Toll-like receptors in rheumatoid arthritis

innate immune sensing of danger signals

Een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

Proefschrift

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CHAPTER 1

General introduction

Modified from:

The orchestra of Toll-like receptors and their role in autoinflammation and autoimmunity

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Rheumatoid arthritis

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease, which is characterized by pain, swelling, loss of strength and loss of mobility and ultimately leads to cartilage damage and destruction of the underlying bone (Figure 1). Many cell types including macrophages, fibroblasts and dendritic cells (DCs) are believed to play a pathogenic role in the inflammatory cascade of synovitis.

DCs are believed to be the most potential antigen presenting cells that regulate the delicate balance between immunity and tolerance (1). The function of DCs depends on their maturation state; immature DCs are located throughout the body, including the synovial tissue. Here they function as sentinels of the immune system that are specialized in antigen recognition, uptake and processing. Upon recognition of antigens, DCs differentiate into so-called mature DCs, a process that is characterized by upregulation of MHC and co-stimulatory molecules (Figure 2), which is essential for antigen presentation to T cells in the lymph nodes or secondary lymphoid organs. This maturation process is accompanied by a large production of pro-inflammatory mediators, such as cytokines, chemokines and metalloproteinases.

Synovial fibroblasts play critical roles in normal joint functioning, for example by production of collagen and other connective tissue molecules that form and maintain the joint capsule. In RA however, synovial fibroblasts are linked to the invasive character of the synovial tissue, by invading surrounding

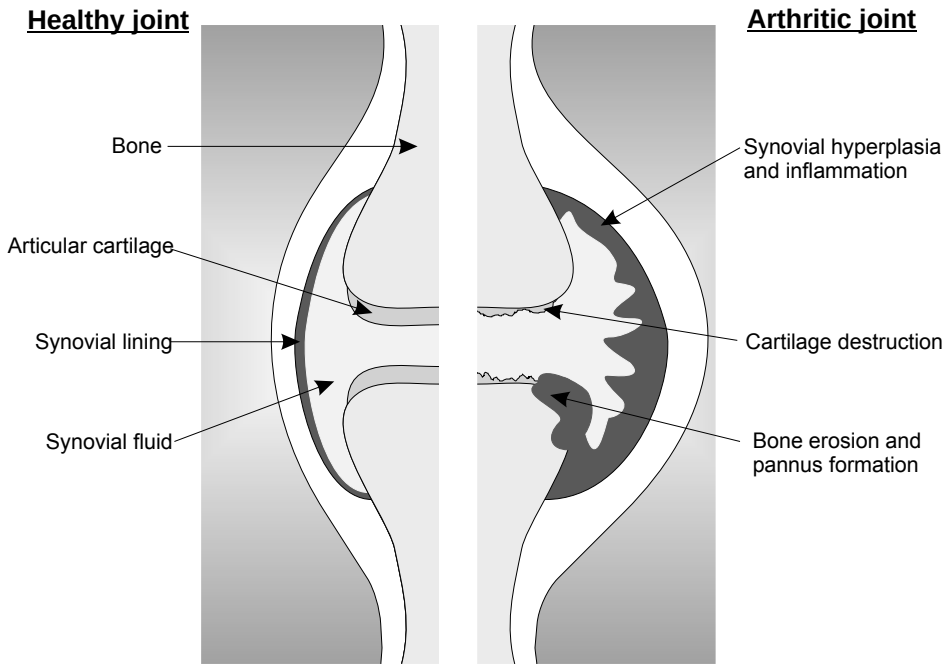
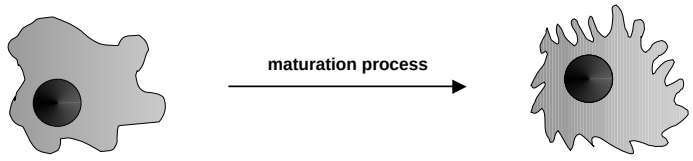


Figure 1. Schematic presentation of a healthy joint and the features that characterize an arthritic joint. This figure was adapted from the thesis of Marije Koenders; Interleukin 17 and its relation to IL-1 and TNF in experimental arthritis, 2007.



Immature DC

reside in periphery
high expression of pathogen recognition receptors
low expression of CD80, CD86 & MHC molecules

Mature DC

reside in lymph nodes and secondary lymphoid organs
high expression of pathogen recognition receptors
low expression of CD80, CD86 & MHC molecules

Figure 2. Specific characteristics for immature and mature DCs; where immature DCs are specialized in antigen recognition, uptake and processing, mature DCs have the capacity for antigen presentation and subsequent T cell stimulation.

bone and cartilage. Furthermore, RA synovial fibroblasts produce and secrete a wide range of pro-inflammatory mediators, such as cytokines and growth factors(2). Macrophages are located in the synovial layers of the synovial tissue and involved in inflammatory responses by production of pro-inflammatory mediators, such as cytokines, chemokines and metalloproteinases, which all are likely to facilitate tissue destruction.

The pro-inflammatory cytokine IL-1β was one of the first cytokines of interest in the field of RA, because of its biological role in joint destruction as shown in models of experimental arthritis. It was followed by TNFα, which acts strongly synergistic with IL-1β (3;4). For several years, anti-TNFα therapy has successfully been used in the clinic to improve the outcome of severe RA by suppression the inflammatory response in a substantial number of RA patients who were resistant for common disease-modifying anti-rheumatic drugs (DMARD). Unfortunately, anti-TNFα therapy is still ineffective in about 35% of the patients. In this light it is essential to identify other factors that are involved in the pathogenesis and the chronicity of RA, which might reveal promising therapeutic targets for the future. Although the exact mechanisms that are responsible for the initiation and perpetuation of the disease remain obscure, recent evidence points to a role for Toll-like receptors (TLRs).

Toll-like receptors (TLRs)

TLRs belong to the family of pattern-recognition receptors (PRRs) and to the Toll/IL-1R (TIR) superfamily. The cytoplasmic region of the TLR shares strong homology with the IL-1 receptor and therefore this region is referred to as the TIR domain. In contrast, the extracellular TLR regions do not contain Ig-like domains like the IL-1R, but several leucine-rich regions (LRR) instead (Figure 3). TLRs are able to recognize microbial components, known as pathogen-associated molecular patterns (PAMPs) and are constitutively expressed by numerous cell types and tissues (5-10), where they detect pathogens leading to the induction of an

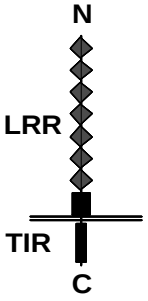


Figure 3. Schematic presentation of the Toll-like receptor structure.

immune response (Table 1). Nowadays, 11 TLRs have been identified in humans. TLR2 (as heterodimer in combination with TLR1 or TLR6) and TLR4 are able to recognize lipid-based structures from gram-positive and gram-negative bacteria, such as lipopeptides and lipopolysaccharides (LPS)(11-13), whereas TLR3, TLR7, TLR8 and TLR9 are involved in the recognition of viral and bacterial nucleic acids, such as double stranded RNA and CpG motifs in DNA (14-16). TLR10, which is believed to originate from the TLR1/TLR6 precursor, has only been identified in human and no specific ligands have been described thus far. TLR5 is able to recognize the bacterial protein flagellin (17) and although profilin has been described as a ligand for mouse TLR11 (18), ligands for human TLR11 have not yet been identified.

Endogenous TLR agonists

Due to extensive research the last few years, it was established that, besides microbial components, TLRs were also able to recognize endogenous agonists (Table 1). From all the potential endogenous TLR agonists described, heat-shock proteins (HSPs) have been studied the most thoroughly thus far. In 2000, Ohashi et al. identified HSP60 as a putative endogenous agonist of the TLR4 complex in mice (24), followed by Vabulas et al who described that both TLR2 and TLR4 were involved in the recognition of self HSP60 in humans (32). Later on, it became clear that cell activation by other members of the HSP family, such as HSP70, gp96 and small HSPB8, were also mediated by TLRs (25;33;34). Where low levels of endogenous HSPs can be detected in the blood of healthy individuals (35), elevated

Table 1: Toll-like receptors and their exogenous as well as endogenous ligands

TOLL-LIKE RECEPTOR	EXOGENOUS & ENDOGENOUS LIGANDS	REFERENCES
TLR1 (+TLR2)	Bacterial tri-acyl lipopeptides	(19)
TLR2	Lipoproteins (gram-positive bacteria) Necrotic cells	(20) (21)
TLR3	Double stranded viral RNA Endogenous RNA from necrotic cells	(14) (22)
TLR4	Lipid-based structures (gram-negative bacteria) Heat-shock proteins Hyaluronan Fibronectin	(20;23) (24;25) (26) (27;28)
TLR5	Bacterial flaggelin	(17)
TLR6 (+TLR2)	Bacterial di-acyl lipopeptides	(13)
TLR7	Single stranded viral RNA	(15;29)
TLR8	Single stranded viral RNA	(30)
TLR9	Bacterial and viral DNA Chromatin IgG complexes	(16;31)
TLR10	-	-
TLR11	-	-

levels of HSPs are produced by cells under stress conditions, which for example occurs during acute infection and trauma- or (autoimmune-induced) inflammation. Be-yond cell stress, infection and inflammation also often coincides with tissue damage, resulting in the release of extracellular matrix (ECM) products. Hyaluronic acid (HA) is one of the major glycosaminoglycans of the ECM, which undergoes degradation at sites of inflammation, leading to increased tissue concentrations of HA fragments. It has been shown that HA is able to induce an immune response upon recognition by TLR4 (26). Otherwise, TLR4 was also found to be important in the recognition of heparan sulphate, which is released from the ECM during tissue damage as well (36).

Nevertheless, as most of the evidence about the ability of endogenous agonists to induce TLR-mediated cell activation was derived using recombinant proteins, the possibility exists that endotoxin contamination interfered with the experiments, as recombinant protein preparations (especially those generated in E. coli) may contain small amounts of endotoxins, such as LPS (37). Therefore further studies are warranted to completely exclude the effects of endotoxin contamination in immune activation induced by endogenous TLR ligands.

TLR driven immune responses

Upon stimulation of all TLRs, except TLR3, myeloid differentiation factor 88 (MyD88) is recruited. Myd88 was the first adaptor molecule described and its activation ultimately leads to the activation of nuclear factor kappa B (NF-κB) (38-41) and the production of pro-inflammatory cytokines such as TNFα, IL-6, IL-1β and IL-12.

Another important adaptor molecule termed TIR-related adaptor protein inducing interferon (TRIF), is involved in the signaling cascade of both TLR3 and TLR4. Recruitment of TRIF eventually leads to the activation of the IRF-3 transcription factor leading to the production of e.g. type I interferon and RANTES (42-43). Furthermore, TRIF activation is also involved in MyD88-independent transcription of NF-κB

For TLR4, it has been described that the MD-2 molecule, which is physically associated with TLR4 on the cell surface, is required to confer an accurate signaling by stabilization of TLR4 dimers (44). In addition, also CD14 (soluble or membrane-bound) that is able to form a physical complex with TLR4, and LPS binding protein (LBP) that is present in serum are essential for the LPS-mediated TLR4 signaling. The requirement of CD14 and LBP are described for TLR2 signaling as well (45) (Figure 4).

