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Stimulation of T cells up-regulates expression of *Ifi202*, an interferon-inducible lupus susceptibility gene, through activation of JNK/c-Jun pathway

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Abstract

Studies have revealed that increased expression of interferon (IFN)-inducible *Ifi202* gene (encoding p202 protein) in splenic B and T cells from B6.Nba2 congenic (congenic for *Nb2* locus derived from NZB mice) female mice is associated with lupus susceptibility. However, signaling pathways that regulate *Ifi202* expression in immune cells remain to be elucidated. Here we report that stimulation of T cells up-regulates the *Ifi202* expression. We found that steady-state levels of *Ifi202* mRNA and protein were detectable in splenic T cells from NZB mice and stimulation of T cells with anti-CD3 and anti-CD28 up-regulated expression of the *Ifi202* gene. Similarly, stimulation of cells of a mouse T-cell hybridoma cell line (2B4.11) also activated transcription of the *Ifi202* gene. Significantly, up-regulation of *Ifi202* expression in stimulated T cells was inhibited by treatment of cells with SP600125, a specific inhibitor of c-Jun N-terminal kinase (JNK). Conversely, treatment of cells with anisomycin, a potent activator of the JNK and c-Jun, up-regulated *Ifi202* expression. Consistent with the activation of JNK/c-Jun pathway by T cell stimulation, forced expression of c-Jun in 2B4 T-cells and in mouse embryonic fibroblasts (MEFs) also up-regulated the *Ifi202* expression. Furthermore, we found that stimulation of T cells increased association of the activated c-Jun to the 5'-regulatory region of the *Ifi202* gene in chromatin immunoprecipitation assays (ChIPs). Together, our observations demonstrate that stimulation of T cells up-regulates the *Ifi202* expression in part through the JNK/c-Jun pathway.

Keywords

T cell stimulation; Interferon-inducible *Ifi202*; Lupus susceptibility; Autoimmunity; JNK; c-Jun/AP-1

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1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterized by production of pathogenic IgG autoantibodies [1–4]. The disease has a gender bias in its development and predominantly affects women of childbearing age [1,5–7]. Genetic linkage studies have indicated that SLE is a polygenic disease [8–11]. Consequently, multiple chromosomal loci (and genes) and signal transduction pathways may be involved in the development of the disease [8,9,12].

Generation of B6.Nba2 mice congenic for *Nba2* interval (derived from NZB strain), which contains the NZB allele of the interferon (IFN)-activatable *Ifi202* gene on the C57BL/6 (B6) background, has identified *Ifi202* as lupus susceptibility gene [13,14]. Studies also showed that B6.Nba2 mice had increased expression of *Ifi202* mRNA and protein (as compared to B6 control mice) in their splenic cells. The increased expression of p202 in splenic B and T cells was associated with the accumulation of B and T cells in spleens (resulting in splenomegaly), apoptosis-resistance in B cells, and increased lupus susceptibility [13].

T cells are thought to play a central role in the pathogenesis of both human and murine lupus [15–17]. For example, compared with normal controls, in which a balance of T cell functions exists, diverse T cell dysfunctions are known to result in an imbalance in the functions of T cell subsets in SLE [15]. Moreover, studies have provided evidence for abnormal T cell signal transduction in SLE [16,17]. The molecular mechanisms that cause T cell dysfunctions in SLE remain largely unknown.

Activation of c-Jun N-terminal kinase (JNK), which is also known as stress-activated protein kinase, is an important downstream event after T cell receptor (TCR) stimulation [18–20]. The JNK family belongs to the mitogen-activated protein kinase (MAPK) super families, which also includes the ERK and p38 families [19]. In T-cells, JNK is implicated in the integration of TCR and CD28 signals, which may play a role in T-cell activation and interleukin-2 production [18]. Furthermore, studies using *jnk1*-null and *jnk2*-null mice have indicated that both JNK1 and JNK2 are required for the differentiation of CD4⁺ T cells into effector Th1 cells [21,22]. These results highlight the importance of the JNK signaling cascade in T cell activation and differentiation.

JNK phosphorylates the transcription factor c-Jun and increases the transcriptional activity of AP-1 [19,23]. Signals from both the TCR-CD3 complex and CD28 are required for JNK and AP-1 activation in T cells, and these signals may be integrated in a such a way as to mediate T cell activation and the induction of IL-2 transcription [19]. Moreover, AP-1 has also been reported to be important for the regulation of Th1 and Th2 cytokine genes [19,24].

The AP-1 transcription factor modulates the immune system through several different mechanisms [24]: (i) by activation of cytokine production in cooperation with transcription factors of the NFAT family in different cell types; (ii) by regulating differentiation of naïve T-cells into T-helper 1 (Th1) or Th2 cells; and (iii) by modulating the activity of the innate immune system. Most cytokine genes (including the IL-2 and IFN- γ) are regulated cooperatively by a transcription factor complex consisting of AP-1 and NFAT [24,25]. However, it remains unclear how these cytokine-induced signaling pathways regulate the expression of genes that affect various functions of the T cells.

To elucidate the molecular mechanisms by which the expression of *Ifi202* is regulated in T-cells, we explored whether stimulation of T cells could regulate expression of the *Ifi202* gene. We report that stimulation of T cells activates *Ifi202* transcription through the JNK/c-Jun signal transduction pathway.

