Expression of Toll-like receptors 2 and 4 is increased in peripheral blood and synovial fluid monocytes of patients with enthesitis-related arthritis subtype of juvenile idiopathic arthritis

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Abstract

Objective. Bacterial trigger possibly causes disease exacerbation in enthesitis-related arthritis (ERA) patients. Microbes initiate immune responses through Toll-like receptors (TLRs). We studied TLR expression on blood and SF monocytes and the effect of TLR ligands on peripheral blood (PB) mononuclear cells (PBMCs) in ERA patients.

Methods. PB from 26 ERA patients and 19 healthy subjects and paired SF from 13 patients were collected. Dual-colour flow cytometry was done for TLR and CD14 expression. Results are expressed as median fluorescence intensity (MFI). Real-time PCR was done for TLRs. PBMCs were stimulated with lipopolysaccharide (LPS) or peptidoglycan and levels of IL-6 and MMP-3 measured in the culture supernatants.

Results. PBMCs from ERA patients had higher expression of TLR-2 [MFI 295.5 (48.1–598) vs 179 (68.7–442); P < 0.05] and TLR-4 [MFI 448 (178–2581) vs 402 (229–569); P < 0.05] as compared with controls. TLR-9 expression showed no significant difference between the two groups. In paired samples, SF mononuclear cells (SFMCs) had higher expression of both TLR-2 [MFI 485 (141–1683) vs 353 (118–598); P < 0.05] and TLR-4 [MFI 1016 (42.4–3159) vs 513 (193–2581); P < 0.05] as compared with PBMCs. Difference in TLR-9 expression was not significant. TLR RNA expression data were similar.

Patients’ PBMCs produced more IL-6 (13.51 vs 6.54 ng/ml) and MMP-3 (61 vs 32.9 ng/ml) as compared with those of the controls, on stimulation by LPS. With peptidoglycan also, IL-6 (30.58 vs 10.84) and MMP-3 (102.54 vs 49.45) were higher than in controls.

Conclusion. Increased TLR-2 and TLR-4 expression on PBMCs and SFMCs may recognize microbial/endogenous ligands and up-regulate IL-6 and MMP-3 leading to disease exacerbation.

Key words: Innate immunity, Juvenile arthritis, Inflammation, Microbial products, Toll-like receptors.

Introduction

JIA is a heterogeneous group of chronic arthritides afflicting children <16 years of age. As per the ILAR criteria, JIA includes seven subtypes [1]. These include systemic-onset JIA, oligoarticular JIA, RF-negative polyarticular JIA, RF-positive polyarticular JIA, enthesitis-related arthritis (ERA), PsA and undifferentiated JIA. Among all the subsets, ERA is most prevalent in India. The pathogenesis of JIA is thought to be an interplay between environmental factors and genetic susceptibility [2]. Microorganisms are one of the important environmental factors suspected to play a role in its pathogenesis. Most microbes interact with the immune system through Toll-like receptors (TLRs).

TLRs are critical components of the innate immune system that help protect the host from infectious disease through the recognition of pathogen-associated molecular patterns [3]. Interaction of TLRs with their ligands results in production of various pro-inflammatory cytokines and effector molecules [4]. Excessive pro-inflammatory
cytokine production coupled with enhanced cell activation can lead to inflammatory diseases affecting a broad range of tissues and organs. Amplification of immune responses by TLRs, while crucial in bringing innate and adaptive immunity together, may also contribute to disease pathogenesis [5]. Experimental models have shown the ability of microbial TLR ligands to trigger arthritis in animals [6].

Ligands for TLRs are found in rheumatoid synovium and may be involved in the pathogenesis and persistence of inflammatory arthritis. TLRs are able to up-regulate TNF-α production in response to endogenous ligands like HSPs [7] released after tissue damage, suggesting that TLRs can maintain an inflammatory response in the absence of pathogen [8]. Work previously done in our lab had suggested that in ERA patients a bacterial trigger may exacerbate the disease [9]. Further, fibroblast-like synoviocytes (FLSs) from ERA patients produce pro-inflammatory cytokines like IL-6, IL-8 as well as matrix-degrading enzymes (FLSs) from ERA patients produce pro-inflammatory cytokines [10]. Thus, TLRs may play a role in immunopathogenesis of JIA.