Besides production of pro-inflammatory cytokines such as TNFα, IL-6, IL-1β and type I IFN, TLR activation also results in maturation of antigen-presenting cells, which in turn, interact with T cells to induce a proper adaptive immune response. Adaptive immune responses of the Th1 type are dri-

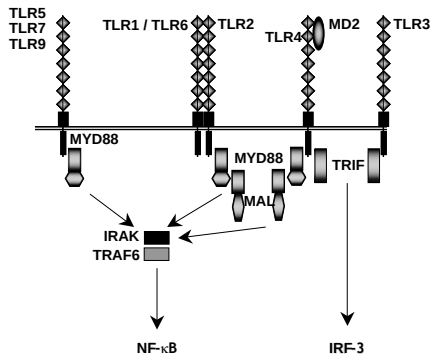


Figure 4. Simplified schematic representation of the human TLR signaling pathway. This figure is adapted from S. Akira et al., Biochem Soc Trans, 2003.

ven by IL-12 and are characterized by the production of IFNγ, whereas IL-10, IL-4 and IL-13 are associated with a Th2 type response. TLR stimulation generally leads to production of IL-12 and thereby favors a Th1 type response (46). Recently, a new T cell subset has been identified, the Th17 cells, which are driven by IL-23, TGFβ and IL-6 and are characterized by production of IL-17 (47;48). Since TLR stimulation also leads to the production of IL-23 and IL-6, it is conceivable that TLR activation also results in a Th17 type response. Furthermore, the Notch ligands Delta-4 and Jagged-1 can be expressed by DCs and selectively induce T helper cell differentiation. Interestingly, co-stimulation of different TLRs results in a synergistically increased Delta-4/Jagged-1 ratio and higher production of IL-12 and IL-23 by human DCs, leading to DCs with enhanced Th1/Th17-polarizing capacity (49).
Although there is lots of evidence for TLRs in Th1-mediated immune responses, only little data suggest that TLRs can regulate Th2-mediated responses as well. Netea et al. demonstrated that TLR2-deficient mice showed severely impaired production of IL-10 accompanied by a 50% decrease in regulatory T cell population (Treg) upon C. Albicans infection, concluding that immunosuppression through TLR2-derived signals acts via increased IL-10 production and survival of Treg (50).

Single nucleotide polymorphisms of Toll-like receptors

Genetic polymorphisms, mainly single nucleotide polymorphisms (SNP), are common variants within a population and are found at a frequency of over 1%. Many genetic variations have been described in TLRs, and since SNP could result in an altered amino acid sequence, one might expect that SNP could affect TLR-mediated immune responses. Several years ago, Arbour et al. described two common co-segregating missense mutations (Asp299Gly and Thr399Ile) affecting the extracellular domain of TLR4, which were associated with hyporesponsiveness to bacterial lipopolysaccharide (LPS). In addition, transfection of THP-1 cells with either wild-type or mutant alleles of TLR4 demonstrated that cells transfected with the Asp299Gly allele showed a decreased response to LPS, whereas cells transfected with the Thr399Ile allele did not (51). Ever since, many studies were performed that investigated the potential association of the TLR4 polymorphisms with the susceptibility to infectious diseases, such as respiratory virus infections and septic shock (52;53), and non-infectious disorders, such as Crohn's disease, acute allograft rejection and asthma (54-56). Also for TLR2 several SNP have been described. Most frequent is the Arg753Gln polymorphism, which is located in the cytoplasmic domain of TLR2 and, as demonstrated for the Asp299Gly SNP in TLR4, showed hyporesponsiveness upon TLR2-induced activation (57;58). Since TLR2 and TLR4 seemed to be the most important receptors involved in infectious diseases, most studies have been focused on these two receptors. However, to a lesser extent, also polymorphisms in other TLRs have been described (59-61).

TLRs in rheumatoid arthritis

The first evidence that demonstrated a role for TLRs in rheumatoid arthritis (RA) originated from Leadbetter et al. who described that B cells from rheumatoid factor positive (RF+) MyD88-deficient mice were completely unresponsive to chromatin-containing immune complexes. In addition, autoimmune sera that effectively stimulated RF+

MyD88 positive B cells, seemed ineffective in the RF+ MyD88-deficient mice, demonstrating that autoantibody-autoantigen immune complexes act via a MyD88-dependent receptor, probably TLR9 (31). The role of TLRs in RA was shown not to be limited to TLR9 since RA synovial fibroblasts, isolated from RA synovial tissue, showed clear upregulation of TLR2 upon stimulation with the pro-inflammatory cytokines TNFα and IL-1β, which were both the first cytokines of interest in the field of RA (62). Furthermore, it was demonstrated that RNA released from necrotic RA synovial fluid cells were able to activate RA synovial fibroblasts via TLR3 (22). The role of TLRs had also been demonstrated in experimental models of arthritis as mice deficient for the adaptor molecule MyD88 did not develop Streptococcal cell wall (SCW)-induced arthritis, shown by complete absence of joint swelling, influx of inflammatory cells as well as cartilage matrix proteoglycan loss (63). Furthermore, soluble ST2 (sST2), which is known as an endogenous inhibitor of TLR4, could attenuate the disease severity in the murine model of collagen-induced arthritis. Moreover, specific RA pathology, such as cellular infiltration in the joints, synovial hyperplasia and joint erosion were deeply reduced in mice treated with sST2. Finally, recently it was demonstrated that a naturally occurring TLR4 antagonist was able to suppress the clinical and histological characteristics of arthritis as demonstrated in several models of experimental arthritis (64). Altogether, preliminary evidence, which indicates a role for TLRs in arthritis, prompted us to introduce a line of research aiming to reveal the involvement of TLRs in the pathogenesis of RA as well as in the chronic phase of the disease.

Aim & outline of this thesis

At the start of this project the evidence that suggested a role for TLRs in RA was very limited and was mainly gathered using several models of experimental arthritis. The aim of this thesis was to investigate the role of TLRs in the pathogenesis and the chronicity of RA in a human setting. This was done to determine whether TLRs could serve as a possible therapeutic target in RA.

In the first part of the thesis (chapter 2 and chapter 3), the presence of TLRs in RA synovial tissue was studied using immunohistochemistry and this was compared to synovial tissue from patients with osteoarthritis (OA) and healthy controls. We found that all TLRs investigated were highly expressed in synovial tissue from RA patients and that this expression was significantly enhanced compared to patients with OA and healthy controls. Subsequently we investigated whether cytokines, which were present in RA synovial tissue, could regulate these TLRs and we found that TLR2 and TLR4 could strongly be upregulated by IL-12 and IL-18 (chapter 2), whereas the expression of TLR3 and TLR7 was strongly regulated by IFNα (chapter 6).

Besides the presence of TLRs in RA synovial tissue, we also investigated the presence of endogenous TLR ligands in serum and synovial fluid from RA patients, using a stably TLR4 transfected CHO reporter cell line, which revealed that presence of endogenous TLR4 ligands was strongly enhanced in serum and synovial fluid of RA patients compared to healthy controls and patients with systemic sclerosis or systemic lupus erythematosus (chapter 3). In addition, using immunohistochemistry and Western blot analysis, we identified small heat-shock protein B8 (HSPB8) as an endogenous ligand present in RA synovial tissue and in

vitro experiments demonstrated that this ligand was able to induce an immune response via TLR4 (chapter 4).

In terms of TLR stimulation, we investigated the effects of TLR co-stimulation and found that simultaneous stimulation of different TLR pathways resulted in a synergistic effect with regard to the production of pro-inflammatory mediators TNFα and IL-6 (chapter 3). Furthermore, we investigated the potential differences in TLR-mediated immune responses between RA patients and healthy controls. Stimulation of monocytes-derived DC using specific ligands for TLR2, TLR3, TLR4 and TLR7/8 showed that cells from RA patients produced much higher amounts of the pro-inflammatory cytokines IL-6 and TNFα upon TLR2- and TLR4-mediated stimulation than cells from healthy controls, whereas cytokine production upon TLR3 and TLR7 stimulation was not enhanced (chapter 3). In chapter 5, using microarray analysis, we identified allograft-inflammatory factor 1 (AIF-1) as a factor that might be involved in the enhanced TLR2/4-mediated immune response in RA patients.

Finally, we investigated the potential effect of the Asp299Gly TLR4 polymorphism in RA. Therefore mononuclear cells (PBMC) from RA patients carrying and not carrying the genetic Asp299Gly TLR4 variant were compared with respect to TLR4 mRNA expression, TLR4 protein expression and cytokine production upon TLR stimulation with exogenous as well as endogenous TLR4 agonists. We demonstrate that cytokine production by cells from patients with the genetic TLR4 variant was significantly diminished upon exogenous as well as endogenous TLR stimulation, despite the fact that the TLR4 expression was unaffected (chapter 7).

In the final chapter (chapter 8) the results described in this thesis are summarized and discussed.

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CHAPTER 2

Expression of Toll-like receptor 2 (TLR2) and TLR4 in rheumatoid synovial tissue and regulation by the pro-inflammatory cytokines IL-12 and IL-18 via IFNγ

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Objective. To study the expression of TLR2 and TLR4 and its association with pro-inflammatory cytokines in synovial tissue from patients with rheumatoid arthritis (RA), osteoarthritis (OA), and healthy individuals.

Methods. Synovial tissue specimens from 29 RA patients were stained for TLR2, TLR4, and pro-inflammatory cytokines IL-1β, IL-12, IL-17, IL-18, and TNFα. The expression of TLR2, TLR4, and cytokines as well as the degree of inflammation in synovial tissue were compared between patients with RA, patients with OA (n = 5), and healthy individuals (n = 3). Peripheral blood mononuclear cells (PBMCs) were incubated with IL-12 and IL-18, and TLR expression was assessed using fluorescence-activated cell sorter analysis. Production of TNFα and IL-6 was measured using Luminex bead array technology.

Results. In RA synovial tissue, the expression of TLR2 was slightly higher than that of TLR4. Interestingly, both TLR2 and TLR4 were expressed at higher levels in moderately inflamed synovium, as compared with synovial tissue with no or severe inflammation. TLR expression in both the lining and the sublining was associated with the presence of IL-12 and IL-18, but no other cytokines, in the lining. The expression of both TLRs was low in synovial tissue from OA patients and healthy donors. Stimulation of PBMCs with IL-12 and IL-18 resulted in increased expression of both TLR2 and TLR4; this could be blocked with anti-IFNγ antibodies, suggesting a role for IFNγ. Lipopolysaccharide- or lipoteichoic acid-mediated triggering of PBMCs incubated with IL-12/IL-18 or IFNγ led to an increased production of both TNFα and IL-6, indicating the functionality of TLR2 and TLR4.

Conclusion. TLR2 and TLR4 are expressed in synovial tissue of patients with clinically active disease and are associated with the levels of both IL-12 and IL-18. The synergistic effect of IL-12 and IL-18 on T cell IFNγ production seems to regulate expression of TLR2 and TLR4 in the synovial tissue of RA patients.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease that predominantly affects synovial joints. Although the exact pathophysiologic mechanisms are still largely unknown, it is generally accepted that numerous inflammatory cells, such as T and B cells, fibroblast-like synoviocytes, and antigen-presenting cells, and their extensive production of pro-inflammatory mediators are implicated (1-4). During RA, the synovial tissue undergoes many changes, including neoangiogenesis, cellular hyperplasia, and influx of inflammatory cells, which are potentially orchestrated by a complex interplay of pro-inflammatory mediators such as cytokines and chemokines (5-8). The recognition of HLA-DR subtypes that are associated with RA, and the presence of autoantibodies, such as rheumatoid factor and citrinullated peptides, suggest an autoimmune component in the pathogenesis of RA (9-11). Furthermore, recent studies have suggested the involvement of Fc receptors and Toll-like receptors (TLRs) in arthritis, and these are of critical importance in autoimmunity (12-16).

TLRs are phylogenetically conserved receptors that recognize pathogen-associated molecular patterns (PAMPs) and are involved in the uptake and processing of various exogenous and endogenous antigens (17). However, TLRs are not only involved in antigen uptake, but also mediate maturation of dendritic cells and promote naive T cells toward a Th0, Th1, or Th2 phenotype. Thus, TLRs play a crucial role

in the regulation of innate and adaptive immune responses (18,19).