2. Materials and methods

2.1. Cells, cell lines, and treatments

Age-matched (9–15 weeks of age) male and female NZB/Ola (NZB) mice (purchased through Harlan, Indianapolis, IN, USA) were housed in the animal facilities of Loyola University Medical Center, Maywood, IL. The experimental protocol to use mice for studies included in this manuscript was approved by the Loyola University's Institutional Animal Care and Use Committee (IACUC).

Total single cell splenocytes were obtained from mice after lysis of red blood cells. Isolated splenocytes were re-suspended in RPMI-1640 (Gibco-BRL) with 10% fetal bovine serum (Gibco-BRL), 1% penicillin/streptomycin/glutamate (Gibco-BRL) and 1x MEM non-essential amino acids/sodium pyruvate (Gibco-BRL). Mouse T-cell hybridoma line 2B4.11 [26] was generously provided by Dr. Michael J. Bevan (University of Washington, Seattle, WA). The 2B4.11 mouse T-cell hybridoma cells (indicated as 2B4 cells) were maintained in RPMI-1640 medium (high glucose) supplemented with 10% fetal bovine serum and antibiotics. Mouse embryonic fibroblasts (MEFs) null for c-Jun were generously provided by Dr. Ron Wisdom [27]. These MEFs were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics.

For separation of pure T- or B-cells from total spleen cells, we used mouse T cell negative isolation kit (cat # 114.13; from Dynal, Carlsbad, CA) or mouse B cell negative isolation kit (cat # 114.21; from Dynal), respectively, as suggested by the supplier. The purified (>95% pure) T- and B-cells were used immediately for further experiments.

For stimulation of T cells, freshly isolated splenocytes ($2-4 \times 10^6$) or 2B4 cells were plated in 60 mm plastic cell culture plates either coated with purified hamster anti-mouse CD3 epsilon ($10 \mu\text{g}/\text{plate}$; cat # 16-0031 from eBioscience, San Diego, CA) antibody or purified golden Syrian hamster IgG ($5 \mu\text{g}/\text{plate}$; cat # 16-4914; from eBioscience) isotype control antibody as suggested by the supplier. Purified anti-mouse CD28 antibody ($2 \mu\text{g}/\text{ml}$; cat # 16-0281; from eBioscience) was added to the medium immediately after plating of fresh splenocytes or 2B4 cells. After stimulation of T cells for the indicated time, cells were collected and processed for isolation of total RNA or protein. When indicated, cells were treated with JNK inhibitor SP600125 ($10 \mu\text{M}$ in DMSO, from Calbiochem, San Diego, CA) [ref. 28] or anisomycin ($20 \mu\text{M}$ in DMSO, from Sigma) [ref. 29] at the indicated concentrations for the indicated duration.

2.2. Plasmids and expression vectors

Mammalian expression vector pCMV-SPORT6-c-Jun (pCMV-c-Jun) encoding mouse c-Jun protein and an empty vector pCMV were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia).

2.3. Nucleofections

2B4 cells ($2-3 \times 10^6$) or c-Jun-null MEFs (2×10^6) were nucleofected with pCMV or pCMV-c-Jun plasmid ($2 \mu\text{g}$; plasmids prepared using a kit from Qiagen that allows endotoxin free preparation) using Nucleofector-II device (Amaxa Biosystems, Germany). Nucleofection procedure for 2B4 cells was optimized using different nucleofection kits and programs. Finally, we chose the Nucleofection Kit VCA-1001 (Amaxa Biosystems, Germany) and the program R-020 to nucleofect 2B4 cells. This procedure consistently gave us relatively high (40–50%) cell survival and allowed us the detection of the exogenous c-Jun expression in cell lysates by immunoblotting. For MEFs, we used a different nucleofection kit (# VPD-1004) with the program A-023. 24–48 h after nucleofections, nucleofected cells were processed for immunoblotting.

2.4. Immunoblotting

Splenocytes or fibroblasts were collected in PBS and re-suspended in a modified RIPA-lysis buffer (50 mM Tris-HCl, pH 8.0; 250 mM NaCl, 1% NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS), supplemented with protease inhibitors (protease inhibitor cocktail, EDTA free from Roche, Mannheim, Germany) and incubated at 4° C for 30 min. Cell lysates were sonicated briefly before centrifugation in a microfuge for 10 min at 4° C. The supernatants were collected and a Bio-Rad protein assay kit measured the protein concentrations. Equal amounts of protein were processed for immunoblotting. Antiserum to p202 has been described previously [30].

2.5. Isolation of RNA and RT-PCR

Total RNA was isolated using Trizol reagent (Gibco-BRL), and was digested with DNase I (to remove any DNA in the preparation). Isolated total RNA (1 µg) was subjected to cDNA synthesis using SuperScript First-Stand Synthesis system (Invitrogen) as suggested by the supplier followed by PCR using a pair of primers specific to the *Ifi202a* cDNA (forward primer: 5'-ggtcattacc aactcagaat-3'; reverse primer: 5'-ctcta ggatgccactgctgttg-3') as described previously [31]. For RT-PCR, we used the Superscript one-step RT-PCR system from Gibco-BRL (Grand Island, NY). A SuperArray (Frederick, MD) MultiGene-12 RT-PCR profiling kit for mouse NF-κB/TNF-α-inducible genes was used as suggested by the supplier to determine alterations in the expression of NF-κB/TNF-α-inducible genes after overexpression of p202 protein in 2B4 cells. After PCR, the amplified DNA fragments were analyzed by agarose gel electrophoresis.

