TLR5, a Novel and Unidentified Inflammatory Mediator in Rheumatoid Arthritis that Correlates with Disease Activity Score and Joint TNF-α Levels

Nathan D. Chamberlain, Olga M. Vila, Michael V. Volin, Suncica Volkov, Richard M. Pope, William Swedler, Arthur M. Mandelin II and Shiva Shahrara

*J Immunol* published online 1 June 2012
http://www.jimmunol.org/content/early/2012/06/01/jimmunol.1102977
TLR5, a Novel and Unidentified Inflammatory Mediator in Rheumatoid Arthritis that Correlates with Disease Activity Score and Joint TNF-α Levels

Nathan D. Chamberlain,* Olga M. Vila,* Michael V. Volin, † Suncica Volkov,* Richard M. Pope, ‡ William Swedler,* Arthur M. Mandelin, II, ‡ and Shiva Shahrara*

The innate immune system plays an important role in rheumatoid arthritis (RA) pathogenesis. Previous studies support the role of TLR2 and 4 in RA and experimental arthritis models; however, the regulation and pathogenic effect of TLR5 is undefined in RA. In this study, we show that TLR5 is elevated in RA and osteoarthritis (OA) ST lining and sublining macrophages and endothelial cells compared with normal individuals. Furthermore, expression of TLR5 is elevated in RA synovial fluid macrophages and RA peripheral blood monocytes compared with RA and normal peripheral blood in vitro-differentiated macrophages. We also found that TLR5 on RA monocytes is an important modulator of TNF-α in RA synovial fluid and that TLR5 expression on these cells strongly correlates with RA disease activity and TNF-α levels. Interestingly, TNF-α has a feedback regulation with TLR5 expression in RA monocytes, whereas expression of this receptor is regulated by IL-17 and IL-8 in RA macrophages and fibroblasts. We show that RA monocytes and macrophages are more responsive to TLR5 ligation compared with fibroblasts despite the proinflammatory response being mediated through the same signaling pathways in macrophages and fibroblasts. In conclusion, we document the potential role of TLR5 ligation in modulating transcription of TNF-α from RA synovial fluid and the strong correlation of TLR5 and TNF-α with each other and with disease activity score in RA monocytes. Our results suggest that expression of TLR5 may be a predictor for RA disease progression and that targeting TLR5 may suppress RA. The Journal of Immunology, 2012, 189: 000–000.

Reumatoid arthritis (RA) is a chronic autoimmune disorder in which the innate immune system plays an important role (1, 2). TLRs are pattern recognition receptors that are present in a number of cells and tissues, which recognize pathogen-associated molecular patterns (PAMPs) or endogenous ligands (3).

Previous studies demonstrate that expression of TLR2 and TLR4 is elevated in RA peripheral blood (PB) monocytes as well as in RA synovial fluid and synovial tissue (ST) macrophages (4–7). Increased TLR2 and TLR4 expression was detected in RA compared with osteoarthritis (OA) ST fibroblasts (8). Further, the data obtained from experimental arthritis models strongly support the role of TLR2 and TLR4 in streptococcal cell wall arthritis (9, 10) while TLR4 has been implicated in collagen-induced arthritis (11) as well as in the IL-1RA/−/− model (11, 12). However, the role of TLR5 in RA and murine models of RA is undefined.

TLR5 is expressed on a variety of cell types such as epithelial cells, neutrophils, monocytes, macrophages, and mast cells and is the receptor for the bacterial structural protein flagellin (13). Flagellin signaling via TLR5 is dependent on MyD88 and IL-1R–associated kinase 1 (14, 15) and subsequent activation of NF-κB, MAPK, and PI3K pathways (16–18). As with other TLR agonists, flagellin has been shown to induce dendritic cell maturation and activation (19), thereby promoting lymphocyte migration to secondary lymphoid sites (20). Others have shown that spontaneous neutrophil apoptosis is delayed by flagellin through induction of McI-1 and inhibition of caspase-3 (21). What remains unclear is whether TLR5 is present in RA synovium and whether ligation of this receptor plays a role in RA pathogenesis.