TLR-2 and TLR-4 are expressed on the synovial tissue in RA as well as in CIA [11]. FLSs from RA patients also have increased expression of TLR-2 mRNA as compared with OA [12]. CD16+ monocytes in peripheral blood (PB) of RA patients had higher expression of TLR-2 as compared with healthy control [13]. However, data on expression of TLR on monocytes/macrophages in JIA are not available; therefore, we studied expression of TLR-2, TLR-4 and TLR-9 on monocytes in PB and SF of ERA patients by dual-colour flow cytometry and real-time PCR. Further, we studied the response of PB mononuclear cells (PBMCs) to TLR ligands to see whether increased expression results in increased production of pro-inflammatory mediators.

Materials and methods

Patients and controls

ERA patients with active disease who satisfied ILAR criteria [1] and whose parents gave informed consent were enrolled in the study. PB and SF (where available) were obtained from ERA patients. PB was collected from similar age and gender non-related healthy controls. One millilitre of blood was drawn in potassium EDTA vials for DNA isolation and HLA-B27 PCR

Isolation of mononuclear cells

PBMCs and SF mononuclear cells (SFMCs) were isolated using density gradient centrifugation by layering on Histopaque 1077-1 (Sigma-Aldrich, St Louis, MO, USA).

DNA isolation and HLA-B27 PCR

DNA was isolated using columns (Qiagen Mini Kit, Valencia, CA, USA) as per manufacturer’s instructions. Primers used for HLA-B27 PCR were [14]: HLA-B27 forward: 5′-GCT ACG TGG ACG ACA AGC T-3′; HLA-B27 reverse 1: 5′-CTC GGT CAT CCT CTT GTG C-3′; HLA-B27 reverse 2: 5′-TCT CGG TAA GTC TGT GGC TT-3′; internal control forward: 5′-TGC CAA GTG GAG CAA CCA A-3′; internal control reverse: 5′-GCA TCT TGC TCT GTG CAG AT-3′.

The PCR conditions were: initial denaturation at 96°C for 1 min; 5 cycles of 96°C (25 s), 70°C (45 s), 72°C (30 s); 21 cycles of 96°C (25 s), 65°C (45 s), 70°C (30 s); 4 cycles of 96°C (25 s), 55°C (60 s), 72°C (120 s); and final elongation at 72°C for 10 min. The PCR products were run on 1.2% agarose gel with a 100-bp ladder (New England Biolabs, Ipswich, MA, USA).

Flow cytometry

PBMCs and SFMCs were dual stained for CD14 (monocyte marker) and TLR-2, TLR-4 or TLR-9. The antibodies used were CD14-FITC (BD Biosciences, San Diego, CA, USA), TLR-2-PE, biotinylated TLR-4, streptavidin-PE (eBiosciences, San Diego, CA, USA) and TLR-9-PE (Imgenex, Bhuneswar, Orissa, India).

For TLR-2 and TLR-4 detection, anti-CD14 antibody and anti-TLR antibody were added to 0.5 x 10⁶ cells suspended in PBS and incubated in the dark at room temperature for 30 min. Cells were washed by centrifuging with 2 ml of PBS for 5 min at 800 g. Streptavidin-PE was added to tubes for TLR-4 detection, incubated for 30 min and washed as above.

For TLR-9 detection, cells were first incubated with anti-CD14 antibody as above. After washing, 20 μl of fixation buffer (AB Serotec, Oxford, UK) was added to the cells and they were incubated for 30 min at room temperature. Following washing, the cells were incubated with 20 μl of permeabilization buffer (AB Serotec) and incubated for 20 min. Subsequently, the cells were incubated with anti-TLR-9 antibody for 30 min. Cells were suspended in stabilization buffer (1% formaldehyde in PBS) and stored at 4°C before acquisition and analysis.

