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Cell type and gender-dependent differential regulation of the p202 and Aim2 proteins: implications for the regulation of innate immune responses in SLE

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Abstract

Upon sensing cytosolic double-stranded DNA (dsDNA), the murine Aim2 (encoded by the *Aim2* gene) protein forms an inflammasome and promotes the secretion of proinflammatory cytokines, such as IL-1β and IL-18. In contrast, the p202 protein (encoded by the *Ifi202* gene) does not form an inflammasome. Previously, we have reported that the interferon (IFN) and female sex hormone-induced increased nuclear levels of p202 protein in immune cells are associated with increased susceptibility to develop a lupus-like disease. However, signaling pathways that regulate the expression of Aim2 protein remain unknown. Here we report that the expression of *Aim2* gene is induced in bone marrow-derived macrophages (BMDMs) by IFN-α treatment and the expression is, in part, STAT1-dependent. However, treatment of splenic T or B cells with IFN-α or their stimulation, which induced the expression of *Ifi202* gene, did not induce the expression of *Aim2* gene. Furthermore, treatment of cells with the male hormone androgen increased levels of Aim2 mRNA and protein. Moreover, treatment of murine macrophage cell lines (RAW264.7 and J774A.1) with IFN-α differentially induced the expression of Aim2 and p202 proteins and regulated their sub-cellular localization. Additionally, activation of Toll-like receptors (TLR3, 4, and 9) in BMDMs and cell lines also differentially regulated the expression of *Aim2* and *Ifi202* genes. Our observations demonstrate that cell type and gender-dependent factors differentially regulate the expression of the Aim2 and p202 proteins, thus, suggesting opposing roles for these two proteins in innate immune responses in lupus disease.

Keywords

Aim2 inflammasome; p202; interferon; inflammation; SLE

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Conflict of interest

None

1. Introduction

Several lines of evidence strongly suggest that sustained production of type I interferon (the IFN-α) contributes to the pathogenesis of systemic lupus erythematosus (SLE) in patients and certain mouse models (Crow et al., 2004; Theofilopoulos et al., 2005). Accordingly, studies have also identified polymorphisms within genes of the IFN pathway (and IFN-regulated genes) that confer an increased risk for the development of SLE (Kozyrev et al., 2007; Korman et al., 2008; Lee et al., 2010). Particularly, the NZB autoimmunity 2 (*Nba2*) interval (~96–100 cM; located on the NZB chromosome 1) has been shown to contain candidate lupus susceptibility genes (Vyse et al., 1997; Wither et al., 2000; Rozzo et al., 2001), which enhance autoantibody production. These genes include the *Fcgr2b* (encoding for the FcγRIIB receptor) (Xiu et al. 2002), *Slam/Cd2* family genes (Wandstrat et al. 2004) and IFN-inducible *Ifi200*-family genes, including the *Ifi202* gene (encoding for the p202 protein) and *Aim2* gene (encoding for the Aim2 protein) (Rozzo et al., 2001; Choubey et al., 2008). The deficiency of *Fcgr2b* (Bolland et al., 2000) or *Slamf1* (Keszei et al., 2011) gene in mice results in a lupus-like disease. Moreover, the *Fcgr2b*-deficiency in human cells (Dhodapkar et al., 2007) and in mice (Panchanathan et al., 2011) up-regulates the expression of the IFN-inducible genes, including the *Ifi202* gene. Notably, Aim2 protein expression is required to suppress the expression of the *Ifi202* gene, production of type I IFN, activation of IFN-signaling, and maintain the expression of the FcγRIIB receptor (Panchanathan et al., 2010, 2011). These observations strongly suggest epistatic interactions among the *Nba2* interval genes in the regulation of the type I IFN pathway and autoantibody production.

One family of the IFN-inducible genes is the *Ifi200*-gene family (Choubey et al., 2008, 2010). Genes in the family encodes for structurally and functionally-related p200-family proteins. Most proteins in the family contain a partially conserved repeat of the 200-amino acid residue (or the HIN-200 domain). The repeat contains two consecutive oligonucleotide/oligo-saccharide-binding folds (OB-folds) (Choubey et al., 2010). Through the repeat, the p200-family proteins can bind to single or double-stranded DNA (dsDNA). Additionally, the repeat is involved in homo and heterodimerization of the p200-family proteins (Choubey et al., 2008). Most p200-family proteins (except the murine p202 protein) also contain a protein-protein interaction domain referred to as the pyrin domain (PYD) to recruit adaptor protein ASC (Choubey et al., 2010).

The p200-protein family includes the murine Aim2 and p202 proteins (Choubey et al., 2008). The Aim2 protein, upon sensing cytosolic dsDNA in bone marrow-derived macrophages (BMDMs), forms an inflammasome (Roberts et al., 2009; Fernandes-Alnemri et al., 2010), which activates caspase-1 and increases the secretion of proinflammatory cytokines, including IL-1β. In contrast, upon sensing cytosolic DNA, p202 protein is unable to form an inflammasome (Roberts et al., 2009). Moreover, the knockdown of p202 expression in BMDMs increased activation of caspase-1 by dsDNA (Roberts et al., 2009).

Expression of *Ifi202* gene is induced by IFNs (Gribaudo et al., 1987) and IL-6 (Pramanik et al., 2004) in immune cells. The expression of the *Ifi202* gene is also induced in T cells upon stimulation with anti-CD3 and anti-CD28 (Chen et al., 2008). Notably, treatment of splenic cells with the female sex hormone estrogen also induces the expression of *Ifi202* gene through the estrogen receptor-α (ERα) (Panchanathan et al., 2009). Given that the p202 and Aim2 proteins initiate different innate immune responses upon sensing cytosolic dsDNA (Roberts et al., 2009; Choubey et al., 2010); that signaling pathways, which regulate the expression of the *Aim2* gene in immune cells remain unknown, to understand the potential role of p202 and Aim2 proteins in the *Nba2* phenotype (autoantibody production), we compared the constitutive and induced expression of the *Ifi202* and *Aim2* genes in immune cells. Our observations revealed that cell type and gender-dependent factors differentially

regulate the expression of Aim2 and p202 proteins, thus, indicating opposing roles for these two proteins in initiating innate immune responses in lupus disease.