To date, 11 human TLRs have been described, of which TLR2 and TLR4 are the most thoroughly investigated. In addition to being involved in the recognition of lipopolysaccharide (LPS), TLR4 also interacts with endogenous ligands such as heat-shock proteins (HSPs) (20), fragments of hyaluronic acid (21), and fibronectin (22). These endogenous ligands are probably released by cells undergoing stress, damage, or necrotic death and are present in the stressed synovium. Increased cell turnover in conditions of minor trauma or inflammation, leading to increased levels of endogenous TLR ligands, is therefore likely to initiate a reaction leading to TLR-mediated triggering of inflammatory cells in RA. Recent evidence underscores such a critical role of TLRs in the onset of autoimmunity (12,23,24).

TLR2 and TLR4 are constitutively expressed on various cell members of the immune system, including macrophages, neutrophils, and dendritic cells. The expression of TLR2 and TLR4 is tightly regulated by several pro-inflammatory cytokines. Stimulation of mouse macrophages and hepatocytes with interleukin-1 beta (IL-1β), interferon-gamma (IFNγ), and tumor necrosis factor-alpha (TNFα) resulted in an up-regulation of both TLR2 and TLR4 messenger RNA (mRNA). In addition, expression of mRNA for TLR2 and TLR4 increased upon stimulation of endothelial cells and renal epithelial cells with IFNγ (25,26). Stimulation with TNFα in renal epithelial cells also led to a clear increase of TLR2 and TLR4 protein expression (25). In contrast, human monocytes seem to regulate TLR4 expression differentially, since stimulation with TNFα resulted in a decreased protein expression of TLR4, whereas stimulation with IL-6 led to an increased expression of TLR4 protein (27).

Although their roles are obviously important in inflammation, clear results with regard to the effects of IL-12 and IL-18 on the expression of TLR2 and TLR4 are lacking. IL-12 generates the development of naive T cells into Th1 cells and stimulates IFNγ secretion by differentiated Th1 cells. IL-12 is produced by phagocytic cells, dendritic cells, B lymphocytes, and natural killer cells upon stimulation with LPS, bacteria, and parasites (28,29). The potentially important role of IL-12 in synovial inflammation was illustrated by the finding that local overexpression of IL-12 transforms an experimental model of acute arthritis into a chronic destructive immune-mediated process (30).

Recently, it was demonstrated that IL-18 can act as a costimulus for IFNγ production and natural killer cell cytotoxicity induced by IL-12 (31). However, IL-18 itself is not a strong inducer of IFNγ in the absence of other cytokines. IL-18 is a pro-inflammatory cytokine member of the IL-1 cytokine superfamily and was originally identified as IFNγ-inducing factor (31). On T cells, IL-18 stimulates Th1 differentiation, promotes secretion of IFNγ, TNFα, and granulocyte-macrophage colony-stimulating factor, and enhances natural killer cell cytotoxicity. A critical role for IL-18 in RA was further substantiated by the finding that the synovial expression of IL-18 was accompanied by the coexpression of IL-1β and TNFα and was associated with local inflammation in RA (6). Since both cytokines are known to

potentiate Th1-driven responses and stimulate the secretion of IFNγ, it is generally accepted that, in conjunction with IL-1β and TNFα, IL-12 and IL-18 are key inflammatory cytokines in the inflammatory cascade of RA.

Seibl et al recently demonstrated an increased expression of TLR2 in the synovial fibroblasts of RA patients, when compared with those of OA patients (15). However, since both TLR2 and TLR4 are potentially critical receptors in the initiation and perpetuation of the inflammatory cycle in arthritis, and fibroblasts are not the only cells considered to be key players in the disease, we investigated the expression of these TLRs in synovial tissue sections from RA patients in comparison with those from OA patients and healthy controls. In addition, we investigated whether synovial expression of TLR2 and TLR4 was associated with the pro-inflammatory cytokines IL-1β, TNFα, IFNγ, IL-12, and IL-18, and we studied the role of IL-12 and IL-18 in the regulation of TLR2 and TLR4 expression on monocytes in vitro.

Our results demonstrate clearly higher expression levels of TLR2 and TLR4 in synovial tissue from RA patients. Moreover, the level of TLR2 and TLR4 expression in both the lining and sublining from moderately inflamed joints was associated with the presence of IL-12 and IL-18 in the lining. In addition, IL-12 and IL-18 induced an upregulation of TLR2 and TLR4 on monocytes, which was inhibited by the blockade of IFNγ. Our findings indicate that IL-12 and IL-18, both abundantly present in the synovium of RA patients, are involved in the regulation of TLR2 and TLR4, which may play a potentially important role in the synovial inflammation during RA.

Methods

Patients.

Twenty-nine consecutive patients with RA were enrolled in the study. All patients met the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (32). The disease activity was calculated by using the Disease Activity Score (DAS), and, for the current study only, patients with a DAS higher than 3.2 were included (33). The therapeutic regimens of all patients were recorded before blood sampling. Patients receiving prednisone within 6 weeks or biologic therapies, including anti-TNFα or IL-1 receptor antagonist, before the study were not included.

Percutaneous biopsies of the knee joint were performed with a Parker-Pearson needle, following local administration of anesthesia. An average of 30 samples was obtained at each procedure. Prior to the biopsy, the erythrocyte sedimentation rate (ESR) was measured and knee joints were scored by an experienced rheumatologist for the absence (score of 0) or presence of local pain, swelling, or effusion (score of 1, respectively, for each of these). The 3 scores were added up and patients were classified as having no (score 0), moderate (score 1-2), or severe (score 3) knee-joint arthritis. The synovial tissue from healthy individuals and patients with OA was isolated during arthroscopic procedures performed by orthopedic surgeons. The Medical Ethics Committee of the University Medical Center Nijmegen approved the study protocol.

Antibodies for detection of TLRs and cytokines.

Diaminobenzidine (DAB) and bovine serum albumin were obtained from Sigma (St.

Louis, MO). Polyclonal antibodies against human TLR2 (H-175) and TLR4 (H-80) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated swine anti-rabbit Ig affinity-isolated $F(ab)_2$ was obtained from Dako (Glostrup, Denmark). Dulbecco's modified Eagle's medium, RPMI-1640 with Glutamax, fetal calf serum (FCS), and sodium pyruvate were purchased from Gibco Invitrogen (Breda, The Netherlands). Monoclonal antibodies against human IL-12 p70 (IgG, MAB 219), human IL-18 (IgG, AF 318), recombinant human IL-12, IL-18, and IFNγ were obtained from R&D Systems (Minneapolis, MN). Antibodies against human CD3 (A0452), CD14 (M0825), and CD68 (M0718) were purchased from Dako. Avidin peroxidase (Elite kit) was obtained from Vector (Burlingame, CA). Lipoteichoic acid (LTA; L3140) and LPS (L4391) were purchased from Sigma. BioPlex kits for determination of IFNγ, IL-6, and TNFα (catalog no. 171-B11921, 171-B10719, and 171-B12236, respectively), using Luminex bead array technology, were purchased from Bio-Rad (Hercules, CA).

Immunohistochemical analysis of synovial tissues.

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. After dewaxing and dehydration, sections were blocked with normal swine serum followed by 60 minutes of incubation with the antibodies against TLR2 and TLR4 at a concentration of 10 μg/ml and 17 μg/ml, respectively. The secondary antibody, biotinylated swine anti-rabbit Ig, was added to the incubation for 30 minutes. Slides were stained with streptavidin peroxidase, developed with DAB, and counterstained with hematoxylin for 30 seconds. A similar amount of biopsy tissue was embedded in Tissue Tek (Miles, Elkart, IN) and then snap-frozen and stored in liquid nitrogen until sectioned. Snap-frozen material was used to assess CD3 and CD68 staining. Control sections were stained with irrelevant primary isotype-specific IgG antibodies.

Sections were coded and randomly analyzed by 2 blinded observers (YMJ and LABJ). Inadequate sections, which lacked synovial lining, were left out of the analysis. Staining for TLR2 and TLR4 was semiquantitatively scored on a 5-point scale (range 0-4) at 200× magnification; a score of 0 represented no or minimal staining, a score of 1 indicated 10-20% positive cells, a score of 2 indicated 30-40% positive cells, a score of 3 indicated 50-60% positive cells, and a score of 4 represented staining of more than 60% of the cells. Staining for IL-1β, IL-18, and TNFα was performed as described previously (8). The number of IL-12-positive cells in the sublining was counted manually in 5 random high-power fields, and the count was then averaged and scored on a 0-4 scale as follows: 0 = 0-1 positive cells, 1 = 2-3 positive cells, 2 = 4-6 positive cells, 3 = 7-10 positive cells, and 4 = >10 positive cells.

Monocyte cultures and fluorescence-activated cell sorter (FACS) analysis.

Monocytes were cultured using standardized protocols as previously described (3). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood using density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Roosendaal, The Netherlands). The interphase was collected and washed with citrated phosphate buffered saline. After washing, PBMCs were adjusted to 0.5×10^6 cells/ml in RPMI-1640 supplemented with antibiotics and 10% heat-inactivated FCS (Life Technologies, Breda, The

Netherlands). Monocytes were isolated from the PBMCs by incubation with magnetic-activated cell sorting beads coated with a monoclonal antibody against human CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany). Immunomagnetic separation was performed according to the manufacturer's instructions. Isolated $CD14^+$ cells were brought to a concentration of 0.5×10^6 cells/ml in RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS and antibiotic-antimycotic. $CD14^+$ monocytes and PBMCs were incubated in medium alone or in medium with either IFNγ (10 ng/ml), IL-12 (10 ng/ml), IL-18 (50 ng/ml), or a combination of IL-12 and IL-18 for 24 hours and 48 hours at 37°C. Neutralizing anti-human IFNγ antibodies (Innogenetics, Ghent, Belgium) were used at a concentration of 10 μg/ml to neutralize IFNγ during PBMC cultures exposed to either IL-12, IL-18, or the combination of both cytokines.

The expression of the cell-surface markers TLR2 and TLR4 on monocytes was measured by indirect immunofluorescence staining, as described in detail previously (3). Briefly, the first layer was attached after incubation of 1×10^5 monocytes for 30 minutes at 4°C with the monoclonal anti-human antibodies against TLR2 (H-175), TLR4 (HTA-125; Santa Cruz Biotechnology), and CD14. After a washing step, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 30 minutes at 4°C in complete darkness.

Cells were gated according to their forward- and side-scattering patterns, using FACS analysis. For each marker, 10^4 cells were counted in the gate. For the functional analysis, PBMCs were preincubated with IL-12 (10 ng/ml), IL-18 (50 ng/ml), and/or the combination of both for 24 hours. Thereafter, the PBMCs were stimulated for 18 hours with TLR2-specific ligands (LTA) and TLR4-specific ligands (LPS).

Statistical analysis.
Results are expressed as the arbitrary score, with the median (25th, 75th percentile [25%, 75%]) or the mean ± SD. Differences between experimental groups were tested using the Mann-Whitney U test. Correlations between the expression of TLRs and cytokines were calculated using Spearman's nonparametric correlation test. Both tests were conducted using GraphPad Prism, version 2.01. Only P values less than 0.05 were considered significant.