A total of 10⁶ cells were acquired on BD FACScalibur using CellQuest software (BD Biosciences). Plots were analysed using FlowJo version 8.4.3 software (Tree Star, Inc., Ashland, OR, USA) and median fluorescence intensities (MFIs) were calculated. Mononuclear cells were gated from the total population. Subsequently, MFI was calculated for CD14+ cells expressing TLRs of interest.

RNA isolation, cDNA conversion and real-time PCR

PBMCs and SFMCs were stored at −80°C in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and RNA was isolated by columns (RNeasy Kit Qiagen, Valencia, CA, USA) using the manufacturer’s protocol with slight modification. After thawing the cells and vortexing to facilitate lysis, 200 μl of chloroform was added per millilitre of Trizol used. After centrifugation at 4°C for 10 min at 12,000 g, the interface was separated and subsequent processing was done using columns. RNA was eluted in RNase-free water and estimated by spectrophotometer (Nanodrop, Wilmington, DE, USA).
cDNA was prepared using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) as per the manufacturer’s instructions. Briefly, 2 μg RNA was added to a master mix comprising RT buffer, dNTP mix, random hexamers and reverse transcriptase. The mixture was incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. Subsequently, real-time PCR was performed using SyBR Green chemistry on Applied Biosystems 7500 Real Time PCR system. Primers were purchased from Sigma-Aldrich (Bangalore, India). Details of primers used are given elsewhere [15].

Each reaction mixture of 20 μl comprised 100 ng cDNA, primers, SyBR Green (Applied Biosystems, Foster City, CA, USA) and sterile water. Reaction conditions were initial denaturation of 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation step was added at the end, comprising 1 cycle of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s.

The 18S rRNA was used as housekeeping gene. Relative fold change was determined using the \( \Delta\Delta C_t \) method \(( C_t = \text{cycle threshold})\), where fold change = \( 2^{-\Delta\Delta C_t} \) and \( \Delta C_t = \Delta C_{\text{TLR}} - \Delta C_{\text{18S}} \), where patient sample − (calibrator sample × control median). Patients’ PBMC Ct was calibrated with PBMCs of healthy controls, while patients’ SFMC Ct was calibrated with the corresponding SFMCs. More than 2-fold increase in expression was considered significant.

### Functional assay
PBMCs \((10^5)\) from eight ERA patients and seven healthy controls were cultured in 10% cRPMI (Sigma-Aldrich) and stimulated with LPS (TLR-4 ligand; 4 μg/ml) or peptidoglycan (TLR-2 ligand; 10 μg/ml). TNF (eBiosciences, 20 ng/ml) was used as positive control and unstimulated cells served as negative control. Supernatants were harvested after 48 h and IL-6 (BD OptEIA Kit, San Jose, CA, USA) and MMP-3 (R&D Systems, Minneapolis, MN, USA) levels were measured by ELISA.

### Statistical analyses
All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Wilcoxon-signed rank test was employed for comparison of PBMC-SFMC pairs, while Mann–Whitney U-test was used to compare data of healthy controls and ERA patients. A \( P < 0.05 \) was considered to be statistically significant.

### Results
Twenty-six patients were recruited after obtaining informed written consent from them/their parents. The patient demographics are given in Table 1. Of these, 22 had enthesitis, 20 had arthritis at the time of inclusion in the study. Nine patients were on MTX, two on prednisolone and the rest on NSAIDs. Median age of controls was 24 years (ranging from 18 to 27 years).

