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J Immunol. Author manuscript; available in PMC 2012 June 15.

Published in final edited form as:
J Immunol. 2011 June 15; 186(12): 6762–6770. doi:10.4049/jimmunol.1003638.

Aim2-deficiency in mice suppresses the expression of the inhibitory Fcγ receptor (FcγRIIB) through the induction of the interferon-inducible p202, a lupus susceptibility protein

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Abstract

Murine *Aim2* and *Ifi202* genes (encoding for the Aim2 and p202 proteins) are members of the interferon (IFN)-inducible *Ifi200*-gene family. The *Aim2*-deficiency in mice activates IFN-signaling and stimulates the expression of lupus susceptibility gene, the *Ifi202*, located within the *Nba2* interval. Given that the deficiency in the expression of the *Fcgr2b* gene (encoding for the inhibitory FcγRIIB receptor) is associated with increased lupus susceptibility in mice, we investigated whether the Aim2 protein could regulate the expression of *Fcgr2b* gene. Here we report that *Aim2*-deficiency in mice suppresses the expression of the FcγRIIB receptor. Interestingly, the *Fcgr2b*-deficient cells expressed increased levels of the IFN-β, activated IFN-signaling, and expressed reduced levels of the Aim2 protein. Treatment of splenic cells with IFN-α or γ reduced levels of the FcγRIIB mRNA and protein, and also decreased the activity of the FcγRIIB p(−729/+ 585) Luc reporter. Moreover, levels of the FcγRIIB receptor were significantly higher in the *Stat1*-deficient splenic cells than the wild type cells. Accordingly, increased expression of IFN-β in lupus-prone B6.*Nba2-ABC* mice, as compared with non lupus-prone B6 or B6.*Nba2*-C mice, was associated with reduced expression of the FcγRIIB receptor. Notably, overexpression of the p202 protein in cells decreased the expression of the *Aim2* gene, activated the IFN-response, and suppressed the expression of the *Fcgr2b* gene. These observations demonstrate that the expression of Aim2 protein is required to maintain the expression of the *Fcgr2b* gene and also predict epistatic interactions between the *Ifi200*-genes and the *Fcgr2b* gene within the *Nba2* interval.

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Disclosures

The authors have no financial conflicts of interest.

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Keywords

Aim2 inflammasome; interferon; FcγRIIB; SLE

Several chromosomal loci appear to contribute to the development of systemic lupus erythematosus (SLE), an autoimmune disease with strong gender bias, in humans and in mouse models of the disease (1–6). In particular, the NZB autoimmunity 2 (*Nba2*)locus (~90–97 cM; locus), which is located on the distal portion of the NZB chromosome 1, has been shown to be a major genetic contribution to the disease susceptibility in the (NZB x NZW)F_1 mice (7, 8). The generation of the B6.*Nba2* congenic (congenic for the *Nba2* interval on the C57BL/6 genetic background) mice identified the interferon (IFN)-inducible *Ifi202* gene (encoding for the p202 protein), a member of the *Ifi200*-gene family, as a candidate lupus susceptibility gene within the *Nba2* interval (8). Increased levels of the p202 protein are associated with defects in apoptosis of the B6.*Nba2* splenic B cells upon ligation with anti-IgM *in vitro.* Moreover, the female mice at the age of ~7 month produce pathogenic antinuclear antibodies and splenomegaly. Interestingly, these congenic female mice do not develop a kidney disease (8), indicating interactions of the *Nba2* locus with other loci for the development of the kidney disease.

Based on the sequencepolymorphisms that are identified in the *Nba2* interval genes, it has been proposed that the interval may contain several candidate lupus susceptibility genes (8–12). The candidate genes include the *Slam*-family genes (12), the *Fcgr2b* gene (encoding for the inhibitory FcγRIIB receptor) (10), and the interferon (IFN)-inducible *Ifi202* gene (8).Generation of B6.*Nba2* sub-congenic lines (B6.*Nba2*-A, B6.*Nba2*-B, and B6.*Nba2*-C) revealed that the subinterval A (comprising the *Fcgr2b* gene) and the subinterval B (comprising the *Slam*-family genes) cooperate with each other to influence the autoantibody production (13). Moreover, the study revealed that genes within the subinterval C (comprising the *Ifi200*-family genes) may negatively regulate the expression of the *Fcgr2b* gene and inhibit the FcγRIIB-induced apoptosis (13).

IFNs, a family of cytokines, exert multiple biological effects on the immune system by regulating the differentiation, proliferation, and survival of immune cells (14, 15). Importantly, the biological activities of IFNs are mediated by the IFN-inducible proteins (16). The IFN-family includes type I (IFN-α and β) and type II (IFN-γ) IFNs (14, 15). Both types of IFNs have been investigated for their role in SLE disease (15). Notably, increased levels of IFN-α have been detected in the sera of SLE patients (15, 17). Moreover, activation of IFN-stimulated genes (ISGs) in peripheral blood mononuclear cells of lupus patients is correlated with severity of the disease (17). Accordingly, mice that are deficient in the type-I IFN receptor (and do not express increased levels of ISGs) do not develop the disease (18, 19). These studies indicate a role for ISGs (and the corresponding effector proteins) in the development of SLE.

One family of the ISGs is the *Ifi200*-gene family, which encodes for structurally-related proteins (the p200-family proteins) (20–22). The family includes the murine (for example, *Ifi202a*, *Ifi202b*, *Ifi203*, *Ifi204*, and *Aim2*) and human (for example, *MNDA*, *IFIX*, *IFI16*, and *AIM2*) genes (22). Most p200-family proteins (except the human AIM2 and murine Aim2 proteins) are detected in the cytoplasmic as well as in the nuclear fractions and the IFN-treatment of certain cell types potentiates the nuclear localization of the p202 protein (23). Interestingly, recent studies revealed that p202 (24), p204 (25), Aim2 (26), IFI16 (25), and AIM2 (27, 28) proteins can sense cytosolic double-stranded DNA (dsDNA) and initiate an innate immune response. Both Aim2 and AIM2 proteins, upon sensing dsDNA, form an inflammasome (20, 30), which through the activation of caspase-1, increases the secretion of

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proinflammatory cytokines, including IL-1β and IL-18, and induces cell death by pyroptosis (caspase-1-dependent death) in macrophages. In contrast to the Aim2/AIM2 proteins, upon sensing dsDNA, the p204 and IFI16 proteins recruit stimulator of interferon genes (STING) protein to stimulate the expression of IFN-β through the activation of interferon-regulated factor 3 (IRF3) and NF-κB (25).