In this study, to our knowledge, we demonstrate for the first time that TLR5 is elevated in RA and OA ST lining and sublining macrophages and endothelial cells compared with normal (NL) controls. Consistently, our data demonstrate that TLR5 expression is greatly elevated in RA synovial fluid macrophages and PB monocytes compared with their NL counterparts. In RA monocytes, patients with higher expression of TNF-α expressed elevated levels of TLR5 and the concentration of both of these factors strongly correlated with increased disease activity score (DAS28). The role of TLR5 expression in RA pathogenesis was documented when the blockade of TLR5 on monocytes significantly reduced synovial fluid-mediated TNF-α transcription by 80%. Interestingly, we demonstrate a feedback modulation between TNF-α production and TLR5 ligation and expression in RA monocytes. Although in RA macrophages, TLR5 expression is induced by IL-17 and IL-8, it is significantly reduced by TLR4 ligation in both RA monocytes and macrophages. Higher expression of TLR5 was detected in RA compared with NL fibroblasts, which was upreg-
ulated by a variety of inflammatory factors excluding LPS. Hence, our data demonstrate the expression of TLR5 in RA and further documents its importance in RA disease activity and TNF-α modulation.

### Materials and Methods

#### Abs and immunohistochemistry

The studies were approved by the Institutional Review Board, and all donors gave informed written consent. Because the RA ST are recruited from the practices of orthopedic surgeons these samples are de-identified; therefore the disease severity and the treatment information is unavailable.

RA, OA, and NL ST were formalin fixed, paraffin embedded, and sectioned in the pathology core facility. ST were immunoperoxidase-stained using Vectastain ABC kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen. Briefly, slides were deparaffinized in xylene for 15 min at room temperature, followed by rehydration by transfer through graded alcohols. Ags were unmasked by incubating slides in proteinase K digestion buffer (DakoCytomation, Carpinteria, CA) for 10 min at room temperature. Tissues were incubated with Abs to human TLR5 (1:50; Santa Cruz Biotechnology), anti-CD68 (1:100; Vector Laboratories), and CD14 (1:1000; Vector Laboratories) Abs. To localize TLR5 to macrophages in RA ST, slides were deparaffinized and unmasked as mentioned above. Using an Invision G2 kit (DakoCytomation), RA ST were stained with anti-TLR5 Ab (1:50 dilution; Santa Cruz Biotechnology) using diaminobenzidine (brown staining) as a chromogen. Thereafter, tissues were blocked (double staining blocker included in the Invision G2 kit) and stained with anti-TLR5 Ab (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) or an IgG control Ab (Beckman Coulter, Brea, CA). Slides were evaluated by blinded observers (22–25) (A.M.M. and M.V.V.). Tissue sections were scored for lining, sublining macrophages and endothelial cell staining on a 0–5 scale (26, 27). Scored data were pooled, and the mean ± SEM was calculated in each data group. To demonstrate location of TLR5 in RA ST serial tissue sections were stained with anti-TLR5 (1:50; Santa Cruz Biotechnology), anti-CD68 (1:100; Vector Laboratories) and anti-CD14 Abs. To localize TLR5 to macrophages in RA ST, slides were deparaffinized and unmasked as mentioned above. Using an Invision G2 kit (DakoCytomation), RA ST were stained with anti-TLR5 Ab (1:50 dilution; Santa Cruz Biotechnology) using diaminobenzidine (brown staining) as a chromogen. Thereafter, tissues were blocked (double staining blocker included in the Invision G2 kit) and stained with anti-CD68 Ab (1:100 dilution; DakoCytomation) using Fast red (red staining) as a chromogen following the manufacturers’ instructions (DakoCytomation).

#### RA patient population

RA specimens were obtained from patients with RA, diagnosed according to the 1987 revised criteria of the American College of Rheumatology (28). PB was obtained from 44 women and 4 men (mean age, 53.7 ± 2.7 y). At the time of treatment, patients were receiving no treatment (n = 7), taking nonbiological disease-modifying antirheumatic drugs (DMARDs) (methotrexate, leflunomide, and sulfasalazine azathioprine) alone (n = 5), taking DMARDs plus hydroxychloroquine (n = 9), taking DMARDs plus prednisone (n = 5), taking DMARDs plus rituximab (n = 3), taking DMARDs plus hydroxychloroquine plus prednisone (n = 2), or taking a TNF-α inhibitor (n = 6), with a DMARD (n = 8), with a DMARD plus hydroxychloroquine (n = 5), with a DMARD plus hydroxychloroquine plus prednisone (n = 2). These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors gave informed written consent. Maximum number of patients was 48; however, please refer to the figure legends for exact number of patients in each experiment.

#### Cell isolation, culture, and procedures

NL and RA PB and RA synovial fluid mononuclear cells were isolated by Histopaque gradient centrifugation (Sigma-Aldrich, St. Louis, MO) as described previously (29, 30). Monocytes/macrophages were isolated from NL and RA PB or RA synovial fluid using a negative selection kit (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer’s instructions (26, 27). Monocytes were subsequently differentiated to macrophages by culturing in RPMI 1640 medium containing 20% FBS for 7 d.

