

# RNA Sensors in Human Osteoarthritis and Rheumatoid Arthritis Synovial Fibroblasts

## Immune Regulation by Vasoactive Intestinal Peptide

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**Objective.** The aim of this study was to analyze both the constitutive and induced expression and function of double-stranded RNA (dsRNA; Toll-like receptor 3 [TLR-3], retinoic acid-inducible gene I [RIG-I], and melanoma differentiation-associated gene 5 [MDA5]) and single-stranded RNA (ssRNA; TLR-7) receptors in osteoarthritis (OA) and rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS), by studying the transcription factors involved and the subsequent effects on antiviral interferon- $\beta$  (IFN $\beta$ ), the proinflammatory CXCL8 chemokine, and matrix metalloproteinase 3 (MMP-3). An additional goal was to study the effect of vasoactive intestinal peptide (VIP).

**Methods.** The expression of TLR-3, TLR-7, RIG-I, and MDA5 in cultured FLS was studied by reverse transcription–polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), immunofluorescence, and Western blotting. Transcription factors were studied using the ELISA-based TransAM transcription factor kit. The expression of IFN $\beta$ , CXCL8 (interleukin-8), and MMP-3 was analyzed by RT-PCR and ELISA.

**Results.** FLS expressed TLR-3, TLR-7, RIG-I, and MDA5. The expression of TLR-3 and RIG-I was higher in RA FLS, while the expression of TLR-7 and MDA5 was higher in OA FLS. Stimulation with poly(I-C) induced the activation of IFN regulatory factor 3 (IRF-3), NF- $\kappa$ B, and activator protein 1 (AP-1) c-Jun as well as the subsequent production of IFN $\beta$ , CXCL8, and MMP-3. VIP reduced the activation of IRF-3 and the production of IFN $\beta$  in both OA and RA FLS. Imiquimod induced the activation of NF- $\kappa$ B, AP-1 c-Fos, and AP-1 c-Jun and the synthesis of CXCL8 and MMP-3. VIP significantly diminished MMP-3 production only in imiquimod-treated RA FLS.

**Conclusion.** The results of this study revealed a prominent function of FLS in the recognition of both dsRNA and ssRNA, which may be present in the joint microenvironment. This study also advances the healing function of the endogenous neuroimmune peptide VIP, which inhibited TLR-3-, RIG-I-, MDA5-, and TLR-7-mediated stimulation of antiviral, proinflammatory, and joint destruction mediators.

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The immune system fights disease through the development of innate and adaptive immune responses. The innate immune system senses the occurrence of infection via the presence of pattern recognition receptors that recognize conserved microbial pathogen-associated molecular patterns as well as endogenous ligands derived from the host. Pattern recognition receptor stimulation produces the expression of proinflammatory cytokines, interferons (IFNs), and costimulatory molecules, which link innate and adaptive immunity (1,2).

There are 2 classes of pattern recognition receptors: transmembrane and cytosolic. Toll-like receptors (TLRs) are transmembrane receptors and are the first

pattern recognition receptors known. Ten TLRs have been identified in humans and are expressed on the cell surface (TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, and TLR-10) and in endosomes (TLR-3, TLR-7, TLR-8, and TLR-9) (3). Helicase retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) are cytosolic pattern recognition receptors that comprise RIG-I and melanoma differentiation-associated gene 5 (MDA5) (4,5). TLR-3, RIG-I, and MDA5 recognize double-stranded RNA (dsRNA). In the cytoplasm, RIG-I binds short dsRNA (length <2 kb) (6,7), whereas MDA5 recognizes complex dsRNA (length >2 kb) (8). The 3 receptors are activated by the synthetic dsRNA poly(I-C) (9). TLR-7 recognizes single-stranded RNA (ssRNA) in endosomes and is activated by the synthetic antiviral compound imiquimod (10). The 4 receptors detect a wide range of RNA viruses, after which TLRs and RLRs initiate signaling cascades that lead to activation and subsequent nuclear translocation of 3 families of transcription factors: IFN regulatory factor 3 (IRF-3) and IRF-7, NF- $\kappa$ B, and activator protein 1 (AP-1) (2).

Rheumatoid arthritis (RA) is an inflammatory/autoimmune disease affecting 1% of the population worldwide (11). Fibroblast-like synoviocytes (FLS) are resident cells involved in RA pathogenesis, with functions in both innate and adaptive immunity (12). RA is caused by the interaction of individual genetic predisposition, environmental factors, and dysregulated immune responses. Among environmental factors, infectious agents are present as pattern recognition receptor ligands that comprise both exogenous factors (bacterial and viral components) (12,13) and endogenous factors (host messenger RNA [mRNA], heat-shock proteins, fibronectin) (14,15). In addition, the expression of TLR-2, TLR-4, and TLR-3 is increased in RA synovial tissue (16–20).

Vasoactive intestinal peptide (VIP) is a molecule shared by the neuroendocrine immune network that is considered a potential candidate for treatment of inflammatory and autoimmune diseases (21–26). Since VIP was described as a beneficial endogenous mediator in collagen-induced arthritis (27), intense interest has been focused on translational research in humans. The prospective therapeutic effects of VIP in human RA cells *ex vivo* have been confirmed (19,20,28–30). VIP and its receptors VPAC<sub>1</sub> and VPAC<sub>2</sub> are expressed in FLS from patients with osteoarthritis (OA) and patients with RA, with a differential expression in both pathologies pointing to a role in the physiopathology of rheumatic diseases (31). The aim of this study was to examine both the expression and function of RNA receptors in FLS and the action of VIP, by studying the

transcription factors involved and the subsequent effects on antiviral, proinflammatory, and matrix-degrading enzyme molecules.

## PATIENTS AND METHODS

**Patients and FLS cultures.** Synovial tissue samples were obtained from patients with RA and patients with OA at the time of knee replacement surgery. All patients with RA fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA (32). The study was performed according to the recommendations of the Declaration of Helsinki and was approved by the ethics committee of the Hospital 12 de Octubre. FLS cultures were established from homogenized synovium in 10% fetal calf serum (FCS)–Dulbecco's modified Eagle's medium (33).

Cultured FLS were used between passages 3 and 9. TLR-3 synthetic ligand poly(I-C) and TLR-7 synthetic ligand imiquimod (both obtained from Invitrogen) were used at 20  $\mu$ g/ml and 15  $\mu$ g/ml, respectively. Stimulated FLS were cultured in the presence or absence of 10 nM VIP (Neosystem).