Fc receptors for IgG are key players in the regulation of innate and adaptive immune responses (31–33). In mice, there are four Fcγ receptors. Upon stimulation, the FcγRI and FcγRIII transduce the stimulatory signals through FcRγ chain associated intracellular immunoreceptor tyrosine-based activating motif (ITAM). In contrast, the FcγRIIB receptor transduces inhibitory signals via intracellular ITIMs. The FcγRIIB is the only Fcγ receptor, which is expressed by B cells (32, 33). Therefore, stringent control of its expression is critical in determining its effects on the immune system. Several isoforms of the inhibitory FcγRIIB receptor have been reported in humans and mice (32, 33). The FcγRIIB1 isoform, which is a membrane protein, is predominantly expressed by B cells. The FcγRIIB2 isoform (a membrane protein) is predominantly expressed by myeloid-derived cells. Both B1 and B2 isoforms are encoded by the same gene through alternative splicing of mRNA (33). When expressed by immune cells, the FcγRIIB receptor inhibits the functions of the activating FcγRs, such as phagocytosis and pro-inflammatory cytokine release (31–33). Therefore, FcγRIIB receptor regulates many different aspects of immune and inflammatory responses. Consistent with the immunoregulatory role of the FcγRIIB receptor, mice that are deficient in the FcγRIIB receptor expression develop SLE disease spontaneously on certain genetic backgrounds (34–36). Interestingly, the selective blockade of the inhibitory FcγRIIB receptor in human dendritic cells (DCs) and monocytes induces a type I interferon response (37). The induction of the type I IFN response results in activation of STAT1 (by phosphorylation on Tyr-701) and increased steady-state levels of mRNAs that encode for the IFN-inducible proteins, including the AIM2 and IFI16 proteins (37). Although, polymorphisms involving short deletions in the promoter and intronic regions of the *Fcgr2b* gene are associated with reduced steady-state levels of mRNA in certain lupus-prone strains of mice (including the NZB mice) (10, 38, 39), the molecular mechanisms that regulate the expression of the *Fcgr2b* gene remain unknown.

Generation of *Aim2*-deficient mice revealed that the Aim2 protein is not needed for IFN-β production after certain bacterial or viral infections (26, 40, 41). Upon infections with certain intracellular pathogens, immune cells (splenic cells and BMDMs) from the *Aim2*-deficient mice are defective in the activation of caspase-1, secretion of IL-1β and IL-18, and induction of cell death *in vitro* (26). Notably, the *Aim2*-deficiency in mice increases the IFN-β expression, stimulates the expression of the *Ifi202* gene, and potentiates the nuclear localization of the p202 protein (42). Given that the p200-family proteins are predicted to negatively regulate the expression of the *Fcgr2b* gene (13), we investigated whether the Aim2-deficiency could regulate the expression of the inhibitory FcγRIIB receptor. Here we report that the expression of Aim2 protein is required to maintain the expression of the inhibitory FcγRIIB receptor.

Materials and Methods

Mice

We have described the generation of the *Aim2*-deficient mice (26). These mice are on the mixed (129 x B6) genetic background. Wild type and *Aim2*-deficient mice were housed in specific pathogen-free animal facilities at the University of Massachusetts Medical School, Worcester, MA. The B6-*Nba2*-ABC (same as B6*Nba2*; ref. 13), B6.*Nba2*-C (13), and C57BL/6 (B6) mice were housed in pathogen-free animal facilities at the University of Virginia, Charlottesville, VA. The *Fcgr2b*-deficient (mice on the C57BL/6 genetic

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background) and *Stat1*-deficient (mice on the 129S6 genetic background) mice and the corresponding wild type mice were purchased from Taconic Farms (Germantown, NY). C57BL/6 and NZB male and female mice were purchased from The Jackson Laboratory (Bar Harbor, Main). Mice were housed in pathogen-free animal facilities at the University of Cincinnati. The Institutional Animal Care and Use Committee (IACUC) at the institution, where the mice were housed, approved the protocol to use mice for studies described here.

Splenocytes isolation, cell culture, and treatments

Total splenocytes that were prepared from age and strain-matched male or female mice were re-suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum. When indicated, splenic B cells ($B220^+$) were purified using magnetic beads (purification kit purchased from Miltenyi Biotec), allowing the positive selection of the cells (43). The purified (90–95% pure) B cells were used immediately for additional experiments. Unless otherwise indicated, splenic cells from two or more mice were pooled to prepare total RNA or protein extracts.

Murine macrophage RAW264.7 and J774.A1 cell lines (purchased from the American Type Culture Collection) were maintained as suggested by the supplier. When indicated, sub-confluent cultures of the murine macrophage cell lines were either left without any treatment or treated with the universal IFN-α (1,000 u/ml; from PBL Biomedical laboratories, Piscataway, NJ), or murine IFN-γ (10 ng/ml; from R & D Systems, Minneapolis, MN) for the time period indicated.

To ectopically overexpress the p202 protein in RAW264.7 cells, cells in a six well plate were transfected with an empty vector (pCMV) or p202 expression plasmid (pCMV-202b) using the FuGene 6 transfection reagent (Roche Applied, Indianapolis, IN) as described previously (43). 24 hours after transfections, cells were split and the transfected cells were selected in G418 (400 μg/ml) for ~10–15 days. Colonies that exhibited G418-resistant were pooled. For experiments, cell cultures were maintained without G418 in the medium for several days.

To knockdown the expression of the *Aim2* or *Ifi202*gene in J774.A1 cells, cells in a six well plate were infected with lentivirus (purchased from Santa Cruz Biotech, Santa Cruz, CA) encoding either shRNA to *Aim2* (sc-140968-V) or *Ifi202* (sc-40698-V) gene. As a control, cells were infected with the lentivirus encoding a control shRNA (sc-108080). 24 hours post-infections, cells were selected in puromycin (1 μg/ml) for a week. Cells that exhibited puromycin-resistance were pooled. For experiments, cell cultures were maintained without puromycin in the medium for several days.

Reporter assays

Reporter assays were performed essentially as described previously (43). In brief, sub-confluent cultures of RAW264.7 cells(in a 6-well plate) were transfected with the reporter plasmid FcγRIIB p(−729/+585)Luc (ref. 44; the plasmid generously provided by Dr. J. E. Gessner, Hanover Medical School, Germany; 2.5 μg) and pRL-TK (purchase from Promega, Madison, WI; 0.5 μg), using FuGENE 6 (Roche Applied, Indianapolis, IN), as suggested by the supplier. The transfected cells were either left untreated (control) or treated with the murine IFN-γ (10 ng/ml for 14 h). Cells were harvested between 40–45 h after transfections and the firefly and *Renilla* dual luciferase activities were determined as described previously (43).

