The Journal of Immunology

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J Immunol 2010; 185:7385-7393; Prepublished online 5 November 2010;
doi: 10.4049/jimmunol.1002468
http://www.jimmunol.org/content/185/12/7385

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Aim2 Deficiency Stimulates the Expression of IFN-Inducible *Ifi202*, a Lupus Susceptibility Murine Gene within the *Nba2* Autoimmune Susceptibility Locus

Ravichandran Panchanathan,*,† Xin Duan,* Hui Shen,*,† Vijay A. K. Rathinam,‡ Loren D. Erickson,§ Katherine A. Fitzgerald,‡ and Divaker Choubey*,†

Murine Aim2 and p202 proteins (encoded by the *Aim2* and *Ifi202* genes) are members of the IFN-inducible p200 protein family. Both proteins can sense dsDNA in the cytoplasm. However, upon sensing dsDNA, only the Aim2 protein through its pyrin domain can form an inflammasome to activate caspase-1 and induce cell death. Given that the p202 protein has been predicted to inhibit the activation of caspase-1 by the Aim2 protein and that increased levels of the p202 protein in female mice of certain strains are associated with lupus susceptibility, we compared the expression of *Aim2* and *Ifi202* genes between *Aim2*-deficient and age-matched wild-type mice. We found that the *Aim2* deficiency in immune cells stimulated the expression of *Ifi202* gene. The increased levels of the p202 protein in cells were associated with increases in the expression of IFN-β, STAT1, and IFN-inducible genes. Moreover, after knockdown of *Aim2* expression in the murine macrophage cell line J774.A1, IFN-β treatment of cells robustly increased STAT1 protein levels (compared with those of control cells), increased the activating phosphorylation of STAT1 on Tyr-701, and stimulated the activity of an IFN-responsive reporter. Notably, the expression of *Aim2* in non-lupus-prone (C57BL/6 and B6.*Nba2*-C) and lupus-prone (B6.*Nba2*-ABC) splenic cells and in a murine macrophage cell line that overexpressed p202 protein was found to be inversely correlated with *Ifi202*. Collectively, our observations demonstrate an inverse correlation between Aim2 and p202 expressions. We predict that defects in Aim2 expression within immune cells contribute to increased susceptibility to lupus. ***The Journal of Immunology*****, 2010, 185: 7385–7393.**

The IFN-inducible *Ifi200* gene family includes several genes that encode structurally and functionally related proteins (the p200 family proteins) (1–4). The gene family includes the murine (e.g., *Ifi202a*, *Ifi202b*, *Ifi203*, *Ifi204*, *Ifi205*, and *Aim2*) and human (e.g., *IFI16*, *MNDA*, *IFIX*, and *AIM2*) genes. The murine *Ifi200* family genes cluster within the New Zealand Black (NZB)-derived *Nba2* lupus susceptibility interval, which is syntenic to the 1q21-23 region in humans (2, 4, 5). Notably, the identification of the murine *Aim2* gene and its sequence analysis revealed that the Aim2 protein sequence is conserved (55% amino acid identities) between mice and humans (6).

Constitutive expression of the human *AIM2* gene is detectable in the spleen, small intestine, and peripheral leukocytes (6, 7). Furthermore, IFN-γ treatment of the human HL-60 cell line (6) or IFN-β treatment of the human THP-1 cell line (8) increases the *AIM2* mRNA levels. The *AIM2* gene contains a microsatellite instability site that results in the inactivation of the gene in certain human cancers (9). Also, the *AIM2* gene is silenced by DNA methylation (9), and reduced levels of the *AIM2* mRNA have been noted in peripheral BMCs from systemic lupus erythematosus (SLE) patients (10). Like the human AIM2 protein, the expression of the murine Aim2 protein is detectable in splenic cells, thioglycolate-elicited macrophages, and bone marrow-derived macrophages (BMDMs) (11). Moreover, the IFN-β treatment of thioglycolate-elicited macrophages further increased the levels of Aim2 protein (11).

We reported earlier that steady-state levels of the *Ifi202* mRNA and protein (the p202 protein) are higher in splenic cells from 4-mo-old (pre-autoimmune) B6.*Nba2* congenic (congenic for the *Nba2* interval on C57BL/6 genetic background) female mice than those in splenic cells from the age-matched C57BL/6 (B6) females (2, 5). In contrast with *Ifi202*, levels of the *Ifi203* mRNA were lower in the congenic female mice than in the B6 female mice, and the levels of *Ifi204* did not differ measurably (5). Levels of the *Aim2* mRNA were not compared (the *Aim2* probe was not present on the microarray GeneChip). Given that the *Ifi202*, *Ifi203*, and *Ifi204* genes are IFN-inducible genes (1, 2) and the activation of IFN signaling is associated with the development of lupus diseases (12), the increased expression of *Ifi202* gene in the B6.*Nba2* congenic female mice (compared with that in B6 female mice) prompted us and others to investigate further the potential role of p202 protein in lupus susceptibility (2, 4).

The expression of the *Ifi202* gene (possibly both *Ifi202a* and *Ifi202b* genes) is upregulated after treatment of cells with type I or type II IFN (2–4). The upregulation is shown to be through the IFN-responsive *cis*-elements (termed IFN-stimulated response elements [ISRE]) in the promoter region of the *Ifi202* gene (2). We have demonstrated that the promoter polymorphisms, which are predicted to affect the transcription of *Ifi202*, contribute to

*Department of Environmental Health, University of Cincinnati, Cincinnati, OH 45267; †Cincinnati Veterans Affairs Medical Center, Cincinnati, OH 45220; ‡Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605; and §Department of Microbiology, University of Virginia, Charlottesville, VA 22908

Received for publication July 21, 2010. Accepted for publication October 5, 2010.

This work was supported by a National Institutes of Health grant (AI066261) to D.C. A grant from the Lupus Research Institute supported work in the laboratory of L.D.E.

Address correspondence and reprint requests to Divaker Choubey, Department of Environmental Health, University of Cincinnati, 3223 Eden Avenue, P.O. Box 670056, Cincinnati, OH 45267. E-mail address: Divaker.choubey@uc.edu

Abbreviations used in this paper: ASC, apoptosis-associated speck-like protein containing a caspase-activating recruitment domain; BMC, bone marrow cell; BMDM, bone marrow-derived macrophage; Cyt, cytoplasmic; FC, fold change in the p202 protein levels; ISRE, IFN-stimulated response elements; MEF, mouse embryonic fibroblast; *NS*, not significant; Nu, nuclear; NZB, New Zealand Black; NZW, New Zealand White; PYD, pyrin domain; SLE, systemic lupus erythematosus.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1002468

increased constitutive expression of the *Ifi202a* gene in certain lupus-prone strains of mice (4, 5). We have noted that steady-state levels of the *Ifi202* mRNA in splenic cells from non-lupus-prone B6 and New Zealand White (NZW) mice are at least ~10- to 100-fold lower than those in splenic cells from the lupus-prone NZB or B6.*Nba2* congenic mice (5). However, steady-state levels of p202 protein in splenic B cells ($B220^+$) from B6.*Nba2* female mice are only 2- to 3-fold higher than those in splenic B cells from age- and gender-matched B6 mice (13). Notably, the B6.*Nba2* congenic female mice develop detectable levels of autoantibodies beginning ~6 mo of age against the nuclear Ags and exhibit increased serum levels of type I IFNs and the expression of *Ifi202* (5, 14). However, the B6.*Nba2* female mice that are deficient in the IFN-α/βR fail to develop autoantibodies and express reduced (~2-fold less) levels of *Ifi202* mRNA (15). Furthermore, ~7-mo-old B6.*Nba2-C* subcongenic female mice do not develop antinuclear Abs and do not exhibit increased serum levels of type I IFNs (14).