**Preparation of nuclear and cytoplasmic extracts.** FLS were cultured in 150-mm petri dishes with TLR-specific ligands in the presence or absence of VIP. A Nuclear Extract Kit (Active Motif) was used for preparing nuclear and cytoplasmic extracts. Briefly, cells were scraped into phosphate buffered saline (PBS) containing phosphatase and protease inhibitors, centrifuged, resuspended in hypotonic buffer, and then kept on ice for 15 minutes. After the addition of detergent, lysates were centrifuged at 14,000g for 30 seconds at 4°C. Supernatants containing cytoplasmic extracts were collected and stored at –80°C. The pellets were resuspended in complete lysis buffer and shaken vigorously. After incubation at 4°C and centrifugation at 14,000g for 10 minutes at 4°C, supernatants were collected and stored at –80°C. Protein content was measured using a QuantiPro BCA Assay Kit (Sigma-Aldrich).

**Semiquantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) assay.** FLS were cultured in 100-mm petri dishes with TLR-specific ligands in the presence or absence of VIP. The expression of TLR-3, TLR-7, RIG-I, MDA5, and IFN $\beta$  mRNA in cultured FLS was analyzed by RT-PCR. The treatment period was 24 hours for TLR-3 and TLR-7 and 2 hours for RIG-I, MDA5, and IFN $\beta$ . Total RNA was obtained from FLS cultures using TRI Reagent (Sigma-Aldrich). Total RNA (2  $\mu$ g) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Complementary DNA (2  $\mu$ l) was PCR-amplified with specific primers for  $\beta$ -actin, TLR-3, and TLR-7. The PCR products were analyzed in agarose gels, and the correct size and sequence of the amplified products were checked. Semiquantitative real-time PCR analysis was also performed with SYBR Green PCR Master Mix (Applied Biosystems) for TLR-3 and TLR-7, using  $\beta$ -actin as reference. The sequence of primers used was as follows: for  $\beta$ -actin, forward 5'-AGAAGGATTCCTATGTGGCG-3', reverse 5'-CATGTCGTCCCAGTTGGTGAC-3'; for TLR-3, forward 5'-ACAACCTAGCACGGCTCTGGA-3', reverse 5'-ACCTCAACTGGGATCTCGTCA-3'; for TLR-7, forward 5'-TGGAATTGCCCTCGTTGTT-3', reverse 5'-GTCAGCG-CATCAAAGCATT-3'. For RIG-I, MDA5, and IFN $\beta$ ,

TaqMan Gene Expression Master Mix (Applied Biosystems) was used, with manufacturer-pre-designed primers. For relative quantification, we compared the amount of target normalized to an endogenous reference ( $\beta$ -actin), using the formula  $2^{-\Delta\Delta C_t}$ .

#### Immunocytochemical detection of TLR-3 and TLR-7.

We performed immunocytochemistry studies using a specific mouse anti-TLR-3 monoclonal antibody and a rabbit anti-TLR-7 polyclonal antibody (both from Imgenex). The specificity of these antibodies has previously been reported. OA or RA FLS were cultured onto glass coverslips, fixed with 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 in PBS at room temperature. Cells were blocked with PBS containing 5% FCS for 15 minutes. Coverslips were incubated for 1 hour at 37°C with primary antibody, at a dilution of 1:100 (for TLR-3) or 1:50 (for TLR-7). After washing, cells were incubated for 1 hour with Alexa Fluor 594-conjugated goat anti-mouse IgG antibody for TLR-3 and Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody for TLR-7 (both from Invitrogen). Coverslips were counterstained with 1  $\mu$ g/ml DAPI to visualize nuclear bodies, mounted with ProLong Gold Antifade Reagent (Invitrogen), and examined under a fluorescence microscope.

**Western blot analysis of TLR-3 and TLR-7.** FLS were cultured and collected, rinsed twice with cold PBS and 1 mM sodium orthovanadate, and pelleted. Proteins were extracted from FLS in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Nonidet P40, 30 mM NaF, 5 mM ethylenediamine tetraacetate, 1 mM dithiothreitol, 0.1% sodium dodecyl sulfate [SDS], 1 mM sodium orthovanadate, and protease inhibitor cocktail). Protein extracts (80  $\mu$ g) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were blocked with Tris buffered saline (pH 7.6) containing 5% bovine serum albumin and 0.1% Tween 20 and then incubated overnight at 4°C with the following primary antibodies: rabbit anti-human TLR-7 (1:200; Acris) and mouse anti-human TLR-3 (2  $\mu$ g; Imgenex), with mouse anti- $\beta$ -actin (1:10,000; Sigma-Aldrich) used as a loading control. Horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) was used for detection. Proteins were detected using an enhanced chemiluminescence Western blot analysis system (Pierce). Protein bands were analyzed with a Bio-Rad gel documentation system, using the Bio-Rad Quantity One program, and normalized against the intensity of  $\beta$ -actin.

**Enzyme-linked immunosorbent assay (ELISA) of IFN $\beta$ , CXCL8 (IL-8), and matrix metalloproteinase 3 (MMP-3) in culture supernatants.** FLS were cultured in 6-well plates with TLR-specific ligands in the presence or absence of VIP for 6 hours (for IFN $\beta$ ) and 24 hours (for CXCL8 and MMP-3). Culture supernatants were concentrated with Amicon Ultra-0.5 Centrifugal Filter devices (Millipore) for IFN $\beta$  and MMP-3. Levels of IFN $\beta$  in concentrated FLS culture supernatants were measured using a VeriKine Human IFN- $\beta$  ELISA kit (PBL InterferonSource). Levels of CXCL8 were determined with a human capture ELISA, as previously described (31). The amount of MMP-3 was determined with a human instant ELISA kit (Bender MedSystems). Both the intraassay and interassay variability were <5% for CXCL8, <8% for IFN $\beta$ , and <10% for MMP-3.

#### ELISA of RIG-I and MDA5 in cytoplasmic extracts.

Eighty micrograms of cytoplasmic extracts of FLS treated with poly(I-C) for 2 hours was isolated as described above, and levels of RIG-I and MDA5 were measured using a sandwich ELISA kit (Apotech; Enzo Life Sciences).