### Table 1 Patient demographics

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<td>Males</td>
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<tr>
<td>Median age at presentation, years</td>
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<tr>
<td>Median disease duration, years</td>
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<td>HLA-B27-positive individuals</td>
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Surface and intracellular TLR expression
The CD14+ PBMCs of ERA patients had significantly higher levels of TLR-2 expression as compared with healthy controls [patient MFI 295.5 (481.1–598) vs control MFI 179 (68.7–442); \( P < 0.05 \); Fig. 1a]. Patients also had higher numbers of CD14+TLR-2+ cells compared with healthy controls [patient median 7.305 (5.34–10.05) vs control median 5.31 (3.93–6.91); \( P < 0.05 \); Fig. 1b]. TLR-4 expression was also significantly elevated in patients [patient MFI 458 (178–2581) vs control MFI 402 (229–569); \( P < 0.05 \); Fig. 1c]. Similar to TLR-2, patients had higher numbers of CD14+TLR-4+ cells compared with healthy controls [patient median 4.27 (3.16–7.45) vs control median 3.5 (2.67–4.58); \( P < 0.05 \); Fig. 1d].

In paired samples \((n = 13)\), TLR-2 expression (MFI) was higher on SF monocytes as compared with PB monocytes \([458 (141–1683) vs 353 (180–598); \( P < 0.05 \); Fig. 2a] and patients also had higher numbers of CD14+TLR-2+ cells \([14.5 (8.8–27.7) vs 6.8 (4.96–8.95); \( P < 0.05 \); Fig. 2b]. Patients’ SFMCs also had elevated TLR-4 expression compared with PBMCs [MFI 1016 (42.4–3159) vs 513 (193–2581); \( P < 0.05 \); Fig. 2c; percentage of CD14+TLR-4+ cells 18.6 (16.31–21.1) vs 3.77 (2.77–4.98); \( P < 0.05 \); Fig. 2d]. TLR-9 expression showed no significant difference between both the two groups (Fig. 2e and f).

Flow cytometry dot plots of PBMCs and SFMCs from a single representative patient are given in supplementary figures S1 and S2 (available as supplementary data at Rheumatology Online).

### RNA levels
Relative to healthy controls (Fig. 3a), patients’ PBMCs had higher amounts of TLR-2 [median (IQR) relative fold change 3.33 (1.77–7.18)] and TLR-4 [median (IQR) relative fold change 3.94 (1.9–5.5)], whereas there was no significant change in levels of TLR-9 transcripts [median (IQR) relative fold change 1.32 (0.87–1.7)].

For paired samples (Fig. 3b), expression in SFMCs was calculated relative to PBMCs, and RNA levels were found to be higher for TLR-2 [median (IQR) relative fold change 2.7 (1.89–3.35)] and TLR-4 [median (IQR) relative fold change 2.36 (1.09–3.18)], while TLR-9 exhibited no change in expression [median (IQR) relative fold change 0.96 (0.03–1.71)]. Thus, protein data were corroborated by RNA data at both PBMC and SFMC levels.
Functional assay

PBMCs from ERA patients produced higher amounts of IL-6 (Fig. 3) and MMP-3 (Fig. 4) in response to stimulation with TLR ligands as compared with healthy controls. Patients’ PBMCs produced a median of 13.5 ng/ml (6.3–19.85 ng/ml) IL-6 and 61 ng/ml (27–66 ng/ml) MMP-3, as opposed to 6.54 ng/ml (5.5–19.5 ng/ml) IL-6 and 32.9 ng/ml (30.1–57 ng/ml) MMP-3 by controls, when stimulated with 4 mg/ml LPS. Stimulation with 10 mg/ml peptidoglycan resulted in production of 30.58 ng/ml (21–40.5 ng/ml) IL-6 and 102.54 ng/ml (82–138 ng/ml) MMP-3 by patients and 10.84 ng/ml (8.04–27 ng/ml) IL-6 and 49.45 ng/ml (41.2–60 ng/ml) MMP-3 by controls. However, there was no significant difference in levels of either MMP-3 or IL-6 on stimulation with TNF-α.

Discussion

Our data suggest that ERA patients have higher expression of TLR-2 and TLR-4 on PB monocytes, which is even higher on the SF monocytes. These findings were confirmed by RNA expression in PBMCs. Further, stimulation by TLR ligands led to increased production of IL-6 and MMP-3 by the PBMCs from ERA patients.