Basal and induced levels of p202 protein are regulated by transcriptional and posttranscriptional mechanisms (2). Agents besides the IFNs that are known to induce the expression of *Ifi202* gene include IL-6 (16) and the female hormone estrogen (17). Notably, basal levels of the p202 protein are detected primarily in the cytoplasm of splenic B cells (13) and mouse embryonic fibroblasts (MEFs) (18) from the B6.*Nba2* congenic mice. Moreover, IFN-α treatment of cells potentiates the nuclear accumulation of p202 protein (13, 18). Consistent with the above observations, the generation of congenic NZB mice that were deficient in the α-chain of IFN-α/βR revealed that the receptor deficiency did not affect the basal levels of p202 protein (19). However, the IFN receptor deficiency ameliorated the symptoms of lupus diseases (19).

Both Aim2 and p202 proteins can form homodimers and heterodimers (6, 20) and recognize dsDNA in the cytoplasm through oligosaccharide/oligonucleotide-binding fold located within the 200-amino-acid repeat (also called the HIN-200 domain) (6). The Aim2 protein contains a homotypic protein–protein interaction pyrin domain (PYD) in the N-terminus (6). After sensing dsDNA in the cytoplasm, the PYD of the human AIM2 and murine Aim2 proteins interacts with an adaptor protein apoptosis-associated speck-like protein containing a caspase-activating recruitment domain (ASC) and forms an inflammasome (6, 8, 21–23). The AIM2/Aim2 inflammasome activates caspase-1 (resulting in the generation of p20 and p10 fragments), which processes the pro–IL-1β and pro–IL-18 for release and induces cell death by pyroptosis (caspase-1–dependent cell death) (6, 23).

In contrast with the Aim2 protein, the p202 protein lacks the PYD (6, 24). Therefore, p202 protein, upon sensing dsDNA in the cytoplasm, cannot form an inflammasome (6). Notably, the knockdown approach has identified p202 protein as an inhibitor of cytosolic DNA-induced caspase-1 (and caspase-3) activation (24). Accordingly, the caspase-1 activation in macrophages in response to dsDNA correlated inversely with the levels of the p202 protein in three strains of mice (24). Thus, it has been predicted that the Aim2 protein promotes and the p202 protein represses the activation of caspase-1 in response to cytoplasmic DNA (24, 25).

Recent studies (11, 26, 27) involving the generation of *Aim2*-deficient mice revealed that *Aim2*-deficient mice are more susceptible to certain bacterial (e.g., *Francisella tularensis* and *Listeria monocytogenes*) and viral (e.g., murine CMV) infections despite increased serum levels of IFN-β. Moreover, immune cells (splenic cells and BMDMs) from the *Aim2*-deficient mice were defective in the activation of caspase-1, secretion of IL-1β and IL-18, and induction of cell death upon infection by certain intracellular pathogens (11, 26, 27). Additionally, these studies indicated that Aim2 expression is not needed for type I IFN production after mice are infected with certain pathogens or when cells are transfected with dsDNA (11, 25–27). Instead, the Aim2 protein appears to act negatively toward regulating the IFN responses (11, 25–27).

Given that the p202 protein cannot form an inflammasome upon sensing dsDNA in the cytoplasm and that increased levels of p202 in female mice of certain strains are associated with increased lupus susceptibility (4, 6), we compared the expression of *Aim2* and *Ifi202* genes between *Aim2*-deficient and age-matched wild-type mice. In this study, we report that *Aim2* deficiency in immune cells stimulates the expression of IFN-inducible *Ifi202* gene.

Materials and Methods

Mice

Generation of *Aim2*-deficient mice on the mixed (129xB6) genetic background has been described (11). Splenic and bone marrow cells were isolated from wild-type or age-matched *Aim2*-deficient male and female mice (age ~4–6 wk) that were housed in pathogen-free animal facilities at the University of Massachusetts (Worcester, MA). Similarly, total splenic cells were isolated from ~4-mo-old B6, B6.*Nba2*-ABC (same as B6.*Nba2*), and B6.*Nba2*-C subcongenic male and age-matched female mice (both B6-*Nba2*-ABC and B6.*Nba2*-C mice were preautoimmune [14]) that were housed in pathogen-free animal facilities at the University of Virginia (Charlottesville, VA). Age-matched male and female non-autoimmune B6 and preautoimmune young (age ~6–8 wk) B6.*Nba2*, NZB, and (NZB × NZW)F_1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in pathogen-free animal facilities at the University of Cincinnati (Cincinnati, OH). The institutional animal care and use committees at the institutions where mice were housed approved the protocol to use mice for studies described in this article.

Splenocytes and bone marrow cells isolation, cell culture, and treatments

Total single-cell splenocytes were prepared from male or age-matched female mice as described previously (17). After the lysis of RBCs, splenocytes were resuspended in RPMI 1640 medium supplemented with 10% FBS. Bone marrow cells (BMCs) were isolated as described previously (28). Unless otherwise indicated, splenic or bone marrow cells from two or more age-matched male or female mice were pooled to prepare total RNA or protein extracts. BMDMs were purified using magnetic beads (purification kit purchased from Miltenyi Biotech, Auburn, CA) allowing the positive selection of $CD11b^+$ cells. The purified (>90–95% pure) BMDMs were cultured in RPMI 1640 supplemented with 10% FBS and GM-CSF (10 ng/ml) for treatments.

Murine macrophage J774.A1 and RAW264.7 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained as suggested by the supplier. When indicated, subconfluent cultures of cells were treated with the murine IFN-β (1000 U/ml; PBL Biomedical Laboratories, Piscataway, NJ) for the indicated times. MEFs that were isolated from wild-type or *Aim2*-deficient embryos were cultured in DMEM (high glucose) cell culture medium (supplemented with 10% FBS and antibiotics).

To overexpress p202 protein in RAW264.7 cells, subconfluent cultures of cells (in a 6-well plate) were transfected with an empty vector (pCMV) or p202 expression plasmid (pCMV-202) using FuGENE 6 transfection reagent (Roche Applied, Indianapolis, IN) as suggested by the supplier. Twenty-four hours after transfections, cells were split, and the transfected cells were selected in G418 (400 μg/ml) for ~10 d. The G418-resistant colonies (>200 colonies) were pooled, and cell cultures were maintained without G418 in the medium for several days before performing experiments that are described in this article.