**Transcription factor activity assay.** Nuclear IRF-3, IRF-7, NF- $\kappa$ B, AP-1 c-Jun, and AP-1 c-Fos activity was quantified using an ELISA-based TransAM kit (Active Motif) according to the manufacturer's protocol. Briefly, the nuclear protein extracts (3–10  $\mu$ g/well) were added to a 96-well plate that was immobilized with oligonucleotide containing IRF-3, IRF-7, NF- $\kappa$ B, and AP-1 elements, respectively. Transcription factor contained in nuclear extracts bound specifically to this oligonucleotide during incubation for 1 hour at room temperature. Primary antibody was then added to each well for 1 hour followed by secondary horseradish peroxidase-conjugated antibody for 1 hour. After adding developing solution and stopping the colorimetric reaction, the transcription factor activity was determined by reading absorbance at 450 nm.

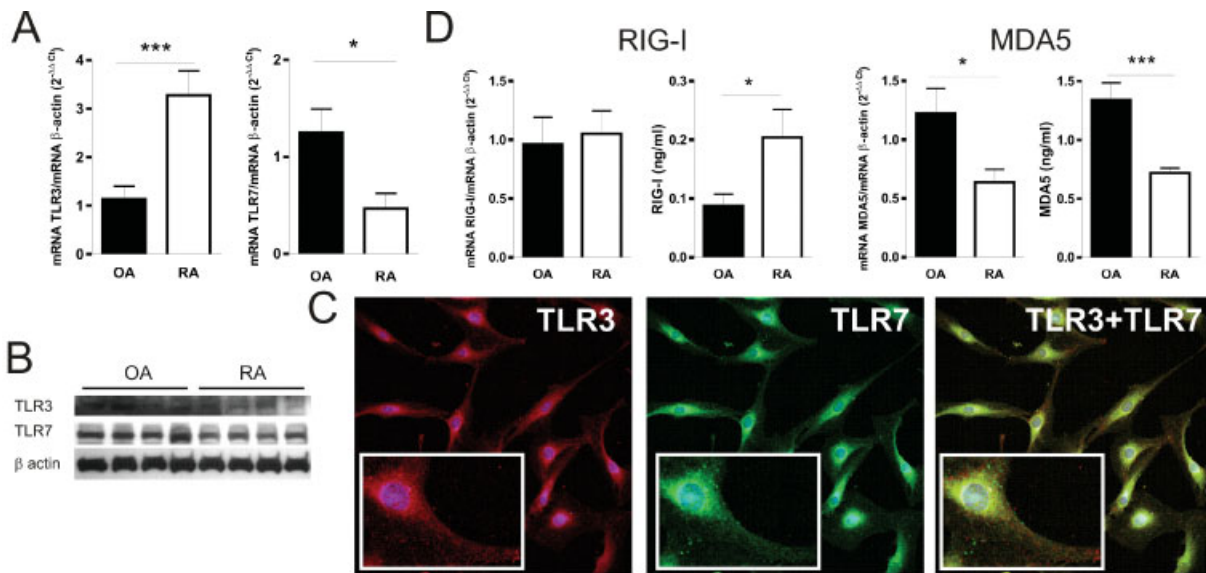
**Statistical analysis.** Results are expressed as the mean  $\pm$  SEM. The significance of the results was analyzed using Student's 2-tailed *t*-test. *P* values less than 0.05 were considered significant.

## RESULTS

**Characterization of ssRNA and dsRNA receptor expression in FLS.** We initially studied the constitutive expression of the membrane receptors TLR-3 (dsRNA receptor) and TLR-7 (ssRNA receptor) on cultured FLS. We observed constitutive mRNA expression of TLR-3 and TLR-7 in both OA and RA FLS. We detected mRNA by means of classic PCR, where we confirmed the identity of PCR products by electrophoretic analysis and subsequent sequencing (data not shown). To quantify the expression, we performed real-time PCR and used  $\beta$ -actin as a reference gene. The pattern was different in both pathologies: the expression of TLR-3 mRNA was 2.9-fold higher in RA FLS than in OA FLS, whereas the expression of TLR-7 mRNA was 2.7-fold higher in OA FLS than in RA FLS (Figure 1A). When comparing TLR-3 expression with TLR-7 expression, we observed that the expression of TLR-3 in OA FLS was less than twice as high as that of TLR-7, whereas the expression of TLR-3 in RA FLS was almost 6 times higher than that of TLR-7.

We then detected TLR-3 and TLR-7 at the protein level by means of Western blotting (Figure 1B). This expression presented great heterogeneity, and we did not observe significant differences between RA FLS and OA FLS. Immunocytochemical analysis showed expression of both TLR-3 and TLR-7 in perinuclear areas, corresponding endosomal localization in the cytoplasm (Figure 1C), with partial colocalization of both receptors (Figure 1C). We observed no staining when isotype controls were used (data not shown). We also studied the constitutive expres-





**Figure 1.** Characterization of Toll-like receptor 3 (TLR-3), TLR-7, retinoic acid-inducible gene I (RIG-I), and myeloid differentiation-associated gene 5 (MDA5) expression in osteoarthritis (OA) fibroblast-like synoviocytes (FLS) and rheumatoid arthritis (RA) FLS. **A**, TLR-3 and TLR-7 mRNA expression was measured by real-time polymerase chain reaction (PCR), using  $\beta$ -actin as the reference gene. **B**, Protein expression of TLR-3, TLR-7, and  $\beta$ -actin was determined by Western blotting, using total protein extracts. **C**, Protein expression and intracellular localization of TLR-3 and TLR-7 were analyzed by immunofluorescence staining. Nuclei were counterstained with DAPI (blue), and receptors were stained with Alexa Fluor 488 (red) and Alexa Fluor 594 (green) for TLR-3 and TLR-7, respectively. Boxed areas show larger-magnification views of individual nuclei. Original magnification  $\times 450$ . **D**, RIG-I and MDA5 mRNA expression was determined by real-time PCR, using  $\beta$ -actin as the reference gene. Protein expression was analyzed by enzyme-linked immunosorbent assay. Results in **B** and **C** are representative of 3 independent experiments. Values in **A** and **D** are the mean  $\pm$  SEM results for at least 8 FLS lines measured in triplicate, with basal OA mRNA expression as the referent. \* =  $P < 0.05$ ; \*\*\* =  $P < 0.001$ .

sion of cytosolic RIG-I and MDA5 and detected similar expression of RIG-I mRNA in OA FLS and RA FLS, whereas the expression of RIG-I in RA FLS was significantly higher than that in OA FLS at the protein level, although the expression measured by ELISA was poor (Figure 1D). We observed higher gene expression of MDA5 in OA FLS compared with RA FLS, which was confirmed at the protein level by ELISA (Figure 1D). When comparing the expression of RIG-I and MDA5 mRNA, that of RIG-I was higher in both OA FLS and RA FLS after poly(I-C) stimulation (Table 1). In summary, TLR-3 and RIG-I were overexpressed in RA FLS (although RIG-I was overexpressed only at the protein level), whereas TLR-7 and MDA5 were overexpressed in OA FLS.

