Juvenile idiopathic arthritis (JIA) is the most common cause of chronic joint damage in children. The pathogenesis of JIA is complex and interaction between environmental factors and genetic susceptibility believed to be responsible for the immune alterations and disease. Of the environmental factors, infections are probably the most important. In most autoimmune arthritis live bacteria have never been demonstrated but bacterial products like antigens, DNA have been found. In JIA an elevated cellular immune response has been demonstrated in synovial compartment against enteric bacteria. The potential role of viruses in the...
pathogenesis of arthritis was demonstrated by the findings that human parvovirus B19 transgenic mice are highly susceptible to polyarthritis\(^5\), and the double stranded viral RNA has overt arthritogenic properties\(^6\).

Like adult rheumatoid arthritis (RA), in JIA, proliferation of synovial membrane following infiltration by immune cells is thought to result in degradation of articular cartilage and bone, causing irreversible damage. The destructive progression is mediated by potent enzymes called matrix metalloproteinases (MMPs)\(^7\). Activity of MMPs, is regulated by tissue inhibitor of MMPs called TIMPs. The relative expression of TIMP versus MMPs in joint tissue may be critical in altering the balance between the maintenance of articular cartilage and its destruction in diseases such as RA and JIA. It has been shown that interleukin (IL1) and tumour necrosis factor (TNF) induce MMPs but not TIMP expression, which may define a pivotal role for these cytokines in the pathogenesis of rheumatoid arthritis\(^8\).

Microbial products are known to activate the innate immune system through pattern recognition receptor called Toll like receptors (TLR). The TLRs recognize determinants that are conserved in prokaryotes but are rarely found in eukaryotes. Different TLRs bind to different pathogen associated molecular patterns (PAMP)\(^9\). Classical animal models of arthritis such as adjuvant arthritis or streptococcal cell wall arthritis are dependent on the TLR pathway activation\(^10\). Further, human synovial tissue of patients with RA expresses TLR2 in the synovial lining\(^11\). Later TLR2, TLR9 and TLR3 expression was found on cultured synovial fibroblasts from RA patients\(^12\). Stimulation of fibroblast like synoviocytes (FLS) by TLR 2 ligand but not TLR 9 ligand induced pro-inflammatory cytokines\(^12\). TLR 3 stimulation resulted in upregulation of chemokines and pro-inflammatory cytokines\(^13\).

Thus it is possible that bacterial and viral products may play an important role in the initiation/perpetuation of disease by arthritogenic pathogens by activating the innate system. None of the previous studies have determined how TLR ligands regulate the balanced expression of metalloproteinases versus TIMP in FLS. Further, there are no data on effect of TLR 5 and TLR7 ligands on FLS. All previous studies have used FLS from patients with RA. Therefore, we assessed whether bacterial peptidoglycan (PG), LPS, flagellin, CpG DNA, and viral (poly I:C, and imiquimod) products have similar stimulatory effect like cytokines IL-1\(\beta\) and TNF-\(\alpha\) on synovial fibroblast from patients with enthesis related arthritis (ERA) subset of JIA to produce MMP3 and MMP1 and its inhibitor TIMP1.

**Material & Methods**

**Patient samples:** Eight patients with ERA subset of JIA satisfying the International League of Associations for Rheumatology (ILAR) criteria\(^14\) were enrolled in the study. Four patients with RA were used as disease control. The study was approved by the institutional ethics committee and the patients gave written informed consent. Synovial fluid was collected from only those patients who required intra-articular corticosteroid injection for their management. The study was conducted at Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow during November 2006 to December 2008.

