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Synoviocyte Innate Immune Responses: II. Pivotal Role of IFN Regulatory Factor 3

Susan E. Sweeney, Trevor B. Kimbler, and Gary S. Firestein

Innate immune responses contribute to synovial inflammation in rheumatoid arthritis. The present study was designed to investigate the contribution of IFN regulatory factor (IRF) 3 and IRF7 to type I IFN-regulated gene expression in synoviocytes. Fibroblast-like synoviocytes were stimulated with polyinosinic-polycytidylic acid (poly [I-C]) after transfection with IRF3 or IRF7 small interfering RNA to knockdown transcription factor expression. Western blots, luciferase assay after transfection with reporter constructs, quantitative PCR, and AP-1 DNA binding assays were performed to evaluate the role of IRF3 and IRF7 in poly (I-C)-induced signaling and synoviocyte gene expression. IRF3 regulates IFN-stimulated response element (ISRE) promoter activity as well as IFN-β, IRF5, IRF7, RANTES, IFN-inducible protein-10, MCP-1, and MIP1α gene expression in response to poly (I-C). IRF7 knockdown modestly decreased a subset of genes and ISRE activity, although the results were not statistically significant. Surprisingly, IRF3 knockdown almost completely blocked expression of additional genes in which the ISRE is not traditionally considered a dominant promoter site in fibroblast-like synoviocytes, including matrix metalloproteinase (MMP)3, MMP9, IL-6, and IL-8. Transcription factor activation studies demonstrated a role for IRF3 in regulation of c-Jun phosphorylation and AP-1 binding. IRF3 rather than IRF7 regulates poly (I-C)-induced type I IFN responses in human synoviocytes by increasing ISRE promoter activity, whereas IRF7 also partially regulates expression of other cytokines and MMP through activation of c-Jun and the AP-1 promoter site. Targeting synoviocyte IRF3 represents a potential approach to suppress diverse mediators while limiting suppression of IRF7-mediated immune responses. The Journal of Immunology, 2010, 184: 7162–7168.

Innate immune responses play a critical role in cell activation and recruitment into the rheumatoid joint (1). TLR and innate sensor recognition of viral and bacterial products can potentially contribute to this sequence of events. In fact, the gene expression profile in rheumatoid arthritis (RA) synovium reflects exposure to TLR ligands and displays characteristic features of the type I IFN signature (2–5). Although the proximal mechanisms involved are not known, the IκB kinase (IKK)-related kinase IKKe appears to participate as a component of the signaling pathway that transduces polyinosinic-polycytidylic acid (poly [I-C])–mediated TLR3 activation in human RA synoviocytes (6). However, IKKe can phosphorylate numerous substrates, and the downstream transcription factors that control expression of type I IFN-regulated genes in RA have not been defined. One possibility is that the IFN regulatory factor (IRF) family, especially IRF3 and IRF7, regulate the IFN response in RA.

Activation of the type I IFN system might contribute to the pathogenesis of many rheumatic diseases. The function and relative hierarchy of kinases and transcription factors that integrate innate responses is cell lineage dependent and varies with the type of stimulus. The synthetic TLR3 ligand poly (I-C) and dsRNA associated with viral infections activate the IKK-related kinases, resulting in phosphorylation, nuclear translocation, and dose-dependent promoter binding of IRF3 and IRF7, NF-κB, and c-Jun (7, 8). Formation of a transcriptional complex, or enhanceosome, results in activation of proinflammatory and IFN-regulated gene expression. In mouse embryonic fibroblasts (MEFs) and plasmacytoid dendritic cells, IRF7 is the master regulator of type I IFN immune responses (9). Constitutive IRF3 mainly controls IFN-β expression, whereas IRF7 is required not only for IFN-α expression but also production of the full IFN signature. Key cell lineage-specific responses to poly (I-C) stimulation of human primary cells of the innate immune system, including dendritic cells, macrophages, endothelial cells, and RA synovial fibroblasts, have been recently described and are more complex than noted in the murine cell systems (10). All of these cell types express TLR3 but differ significantly in their response to stimulation. Of interest, TLR3 stimulation of RA synovial fibroblasts results in activation of IRF3, MAPK, and NF-κB and also TNF-α production.