**Differential induction of TLR-3, TLR-7, RIG-I, and MDA5 in FLS by poly(I-C) or imiquimod.** In order to mimic the effect of endogenous RNAs present in the joint, we examined the effect of the agonists poly(I-C) and imiquimod on the expression of these receptors. Stimulation with poly(I-C) significantly increased TLR-3, RIG-I, and MDA5 gene expression in FLS,

**Table 1.** Relative induced expression of single-stranded RNA and double-stranded RNA sensor mRNA in fibroblast-like synoviocytes\*

	OA	RA
<b>TLR-3</b>		
Basal	1.13 $\pm$ 0.13	1.01 $\pm$ 0.05
Poly(I-C)-stimulated	16.62 $\pm$ 2.30 <sup>†</sup>	9.03 $\pm$ 0.67 <sup>†</sup>
<b>RIG-I</b>		
Basal	1.02 $\pm$ 0.13	1.04 $\pm$ 0.10
Poly(I-C)-stimulated	5.79 $\pm$ 0.62 <sup>†</sup>	16.51 $\pm$ 2.52 <sup>†</sup>
<b>MDA5</b>		
Basal	1.06 $\pm$ 0.15	0.98 $\pm$ 0.13
Poly(I-C)-stimulated	2.21 $\pm$ 0.40 <sup>‡</sup>	9.02 $\pm$ 1.37 <sup>†</sup>
<b>TLR-7</b>		
Basal	1.25 $\pm$ 0.25	1.07 $\pm$ 0.10
Imiquimod-stimulated	2.87 $\pm$ 0.51 <sup>§</sup>	2.20 $\pm$ 0.44 <sup>‡</sup>

\* Values are the mean  $\pm$  SEM from at least 3 individual experiments performed in triplicate. Osteoarthritis (OA) and rheumatoid arthritis (RA) fibroblast-like synoviocytes were stimulated for 24 hours with poly(I-C) 20  $\mu$ g/ml or imiquimod 15  $\mu$ g/ml, and mRNA expression was determined by relative real-time quantitative polymerase chain reaction and corrected for mRNA expression of  $\beta$ -actin in each sample. The amount of target normalized to  $\beta$ -actin was compared using the formula  $2^{-\Delta\Delta C_t}$ . TLR-3 = Toll-like receptor 3; RIG-I = retinoic acid-inducible gene I; MDA5 = myeloid differentiation-associated gene 5.

<sup>†</sup>  $P < 0.001$  versus basal.

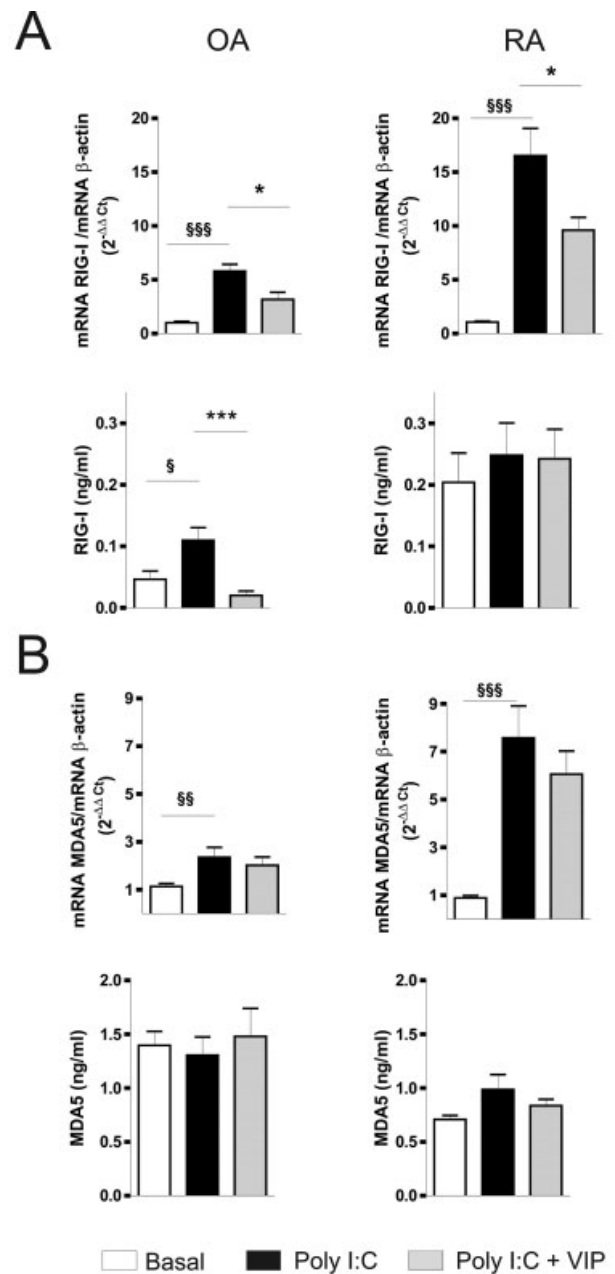
<sup>‡</sup>  $P < 0.01$  versus basal.

<sup>§</sup>  $P < 0.05$  versus basal.

although with different intensities (Table 1 and Figure 2). Although the increase in TLR-3 mRNA expression was higher in OA FLS than in RA FLS, the increase in RIG-I and MDA5 mRNA expression was higher in RA FLS than in OA FLS. Analysis of protein in cytoplasmic extracts showed a significant increase in RIG-I only in poly(I-C)-stimulated OA FLS and a trend toward higher expression of MDA5 in RA FLS (Figure 2). Stimulation with imiquimod increased TLR-7 mRNA expression, with similarly low, although significant, intensity in both OA FLS and RA FLS. Although poly(I-C) is the main inducer of RIG-I and MDA5 responses, a recent study showed up-regulated TLR-7-induced expression of both cytoplasmic receptors in human plasmacytoid dendritic cells (DCs) (34). Surprisingly, in FLS, imiquimod was able to significantly induce MDA5 at both the protein and mRNA levels, and a similar tendency was observed for RIG-I expression (data not shown). Regarding TLR-3 and TLR-7, we did not detect significant differences at the protein level (data not shown). Thus, agonists up-regulated the expression of the ssRNA and dsRNA receptors tested, in both OA and RA FLS.