**Culture of cells from synovial fluid:** Synovial fluid was collected in vials containing lithium heparin 1000units/ml (Sigma, St. Louis, USA) from patients with ERA and RA. FLS were cultured from synovial fluid according to the method described by Stebulis et al\(^15\). Synovial fluid was centrifuged at 1500 g for 10 min. The resulting cell pellet was re-suspended in 5 ml of minimum essential medium (MEM) (HyClone, Logan, Utah) with 15 per cent heat inactivated foetal bovine serum (FBS), 1 per cent nonessential amino acid, 1 per cent penicilline streptomycin solution and plated in 25 cm\(^2\) tissue culture flasks. Cells were incubated at 37\(^{\circ}\)C with 5 per cent CO\(_2\) for 24 h after which medium was aspirated and cells were washed with phosphate buffered saline (PBS, 0.15 M pH 7.4) to remove non-adherent cells. Growth medium was replaced every 3 to 4 days. After 10 to 14 days adherent cells were removed from flasks by trypsinization, washed and split 1:2 in 25 cm\(^2\) flasks. Cell in passages 3 to 6 were used for experiments.

**Stimulation of FLS with cytokines and TLR ligands:** Fibroblast like synoviocytes (10,000 cells per well) from four patients with JIA and two patients with RA were stimulated with 10 \(\mu\)g/ml of peptidoglycan from streptococci species (Sigma, St. Louis, MO, USA), 2 \(\mu\)g/ml of LPS *Escherichia coli* (Sigma, St. Louis, MO, USA), 20 \(\mu\)g/ml of unmethylated cytosine guanosine dinucleotide (CpG) oligonucleotide (sequence 5`-ttgctggattttggttgc-3`) (Operon biotechnologies, Germany), 1 ng/ml of IL-1 beta (BD Pharmingen, San Diego, CA), 1ng/ml of TNF-alpha (BD Pharmingen, San Diego, CA). In another set of experiment FLS from 4 patients with JIA and two patients with RA were stimulated with 5 \(\mu\)g/ml of flagellin (Invivogen, San Diego, CA).
Diego, CA, USA), 20 µg/ml of poly I:C (Invivogen, San Diego, CA, USA) and 10 µg/ml of imiquimod (Invivogen, San Diego, CA, USA) in 24 well culture plates for 48 h. Culture supernatant was collected and stored at -80°C for subsequent ELISA experiments.

**ELISA:** Levels of MMP1 and MMP3 in culture supernatant were measured using enzyme immunoassay (Quantikine, R & D systems, Minneapolis, USA). The quantikine MMP3 immunoassay measures total MMP3 (Pro and active MMP3). Briefly, diluted culture supernatant was added to pre-blocked plate and incubated for 2 h. After washing the antibody conjugated with HRP was added. TIMP and IL6 levels were measured using Duo set ELISA development kits (R & D systems, Minneapolis, USA). For TIMP and IL6, assays were carried out as follows. In brief, capture antibodies against the TIMP, IL6 were coated overnight on 96-well ELISA plates (Nunc, Roskilde, Denmark). Plates were then blocked using phosphate buffered saline (0.15 M, pH 7.2) with 1 per cent BSA. After washing the wells were incubated with tests specimen for 2 h. Bound TIMP, IL6 were then detected using a biotinylated anti TIMP, anti-IL6, antibody and streptavidin-horse radish peroxidase conjugate. Tetra-methyl-benzidine was used as the substrate, and absorbance was measured at 450 nm using an ELISA reader (Tecan, Germany). The minimum detection limits of the assays were as follows: MMP3, MMP1 0.156 ng/ml; TIMP1 31.2 pg/ml; IL6 9.3 pg/ml.

IL-8 in culture supernatant was detected by ELISA using the Opt EIA human IL-8 kit according to the manufacturer’s instructions (BD PharMingen, San Diego, CA, USA). The minimum detection limit for the assay was 3.125 pg/ml.

**Reverse transcription-polymerase chain reaction (RT-PCR):** RNA was isolated from 3 X 10⁵ cultured FLS, using Qiagen column (RNeasy mini kit, Qiagen, Germany) according to the manufacturer’s instructions. Five micrograms of total RNA was used in first strand-cDNA using SuperScript™ II RNase H reverse transcriptase (Invitrogen, Life technologies, USA) and Oligo (dT)₁₂₋₁₈ (Bangalore Genei, India). PCR for TLR2, TLR4, TLR7, TLR9 was performed using specific primers for each TLR gene (MWG Biotech, Bangalore, India). The PCR primers were as follows: TLR2, forward primer 5’-GAC TTT GAA ACT CAA GCC -3’, reverse primer 5’-GTT CCT GGA GTT TGT TGA TGT TC -3’; TLR9, forward primer 5’-TAC CAA CAT CCT GAT GCT AGA CTC -3’, reverse primer 5’-TAG GAC AAC AGC AGA TAC TCC AGG -3’; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward primer 5’-GAA GGT GAA GGT CGG AGT C-3’, reverse primer 5’-GGA GGT GAA GGT CGG AGT C-3’, reverse primer 5’-GAA GAT GGT GAT GGG ATT TC-3’. 10 µl of PCR products were analyzed on 1.5 per cent agarose gels and visualized by UV fluorescence after staining with ethidium bromide.