To knock down *Aim2* or *Ifi202* expression in J774.A1 cells, subconfluent cultures of cells (in a 6-well plate) were infected with lentivirus (purchased from Santa Cruz Biotechnology, Santa Cruz, CA) encoding short hairpin RNA (shRNA) to either *Aim2* (sc-140968-V) or *Ifi202* (sc-40698-V) genes. As a control, cells were infected with the lentivirus encoding a control shRNA (sc-108080). Twenty-four hours after infections, cells were selected in puromycin (1 μg/ml) for at least 5 d. Puromycin-resistant cells were pooled, and cell cultures were maintained without puromycin in the medium for 3 d before experiments.

Reporter assays

For reporter assays, subconfluent cultures of J774.A1 cells (in a 6-well plate) were transfected with the reporter plasmid ISRE-luc (purchased from

Clontech, Mountain View, CA; 2.5 μg) and pRL-TK (purchased from Promega, Madison, WI; 0.5 μg), using FuGENE 6 (Roche Applied), as suggested by the supplier. When indicated, cells were either left untreated (control) or treated with the murine IFN-β (1,000 U/ml for 15 h). After transfections, cells were harvested between 40 and 45 h. Cells were lysed, and the firefly and *Renilla* dual luciferase activities were determined as described previously (17).

Isolation of RNA from splenocytes, BMCs, and RT-PCR

Total splenocytes or BMCs (5×10^6 to 8×10^6 cells) were used to prepare RNA using TRIzol (Invitrogen, Carlsbad, CA) method (17). Total RNA preparation was digested with DNase I (to remove the contaminating genomic DNA) and 0.5–2 μg RNA was used for RT-PCR reaction using the Superscript one-step RT-PCR system (Invitrogen). PCR reactions were performed using a pair of the *Ifi*202 (primers: forward, 5′-GGTCATCTACCAACTCAGAAT-3′; reverse, 5′-CTCTAGGATG CCACTGCTGTTG-3′), *Ifi203* (primers: forward, 5′-GATGGCTGAATACAAGAATATTG-3′; reverse, 5′-TCAGAAGTATGGTTCCAGAGA-3′), or the *Aim2* (primers: forward, 5′-ACAGTGGCCACGGAGA-3′; reverse, 5′-AGGTGACTTCACTCCACA-3′) gene-specific primers. The conditions for the regular PCR have been described previously (17).

Quantitative real-time TaqMan PCRs were performed using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) and the commercially available real-time TaqMan gene expression assays. The PCR cycling program consisted of denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 s, followed by annealing and elongation at 60°C for 1 min. The TaqMan assays for *Ifi202* (Assay ID No. Mm0304 8198_m1; the assay allowing the detection of both *Ifi202a* and *Ifi202b* mRNA levels), *Aim2* (Assay ID No. Mm01295719_m1), *Ifnb* (Assay ID No. Mm00439552_s1), *Rsad2* (Assay ID No. Mm00491265_m1), *Syn25* (Assay ID No. Mm00836412_m1), and the endogenous *Actb* control (Cat. No. 4352933E) were purchased from Applied Biosystems and used as suggested by the supplier.

Immunoblotting and cell fractionation

As described previously (17), total cell lysates from splenocytes or J774.A1 cells were prepared in a modified radioimmune precipitation assay lysis buffer, and the lysates containing equal amounts of protein were processed for immunoblotting. The p202 antiserum, which detects both p202a and p202b proteins in immunoblotting, has been described previously as well (29). To detect p202 protein in extracts from J774.A1 cells, we also used monoclonal Abs to p202 (sc-166253) from Santa Cruz Biotechnology. Abs to detect mouse Aim2 protein were raised in rabbits using a C-terminal peptide (KVIKAAKPKTDMKSVE). The specificity of the Abs was confirmed through immunoblotting that used bacterially expressed murine recombinant Aim2 protein. Abs to STAT1 (No. 9172), p-STAT1$^{Tyr\text{-}701}$ (No. 9171), and β-actin (No. 4967) were purchased from Cell Signaling Technology (Danvers, MA). Abs to viperin (sc-102099) and ASC (sc-22514) were purchased from Santa Cruz Biotechnology. Abs (AHZ0082) to detect caspase-1 and cleaved caspase-1 were purchased from Invitrogen.

Wild-type or *Aim2*-deficient MEFs were lysed using a detergent lysis procedure to prepare cytoplasmic and nuclear fractions as described previously (29). Fractions containing equal amounts of proteins were analyzed by immunoblotting.

Statistical analyses

The measurement values are presented as means ± SEM. The statistical significance of differences in the measured mean frequencies between the two groups was calculated using the Student two-tailed *t* test. A p value <0.05 was considered significant.

Results

Aim2 deficiency stimulates Ifi202 expression

The generation of *Aim2*-deficient mice indicated that the Aim2 protein negatively regulates type I IFN responses (11, 25–27). Therefore, we compared steady-state levels of the IFN-inducible *Ifi202* mRNA and protein between wild-type and age-matched *Aim2*-null mice at an early age (4–6 wk). Because the expression of the *Ifi202* gene is gender dependent (17), consequently we included both male and age-matched female mice in our studies. As shown in Fig. 1*A*, *Aim2*-null splenic cells had higher basal levels of STAT1, p-STAT1, viperin (an IFN-inducible protein) (11), and p202 compared with those of the wild-type cells. Consistent with our previous observations (17), the p202 protein levels were higher in females than in the age-matched males. Correspondingly, we noted increased steady-sate levels of the *Ifi202* mRNA in *Aim2*-null splenic and bone marrow-derived cells in the female mice compared with those in the male mice (Fig. 1*B–D*). Moreover, consistent with our above observations, *Aim2*-deficient MEFs had higher levels of *Ifi202* mRNA (Fig. 1*E*) and protein (Fig. 1*F*) compared with those of the wild-type MEFs, and the p202 protein was detected in cytoplasmic as well as nuclear fractions (Fig. 1*F*). Furthermore, consistent with our previous observations (5), levels of *Ifi203* mRNA were inversely correlated with the *Ifi202* mRNA (Fig. 1*G*). Notably, no appreciable difference in the levels of the *Ifi203* mRNA was evident between males and females. Collectively, our above observations indicated that *Aim2* deficiency in splenic cells, BMCs, and MEFs results in increased levels of *Ifi202* mRNA and protein.