Most data on TLR-dependent and -independent signaling pathways are derived from murine knockout cells and immortalized cell lines and does not necessarily reflect cell and species-specific responses in primary human cells. We have begun dissecting the poly (I-C)-activated signaling pathway in human fibroblast-like synoviocytes (FLS); initial studies showed that the IKK-related kinase IKKe regulates IRF3 phosphorylation in cultured RA FLS (6). IRF3 phosphorylation is significantly increased in RA compared with osteoarthritic synovial tissue. Although IRF3 is expressed and activated in rheumatoid synovial tissue, the relative contribution of IRF3 and IRF7 to synovial gene expression is not known. The present study was designed to determine whether IRF3 or IRF7 is the primary regulator of type I IFN responses in poly (I-C)-stimulated FLS. In contrast to many other cell types, IRF3 is the dominant transcription factor in primary human RA synoviocytes, whereas the contribution of IRF7...
is relatively modest. In addition, IRF3 regulates other cytokines, chemokines, and matrix metalloproteinases (MMPs) through a novel mechanism that involves c-Jun and the AP-1 promoter site.

**Materials and Methods**

**Preparation of human FLS**

This study was approved by the Institutional Review Board of University of California from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti–phospho-IRF3 Ser396 (9475S) was purchased from Cell Signaling Technology (Danvers, MA). Dr. M. David (University of California San Diego, La Jolla, CA) provided the IFN-β and polyclonal rabbit anti-IRF3 serum. IL-1 was purchased from R&D Systems (Minneapolis, MN).

**Small interfering RNA transfection**

Dose-response and kinetic studies were performed using 1, 3, and 5 μg siRNA at days 3, 5, and 7 to confirm knockdown. A total of 5 × 10^5 FLS (passages 4–6) were transfected with 5 μg IRF3, IRF7, or scramble (sc) control Smartpool small interfering RNA (siRNA; Dharmacon, Lafayette, CO), using normal human dermal fibroblast Nucleofector kit, according to the manufacturer’s instruction (Amaxa, Gaithersburg, MD). Approximately 75–95% decrease in protein expression is achieved using this method. Transfected FLS were allowed to recover overnight, synchronized 24 h, and then stimulated over-night with poly (I-C) prior to lysis on day 5 posttransfection.

**Western blot analysis**

Western blot analysis was performed as described previously (13). Dose-response studies were performed, and FLS were incubated with 0.1% FBS medium, TNF-α (100 ng/ml), IL-1 (1 ng/ml), LPS (1 μg/ml), PGN (50 μg/ml), poly-I-C (20 μg/ml), or IFN-β (1000 U/ml) for various time points up to 24 h. Samples containing 50 μg protein were resolved via 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked and incubated with primary Ab at 4°C overnight, followed by HRP-conjugated secondary Ab. The assay measures phospho-c-Jun binding AP-1 promoter site.

**Statistical analysis**

Statistics were generally performed using the paired Student t test. A comparison was considered significant if p < 0.05.

**Results**

**Activation of IRF3 and IRF7 in human FLS**

We previously showed that IKKε and IRF3 are expressed by FLS and that IKKε can regulate IRF3 phosphorylation (6). To characterize the relative functional hierarchy of IRF3 and IRF7 in synovioyte innate immune responses, FLS were stimulated with cytokines or TLR ligands, followed by Western blot analysis to detect IRF3 phosphorylation (Fig. 1, top panel). Because IRF7 is inducible rather than constitutively expressed, we also measured total IRF7 protein expression. Quantification of phosphorylated IRF3 (P-IRF3) and IRF7 expression in FLS stimulated with each ligand is also shown in Fig. 1, bottom panel. Poly (I-C), IFN-β, IL-1, LPS, and PGN induced IRF7 expression in FLS, and poly (I-C) was the most potent. On the basis of these results, this synthetic TLR3 ligand poly (I-C) was used for subsequent studies of the type I IFN response.