Results

TLR2 and TLR4 expression in synovial tissue of RA and OA patients and healthy individuals.
Synovial sections obtained from 29 patients with RA were stained with antibodies against TLR2 and TLR4, or with irrelevant primary isotype-specific IgG antibodies as a negative control. TLR2 was detected in 78% of the RA patients, whereas TLR4 was present in 70%. TLR2 and TLR4 were detected in the lining, the sublining, and the perivascular region of the RA synovium (Figure 1). Both TLR2 and TLR4 were relatively overexpressed in the lining (mean ± SD scores 1.9 ± 1.5 and 1.4 ± 1.4, respectively) compared with expression in the sublining (1.3 ± 1.3 and 0.8 ± 1.2, respectively) or the endothelial cells (0.8 ± 1.1 and 1.1 ± 1.6, respectively). Moreover, in moderately inflamed synovium, expression of TLR2 in the lining was higher when compared with that of TLR4 (median [25%, 75%] score 3 [2, 4] versus 2 [1, 4]). In

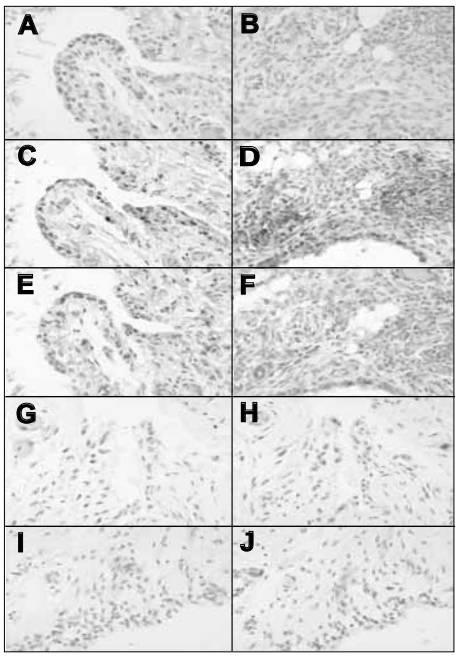

Figure 1. Immunohistochemical detection of Toll-like receptor 2 (TLR2) and TLR4 in synovial biopsy tissues from patients with rheumatoid arthritis (RA), osteoarthritis (OA), and healthy individuals. As a control, staining was performed with nonrelevant isotype control antibody (A and B). Expression of TLR2 (C and D) and TLR4 (E and F) was examined in RA synovial tissue, as compared with TLR2 and TLR4 expression in synovial tissue from patients with OA (G and H) and healthy individuals (I and J). Hematoxylin counterstained; original magnification 400×.

contrast, the expression of both TLR2 and TLR4 was markedly lower in synovial tissue obtained from patients with OA and was hardly detectable in synovial tissue samples from healthy controls (Figure 1).

Expression of TLR2 and TLR4 predominantly in moderately inflamed knee joints.

Prior to the biopsy, the 29 RA patients were classified into 3 groups on the basis of the ESR and clinical scoring of the knee, with categories as follows: no (n = 5), moderate (n = 12), or severe (n = 12) knee-joint arthritis. The degree of inflammation along with the scores for TLR2 and TLR4 expression are shown in Figure 2. In the lining, both TLR2 (median [25%, 75%] 3 [2, 4] versus 1 [0, 2]; P = 0.007) and TLR4 (median 2 [1, 4] versus 0 [0, 0.5]; P = 0.03) were expressed at significantly higher levels in synovial tissue with moderate inflammation when compared with synovial tissue with no inflammation. In contrast, TLR2 and TLR4 were equally distributed in the sublining and perivascular regions of synovial tissue from joints with severe, moderate, or no inflammation.

The predominant expression of TLR2 and TLR4 in the synovial tissue of moderately inflamed joints was not correlated with the presence of T cells (CD3) or macrophages (CD68) (data not shown). In contrast, the expression of CD3 was lowest in the synovial tissue from moderately inflamed joints, whereas CD68 was most abundant in severely inflamed knee joints (data not shown).

Association of TLR2 and TLR4 expression with presence of pro-inflammatory cytokines.

The biopsy samples used in this study were previously used to detect the presence of IL-1β, IL-12, IL-17, IL-18, and TNFα (6). Since we were interested in the potential association between the presence of pro-inflammatory cytokines and the levels of TLR2 and TLR4, we studied this relationship. Expression of the cytokines and receptors was scored on a 5-point scale (range 0-4) and correlations were determined.

The overall presence of IL-1β, IL-17, and TNFα was not associated with either TLR2 or TLR4. Interestingly, IL-12 levels in the li-

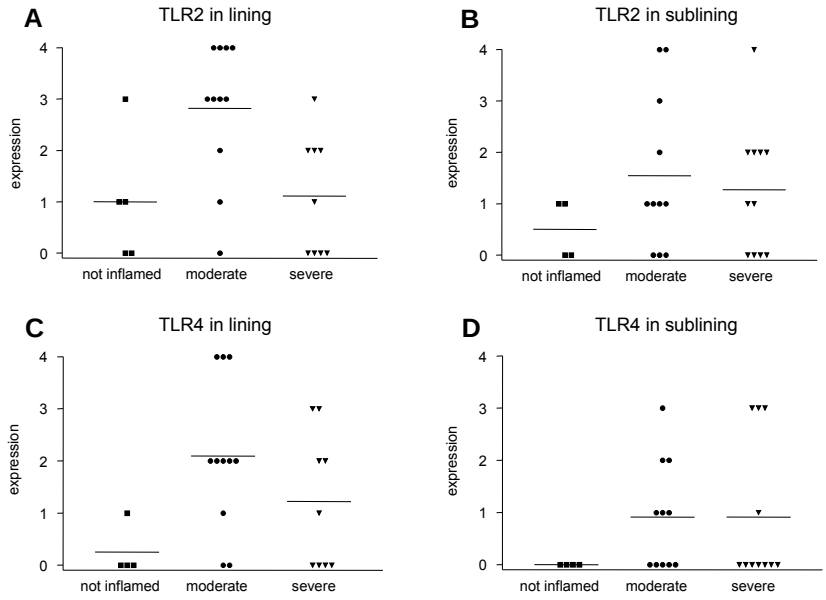

Figure 2. Expression of TLR2 and TLR4 in relation to the severity of arthritis in synovial tissues from RA patients. On macroscopic scoring, synovial samples were categorized as noninflamed, moderately inflamed, or severely inflamed. Microscopic inflammation and expression of TLR2 in the lining (A) and sublining (B) together with TLR4 in the lining (C) and sublining (D) were scored using semiquantitative scales ranging from 0 to 4 (0 = no or minimal staining; 1 = 10-20% positive cells; 2 = 30-40% positive cells; 3 = 50-60% positive cells; 4 = >60% positive cells). Bars show the mean score for synovial sections without inflammation (n=5), with moderate inflammation (n=12), or with severe inflammation (n=12).

ning were significantly associated with the expression of TLR2 (for lining, r = 0.53, P < 0.005; for sublining, r = 0.60, P = 0.001) and TLR4 (for lining, r = 0.46, P = 0.01; for sublining, r = 0.44, P = 0.03) (Table 1). The same trend, albeit not significant, was observed between the levels of IL-18 and the expression of TLR2 (for lining, r = 0.27, P = 0.06; for sublining, r = 0.23, P = 0.07) and TLR4 (for lining, r = 0.24, P = 0.07; for sublining, r = 0.27, P = 0.08).

Association of TLR2 and TLR4 expression on monocytes with indirect regulation by IL-12 and IL-18.

To explore the potential effects of IL-18 and IL-12 on the regulation of TLR2 and TLR4 protein expression on monocytes, purified $CD14^+$ monocytes from RA patients and healthy individuals were incubated with IL-12 and IL-18. In contrast to the results in monocyte cultures with IFNγ (positive control), both IL-12 and IL-18 did not affect the expression of TLR2 or TLR4 on purified monocytes (Table 2).

Because the presence of IL-12, IL-18, and, especially, the combination of both cytokines results in production of IFNγ by T cells, we sought evidence to indicate whether IL-12, IL-18, or both were able to increase the expression of TLR2 and TLR4 on monocytes in the presence of T cells. We therefore incubated PBMCs with IFNγ, IL-12, IL-18, and

Table 1: Individual scores of IL-12/IL-18 and TLR2/TLR4 expression in synovial tissue samples from RA patients

	IL-12		IL-18		TLR2		TLR4	
PATIENT	LINING	SUBLINING	LINING	SUBLINING	LINING	SUBLINING	LINING	SUBLINING
1	1.0	2.0	1.0	0.5	4.0	1.0	1.0	0.0
2	0.0	0.0	0.0	0.0	3.0	2.0	2.0	0.0
3	0.0	0.0	0.0	0.0	1.0	1.0	2.0	3.0
4	0.0	0.0	1.0	1.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0
6	3.0	4.0	3.0	0.5	4.0	4.0	4.0	2.0
7	0.0	2.0	3.0	0.5	3.0	1.0	4.0	3.0
8	2.0	3.0	1.0	1.0	2.0	2.0	1.0	1.0
9	0.0	0.0	0.0	0.0	3.0	2.0	0.0	3.0
10	0.0	2.0	0.5	0.5	ND	ND	ND	ND
11	0.0	2.0	1.0	3.0	0.0	0.0	3.0	3.0
12	0.0	1.0	0.0	0.0	1.0	ND	2.0	ND
13	0.0	1.0	2.0	1.0	0.0	2.0	0.0	0.0
14	1.0	2.0	2.0	3.0	3.0	3.0	2.0	1.0
15	0.0	1.0	1.0	1.0	3.0	0.0	2.0	2.0
16	0.0	0.0	1.0	1.0	ND	ND	ND	ND
17	0.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0
18	0.0	1.0	1.0	1.0	ND	4.0	ND	0.0
19	0.0	1.0	0.0	0.0	3.0	1.0	1.0	0.0
20	1.0	2.0	2.0	1.0	1.0	1.0	2.0	1.0
21	0.0	0.0	0.0	0.0	2.0	0.0	4.0	0.0
22	0.0	0.0	1.0	4.0	ND	ND	ND	0.0
23	2.0	4.0	3.0	1.0	4.0	4.0	0.0	1.0
24	1.0	1.0	4.0	2.0	4.0	1.0	2.0	0.0
25	0.0	0.0	1.0	2.0	1.0	1.0	0.0	0.0
26	0.0	0.0	4.0	4.0	0.0	0.0	0.0	0.0
27	0.0	0.0	1.0	3.0	0.0	0.0	2.0	0.0
28	0.0	2.0	1.0	2.0	2.0	2.0	3.0	0.0
29	0.0	0.0	0.0	1.0	2.0	0.0	0.0	0.0

In total, 29 synovial tissue samples from rheumatoid arthritis (RA) patients with active disease were analyzed for IL-12, IL-18, TLR2, or TLR4 expression. Expression was scored in the lining and sublining by 2 blinded observers (YMJ and LABJ) on a scale ranging from 0 to 4 (for details, see Patients and Methods). Correlations were calculated using Spearman's nonparametric correlation test, with GraphPad Prism, version 2.01. Biopsy samples were obtained from noninflamed (n=5; patients 4, 12, 19, 25, and 26), moderately inflamed (n=12; patients 1, 2, 6, 7, 14-17, 20, 21, 23, and 24), and severely inflamed (n=12; patients 3, 5, 8-11, 13, 18, 22, and 27-29) knee joints. ND = not detectable).

a combination of IL-12 and IL-18 and studied the expression of both receptors on monocytes. Incubation with IFNγ resulted in a marked increase of both TLR2 and TLR4 on $CD14^+$ monocytes, which was equally distributed among healthy individuals (increase in TLR2, 126%; increase in TLR4, 250%) and RA patients (increase in TLR2, 127%; increase in TLR4, 222%), and reached a peak at 48 hours (Figure 3A). Intriguingly, stimulation with IL-12 and IL-18 and the combination of both IL-12 and IL-18 similarly resulted in an increase in TLR2 and TLR4 expression; however, their peaks were reached within 24 hours (data not shown). These data suggest that IL-12 and IL-18 alone and in combination promote IFNγ secretion by T cells, which in turn induces enhancement of TLR2 and TLR4 expression on monocytes.