2. Materials and methods

2.1 Mice

The *Stat1*-deficient (mice on the 129S6 genetic background) mice and the corresponding wild type mice were purchased from the Taconic Farms (Germantown, NY). C57BL/6 (B6) and NZB male and female mice were purchased from The Jackson Laboratory (Bar Harbor, Main). The B6.*Nba2* mice were originally purchased from The Jackson Laboratory and bred at the Laboratory Animal Medical Services (LAMS) facilities at the University of Cincinnati. All mice were housed in pathogen-free animal facilities at the University of Cincinnati. The Institutional Animal Care and Use Committee (IACUC) at the institution approved the protocol to use mice for studies described here.

2.2 Splenocytes isolation, cell purification, cell culture, and treatments

Splenocytes were prepared from male or age-matched female mice as described previously (Panchanathan et al., 2009). In brief, cells were resuspended in RPMI 1640 cell culture medium, which was supplemented with 10% fetal bovine serum and antibiotics. When indicated, splenic B cells ($B220^+$), T cells (pan T cells), or bone marrow-derived macrophages (BMDMs; $Cd11b^+$) were purified using cell purification kits (kits purchased from Miltenyi Biotec) involving the positive selection of cells. The purified (90–95% pure) cells were either used immediately or incubated with the indicated agents. Unless indicated otherwise, cells from two or more age and gender-matched mice were pooled to prepare total RNA or protein extracts.

Purified splenic T cells were stimulated as described previously (Chen et al. 2008). In brief, freshly isolated splenic cells ($2–4 \times 10^6$) were plated in 60 mm plastic cell culture plates either coated with purified hamster anti-mouse CD3 epsilon (10 μg/plate; from eBioscience, San Diego, CA) antibody or purified golden Syrian hamster IgG (5 μg/plate; from eBioscience) isotype control antibody. Purified anti-mouse CD28 antibody (2 μg/ml; from eBioscience) was added to the culture medium after cell plating. T cells were stimulated for 22 h. Similarly, for the stimulation of B cells, splenic cells ($2–4 \times 10^6$) were plated in 60 mm plastic cell culture plates and cells were either incubated with goat anti-mouse IgM (2 μg/ml; from Southern Biotechnology Associates Inc., Birmingham, AL) or, as a control, with an isotype antibody for 20 h. After stimulation of cells for the indicated time, cells were collected and processed for the isolation of total RNA.

Murine macrophage cell lines RAW264.7 and J774.A1 were purchased from the American Type Culture Collection. Cells were maintained as suggested by the supplier. When indicated, sub-confluent cultures of cells were either left without any treatment (control) or treated with the indicated agents. When indicated, cells were 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 stimulate the TLR-induced signaling in macrophage cell lines, cells were incubated with indicated TLR ligand (using the mouse TLR1-9 agonist kit from InvivoGen, San Diego, CA) as suggested by the supplier.

Androgen-responsive mouse breast cancer cell line WT276 (Zinser et al., 2003) was generously provided by Dr. JoEllen Welsh, University of Notre Dame, Notre Dame, IN. For treatment of WT276 cells with dihydrtestosterone (DHT; 0, 5, or 10 nM), cells were cultured

in the phenol red-free RPMI 1640 medium (Invitrogen) and the medium was supplemented with 10% charcoal-stripped fetal bovine serum (Invitrogen).

2.3 Preparation of RNA and RT-PCR

Splenocytes, BMDMs, or macrophage cell lines were used to prepare total RNA using TRIzol (Invitrogen, Carlsbad, CA) method. Isolated RNA (0.5–2 μg) 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′-ggtcatctaccaactcag aat-3′; reverse primer: 5′-ctctaggatg ccactgctgttg-3′) or *Aim2* (primers: forward: 5′-acagtggccacggaga- 3′; reverse: 5′-aggtgacttcactccaca-3′) gene. The conditions for the regular PCR were the same as described previously (Panchanathan et al., 2009).

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 (Panchanathan et al., 2009). The TaqMan assays for the *Ifi202* (Assay Id# Mm0304 8198_m1; the assay allows the detection of both the *Ifi202a* and *Ifi202b* mRNA levels), *Ifi204* (Assay Id# Mm00492602_m1), *Aim2* (Assay Id# Mm01295719_ m1), the endogenous *Actb* control (cat # 4352933E) and β2-microglubulin (Assay Id# Mm00437762_m1) were purchased from the Applied Biosystems (Foster City, CA) and used as suggested by the supplier.

2.4 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 (Panchanathan et al., 2009). The p202 antiserum, which allows the detection of both p202a and p202b proteins in immunoblotting, has been described (Choubey et al., 1993). 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. Antibodies to β2-mcroglobulin (sc-13565) and AR (sc-816) were from Santa Cruz Biotech. Polyclonal antibodies that were raised against murine Aim2 protein have been described previously (). Antibodies to STAT1 (# 9172), p-STAT1$^{Tyr\text{-}701}$ (# 9171), IκBα (#9242), histone H3 (# 9715), and β-actin (# 4967) were purchased from Cell Signaling Technology (Danvers, MA).

2.5 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.