Aim2 expression is inversely correlated with the expression of IFN-β and the IFN-inducible genes

As noted earlier (Fig. 1*A*), our observations revealed that the *Aim2*-deficient splenic cells showed constitutive activation of IFN signaling, as determined by increased levels of STAT1, p-STAT1, and IFN-inducible proteins (such as viperin and p202). Therefore, we compared steady-state levels of IFN-β mRNA between the wild-type and *Aim2*-null splenic cells. As shown in Fig. 2*A*, steady-state levels of IFN-β (but not IFN-γ; data not shown) mRNA were higher in splenic cells from both male and female *Aim2*-deficient mice (in females more than males) compared with those in splenic cells from the wild-type mice. Similarly, levels of the IFN-inducible viperin protein encoding mRNA (encoded by the *Rsad2* gene) (Fig. 2*B*) were significantly higher in *Aim2*-null cells than those in the wild type cells. Notably, levels of 2′-5′ synthetase mRNA were higher in *Aim2*-null male mice compared with those in the wild-type mice (Fig. 2*C*). However, no increase in mRNA levels was evident in the *Aim2*-null female mice. Although it is not known whether gender-dependent factors regulate the expression of 2′-5′ synthetase gene, our above observations warrant further investigation. Collectively, the above observations indicated that the *Aim2* expression in splenic cells is inversely correlated with the IFN-β expression, the activation of IFN signaling, and increased expression of certain IFN-inducible genes, including *Ifi202*.

Knockdown of Aim2 expression potentiates the activating phosphorylation of STAT1 and the expression of IFN-inducible proteins

Our earlier observations that *Aim2*-deficient cells exhibit increased levels of IFN-β mRNA and p202 protein prompted us to investigate the molecular mechanisms by which the Aim2 protein suppresses the expression of IFN-inducible genes. For this purpose, we knocked down the expression of the *Aim2* gene in the murine macrophage cell line J774.A1. We chose these cells because they express detectable basal levels of Aim2 protein and the levels increased further (~2-fold) by type I or type II IFN treatment of cells (Fig. 3*A*). As shown in Fig. 3*B* and 3*C*, stable infection of cells with lentivirus encoding shRNA to *Aim2* gene efficiently reduced basal levels of Aim2 protein and mRNA compared with those in cells infected with control shRNA. Unexpectedly, treatment of cells with IFN-β induced levels of the Aim2 protein (Fig. 3*B*) as well as mRNA (Fig. 3*C*) in *Aim2* knocked down cells. These observations are consistent with the possibility that the type I IFN treatment of J774.A1 cells, by inhibiting the expression of Dicer (RNase III enzyme) (30), interferes with the knockdown of the *Aim2* gene by small interfering RNA approach. Notably, the IFN-treatment resulted in the robust activating phos-

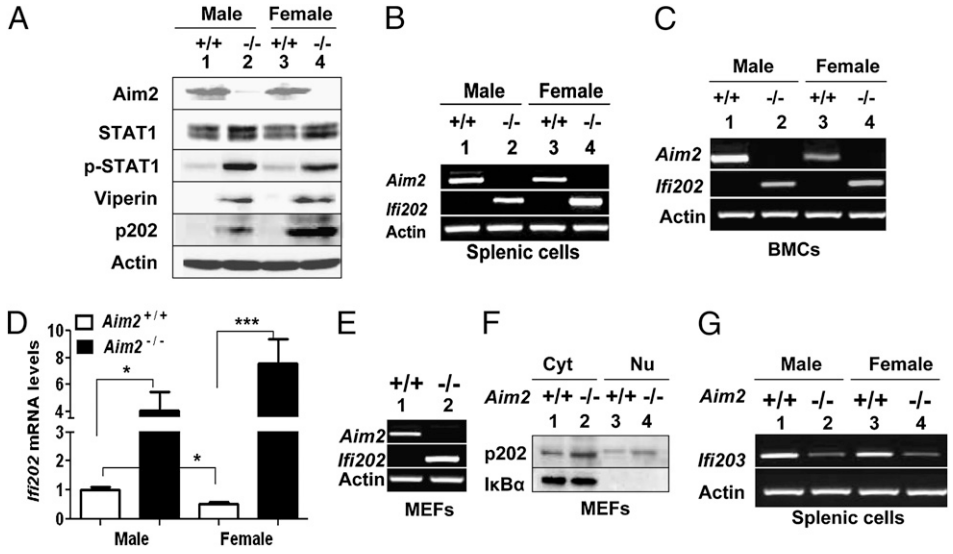

FIGURE 1. *Aim2* deficiency stimulates *Ifi202* expression. *A*, Total cell lysates were prepared from splenocytes isolated from wild-type (*lanes 1* and *3*) and age-matched *Aim2*-deficient (*lanes 2* and *4*) male (*lanes 1* and *2*) or female (*lanes 3* and *4*) mice (age ~4 wk). Extracts containing equal amounts of proteins were analyzed by immunoblotting using Abs specific to the indicated proteins. *B*, Total RNA was prepared from splenocytes isolated from wild-type and age-matched *Aim2*-deficient male or female mice (age ~5 wk). Steady-state levels of *Aim2* and *Ifi202* mRNA were analyzed by semiquantitative PCR using a pair of primers specific to the indicated gene. *C*, Total RNA was prepared from BMCs isolated from wild-type and age-matched *Aim2*-deficient male or female mice (age ~5 wk). Steady-state levels of *Aim2* and *Ifi202* mRNA were analyzed by semiquantitative PCR as described in *B*. *D*, Total RNA isolated from BMCs from wild-type and age-matched *Aim2*-deficient male or female mice (age ~5 wk) was analyzed by quantitative TaqMan real-time PCR using the assay specific to the *Ifi202* gene. The ratio of the 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 relative steady-state levels of *Aim2* or *Ifi202* mRNA in male wild-type mice are indicated as 1. Results are mean values of triplicate experiments, and error bars represent SD. $*p < 0.05$; $***p < 0.001$. *E*, Total RNA was prepared from MEFs isolated from wild-type and *Aim2*-deficient embryo. Steady-state levels of *Aim2* and *Ifi202* mRNA were analyzed by semiquantitative PCR using a pair of primers specific to the indicated gene. *F*, MEFs isolated from wild-type and *Aim2*-deficient embryos were fractionated into the cytoplasmic (Cyt) and nuclear (Nu) fractions, and fractions containing equal amounts of proteins were analyzed by immunoblotting using specific Abs to p202 protein and the cytoplasmic IκBα protein, which served as a quality control for the fractionations. *G*, Total RNA, which was isolated as described in *B*, was analyzed for steady-state levels of the *Ifi203* mRNA by semiquantitative PCR using a pair of specific primers.

phorylation of STAT1 (Tyr-701) and increases in STAT1 and viperin protein levels. Given that the STAT1 expression is autostimulatory (31), these observations revealed that Aim2 expression in J774.A1 cells inhibits a step upstream to the activating phosphorylation of STAT1. Accordingly, the activity of an IFN-responsive reporter (the ISRE-luc) was stimulated ~5-fold more by IFN-β (1000 U/ml) treatment in the Aim2 knocked down cells compared with that in control cells (Fig. 3*D*). Importantly, the knockdown of Aim2 expression in J774.A1 cells also moderately (2- to 2.5-fold) increased basal levels of the *Ifi202* mRNA (Fig. 3*E*) and protein (Fig. 3*F*). This moderate increase in the p202 mRNA and protein levels is consistent with several previous observations (2, 4, 15). Collectively, our above observations revealed that knockdown of *Aim2* expression in J774.A1 cells potentiate the activating phosphorylation of STAT1 and the expression of IFN-inducible proteins, including p202.