To examine whether the IL-12- and/or IL-18-driven up-regulation of TLR2 and TLR4 protein expression was dependent on IFNγ, we determined IFNγ levels in culture super-

Table 2: TLR2 and TLR4 expression on purified $CD14^+$ monocytes

TIME POINT STIMULUS	TLR2	TLR4
0	63 ±14	16 ± 6
24 hours		
medium	70 ± 4	17 ± 2*
IFNγ	81 ± 23	64 ± 34
IL-12	55 ± 20	20 ± 10
IL-18	59 ± 19	18 ± 8
IL-12 / IL-18	60 ± 25	19 ± 13
48 hours		
medium	99 ± 10*	13 ± 3*
IFNγ	149 ± 21*	34 ± 12*
IL-12	108 ± 12	19 ± 8
IL-18	107 ± 18	17 ± 7
IL-12 / IL-18	104 ± 17	20 ± 12

$CD14^+$ monocytes were isolated from heparinized venous blood by density-gradient centrifugation over Ficoll-Hypaque and positive selection using MACS CD14 beads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$ monocytes were cultured for 24 hours with IFNγ (10 ng/ml), IL-12 (10 ng/ml), IL-18 (50 ng/ml), or the combination of IL-12/IL-18. Thereafter, TLR2 and TLR4 expression was determined by fluorescence-activated cell sorter analysis (see Patients and Methods). Values are the mean ± SD of 3 healthy controls. *$P < 0.01$ versus medium control group, by Mann-Whitney U test.

natants after exposure to IL-12, IL-18, or the combination of both (Figure 3B). Using the Luminex bead array system, we found high levels of IFNγ in supernatants of PBMCs that were incubated with the combination of IL-12 and IL-18. Interestingly, the in vitro IFNγ production induced by the combination of IL-12 and IL-18 is significantly lower in PBMCs from RA patients compared with that in PBMCs from healthy controls.

To confirm the finding that IFNγ regulates the enhancement of TLR expression, we performed studies in the presence or absence of neutralizing antibodies directed against human IFNγ. Figure 3C shows clearly that the up-regulation of TLR2 and TLR4 was largely IFNγ-dependent, since it was almost fully inhibited by the neutralization of IFNγ. Remarkably, although we could not detect IFNγ in the culture supernatant after 24 hours of stimulation of PBMCs with either IL-12 or IL-18, the slight up-regulation of both TLR2 and TLR4 seen after stimulation with both IL-12 and IL-18 combined was IFNγ dependent (Figure 3C).

IL-12/IL-18-mediated up-regulation of TLR2 and TLR4 and TLR-specific triggering of increased production of TNFα and IL-6.

For a better understanding of the impact of IL-12/IL-18- and TLR2/TLR4-mediated effects on RA initiation and/or progression, the functionality of the increased expression of TLR2 and TLR4 after stimulation with IL-12 and IL-18 was tested. To this aim, we incubated IL-12/IL-18-prestimulated PBMCs with TLR2- and TLR4-specific ligands (LTA and LPS, respectively) and measured the secretion of TNFα and IL-6; we then compared the findings with those in PBMCs stimulated with IFNγ alone. As expected, stimulation of both PBMC supernatants, preincubated with either IL-12/IL-18 or IFNγ together with LPS, resulted in a clearly higher secretion of TNFα (mean ± SD 3,333 ± 249 pg/ml [$P < 0.001$] and 2,582 ± 289 pg/ml [$P < 0.001$], respectively) when compared with PBMCs without any preincubation (150 ± 79 pg/ml TNFα) (Figure 3D). Similarly, the production of IL-6 was higher by PBMCs preincubated with IL-12/IL-18 (18,989 ± 3,739 pg/ml) or IFNγ (13,767 ± 742 pg/ml) than in PBMCs stimulated with LPS alone (6,258 ± 245 pg/ml); this same phenomenon was observed for IL-6 production upon stimulation with LPS. The triggering of TLR2-mediated pathways by LTA resulted in the same effects, indicating that the increased expression of both TLR2 and TLR4 mediated by IL-12 and IL-18 was functional.

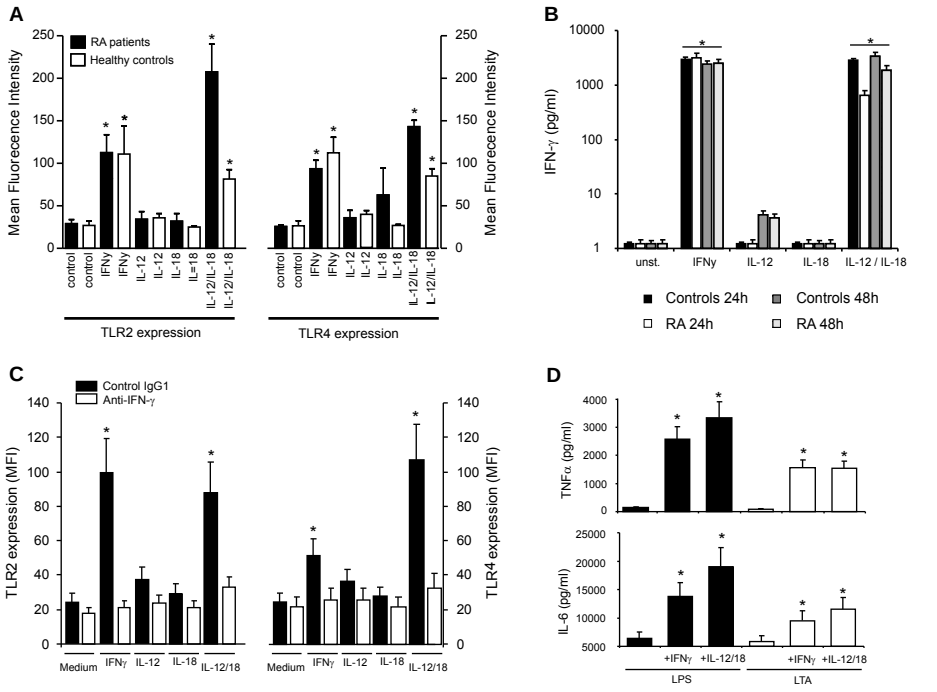

Figure 3. Cytokine regulation of TLR2 and TLR4 expression and function. A. To measure TLR2 and TLR4 protein expression on $CD14^+$ monocytes, freshly isolated PBMCs from healthy controls (n=6) or RA patients (n=3) were cultured for 48 hours, with either IL-12 (10 ng/ml), IL-18 (50 ng/ml), or the combination of both cytokines. IFNγ (10 ng/ml) was included as a positive control. TLR expression on $CD14^+$ cells was examined by fluorescence-activated cell sorter (FACS) analysis, using specific antibodies for CD14, TLR2, and TLR4. *P < 0.05 versus controls, by Mann-Whitney U test. B. To demonstrate stimulation of IFNγ production by IL-12 and IL-18, supernatants of cultured PBMCs were analyzed for IFNγ levels. Note the slightly enhanced levels of IFNγ after 48 hours of IL-12 exposure, both in PBMC culture supernatants from healthy controls (n = 6) and in those from RA patients (n = 3). Remarkably higher levels of IFNγ were found after exposure of PBMCs to the IL-12/IL-18 combination. C. To show that blockade of IFNγ prevents IL-12/IL-18-driven up-regulation of TLR2 and TLR4 expression, PBMCs from healthy controls (n=3) were cultured for 24 hours with IFNγ, IL-12, IL-18, or the IL-12/IL-18 combination. Previously, neutralizing mouse anti-human IFNγ antibodies (10μg/ml) or control IgG1 antibodies were added to the PBMC cultures. TLR expression on $CD14^+$ cells was examined by FACS analysis. Note that blockade of IFNγ prevents enhanced TLR2 and TLR4 expression induced by IL-12, IL-18, and the combination of both cytokines. D. To show that enhanced TLR2/TLR4 expression by IL-12/IL-18 exposure promotes production of cytokines, PBMCs from healthy controls (n=3) were cultured for 24 hours either in medium alone or with IFNγ or with the IL-12/IL-18 combination. Thereafter, the cells were cultured for another 24 hours in medium, TLR2 agonist (lipoteichoic acid 3μg/ml), or TLR4 agonist (lipopolysaccharide 20 ng/ml). The sensitivity of the Luminex bead array was below 10 pg/ml for both IL-6 and TNFα. *P < 0.01 versus medium alone, by Mann-Whitney U test. Bars show the mean and SD. MFI = mean fluorescence intensity.

Discussion

In the present study we demonstrate that the expression of both TLR2 and TLR4 in synovial tissue from RA patients is significantly increased compared with that in synovial tissue from OA patients and their healthy counterparts. In addition, we provide evidence of a critical role of IL-12 and IL-18 in the regulation of TLR2 and TLR4 expression in synovial tissue. The up-regulation of TLR2 and TLR4, however, was indirect, since monocytes alone were not able to up-regulate TLR2 and TLR4 when cultured with IL-12, IL-18, or the combination of both. In contrast, the addition of IFNγ to monocytes did result in a clear up-regulation of TLR2 and TLR4. The indirect role of IL-12 and IL-18 on TLR expression was shown by the fact that stimulation of PBMCs with IL-12 and IL-18 resulted in an increased expression of the TLRs. As expected, the concentration of IFNγ was significantly increased in these cultures and was most likely produced by T cells. Blockade studies with anti-IFNγ antibodies confirmed that IL-12/IL-18-induced TLR expression on $CD4^+$ monocytes is IFNγ-dependent. Thus, our data suggest that the higher expression of IL-12 and IL-18 in the synovium might be responsible for the increased synovial expression of TLR2 and TLR4.

Recently, we and other investigators showed that IL-18 was abundantly present in the synovial tissue of RA patients and demonstrated that this pro-inflammatory cytokine was related to local and systemic parameters of inflammation in patients with RA (6,34). IL-12, another pro-inflammatory cytokine, is a potent stimulator of TNFα and IFNγ. Together with IL-18, IL-12 has a unique synergistic effect on the induction of IFNγ secretion and Th1 responses (35).

In vitro exposure of PBMCs to IL-12/IL-18 resulted in synergistic IFNγ production by T cells. Consistent with very recent findings, PBMCs of RA patients produced remarkably lower levels of INFγ after stimulation with the IL-12/IL-18 combination (36). In addition to IL-18, IL-12 was found to be highly expressed in synovial tissue from patients with RA, suggesting an important role for these cytokines in RA (6). Evidence of a critical role of both IL-12 and IL-18 in arthritis was further substantiated by animal studies. The administration of IL-12 accelerated onset of disease and increased severity of collagen-induced arthritis (CIA) (37), whereas mice deficient in IL-18 exhibited a clearly reduced incidence of CIA, characterized by less inflammation and attenuated cartilage destruction (38). Although not previously explored, one of the mechanisms by which IL-12 and IL-18 deploy their effects on the immune system might be regulation of TLRs either directly or indirectly via secretion of IFNγ.

TLRs have recently emerged as key receptors responsible for the recognition of specific bacterial components, including LPS, CpG DNA, and flagellin, which are also known as PAMPs (13,17). In addition, TLRs recognize endogenous ligands, including fragments of the cartilage, such as fibronectin fragments, and hyaluronic acid and HSPs. It was recently demonstrated that these endogenous ligands (natural adjuvants) can activate dendritic cells in conditions of cell stress and/or structural cell damage, leading to primary immune responses and initiation of autoimmunity (13,39,40). Consistent with this scenario, several pathways of stress-signaling molecules are found to be activated in the synovial membrane and fluid of RA patients (41). It is conceivable that these local endo-

genous ligands activate TLR2- and TLR4-bearing dendritic cells, macrophages, and fibroblasts, which leads to an increased secretion of pro-inflammatory cytokines such as IL-12, IL-18, IL-1β, and TNFα.