#### Quantification of chemokines and cytokines

Human TNF-α, IL-6, and CCL2 (R&D Systems, Minneapolis, MN) ELISA kits were used according to the manufacturer’s instructions.

#### Isolation of RA ST fibroblasts

ST fibroblasts were isolated from fresh RA ST by mincing and digestion in a solution of dispase, collagenase, and DNase (30). Cells were used between passages 3 and 9 and cultured in DMEM containing 10% FBS, and cell purity was validated by CD90 staining.

#### Cell treatment

RA PB monocytes and in vitro-differentiated macrophages or RA ST fibroblasts were treated with polyinosinic-polycytidylic acid (poly I:C) (10 ng/ml; only in RA monocytes; InvivoGen, San Diego, CA), LPS (10 ng/ml; Sigma-Aldrich), IL-1β (10 ng/ml; R&D Systems), TNF-α (10 ng/ml; R&D Systems), IL-17 (50 ng/ml; R&D Systems), IL-6 (10 ng/ml; R&D Systems), IL-8 (10 ng/ml; R&D Systems), or RA synovial fluid (10%). Cells were harvested after 6 h, and the TLR5 mRNA levels were quantified by real-time RT-PCR. RA ST fibroblasts, RA PB monocytes, and differentiated macrophages were treated with flagellin Ultra pure (10 and 100 ng/ml) (endotoxin levels < 50 EU/mg) (InvivoGen), cells (6 h; for real-time RT-PCR) or conditioned media (24 h; for ELISA) were harvested following treatment, and TNF-α, IL-6, and CCL2 mRNA production was quantified. In a different experiment, RA monocytes from six different patients were treated with anti-TLR5 Ab or IgG (10 μg/ml; InvivoGen) for 1 h prior to being treated with RA synovial fluid (10%; n = 6) for 6 h. To demonstrate that reduction of RA synovial fluid-mediated TNF-α levels are due to blockade of TLR5 and not to the necrotic effect of this Ab in RA monocytes, cells were pretreated with anti-TLR5 Ab or IgG control 1 h prior to treating the cells with PBS or flagelin for 6 h. Subsequently, the TNF-α mRNA levels were quantified by real-time RT-PCR for experiments performed for Fig. 3A and 3B.

#### Real-time RT-PCR

Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from the different cell types. Subsequently, reverse transcription and real-time RT-PCR were performed to determine TLR5, TNF-α, IL-6, and CCL2 expression levels as described previously (29–31). Relative gene expression was determined by the ΔΔCt method, and results were expressed as fold increase above conditions indicated in the figure legends.

#### Flow cytometry

To determine TLR5+ cells, NL and RA monocytes and differentiated macrophages were washed with FACS buffer (5% FBS in PBS). Thereafter, cells were blocked with 50% human serum and 0.5% BSA. Cells were then stained for PE-conjugated anti-TLR5 (Imgenex, San Diego, CA) or FITC-labeled anti-CD14 (BD Immunocytometry, Franklin Lakes, NJ) or IgG control Abs (BD Pharmingen). Percent TLR5+ cells were identified as those that were CD14−TLR5+. Because of limited access to RA synovial fluid macrophages, these cells were not included in the FACS analysis.

#### Flagellin signaling pathways in RA macrophages or RA fibroblasts

RA ST fibroblasts and macrophages (2 × 10^7/ml) were untreated or treated with flagellin (100 ng/ml) for 0–65 min. Cell lysates were examined by Western blot analysis (30). Blots were probed with p-ERK, p-p38 MAPK, p-AKT, p-JNK (1:1000 dilution; Cell Signaling Technology), or degradation of IκBα (1:3000 dilution; Cell Signaling Technology), or degradation of its GAPDH mass, and values were correlated with DAS28 score. The data were analyzed using one-way ANOVA, followed by a post hoc two-tailed Student test for paired and unpaired samples. In RA monocytes, TLR5 and TNF-α mRNA expression was correlated with each other using the ΔΔCt method. Further TLR5 or TNF-α mRNA was normalized to its GAPDH mass, and values were correlated with DAS28 score using linear regression analysis in RA monocytes. The p values < 0.05 were considered significant.

#### Inhibition of the signaling pathways in RA ST fibroblasts and macrophages

To define which signaling pathways mediate flagellin-induced CCL2 secretion, RA macrophages and fibroblasts were incubated with DMSO or 10 μM inhibitors to p38 (SB203580), ERK (PD98059), JNK (SP600125), or NEM inhibitors to p-ERK, p-p38 MAPK, p-AKT, p-JNK, and JNK or actin (1:3000 dilution; Cell Signaling Technology or Sigma-Aldrich).