Lupus-susceptible mice express reduced levels of the Aim2 protein

Given that the increased expression of p202 in immune cells is associated with increased lupus susceptibility in certain strains of female mice (4), the above observations that *Aim2* deficiency stimulates the expression of p202 protein prompted us to compare steady-state levels of *Aim2* and *Ifi202* mRNAs and proteins in immune cells from non-lupus-prone and lupus-prone mice. As shown in Fig. 4*A* and 4*B*, basal steady-state levels of *Aim2* mRNA

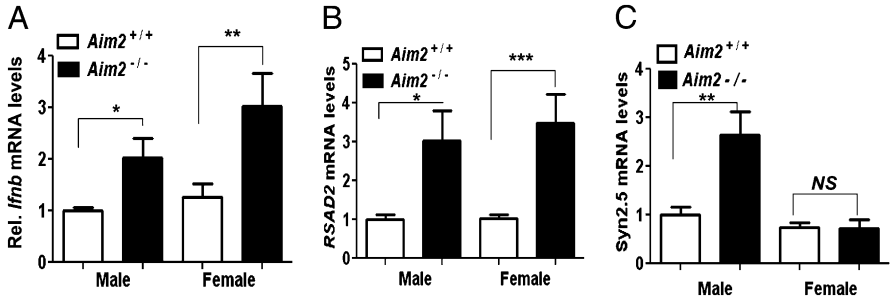

FIGURE 2. *Aim2* expression is inversely correlated with the expression of IFN-β and the IFN-inducible genes. Total RNA isolated from splenocytes from wild-type and age-matched *Aim2*-deficient male or female mice (age ~5 wk) was analyzed by quantitative TaqMan real-time PCR using the assay specific to the *Ifnb* (*A*), *Rsad2* (*B*), or *Syn2,5* (*C*) gene. The ratio of the test gene to β_2-*microglobulin* mRNA was calculated in units (one unit being the ratio of test gene to β_2-*microglobulin* mRNA). The relative levels of the *Ifnb* (*A*), *Rsad2* (*B*), or *Syn2,5* (*C*) mRNA in the wild-type male mice are indicated as 1. Results are mean values of triplicate experiments, and error bars represent SD. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

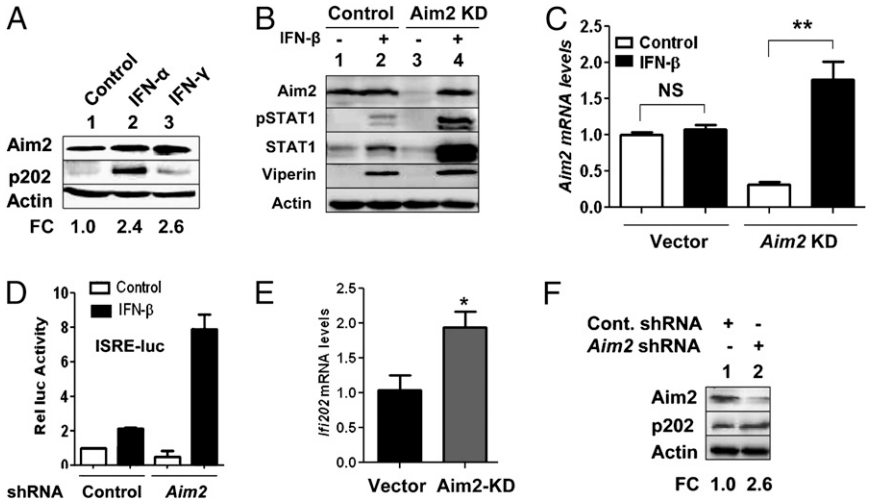

FIGURE 3. Knockdown of *Aim2* expression in J774.A1 cells potentiates the activating phosphorylation of STAT1 and the expression of IFN-inducible proteins. *A*, Subconfluent cultures of J774.A1 cells were either left untreated (control) or treated with IFN-α (1000 U/ml for 14 h) or IFN-γ (10 ng/ml for 14 h). After the treatment, total cell extracts containing equal amounts of proteins were analyzed by immunoblotting using specific Abs to the indicated proteins. *B*, J774.A1 cells were either infected with lentivirus expressing control shRNA (*lanes 1* and *2*) or sh*Aim2* RNA (*lanes 3* and *4*), and cells were selected in puromycin for a week. Puromycin-resistant colonies were pooled and allowed to grow without the drug for 3 d. Total cell lysates prepared from cells either left untreated (control; *lanes 1* and *3*) or treated with IFN-β (*lanes 2* and *4*) for 18 h were analyzed by immunoblotting using Abs specific to the indicated proteins. *C*, J774.A1 cells stably infected with control lentivirus (Vector) or shAim2 lentivirus (Aim2 KD) were either left untreated or treated with IFN-β for 18 h. After the treatment, total RNA was analyzed by quantitative TaqMan real-time PCR using the assay specific to the *Aim2* gene. The ratio of the 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 relative steady-state levels of *Aim2* mRNA in vector-untreated cells are indicated as 1. Results are mean values of triplicate experiments, and error bars represent SD. $^{**}p < 0.005$. *D*, Subconfluent cultures of J774.A1 cells (control or shAim2 RNA), as described in *A*, were transfected with the ISRE-luc reporter plasmid (2.5 μg) along with pRL-TK (0.5 μg) reporter plasmid using FuGENE 6 transfection reagent. Twenty-four hours after transfections, cells were either left untreated or treated with IFN-β for 18 h. Forty to Forty-five hours after transfections, cells were processed for dual luciferase activity. *E*, Total RNA from stably infected with control lentivirus (Vector) or shAim2 lentivirus (Aim2 KD) J774.A1 cells was analyzed by quantitative TaqMan real-time PCR using the assay specific to the *Ifi202* gene. The ratio of the test gene to β_2-*microglobulin* mRNA was calculated in units as described in *C*. The relative steady-state levels of *Ifi202* mRNA in vector cells are indicated as 1. Results are mean values of triplicate experiments, and error bars represent SD. $^{*}p < 0.05$. *F*, Extracts described in *B* were analyzed for levels of the p202 protein by immunoblotting. FC, fold change in the p202 protein levels.

