp-1. Because IRF5 expression decreases in plasma cells (4) and Blimp-1 promotes B cell differentiation (12), we also compared levels of IRF5, Blimp-1, and p202 proteins in splenic CD138$^+$ plasma cells. As shown in Fig. 6*D*, we found that levels of IRF5 and p202 proteins were higher in lupus-prone B6.*Nba2*-ABC females than in age-matched non–lupus-prone B6 females. However, levels of Blimp-1 were comparable between the B6 and B6.*Nba2* plasma cells, and levels of Aim2 protein were lower in the B6.*Nba2*-ABC females than in B6 females.

We have noted earlier that expression levels of IRF5, Blimp-1, and p202 are higher in splenic cells from females than from males and that the female sex hormone estrogen through estrogen receptor (ER)α upregulates their expression (11, 41). Therefore, we also compared levels of these proteins in splenic CD138$^+$ plasma cells isolated from B6.*Nba2*-ABC males and age-matched females. As shown in Fig. 6*E*, we could detect the expression of ERα in CD138$^+$ plasma cells and the levels were higher in females than in males. Consistent with our previous observations (11, 41), we detected much higher levels of IRF5, Blimp-1, and p202 in cells from females than males. Interestingly, we also detected much higher levels of STAT1 in females than in males. Taken together, these observations indicated that the gender-dependent increased levels of IRF5 and Blimp-1 proteins in the B6.*Nba2*-ABC female CD138$^+$ plasma cells, as compared with age-matched males, are associated with increased expression levels of p202 protein.

B6.*Sle123* congenic (congenic for the NZM2410 lupus-prone strain-derived *Sle1*, *Sle2*, and *Sle3* loci) mice spontaneously develop lupus-like disease, which is characterized by autoantibody production, lymphosplenomegaly, and glomerulonephritis (44). As noted above, the *Sle1* locus (derived from chromosome 1 from NZW strain) contains the genomic region corresponding to the *Nba2* interval (43, 44). Because splenic cells from NZW females express higher levels of *Ifi202* mRNA than do the age-matched B6 females (28), and the B6.*Sle123* congenic females develop de-

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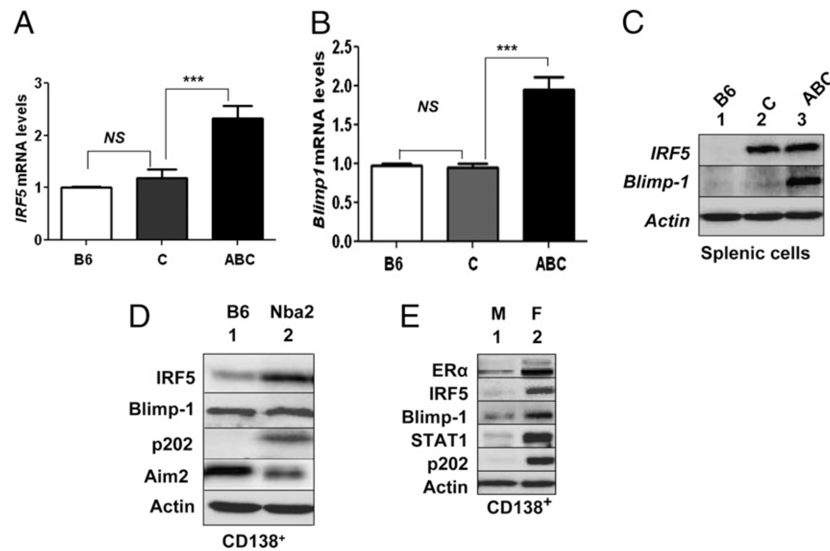