#### Statistical analysis

The data were analyzed using one-way ANOVA, followed by a post hoc two-tailed Student t test for paired and unpaired samples. In RA mono-

{downloaded from http://www.jimmunol.org/ by guest on July 28, 2017}
Results

**TLR5 elevated in RA and OA ST**

To characterize the expression pattern of TLR5 in RA and OA compared with controls, ST were stained with Abs against TLR5. We found that both in RA and OA, TLR5 immunostaining was significantly higher on ST lining and sublining macrophages and endothelial cells compared with NL ST (Fig. 1A–D). Consistently, TLR5 staining was colocalized to RA ST CD68⁺ (Fig. 1E, 1F, 1H) and VWF⁺ cells (Fig. 1E, 1G). Although previous studies demonstrate that fibroblasts in the lining and macrophages in the lining and sublining express TLR2 and 4 (32), expression of these receptors has not been reported in endothelial cells. Therefore, TLR5 may be a member of the TLR family that is uniquely elevated on RA and OA endothelial cells.

**RA synovial fluid macrophages and RA PB monocytes express upregulated levels of TLR5**

Because TLR5 immunostaining was elevated in RA ST lining and sublining macrophages, we asked whether mRNA and/or cell surface expression of this receptor was increased in RA synovial fluid macrophages compared with RA and NL PB monocytes or differentiated macrophages. Using microarray analysis, TLR5 was identified as one of the genes (3.2-fold higher; \( p = 1.58 \times 10^{-10} \)) that was greatly increased in RA synovial fluid macrophages compared with NL macrophages. These results were confirmed when real-time RT-PCR demonstrated that the expression of TLR5 was elevated 9- and 35-fold in RA synovial fluid macrophages compared with RA and normal PB-differentiated macrophages, respectively (Fig. 2A). Furthermore, levels of TLR5 were 7- and 3-fold greater in RA PB monocytes compared with RA PB-differentiated macrophages and normal monocytes (Fig. 2A). Consistent with our mRNA results, FACS analysis demonstrated that percent TLR5 was significantly reduced both in RA and NLs when monocytes were differentiated into macrophages; however, percent TLR5 was 4-fold higher in RA monocytes and macrophages compared with NL counterpart cells (Fig. 2B, 2C). Despite reduction of TLR5 expression during monocyte to macrophage differentiation, TLR5 expression is significantly increased in macrophages isolated from RA joints, compared with control or RA PB macrophages. Altogether, our results suggest that RA ST and fluid macrophages as well as RA PB monocytes may be an important source for TLR5 response.

**TLR5 ligation can modulate synovial fluid-induced TNF-α transcription in RA monocytes and expression of TLR5 on these cells strongly correlates with DAS28 and TNF-α**

Because expression of TLR5 was higher in RA PB monocytes compared with differentiated macrophages, we asked whether ligation of TLR5 in RA monocytes may affect disease pathogenesis. Previous studies have identified a number of endogenous TLR2 and/or TLR4 ligands in RA synovial fluid (33, 34). Hence, synovial fluid-mediated TNF-α transcription in RA monocytes was examined to determine whether endogenous TLR5 ligand(s) were present in RA synovial fluid. Our results show that blockade of TLR5 on RA monocytes greatly downregulates (5-fold decrease; 80% reduction) TNF-α transcription activated by RA synovial fluid (Fig. 3A), suggesting that ligation of TLR5 by potential endogenous ligands expressed in RA synovial fluid may be partially responsible for joint TNF-α modulation. We further validated that the inhibitory effect of anti-TLR5 Ab on RA synovial fluid-mediated TNF-α was specifically due to blockade of TLR5 ligation and had no effect on cell necrosis (Fig. 3B). Given that ligation of TLR5 plays a role in joint TNF-α regulation, we asked whether expression of these two factors correlate with each other.

**FIGURE 1.** TLR5 expression is increased in RA and OA ST lining and sublining macrophages and endothelial cells compared with NL ST. NL (A), OA (B), and RA (C) ST were stained with anti-human TLR5 (A–C) (original magnification ×200), and positive immunostaining was scored on a 0–5 scale (D). ST lining and sublining macrophage (Mac) and endothelial (Endo) immunostaining are shown as mean ± SEM; \( n = 5–7 \). *\( p < 0.05 \). RA serial sections were stained with anti-TLR5 (E), anti-CD68 (F), and anti-VWF (G) Abs to distinguish TLR5 immunostaining on RA ST macrophages and endothelial cells (original magnification ×400). Black (E, F) and yellow arrows (E, G) demonstrate colocalization of TLR5 on CD68⁺ and VWF⁺ cells. (H) RA ST were stained for anti-TLR5 (brown staining) and anti-CD68 Abs (fast red staining) (original magnification ×800) to demonstrate TLR5 costaining on lining macrophages.
and/or DAS28. We found that the levels of TLR5 and TNF-α in RA monocytes were closely related ($R^2 = 0.71; p = 4.62 \times 10^{-14}$) (Fig. 3C). Furthermore, data analyzed by regression analysis demonstrated that patients with greater levels of DAS28 had increased expression of TLR5 ($R^2 = 0.57; p = 1.88 \times 10^{-9}$) (Fig. 3D) and TNF-α ($R^2 = 0.62; p = 1.34 \times 10^{-10}$) (Fig. 3E) in RA