**Effect of VIP on poly(I-C)- and imiquimod-stimulated expression of TLR-3, TLR-7, RIG-I, and MDA5.** Because we previously observed that VIP exerts its immunomodulatory action on TLR-2 and TLR-4 by down-regulating their expression in human FLS (20), we next studied the effect of VIP after stimulation with poly(I-C) and imiquimod. VIP significantly inhibited the expression of RIG-I mRNA stimulated with poly(I-C) in OA FLS and RA FLS. This effect was observed only at the protein level in OA FLS, where the unstimulated expression of RIG-I was less than that in RA FLS, and enhanced expression was observed only in OA FLS after poly(I-C) stimulation (Figure 2A). In the case of MDA5 mRNA, we observed a nonsignificant decrease in expression in both OA and RA FLS; a similar tendency was detected at the protein level in RA FLS. In OA FLS, poly(I-C) was not able to increase MDA5 expression, and the basal expression of MDA5 was higher than that in unstimulated RA FLS (Figure 2B). TLR-3 and TLR-7 agonist-enhanced expression and imiquimod-enhanced expression of RIG-I and MDA5 were not altered by VIP treatment (data not shown). Taken together, these data indicate that, with the exception of the VIP-mediated decrease in RIG-I expression, the potential action of VIP was not mediated by reduced expression of the receptors studied.

**Different patterns of nuclear translocation of transcription factors in FLS following stimulation with poly(I-C) or imiquimod.** Stimulation of ssRNA and dsRNA receptors leads to the activation of different



**Figure 2.** Characterization of induced expression of RIG-I and MDA5 after poly(I-C) stimulation, and analysis of vasoactive intestinal peptide (VIP) treatment. Messenger RNA and protein expression of RIG-I (A) and MDA5 (B) from OA and RA FLS was measured by real-time PCR and enzyme-linked immunosorbent assay, respectively. Messenger RNA and protein expression of both genes was analyzed under 3 conditions: basal, stimulation with poly(I-C), and treatment with poly(I-C) plus VIP. Values for the relative expression of both genes were corrected using mRNA expression of  $\beta$ -actin. Values are the mean  $\pm$  SEM results from at least 3 FLS lines measured in triplicate, with basal OA or RA mRNA expression as the referent. \* =  $P < 0.05$ ; \*\*\* =  $P < 0.001$ ; § =  $P < 0.05$ ; §§ =  $P < 0.01$ ; §§§ =  $P < 0.001$ . See Figure 1 for other definitions.

**Table 2.** Intensity of transcription factor stimulation in OA and RA fibroblast-like synoviocytes\*

Transcription factor/ stimulation	OA	RA
IRF-3		
Poly(I-C), 90 minutes	15.02 ± 1.66	10.89 ± 1.67
NF-κB		
Poly(I-C), 1 hour	9.86 ± 1.07	75.88 ± 26.4†
Imiquimod, 1 hour	4.22 ± 0.62	38.29 ± 13.02†
c-Jun		
Poly(I-C), 90 minutes	3.95 ± 0.95	3.91 ± 0.83
Imiquimod, 90 minutes	3.65 ± 1.31	4.64 ± 0.92
c-Fos		
Imiquimod, 2 hours	36.79 ± 8.42	67.65 ± 1.09‡

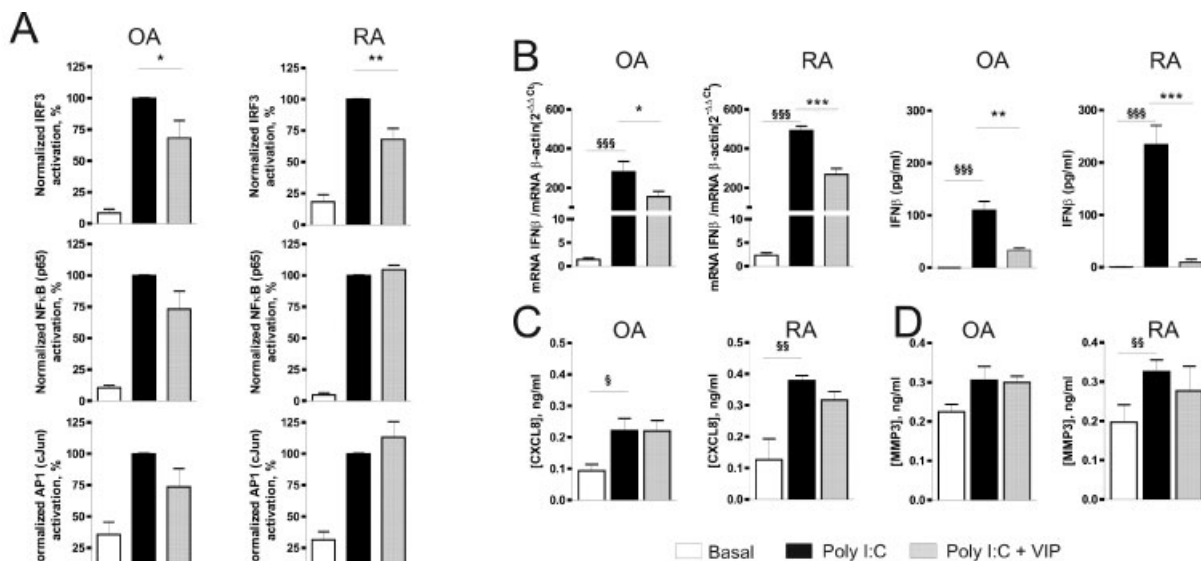
\* Values are the mean ± SEM results from triplicate experiments. Binding of the indicated active nuclear transcription factor to an oligonucleotide was determined using a TransAM kit. Osteoarthritis (OA) and rheumatoid arthritis (RA) fibroblast-like synoviocytes were treated with 20 μg/ml poly(I-C) or 15 μg/ml imiquimod. After stimulation for the periods of time indicated, cells were re-collected, and nuclei were isolated. The amount of active transcription factor was normalized to the levels in basal lysates; IRF-3 = interferon regulatory factor 3.

†  $P < 0.05$  versus OA.