FIGURE 6. Increased expression of IRF5 and Blimp-1 proteins in lupus-susceptible mice is associated with increased expression levels of the p202 protein. *A* and *B*, Total RNA was prepared from splenic cells, which were isolated from B6, B6.*Nba2*-C (C), or B6.*Nba2*-ABC (ABC) females (age, ~4 mo). Steady-state levels of mRNA for IRF5 (*A*) or Blimp-1 (*B*) were analyzed by quantitative real-time PCR. The ratio of the IRF5 mRNA levels to β_2-*microglubulin* mRNA was calculated in units (one unit being the ratio of the test gene to β_2-*microglubulin* mRNA). The ratio of the mRNA levels in the B6 splenic cells is indicated as 1. The error bars represent the SD. ***$p < 0.001$. *C*, Total cell extracts were prepared from splenic cells, which were isolated from B6, B6.*Nba2*-C (C), or B6.*Nba2*-ABC (ABC) females (age, ~4 mo). Steady-state levels of the indicated proteins were analyzed by immunoblotting. *D*, Total cell lysates prepared from purified splenic plasma cells (CD138$^+$) that were isolated from B6 (*lane 1*) or age-matched B6.*Nba2*-ABC (Nba2) female mice (age, ~9 wk) were analyzed by immunoblotting using Abs specific to the indicated proteins. *E*, Total cell lysates prepared from the purified splenic plasma cells (CD138$^+$) that were isolated from B6.*Nba2*-ABC male (M) or female (F) mice (age, ~9 wk) were analyzed by immunoblotting using Abs specific to the indicated proteins.

tectable levels of autoantibodies beginning ~6 mo of age (44), we decided to compare levels of IRF5, Blimp-1, p202, Aim2, and FcγRIIB proteins in splenic cells from age-matched (age, 8–10 wk; much before detection of autoantibodies) B6, B6.*Nba2*, NZB, and B6.*Sle123* females. As shown in Fig. 7*A*, we detected increased levels of IRF5 and Blimp-1 in B6.*Nba2*, NZB, and B6.*Sle123* cells as compared with B6 cells. Similarly, levels of p202 protein were higher in B6.*Nba2*, NZB, and B6.*Sle123* cells than in B6 cells. As expected from our experiments above (Fig. 1), steady-state levels of Aim2 and FcγRIIB proteins in these four strains of female mice were inversely correlated with the levels of IRF5 and p202 proteins. Furthermore, we detected significantly higher steady-state levels of IRF5 mRNA in all lupus-prone strains of female mice (Fig. 7*B*). Accordingly, we also detected increased levels of *Ifnb* and *Ifi202* mRNAs (Fig. 7*C*, 7*D*). Taken together, these observations indicated that increased expression levels of IRF5 and Blimp-1 in lupus-susceptible strains of mice are associated with increased levels of the p202 protein.

Discussion

Genetic studies involving SLE patients have identified *IRF5* and *PRDM1* genes as candidate lupus susceptibility genes (1, 2). Correspondingly, the murine *Irf5* and *Prdm1* genes have been shown to play a role in lupus disease in FcγRIIB$^{-/-}$ Yaa and FcγRIIB$^{-/-}$ mice (3), as well as in MRL/*lpr* mice (7). These studies suggest that the IRF5 transcription factor plays an important role in the development of lupus disease. Because the "IFN signature" in SLE patients is associated with the disease activity (20, 21) and a variant of human IRF5 is linked to increased serum levels of IFN-α in SLE patients (23), we tested whether the IRF5/Blimp-1 axis could regulate the expression of IFN-regulated lupus susceptibility genes within the *Nba2* autoimmunity locus. Our observations revealed that: 1) an *Irf5* gene deficiency in mice reduced the expression of Blimp-1 and p202 proteins, whereas it increased the expression of Aim2 and FcγRIIB proteins (Fig. 1); 2) overexpression of murine IRF5 in RAW264.7 cells upregulated expression of Blimp-1, IFN-β, and

