Expression of Toll-like Receptors (TLRs) and Their Signaling Pathways in Rheumatoid Synovitis.

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Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects multiple small joints simultaneously causing a chronic and destructive synovitis.

In ordinary host defense, neutrophils and macrophages mediate innate host responses by recognizing micro-organisms, followed by their phagocytosis and clearance. Further, macrophages and in particular dendritic cells (DC) can present pathogen antigens to initiate T cell-mediated adaptive immune responses [1], but can also activate autoreactive T cells via abnormal presentation of self-antigens.

Toll-like receptors (TLRs) are trans-membrane proteins of inflammatory resident and stromal cells, which share leucine-rich extracellular repeats and intracellular Toll/interleukin (IL)-1 receptor (TIR) domains [2]. The innate immune system recognizes conserved pathogen-associated molecular patterns (PAMPs) through TLRs. It was later found that TLRs also bind endogenous alarmins [3], such as monosodium urate crystals. Together with PAMPs they form the so-called “danger signals” or danger-associated molecular patterns (DAMPs) [4]. DAMPs stimulate innate inflammatory responses, but they also provide the danger or second signal to adaptive immune responses. TLRs recognizing endo-/exogenous danger signals could play a significant role in RA. The aim was to elucidate the eventual presence, localization and extent of expression of TLRs and their adapters.

Materials and Methods
Synovial membranes were obtained from RA (n=10) and osteoarthritis (OA) (n=5).

mRNA analysis (quantitative real-time RT-PCR)
Total RNA was isolated from each frozen tissue, following to convert into cDNA and enzymatic amplification of specific cDNA sequences was performed on Light Cycler system (Roche, Germany). TLR 1, 2, 3, 4, 5, 6, and 9 receptors, and MyD88, TIRAP/Mal, TRIF/TICAM-1 adapters were analyzed. Quantitative analysis of the mRNAs was performed with the use of Light Cycler Software. β-actin was used as control and statistical analysis was performed by Fisher’s PLSD test.

Immunohistochemical analysis
TLR 1, 2, 3, 4, 5, 6, and 9 receptors, and MyD88, TIRAP/Mal, TRIF/TICAM-1 adapters were analyzed by conventional immunohistochemical study. Their co-localization with CD68, CD15, CD3, CD4, CD8, CD20, DC-LAMP, CD123 and 5B5 cellular markers was analyzed in double immunofluorescence staining using Alexa fluorescent system (Molecular Probes Inc., OR, USA).

Results
mRNA analysis (quantitative real-time RT-PCR)
In RA β-actin standardized mRNAs of TLR 2, 3, and 9 (p<0.001) were particularly high, TLR 5 and 6 were also elevated (p<0.05), but TLR 1 and 4 & adapters did not differ between RA and OA (Figure 1). Immunohistochemical analysis
In double staining TLRs and adapters were observed in DC-LAMP+ mDCs. In addition, co-localization of TLR 2, 4, and 9 was observed in CD123+ pDC identified in perivenular areas, large and small lymphoid aggregates, and around lymphoid follicles. Moderately labeled TLR 1, 2, 3, 4, 5, 6, and 9 and their adapters were seen in CD68+ type A lining cells and infiltrating macrophages. TLRs and their adapters were found in 5B5+ type B lining cells and fibroblasts of the sublining stroma, with weak and/or at most moderate reactivity. CD3+/CD4+ and CD3+/CD8+ T cells and CD20+ B cells in perivenular areas and in lymphoid follicles were moderately TLR and weakly adapter positive. In OA, TLRs and adapters were only very weakly immunolabeled in vascular, lining and inflammatory cells (Figure 2).

Conclusion
This study demonstrated that in RA synovitis tissue the β-actin standardized mRNA levels of TLR 2, TLR 3 and TLR 9 are very high, TLR 5 and TLR 6 are elevated but TLR 1 and TLR 4 do not differ between RA and OA. This suggests that RA synovitis tissue is particularly responsive to TLR 2, TLR 3 and TLR 9 stimulation. It was more likely that TLR 2, TLR 3 and TLR 9 were stimulated by some exogenous PAMPs and endogenous alarmins.

Increased expression of TLR 2, TLR 3, and TLR 9 in general suggests a highly potent responsiveness of RA synovial tissue to various TLR 2, TLR 3, and TLR 9 binding DAMPs, in particular endogenous alarmins but possibly also some exogenous PAMPs. Further, when this analysis was extended to cell subtypes, the strong expression of TLR 1, 2, 3, 4, 5, 6, and 9 in mDC and of TLR 2, 4, and 9 in pDC, together with the associated adapters, suggests intense potential involvement of DCs in adaptive autoimmune immune responses, although type A cells/macrophages and type B cells/fibroblasts may be mostly responsible for cytokine and proteinase production and autoinflammatory responses. Less intense inflammation and lower TLR mRNA and protein levels in OA suggest less potent responsiveness compared to RA. Similar expression of MyD88 and TIRAP/Mal adapters suggests competent downstream signal transduction pathways in both conditions and that the main difference between RA and OA lies in the general and cell subtype specific levels of certain TLRs rather than their adapters.

Danger-associated molecular patterns-to-TLR interactions may drive not only DCs but also synovial macrophages and fibroblasts, to autoinflammatory and autoimmune cascades/synovitis in RA.

References

Figure 1. Quantitative real-time RT-PCR

![Figure 1](image1.png)

**Figure 1.** Quantitative real-time RT-PCR

Figure 2. Co-localization of TLRs and CD68/Fibroblast (5B5) in RA.

![Figure 2](image2.png)

**Figure 2.** Co-localization of TLRs and CD68/Fibroblast (5B5) in RA.