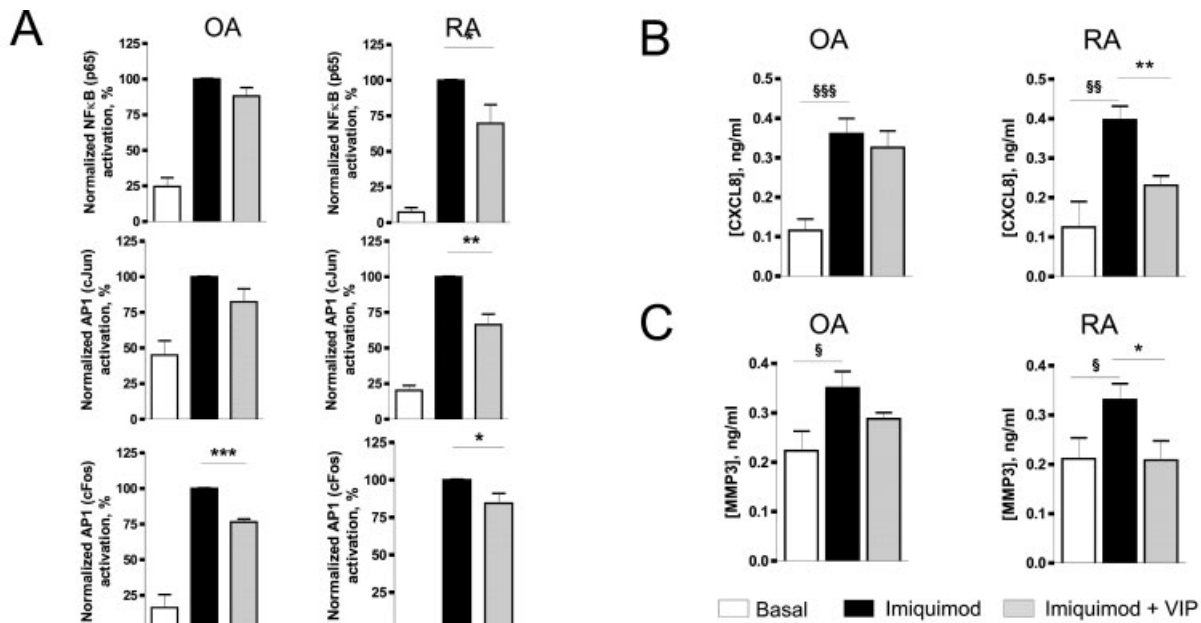
‡  $P < 0.01$  versus OA.

transcription factors, such as IRF-3, IRF-7, NF-κB, and AP-1, which induce the production of type I IFNs and inflammation mediators (2). We thus next studied the time course of translocation to the nucleus of these transcription factors after the recognition of the synthetic analogs.

We observed different timing of activation in poly(I-C)- and imiquimod-treated OA FLS (data not shown). Poly(I-C)-induced activation of NF-κB p65 and AP-1 c-Jun was observed within 5 minutes of treatment, whereas activation of IRF-3 was observed after 60 minutes. The highest activation of IRF-3 and AP-1 c-Jun was observed after 90 minutes, while maximum NF-κB p65 activation was reached after 120 minutes. No activation of AP-1 c-Fos was observed after poly(I-C) treatment (data not shown). After the highest activation was achieved, prolonged treatment diminished transcription factor activation, which disappeared when treatment lasted 24 hours (data not shown).



**Figure 3.** Determination of induced activation of interferon (IFN) regulatory factor 3 (IRF-3), NF-κB, activator protein 1 (AP-1), IFNβ, CXCL8, and matrix metalloproteinase 3 (MMP-3) by poly(I-C) stimulation (for the indicated periods of time), and analysis of vasoactive intestinal peptide (VIP) treatment in OA and RA FLS. **A**, Activation of IRF-3 (90 minutes), NF-κB p65 (120 minutes), and AP-1 c-Jun (90 minutes) in OA and RA FLS was determined with the enzyme-linked immunosorbent assay (ELISA)-based TransAM kit, using nuclear protein extract. Transcription factor activity was normalized to the levels in poly(I-C)-stimulated nuclear extracts (% of stimulated FLS). **B**, Messenger RNA (2 hours) and protein (6 hours) expression of IFNβ from OA and RA FLS was analyzed by real-time PCR using β-actin as the reference gene and ELISA, respectively. **C** and **D**, The presence of CXCL8 and MMP-3, respectively, in the supernatants of OA and RA FLS cultures was determined by ELISA. Every assay was done under 3 conditions: basal, stimulation with poly(I-C), or treatment with poly(I-C) plus VIP. Values are the mean ± SEM from at least 3 FLS lines measured in triplicate. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ; § =  $P < 0.05$ ; §§ =  $P < 0.01$ ; §§§ =  $P < 0.001$ . See Figure 1 for other definitions.



**Figure 4.** Determination of induced activation of NF- $\kappa$ B, AP-1, CXCL8, and MMP-3 protein expression by imiquimod stimulation and analysis of VIP treatment in osteoarthritis (OA) and rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS). **A**, Activation of NF- $\kappa$ B p65 (60 minutes), AP-1 c-Jun (90 minutes), and AP-1 c-Fos (120 minutes) in OA and RA FLS was determined with the ELISA-based TransAM kit, using nuclear protein extract. Transcription factor activity was normalized to the levels in imiquimod-stimulated nuclear extracts (% of stimulated FLS). **B** and **C**, The presence of CXCL8 and MMP-3, respectively, in the supernatants of OA and RA FLS cultures was determined by ELISA. Activation of NF- $\kappa$ B p65 and AP-1 c-Jun and c-Fos and protein expression of CXCL8 and MMP-3 were analyzed under 3 conditions: basal, treatment with imiquimod, or treatment with imiquimod plus VIP. Values are the mean  $\pm$  SEM from at least 3 FLS lines measured in triplicate. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ; § =  $P < 0.05$ ; §§ =  $P < 0.01$ ; §§§ =  $P < 0.001$ . See Figure 3 for other definitions.

Imiquimod produced less and earlier activation of NF- $\kappa$ B p65 than poly(I-C) stimulation. The highest activation of NF- $\kappa$ B p65 was observed at 60 minutes before AP-1 c-Jun and c-Fos. Moreover, imiquimod triggered the activation of both AP-1 c-Jun and AP-1 c-Fos, with maximum activation observed at 90 minutes and 120 minutes, respectively. AP-1 c-Jun was activated sooner and with more intensity with imiquimod than with poly(I-C), which was not able to activate AP-1 c-Fos. IRF-3 was not activated by imiquimod, and we did not observe IRF-7 translocation after imiquimod or poly(I-C) treatment, even when treatment was prolonged to 2, 4, 6, or 24 hours (data not shown).