3. Results

3.1 The IFN-inducibility of Aim2 gene is cell type-dependent

Expression of *Ifi202* gene is induced by activation of the IFN-signaling (Choubey et al., 1993). Accordingly, mice that are deficient in a type I IFN receptor-signaling express reduced levels of *Ifi202* mRNA (Jorgensen et al., 2007). Moreover, a previous study noted the lack of Aim2 induction in BMDMs after treatment with the type I IFN or TLR ligands (which induces type I IFN induction) (Fernandes-Alnemri et al., 2010). These observations prompted us to test the IFN-inducibility of the *Aim2* gene in immune cells. As shown in Fig. 1A, incubation of BMDMs from C57BL/6 (B6) mice with IFN-α increased levels of the Aim2 protein in a time-dependent manner and the maximal increase was seen after 14 h of

treatment. Accordingly, we also noted appreciable increases in steady-state levels of Aim2 mRNA (Fig. 1B). Furthermore, consistent with these observations, we noted reduced steady-state levels of Aim2 mRNA in STAT1-deficient male and female mice as compared to age-matched wild-type mice (Fig. 1C). Interestingly, we also noted some differences in the levels of Aim2 mRNA between male and female mice (Fig. 1C).

Encouraged by the above observations, we also examined IFN-inducibility of *Aim2* gene in splenic cells. As shown in Fig. 1D, treatment of B6 female splenic cells with IFN-α, which increased steady-state levels of the *Ifi202* and *Ifi204* mRNA, did not increase steady-state levels of *Aim2* mRNA. In fact, we noted about 50% decrease in the mRNA levels. These observations prompted us to test IFN-inducibility of *Aim2* gene in purified splenic cells. As shown in Fig. 1E, treatment of purified T or B cells with IFN-α, which again increased levels of *Ifi202* mRNA, reduced levels of *Aim2* mRNA. Together, these observations revealed that the IFN-inducibility of the *Aim2* gene in immune cells depends on the cell type.

3.2 Stimulation of T and B cells down-regulates the expression of Aim2

Stimulation of splenic T cells from the NZB female mice by anti-CD3 and anti-CD28 up-regulates the expression of *Ifi202* gene (Chen et al., 2008). Therefore, we investigated whether stimulation of splenic T or B cells regulates the expression of *Aim2* gene. As shown in Fig. 2A, stimulation of T cells from B6 or B6.*Nba2* female mice with anti-CD3 and anti-CD28, which increased levels of the *Ifi202* mRNA, decreased steady-state levels of *Aim2* mRNA. Similarly, stimulation of splenic B cells from the B6 or B6.*Nba2* female mice with the anti-IgM antibodies, which increased steady-state levels of *Ifi202* mRNA, decreased levels of *Aim2* mRNA (Fig. 2B). Together, these observations revealed that the signaling pathways that are activated by stimulation of T or B cells differentially regulate the expression of the *Ifi202* and *Aim2* genes.

3.3 Gender-dependent regulation of Aim2 gene

The female sex hormone estrogen through the estrogen receptor-α (ERα) up-regulates the expression of the *Ifi202* gene (Panchanathan et al., 2009). Moreover, treatment of mice with the male hormone androgen reduced levels of the *Ifi202* mRNA (Panchanathan et al., 2009). Because we have noted increased levels of Aim2 mRNA and protein in splenic cells from the male mice as compared to age-matched females (Panchanathan et al., 2010), we further investigated whether the gender-dependent factors could regulate the expression of *Aim2* gene. As shown in Fig. 3A, steady-state levels of *Aim2* mRNA were 30–70% lower in splenic cells from female B6, NZB, and B6.*Nba2* mice as compared to age-matched males. Encouraged by this interesting observation, we compared levels of Aim2 protein between B6 splenic cells isolated from males and age-matched females. As shown in Fig. 3B, levels of Aim2 protein were appreciably higher in the B6 males than age-matched females. Accordingly, levels of Aim2 protein in purified T and B cells from B males were higher than age matched females (Fig. 3C). Moreover, treatment of WT-276 mouse breast epithelial cells, which express the androgen receptor (Fig. 3D), with increasing concentrations (0, 5, or 10 nM) of the male hormone dihydrotestosterone (DHT) increased levels of the Aim2 protein (Fig. 3D) and mRNA (Fig. 3E). Together, these observations indicated that the male hormone androgen positively regulates the expression of the *Aim2* gene in immune cells.

3.4 IFNs differentially regulation the expression of Aim2 and Ifi202 in macrophage cell lines

Introduction of dsDNA into the murine J744A.1, but not in RAW264.7, macrophage cell line activated the Aim2 inflammasome, resulting in the activation of caspase-1 and induction of apoptosis (Roberts et al., 2009). This difference in the innate immune response to dsDNA

between these two cell lines prompted us to compare the constitutive and IFN-induced expression of Aim2 and p202 proteins and their sub-cellular localization. As shown in Fig 4, the constitutive levels of the Aim2 mRNA (Fig. 4A) and protein (Figs 4B and C) were detectable in both the cell lines and the type I IFN treatment of cells appreciably increased the levels of Aim2 protein in RAW264.7 cell line. However, the IFN-γ treatment increased levels appreciably in J744A.1 cell line (Fig. 4C). Similarly, constitutive levels of p202 protein were detectable in RAW264.7 cells. However, p202 was not detectable in J744A.1 cells. Interestingly, type I IFN treatment of cells increased p202 protein levels in both RAW264.7 and J744A.1 cell lines (Fig. 4B and C). These observations prompted us to compare sub-cellular localization of Aim2 and p202 proteins. As seen in Fig. 4D, 50% of the constitutive levels of the Aim2 protein were detectable in the nuclear fraction in RAW264.7 cells. In contrast to Aim2 protein, constitutive levels of p202 protein were not detectable in RAW264.7 cells. Moreover, upon IFN-α-treatment of RAW264.7 cells, the induced levels of Aim2 protein were primarily detected in the cytoplasmic fraction whereas p202 was detected in the nuclear fraction. In contrast, in J774.1 cells, constitutive levels of Aim2 protein were not detectable under the conditions used. However, the IFN-induced levels were detected in both cytoplasm and nucleus (more in the nucleus than cytoplasm; Fig. 4E). Surprisingly, the p202 protein was detected primarily in the cytoplasmic fraction of untreated and IFN-α-treated cells. These observations indicated that the IFN-treatment of RAW264.7 and J774.1 cells differentially regulates the expression levels and sub-cellular localization of the Aim2 and p202 proteins.