were relatively higher in splenic cells from non-lupus-prone B6 female mice than those in splenic cells from the age-matched NZB, B6.*Nba2* (same as B6.*Nba2*-ABC), and (NZB × NZW)F_1 females. In contrast, steady-state levels of the *Ifi202* mRNA were significantly higher in preautoimmune female mice than those in the age- and gender-matched B6 mice. Because generation of the B6.*Nba2*-C mouse line (14), a subcongenic line of the *Nba2* congenic mouse line (5), indicated that these mice do not produce antinuclear autoantibodies and type I IFNs at the age of ~7 mo (14), our above observations that the expression of *Aim2* is inversely correlated with the expression of *Ifnb* and *Ifi202* genes made it conceivable that B6.*Nba2*-C mice express *Aim2* but not *Ifi202* gene. Therefore, we compared basal levels of the *Aim2*, *Ifi202*, and *Ifnb* mRNA among the B6, B6.*Nba2*-C, and B6.*Nba2*-ABC splenic cells isolated from ~4-mo-old (preautoimmune) females. As shown in Fig. 4*C* and 4*D*, steady-state levels of *Aim2* mRNA were inversely correlated with both *Ifi202* and *Ifnb* mRNA in three strains of mice. Accordingly, the expression of the Aim2 protein was detectable in splenic cells from the B6 or B6.*Nba2*-C, but not B6.*Nba2*-ABC, female mice. Consistent with the post-transcriptional mechanisms regulating the p202 protein levels in a variety of cells (2, 4), we did not detect the p202 protein levels in the B6.*Nba2*-C mice (Fig. 4*E*). Because both B6.*Nba2*-C and B6.*Nba2*-ABC females were preautoimmune (not expected to produce detectable levels of autoantibodies [5, 14]), we decided to compare levels of procaspase-1 and pro–IL-1β among B6, B6.*Nba2*-C, and B6.*Nba2*-ABC splenic cells. As shown in Fig. 4*E*, levels of the procaspase-1 protein were comparable among the three strains of female mice. However, levels of pro–IL-1β protein were significantly higher in the B6 mice compared with those in the B6.*Nba2*-C or B6.*Nba2*-ABC females (Fig. 4*E*). Collectively, these observations indicated that lupus-susceptible B6.*Nba2*-ABC female mice express reduced levels of Aim2 protein in splenic cells before detection of any autoantibodies and that the reduced levels of the Aim2 protein in splenic cells are associated with increased levels of IFN-β mRNA, potentiation of IFN signaling (as measured by the activating phosphorylation of STAT1), and increased steady-state levels of the p202 protein.

Expression levels of p202 protein are inversely correlated with Aim2 protein and the activation of caspase-1

Caspase-1 activation in macrophages, in response to dsDNA, inversely correlated with the levels of the *Ifi202* mRNA in three strains (B6, BALB/c, and NZB) of female mice (24). Given that the expression of p202 depends on gender (17), we compared the activation of caspase-1 in BMDMs between NZB male and age-matched female mice. We chose the NZB mice because basal levels of the p202 protein are readily detectable (5), and these mice generate detectable levels of the anti-DNA Abs at an early age (19). As shown in Fig. 5*A*, the basal levels of p202 protein were several folds higher in BMDMs isolated from NZB females (age ~12 wk) than those in BMDMs isolated from the age-matched males. Notably, basal levels of Aim2 protein were inversely correlated with the p202 protein: detectable in BMDMs

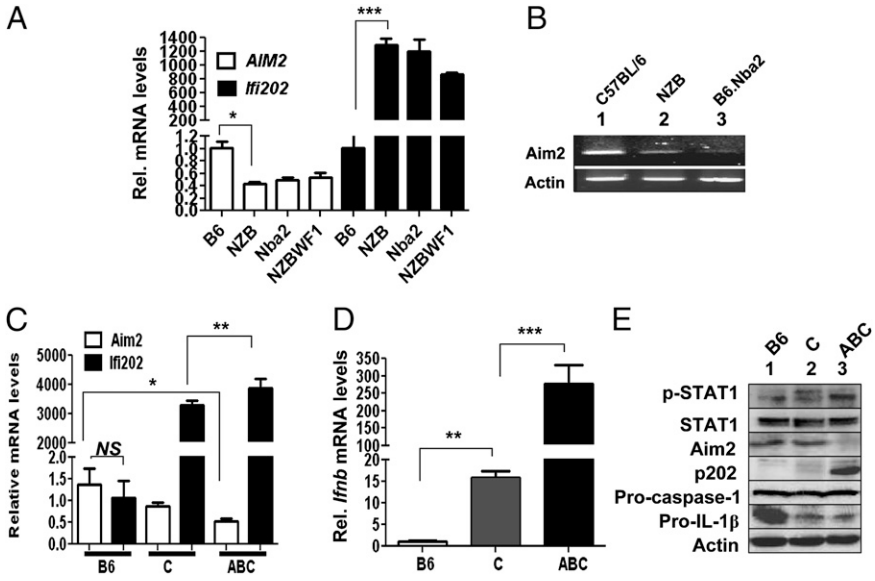

FIGURE 4. Lupus-susceptible mice express reduced levels of the *Aim2* protein. *A*, Total RNA prepared from splenocytes isolated from C57BL/6 (B6), NZB, B6.*Nba2* (Nba2), or (NZB × NZW)F_1 (NZBWF1) female mice (age ∼9 wk) was analyzed by quantitative TaqMan real-time PCR using an assay either specific to the *Aim2* or the *Ifi202* genes. The ratio of the 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 relative levels of *Aim2* or *Ifi202* mRNA in the B6 female mice are indicated as 1. Results are mean values of triplicate experiments, and error bars represent SD. $*p < 0.05$; $***p < 0.001$. *B*, Total RNA isolated in *A* was also analyzed by semiquantitative PCR using a pair of primers specific to the indicated gene. *C*, Total RNA prepared from splenocytes isolated from B6, B6.*Nba2*-C (C), or B6.*Nba2*-ABC (ABC) female mice (age ∼4 mo) was analyzed by quantitative TaqMan real-time PCR using assay specific to either *Aim2* or *Ifi202*. The ratio of the 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 in splenocytes). The relative levels of *Aim2* or *Ifi202* mRNA are indicated. Results are mean values of triplicate experiments, and error bars represent SD. *NS*, not significant; $*p < 0.05$; $**p < 0.01$. *D*, Total RNA as described in *C* was analyzed by quantitative TaqMan real-time PCR using assay specific to the *Ifnb* gene. The ratio of *Ifnb* mRNA to β_2-*microglobulin* mRNA was calculated in units (one unit being the ratio of the *Ifnb* to β_2-*microglobulin* mRNA in splenocytes). The relative levels of *Ifnb* mRNA in the B6 female mice are indicated as 1. Results are mean values of triplicate experiments, and error bars represent SD. $**p < 0.01$; $***p < 0.001$. *E*, Total cell extracts prepared from splenocytes isolated from B6, B6.*Nba2*-C (C), or B6.*Nba2*-ABC (ABC) female mice (age ∼4 mo) were analyzed by immunoblotting using Abs specific to the indicated proteins.

from the male mice but not in those from female mice. Furthermore, IFN-β treatment of cells, which increased levels of the p202 protein, did not increase Aim2 protein levels. Moreover, basal levels of the caspase-1 p20 were lower in females than those in males (Fig. 5*A*), and IFN-β treatment of BMDMs from females, which further increased (∼6-fold) the p202 protein levels, reduced the levels of caspase-1 p20 subunit.