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Isolation of RNA from splenocytes and RT-PCR

Splenocytes (5–8 × 10^6 cells) were used to prepare total RNA using TRIzol (Invitrogen, Carlsbad, CA) method (43) and 0.5–2 μg of RNA was used for RT-PCR reaction using the Superscript one-step RT-PCR system (from Invitrogen). Semi-quantitative PCR was performed using a pair of primers specific to the *Ifi*202 (primers: forward: 5′-ggtcatctaccaactcagaat-3′; reverse primer: 5′-ctctaggatg ccactgctgttg-3′), *Aim2* (primers: forward: 5′-acagtggccacggaga- 3′; reverse: 5′-aggtgacttcactccaca-3′), *Fcgr2b* (primers: forward: 5′-aagtctaggaaggacactgc-3′; reverse: 5′-atcctggcctttctggcttgc-3′) or the murine *Ifnb* (primers: forward: 5′-ctgcgttcctgctgtgcttctcca- 3′; reverse: 5′-ttctccgtcatctccatagggatc-3′) gene. The conditions for the regular PCR were the same as described previously (43).

To perform quantitative real-time TaqMan PCRs, we used the 7300 Real-Time PCR System (from Applied Biosystems, Foster City, CA, USA) and the commercially available real-time TaqMan gene expression assays. The PCR cycling program has been described previously (43). The TaqMan assays for *Ifi202* (Assay Id# Mm0304 8198_m1; the assay allows the detection of both the *Ifi202a* and *Ifi202b* mRNA levels), *Aim2* (Assay Id# Mm01295719_m1), *Ifnb* (Assay Id# Mm00439552_s1), *Fcgr2b* (Assay Id# Mm00438875_m1; the assay allows the detection of mRNA encoding both the B1 and B2 isoforms of the FcγRIIB receptor), *2,5AS* (Assay Id# Mm00836412_m1), *Mx1* (Assay Id# Mm00487796_m1), *Rsad2* (Assay Id# Mm00491265_m1), the endogenous *Actb* control (cat # 4352933E), and *β2-microglobulin* (Assay Id# Mm00437762_m1) were purchased from the Applied Biosystems (Foster City, CA) and used as suggested by the supplier.

Immunoblotting

Total cell lysates containing approximately equal amounts of proteins prepared from splenocytes or murine macrophage cell lines were subjected to immunoblotting as described previously (43). The p202 antiserum, which allows the detection of both p202a and p202b proteins in immunoblotting, has been described (43). When indicated, we also used monoclonal antibodies to p202 (sc-166253) from Santa Cruz Biotech (Santa Cruz, CA) to detect the p202 protein in extracts from the murine cell lines. Polyclonal antibodies that were raised against murine Aim2 protein have been described previously (42). Antibodies to detect the murine FcγRIIB receptor (sc-28842) in immunoblotting were purchased from Santa Cruz Biotec. Antibodies to STAT1 (# 9172), p-STAT1$^{Tyr-701}$ (# 9171), and β-actin (# 4967) were purchased from Cell Signaling Technology (Danvers, MA).

Statistical analyses

The statistical significance of differences in the measured mean frequencies between the two groups of observations was calculated using the Student's two-tailed *t* test. A p value <0.05 was considered significant.

Results

Aim2-deficiency or knockdown decreases the expression of the Fcgr2b gene

Aim2-deficiency in splenic and bone marrow-derived cells increases the constitutive expression of type I IFN (IFN-β) and certain IFN-inducible proteins, including the p202 protein, in gender-dependent manner (42). Given that the *Ifi200*-family genes that are located within the C-region of the *Nba2* interval may negatively regulate the FcγRIIB receptor expression (13), we investigated whether the lack of *Aim2* expression in the *Aim2*-knockout mice (26) could regulate the expression of the FcγRIIB receptor. As shown in Fig. 1, *Aim2*-deficient B cells had significantly lower basal levels of the FcγRIIB receptor mRNA (Fig. 1A and B) and protein (Fig. 1C) as compared to cells isolated from age and gender-matched wild-type mice. Interestingly, the FcγRIIB receptor protein levels were

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measurably higher in the splenic B cells from males than the age-matched females (Fig. 1C). Moreover, the knockdown of *Aim2* expression in the murine macrophage J774.A1 cell line also reduced steady-state levels of FcγRIIB receptor mRNA (Fig. 1D) and protein (Fig. 1E). Collectively, these observations revealed that the *Aim2*-deficiency reduces the expression levels of the inhibitory receptor FcγRIIB.

Fcgr2b *gene* deficiency induces a type I IFN response

Selective blockade of the inhibitory FcγRIIB receptor in human dendritic cells and monocytes induces a type I interferon response that results in the activating phosphorylation of STAT1 and increased steady-state levels of mRNAs that code for the IFN-inducible proteins (37). However, it remains unknown whether a similar response is generated in the murine cells that are deficient in the FcγRIIB receptor. Given that the *Aim2*-deficiency significantly decreased the expression of FcγRIIB in splenic cells (Fig. 1), we investigated whether the *Fcgr2b*-deficiency could induce a type I interferon response. As shown in Fig. 2, the deficiency of the *Fcgr2b* gene in splenic cells (Fig. 2A and B), which was associated with reduced steady-state levels of the Aim2 protein (Fig. 2B) and mRNA (Fig. 2C), increased basal levels of IFN-β mRNA (Fig. 2D). Accordingly, we detected higher basal levels of the activated STAT1 (phosphorylated on Tyr-701) and STAT1 proteins in FcγRIIB-null than the age and gender-matched wild type cells (Fig. 2E; a very long exposure showed bands in lanes 1 and 3). Furthermore, consistent with the activation of STAT1 in the *Fcgr2b*-deficient cells, we also noted increased steady-state levels of mRNAs encoded by known IFN-inducible genes, including the *2,5 AS* (encoding for 2′-5′ oligoadenylate synthetase) *Mx1*, *Rsad2* (encoding for the viperin protein), and *Ifi202* than the wild-type cells (Fig. 3). These observations demonstrated that the FcγRIIB receptor deficiency in murine splenic cells induces a type I IFN response, which results in the activation of STAT1 and the induction of the IFN-inducible genes.