2.6. Chromatin immunoprecipitation assays

2B4 cells (5×10^6) were either stimulated with anti-CD3e and anti-CD28 or an isotype control antibody as described above. After 24 hours of stimulation, cells were cross-linked for 10 min at room temperature by directly adding 1/10th medium volume of cross-linking reagent (11% formaldehyde, 100 mM NaCl, 0.5 mM EGTA, 50 mM HEPES, pH 8.0) to the plate. The cross-linking reaction was quenched by adding 1/10th volume of 1.25 M Glycine and incubating cells at room temperature for 5 min. Cells were centrifuged and washed (twice) with PBS. Cells were re-suspended in 500 µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, supplemented with the protease inhibitors) and tubes were incubated on ice for 10 min with occasional mixing. The cell lysates were sonicated (4 times, 15 seconds each) to break chromatin and processed for the reversal of cross-linking as described previously (33). The cell lysates were diluted to a final volume of 1 ml with a mixture of 9-parts of the dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH8.0, plus protease inhibitors) and 1 part of cell lysis buffer. The diluted cell lysates were first pre-cleared by incubating with protein A/G beads (Pierce) for 30 min at 4°C. After pre-clearing, the cell lysates (containing equal amounts of proteins) were divided into two fractions: one fraction was subjected to immunoprecipitation using mouse monoclonal anti-phosphor-c-Jun antibody (4 µg/ml), another fraction, was immunoprecipitated with the same amount of mouse normal IgG antibody as a control. The immunoprecipitates were collected using protein A/G beads. Beads were centrifuged, washed (5-times) in 1 ml of washing buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH8.0). The final wash was with a buffer containing 1% Triton X-100, 0.1 % SDS, 500 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0). Immune complexes were eluted from beads by adding 450 µl of elution buffer containing proteinase-K and RNase A (500 µg/ml each), and by incubating tubes at 37°C for 30 min. Tubes were centrifuged and the supernatants containing DNA were collected. The DNA was purified, precipitated, washed, and dissolved in water. Equal volumes of DNA were used to perform PCR to detect the promoter region of the *Ifi202* gene. The following primers were used: region 383 to 402 forward primer (5'-gtgtctagtggccagtgtac-3') and region 804 to 784 reverse primer (5'-tctgcagtgtat gtacagatcc-3') (see Fig. 7 in ref. 32). The PCR amplification

conditions were: incubation at 94°C for 5 min, followed by 94° C for 30 seconds, 45° C for 30 seconds, 72° C for 45 seconds. Total 40 amplification cycles were performed. The PCR products were analyzed by agarose gel electrophoresis.

2.7. Flow-cytometric analysis

Flow-cytometry was performed on single cell suspensions of 2B4 cells. Briefly, to measure accumulation of cells with sub-G₁ DNA content after stimulation of TCR-signaling, cells were stained with propidium iodide (50 µg/ml, Sigma) and subjected to flow-cytometry as described previously [33].

3. Results

3.1. Expression of *Ifi202* is detectable in splenic T-cells

Our previous study [13] indicated that expression of *Ifi202* mRNA is detectable in splenic B and T cells from B6.Nba2 congenic female mice. The study also indicated that levels of the *Ifi202* mRNA were relatively lower in the T-cells than in the B-cells. Because expression levels of p202 protein in cells are regulated by both transcriptional and post-transcriptional mechanisms [14,34], we sought to determine whether splenic T-cells from the NZB mice (a high p202 expressing strain, ref. 13) express detectable levels of *Ifi202* mRNA and protein. Consistent with our previous observations [13], we were able to detect (Fig. 1A) steady-state levels of *Ifi202* mRNA in purified T-cells from the NZB mice. Moreover, we found that p202 protein levels were detectable (Fig. 1B) in purified splenic T- cells. Interestingly, the steady-state levels of the p202 protein in purified splenic T cells were reproducibly lower than B-cells (compare lane 3 with 4). Furthermore, consistent with our previous observations (13), in extracts from NZB splenocytes, but not in extracts from B6.Nba2 splenocytes (and other cell lines), the p202 protein band was not sharp, but diffused (Fig. 1B, and data not shown). Because p202 protein is known to be modified by post-translational modifications [14,30], it seems likely that the NZB mouse strain-specific post-translational modifications of p202 protein affect its mobility on SDS-PAGE. Significantly, in three independent experiments, we found a good correlation between the *Ifi202* mRNA levels and the p202 protein levels in splenic T cells from the NZB mice.