**Kinetics of IRF activation in cultured human FLSs**

To determine the time course of IRF3 and IRF7 activation, we stimulated human FLS with poly (I-C) for up to 24 h (Fig. 2). Phosphorylation of constitutively expressed IRF3 was detected within 2 h of poly (I-C) exposure and persisted for 24 h. Inducible IRF7 protein expression began within 4 h and persisted for the 24-h culture period with peak induction at 16 h. As anticipated, total IRF3 protein did not change significantly during the experiment. IRF7 is not constitutively expressed in the most human FLS lines, and there is minimal baseline protein expression in unstimulated cells.
Targeted knockdown of IRF3 and IRF7

The relative contribution of IRF3 and IRF7 to the type I IFN response was examined by transfecting FLS with IRF3, IRF7, or IRF3 and IRF7 combined (IRF3/7) siRNA or control siRNA (sc), followed by poly (I-C) stimulation. Western blot analysis confirmed effective knockdown of IRF3 and IRF7 protein expression (Fig. 3). IRF3 siRNA completely blocked IRF3 and P-IRF3 protein expression as well as partially inhibited IRF7 protein to the basal unstimulated level. Of interest, knockdown of IRF3, IRF7, or a combination of IRF3 and IRF7 prevented IRF7 induction. Complete inhibition of phosphorylation of IRF3 was observed in cultures treated with combined IRF3/7 siRNA or IRF3 siRNA alone. However, IRF7 siRNA results in less inhibition of IRF3 phosphorylation compared with IRF3 siRNA. In the experiment shown in Fig. 3, IRF7 expression was increased ~2-fold by poly (I-C), compared with an average of 3-fold in other experiments (see Fig. 1).

Role of IRF3 and IRF7 in ISRE promoter activity

The upstream binding of IRF3 and IRF7 to the ISRE in the promoter of the classic IFN response gene IFN-stimulated gene 15 kD protein, dependent only on the ISRE, was evaluated using an ISRE luciferase reporter construct. ISRE promoter activity in poly (I-C)–stimulated FLS was significantly lower in IRF and IRF3/7-deficient FLS (p < 0.03; n = 4 independent experiments; Fig. 4). IRF7 siRNA modestly decreased ISRE activity, but the effect of IRF3 and IRF7 siRNA together was the same as IRF3 siRNA alone. These data demonstrate a primary role for IRF3 in ISRE transcriptional activity in human FLS stimulated with poly (I-C).

Decreased IFN-regulated gene expression in IRF3-deficient synoviocytes

We then studied whether the differences in IRF3 and IRF7 ISRE promoter activity were reflected in poly (I-C)–induced gene expression. IRF3, IRF7, and combined IRF3 plus IRF7 siRNA knockdown was performed, and cells were stimulated with poly (I-C). Quantitative PCR was used to measure IFN-regulated genes IFN-β, IFN5, IRF7, RANTES, IFN-inducible protein-10 (IP-10), MCP-1, and MIP1α mRNA levels. These genes are considered IFN-regulated or IFN-stimulated because they are partially controlled by the upstream ISRE, although other promoter sites are present. Fig. 5A shows that IRF3 deficiency profoundly decreased IFN-β, IFN5, RANTES, IP-10, MCP-1, and MIP1α gene expression (90–100% inhibition; p < 0.01). IRF7 deficiency decreased IRF7 mRNA levels as expected but resulted in only partial inhibition of a subset of IFN-stimulated genes. Of interest, RANTES and IP5 gene expression was not decreased by IRF7 knockdown. The combination of IRF3 and IRF7 siRNA knockdown was similar to IRF3 siRNA alone. IRF3 siRNA appeared slightly more effective compared with IRF7 siRNA, although this was not a statistically significant difference. Because IRF7 is an inducible gene, its siRNA might not be as effective as using IRF3 siRNA to prevent the signal responsible for induction. IRF7 is not constitutively expressed in the majority of human FLS lines, and there is only trace baseline expression in unstimulated cells. This low level of constitutive IRF7 protein was not modified by siRNA.