Activation of IFN-signaling decreases FcγRIIB receptor expression

Given that the *Aim2*-deficiency induces a type I IFN response (42) and also decreases steady-state levels of FcγRIIB mRNA and protein (Fig. 1), we explored whether the activation of IFN-signaling could negatively regulate the expression of the *Fcgr2b* gene. As shown in Fig. 4, treatment of B6 splenic cells with IFN-α or IFN-γ (we also used IFN-γ because it suppresses the transcription from the *Fcgr2b* gene promoter; ref. 44) significantly (>50%) reduced steady-state levels of FcγRIIB mRNA (Fig. 4A) and protein (Fig. 4B). However, levels of the FcγRI mRNA did not decrease (data not shown). Interestingly, the decrease in FcγRIIB protein levels was time-dependent and more appreciable after 48 h of the treatment with IFN-α. This decrease in the FcγRIIB receptor protein levels after a prolonged treatment with IFN-α is consistent with a long predicted half life (>30 h) of the protein. Moreover, as expected (44), the treatment of RAW264.7 cells with IFN-γ significantly decreased the activity of the FcγRIIB p(−729/+585)-Luc reporter gene, the expression of which was driven by the 5′-regulatory region (−729 to +585 base pairs) of the murine *Fcgr2b* gene (ref. 44; Fig. 4C). The above observations that the activation of the IFN-signaling in splenic cells reduced the expression of *Fcgr2b* gene prompted us to compare steady-state levels of FcγRIIB mRNA and protein between the *Stat1*-deficient and wild type cells. Expectedly, levels of the FcγRIIB mRNA (Fig. 4D; these cells express detectable levels of mRNA encoding for the B1 and B2 isoforms of the FcγRIIB receptor) and protein (Fig. 4E) were significantly higher in splenic cells from *Stat1*-deficient than the age and gender-matched wild-type mice. In summary, these observations demonstrate that the activation of IFN-signaling in immune cells, which resulted in the activating phosphorylation of the STAT1, negatively regulates the expression of the inhibitory FcγRIIB receptor.

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Increased expression of IFN-β in lupus susceptible mice is associated with reduced expression of the FcγRIIB receptor

Lupus susceptible preautoimmune (age ~9 weeks) NZB and B6.*Nba2*-ABC (same as B6.*Nba2*) female mice express lower levels of the Aim2 mRNA as compared with age-matched B6 females (42). Therefore, our above observations that the *Aim2*-deficiency is associated with reduced levels of FcγRIIB mRNA and protein (Fig. 1) prompted us to compare the expression of FcγRIIB between non lupus-prone and lupus-prone mice. As shown in Fig. 5, basal steady-state levels of FcγRIIB mRNA (Fig. 5A) and protein (Fig. 5B) were measurably lower in the splenic cells from the NZB and B6.*Nba2-ABC* female mice as compared with the age-matched B6 females. Notably, levels of the FcγRIIB receptor were appreciably (~50%) lower in the B6.*Nba2* females than the age-matched NZB females (Fig. 5B, compare lane 3 with 2).

Earlier, we noted that reduced expression of the Aim2 protein in preautoimmune (age ~4 month-old) B6.*Nba2*-ABC female mice is associated with increased expression of IFN-β and p202 protein as compared to age-matched B6 mice (42). Therefore, we compared steady-state levels of IFN-β mRNA in female mice (B6, B6.*Nba2*-C, and B6.*Nba2*-ABC), which differ with respect to the expression of Aim2 and p202 proteins (42). As shown in Fig. 5C, consistent with our previous observations (42), steady state levels of IFN-β mRNA were readily detectable in the B6.*Nba2-ABC* female mice. However, the levels were either much lower in the B6.*Nba2-C* female mice or not detectable in the B6 females. Importantly, the steady-state levels of FcγRIIB mRNA were significantly lower in the B6.*Nba2-ABC* mice as compared to age-matched B6 females (Fig. 5D and E). Notably, the difference in the mRNA levels was minimal between the B6 and B6.*Nba2-C* mice. Accordingly, levels of the FcγRIIB receptor were ~70% lower in the B6.*Nba2-ABC* cells than the age-matched B6 mice (Fig. 5F). Collectively, the above observations indicated that increased levels of type I IFNs in the lupus-prone female mice are associated with decreased expression of the inhibitory FcγRIIB receptor.

The expression levels of the p202 protein are inversely correlated with the Aim2 and FcγRIIB receptor levels

Aim2-deficiency increases the expression of IFN-β, activates IFN-signaling, and increases levels of IFN-inducible p202 protein (42). Given that increased expression of the IFN-β in the B6.*Nba2-ABC* mice is inversely correlated with the FcγRIIB receptor levels (Fig. 5), we explored whether the increased levels of the p202 protein could negatively regulate the expression of the *Aim2* and *Fcgr2b* genes. For this purpose, we chose to overexpress p202 protein in murine macrophage cell line RAW264.7. We chose these cells because they lack the expression of an adaptor protein ASC (24), thus, allowing stable transfection of an expression plasmid and expression of a transgene. As shown in Fig. 6A, increased expression of the p202 mRNA in RAW264.7 cells was associated with significantly reduced steady-state levels of Aim2 mRNA, increased levels of IFN-β mRNA, and reduced levels of FcγRIIB mRNA (these cells express both the B1 and B2 isoforms of the FcγRIIB mRNA). Accordingly, increased levels of the p202 protein in RAW264.7 cells significantly decreased basal levels of the Aim2 protein, increased the activating phosphorylation of STAT1 and levels of STAT1 protein, and reduced FcγRIIB receptor levels (Fig. 6B). Moreover, consistent with these observations, the knockdown of p202 expression in the murine J774.A1 cell line increased (~2.6-fold) steady-state levels of FcγRIIB protein (Fig. 6C). Collectively, these observations, which are consistent with our previous observations that the *Aim2*-deficiency stimulates the expression of the *Ifi202* gene (42), demonstrate that the expression levels of the p202 protein in immune cells are inversely correlated with the expression levels of the Aim2 and FcγRIIB proteins.

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Discussion

Our previous studies (and studies by others) indicated a role for the IFN-inducible p202 protein in lupus susceptibility (8, 20, 22). Furthermore, the generation of sub-congenic lines (the B6.*Nba2*-A, B6.*Nba2*-B, and B6.*Nba2*-C) and their comparisons with the control mice (B6 and B6.*Nba2*-ABC mice) reveled that the p200-family proteins may negatively regulate the expression of the *Fcgr2b* gene (13). Because our recent study revealed that the *Aim2*-deficiency in mice activates the IFN-responses and stimulates the expression of the p202 protein (42), we investigated whether the *Aim2*-deficiency in mice could regulate the expression of the inhibitory receptor FcγRIIB. Our experiments revealed that: (i) *Aim2*-deficiency in mice or the knockdown of the *Aim2* expression in macrophage cell lines, which stimulates the *Ifi202* expression (42), decreases the *Fcgr2b* expression (Fig. 1); and (ii) in lupus-prone mice, reduced expression of the *Aim2* gene is associated with activation of a type I IFN response (42) and decreased expression of the FcγRIIB receptor (Fig. 5); Collectively, these observations demonstrate that the expression of Aim2 protein is required to maintain the expression of the inhibitory FcγRIIB receptor.