3.2. Stimulation of splenic T cells up-regulates *Ifi202* expression

To test whether stimulation of T cells regulates *Ifi202* expression, we incubated splenocytes from age-matched NZB male or female mice with either control isotype antibody or anti-CD3 and anti-CD28 antibodies (because signals from both the TCR-CD3 complex and CD28 activate T cells; ref. 19) for 24 h. As shown in Fig. 2A, we found that steady-state levels of *Ifi202* mRNA were relatively low in male mice than female mice (Fig. 2A, compare lane 1 with 3) and stimulation of T-cells with anti-CD3 and anti-CD28 resulted in increases in *Ifi202* mRNA levels. Interestingly, the extent of increase in *Ifi202* mRNA levels was more pronounced in male splenocytes (compare lane 2 with 1) than female splenocytes (compare lane 4 with 3). Consistent with mRNA levels, we also noted that stimulation of T cells resulted in increases in p202 protein levels (Fig. 2B). Again, the increase in p202 protein levels was relatively more pronounced in male splenocytes (compare lane 4 with 3) than female splenocytes (compare lane 6 with 5). Together, these observations suggested that stimulation of splenic T-cells up-regulates *Ifi202* expression.

3.3. Stimulation of a T-cell hybridoma cell line up-regulates *Ifi202* expression

The mouse 2B4 T-cell hybridoma cell line has been extensively used to study signaling pathways after stimulation of cells [26,35]. Therefore, to identify signaling pathways that contribute to transcriptional activation of *Ifi202* gene in stimulated T cells, we explored whether

stimulation of 2B4 cells up-regulates the expression of *Ifi202*. As shown in Fig. 3A, stimulation of 2B4 cells with α -CD3 and α -CD28 resulted in up-regulation of *Ifi202* mRNA after ~12 h. However, the maximum increase in *Ifi202* mRNA levels was evident after 24 h of stimulation. Of note, levels of *Ifi202* mRNA remain relatively high even after 36 h of stimulation. Consistent with our previous observation (Fig. 2), increases in the *Ifi202* mRNA levels in 2B4 cells after stimulation also correlated with increases in p202 protein levels (Fig. 3B). Furthermore, we noted that p202 protein levels decreased about 50% (as compared to stimulation after 24 h; compare lane 6 with 5) after 36 h of stimulation of cells in three independent experiments. Together, these and the above observations clearly demonstrated that stimulation of splenic T and 2B4 cells resulted in transcriptional activation of the *Ifi202* gene.

3.4. Up-regulation of *Ifi202* expression after T cell stimulation depends on the activation of the JNK/c-Jun pathway

Stimulation of mouse splenic T cells is known to activate the Jun N-terminal Kinase (JNK) [18–20], resulting in activating phosphorylation of c-Jun and up-regulation of c-Jun protein levels by auto-stimulation of its expression [19,23]. Therefore, we tested whether the stimulation of 2B4 cells by α -CD3 and α -CD28 results in phosphorylation of c-Jun. As shown in Fig. 4A, the stimulation of 2B4 cells resulted in activating phosphorylation of c-Jun after 12 h (compare lane 4 with lane 2 or 3), which correlated with up-regulation of c-Jun protein levels (compare lanes 4–6 with lane 2 or 3).

Next, to examine the role of JNK/c-Jun in up-regulation of *Ifi202* expression, we stimulated the 2B4 cells in the absence and the presence of SP600125 (SP), a specific inhibitor of JNK [28], and examined the steady-state levels of *Ifi202* mRNA and protein. Consistent with our above observations (Fig. 3), we were able to detect the basal levels of *Ifi202* mRNA levels in control 2B4 cells (Fig. 4B, lane 1) and stimulation of cells for 12 or 24 h resulted in up-regulation of *Ifi202* mRNA levels (compare lane 2 or 3 with lane 1). Furthermore, treatment of cells with the inhibitor strongly inhibited the up-regulation of *Ifi202* mRNA after stimulation of cells (compare lane 6 with 3). Similarly, consistent with inhibition of *Ifi202* mRNA up-regulation by SP inhibitor, we also observed that treatment of cells with SP inhibitor also resulted in inhibition of up-regulation of p202 protein levels after stimulation of 2B4 cells for 12 or 24 h (Fig. 4C).

Treatment of cells with anisomycin activates JNK and the activated JNK activates c-Jun by phosphorylation [19,29]. Therefore, to further examine the potential role of JNK/c-Jun signal transduction pathway in up-regulation of *Ifi202* expression in stimulated T cells, we treated 2B4 cells with anisomycin for increasing length of time and examined the p202 protein levels in cell extracts. As shown in Fig. 4D, treatment of cells with anisomycin, which resulted in activating phosphorylation of c-Jun within 30 min (compare lane 4 with 3), transiently up-regulated the expression levels of p202 (compare lanes 4–6 with lane 3).

Up-regulation of *Ifi202* expression through the activation of JNK and c-Jun, prompted us to determine whether stimulation of splenic T cells, which resulted in up-regulation of p202 (Fig. 2), also resulted in activation of c-Jun. As shown in Fig. 4E, the stimulation of splenic T cells from NZB mice resulted in activating phosphorylation of c-Jun in 16 h, which was associated with up-regulation of c-Jun protein levels after 16 and 24 h of stimulation. Together, these observations are consistent with up-regulation of *Ifi202* expression through the JNK/c-Jun signal transduction pathway in splenic T cells after their stimulation.

3.5. Ectopic expression of c-Jun up-regulates *Ifi202* expression

To further examine whether stimulation of T cells up-regulates the expression of *Ifi202* through up-regulation of c-Jun protein expression, we chose to overexpress c-Jun protein in 2B4 cells.

As shown in Fig. 5A, nucleofection of 2B4 cells with a plasmid encoding mouse c-Jun, but not pCMV vector, resulted in up-regulation of *Ifi202* mRNA in a dose-dependent manner. Moreover, forced expression of c-Jun in 2B4 cells also resulted in up-regulation of p202 protein (Fig. 5B).