**Statistical analysis:** Paired t test was used for analysis.

**Results**

**Production of IL6 and IL8 by FLS:** Both IL-1β and TNF-α significantly increased the production of IL6 over baseline by 15- and 8.2-fold respectively and IL8 by 24- and 22.9- fold respectively (Fig. 1a and b). Bacterial and viral products PG, PolyI:C, LPS, Flagellin also significantly increased the production of IL6 compared to baseline levels. However imiquimod (TLR7 ligand) and CpG Oligodeoxynucleotide (TLR9 ligand) had no stimulatory effect on IL6 and IL8 production (Fig. 1). The results of JIA FLS were similar to RA FLS.

**Production of MMP3 by FLS:** Initially the effect of pro-inflammatory cytokine on FLS MMP production was assessed. Both IL-1β and TNF-α significantly increased the production of MMP3 over baseline (P<0.01) by 14- and 6.7-fold respectively. MMP3 production was increased on stimulation by PG and LPS as compared to basal levels. Poly I:C and flagellin also induced production of MMP3 in FLS derived from patients with JIA and results were similar to FLS derived from RA. In contrast, CpG DNA and imiquimod had no stimulatory effect on the production of MMP3 by FLS (Fig. 2).

**Production of pro-MMP1 by FLS:** Both IL-1 beta and TNF-alpha significantly increased the production of MMP1 over baseline by 2.8- and 3.4-fold respectively. IL1 beta had more effect on the production of MMP3 while TNF had more effect on MMP1 production. PG, LPS, PolyI:C and flagellin induced production of Pro-MMP1 in FLS derived from patients with JIA and the results were similar for FLS derived from RA. In contrast, CpG DNA and imiquimod had no stimulatory effect on the production of MMP1 by FLS (Fig. 2).

**Effect on TIMP production by synovial fibroblast:** No effect on TIMP production was observed after stimulation by IL1, TNF or by different TLR ligands.
Fig. 1. Production of IL-6 (a,c,e) and IL-8 (b,d,f) in culture supernatants of JIA and RA FLS cultured in the presence of cytokines and TLR ligands. Four different JIA FLS cultures and two different RA FLS cultures were stimulated with IL-1, TNF-alpha (1a,1b), PGN, LPS, CpG ODN or were left untreated (1c, 1d). In another set of experiments 4 different JIA FLS and 2 different RA FLS cultures were stimulated with PolyI:C, flagellin, Imiquimod or were left untreated (1e, 1f). P<0.05, **<0.01, ***<0.001 compared to baseline.
Fig. 2. Production of MMP3 (a,c,e) and MMP1 (b,d,f) in culture supernatants of JIA and RA FLS cultured in the presence of cytokines and TLR ligands. Four different JIA FLS cultures and two different RA FLS cultures were stimulated with IL-1, TNF-alpha (2a,2b), PGN, LPS, CpG ODN or were left untreated (2c, 2d). In another set of experiments 4 different JIA FLS and 2 different RA FLS cultures were stimulated with PolyI:C, flagellin, Imiquimod or were left untreated (2e, 2f). $P^*<0.05$, $**<0.01$, $***<0.001$ compared to baseline.
Fig. 3. Production of TIMP in culture supernatants of JIA and RA FLS cultured in the presence of cytokines and TLR ligands. Four different JIA FLS cultures and two different RA FLS cultures were stimulated with IL-1, TNF-alpha (3a), PGN, LPS, CpG ODN or were left untreated (3b). In another set of experiments 4 different JIA FLS and 2 different RA FLS cultures were stimulated with PolyI:C, flagellin, Imiquimod or were left untreated (3c).