Decreased proinflammatory and MMP gene expression in IRF3-deficient synoviocytes

We then evaluated the contribution of IRF3 and IRF7 to expression of MMP3 and MMP9 as well as the cytokines IL-6 and IL-8, because
of their role in inflammation and joint destruction in RA (Fig. 5B). The upstream regulatory regions of these genes contain multiple promoter sites. The dominant promoter element involved in MMP gene expression is AP-1 in most cell types including human FLS. IL-6 and IL-8 gene expression is more directly under the control of NF-κB. Interestingly, IRF3 inhibition decreased poly (I-C)–induced expression of MMPs, IL-6, and IL-8 gene expression, whereas the effect of IRF7 knockdown was not significant. Because we have previously demonstrated that IKKe, directly upstream of IRF3, can regulate production of MMPs in synoviocytes (15), we hypothesized that IRF3 contributed to MMP production through modulation of c-Jun binding AP-1.

**IRF3 knockdown decreases binding of phosphorylated c-Jun to AP-1**

Because MMP3 and MMP9 were inhibited by IRF3 knockdown, we investigated a role for IRF3 in AP-1 promoter binding (Fig. 6). Because IRF7 knockdown did not significantly decrease ISRE promoter activity or IFN-regulated gene expression, we studied the effect IRF3 knockdown on AP-1 activity. AP-1 is the dominant promoter element involved in regulation of MMP gene expression. Transcription factor ELISA (TransAM) using an AP-1 oligonucleotide is a quantitative and sensitive method for measuring phosphorylated c-Jun binding to the AP-1 promoter site. This approach can be used for quantitative analysis of transcription factor activation rather than phospho-Western blot, EMSA, or reporter gene assay. IRF3 deficiency decreased poly (I-C)–induced AP-1 binding by 52% compared with control (n = 3; p < 0.02).

**IRF3 activation of c-Jun/AP-1 and MMP gene expression is not mediated by IKKe phosphorylation**

A powerful amplification loop in the type I IFN response can proceed through the IKK-related kinase IKKe (16). This pathway involves IFN-β–mediated activation of IKKe, followed by IKKe phosphorylation of STAT1. The binding of phosphorylated c-Jun to the AP-1 site was inhibited by IRF3 knockdown. We hypothesized that IKKe could mediate activation of c-Jun and MMP gene expression in RA synoviocytes in response to innate receptor activation by poly (I-C). After siRNA transfection of IRF3 or IRF7, poly (I-C)–stimulated FLS lysates were evaluated for IKKe activation by P-IKKε Western blot analysis (Fig. 7). The regulation of c-Jun activation does not involve IKKe phosphorylation (Fig. 7). Inhibition of type I IFN signaling through IRF3/IRF7 knockdown does not alter poly (I-C)–induced phosphorylation of IKKe, suggesting that the amplification loop does not contribute to the IFN response in RA FLS. Other kinases such as TANK-binding kinase 1 (TBK1) or the MAPK activated by poly (I-C) could potentially participate. In addition, IRF3 deficiency might alter formation of the IFN enhanceosome, resulting in decreased c-Jun activation. These possibilities are currently being explored.
experiments.

IRF7, or IRF3 plus IRF7 (IRF3/7). FLS were stimulated with poly (I-C) to produce IFN-

ISRE to produce IFN-

formation, dimerization, nuclear localization, and DNA binding to the

IKK-related kinase IKK

formed cell lines, poly (I-C) stimulation or viral infection results in

inflammation (19–21). In other cell types such as MEFs and trans-

known to be present in the rheumatoid joint contribute to synovial

also possible that endogenous ligands, necrotic debris, and cytokines

identified. Although virus exposure or infection could participate, it is

response in RA synoviocytes or peripheral blood cells have not been

seen in human RA synoviocytes.