The above observations that forced expression of c-Jun protein in 2B4 cells up-regulated the expression of *Ifi202* mRNA and protein prompted us to compare basal un-induced levels of *Ifi202* mRNA between mouse embryonic fibroblasts (MEFs) from wild-type (c-Jun^{+/+}) and c-Jun-null (c-Jun^{-/-}) mice. These experiments revealed that the basal levels of *Ifi202* mRNA were lower in c-Jun-null than the wild-type MEFs (data not shown). This observation prompted us to determine whether the overexpression of c-Jun expression in MEFs could up-regulate the expression of *Ifi202*. As shown in Fig. 5C, nucleofection of MEFs with pCMV-c-Jun plasmid, but not pCMV vector, resulted in up-regulation of p202 protein (compare lane 4 with 2). Furthermore, treatment of cells (that were expressing c-Jun protein) with anisomycin further increased p202 protein levels (compare lane 5 with 4). Together, these observations demonstrated that increased expression of c-Jun in 2B4 and splenic T cells after their stimulation contributes to the up-regulation of *Ifi202* expression.

3.6. The c-Jun associates with the 5'-regulatory region of the *Ifi202* gene after stimulation of T cells

Our previous study had revealed that the transcription of *Ifi202* gene is activated by expression of the JunD protein [36], a member of the c-Jun family [37]. Moreover, we found that site-directed mutagenesis of the two potential c-Jun/AP-1 DNA-binding sites in the 5'-regulatory region of the *Ifi202* gene resulted in the lack of stimulation of the activity of 202-luc-reporter after ectopic expression of JunD protein [36]. Because stimulation of splenic T and 2B4 cells up-regulated the expression of *Ifi202* through activation of the JNK/c-Jun signal transduction pathway, we sought to determine whether activated c-Jun increases the expression of *Ifi202* by associating with the 5'-regulatory region of *Ifi202* gene, which contains two AP-1 DNA-binding sites (Fig. 6A), after stimulation of 2B4 cells. As shown in Fig. 6B, we were able to detect association of activated c-Jun (p-c-Jun) in control 2B4 cells with the 5'-regulatory region of the *Ifi202* gene, which contains the two AP-1 DNA-binding sites [36] in chromatin immunoprecipitation assays. Furthermore, stimulation of 2B4 cells by α -CD3 and α -CD28 for 24 h resulted in a 3–4-fold increased association of p-c-Jun with the regulatory region (compare lane 8 with 7) of the *Ifi202* gene. Together, these observations indicated that the increased expression of *Ifi202* in 2B4 cells after the stimulation is associated with increased binding of p-c-Jun to the 5'-regulatory region of the *Ifi202* gene.

3.7. Up-regulation of p202 expression in stimulated 2B4 cells is associated with decreases in levels of I κ B- α and overexpression of p202 in 2B4 cells down-regulates expression of I κ B- α

Increased expression of p202 in a variety of cells is known to modulate cell survival [14,34]. Therefore, we explored whether increased expression of p202 in α -CD3 and α -CD28 stimulated 2B4 cells affects cell survival. Firstly, we noted that transcriptional activation of the *Ifi202* gene in stimulated 2B4 cells was associated with increases (from 14.2% after 6 h of stimulation to 40% after 24 h and 43.8% after 36 h of stimulation) in cell death by apoptosis as determined by propidium iodide staining of cells followed by flow-cytometry to detect the accumulation of cells in the sub-G₁ state (an indicator of apoptosis). Secondly, we noted that the increased expression of p202 in stimulated 2B4 cells was associated with decreases in expression of I κ B- α protein (Fig. 7A), a transcriptional target and an inhibitor of NF- κ B transcription factor [43]. Because increased expression of p202 in stimulated cells was associated with decreases in I κ B- α protein levels and apoptosis, we tested whether overexpression of p202 in 2B4 cells down-regulates expression of I κ B- α . As shown in Fig. 7B, forced expression of p202 in 2B4

cells appreciably down-regulated the expression of I κ B- α mRNA (compare lane 8 between top and bottom panels). Together, these observations provide support for the idea that increased expression of p202 in 2B4 cells decreases cell survival, in part, by inhibiting anti-apoptotic signals, such as NF- κ B.

4. Discussion

In our attempts to elucidate the molecular mechanisms by which the expression of *Ifi202* is regulated in immune cells, we explored whether stimulation of T cells could regulate expression of the *Ifi202* gene. Our experiments revealed that: (i) steady-state levels of *Ifi202* mRNA and protein were detectable in splenic T cells from NZB mice (Fig. 1); (ii) stimulation of splenic T and 2B4.11 murine T cells up-regulated the *Ifi202* expression (Figs. 2 and 3); (iii) treatment of cells with SP600125, a known specific inhibitor of the JNK, inhibited up-regulation of *Ifi202* expression (Fig. 4); (iv) treatment of cells with anisomycin, a potent activator of the JNK and c-Jun, up-regulated the expression of *Ifi202* gene (Fig. 4); (v) ectopic expression of the c-Jun in cells up-regulated *Ifi202* expression (Fig. 5); and (vi) stimulation of T cells increased association of activated c-Jun to the 5'-regulatory region of the *Ifi202* gene in chromatin immunoprecipitation assays (ChIPs) (Fig. 6). Together, these observations demonstrated that stimulation of splenic T cells activates transcription of the *Ifi202* gene through the JNK/c-Jun signal transduction pathway.