Knowing the maximum activation time, we studied the different stimulation intensity in OA FLS and RA FLS. All of the transcription factors studied were activated more strongly in RA FLS, except for IRF-3 and AP-1 c-Jun, for which activation was similar (Table 2). These differences were statistically significant for NF- $\kappa$ B and AP-1 c-Fos. In summary, we observed maximum transcription factor activation in FLS between 60 minutes and 120 minutes, and poly(I-C) strongly

activated IRF-3 and NF- $\kappa$ B, whereas AP-1 was more strongly activated by imiquimod.

**Effect of VIP on transcription factor activation after poly(I-C) or imiquimod treatment.** We investigated whether the potential immunoregulatory role of VIP could be mediated by its effect on nuclear translocation of these transcription factors. VIP significantly diminished poly(I-C)-induced activation of IRF-3 in both OA FLS and RA FLS, but it had little if any effect on the other transcription factors examined in the presence of poly(I-C) (Figure 3A). In imiquimod-treated FLS, VIP decreased activation of every transcription factor (NF- $\kappa$ B, AP-1 c-Jun, and AP-1 c-Fos), with a significant reduction in all of them in RA FLS and significant inhibition only for AP-1 c-Fos in OA FLS (Figure 4A). Taken together, these results indicate that, after stimulation of dsRNA receptors, VIP produced similar effects in both OA FLS and RA FLS in reducing IRF-3 translocation, whereas after the engagement of ssRNA receptors, VIP was more effective in RA FLS than in OA FLS in terms of down-regulating transcription factors.



**Effect of VIP on agonist-stimulated production of IFN $\beta$ , CXCL8, and MMP-3.** Transcription factor activation in response to synthetic agonists has consequences in the production of antiviral agents such as IFN $\beta$ , proinflammatory chemokines such as CXCL8, and MMPs involved in joint destruction, such as MMP-3. First, we studied the expression of IFN $\beta$ , which is augmented in response to IRF-3 activation. Poly(I-C) treatment produced a significant increase in IFN $\beta$  mRNA expression, by almost twice as much in RA FLS compared with OA FLS (Figure 3B, left panels). To determine whether treatment with poly(I-C) could result in increased IFN $\beta$  secretion by these cells, we measured it by means of ELISA. We did not detect unstimulated IFN $\beta$  in the supernatants of FLS, but this protein was detected in both OA FLS and RA FLS supernatants after 6 hours of culture, with double secretion in RA FLS compared with that in OA FLS (Figure 3B, right panels). In addition, after poly(I-C) treatment, VIP significantly decreased IFN $\beta$  mRNA expression, in both OA FLS and RA FLS. At the protein level, VIP significantly reduced IFN $\beta$  secretion in both RA and OA FLS and nearly eliminated it in RA FLS supernatants (Figure 3B). In contrast, after imiquimod treatment, there was no increase in IFN $\beta$ , IFN $\alpha$ 1, or IFN $\alpha$ 4 expression at either the mRNA or protein level (data not shown).

CXCL8 is one of the chemokines secreted in response to NF- $\kappa$ B activation. Therefore, we measured the secretion of CXCL8 in FLS supernatants. Stimulation with poly(I-C) significantly augmented CXCL8 secretion in FLS, but VIP still did not have any effect on this increase (Figure 3C). Imiquimod also significantly enhanced CXCL8 secretion in both RA and OA FLS, and VIP significantly reduced this secretion in RA FLS but not in OA FLS. AP-1 is a key transcription factor for MMP-3 gene expression in FLS. Both poly(I-C) and imiquimod stimulation significantly increased MMP-3 secretion in FLS, except in poly(I-C)-treated OA FLS, where this increase was not significant. VIP significantly diminished MMP-3 production only in imiquimod-treated RA FLS, and a tendency toward diminished production was noted in poly(I-C)-treated RA FLS and imiquimod-treated OA FLS (Figures 3D and 4C). In summary, VIP treatment variably reduced inflammation mediators such as CXCL8, the matrix-degrading enzyme MMP-3, and antiviral IFN $\beta$ .

## DISCUSSION

Rheumatic diseases are a diverse group of disorders, with an unknown etiology and poorly understood

pathogenesis, involving the musculoskeletal system. The innate immune pathways in FLS play a crucial role not only in the initiation but also in the perpetuation of rheumatic diseases. This study is the first to show the constitutive expression, activation, and function of RIG-I, MDA5, and TLR-7 in FLS and also confirms the presence and functionality of TLR-3 described previously by other investigators. Since the initial description of TLR-2 in 2003 (16), it has been generally accepted that the expression of TLRs 2, 3, 4, and 7 and RIG-I is increased in the synovial tissue of patients with RA compared with healthy controls and patients with OA (15,35,36). However, the data regarding their constitutive expression and their activation in isolated FLS are dissimilar, and the differences between both pathologies are not as apparent.

A recent study demonstrated a tendency toward higher basal expression of TLR-3 mRNA in RA FLS when compared with OA FLS, normal FLS, and skin fibroblasts (37), whereas our results demonstrated that these differences are significant at the mRNA level and with great heterogeneity between OA FLS and RA FLS at the protein level. In contrast with a previous study (36), we detected constitutive expression of RIG-I in FLS, which was increased at the protein level in RA FLS. Interestingly, MDA5 and RIG-I have been involved in the induction of apoptosis, and MDA5 has been involved in growth cell suppression (38), 2 pivotal functions of FLS that contribute to RA pathogenesis. The presence of TLR-7 in synovial tissue has been described previously (18,39). This report is the first to describe the constitutive and imiquimod-stimulated expression of TLR-7 in isolated FLS and its coexpression with TLR-3.

TLR-3 stimulation activates the TRIF-dependent signaling pathways activating NF- $\kappa$ B and AP-1 transcription factors, which induce production of inflammation mediators and MMPs. TRIF also participates in the activation of IRF-3 and IRF-7, which regulate type I IFN, chiefly inducing production of IFN $\beta$ . IFN $\beta$  gene transcription is complex and requires IRF-3, NF- $\kappa$ B, and activating transcription factor 2/c-Jun complexes or enhanceosomes (40).