Selective blockade of the inhibitory FcγRIIB receptor in human dendritic cells and monocytes induces a type I interferon response and increased expression of certain ISGs, including the *IFI16* and *AIM2* (37). Therefore, our observations that *Fcgr2b*-deficiency in mice activates a type I IFN response (increased levels of IFN-β and activation of STAT1) are consistent with the above report. Moreover, our observations revealed that: (i) the activation of IFN-signaling in the B6 splenic cells decreases the expression of *Fcgr2b* gene (Fig. 4); (ii) in lupus susceptible B6.*Nba2-ABC* mice, increased expression of the IFN-β and increased levels of the p202 protein (as compared to the B6 mice) are associated with decreases in the expression *Fcgr2b* (Fig. 5 and ref. 42); and (iii) overexpression of p202 protein in a murine macrophage cell line decreased levels of the Aim2 protein, activated STAT1, and reduced the FcγRIIB receptor levels (Fig. 6). Collectively, these observations are consistent with the idea that increased levels of the IFN-inducible p202 protein in certain strains of female mice contribute to lupus susceptibility in part by negatively regulating the expression of the *Aim2* and *Fcgr2b* genes.

The expression of p202 protein is gender-dependent: higher in the females than the age-matched males (43). Therefore, our observation that increased levels of p202 protein in Aim2-deficient cells are associated with reduced levels of FcγRIIB levels raise the possibility that the expression of the *Fcgr2b* gene is also regulated by the gender-dependent factors. In this regard, a study noted higher steady-state levels of the *Fcgr2b* mRNA in 4-month-old pre-lupus (NZB x NZW)F_1 males than females (45). Additionally, it has been reported that healthy women had overall lower FcγRIIb expression on B cells than men (46). Given that the expression levels of the inhibitory FcγRIIb receptor contribute to the regulation of survival and functions of the immune cells (31, 32), including the B cells, it is likely that the gender and mouse strain-dependent decreased levels of the receptor in lupus-prone female mice (for example, NZB and B6.*Nba2*-ABC) as compared to the B6 mice affect the sub-populations of the immune cells that express the FcγRIIB receptor. Therefore, further work will be needed to determine whether the observed differences in the levels of the FcγRIIb receptor between various strains of male and the age-matched female mice are associated with increases (or decreases) in certain sub-populations of the immune cells.

Polymorphisms in the promoter region of the *FCGR2B* gene reduce the expression of the receptor in SLE patients (47) and in certain strains of mice (38, 39). As compared to normal healthy B6 mice, autoimmune-prone NZB and MRL mice share three deletions: two in the promoter region and one in the third intronic region (39). Importantly, these polymorphisms correlate well with the extent of down-regulation of the *Fcgr2b* expression in germinal

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center B cells (39). Notably, very low-level expression of FcγRIIB receptor on macrophages expressing the NZB-type *Fcgr2b* allele, as compared with those expressing the B6-type allele, is associated with the development of monocytosis (an indicator for severe SLE disease in mice) in the FcγRIIB haploinsufficient B6 mice carrying the *Yaa* mutation (48). Consistent with these observations, we have noted lower levels of FcγRIIB receptor expression in purified plasma cells and germinal center B cells from 4-month-old B6.*Nba2* female mice, which express the NZB-type *Fcgr2b* allele, than the age-matched B6 mice (13). In the present study, we provide evidence that at least two mechanisms contribute to reduced steady-state levels of the FcγRIIB receptor in the B6.*Nba2-ABC* mice (as compared to age and gender-matched B6 mice): (i) polymorphisms involving deletions in the NZB allele (the NZB genotype); and (ii) the *Aim2* deficiency, which activates type I IFN responses and induces the expression of the p202 protein (42), which suppresses the expression of the *Fcgr2b* gene (Fig. 5).

The regulation of the expression of the human and murine FcγRIIB receptor gene is complex and depends on the cell type (32). Interestingly, the transcription of the human *FCGR2B* gene is activated by the c-Jun/AP-1 transcription factor (49). Moreover, the expression of the murine *Fcgr2b* gene is negatively regulated by IFN-γ (44). Given that the transcription of the human *FCGR2B* gene is activated by the c-Jun/AP-1 transcription factor (49), we have analyzed the 5′-regulatory region (nucleotides from −729 to +585; ref. 44) of the mouse *Fcgr2b* gene for the presence of potential c-Jun/AP-1 DNA-binding sites. The search revealed that the regulatory region contains at least two perfect (tgag/ctca) potential c-Jun/AP-1 DNA-binding sites. Because increased levels of the p202 protein in cell lines inhibit c-Jun/AP-1-stimulated transcription (50) and increased levels of p202 protein in B6.*Nba2* splenic cells are associated with reduced steady-state levels of c-Jun mRNA (a transcriptional target of the AP-1) after stimulation of B cells (with anti-IgM for 20 h) as compared to B6 cells (data not shown), it is likely that p202 protein down-regulates the expression of the *Fcgr2b* gene in part by inhibiting the c-Jun/AP-1-mediated transcription. Further work will be needed to test this interesting possibility.

Presently, it is not known whether polymorphisms in the promoter region of the *Aim2* gene (or other mechanisms) contribute to its differential expression between non lupus-prone and lupus-prone strains of mice. Moreover, a comparison of the Aim2 protein levels in splenic B ($B220^+$), T ($CD3^+$), and bone marrow-derived macrophage (BMDMs; $CD11b^+$) from B6 female mice (age ~8-weeks) indicated that the levels of Aim2 protein are higher in the B cells than T or BMDMs (B>T>BMDM; data not shown). Therefore, although the expression of the Aim2 protein is detectable in both lymphoid and myeloid cells, it remains to be determined whether the Aim2 protein-mediated regulation of the *Fcgr2b* gene is cell-type dependent.

Cross-linking of the FcγRIIb receptor induces apoptosis of plasma cells (51). Therefore, reduced expression of the inhibitory receptor is predicted to increase cell survival and the production of antibodies. Consistent with the above prediction, plasma cells from lupus-prone mice express reduced levels of FcγRIIb receptor and are protected from apoptosis (51). Therefore, our observations that the Aim2-deficiency results in reduced levels of FcγRIIb receptor in immune cells support the possibility that defects in the expression of the *Aim2* gene are likely to contribute to increased survival of plasma cells that produce the pathogenic autoantibodies. Further work is in progress to test this possibility.