3.5 Toll-like receptor signaling regulates the expression of Aim2 and Ifi202 genes

A previous study (Fernandes-Alnemri et al., 2010) noted that transfection of BMDMs from C57BL/6J mice with the synthetic DNA poly(dA:dT) or plasmid DNA (pcDNA) (which activates the TLR9-mediated signaling), or treatment with LPS (which activates the TLR4-mediated signaling) did not appreciably change levels of Aim2 protein. Given that the activation of TLR4 or 9 signaling in BMDMs induces the expression of type I IFNs, we explored whether the activation of TLR-signaling in BMDMs could induce the expression of *Aim2* and/or *Ifi202*. Treatment of BMDMs from the female mice with TLR3, 4, or 9-specific ligand for 6 h increased steady-state levels of the Aim2 mRNA between 60% to 2-fold as determined by quantitative real-time PCR (Fig. 5A) and regular PCR (Fig. 5C). The maximum induction was seen after treatment with the TLR9 ligand. Similarly, the treatment resulted in increases in levels of *Ifi202* mRNA between 50% to 3.5-fold (Fig. 5B and C). Again, the maximum induction was noted after activation of TLR9-signaling. Consistent with these observations, we also noted induction of Aim2 and p202 protein (Fig. 6A) and mRNA (Fig. 6B) in RAW264.7 cells after treatment with TLR3 or TLR4-specific ligand for 6 h. Interestingly, the induction of the p202 protein was accompanied by the induction of STAT1 protein levels (Fig. 6A) and the activation of STAT1 (data not shown). Similarly, treatment of J774.1 cells with TLR3 ligand polyI:C or TLR4 ligand LPS for increasing length of time (0, 6, or 9 h), which activated the IFN-signaling (as determined by an activating phosphorylation of STAT1 on Tyr-701 residue), also increased the levels of both Aim2 and p202 proteins (Fig. 6C and D). Interestingly, the LPS treatment induced p202 protein earlier than Aim2 protein. Together, these observations indicated that the activation of TLR-signaling in BMDMs and macrophage cell lines induces the expression of both *Aim2* and *Ifi202* genes to the different extents.

4. Discussion

Promoter polymorphisms contribute to increased expression of *Ifi202* gene in certain lupus-prone strains (for example, NZB, (NZB × NZW)F_1, and B6.*Nba2*) as compared to non lupus-prone strains (for example, C57BL/6) of female mice (Rozzo et al., 2001; Choubey et

al., 2008). Moreover, an inverse correlation has been noted between the *Ifi202* and *Aim2* genes with respect to their expression in splenic cells from the above strains of mice (Panchanathan et al., 2010). Consistent with the above observations, the *Aim2*-deficiency in mice on a mixed (B6 x 129sv) genetic background increased steady-state levels of the *Ifi202* mRNA and protein in splenic cells (Panchanathan et al., 2010). Because Aim2 and p202 proteins differ in their ability to initiate innate immune responses after sensing cytosolic dsDNA (Roberts et al., 2009; Choubey et al., 2010) and the knockdown of the *Ifi202* expression in BMDMs stimulated the activity of caspase-1 (Roberts et al., 2009), we decided to compare the expression of *Aim2* and *Ifi202* genes in immune cells in order to understand their relative contributions in the *Nba2* interval-associated phenotype (autoantibody production) in B6.*Nba2* congenic mice. Our observations revealed that: (i) the IFN-inducibility of *Aim2* gene is cell type-dependent (Fig. 1); (ii) stimulation of T and B cells down-regulates the expression of *Aim2* gene whereas the expression of *Ifi202* gene is up-regulated (Fig. 2); (iii) the male sex hormone androgen up-regulates the expression of *Aim2* gene (Fig. 3); (iv) IFNs differentially induce the expression of Aim2 and p202 proteins and their sub-cellular localization in macrophage cell lines (Fig. 4); and (v) TLR (TLR3, 4 and 9)-induced signaling in BMDMs and macrophage cell lines differentially regulates the expression of the *Aim2* and *Ifi202* genes (Figs. 5 and 6). These observations suggested that relative expression levels of the Aim2 and p202 proteins and their co-localization in the cytoplasm of immune cells contributes to the regulation of the innate immune responses that are initiated after sensing cytosolic dsDNA.

Generation of *Aim2*-deficient mice on different genetic backgrounds has indicated that the *Aim2* gene is not needed for the expression of type I IFN after certain infections or an introduction of cytosolic dsDNA in BMDMs (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010). Interestingly, we noted that the deficiency of *Aim2* gene in mice on a mixed (B6 x 129sv) genetic background increased levels of p202 protein (and its nuclear localization), increased the expression of IFN-β, and activated the type I IFN-signaling (Panchanathan et al., 2010). Additionally, the deficiency also reduced levels of the inhibitory IgG receptor, the FcγRIIB (encoded by the *Fcgr2b* gene) on B cells (Panchanathan et al., 2011). Because increased levels of the p202 protein in macrophage cell lines suppressed the expression of *Aim2* and *Fcgr2b* genes (Panchanathan et al., 2011), our observations suggest that stimulation of B cells, which increased the expression of the *Ifi202* gene but not *Aim2* gene (Fig. 2), increases the nuclear levels of the p202 protein. Thus, it is likely that the increased nuclear levels of p202 protein in B cells contribute to defects in B cell functions, including increased cell survival (Rozzo et al., 2001, Choubey et al., 2002), through modulation of the transcriptional activity of factors, such as p53, E2Fs, NF-κB, and AP-1, which regulate cell survival (Choubey et al., 2008).