**FIGURE 2.** TLR5 is upregulated in RA synovial fluid (SF) compared with RA and NL PB macrophages. (A) TLR5 mRNA levels were determined in NL (n for monocytes [mono] or macrophages [mac] = 11 or 18) and RA PB monocytes (n = 11) and differentiated macrophages (n = 15) as well as in RA SF macrophages (n = 10) by using real-time RT-PCR. The data are shown as fold increase above NL PB monocytes and are normalized to GAPDH. (B) Normal and RA PB monocytes and differentiated macrophages were immunostained with CD14 labeled with FITC and TLR5 conjugated with PE to determine percent TLR5+ cells (n = 6–10). The values are presented as mean ± SEM of percent CD14+TLR5+ in each cell population. (C) Representative flow cytometry histograms showing CD14+TLR5+ in NL and RA PB monocytes and differentiated macrophages. *p < 0.05.

**FIGURE 3.** TLR5 ligation can regulate synovial fluid (SF)-induced TNF-α transcription in RA monocytes and expression of TLR5 on these cells strongly correlates with DAS28 and TNF-α levels. (A) RA monocyte from six different patients were treated with anti-TLR5 Ab or IgG (10 μg/ml; InvivoGen) for 1 h prior to being treated with RA SF (10%; n = 6) for 6 h. (B) RA monocytes were pretreated with anti-TLR5 Ab or IgG control 1 h prior to treating the cells with PBS or flagellin for 6 h. Subsequently, the TNF-α mRNA levels were quantified in (A) and (B) by real-time RT-PCR and normalized to GAPDH value. In (A), the data are shown as fold increase above RA monocytes treated with RA SF plus anti-TLR5 Ab. Whereas in (B), the data are shown as fold increase above the IgG-pretreated PBS group. Linear regression analysis was used to compare TNF-α levels with TLR5 (C) (n = 48 RA patients) as well as DAS28 score with expression of TLR5 (RNA mass normalized to GAPDH mass) (D) (n = 45 RA patients) or TNF-α (RNA mass normalized to GAPDH mass) (E) (n = 45 RA patients) in RA monocytes. The mRNA expression in RA monocytes is shown as a fold increase above NL PB monocytes and is normalized to GAPDH. *p < 0.05.
monocytes. These results suggest that RA disease expression is related to ligation of TLR5 and production of TNF-α from RA monocytes.

**Proinflammatory factors regulate expression of TLR5 in RA monocytes and macrophages**

To determine which factors modulate expression of TLR5 in RA PB monocytes or in vitro-differentiated macrophages, cells were either untreated or treated with poly I:C (only in RA monocytes), LPS, IL-1β, TNF-α, IL-17, IL-6, IL-8, or RA synovial fluid. Results from these experiments demonstrate TLR5 expression was modulated by TNF-α in RA monocytes and by IL-17 and IL-8 in RA macrophages; however, expression levels of TLR5 were suppressed by TLR3 and TLR4 ligation in RA monocytes and/or differentiated macrophages (Fig. 4). Hence, the data suggest that with the exception of LPS, the expression of TLR5 in RA monocytes and macrophages is differentially regulated in RA monocytes and differentiated macrophages.

**Ligation of TLR5 induces production of proinflammatory factors in RA PB monocytes and macrophages**

Next, we asked whether RA monocytes and differentiated macrophages respond to ligation of flagellin to TLR5. For this purpose, RA monocytes and differentiated macrophages were activated with different doses of flagellin, and cells were screened for transcription (6 h) and production (24 h) of proinflammatory factors such as TNF-α, IL-6, and CCL2. Generally, transcription but not the secretion of TNF-α, IL-6, and CCL2 was dose dependently increased with flagellin stimulation in RA monocytes and differentiated macrophages (Figs. 5, 6A–C). Although TLR5 expression was greatly elevated in RA monocytes compared with RA-differentiated macrophages, TLR5 ligation resulted in higher
(TNF-α) or comparable production of proinflammatory factors (IL-6 and CCL2) in RA macrophages compared with that of monocytes. These results suggest that despite lower expression of TLR5 in RA macrophages, both monocytes and macrophages respond comparably to TLR5 ligation.