Previously, we have noted that activation of the members of the c-Jun family, such as JunD [36] or c-Jun [39], by IFN-independent signaling pathways activates transcription of the *Ifi202* gene. Moreover, we demonstrated that binding of the c-Jun/AP-1 transcription factor to two AP-1 DNA-binding sites (the AP-1CS1 and AP-1CS2) in the 5'-regulatory region of the *Ifi202* gene activates the transcription of *Ifi202* gene in H-Ras transformed NIH 3T3 cells [39]. Therefore, our observations that: (i) stimulation of splenic T and 2B4 cells, which up-regulated the *Ifi202* expression, is associated with activation of the c-Jun/AP-1; and (ii) stimulation of T cells, which activated the JNK/c-Jun pathway, increased the association of c-Jun with the 5'-regulatory region of the *Ifi202* gene in ChIPs, are consistent with our previous observations [36,39] that activation of the AP-1 family of transcription factors in a variety of cells regulates transcription of the *Ifi202* gene.

Increased expression of the p202 (52-kDa) in cell lines inhibits the transcriptional activity of factors, such as E2Fs, p53, NF- κ B, and AP-1 [14,26]. These transcription factors, by modulating transcription of target genes, are known to regulate cell proliferation and survival [14,34]. Consistent with inhibition of the transcriptional activity of the above factors by p202, the increased expression of p202 in cell lines, depending on the cell context, either sensitizes cells to apoptosis [40] or decrease the susceptibility to apoptosis [41,42]. Therefore, our observations that: (i) increased expression of p202 in stimulated 2B4 cells was associated with reduced levels of I κ B- α protein (Fig. 7A) and apoptosis, and (ii) forced expression of p202 in 2B4 cells resulted in down-regulation of I κ B- α mRNA levels (Fig. 7B) provide support for the idea that increased expression of p202 in stimulated 2B4 cells decreases cell survival, in part, by inhibiting anti-apoptotic signals, such as NF- κ B.

We have reported [45] that increased expression of p202 in splenic cells from B6.Nba2 mice is associated with inhibition of p53-mediated transcription of pro-apoptotic genes and inhibition of p53-mediated apoptosis. Because control of cell cycle progression by the c-Jun, a proto-oncogene, is p53-dependent [46], and c-Jun inhibits the p53-mediated transcription [46], our observation that stimulation of splenic T cells up-regulates expression of the *Ifi202* through JNK/c-Jun signal transduction pathway raises the possibility that T cell receptor-mediated activation of the c-Jun/AP-1 transcription factor promotes cell survival through up-regulation of the p202 expression, a negative regulator of p53-mediated apoptosis [33].

Increased expression of *Ifi202* gene in splenic B and T cells derived from female B6.Nba2 congenic (congenic for the *Nba2* interval derived from NZB mice on C57BL/6 mice) mice is associated with production of autoantibodies and defects in apoptosis of B cells [13,14]. Interestingly, a recent study [47] has noted differences between male and female B6.Nba2 mice with respect to production of autoantibodies, raising the possibility that expression of *Ifi202* gene in the B6.Nba2 mice is regulated by gender-dependent factors. Consistent with this idea, we noted that steady-state levels of *Ifi202* mRNA were relatively low in male mice as compared to age-matched female NZB mice (Fig. 2A). Although, gender-dependent factors that contribute to the regulation of *Ifi202* expression remain unknown, our observations that activation of T cells stimulates the expression of *Ifi202* raise the possibility that differential constitutive (or basal) activation of splenic T cells between male and female NZB mice could account for observed differences in expression of *Ifi202* in our experiments. Further work will be needed to examine this exciting possibility.

T cells from SLE patients have defects in transcriptional activity of the NF- κ B (p65/p50 heterodimer) as compared to T cells from normal individuals [46]. Because binding of p202 to NF- κ B (p65/p50 heterodimer) inhibits the transcriptional activity [48], our observation that increased expression of p202 in 2B4 cells inhibits I κ B- α expression will serve the basis to identify the human functional homologue(s) of p202 that inhibit the NF- κ B activity in T cells in human SLE patients.

In summary, our observations provide support for our model (Fig. 8). The model predicts that activation of the JNK/c-Jun pathway in T cells after their stimulation contributes to induction of *Ifi202* mRNA and protein. Our observations will serve basis to understand the molecular mechanisms that contribute to lupus susceptibility.

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Fig. 1. Expression of *Ifi202* is detectable in splenic purified T cells from NZB mice

(A) Total RNA was isolated from freshly prepared purified splenic T cells (lane 3) or splenic cells depleted of T cells (lane 4) as described in methods. The isolated RNA was subjected to semi-quantitative RT-PCR using a pair of primers specific to the *Ifi202* gene or actin. As a control for *Ifi202* expression, total RNA from mouse L929 cells, either left untreated (lane 1) or treated with IFN- α (1,000 u/ml for 24 h) (lane 2), was also subjected to RT-PCR.