Mice deficient in FcγRIIB receptor expression develop antinuclear antibodies and glomerulonephritis (34–36). Interestingly, dendritic cells (DCs) from these mice that develop lupus-like disease exhibit altered NF-κB expression (compared with wild-type mice): reduced expression of the inhibitory protein IκBα (which indicates the activation of

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NF-κB) (52). Moreover, pharmacological inhibition of the activity of NF-κB in FcγRIIB-deficient mice reduces the susceptibility to develop SLE and prevents the symptoms (production of antinuclear antibodies and glomerulonephritis) (52). Given that increased expression of p202 protein in bone marrow-derived DCs from (NZB x NZW)F_1 mice stimulates the activity of NF-κB (53), our observations that overexpression of p202 in RAW264.7 cells decreases levels of FcγRIIB receptor (Fig. 5) make it likely that the p202-mediated decrease in the expression of FcγRIIB receptor in certain strains of mice contribute to the activation of the transcriptional activity of NF-κB in DCs. Consistent with this prediction, we have noted reduced expression of IκBα in splenic cells from the B6.*Nba2* female mice as compared to age-matched B6 mice (data not shown).

A recent study noted that type I IFN-signaling inhibits IL-1 (both IL-α and β) production and inflammasome activation (54). Therefore, our observation that the *Aim2*-deficiency activates type I IFN response and the expression of IFN-inducible genes are consistent with the idea that the Aim2 protein and the activation of type I IFN-responses are part of a regulatory negative feedback mechanism. Consequently, further work will be needed to understand how defects in the expression of *Aim2* gene contribute to increased production of type I IFNs and associated autoimmune diseases, including lupus.

In summary, our observations support our model (Fig. 7), which predicts that the expression levels of the Aim2 protein above certain thresholds are required to maintain the expression of the *Fcgr2b* gene in immune cells. Given that the Aim2 and p202 proteins are part of a mutually negative feedback loop (42), it is conceivable that the mouse-strain and gender-dependent increases in the levels of p202 protein in immune cells reduce the *Aim2* expression below the threshold, resulting in auto-stimulation of the *Ifi202* expression and suppression of the *Fcgr2b* gene expression. Our observations will serve basis to understand the role of the human AIM2 protein in lupus susceptibility.

Acknowledgments

We would like to thank Dr. J. E. Gessner for generously providing the FcγRIIB p(−729/+585)-Luc reporter plasmid.

This work was supported by a grant (AI066261) from the National Institutes of Health (NIH) to D.C. A grant from the Lupus Research Institute supported work in the laboratory of L. D. E. V.A.R is supported by a postdoctoral fellowship from the New England Regional Center of Excellence for Biodefense and Emerging Infectious Diseases (NERCE; NIH/NIAID AI057159). K.A.F is supported by a NIH grant AI083713.

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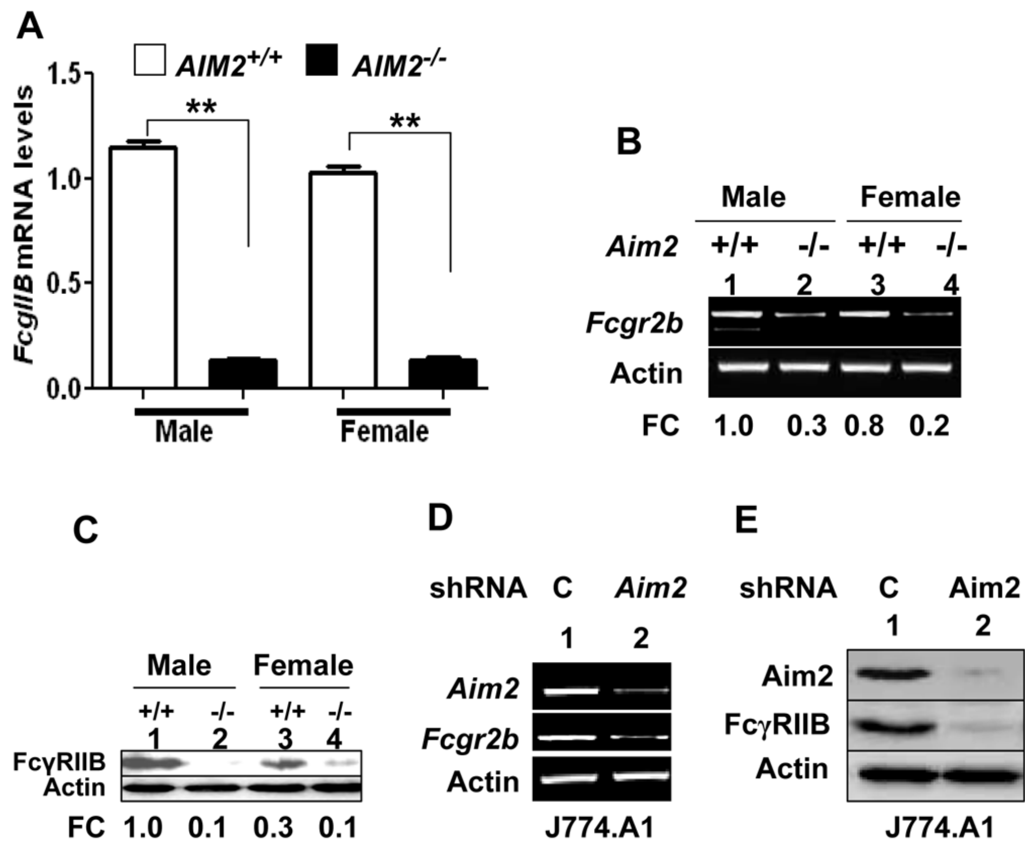

FIGURE 1. *Aim2*-deficiency or knockdown decreases the *Fcgr2b* expression

(**a**) Total RNA was extracted from the purified splenic B cells that were isolated from wild-type and age-matched *Aim2*-deficient male or female mice (age 6–8 weeks). Steady state levels of *Fcgr2b* mRNA were analyzed by quantitative TaqMan real-time PCR. The ratio of the test gene to *β2-microglobulin* mRNA was calculated in units (one unit being the ratio of the test gene to *β2-microglobulin* mRNA). Results are mean values of triplicate experiments and error bars represent standard deviation (** $p < 0.005$). (**b**) Total RNA as described in panel (**a**) was also analyzed by semi-quantitative PCR using a pair of primers specific to the indicated genes. (**c**) Total cell lysates from purified splenic B cells that were prepared from wild-type (lanes 1 and 3) and age-matched *Aim2*-deficient (lanes 2 and 4) male or female mice (age 6–8 weeks) were analyzed by immunoblotting using antibodies specific to the indicated proteins. The fold change (FC) in the levels of the FcγRIIB receptor protein after normalization with actin protein levels is also indicated. (**d**) Total RNA was extracted from stably infected with control lentivirus (C) or sh*Aim2* lentivirus (*Aim2*) J774.A1 cells. The RNA was analyzed by semi-quantitative PCR using a pair of primers specific to the indicated gene. (**e**) Total cell lysates prepared from J774.A1 cells described in panel (**d**) were subjected to immunoblotting to detect the indicated proteins.