**Flagellin-induced CCL2 is regulated by NF-κB and PI3K pathways in RA macrophages**

We next inhibited flagellin-activated pathways in RA-differentiated macrophages to determine signaling pathways contributing to flagellin-mediated proinflammatory factor production. We found that p38 (5 min), AKT1 (5 min), ERK (35 min), and NF-κB (15 min) pathways (Fig. 6D–G) were activated by flagellin stimulation in RA-differentiated macrophages. We chose to examine the regulation of flagellin-induced CCL2 because this chemokine was detected both in RA-differentiated macrophages and fibroblasts. Although chemical inhibitors to NF-κB and PI3K suppressed flagellin-induced CCL2 secretion by 3- to 6-fold \( (p < 0.05; \text{Fig. 6H}) \), inhibition of p38 or ERK pathway did not reduce the levels of CCL2 secretion by RA-differentiated macrophages. Our results suggest that activation of NF-κB and PI3K by flagellin regulates CCL2 production in RA-differentiated macrophages.

**TLR5 is elevated in RA fibroblasts and its expression is responsive to stimulation**

On the basis of our histological data, we asked whether expression of TLR5 was elevated in RA compared with NL ST fibroblasts. Results obtained from real-time RT-PCR demonstrate that TLR5 (Fig. 7A) expression was 23-fold greater in RA compared with NL ST fibroblasts. We next show that with the exception of LPS, all other proinflammatory factors such as TNF-α (14-fold), IL-1β (7-fold), IL-17 (31-fold), IL-8 (20-fold), and RA synovial fluid (28-fold) greatly upregulate the expression of TLR5 in RA fibroblasts (Fig. 7B). To determine whether RA fibroblasts respond to TLR5 ligation, flagellin-activated cells (at two different doses) were screened for a variety of proinflammatory factors. Unlike RA monocytes and differentiated macrophages that are very responsive to flagellin stimulation, RA fibroblasts produce increased levels of IL-6 and CCL2 only when activated with a higher dose of flagellin (100 ng/ml). We found that flagellin activates JNK (15 min), ERK (15 min), AKT1 (35 min), and degradation of IκB (Fig. 7G) and/or equal loading control. To examine which of the signaling pathways were associated with TLR5-induced CCL2 production, in RA macrophages, cells were untreated (DMSO) or treated with 10 μM inhibitors to ERK (PD98059), p38 (SB203580), PI3K (LY294002), or NF-κB (MG-132) for 1 h. Cells were subsequently activated with flagellin (100 ng/ml) for 24 h, and the conditioned media were collected to quantify the levels of CCL2 using ELISA (H). Values are the mean ± SEM; \( n = 4 \). \( *p < 0.05 \).

---

**FIGURE 6.** CCL2 levels are increased following TLR5 ligation in RA monocytes and macrophages. Furthermore, in RA macrophages, flagellin-induced CCL2 production is modulated by PI3K and NF-κB pathways. RA monocytes (A) and differentiated macrophages (B) were either untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 6 h, and expression levels of CCL2 (A, B) were quantified by real-time RT-PCR; \( n = 6–10 \). The data are shown as fold increase above untreated cells and were normalized to GAPDH. Supernatants were harvested from RA monocytes (mono) or differentiated macrophages (mac) untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 24 h, and CCL2 (C) levels were determined by ELISA. Values are the mean ± SEM; \( n = 5–8 \). To determine the mechanism of TLR5 activation in RA macrophages, cells were stimulated with flagellin at 100 ng/ml for 0–65 min, and the cell lysates were probed for, p-ERK (D), p-p38 (E), p-AKT1 (F), and degradation of IκB (G) and/or equal loading control. To examine which of the signaling pathways were associated with TLR5-induced CCL2 production, in RA macrophages, cells were untreated (DMSO) or treated with 10 μM inhibitors to ERK (PD98059), p38 (SB203580), PI3K (LY294002), or NF-κB (MG-132) for 1 h. Cells were subsequently activated with flagellin (100 ng/ml) for 24 h, and the conditioned media were collected to quantify the levels of CCL2 using ELISA (H). Values are the mean ± SEM; \( n = 4 \). \( *p < 0.05 \).
ligation of TLR5 can induce production of proinflammatory factors through the same signaling pathways in both RA macrophages and fibroblasts, macrophages are comparatively more sensitive to TLR5 activation.