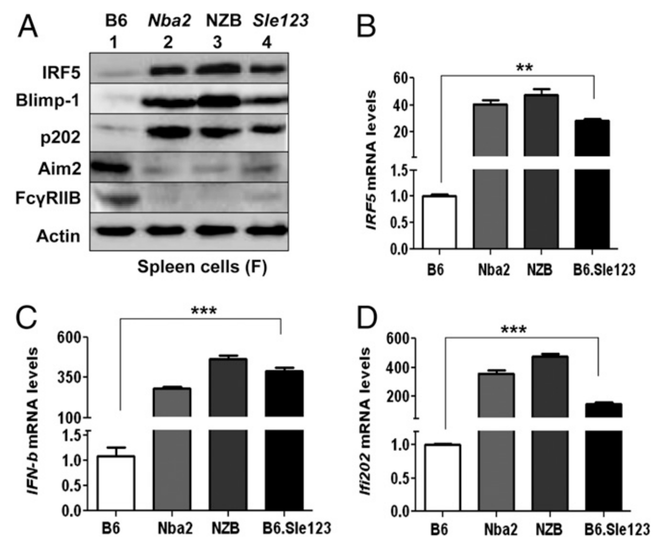

FIGURE 7. Increased expression of IRF5 in lupus-susceptible female mice is associated with increased expression levels of the p202 protein and decreased levels of Aim2 and FcγRIIB. *A*, Total cell lysates prepared from splenic cells that were isolated from B6 (*lane 1*) or age-matched B6.*Nba2*, NZB, and B6.*Sle123* females were analyzed by immunoblotting using Abs specific to the indicated proteins. *B–D*, Total RNA prepared from splenic cells that were isolated from B6 or age-matched B6.*Nba2*, NZB, and B6.*Sle123* female mice was analyzed by quantitative real-time PCR for steady-state levels of *Irf5* (*B*), *Ifnb* (*C*), and *Ifi202* (*D*) mRNAs. The ratio of the indicated mRNA levels to β_2-*microglubulin* mRNA was calculated in units. The ratio of the mRNA levels in the B6 splenic cells is indicated as 1. The error bars represent the SD. **$p < 0.005$, ***$p < 0.001$.

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p202 proteins and also stimulated the activity of the 202-luc-reporter (Fig. 2); 3) overexpression of Blimp-1 protein in RAW264.7 cells induced expression of *Ifi202* gene (Fig. 3); 4) increased levels of p202 protein induced expression of Blimp-1 (Fig. 3); 5) gender-dependent factors increase steady-state levels of IRF5 mRNA and protein in *Aim2*-deficient mice (Fig. 4); 6) deficiency of the inhibitory receptor FcγRIIB in immune cells increases IRF5 mRNA and protein levels (Fig. 5); 7) increased expression of IRF5 and Blimp-1 proteins in lupus-susceptible B6.*Nba2* (Fig. 6) and B6.*Sle123* (Fig. 7) congenic female mice, as compared with age-matched B6 female mice, is associated with increased expression of p202 protein. These observations revealed that the IRF5/Blimp-1 axis differentially regulates the expression of lupus susceptibility genes within the *Nba2* interval and suggest that the axis contributes to lupus susceptibility in part by downregulating the expression of *Aim2* and *Fcgr2b* genes (Fig. 8).

Notably, mice that are deficient in the *Irf5* gene do not exhibit any significant change in the number of $CD4^+$ and $CD8^+$ cells (4). However, in these mice, some increase in the number of B cells ($CD19^+$) was evident with the age: the old mice (∼14 mo) exhibited an expansion of $CD19^+B220^-$ cells presumably due to plasma blasts. Given that we used cells from ∼12-wk-old *Irf5*-deficient female mice (Fig. 1), it is unlikely that changes in the expression of Blimp-1, IFN-β, p202, Aim2, and FcγRIIB proteins that are detected in splenic cells from the *Irf5*-deficient mice (as compared with age-matched wild-type mice) are due to changes in the composition of the lymphoid compartment.

Type I IFN receptor subunit 1-deficient FcγRIIB$^{-/-}$ Yaa mice maintain a substantial level of residual disease (3), thus raising the possibility that IRF5 contributes to lupus susceptibility independent of its role in type I IFN expression and activation of IFN signaling. Accordingly, a recent study (5) has noted that the IRF5 contributes to murine SLE-like disease through its direct control of class switch recombination of the γ2a locus in B cells. Given that increased levels of p202 protein in B6.*Nba2*-ABC lupus-prone female mice are associated with inhibition of the transcriptional activity of p53 (50) and that p53 represses class switch recombination (51), further work is needed to test whether the IRF5-induced p202 protein levels promote the class switch recombination by inhibiting the p53-mediated transcription.

Expression of FcγRIIB receptor is detectable in B cells and plasma cells (both splenic and bone marrow), and the receptor controls persistence and apoptosis of plasma cells in the bone marrow (52, 53). Consequently, mice that are deficient in the FcγRIIB receptor expression exhibit B cell hyperactivity and develop SLE disease spontaneously on certain genetic backgrounds (53). Interestingly, reduced expression of the *Fcgr2b* gene in the B6.*Nba2*-ABC congenic mice is associated with defects in apoptosis of germinal center B cells and plasma cells (32). Moreover, increased levels of p202 protein suppress the expression of the FcγRIIB receptor (42). Therefore, our observations that an IRF5 deficiency in mice decreased levels of both Blimp-1 and p202 proteins but increased levels of the FcγRIIB receptor are consistent with the negative regulation of the *Fcgr2b* gene by the IRF5/Blimp-1/p202 axis.