We observed that poly(I-C) induced the translocation of NF- $\kappa$ B p65, AP-1 c-Jun, and IRF-3 in FLS. NF- $\kappa$ B p65 activation was significantly stronger in RA FLS than in OA FLS. Activation of IRF-7 and AP-1 c-Fos was not detected. In a recent study, Sweeney et al observed a primary role of IRF-3 not only in the inhibition of IFN-regulated genes but also in the inhibition of AP-1-regulated genes, while being irrelevant in



the presence of IRF-7 for the production of IFN $\beta$  and other proinflammation mediators (41). Those investigators reported inducible IRF-7 protein expression at 2 hours that continued for 24 hours. We did not observe the translocation of IRF-7 to the nucleus in our cultures even after 24 hours, confirming the unrelated role of this factor in poly(I-C) stimulation, although we cannot rule out insufficient activation of IRF-3 as a requirement for IRF-7 activation. NF- $\kappa$ B p65 and AP-1 c-Jun were activated rapidly (in 5 minutes), whereas activation of IRF-3 was observed after 60 minutes, consistent with the results of studies by Sweeney et al (41). Our results confirm those of previous studies of the main role played by IRF-3, NF- $\kappa$ B p65, and AP-1 c-Jun as components of the enhanceosome for IFN $\beta$  synthesis, as well as for the production of mediators such as CXCL8 and MMP-3 that are involved in joint destruction (12).

Although dsRNA has arthritogenic properties, TLR-3-knockout mice experienced the development of arthritis, supporting the finding that other receptors such as cytoplasmic receptors are involved in the induction of inflammation (42). Both RIG-I and MDA5 stimulation with poly(I-C) activated IRF-3 and NF- $\kappa$ B, thus sharing signaling pathways with TLR-3. Although our study provides the first description of the basal expression of cytoplasmic receptors and activation of MDA5 in FLS, the final contribution of TLR-3, RIG-I, and MDA5 to the activation of transcription factors could not be dissected and remains to be elucidated. IRF-3 is involved in the production of the antiviral IFN $\beta$ , NF- $\kappa$ B mediates the synthesis of the proinflammatory chemokine CXCL8, and AP-1 is involved in the production of MMP-3. Poly(I-C) induced an increase in gene and protein expression of IFN $\beta$ , CXCL8, and MMP-3 in RA FLS compared with OA FLS. Again, we cannot dissect the contribution of RIG-I and MDA5 in the production of IFN $\beta$  and CXCL8. The system is further complicated by the fact that IRF-3 is also involved in the production of MMP-3 and CXCL8 gene expression (41).

TLR-7 responses require myeloid differentiation factor 88, which activates NF- $\kappa$ B and MAP kinase, as well as IRF-7 signaling in plasmacytoid DCs for the production of type I IFNs, particularly IFN $\alpha$  (2). High levels of this receptor have been reported in plasmacytoid DCs, monocytes, and B cells (34), along with the involvement of various transcription factors such as AP-1 c-Jun and IRF-7. The molecular pathways of TLR-7-mediated activation in FLS have not been characterized to date. Treatment with imiquimod produced activation of NF- $\kappa$ B, AP-1 c-Jun, and AP-1 c-Fos in FLS, with the consequent up-regulation of CXCL8 and

MMP-3, whereas IRF-7 was not detected in the nucleus on any occasion studied, nor was production of IFN $\alpha$ 1, IFN $\alpha$ 4, and IFN $\beta$  observed. The up-regulation of CXCL8 has also been reported in human plasmacytoid DCs after treatment with the same agonists (34). It is of interest that we observed an increase in both gene expression and protein production of RIG-I and MDA5 after imiquimod treatment, in accordance with the TLR-7-mediated gene overexpression observed in human plasmacytoid DCs (34), showing the ability of the innate immune system to intensify itself. We also report here, for the first time, the TLR-7-mediated production of MMP-3. Thus, in FLS, TLR-7 stimulation leads to the production of mediators of inflammation and joint destruction, together with amplification of the dsRNA cytoplasmic receptors.

We previously reported the VIP-mediated down-regulated expression of TLR-2 and TLR-4 in RA FLS associated with reduced production of IL-6, CXCL8, and CCL2 (43). In the current study, we showed a VIP-mediated decrease in the expression of RIG-I and MDA5 receptors and no effect of VIP on TLR-3 and TLR-7. VIP treatment reduced the poly(I-C)-induced activation of IRF-3 and consequently the production of IFN $\beta$  in FLS. The role of IFN $\beta$  in rheumatic diseases is controversial. It decreases the expression of inflammation mediators produced by synoviocytes, but clinical trials with IFN $\beta$  have demonstrated negligible effectiveness (44). Moreover, autoimmunity is precipitated by the overproduction of type I IFN and inflammatory cytokines. Roelofs et al reported that in RA tissue, TLR-3/TLR-7 signaling through IFN $\beta$ /IL-18 expression contributes to the activation of TLR-4, maintaining the proinflammatory cascade (39). The VIP-mediated decrease in IFN $\beta$  could also contribute to the previously reported VIP-mediated inhibition of TLR-4 expression and the inhibition of proinflammation mediators in FLS.

The effect of VIP on imiquimod-treated FLS was limited to RA, resulting in reduced activation of NF- $\kappa$ B, AP-1 c-Jun, and AP-1 c-Fos and a decrease in CXCL8 and MMP-3 production. The effect of VIP on FLS is mediated by 2 protein G-coupled receptors, VPAC<sub>1</sub> and VPAC<sub>2</sub>, with overexpression of VPAC<sub>2</sub> in RA FLS (31). An important point to note is that imiquimod triggered a potent up-regulation of VPAC<sub>2</sub> gene expression in macrophages (45). In accordance with our results, the VIP-mediated inhibition of inducible nitric oxide synthase gene expression through the inhibition of poly(I-C)-induced AP-1 activation in Schwann cells has been reported (46). The precise ligands for the pattern recognition receptors studied in rheumatic diseases have not been identified. Virus occurrence was mainly studied

in RA, where dsRNA such as cytomegalovirus, a ligand for TLR-3, and Epstein-Barr virus, a ligand for RIG-I that induces anti-cyclic citrullinated protein antibodies in patients with RA, were identified, and ssRNA, such as parvovirus B19, were also observed (13,47,48). However, endogenous ligands, such as the necrotic debris present in the joint microenvironment as a result of inflammation (14), and high levels of circulating (49) and synovial fluid nucleic acids (50) could also contribute to both the initiation and maintenance of disease.

Understanding the molecular mechanisms regulating the induction of inflammation and joint destruction mediators, as well as type I IFNs by pattern recognition receptors, is crucial for the development of novel therapeutic immune modulators. In recent years, it has been demonstrated that among these, VIP could be an excellent candidate.

#### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Gomariz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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