Discussion

In the current study, we show that RA and OA ST lining and sublining macrophages and endothelial cells express higher levels of TLR5 than tissues of normal controls. We found that transcription levels of TLR5 were elevated in RA synovial fluid macrophages and RA monocytes compared with RA and NL-differentiated macrophages. Confirming histological studies, TLR5 levels were also elevated in RA compared with NL fibroblasts. We show that in RA fibroblasts and macrophages, the TLR5 mRNA concentration was modulated by IL-17 and IL-8. Despite elevated cell surface levels of TLR5 in RA PB monocytes compared with differentiated macrophages, production of proinflammatory factors was comparable in both cell types, which was higher than what was secreted by RA fibroblasts following ligation. Most importantly, we document that in RA monocytes, TLR5 is a regulator of synovial fluid-mediated TNF-α transcription, and levels of this receptor are strongly correlated to TNF-α and DAS28 score. These results suggest that TLR5 endogenous ligand(s) in the RA joint may potentially activate TLR5+ RA monocytes and contribute to production of joint TNF-α and perpetuation of disease activity.

To our knowledge, we show for the first time that TLR5 expression is elevated in RA and OA ST lining and sublining macrophages and endothelial cells compared with normal individuals. However, expression of TLR5 has not been associated with systemic lupus erythematosus (35). Previous studies demonstrate that TLR5 is expressed in dendritic cells (36), neutrophils (37), and synovial fibroblasts from patients with juvenile idiopathic arthritis (38) and in a number of endothelial cell lines (39) however its expression is undefined in RA ST and blood cells.

Interestingly, we found that differentiation of RA monocytes to macrophages reduces TLR5 expression, as confirmed by both real-time RT-PCR and FACS studies. The same trend was also observed in normal cells. As with TLR5, expression of TLR2 was greater in normal monocytes compared with PB-differentiated macrophages,

![Figure 7: Expression of TLR5 in RA ST fibroblasts is very responsive to stimulation; however, only higher concentrations of flagellin can induce production of IL-6 and CCL2, which is modulated by PI3K and NF-kB pathways. (A) TLR5 mRNA levels were determined in NL and RA ST fibroblasts using real-time RT-PCR (n = 7). The data are shown as fold increase above NL ST fibroblasts and are normalized to GAPDH. (B) RA ST fibroblasts were untreated (PBS) or treated with LPS (10 ng/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml), IL-17 (50 ng/ml), IL-6 (10 ng/ml), IL-8 (10 ng/ml), or RA SF (10%) for 6 h, and the expression of TLR5 was measured by real-time RT-PCR (n = 5–12). The data are shown as fold increase above untreated RA fibroblasts and are normalized to GAPDH. (C) Supernatants were harvested from RA fibroblasts untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 24 h, and IL-6 and CCL2 levels were determined by ELISA (n = 4). Values demonstrate mean ± SEM; *p < 0.05. To determine the mechanism of TLR5 activation in RA fibroblasts, cells were stimulated with flagellin 100 ng/ml for 0–65 min, and the cell lysates were probed for p-ERK (D), p-JNK (E), p-AKT1 (F), and degradation of IκB (G) and/or equal loading control. To examine which of the signaling pathways were associated with TLR5-induced CCL2 production, in RA fibroblasts, cells were untreated (DMSO) or treated with 10 μM inhibitors to ERK (PD98059), JNK (SP600125), PI3K (LY294002), or NF-κB (MG-132) for 1 h. Cells were subsequently activated with flagellin (100 ng/ml) for 24 h, and the conditioned media were collected to quantify the levels of CCL2 using ELISA (H). Values are the mean ± SEM; n = 4. *p < 0.05.
whereas similar levels of TLR4 were detected in normal PB monocytes and differentiated macrophages (7). Furthermore, elevated expression levels of TLR2 and TLR4 in RA synovial fluid macrophages compared with normal macrophages (7) is consistent with our findings with TLR5. In contrast to our results, others have shown that TLR5 is similarly expressed in normal PB monocytes and macrophages (37). The discrepancy in the data may be due to monocyte isolation technique as well as using 100 ng/ml M-CSF for macrophage differentiation studies (37). In addition, we demonstrate that TLR3 and TLR4 ligation reduced TLR5 expression on RA monocytes and/or macrophages or fibroblasts. TRIF is an adaptor protein that is shown to degrade TLR5 expression through a caspase-dependent manner (40). Hence, suppression of TLR5 expression in RA cells may be due to activation of TRIF by TLR3 or TLR4 ligation. In RA fibroblasts, although expression of TLR5 is reduced by TLR4 ligation, stimulation with IL-1β has a reverse effect, and this may be due to its lack of association with the TRIF pathway (41). With the exception of LPS, TLR5 expression is differentially regulated in monocytes and macrophages. Others have shown that in human monocytes, expression of TLR5 is suppressed by TLR2 ligation as well as stimulation with IFN-γ and GM-CSF; however, TLR5 expression is greatly increased by flagellin ligation (37, 42). In RA macrophages and fibroblasts, expression of TLR5 was modulated by IL-17. Previous studies have shown that TLR5 ligation can induce Th17 cell differentiation in normal PB mononuclear cells (43) as well as the production of IL-17 in splenocytes (44). IL-17 can also enhance TLR5-induced TNF-α and IL-1β production in epithelial cells (45). These results suggest that expression and ligation of TLR5 on cells present in RA ST lining may be in feedback regulation with Th17 cell differentiation and production of joint IL-17.