The specific ligands or cytokines that activate the type I IFN re-

sponse in RA synovium or peripheral blood cells have not been

identified. Although virus exposure or infection could participate, it is

also possible that endogenous ligands, necrotic debris, and cytokines

known to be present in the rheumatoid joint contribute to synovial

inflammation (19–21). In other cell types such as MEFs and trans-

formed cell lines, poly (I-C) stimulation or viral infection results in

IKK-related kinase IKKe controls distal transcription factors such as

IRF3 and c-Jun in human synoviocytes (6). In the current study, we extended these observations by dissecting how two key IRFs, namely IRF3 and IRF7, contribute to the synoviocyte type I IFN response. In contrast to hematopoietic cells, these experiments identified IRF3, rather than IRF7, as a pivotal transcription factor in human RA synoviocytes.

Discussion

Innate pathways can potentially activate viral and stress-inducible
gene expression of chemokines and cytokines that promote inflam-

ation, cell recruitment, and joint destruction in RA. These signaling

pathways have been implicated in inflammatory arthritis, and the IFN signature induced by innate receptor activation has been observed in diverse autoimmune diseases. In addition to the syno-

vium, an IFN profile has been reported in peripheral blood cells of

a subset of RA patients (17). The relevance of this observation to
disease activity and progression of RA is unknown, because the IFN pathway can be either detrimental or beneficial depending on the relative balance of IFN-β, IL-1 receptor antagonist, and proin-

flammatory chemokines (18). To determine the contribution of this

signaling cascade in RA, we previously examined how the IKK-

related kinase IKKe controls distal transcription factors such as

IRF3 and c-Jun (22, 23). Assembly of a transcription

factor complex in the enhancer region, including c-Jun/ATF2, NF-

κB, and IRFs, amplifies IFN-stimulated gene expression (7, 8). This initial response is followed by activation of IFN-α/β receptor signaling, inducing IRF7 transcription (24). A powerful amplification loop can proceed through the IKK-related kinases, especially IKKe (16). Certain type I IFN-stimulated genes are not activated in the absence of IKKe because the IFN-stimulated gene factor 3 complex does not bind to promoter elements of the affected genes. This pathway involves IFN-β–mediated activation of IKKe, followed by IKKe phosphorylation of STAT1, a component of IFN-

stimulated gene factor 3.

For in vitro synoviocyte studies that could mimic the in vivo environment, we focused on the TLR3 ligand poly (I-C) because it was the most potent activator of IRF3 and IRF7 in cultured FLS. In-

nate TLR-independent sensors in the cytoplasm might also recognize

synthetic dsRNA; however, the concentration used mainly activates

surface TLR3 in human FLS. Activation of RA FLS by poly (I-C) stimulation has been previously demonstrated to result in induction of IFN-regulated and proinflammatory gene expression (1). Previous synoviocyte studies indicate that IKKe regulates c-Jun activation of MMP expression and IRF3-induced transcription of IFN-β and RANTES (6, 15). The sequence of events in RA synoviocytes is similar to other cell types because targeted inhibition of IRF3, IRF7, as well as IRF3 plus IRF7 blocked IRF7 induction. Thus, as anticipated, the IFN-stimulated gene IRF7 requires IRF3 in primary human synoviocytes. No synergy is apparent when comparing IRF3 with the combination IRF3 and IRF7 siRNA. The in vitro knock-
down studies were performed at a relatively early time point, even

though IRF7 expression remains high for at least 48 h (data not shown). We focused on shorter periods of activation to minimize the likelihood of secondary events and amplification through endog-

enously produced cytokines and type I IFNs.