(B) Total cell extracts prepared from purified splenic T- (lane 3) or B-cells (lane 4) were subjected to immunoblotting using antibodies specific to the indicated proteins. As a negative and a positive control for immunoblotting, total cell extracts from mouse L929 cells, either left untreated (lane 1) or treated with IFN- α (1,000 u/ml for 24 h) (lane 2), were also subjected to immunoblotting.

Fig. 2. Stimulation of splenic T-cells up-regulates *Ifi202* expression

(A) Freshly isolated splenocytes ($2-4 \times 10^6$) from age-matched (~ 12-weeks) male (lanes 1 and 2) or female (lanes 3 and 4) mice were plated in 60 mm plastic cell culture plates either coated with purified hamster anti-mouse CD3 epsilon ($10 \mu\text{g}/\text{plate}$) antibody or purified golden Syrian hamster IgG ($5 \mu\text{g}/\text{plate}$) isotype control antibody. Purified anti-mouse CD28 antibody ($2 \mu\text{g}/\text{ml}$) was added to the medium immediately after plating of splenocytes. After incubation of cells for 24 h, cells were collected and processed for isolation of total RNA. Isolated RNA was subjected to semi-quantitative RT-PCR using a pair of primers specific to *Ifi202* gene or actin. (B) Freshly isolated splenocytes ($2-4 \times 10^6$) from age-matched male (lanes 3 and 4) or female (lanes 5 and 6) mice were subjected to stimulation of T cells for 24 h as described in (A) above and cells were processed for isolation of total proteins. Total cell lysates were subjected to immunoblotting using antibodies specific to the indicated proteins. As a control, we also analyzed extracts from untreated (lane 1) or IFN-alpha treated (lane 2) L929 cells.

Fig. 3. Stimulation of 2B4 cells up-regulates the expression of *Ifi202* gene

(A) The mouse 2B4 cells ($2-4 \times 10^6$) were incubated with anti-mouse CD3 epsilon ($10 \mu\text{g}/\text{plate}$) and anti-mouse CD28 antibody ($2 \mu\text{g}/\text{ml}$) for either 0 (lane 1), 0.5 (lane 2), 3 (lane 3), 6 (lane 4), 12 (lane 5), 24 (lane 6), or 36 h (lane 7) for stimulation. After the stimulation, cells were collected and processed for isolation of total RNA. The RNA was subjected to semi-quantitative RT-PCR using a pair of primers specific to *Ifi202* gene or actin.

(B) 2B4 cells ($2-4 \times 10^6$) were stimulated with anti-mouse CD3 epsilon ($10 \mu\text{g}/\text{plate}$) and anti-mouse CD28 antibody ($2 \mu\text{g}/\text{ml}$) for either 0 (lane 1), 0.5 (lane 2), 6 (lane 3), 12 (lane 4), 24 (lane 5), or 36 h (lane 6). After the stimulation, cells were collected and lysed. Total cell lysates were subjected to immunoblotting using antibodies specific to the indicated proteins.

Fig. 4. Up-regulation of the *Ifi202* expression after T cell stimulation depends on activation of the JNK/c-Jun pathway

(A) 2B4 cells ($2-4 \times 10^6$) were incubated with either an isotype control antibody (lane 1) or stimulated with anti-mouse CD3 epsilon (10 μ g/plate) and anti-mouse CD28 antibody (2 μ g/ml) for 0.5 (lane 2), 6 (lane 3), 12 (lane 4), 24 (lane 5), or 36 h (lane 6). After stimulation of cells, cells were collected and lysed. Total cell lysates were subjected to immunoblotting using antibodies specific to the indicated proteins.

(B) 2B4 cells ($2-4 \times 10^6$) were incubated with either an isotype control antibody (lane 1 and 4) or stimulated with anti-mouse CD3 epsilon (10 μ g/plate) and anti-mouse CD28 antibody (2 μ g/ml) for 12 h (lanes 2 and 5) or 24 h (lanes 3 and 6) in the absence (lanes 1–3) or presence (lanes 4–6) JNK inhibitor SP600125 (20 μ M). After incubations, cells were collected and processed for isolation of total RNA. The RNA was subjected to semi-quantitative RT-PCR using a pair of primers specific to *Ifi202* gene or actin.

(C) 2B4 cells ($2-4 \times 10^6$) were incubated with either an isotype control antibody (lane 1 and 4) or stimulated with anti-mouse CD3 epsilon ($10 \mu\text{g}/\text{plate}$) and anti-mouse CD28 antibody ($2 \mu\text{g}/\text{ml}$) for 12 h (lanes 2 and 5) or 24 h (lanes 3 and 6) in the absence (lanes 1–3) or presence (lanes 4–6) of JNK inhibitor SP600125 ($10 \mu\text{M}$). After incubations, cells were lysed. Total cell lysates were subjected to immunoblotting using antibodies specific to the indicated proteins.

(D) 2B4 cells ($2-4 \times 10^6$) were incubated without any treatment (lane 1) or, as a control, with DMSO (lane 2), or anisomycin ($20 \mu\text{M}$ in DMSO) for the indicated length of time. After incubations, cells were lysed. Total cell lysates were subjected to immunoblotting using antibodies specific to the indicated proteins.