The presence of endogenous ligands together with the up-regulated expression of TLR2/TLR4, mediated by IL-12/IL-18, does perhaps lead to a vicious circle of inflammation that ultimately leads to irreversible joint destruction. A potential role for TLRs in RA and experimental models of arthritis has recently been suggested by a sequence of intriguing findings. First, the identification of a critical link with DNA-bound immune complexes, which are abundantly present in RA and trigger TLRs, sheds new light on the potential pathogenic mechanisms involved (12,24). Second, the vaccination against adjuvant arthritis, using Hsp60, clearly showed the importance of TLR4 in the regulation of the immune balance (42). Finally, a role for TLR2 and TLR4 was suggested by our group by the finding that a TLR4 variant was associated with RA disease susceptibility (14). Furthermore, we found evidence that stimulation via TLR ligands leads to distinct cytokine patterns produced by dendritic cells from RA patients as compared with healthy individuals (Radstake RDJ, et al: unpublished observations).

The enhanced expression of TLR2 and TLR4 in RA synovial tissue, together with the new regulatory pathway associated with IL-12 and IL-18, prompted us to postulate the following hypothesis for a new pathogenic mechanism in RA. Endogenous or exogenous ligands, released in RA synovial tissue upon cell stress, minor trauma, or undetermined bacterial or viral infection, lead to a TLR-mediated activation of the innate immune response. In the absence of an efficient contraregulatory mechanism, this initial response might lead to a vicious circle in which mass production of pro-inflammatory mediators, including cytokines and chemokines, results in influx of inflammatory cells, more cartilage damage, and the subsequent release of more endogenous ligands. When not efficiently counteracted, this reaction leads to the detrimental effect seen in sustained RA.

In conclusion, our data support previous evidence of an important role of TLR2 and TLR4 in the pathogenesis of RA. Furthermore, we present evidence that IL-12 and IL-18 are indirectly associated with the regulation of TLR2 and TLR4 expression. Detailed insight into the precise role of TLRs in RA pathogenesis might lead to new therapeutic strategies.

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CHAPTER 3

The expression of TLR3 and TLR7 in rheumatoid arthritis synovium is increased and costimulation of TLR3, TLR4 and TLR7/8 results in synergistic cytokine production by dendritic cells

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Objective. To evaluate the expression of TLR3 and TLR7 in synovial tissue and to study potential differences in the maturation and cytokine production mediated by TLR2, TLR3, TLR4, and TLR7/8 by dendritic cells (DCs) from rheumatoid arthritis (RA) patients and DCs from healthy controls.

Methods. Synovial expression of TLR3 and TLR7 in RA synovial biopsies was studied using immunohistochemistry. Monocyte-derived DCs from RA patients and healthy controls were cultured for 6 days and subsequently stimulated for 48 hours via TLR-mediated pathways (lipoteichoic acid, Pam_3Cys, and fibroblast-stimulating lipopeptide 1 for TLR2, poly(IC) for TLR3, lipopolysaccharide and extra domain A for TLR4, and R848 for TLR7/8). Phenotypic DC maturation was measured using flow cytometry. The secretion of TNFα, IL-6, IL-10, and IL-12 was measured using the Bio-Plex system. Cell lines expressing TLR2 and TLR4 were used for the detection of TLR2 and TLR4 ligands in serum and synovial fluid from RA patients.

Results. TLR3 and TLR7 were highly expressed in RA synovial tissue. All TLR ligands elicited phenotypic DC maturation equally between DCs from RA patients and those from healthy controls. TLR2- and TLR4-mediated stimulation of DCs from RA patients resulted in markedly higher production of inflammatory mediators (TNFα and IL-6) compared to DCs from healthy controls. In contrast, upon stimulation of TLR3 and TLR7/8, the level of cytokine production was equal between DCs from RA patients and those from healthy controls. Remarkably, both TLR3 and TLR7/8 stimulation resulted in a skewed balance towards IL-12. Intriguingly, the combined stimulation of TLR4 and TLR3-7/8 resulted in a marked synergy with respect to the production of inflammatory mediators. As a proof of concept, TLR4 ligands were increased in the serum and synovial fluid of RA patients.

Conclusion. TLRs are involved in the regulation of DC activation and cytokine production. We hypothesize that various TLR ligands in the joint trigger multiple TLR simultaneously, favoring the breakthrough of tolerance in RA.

Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that leads to the destruction of synovial joints. Although the exact mechanisms that are responsible remain obscure, it is generally accepted that besides genetic factors, environmental triggers contribute to the disease pathogenesis (1,2). An infectious etiology of RA has been a longstanding hypothesis, which has been examined with greater sophisticated scientific rigor recently. Currently, evidence points towards a potential role of Epstein-Barr virus (3-5), parvovirus B19 (6,7), and cytomegalovirus (8,9), all of which are found in RA synovial tissue. The potential role of viruses in the pathogenesis of RA was further substantiated by the findings that human parvovirus B19-transgenic mice are highly susceptible to polyarthritis (10), the murine herpesvirus induces relapsing experimental autoimmune arthritis (11), and that double-stranded (viral) RNA (dsRNA) has overt arthritogenic properties (12). Several mechanisms have been attributed to the actual link between the presence of viral material and the observed immunohistopathologic changes during inflammatory synovitis; however, the precise mode of action has not been elucidated thus far.

Synovial inflammation is characterized by a massive influx of numerous inflammatory cells, including T cells, B cells, macrophages, and dendritic cells (DCs). Potentially, all of these contribute to the progressive breakdown of cartilage and destruction of the underlying bone (13-15). DCs are pro-

fessional antigen-presenting cells that are highly adept at stimulating T cells and are therefore intricately involved in the balance between immunity and tolerance (16). DCs can produce large quantities of pro-inflammatory cytokines, chemokines, and metalloproteinases that potentially contribute to the detrimental processes during synovial inflammation. In fact, several observations indicate the potential role of DCs in RA disease pathogenesis. First, DCs are abundantly present at strategic locations in RA synovial tissue (17-19). Second, models of experimental arthritis have shown that presentation of collagen-derived peptides by mature DCs is sufficient for the induction of arthritis (20), and finally, genetically modulated DCs were able to abrogate experimental arthritis (21).

The function of DCs is time and space dependent. In the immature state, DCs reside in the periphery and function as sentinels of the immune system, specialized in antigen uptake and processing. To execute antigen recognition with utmost efficiency, DCs are equipped with several antigen recognition receptors, including Toll-like receptors (TLRs).

TLRs are involved in the detection of environmental signals and the regulation of DC function and are therefore the subject of investigations in autoimmunity. The signals that elicit TLRs are often called danger signals and include both exogenous ligands, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), flagellin, CpG motifs, and dsRNA, often referred to as pathogen-associated molecular patterns (22,23), and endogenous ligands, such as heat-shock proteins (24), fibronectin (25), hyaluronic acid (26), and messenger RNA (mRNA) (27). Endogenous ligands are released upon normal cell turnover and stressful events and are likely to be abundant in the synovial compartment. The pivotal role of endogenous ligands was substantiated by the finding that such signals lead to primary immune responses under conditions of cell stress and precipitate autoimmunity in the presence of self antigens (28-31). Therefore, endogenous ligands are considered to be natural initiators of autoimmunity.

Evidence of a role of TLRs in the pathogenesis of RA originates from the finding that inducible Hsp70, generally accepted as a TLR4 ligand, was increased in RA synovial fluid and on DCs isolated from RA synovial fluid (32). Expression of TLR2 and TLR4 is increased and regulated by pro-inflammatory cytokines that are present in the synovial compartment (33,34) and by the identification of the TLR4 (Asp299Gly) functional variant as a marker for RA disease susceptibility (35). In addition, the link between DNA and TLRs in RA, as recently demonstrated by Leadbetter and colleagues, further substantiated the potential role of TLRs in arthritis (36).

Taken together, it is tempting to speculate that TLRs play a role in the onset and/or severity of RA. This prompted us to investigate the expression of TLRs other than TLR2 and TLR4 in RA synovial tissue and to study potential differences in TLR-mediated DC activation between RA patients and healthy controls using both exogenous and endogenous ligands. Furthermore, we tested the hypothesis that simultaneous stimulation of different TLRs resulted in a synergistic effect on cytokine production by DCs.

Here we demonstrate that TLR3 and TLR7 are increased in the synovial tissue of RA patients. Furthermore, we show that the cy-

tokine repertoire produced by DCs depends heavily upon the TLR pathways triggered. Simultaneous stimulation of TLR4, in combination with TLR3-7/8 pathways, leads to a synergistic effect with regard to cytokine production, suggesting that the presence of different TLR agonists in the joint compartment, including viral RNA, favors a proinflammatory environment that leads to a disturbed balance between tolerance and immunity.

Methods

Study population

A total of 22 patients with RA and 21 healthy volunteers were enrolled in this study. Patients attended the Radboud University Nijmegen Medical Centre and fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria for RA (37). The treatment regimens of all patients were recorded before blood sampling, and patients who used high-dose prednisolone or anticytokine therapies (anti-tumor necrosis factor α [anti-TNFα] and/or interleukin-1 receptor antagonist) were excluded. All patients provided informed consent.

For immunohistochemical analysis, percutaneous biopsies of the knee joint were performed with a Parker-Pearson needle following administration of local anesthesia. On average, 30 samples were obtained during each procedure. The synovial tissue from healthy individuals and from patients with osteoarthritis (OA) was isolated during arthroscopic procedures performed by the orthopedic surgeons. The Medical Ethics Committee of Radboud University Nijmegen Medical Centre approved the study protocol.

Antibodies used for TLR immunohistochemical analysis and cytokine detection

Antibodies against human TLR2 (H-175), TLR4 (H-80), TLR3 (T-17), and TLR7 (V-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated swine anti-rabbit Ig affinity-isolated F(ab)2 was obtained from Dako (Glostrup, Denmark). Biotinylated mouse anti-goat IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Vectastain ABC reagent (Elite kit) was obtained from Vector (Burlingame, CA)-and streptavidin peroxidase was obtained from Dako. Diaminobenzidine (DAB) was obtained from Sigma (St. Louis, MO). Bio-Plex kits using Luminex bead array technology for the determination of IL-6, TNFα, IL-10, and IL-12 were purchased from Bio-Rad (Hercules, CA).

Immunohistochemical analysis of synovial tissue

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. For TLR2 and TLR4 staining, after dewaxing and dehydration, sections were blocked with normal swine serum followed by 60 minutes of incubation with antibodies against TLR2 and TLR4 at concentrations of 10 μg/ml and 17 μg/ml, respectively. After this, endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 15 minutes, and the secondary antibody, biotinylated swine anti-rabbit IgG, was incubated for 30 minutes. Slides were stained with streptavidin-peroxidase, developed with DAB, and counterstained with hematoxylin for 30 seconds. For TLR3 and TLR7 staining, sections were incubated with antibodies against human TLR3 and human TLR7 for 60 minutes. After this, endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 15 minutes, and subse-

quently, the secondary antibody, biotinylated mouse anti-goat IgG, was incubated for 30 minutes. Vectastain ABC reagent was incubated for 30 minutes, developed with DAB, and counterstained with hematoxylin for 30 seconds. Staining of TLR2, TLR4, TLR3, and TLR7 at 200× magnification was scored semiquantitatively on a 5-point scale. A score of 0 represented no or minimal staining, 1 represented 10-20% positive cells, 2 represented 30-40% positive cells, 3 represented 50-60% positive cells, and a score of 4 represented staining of >60% of the cells.