Our observations that treatment of BMDMs with type I IFN (IFN-α) (Fig. 1) or TLR ligands, such as polyI:C, LPS, or stimulatory CpG ODN (but not control ODNs) (Fig. 5), increased levels of *Aim2* mRNA are not consistent with the previous report (Fernandes-Alnemri et al., 2010) in which the treatment of BMDMs with the synthetic DNA poly(dA:dT) (TLR9 ligand), LPS (TLR4 ligand), or infection with *Francisella tularensis* and treatment with IFN-β (which activated the IFN-signaling) did not increase levels of the Aim2 protein. These observations raise the possibility that post-transcriptional mechanisms contribute to the regulation of steady-state levels of Aim2 protein in BMDMs. Therefore, further work will be needed to test this possibility.

We have noted earlier that in splenic cells and in bone marrow-derived cells levels of Aim2 mRNA and proteins are higher in B6 males than the age-matched females (Panchanathan et al., 2010). Because the expression of *Ifi202* gene is stimulated by the female sex hormone estrogen (Panchanathan et al., 2009), our observations that steady-state levels of Aim2

mRNA and protein are higher in splenic cells from male mice as compared to age-matched females (Fig. 3) and treatment of an AR-responsive cell line with the male hormone DHT increased levels of both Aim2 protein and mRNA suggest that the expression of *Aim2* and *Ifi202* gene is differentially regulated by the male and female sex hormones, such as estrogen and androgen, in immune cells.

Previous studies (Choubey et al., 2003, 2010) have demonstrated that the activation of IFN-signaling potentiates the nuclear localization of p202 protein in immune cells. Given that the previous studies have provided support for the idea that sub-cellular localization (cytoplasmic versus nuclear) of the human Aim2 protein depends on the cell type (Choubey et al., 2010), we investigated the sub-cellular localization of both p202 and Aim2 proteins in macrophage cell lines. Our observations revealed that sub-cellular localization of both the proteins is cell type-dependent (Fig. 4). Interestingly, more Aim2 protein was detected in the nuclear fraction of J774.A1 cells than in the cytoplasm. Currently, it is not known whether the Aim2 protein has any role in the nucleus. However, given that the p200-family proteins have the ability to form heterodimers (Choubey et al., 2008), it is likely that heterodimerization of the nuclear Aim2 protein with other p200-family proteins regulates cell survival.

In summary, our observations demonstrate that the constitutive and induced (induced by type I IFN or TLR ligands) levels of Aim2 and p202 proteins depend on cell type (Table 1). Additionally, our observations demonstrate that gender-dependent factors differentially regulate the expression of the Aim2 and p202 proteins. These observations suggest opposing roles for these two innate immune sensors for cytosolic dsDNA in innate immune responses that are initiated in lupus disease.

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Highlights

- p202 and Aim2 proteins are newly identified innate immune sensors for cytosolic DNA.
- Increased levels of the p202 protein are associated with lupus susceptibility.
- Expression of p202 and Aim2 proteins is cell type and gender-dependent.
- Expression of p202 and Aim2 in immune cells is inversely correlated.
- Observations suggest opposing roles for the p202 and Aim2 proteins in lupus.

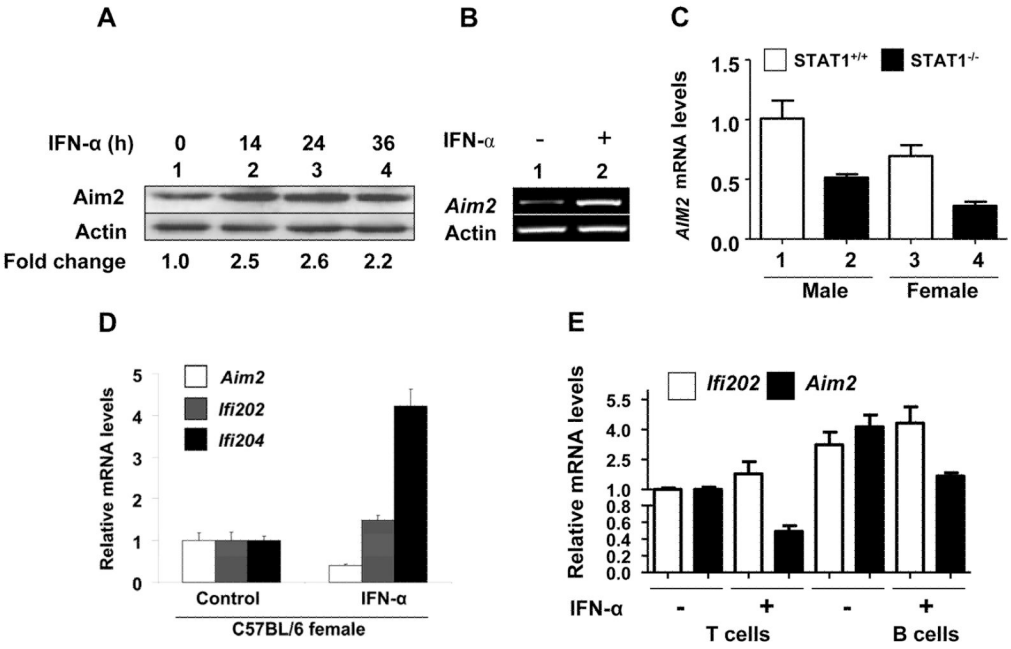

Fig. 1.