IRF3 and IRF7 bind to the ISRE and regulate transcription of IFN-

stimulated genes that are expressed in rheumatoid joints, including

IFN-β, RANTES, and IP-10 (2–4). Many of these proteins contribute to cell recruitment in RA because of their chemotactic activity. IFN-β, however, might play a more complex role and could potentially suppress inflammation. Some investigators have suggested that IFN-β itself could be used as a therapeutic agent in RA because it decreases MMP, IL-1, and TNF production by synoviocytes (25).

Mice with collagen-induced arthritis injected with fibroblasts expressing IFN-β have less severe disease and decreased bone and cartilage destruction (26). However, a clinical trial using IFN-β in patients with RA showed minimal efficacy despite decreased synovial IL-1, IL-6, and MMP1 (27). An alternative approach in a murine model combined IKKe deficiency with low-dose “replacement” IFN-β, which amplified the anti-inflammatory effects of this pathway (18). Thus, careful dissection of the signaling pathways that regulate the type I
IFN response and identification of the key regulatory IFN response genes could shed light on news ways to enhance anti-inflammatory effects without markedly suppressing host defense in RA patients. We also evaluated the hierarchy of IRF3 and IRF7 in synovioocyte gene expression. In contrast to MEFs and bone marrow-derived cells where IRF7 is paramount, IRF3 is the master regulator of the type I IFN response in human synovioocytes stimulated overnight with poly (I-C). One caveat is that IRF3 could potentially act by blocking IRF7 induction. However, this is unlikely because selective IRF7 knockdown had only a modest effect on expression of IFN-stimulated genes, whereas IRF3 deficiency profoundly blocked all of these same genes. In addition, IRF7 might play a more important role in the type I IFN response at later time points or in response to alternative ligand stimulation. Of interest, IRF3 also controlled IRF5 expression. Similar to IRF7, IRF5 is an IFN-inducible gene, and protein expression is not constitutive but is upregulated by stimulation of FLS with poly (I-C). It is constitutively expressed in subsets of dendritic cells and B cells but inducible in other cell types. IRF5 is also interesting because polymorphisms have been associated with SL (28). However, the genetic contribution of IRF5 variants to RA is controversial (29, 30).

IRF3 deficiency also suppressed expression of some genes implicated in RA FLS that are predominantly regulated by AP-1 (MMPs) or NF-kB (IL-8 and IL-6) promoter elements rather than an ISRE site. As with the classical IFN response genes, IRF7 was less effective than IRF3 in the regulation of these cytokine and MMP genes. Although all three sites are present in the positive regulatory domains of IFN-stimulated genes and interact to amplify responses via formation of a transcription factor enhanceosome, we hypothesized that predominantly AP-1-regulated genes such as MMPs might be directly induced by IRF3 via activation of c-Jun. The quantitative binding of phosphorylated c-Jun to the AP-1 site was inhibited by IRF3 knockdown. The mechanism is uncertain but does not appear to require the IKK-related kinase IKKe (Fig. 7). Other signaling kinases activated by poly (I-C), including JNK, p38, or TBK1, could potentially participate. Alternatively, IRF3 deficiency might prevent complete formation of the IFN enhanceosome, resulting in decreased c-Jun activation. These possibilities are currently being explored.

Because IRF3 is constitutively expressed and involved in immediate antiviral responses, inhibition might alter early innate immunity. IRF7 is expressed in a more limited fashion and is induced transiently in most cells after IFN-β production is initiated. IRF7 can also serve as a critical checkpoint in adaptive immune responses and Ag presentation and might be more important in later stages of arthritis. Thus, targeting the innate signaling pathways and IFN signature can be complex and requires a detailed understanding of each component. Considering the potential pathogenic role of IFN in autoimmune disease and the delicate balance between anti- and proinflammatory effects, dissecting the IFN response could have important therapeutic and safety implications. Our studies are limited to human FLS, and the overall effect of blocking IRF3 might depend on species, cell lineage, and the microenvironment. On the basis of the FLS data, targeting IRF3 in could potentially decrease synovial inflammation while sparing the critical functions of IRF7 in immune cells.

Disclosures

The authors have no financial conflicts of interest.

References