Fig. 4. Expression of TLR2, TLR4, TLR7 and TLR9 in FLS derived from patients with JIA. Lane 1 shows 100 bp ladder, Lane 2 shows 225 bp product of GAPDH, lane 3 shows 294 bp product of TLR2, lane 4 shows 439 bp product of TLR4, lane 5 does not show any product of TLR7 and lane 6 shows a faint band at 233 bp of TLR9. Primer dimers are seen at the bottom in lane 3-6.

Fig. 4. Expression of TLR2, TLR4, TLR7 and TLR9 in FLS derived from patients with JIA. Lane 1 shows 100 bp ladder, Lane 2 shows 225 bp product of GAPDH, lane 3 shows 294 bp product of TLR2, lane 4 shows 439 bp product of TLR4, lane 5 does not show any product of TLR7 and lane 6 shows a faint band at 233 bp of TLR9. Primer dimers are seen at the bottom in lane 3-6.

Like PG, LPS, Poly I:C, CpG DNA, flagellin and imiquimod (Fig. 3).

Detection of TLR 2, 4, 7 and 9 expression in JIA FLS: TLR2, TLR4 and low expression of TLR9 was found but, no expression of TLR7 was found in FLS derived from JIA patients (Fig. 4).

Discussion

In this study, increase in the production of IL6, IL8, MMP3 and MMP1 was found in culture supernatant after stimulation by bacterial and viral products like peptidoglycan (TLR2 ligand), LPS (TLR4 ligand), PolyI:C (TLR3 ligand), flagellin (TLR5 ligand); however, CpG DNA (TLR9 ligand) and imiquimod (TLR7 ligand) had no effect on synovial fibroblasts. Bacterial and viral products had no effect on TIMP production.

The observation of TLR2 and TLR4 ligand stimulation leading to production of IL-6, IL-8 and MMPs in juvenile idiopathic arthritis and RA is supported by previous observation of induction of IL-6, MMPs and adhesion molecules at mRNA level following activation of TLR2 with peptidoglycan but not with CpG ODN. But in the study by Kyburz et al., effect of these TLR ligands on the production of TIMP was not studied. Further, chemokine production by FLS has been reported on stimulation by TLR-2 ligand i.e., bacterial peptidoglycan. Higher expression of TLR2 and TLR4 is found in rheumatoid synovial tissue compared to OA patients and healthy donors. We also found TLR2 and TLR4 expression in JIA FLS.
Synovial fibroblasts cultured in vitro can upregulate the expression of TLR2 upon stimulation with IL1 and peptidoglycan. Thus TLR2 mediated activation can act in an autocrine fashion and amplify the response.

Flagellin induced production of IL6, IL8, MMP1, and MMP3 after stimulation in FLS. However, it had no effect on TIMP production. Thus the balance was tilted in favour of MMPs after stimulation. TLR5 is expressed by a variety of cells including monocytes, dendritic cells, epithelial cells and mast cells. Stimulatory effect of flagellin on dendritic cells is well established, but their effect on synovial fibroblasts is not known. It was found that flagellin induce human DC maturation in vitro and this maturation was accompanied by chemokine and cytokine production. These observations help to explain the high antigenicity and adjuvant activity of bacterial flagellin that may contribute to immune-inflammation in chronic synovitis. Presently no endogenous ligands are known for TLR5.

TLR 3 ligand polyI:C stimulated FLS to produce IL6, IL8, MMP3 and MMP1 but not TIMP1. MMP3/ TIMP1 ratio was also higher after stimulation by TLR3. ligand polyI:C. Synovial fibroblasts from RA patients are shown to express TLR3 and these can produce high levels of chemokines and IL6 after stimulation by polyI:C and necrotic synovial fluid cells. But its effect on MMP production was not studied in the above report. Another study found increased expression of TLR3 in synovium from RA patients compared with that of healthy control and co-stimulation of TLR3, 4, 7/8 resulted in synergistic cytokine production by dendritic cells.