Culture of monocyte-derived DCs

Monocyte-derived DCs were cultured using standardized protocols, as previously described (18). Briefly, peripheral blood mononuclear cells were isolated from heparinized venous blood by density gradient centrifugation over Ficoll-Paque (Amersham Biosciences, Roosendaal, The Netherlands). Low-density cells were collected and washed with citrated phosphate buffered saline and 5% fetal calf serum (FCS), after which the cells were allowed to adhere for 1 hour at 37°C in RPMI 1640 (Dutch modification) (Invitrogen, Carlsbad, CA) supplemented with 2% human serum (PAA Laboratories, Linz, Austria) in 6-well culture plates or 25cm^2 cell culture flasks (Corning, Corning, NY). Adherent monocytes were cultured in RPMI 1640 (Dutch modification) supplemented with 10% FCS and antibiotic/antimycotic (Life Technologies, Gaithersburg, MD) in the presence of IL-4 (500 units/ml; Schering-Plough, Amstelveen, The Netherlands) and granulocyte-macrophage colony-stimulating factor (800 units/ml; Schering-Plough) for 6 days. Fresh culture medium with the same supplements was added on day 3, and then immature DCs were harvested on day 6. To generate mature DCs, immature DCs were resuspended in fresh cytokine-containing culture medium and transferred to new culture plates at a concentration of 0.5 × 10^6/ml. To induce DC maturation, cells were stimulated as described below.

TLR stimulation of monocyte-derived DCs

Triggering via TLR-mediated pathways was achieved with various TLR-specific ligands. To trigger TLR2, 10 μg/ml of LTA (Sigma) was used. To correct for potential LPS contamination in the LTA, Pam_3Cys and fibroblast-stimulating lipopeptide 1 (FSL-1), which are known to be highly specific for the TLR1/TLR2 or TLR2/TLR6 pathways, were used. For stimulation of TLR4, we used 2μg/ml of Escherichia coli LPS (Sigma). As a known endogenous TLR4 ligand, we used 1 M recombinant extra domain A (EDA) proteins. As a control for this, recombinant extra domain B and III_{11} domain proteins (all kindly provided by Dr. J. Strauss, University of Pennsylvania, Philadelphia, PA) were used, which are also fibronectin fragments but lack TLR4 binding capacity.

To trigger TLR3 and TLR7/8, we used poly(IC) and R848, both obtained from InvivoGen (San Diego, CA). All TLR ligands that were used were checked for potential LPS contamination using a TLR4 antagonist (E5564), which was kindly provided by Eisai Research Institute, Andover, MA (38). After culture for 48 hours at 37°C in the presence of 5% CO_2, the supernatants were collected.

Simultaneous stimulation of different TLR pathways

To test whether simultaneous stimulation of different TLR pathways resulted in synergistic cytokine production, immature DCs were generated as described above. On

day 6, cells were stimulated with combinations of TLR2/TLR3, TLR2/TLR4, TLR2/TLR7/8, TLR3/TLR4, TLR3/TLR7/8, and TLR4/TLR7/8 ligands and compared to cells stimulated with each of these substances alone. Furthermore, the combinations of TLR2/TLR3/TLR7 and TLR4/TLR3/TLR7/8 were used to test a potential additional effect of 3 TLR pathways. After culture for 24 hours at 37°C in the presence of 5% CO_2, the supernatants were collected.

Phenotype analysis of monocyte-derived DCs

Flow cytometry was used to determine the expression of cell surface markers on both immature and mature DCs. First, DCs (1×10^5) were incubated with monoclonal antibodies against human CD14 (Dako), CD80 (Becton Dickinson, Mountain View, CA), CD83 (Beckman Coulter, Mijdrecht, The Netherlands), CD86 (PharMingen, San Diego, CA), type I major histocompatibility complex (MHCI) (clone W6/32), and MHCII HLA-DR/DP (clone Q1514) for 30 minutes at 4°C. Cells were then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Zymed, South San Francisco, CA) for 30 minutes at 4°C in complete darkness. Subsequently, cells were washed and analyzed using a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson) to determine the proportion of positive cells relative to cells stained with relevant IgG isotypes. For TLR2 and TLR4 expression, cells were incubated with monoclonal antibodies against human TLR2 (H-175) and TLR4 (H-80), both obtained from Santa Cruz Biotechnology, and subsequently incubated with goat anti-rabbit IgG and goat anti-mouse IgG, respectively (both obtained from Zymed).

Measurement of cytokines in culture supernatants

TNFα, IL-6, IL-10, and IL-12 levels were measured in the supernatants of the DC cultures, using commercially available kits according to the manufacturer's instructions (Bio-Rad) (39). Cytokine levels were measured and analyzed using the Bio-Plex system (Bio-Rad). Data were analyzed using Bio-Plex Manager software (Bio-Rad).

Stimulation of TLR2 and TLR4 transgenic Chinese hamster ovary (CHO) cell lines

The CHO fibroblast CD14 reporter line (clone 3E10) is a stably transfected CD14-positive CHO cell line that expresses inducible membrane CD25 (Tac antigen) under transcriptional control of the human E-selectin promoter (40). The promoter fragment contains an essential NF-κB binding site, which, upon stimulation with LPS, results in a 3-10-fold increase in CD25 surface expression. CHO/CD14/human TLR2 (HuTLR2) reporter cell lines were constructed by stable cotransfection with complementary DNA for the human TLR2 and pcDNA3 (Invitrogen). The CHO/CD14/HuTLR4 reporter cell lines were derived in the same manner. (Both reporter cell lines were kindly provided by Dr. R. Ingalls and Dr. D. Golenbock from the Boston Medical Center, Boston, MA.) All of the experiments with the CHO cell lines were performed at least twice.

CHO/CD14 cells were cultured in Ham's F12 medium with L-glutamine (BioWhittaker, Walkersville, MD), gentamicin (Centrafarm, Etten-Leur, The Netherlands), 2.5% FCS, and 400 units/ml hygromycin B (Calbiochem, Amsterdam, The Netherlands). G418 sulfate (0.5 mg/ml) and puromycin (50 μg/ml) (both obtained from Sigma) were added to the CHO/CD14/TLR2 and CHO/CD14/TLR4 cells, respectively. Cells at a

concentration of 1 × 10^5/ml were stimulated with 10% serum or synovial fluid from RA patients, 10% serum from patients with systemic sclerosis (SSc) or systemic lupus erythematosus (SLE), or 10% normal human serum (PAA Laboratories). To check for potential endotoxin contamination of the synovial fluids used, some of the experiments were performed in the presence or absence of polymyxin B, a natural LPS antagonist. The synovial fluids were also measured for gram-negative bacterial endotoxins using the Limulus amebocyte lysate assay, performed according to the manufacturer's instructions (Pyrogent Plus multitest kit; BioWhittaker).

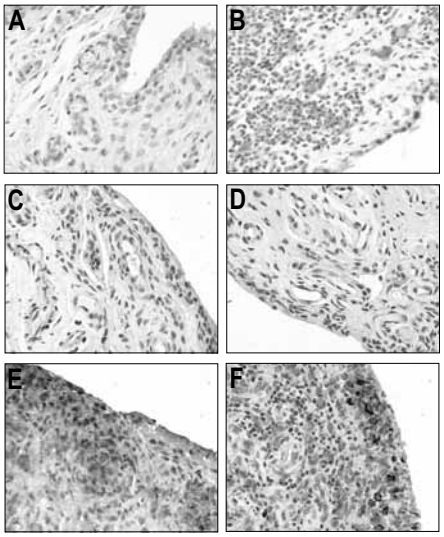

Figure 1. Immunohistochemical detection of TLR3 (left panel) and TLR7 (right panel) in synovial biopsy samples from RA patients and healthy controls. Top panel shows staining with an isotype control antibody. C, E. TLR3 expression in synovial tissue from healthy individuals and RA patients respectively. D, F. TLR7 expression in synovial tissue from healthy individuals and RA patients respectively.All tissues were counterstained with hematoxylin (original magnification × 400).

Analysis of CD25 surface expression by CHO cells

Flow cytometry was performed to determine the surface expression of CD25 on the transgenic cell lines. First, 1 × 10^5 cells were incubated with a monoclonal antibody against human CD25 (Dako) for 30 minutes at 4°C. Cells were then washed and incubated with FITC-conjugated goat anti-mouse IgG (Zymed) for 30 minutes at 4°C in complete darkness. Cells were washed and analyzed by FACSCalibur to determine the proportion of positive cells relative to cells stained with a relevant IgG isotype.

Statistical analysis

Differences in cytokine production between cells from healthy volunteers and those from RA patients were analyzed using the Mann-Whitney U test. P values were 2-sided, and P values less than 0.05 were considered significant.

Results

Increased expression of TLR3 and TLR7 in RA synovial tissue.

To study the potential role of viral RNA in synovial inflammation, we tested the expression of TLR3 and TLR7 in RA synovial tissue. To this end, we stained synovial tissue sections from 5 RA patients with antibodies against TLR3 and TLR7 and compared them with synovial tissue sections from 5 OA patients and 5 healthy controls (Figure 1). Intriguingly, both TLR3 and TLR7 were abundantly expressed in RA synovial tissue. Whereas TLR3 was predominantly located in the lining, sublining, and perivascular regions, TLR7 was scattered throughout the lining and expressed in the lining of RA synovial tissue (mean ± SEM TLR3 score 0.7

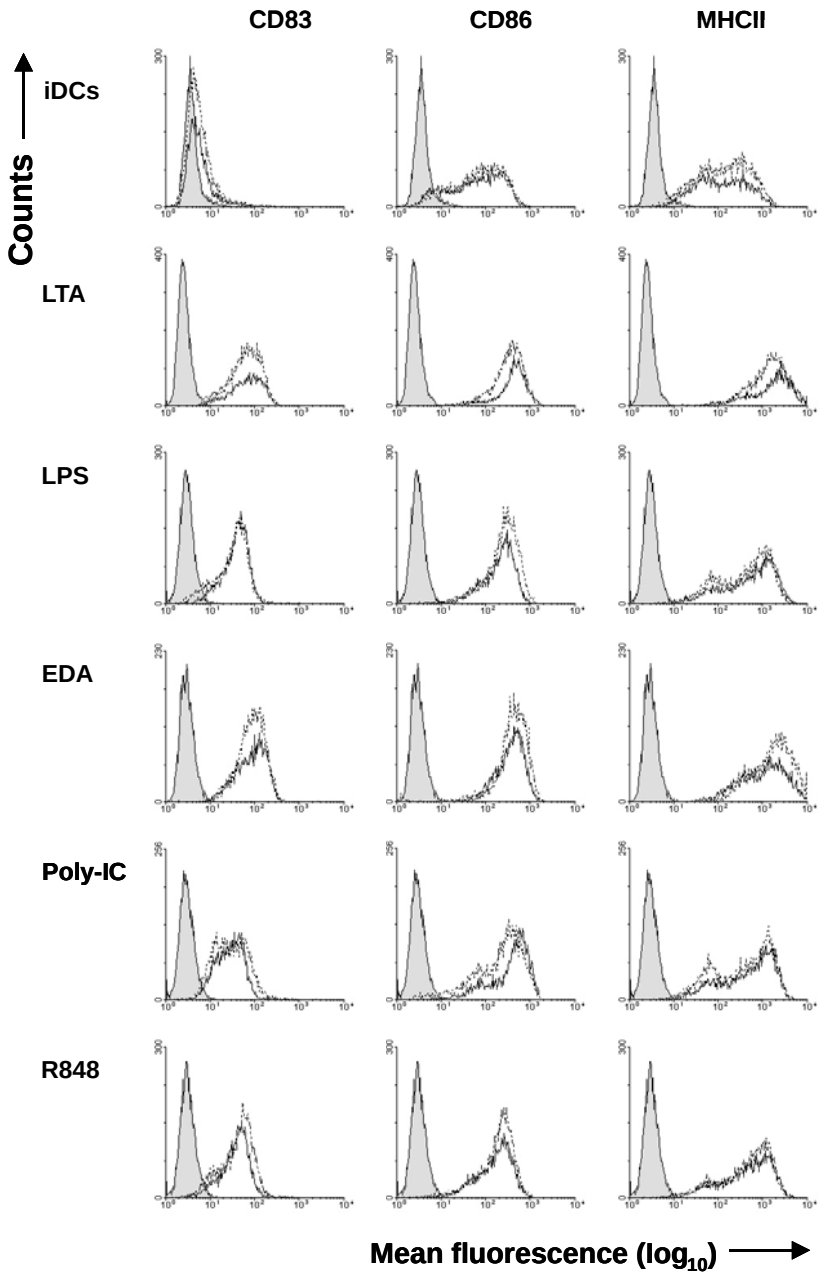

Figure 2. Expression of cell surface markers CD83, CD86, and MHCII on DCs before and after Toll-like receptor (TLR)-mediated maturation with various TLR ligands. The indicated markers or isotype controls (gray peak) of DCs within the life gate were determined using fluorescence-activated cell sorting analysis. Each graph displays data from a representative rheumatoid arthritis (RA) patient (dotted line) and a healthy control (solid line). Top panel shows the expression of CD83, CD86, and MHCII by immature DCs (iDCs) from a RA patient and a healthy control. Other panels show the expression of these markers upon stimulation with the TLR ligands indicated on the y-axis.