The IFN-inducibility of *Aim2* gene is cell type-dependent. (A) Bone marrow-derived macrophages (BMDMs) were either left without any treatment (lane 1) or treated with IFN-α (1,000 u/ml) for the indicated time (h). At the end of the treatment, total cell lysates were analyzed by immunoblotting using antibodies specific to the indicated proteins. Fold change indicates increases in the levels of Aim2 protein. (B) BMDMs as in panel (A) were either left untreated or treated with IFN-α for 14 h. After the treatment, total RNA was analyzed for the levels of the indicated mRNAs by regular RT-PCR using specific primers. (C) Total RNA was prepared from splenocytes isolated from wild-type and age-matched *Stat1*-deficient male or female mice (age ~9 weeks). Steady state levels of *Aim2* mRNA were analyzed by analyzed by quantitative TaqMan real-time PCR using the assay specific to the *Aim2* gene. 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). The relative steady-state levels of *Aim2* mRNA in male wild-type mice are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation. (D) Splenocytes isolated from B6 female mice (age ~9 weeks) were either left untreated or treated with IFN-α for 14 h. Steady state levels of mRNAs corresponding to the *Aim2*, *Ifi202*, and *Ifi204* genes were analyzed by quantitative TaqMan real-time PCR as described in the panel (C). The relative steady-state levels of mRNAs for all genes in control untreated Splenocytes are indicated as 1. (E) Purified splenic T or B cells from B6 female mice (age ~9 weeks) were either left untreated or treated with IFN-α for 16 h. Steady state levels of mRNAs corresponding to the *Aim2* and *Ifi202* genes were analyzed by quantitative TaqMan real-time PCR as described in the panel (D). The relative steady-state levels of mRNAs for both genes in control untreated T cells are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation.

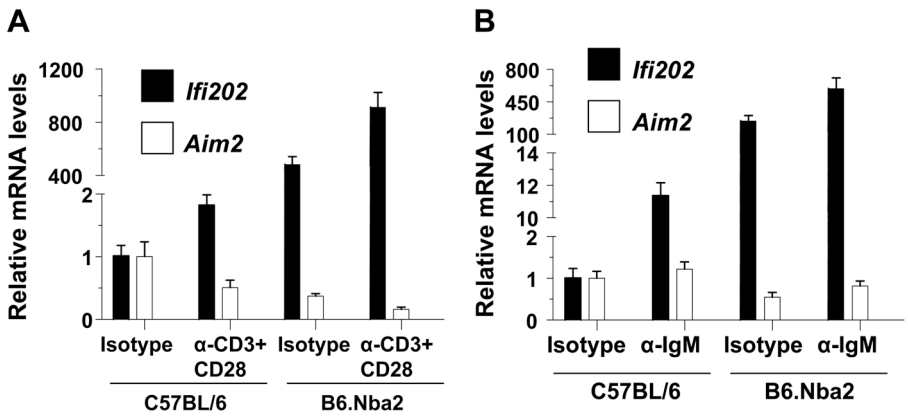

Fig. 2.

Stimulation of T and B cells down-regulates the expression of *Aim2* gene. (A) Splenic cells (cells pooled from two or more age-matched mice) from the B6 or B6.*Nba2* female mice (age ~9-weeks) were either incubated with an isotype control antibody or with α-CD3 and α-CD28 for 22 h. After the incubations, total mRNA were isolated and the steady-state levels of mRNA were analyzed by quantitative TaqMan real-time PCR for *Ifi202* and *Aim2* genes. The relative steady-state levels of mRNAs for both genes in control isotype treated cells are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation. (B). Splenic cells from B6 or B6.*Nba2* female mice as noted in the panel (A) were either incubated with an isotype control antibody or with α-IgM for 20 h and the steady-state levels of mRNA were analyzed by quantitative TaqMan real-time PCR for *Ifi202* and *Aim2* genes. The relative steady-state levels of mRNAs for both genes in control isotype treated splenic cells are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation.

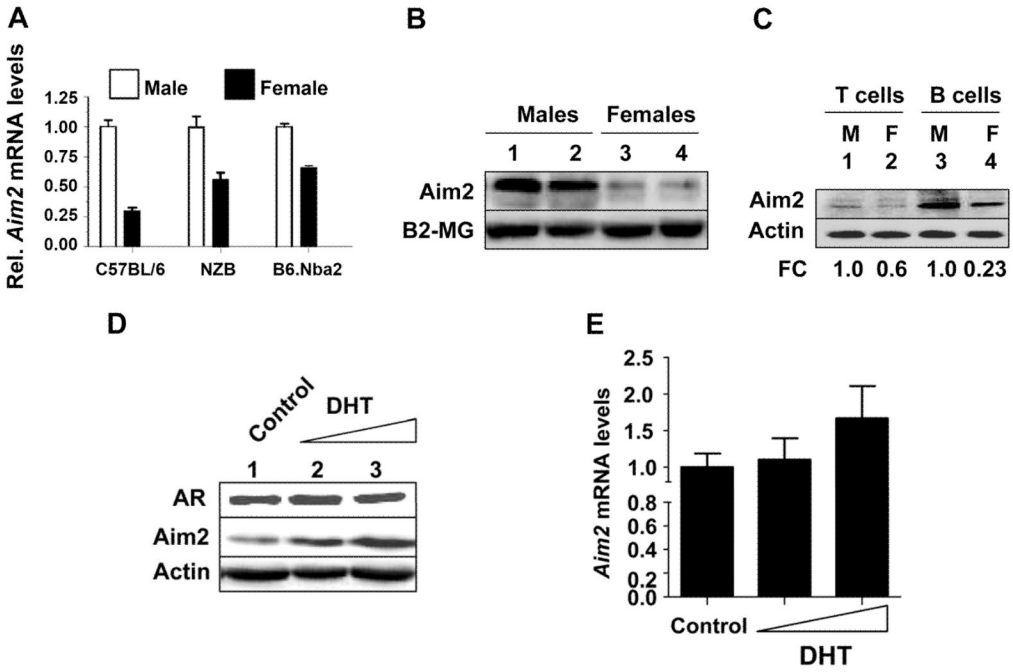

Fig. 3.