Our above observations that increased basal and IFN-β–induced levels of the p202 protein in the NZB BMDMs inversely correlated with Aim2 protein levels prompted us to test whether the increased levels of p202 protein could regulate the expression of the *Aim2* gene. For this purpose, we overexpressed p202 protein in RAW264.7 cells, a murine macrophage cell line. We chose these cells because they do not express the adapter protein ASC (24) and therefore allow stable overexpression of a gene of interest from a transfected plasmid. As shown in Fig. 5*B* and 5*C*, increased expression of *Ifi202* mRNA and protein in RAW264.7 cells reduced basal levels of *Aim2* mRNA and protein. Accordingly, knockdown of p202 expression in murine macrophage cell line J774.A1 also resulted in moderate (∼2-fold) increases in Aim2 protein levels (Fig. 5*D*) and activation of the caspase-1 (as measured by the detection of p20 and p10 proteins) in response to transfection of synthetic DNA (poly dA.dT) into cells (Fig. 5*D*). Notably, under our experimental conditions, transfection of the synthetic DNA into J774.A1 cells increased levels of p202 protein, which slightly reduced levels of the activated caspase-1. Again, it is conceivable that the production of type I IFN in response to transfection of the synthetic DNA (poly dA.dT) into J774.A1 cells inhibited the efficiency of knockdown by small interfering RNA approach through decreasing the levels of Dicer (30). Collectively, these observations indicated that the expression levels of p202 protein in cells are inversely correlated with Aim2 protein levels and the activation of caspase-1.

Discussion

Recent studies (11, 26, 27) demonstrated that *Aim2*-deficient mice are more susceptible than the wild-type mice to certain infections that result in the release of pathogen-derived DNA into the cytoplasm of BMDMs. These studies also indicated that *Aim2*-deficient mice are not defective in producing IFN-β after infections. Therefore, it has been proposed that the Aim2 inflammasome function is needed for the elimination of infected cells (through caspae-1–dependent cell death) to clear certain infections in mice and increase chances for survival.

Earlier, the knockdown approach identified the p202 protein as a potential inhibitor of cytosolic DNA-induced caspase-1 (and caspase-3) activation by Aim2 inflammasome (24, 25). Therefore, we compared the expression of *Aim2* and *Ifi202* genes between *Aim2*-deficient and age-matched wild-type mice. Our experiments reveal that i) *Aim2* deficiency in mice increased levels of the IFN-β mRNA, levels of STAT1, p-STAT1 proteins, and the expression of IFN-inducible genes, including the *Ifi202*, compared with those in the wild-type mice (Figs. 1 and 2); ii) IFN-β treatment of J774.A1 macrophage cells after knockdown of *Aim2* expression robustly increased levels of STAT1, its activating phosphorylation on Tyr-701, and stimulated the activity of an IFN-responsive reporter above those of the control cells (Fig. 3); iii) the expression of *Aim2* in immune cells from certain strains of female mice (B6,

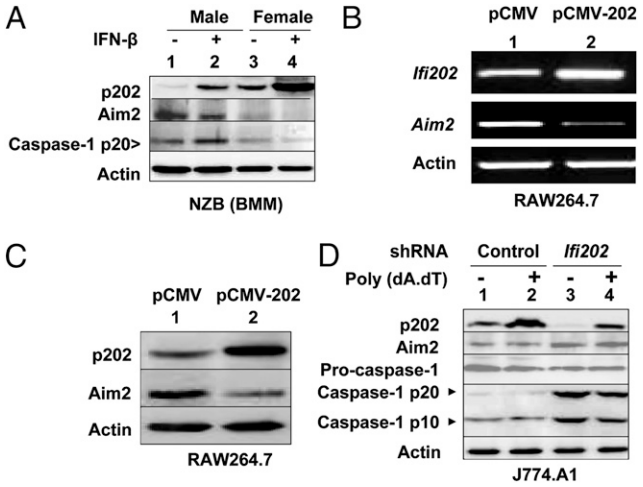

FIGURE 5. Expression levels of p202 protein are inversely correlated with the activation of caspase-1. *A*, Purified BMDMs from NZB males (*lanes 1* and *2*) or age-matched females (*lanes 3* and *4*) (age ~12 wk) were either left untreated (*lanes 1* and *3*) or treated with IFN-β (*lanes 2* and *4*) for 15 h in the culture medium (supplemented with GM-CSF). After the treatment, total cell lysates containing equal amounts of protein were analyzed by immunoblotting using Abs specific to the indicated proteins. Levels of the cleaved procaspase-1 (the p20) were detected in total cell lysates as described previously (21). *B*, Total RNA isolated from RAW264.7 cells either stably transfected with a control vector (pCMV) or a plasmid (pCMV-202) that allowed the expression of the *Ifi202* gene was analyzed by semiquantitative PCR using a pair of primers specific to the indicated gene. *C*, Total cell lysates prepared from RAW264.7 cells either stably transfected with pCMV plasmid or the pCMV-202 plasmid were analyzed by immunoblotting using Abs specific to the indicated proteins. *D*, J774.A1 cells were either infected with lentivirus expressing control shRNA (*lanes 1* and *2*) or sh*Ifi202* RNA (*lanes 3* and *4*), and cells were selected in puromycin for a week. Puromycin-resistant cells were pooled and allowed to grow without drug for 3 d. Total cell lysates prepared from cells either left untreated (control; *lanes 1* and *3*) or transfected with synthetic DNA poly (dA.dT) (*lanes 2* and *4*) and incubated for 18 h were analyzed by immunoblotting using Abs specific to the indicated proteins.

B6.*Nba2*-C, and B6.*Nba2*-ABC) inversely correlated with the expression of *Ifnb* and *Ifi202* genes (Fig. 4); and iv) expression levels of p202 protein in BMDMs from the NZB mice or RAW264.7 cells inversely correlated with Aim2 protein levels and the activation of caspase-1 (Fig. 5). These observations indicate that the activation of the type I IFN signaling in *Aim2*-deficient immune or J774.A1 cells increases the expression of IFN-inducible proteins, including p202. Moreover, these observations support the idea that increased levels of the p202 protein, a negative regulator of caspase-1 activation by Aim2 (24, 25), increase the chances of survival for cells with cytosolic DNA, resulting in chronic activation of the innate immune responses that are often associated with the development of SLE.

SLE is an autoimmune disease characterized by chronic stimulation of the innate immune system by endogenous nucleic acids, resulting in increased levels of type I IFNs and associated defects in immune cells (32–34). SLE patients and murine models of the disease develop a peripheral blood gene expression profile, which is characterized by an "IFN-α signature" (33, 34). Moreover, the severity of SLE is correlated with high levels of type I IFNs and pathogenic autoantibodies against the nuclear Ags (32–34). Because gender-dependent increased levels of the p202 protein in immune cells of certain strains of female mice are associated with increased production of antinuclear autoantibodies (4, 17) and associated kidney pathology (35), our observations described here support the idea that cellular levels of the Aim2 protein at certain thresholds are necessary to suppress type I IFN response after sensing cytosolic DNA (either pathogen or self-derived). Given that defects in caspase-dependent cell death are associated with the development of lupus disease (36, 37), our observations also support the idea that elimination of cells with cytosolic DNA through pyroptosis (caspase-1–dependent cell death) may be important to reduce the production of type I IFNs and chronic stimulation of the innate immune system.