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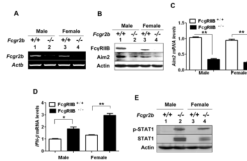

FIGURE 2. *Fcgr2b*-deficiency induces a type I IFN response

(**a**) Total RNA was extracted from splenocytes isolated from wild-type (lanes 1 and 3) and age-matched *Fcgr2b*-deficient (lanes 2 and 4) male or female mice (age ~8 weeks). The RNA was analyzed by semi-quantitative PCR using a pair of primers specific to the indicated genes. (**b**) Total protein extracts were prepared from splenocytes isolated from wild-type (lanes 1 and 3) and age-matched *Fcgr2b*-deficient (lanes 2 and 4) male or female mice (age ~8 weeks). Extracts containing approximately equal amounts of proteins were analyzed by immunoblotting for the indicated proteins. (**c**) The RNA samples described in the panel (**a**) were subjected to quantitative real-time PCR to detect levels of the *Aim2* mRNA. The ratio of the *Aim2* mRNA to the *β2-microglobulin* mRNA was calculated in units (one unit being the ratio of the test gene to *β2-microglobulin* mRNA). The relative steady-state levels of *Aim2* mRNA in male mice are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation (** $p < 0.01$). (**d**) The RNA samples described in the panel (**a**) were subjected to quantitative real-time PCR to detect levels of the *Ifnb* mRNA. The ratio of the *Ifnb* mRNA to the *β2-microglobulin* mRNA was calculated in units (one unit being the ratio of the test gene to *β2-microglobulin* mRNA). The relative steady-state levels of *Ifnb* mRNA in male mice are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation (* $p < 0.05$; ** $p < 0.01$). (**e**) Total protein extracts described in panel (**b**) were analyzed by immunoblotting for the indicated proteins.

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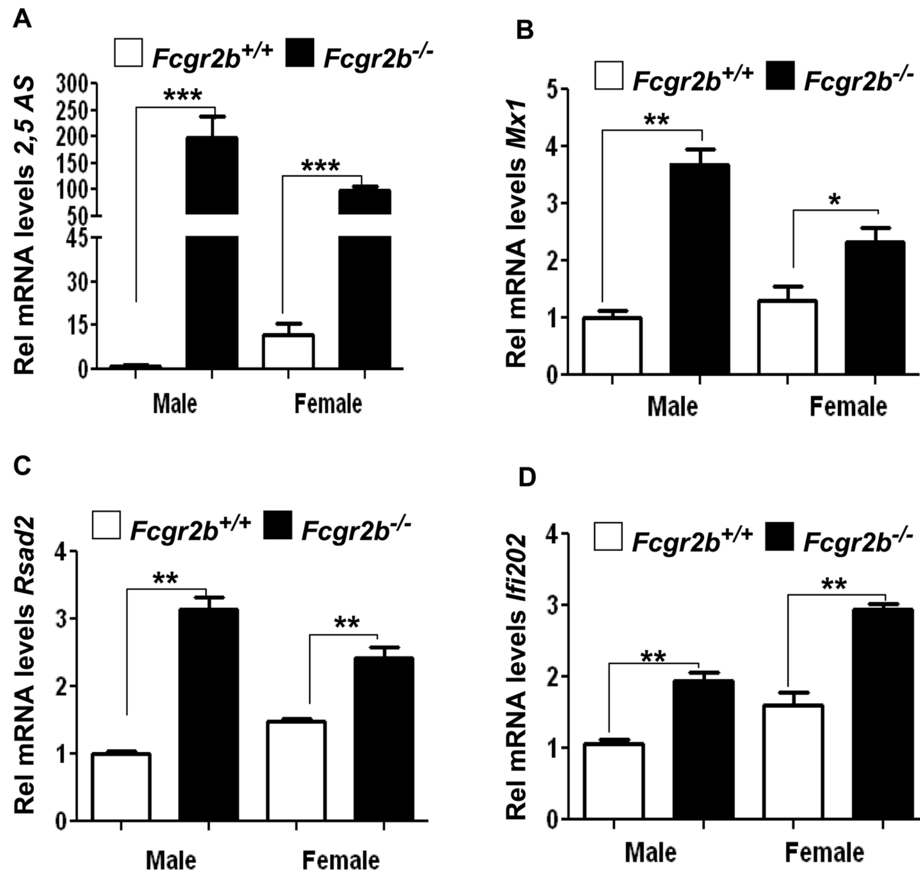

FIGURE 3. *Fcgr2b*-deficiency induces the expression of IFN-inducible genes

Total RNA was isolated from total splenocytes isolated from wild-type and age-matched *Fcgr2b*-deficient male or female mice (age ~8 weeks). Steady state levels of mRNA corresponding to the IFN-inducible *2,5 AS* (**a**), *Mx1* (**b**), *Rsad2* (**c**), and *Ifi202* (**d**) genes were analyzed by quantitative TaqMan real-time PCR. The ratio of the test gene to *β2-microglobulin* mRNA was calculated in units (one unit being the ratio of the test gene to *β2-microglobulin* mRNA). Results are mean values of triplicate experiments and error bars represent standard deviation (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$).

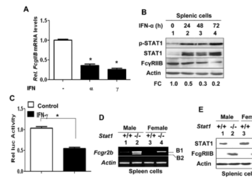

FIGURE 4. Activation of IFN-signaling decreases FcγRIIB receptor expression

(**a**) Splenocytes isolated from the B6 female mice (age ~10 weeks) were either left without any treatment or treaded with IFN-α (1,000 u/ml) or IFN-γ (10 ng/ml) for 18 h. Total RNA was extracted after the treatment and analyzed by quantitative real-time PCR for FcγRIIB mRNA levels. The ratio of the FcγRIIB mRNA mRNA to the *β2-microglobulin* mRNA was calculated in units (one unit being the ratio of the test gene to *β2-microglobulin* mRNA). The relative steady-state levels of FcγRIIB mRNA in untreated control cells are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation (* $p < 0.05$). (**b**) Splenocytes isolated from the B6 female mice (age ~10 weeks) were either left without any treatment or treaded with IFN-α (1,000 u/ml) for the indicated time (h). Total cell extracts were subjected to immunoblotting using specific antibodies to the indicated proteins. FC, indicates fold change in the FcγRIIB receptor levels. (**c**) Cultures of RAW264.7 cells in a 6-well plate were transfected with the FcγRIIB p(−729/+ 585) Luc-reporter plasmid (2.5 μg) along with pRL-TK (0.5 μg) reporter plasmid using FuGENE 6 transfection reagent. 24 h after transfections, cells were either left untreated or treated with IFN-γ. 40–45 h after transfections, cells were processed for the dual luciferase activity. Results are mean values of triplicate experiments and error bars represent standard deviation (* $p < 0.05$). (**d**) Total RNA was prepared from splenocytes isolated from wild-type (lanes 1 and 3) and age-matched *Stat1*-deficient (lanes 2 and 4) male or female mice (age ~9 weeks). The RNA was analyzed by semi-quantitative PCR using a pair of primers specific to the indicated genes. (**e**) Protein extracts containing equal amounts of proteins that were prepared from wild-type (lanes 1 and 3) and age-matched *Stat1*-deficient (lanes 2 and 4) male or female mice (age ~9 weeks) were subjected to immunoblotting to analyze the levels of the indicated proteins.