(E) Freshly isolated splenocytes ($2-4 \times 10^6$) from male mice (~12 weeks of age) were incubated with either control isotype antibody (lanes 1, 3, and 5) or anti-mouse CD3 epsilon and anti-mouse CD28 antibody (lanes 2, 4, and 6) for the indicated time as described in Fig. 2B above and cells were processed for isolation of total proteins. Total cell lysates were subjected to immunoblotting using antibodies specific to the indicated proteins.

Fig. 5. Forced expression of c-Jun in cells up-regulates the *Ifi202* expression and increased levels of the c-Jun in splenic cells from the female mice are associated with increased levels of the p202 (A) 2B4 cells ($2-4 \times 10^6$) were mock nucleofected with the nucleofection reagent (lane 1), pCMV vector (2 μ g; lane 2) or increasing amounts (2 μ g, lane 3; 4 μ g lane 4) of pCMV-c-Jun plasmid. Cells were harvested after 24 h of nucleofections and processed for isolation of total RNA. The RNA was subjected to semi-quantitative RT-PCR using a pair of primers specific to mouse *c-Jun*, *Ifi202* gene or actin.

(B) 2B4 cells ($2-4 \times 10^6$) were either nucleofected with pCMV vector (2 μ g; lane 1) or equal amounts of pCMV-c-Jun plasmid (lane 2). Cells were harvested after 24 h of nucleofections and lysed. Total cell lysates were subjected to immunoblotting using antibodies specific to the indicated proteins.

(C) Immortalized MEFs (c-Jun^{+/+}; $2-4 \times 10^6$ cells) were either nucleofected with pCMV vector (2 μ g; lanes 1 and 2) or equal amounts of pCMV-c-Jun plasmid (lanes 3 and 4). 24 h after nucleofections, cells were either treated with DMSO (lanes 1 and 3) or treated with anisomycin (20 μ M in DMSO; lanes 2 and 4). After 3 h, cells were lysed and total cell lysates were subjected to immunoblotting using antibodies specific to the indicated proteins.

Fig. 6. Activated c-Jun associates with the 5'-regulatory region of the *Ifi202* gene

(A) Schematic presentation of two AP-1 DNA-binding sites in the 5'-regulatory region of the *Ifi202* gene and the relative locations of the PCR primers used after chromatin immunoprecipitations.

(B) Soluble chromatin was prepared from cells either incubated with control isotype antibody (lanes 3, 5, and 7) or stimulated with anti-mouse CD3 epsilon (10 μ g/plate) and anti-mouse CD28 antibody (2 μ g/ml) for 24 h (lanes 4, 6, and 8). Chromatin was incubated with antibodies to p-c-Jun (lanes 7 and 8) or, as a negative control, with an isotype antibody (lanes 5 and 6). DNA was extracted from immunoprecipitates and PCR-amplified using a pair of primers that cover the AP-1 DNA-binding sites in the 5'-regulatory region of *Ifi202* gene (see panel A). As a positive control, we also amplified the input chromatin DNA from control (lane 3) and stimulated T cells (lane 4). As a negative and a positive control for PCR, we also analyzed PCR reactions without addition of any template (lane 1) or after addition of *Ifi202* cDNA (lane 2).

Fig. 7. Increased expression of *Irf202* in stimulated 2B4 cells is associated with decreases in protein levels of IκB-α and forced overexpression of p202 in 2B4 cells down-regulates expression of IκB-α (A) 2B4 cells ($2-4 \times 10^6$) were incubated with either an isotype control antibody (lane 1, for 24 h) or stimulated with anti-mouse CD3 epsilon (10 μg/plate) and anti-mouse CD28 antibody (2 μg/ml) for 0 (lane 2), 6 (lane 3), 12 (lane 4), 24 (lane 5), or 36 h (lane 6). After stimulation of cells, cells were collected and lysed. Total cell lysates were subjected to immunoblotting using antibodies specific to the indicated proteins.

(B) Total RNA was isolated from 2B4 cells (after 24 h) either nucleofected with empty pCMV plasmid (top panel) or pCMV-202 plasmid (bottom panel). The RNA was subjected to RT-PCR using the SuperArray MultiGene-12 RT-PCR profiling kit for NF-κB/TNF-α-responsive genes as described in Material and Methods. After PCR, the amplified DNA fragments were analyzed by agarose gel electrophoresis. Lane M, DNA molecular weight markers (100 bp-ladder); lane 1, Cxcl1 (Chemokine C-X-C motif ligand 1; 166 bp); lane 2, Dusp1 (Dual specificity phosphatase-1, 84 bp); lane 3, Egr1 (Early growth response-1, 180 bp); lane 4, Ier2 (Immediate early response-2, 188 bp); lane 5, il6 (Interleukin-6, 178 bp); lane 7, Nfkb1a (NF-κB inhibitor-α, 146 bp); lane 8, Ptgs2 (Prostaglandin peroxidase synthase-2, 193 bp); lane 9, Sod2 (Superoxide dismutase 2, mitochondrial, 156 bp); lane 10, Klf10 (Kruppel-like factor 10, 135 bp); lane 11, Tnfaip3 (Tumor necrosis factor-α-inducible protein 3, 160 bp); and lane 12, Gapd (Glyceraldehyde-3-phosphate dehydrogenase, 203 bp).

T-cell stimulation up-regulates the *Ifi202* expression

Fig. 8. Stimulation of splenic T cells activates transcription of the *Ifi202* gene through the JNK/c-Jun signaling pathway