Ours is the first study that has shown TLR overexpression in immune cells in ERA. Previous studies...
have demonstrated over-expression of TLRs on monocytes from RA [13], AS [16] and PsA [17] patients. Similar to our data, TLR-2 has been found to be the most commonly over-expressed TLR [13]. TLR-2 recognizes peptidoglycan, a glycan found in many bacteria. TLR-9 expression, though found to be increased in SLE [18], has not been found to be increased in inflammatory arthritis [19] similar to our observations. As RNA levels were also increased it suggests regulation at the level of transcription.

Over-expression of TLR on SF monocytes as compared with PB suggests that the local milieu in the synovium further enhances the expression of TLR or cells with higher expression selectively home to the synovium.

Synovial biopsy tissue from RA [20] as well as CIA model [11] shows increased expression of TLRs. Later, FLSs derived from RA synovial tissue were found to have increased expression of TLRs [19, 21]. This suggests that the pro-inflammatory milieu may be responsible for this over-expression. Cytokines are known to increase TLR transcription and their expression [22]. Synovial fibroblasts cultured in vitro can up-regulate the expression of TLR-2 upon stimulation with IL-1 and peptidoglycan [23].

What is the functional relevance of this over-expression? TLR ligands should lead to increased activation of cells having higher TLR expression and thus result in production of pro-inflammatory cytokines and other...
mediators. Our PBMC stimulation data indeed suggest that patients’ PBMCs produce more IL-6 as compared with healthy controls, when stimulated with TLR-2 and TLR-4 ligands, whereas there is no difference on TNF stimulation. Even among TLR-2 and TLR-4, TLR-2 stimulation led to higher IL-6 and MMP-3 production paralleling the level of expression. In another study, PBMCs from RA patients had higher fold increase in IL-6 and TNF production on stimulation by LPS [24].

For the TLR pathway to be activated in vivo, TLR ligands are needed. Indeed, bacterial products like LPS and peptidoglycan are found in patients with JIA, ReA, etc. [25]. Further endogenous ligands of TLR are also over-expressed in inflammatory arthritis. The RA synovium contains a large repertoire of TLR ligands, namely, hyaluronan oligosaccharides, fibronectin fragments, antibody–DNA complexes, HSPs and necrotic cells, along with several bacterial products (potential TLR ligands) such as LPS and unmethylated CpG motifs [26]. In ERA, increased proliferation of SFMCs to bacterial antigen has been shown suggesting that bacterial antigens may be present in the synovium, which leads to generation of bacterial antigen-specific T cells [9]. In a streptococcal cell wall arthritis model of RA, arthritis was Myd88 and TLR dependent, again highlighting the role of the TLR pathway in chronic arthritis [6]. Arthritis can be induced by unmethylated CpG motifs (found in bacterial DNA) that bind to TLR-9 [27] and by peptidoglycan [28].

Blocking the TLR pathway may be an attractive therapeutic tool. Inhibition of TLR-4 suppresses the severity of experimental arthritis and results in lower IL-1 expression in arthritic joints [29]. The use of TLR-9-specific inhibitory oligodeoxynucleotides to ameliorate autoimmune diseases like RA and SLE has also been reported [30]. DMARDs such as HCQ used in RA and JIA inhibit the TLR-3-mediated pathway [31]. In other words, not only have the TLRs been shown to play a vital role in several inflammatory disorders, but their inhibition also modulates the disease.

Thus, in conclusion, our data suggest that TLR-2 and TLR-4 are over-expressed in JIA and stimulation of these TLRs by potential ligands (microbial or endogenous) may contribute to disease pathogenesis in JIA. TLR-2 and TLR-4 pathways are potential targets of therapeutic intervention in JIA.
### Rheumatology key messages
- ERA patients have higher TLR-2 and TLR-4 expression on blood and SF monocytes.
- Patients’ PBMCs produce higher IL-6 and MMP-3 on stimulation by TLR-2 and TLR-4 agonists.
- TLRs by recognizing microbial ligands/endogenous products may have a role in the pathogenesis of ERA.

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### Supplementary data

Supplementary data are available at *Rheumatology* Online.

### References