The activation of caspase-1 in BMDMs, in response to dsDNA, inversely correlated with the levels of p202 protein in three strains (B6, BALB/c, and NZB) of female mice (24). Notably, basal (spontaneous) activation of caspase-1 was only detected in macrophages from NZB females (and not in other strains of mice), and introduction of synthetic DNA into macrophages did not result in appreciable further increase in levels of activated caspase-1 (24). Therefore, our observations that the basal activation of caspase-1 was detectable in macrophages from NZB mice are consistent with the previous report (24). Moreover, our observations (Fig. 5) that macrophages from NZB males had higher basal levels of activated caspase-1 than those of macrophages from age-matched females are consistent with basal and IFN-β–induced increased levels of p202 protein in females compared with those in age-matched males. Collectively, these observations indicated the mouse gender-dependent differential activation of caspase-1 in immune cells by the expression levels of the Aim2 and p202 proteins.

Levels of IL-1β mRNA are reported to be significantly lower in peritoneal macrophages isolated from lupus-prone strains of mice (including the NZB mice) than those in peritoneal macrophages isolated from nonautoimmune mice (including the B6 mice) when cultured under certain conditions (38). Given that the expression of pro–IL-1β is, in part, regulated by the transcription factor NF-κB (39) and defects in the activity of NF-κB in immune cells are associated with lupus disease (40), our observation that steady-state levels of pro–IL-1β are reduced in splenic cells from pre-autoimmune B6.*Nba2*-ABC and B6.*Nba2*-C females compared with those in splenic cells from B6 mice (Fig. 4*E*) are of some interest and warrant further investigation to determine whether Aim2 and/or p202 regulate the expression of pro–IL-1β by modulating the activity of NF-κB in a particular splenic cell type (6).

Currently, it is not clear how defects in the expression of the *Aim2* gene in immune or J774.A1 cells increase steady-state levels of *Ifi202* mRNA and protein. Increased steady-state levels

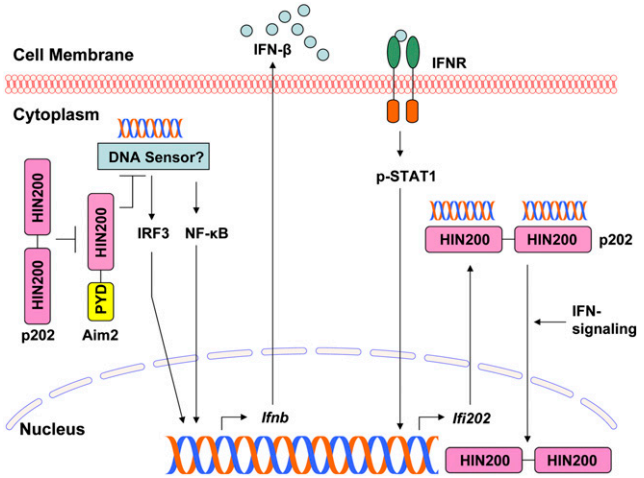

FIGURE 6. Proposed role of Aim2 protein in cytosolic DNA-induced regulation of type I IFN responses and the expression of the IFN-inducible genes.

of IFN-β (but not IFN-γ) mRNA, STAT1 protein, and p-STAT1 in *Aim2*-deficient or knocked down cells make it likely that increased production of IFN-β through an autocrine or paracrine mechanism activates the transcription of the *Ifi202* gene in cells (Fig. 6). Notably, gender-dependent expression of *Ifi202* in splenic B and T cells (17) makes it likely that *Aim2* expression in immune cells is also regulated in a gender-dependent manner. Consistent with this prediction, we have noted 4-fold higher levels of the Aim2 protein in purified splenic B cells ($B220^+$) from B6 male mice than those in purified splenic B cells from the age-matched females (data not shown). However, further work will be needed to determine whether the gender-dependent factors also regulate the expression of *Aim2* in other cell types.

It has been proposed (27) that an unidentified sensor of cytosolic DNA, which has a relatively lower threshold than the Aim2 protein to sense cytosolic DNA, may induce the expression of IFN-β. Because *Aim2*-deficient cells appear to express more IFN-β than that of the wild type cells and because the Aim2 protein has the ability to homodimerize and heterodimerize (6), it is conceivable that the Aim2 protein interacts with this cytosolic DNA sensor and negatively regulates its ability to induce IFN-β expression through the activation of IFN-regulated factor 3 and/or NF-κB (Fig. 6). Further work will be needed to identify the molecular mechanisms by which the Aim2 protein suppresses a type I IFN response and the expression of IFN-inducible genes.

Overexpression of the human AIM2 or the murine Aim2 protein in transfected human embryonic kidney cells indicated that both proteins are detected in the cytoplasm (8, 21, 22). Likewise, uninduced basal levels of the p202 protein are also detected primarily in the cytoplasm of splenic B cells (13) and MEFs (18) from the B6.*Nba2* congenic mice. Notably, the IFN-α treatment of cells potentiates the nuclear accumulation of the p202 protein (13, 18). Consistent with these observations, we found that increased levels of p202 protein in *Aim2*-deficient MEFs were detected both in the cytoplasm and nucleus (Fig. 1*F*). Therefore, it is likely that age- and gender-dependent increased serum levels of type I IFNs in lupus-prone or *Aim2*-deficient mice potentiate the nuclear localization of the p202 protein in immune cells.

Our observations that overexpression of p202 in RAW264.7 cells decreases levels of *Aim2* mRNA and protein (Fig. 5) raise the possibility that promoter polymorphisms and gender-dependent increased levels of the p202 protein in immune cells of certain strains of female mice, such as B6.*Nba2*-ABC, downregulate the expression of *Aim2*. Although it remains to be determined how increased levels of p202 protein negatively regulate the levels of the *Aim2* mRNA and protein, the demonstrated ability of p202 protein to act as a transcriptional modulator for a number of transcription factors makes it conceivable that p202 protein represses the transcription of the *Aim2* gene. However, our observations do not rule out the possibility that polymorphisms in the *Aim2* gene contribute to its differential expression in certain strains of mice. Further work is in progress to elucidate the molecular mechanisms that regulate the differential expression of the *Aim2* gene in certain strains of mice.

In conclusion, our observations provide support for our model (Fig. 6). The model predicts that levels of the Aim2 protein at certain thresholds are necessary for the suppression of a type I IFN response and the expression of the IFN-inducible proteins (including the p202). Additionally, the increased levels of the p202 protein in immune cells inhibit the formation of the Aim2 inflammasome in response to cytosolic DNA and the activation of caspase-1, thus, contributing to chronic stimulation of the innate immune responses. Given that the sequence of the murine Aim2 protein is conserved between mice and humans (6), our observations will serve as a basis to understand the role of the human AIM2 protein in defective innate immune responses that are associated with certain autoimmune diseases, including SLE.

Disclosures

The authors have no financial conflicts of interest.

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