Our results suggest that TLR5 endogenous ligand(s) may be present in synovial fluid because blockade of this receptor on monocytes significantly reduces TNF-α transcription induced by synovial fluid. Interestingly, a number of endogenous TLR ligands have been identified in RA ST and fluid including fibrinogen, heat shock protein (HSP)60, 70, and 96 and ED fibronectin that bind to TLR2 and/or TLR4 (33, 34). Previous studies demonstrate that TLR5-transfected reporter HEK 293T cells stimulated with full-length HSP70 had enhanced flagellin-induced NF-κB-mediated luciferase activity; however, this effect was not detected with HSP70 treatment alone (46). These findings suggest that HSP70 expressed in RA synovial fluid (47), ST macrophages, and fibroblasts (33) may be a chaperone protein for TLR5 endogenous ligand(s) (48). Lectins have also been identified as novel agonists for cell surface-bound TLRs (49). On the basis of earlier investigations, there is a possibility that HSPs (33, 34) and/or lectins (49) may be potential TLR5 endogenous ligands in RA joints. Therefore, studies are currently being conducted to identify RA synovial fluid TLR5 endogenous ligands (not within the scope of this study).

Our results suggest that ligation of synovial fluid TLR5 endogenous ligands to TLR5+ monocytes can contribute to production of joint TNF-α, which in turn can further upregulate expression of TLR5 on these cells. Once RA monocytes reach their destination in the joint and differentiate to macrophages, TLR5 expression is no longer modulated by TNF-α, and their levels are reduced; however, they remain at least as responsive to ligation as RA monocytes. Perhaps in RA monocytes, TLR5 levels correlate with DAS28 and TNF-α and are in a feedback regulation with TNF-α by producing and responding to this factor to perpetuate disease.

When RA monocytes and differentiated macrophages were stimulated with flagellin, similar levels of IL-6 and CCL2 were produced despite RA macrophages having lower TLR5 expression compared with RA monocytes. This may be due to monocytes being in circulation, whereas macrophages are immobilized in the inflammatory milieu of RA ST in cell-to-cell contact with other macrophages or RA fibroblasts, therefore amplifying the activation response. It is also possible that macrophages from RA ST, like those from RA synovial fluid, have higher TLR5 expression compared with RA monocytes, and maybe the presence of proinflammatory factors is required to enhance TLR5 expression during the differentiation process, which is available in the RA joint and unavailable in the culture system. In contrast to our results, other studies were unable to detect TNF-α production when normal monocytes were activated with flagellin (50). This may be due to lower levels of TLR5 expression in normal cells compared with RA monocytes as well as isolation and culturing methods. However, consistent with our data, they were able to demonstrate high levels of CCL2 following TLR5 ligation in normal monocytes (50).

Unlike RA fibroblasts where only higher concentrations of flagellin (100 ng/ml) are capable of inducing expression of IL-6 and CCL2, in RA monocytes and macrophages, ligation of TLR5 with lower concentrations (10 ng/ml) can produce these factors. Conversely, in skeletal muscle cells, ligation of TLR5 was unable to produce significant levels of CCL2 without IFN-γ priming (51). Despite activation of nonoverlapping pathways in RA fibroblasts and macrophages by TLR5 ligation, CCL2 production was modulated by inhibition of NF-κB and PI3K in both cell types. In contrast to our results, blockade of PI3K or use of PI3K/AKT-deficient mice resulted in marked increase in flagellin-induced IL-6 or IL-8/KC levels (52), indicating that the proinflammatory factors produced as a result of TLR5 ligation are differentially regulated in mice and humans.

In conclusion, to our knowledge, we demonstrate for the first time that TLR5 is expressed in RA ST macrophages and fibroblasts as well as RA PB monocytes. We further document modulating factors and pathways contributing to TLR5 inflammatory response. Moreover, our study highlights that there is a strong correlation between TNF-α and TLR5 expression with disease activity in RA monocytes suggesting that TLR5 may be a TNF-α responsive gene that is linked to RA progression.

Disclosures

The authors have no financial conflicts of interest.

References