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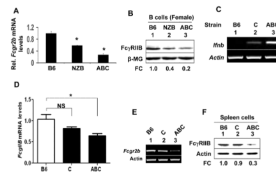

FIGURE 5. Increased expression of IFN-β in lupus susceptible mice is associated with reduced expression of FcγRIIB receptor

(**a**) Total RNA isolated from splenocytes prepared from age-matched (~9-weeks) B6, NZB, or B6.*Nab2* (ABC) female mice was analyzed by quantitative real-time PCR for FcγRIIB receptor mRNA levels. The ratio of the FcγRIIB mRNA to the *β2-microglobulin* mRNA was calculated in units (one unit being the ratio of the test gene to *β2-microglobulin* mRNA). The relative steady-state levels of FcγRIIB mRNA in the B6 female mice are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation (* $p < 0.05$). (**b**) Total protein extracts from purified B cells ($B220^{+}$) were subjected to immunoblotting for the indicated protein using the specific antibodies. FC, indicates fold change in the FcγRIIB receptor levels. (**c**) Total RNA extracted from age-matched (~4-month-old) female B6, B6.*Nba2*-C (C), or B6.*Nba2*-ABC (ABC) splenic cells was analyzed by semi-quantitative PCR for *Ifnb* mRNA levels. (**d**) Total RNA in the panel (**c**) was analyzed by quantitative TaqMan real-time PCR using the specific to *Fcgr2b.* The ratio of the *Fcgr2b* mRNA levels to *β2-microglobulin* mRNA levels was calculated in units (one unit being the ratio of the test gene to *β2-microglobulin* mRNA in splenocytes). The relative levels of *Fcgr2b* mRNA levels in the B6 females are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation (*NS*, not significant; * $p < 0.05$). (**e**) Total RNA in the panel (**c**) was also analyzed by semi-quantitative PCR for *Fcgr2b* mRNA levels. (**f**) Total cell extracts from splenocytes prepared from age-matched (~10-weeks) B6, NZB, or B6.*Nab2* (ABC) female mice were analyzed by immunoblotting using antibodies specific to the indicated proteins. FC, indicates fold change in the FcγRIIB receptor levels.

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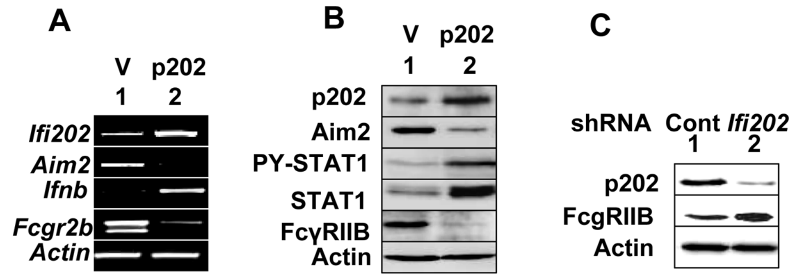

FIGURE 6. The expression levels of the p202 protein are inversely correlated with FcγRIIB receptor

(**a**) Total RNA prepared from RAW264.7 cells either stably transfected with a control vector (pCMV; indicated as V) or a plasmid (pCMV-202; indicated as p202) that allowed the expression of the *Ifi202* gene was analyzed by semi-quantitative PCR using a pair of primers specific to the indicated genes. (**b**) Total cell extracts from RAW264.7 cell clones described in the panel (**a**) were analyzed by immunoblotting using antibodies specific to the indicated proteins. (**c**) Total cell extracts from J774.A1 cells infected with control lentivirus (lane 1) or sh*Ifi202* lentivirus (lane 2) were subjected to immunoblotting for the indicated proteins.

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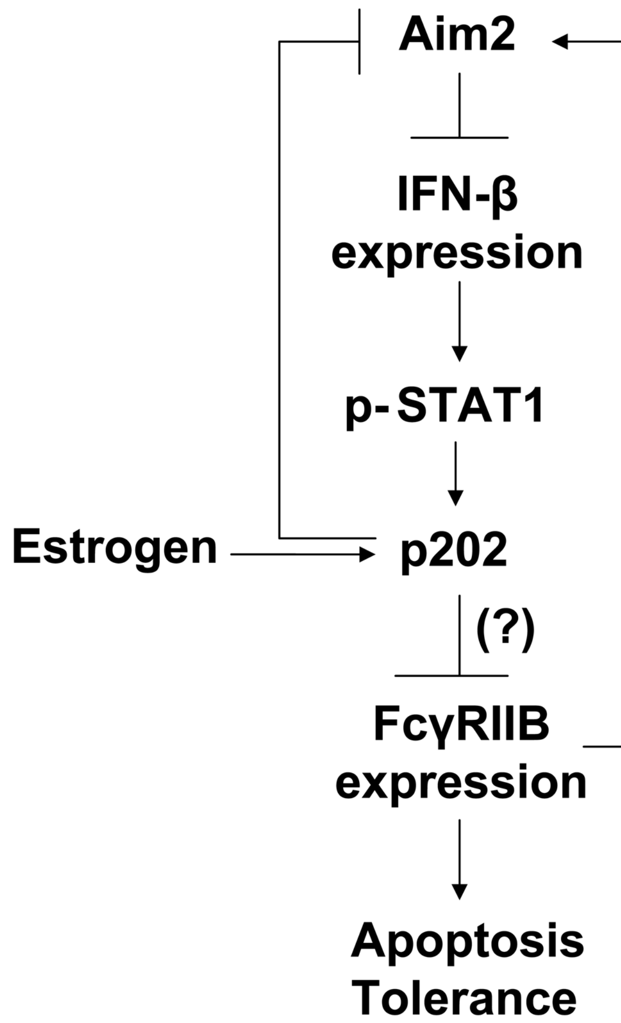

FIGURE 7. Proposed regulatory role of the Aim2 protein in the maintenance of the FcγRIIB receptor expression through down-regulation of the p202 protein expression