Gender-dependent factors regulate the expression of *Aim2* gene. (A) Total RNA was prepared from splenocytes isolated from the indicated strain of male or age-matched female mice (age ~9 weeks). Steady state levels of *Aim2* mRNA were analyzed by 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). The relative steady-state levels of *Aim2* mRNA in male mice of all strains are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation. (B) Total cell lysates prepared from B6 splenic cells isolated from male or age-matched female (age ~8 weeks) mice were analyzed by immunoblotting using antibodies specific to the indicated proteins. (C) Total cell lysates prepared from B6 T or B cells purified from male or age-matched female (age ~8 weeks) mice were analyzed by immunoblotting using antibodies specific to the indicated proteins. (D) Sub-confluent cultures of WT-276 cells were either left untreated (lane 1), treated with 5 nM (lane 2), or 10 nM DHT in phenol-free DMEM medium (supplemented with 10% charcoal stripped fetal bovine serum) for 18 h. Total cell lysates were analyzed by immunoblotting using antibodies specific to the indicated proteins. (E) Sub-confluent cultures of WT-276 cells were either left untreated or treated with DHT as described in panel (D). Total RNA was analyzed for levels of *Aim2* MRNA by real-time PCR. The relative steady-state levels of *Aim2* mRNA in untreated cells are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation.

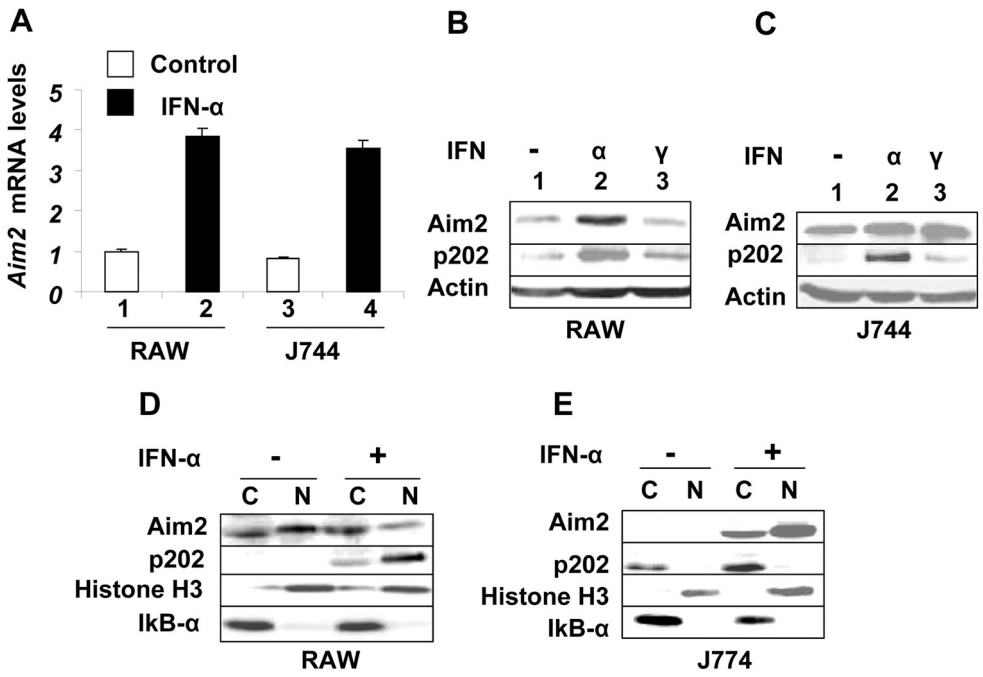

Fig. 4.

Differentially regulation of *Aim2* and *Ifi202* expression by IFNs in macrophage cell lines. (A) Sub-confluent cultures of macrophage RAW264.7 or J774.A1 cell lines were either left untreated or treated with IFN-α (1,000 u/ml) for 16 h. Total RNA was isolated and analyzed by real-time PCR for increases in *Aim2* mRNA levels. The relative steady-state levels of *Aim2* mRNA in untreated cells are indicated as 1. (B and C) Cells of RAW264.7 (B) or J774.A1 (C) macrophage cell lines were either left untreated (lane 1), treated with IFN-α (lane 2), or IFN-γ (10 ng/ml) for 16 h. Cells were harvested and total cell lysates were analyzed by immunoblotting for the indicated proteins. (D and E) Sub-confluent cultures of RAW264.7 (D) or J774A.1 (E) macrophage cell lines were either left untreated or treated with IFN-α (1,000 u/ml) for 16 h. Cells were fractionated into the cytoplasmic (Cyt) and nuclear (Nu) fractions and fractions containing equal amounts of proteins were analyzed by immunoblotting using specific antibodies to the indicated proteins. Levels of IκBα protein in the cytoplasm and histone H3 in the nucleus served as a quality control for the cell fractionations.