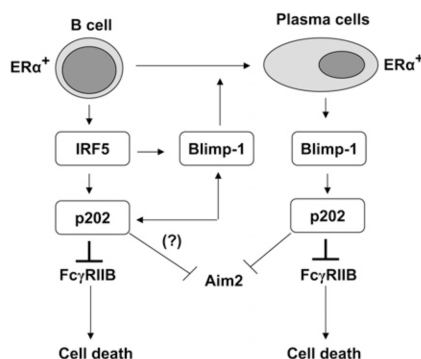

FIGURE 8. Proposed roles of IRF5 and Blimp-1 proteins in differential regulation of the p202, Aim2, and FcγRIIB proteins encoded by the *Nba2* lupus susceptibility genes.

Previous studies indicate that Aim2 protein is not needed for type I IFN expression after sensing cytosolic dsDNA (40). Moreover, *Aim2* deficiency in female mice increased steady-state levels of mRNA encoding for *Ifnb* and *Ifi202* and also stimulated the expression of IFN-inducible genes (36). These observations supported the idea that Aim2 protein suppresses type I IFN responses. Therefore, our observation that the *Aim* deficiency increased levels of IRF5 in female mice, as compared with age-matched wild-type female mice (Fig. 4), provides a potential molecular mechanisms by which the Aim2 protein suppresses the type I IFN responses in immune cells.

Expression levels of p202 and Aim2 mRNA and proteins are inversely correlated in immune cells derived from certain strains of male and female mice (36). Moreover, increased levels of p202 protein in RAW264.7 cells reduced levels of Aim2 protein (42). Given that Blimp-1 is reported to suppress transcription of the *Aim2* gene (14), our observation that increased levels of the p202 protein induce expression of Blimp-1 (Fig. 3) supports the possibility that p202-mediated increased levels of Blimp-1 negatively regulate transcription of the *Aim2* gene. Further work is in progress to test this exciting possibility.

The 5′ regulatory region of the *Ifi202* gene contains a GA box (49), which contains a potential DNA-binding consensus sequence (GAAAG) for the IRF5 and Blimp-1 transcription regulators (14). Therefore, our observations that increased expression of IRF5 or Blimp-1 protein in RAW264.7 cells stimulated the expression of *Ifi202* gene and increased the activity of the 202-luc-reporter in promoter reporter assays (Figs. 2 and 3, respectively) are consistent with the transcriptional regulation of the *Ifi202* gene by these two regulators. Because Blimp-1 negatively regulates expression of c-Myc and E2F1 transcription factors (12), which negatively regulate the expression of *Ifi202* gene (47, 48), our observations do not rule out the possibility that Blimp-1 stimulates the expression of *Ifi202* gene through suppression of c-Myc and E2F1 activity. Therefore, further studies are in progress to investigate the molecular mechanisms.

Expression levels of p202 protein in immune cells are regulated by sex hormones (41): levels are higher in B cells, which express increased levels of ERα, whereas levels are lower in T cells, which express increased levels of androgen receptor. Therefore, our observations that increased levels of ERα in $CD138^+$ plasma cells from female B6.*Nba2* than from age-matched males are associated with increased levels of IRF5, Blimp-1, and p202 proteins are consistent with our previous observations that levels of IRF5, Blimp-1, and p202 proteins are upregulated by the female sex hormone estrogen through ERα (11, 41). Moreover, these observations suggest that the IRF5/Blimp-1/p202 axis contributes to sex bias in lupus disease in mice in part through downregulation of Aim2 and FcγRIIB expression.

In summary, our observations support our model (Fig. 8), which predicts that gender-dependent increased levels of IRF5 in mature B cells and Blimp-1 in plasma cells contribute to lupus susceptibility in part by differentially regulating the expression of *Nba2* lupus susceptibility genes, such as *Ifi202* and *Fcgr2b*. Our observations will serve as a basis to understand the role of the IRF5/Blimp-1/p202 axis in lupus susceptibility in mice.

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Disclosures

The authors have no financial conflicts of interest.

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