No stimulatory effect of TLR7 and TLR9 ligands was seen on FLS. Data on effect of TLR7 ligation on FLS are not available. TLR7 ligation could not induce IFN-γ dependent CXCR3 ligand production in human microvascular endothelial cells (HMVEC) and TLR7 was not expressed on these cells. CpG ODN, a TLR9 ligand failed to induce MMP and cytokines at mRNA level in a previous study thus supporting our observation. The lack of stimulatory effect of TLR7 and TLR 9 ligand in our experiments could have been due to insufficient expression of these receptors on synovial fibroblast, inability of the ligand to reach the intracellular localization of these TLRs or true lack of effect. On synovial tissue TLR 7 expression was found however, the cell type expressing TLR7 was not studied. Later it was found that TLR7 are not expressed in RA synovial fibroblasts. We have reconfirmed the lack of TLR7 expression on FLS. However, the data are different for TLR9 where a low level expression similar to that reported earlier was found in this study. Thus the exact reason behind lack of TLR9 effect is still not clear.

Results from animal model of arthritis suggest an involvement of TLR signalling pathways in the pathogenesis of arthritis. Adjuvant arthritis or streptococcal cell wall arthritis are dependent on the activation of the innate immune system by TLR ligands. MyD88 deficient mice did not develop streptococcal cell wall induced arthritis and significantly reduced severity of arthritis was found in TLR2 deficient mice. Transient arthritis was induced in mice following intra-articular injection of CpG oligonucleotides, bacterial proteoglycans and by ds RNA. Recent data suggest that TLR2 and TLR4 but not TLR9 are involved in the pathogenesis of autoimmune arthritis and play distinct role in the regulation of T cells and cytokines. These results also suggest that availability of TLR ligands might be sufficient to initiate arthritis in a susceptible host.

Even though live bacteria or viruses have not been found in autoimmune arthritis, various TLR ligands like LPS, peptidoglycan, bacterial DNA have been demonstrated in the joint in chronic arthritis. Besides bacterial products many endogenous ligands for various TLRs have been recently described and they may have a potential role in autoimmune synovitis. Some of these endogenous ligands are found in joints of patients with inflammatory arthritis like RA. Heat shock proteins have previously been implicated in the pathogenesis of inflammatory arthritis. These proteins activate macrophages via TLR2 and RNA released from necrotic synovial fluid cells can activate RA synovial fibroblast via TLR3. It is likely that dsRNA sequences present in mRNA are the possible ligands for TLR3. Therefore, these endogenous ligands of TLR2, 4 and 3 might be responsible for activation of synovial fibroblasts in JIA and RA.

Blocking ligand-TRL interaction and its intracellular activation pathway can be attractive targets for therapy. These include, firstly, neutralizing antibodies to either TLRs or TLR ectodomains to prevent ligand binding; secondly, small molecules that block enzymes in the common signalling pathway, such as interleukine-1 receptor associated kinase -4; thirdly, small molecules that would act as agonists,
such as imiquimod, and have adjuvant effects for
immune-stimulation (e.g., in anti-tumour or anti-
viral therapies); and finally, agents that might block
protein-protein interactions in signalling cascades.
Indeed, a peptide based on the TIR domain of Mal
that specifically interferes with Mal signalling has
been described\(^\text{27}\). A variety of TLR antagonists have
been identified which modify the immune response
and reduce arthritis\(^\text{28,30}\). Conventionally used drug in
RA and JIA like hydroxychloroquin has been shown
to inhibit TLR3 mediated pathway.

In conclusion, ability of bacterial and viral
products to specifically induce MMP1, MMP3 and
cytokine production in JIA synovial fibroblast, without
inducing TIMP, may suggest a role of these products in
the pathogenesis of juvenile idiopathic arthritis and
cartilage destruction. In addition, endogenous ligands
generated during joint inflammation can activate TLR
pathway and perpetuate joint damage. Therefore TLR
signaling pathways are a potential target for control of
disease.

**Acknowledgment**

Authors acknowledge the financial support provided by the
Indian Council of Medical Research, New Delhi.

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