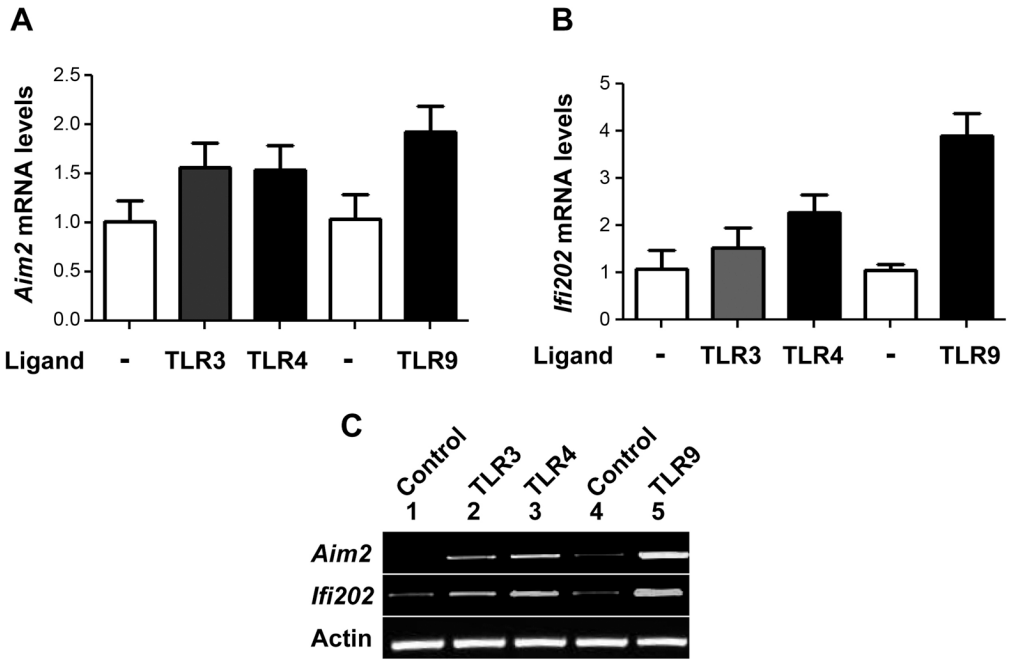

Fig. 5.

Toll-like receptor signaling differentially regulates the expression of *Aim2* and *Ifi202* genes in BMDMs. (A-C) BMDMs from B6.*Nba2* female mice were either left untreated or treated with the indicated TLR ligand for 16 h. Total RNA was isolated and analyzed by real-time PCR (A and B) or regular PCR (C) for steady-state levels of *Aim2* and *Ifi202* mRNA. Levels of mRNA in untreated cells or cells treated with non-stimulatory TLR9 control ODN are indicated as 1. Results are mean values of triplicate experiments and error bars represent standard deviation.

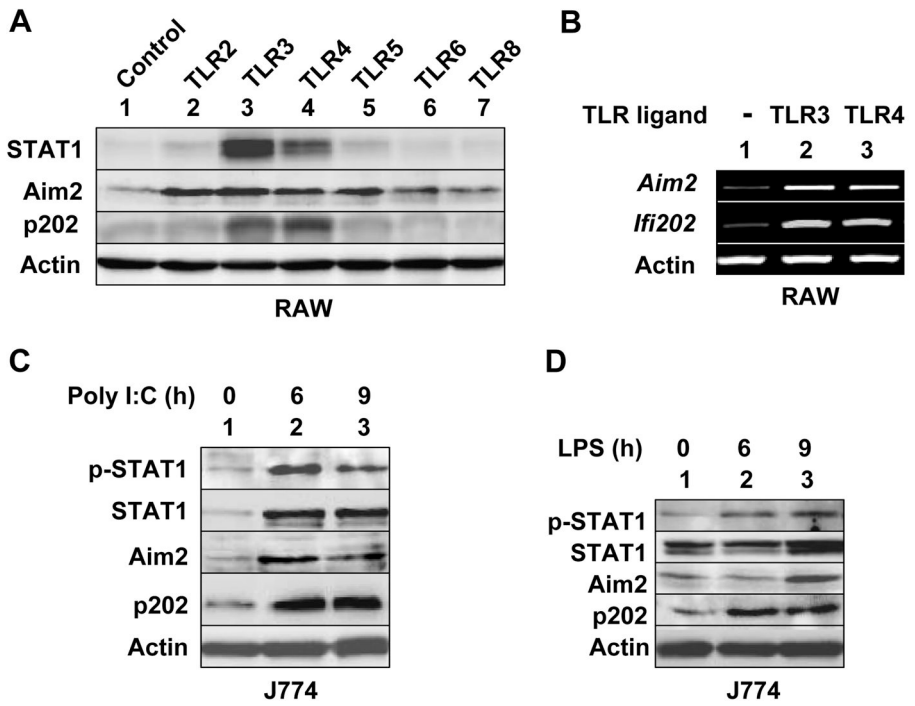

Fig. 6.

Toll-like receptor signaling differentially regulates the expression of Aim2 and p202 proteins in macrophage cell lines. (A) Sub-confluent cultures of RAW264.7 cells were either left untreated or treated with the indicated TLR ligand for 6 h. After the treatments, cells were harvested and total cell lysates were analyzed by immunoblotting. (B) Raw264.7 cells were either left untreated or treated with the indicated TLR ligand for 6 h. Total RNA was isolated and analyzed by regular PCR. (C and D) Sub-confluent cultures of J774.A1 cells were either left untreated or treated with the TLR3 ligand (polyI:C ; 10 μg/ml) (C) or TLR4 ligand (LPS; 100 ng/ml) for the indicated time. Cells were harvested and total cell lysates were analyzed by immunoblotting using antibodies specific to the indicated proteins.

Table 1

Differential regulation of *Aim2* and *Ifi202* genes in immune cells

Treatment	**Cell type**	***Aim2***	***Ifi202***
IFN-α	BMDMs	Increase	NT
IFN-α	T and B cells	Decrease	Increase
α-CD3+ α-CD28	T cells	Decrease	Increase
α-IgM	B cells	Decrease	Increase
Androgen	WT-276	Increase	NT
Poly(I:C)	BMDMs	Increase	Increase
LPS	BMDMs	Increase	Increase
TLR9 ligand	BMDMs	Increase	Increase

BMDMs, Bone marrow-derived macrophages; NT, Not tested, LPS, Lipopolysaccharide