± 0.4, TLR7 score 1.8 ± 0.7) as compared to synovial tissue from OA patients (TLR3 score 0.2 ± 0.2, TLR7 score 0.5 ± 0.4) and healthy controls (TLR3 score 0.2 ± 0.2, TLR7 score 0.3 ± 0.2). Remarkably, TLR3 and TLR7 were hardly detectable in the sublining of synovial tissue from OA patients and healthy controls; in contrast, both TLR3 and TLR7 were abundant in the sublining of RA synovial tissue (TLR3 score 0.8 ± 0.4, TLR7 score 1.8 ± 0.7).

Maturation of DCs from RA patients and healthy controls upon stimulation with TLR2, TLR3, TLR4, and TLR7/8 agonists.

Since TLR2, TLR3, TLR4, and TLR7 are highly expressed in RA synovial tissue (33) and important in DC maturation, we used monocyte-derived DCs to test the capacity of various TLR ligands to induce DC maturation. In addition, we studied potential differences between DCs from RA patients and those from healthy controls. Consistent with the literature, immature DCs were characterized by the absence of CD14 (data not shown), intermediate expression of CD86 and MHCII, and low expression of the mature DC marker CD83 (Figure 2, top panel). TLR-mediated DC maturation was reflected by the up regulation of CD83, CD86, and MHCII (Figure 2) combined with the down-regulation of CD14. Stimulation of the TLR2 (LTA), TLR3 (poly[IC]), TLR4 (LPS and EDA), and TLR7/8 (R848) pathways resulted in full DC maturation, which was found to be equally efficient for DCs from RA patients

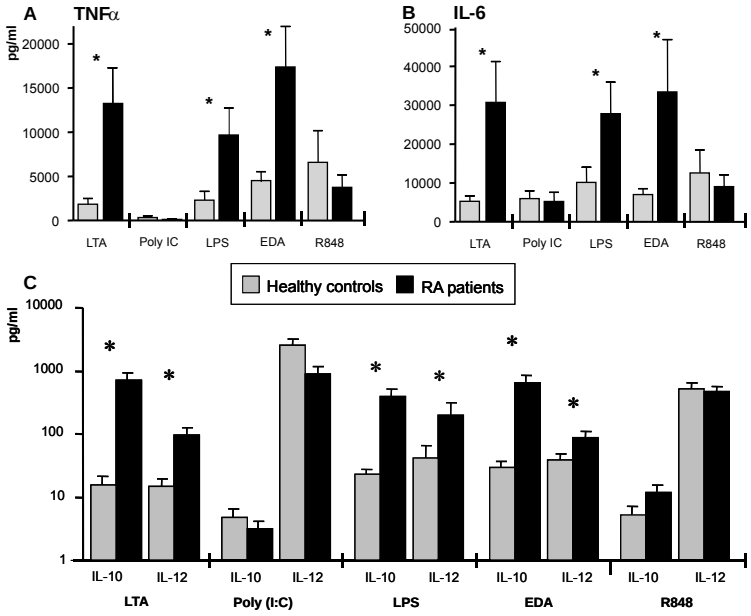

Figure 3. Production of pro- and anti-inflammatory cytokines by DCs after incubation for 48 hours with TLR2-, TLR3-, TLR4-, and TLR7/8-specific ligands. Production of A. TNFα, B. IL-6, and C. IL-10 and IL-12 by DCs from healthy controls (n=8) and from RA patients (n=10) upon stimulation with LTA, poly(IC), LPS, EDA, and R848 is shown. Values represent the mean and SEM (corrected for cytokine production by unstimulated cells). The sensitivity of the cytokine assay was <5 pg/ml for each measured cytokine. $P < 0.05$.

(n = 10) and healthy controls (n = 8) (Figure 2). No effect was seen upon stimulation with EDB and III_{11}, which were used as negative controls for EDA, which lack the capacity to elicit TLR4-mediated triggering (data not shown) (25).

Different cytokine patterns upon DC stimulation via TLR2, TLR3, TLR4, and TLR7/8 pathways.

The type of immune response is largely dependent on the production of cytokines. Therefore, we next investigated the production of TNFα, IL-6, IL-10, and IL-12 by monocyte-derived DCs from RA patients (n = 10) and healthy controls (n = 8) upon stimulation via TLR2, TLR3, TLR4, and TLR7/8 pathways. The cytokine levels, as shown in Figure 3, were corrected for cytokine production by unstimulated cells. For all stimuli used, cytokine production was enhanced compared to unstimulated DCs. Intriguingly, the production of TNFα by DCs from RA patients upon stimulation with the TLR2 ligand LTA ($P < 0.01$) and the TLR4 ligands LPS ($P = 0.03$) and EDA ($P = 0.02$) was significantly higher than that of DCs from healthy controls (Figure 3A). Similarly, the production of IL-6 was significantly increased by DCs from RA patients upon stimulation with LTA ($P = 0.01$), LPS ($P = 0.03$), and EDA ($P = 0.03$) (Figure 3B). In contrast to TLR2/4 triggering, the level of cytokine production upon TLR3 and TLR7/8 stimulation was equal between DCs from RA patients and those from healthy controls (Figures 3A and B).

The balance between IL-10 and IL-12 largely determined the type of immune response. Upon stimulation of the TLR2 and TLR4 pathways, the IL-10 levels tended to be higher than the IL-12 levels, which occurred exclusively in DCs from RA patients. In contrast, TLR3 and TLR7/8 stimulation resulted in a skewed balance towards IL-12, which occurred in DCs from RA patients as well as healthy controls. Taken together, these data suggested that TLR3- and TLR7/8-mediated DC stimulation in RA results in a skewed balance towards IL-12 and strongly favors Th1-mediated responses.

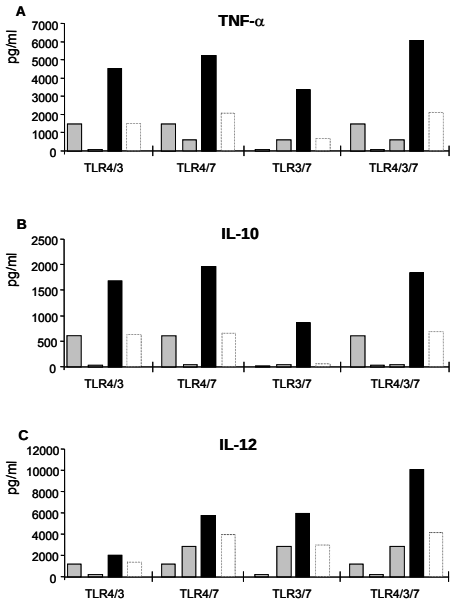

Figure 4. Production of A. TNFα, B. IL-10, and C. IL-12 by DCs upon triggering of single and multiple TLR pathways. DCs were stimulated with TLR3, TLR4, and TLR7/8 ligands alone (shaded bars; each individual shaded bar represents 1 TLR ligand as indicated) and with a combination of different TLR pathways simultaneously (solid bars). These values were compared with the cytokine production that would be estimated to occur if the combination of TLR ligands had an additive effect over the effect with one of the TLR pathways alone (open bars). TNFα, IL-10, and IL-12 were measured in the supernatants after 24 hours of coincubation. Each graph displays data from a representative RA patient. The sensitivity of the cytokine assay was <5 pg/ml for each measured cytokine. See Figure 2 for other definitions.

Table 1. Median level of synergy of TNFα, IL-12, and IL-10 production by dendritic cells from rheumatoid arthritis patients upon stimulation with a combination of TLR pathways

COMBINATION	TNFα	IL-12	IL-10
TLR4/3	23	209	5
TLR4/7	128	478	428
TLR3/7	116	116	299
TLR3/4/7	37	211	210

Synergy is expressed as the median percentage increase compared with the value that would be expected when the combination of TLR pathways was considered to have an additive effect.

Synergistic increase in cytokine production upon simultaneous stimulation of TLR4 and TLR3-7/8 pathways.

To test the hypothesis that simultaneous stimulation of different TLR pathways results in an augmented DC activation, we stimulated DCs with combinations of several TLR ligands. To this end, we cocultured DCs from 5 healthy controls and 5 RA patients with combinations of TLR3/4, TLR4-7/8, TLR3-7/8, and TLR3-4-7/8 for 24 hours. As shown in Figure 4 and Table 1, the production of TNFα, IL-10, and IL-12 dramatically increased in a synergistic manner when 2 or more TLR pathways were stimulated simultaneously. Similar patterns of cytokine production were observed for DCs from RA patients and healthy controls (data not shown). Intriguingly, the highest absolute levels of TNFα (mean 10,152 pg/ml), IL-12 (3,089 pg/ml), and IL-10 (686 pg/ml), and the highest percentage increase in synergistic effect on TNFα (median 128%), IL-12 (478%), and IL-10 (428%) were reached upon stimulation using the combination of TLR4 and TLR7/8 agonists. Moreover, the addition of a third TLR stimulus resulted in a further increase in the absolute levels of TNFα, IL-10, and IL-12 compared to the combinations of 2 TLRs. However, the percentage increase in these cytokines was similar, or even lower, when DCs were stimulated with a combination of 3 TLR ligands as compared to 2 TLR ligands. Remarkably, the addition of TLR2 ligands to any other TLR pathway did not result in a clear synergistic effect (data not shown). Taken together, these data underscore the potential role of simultaneous TLR stimulation in DC activation, with special emphasis on synergy between the TLR4 and TLR3-7/8 pathways.

Presence of TLR4 ligands in RA serum and synovial fluid.

Since we demonstrated that the combination of TLR4 ligands with TLR3 and/or TLR7/8 resulted in a synergistically elevated produc-

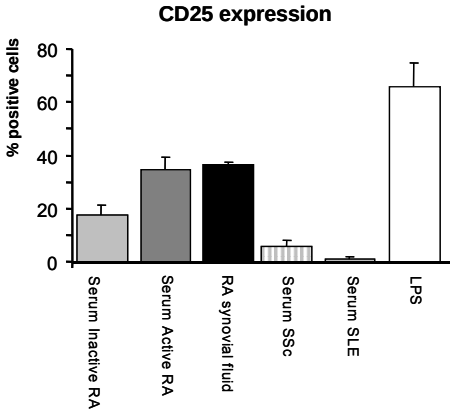

Figure 5. Expression of CD25 on Chinese hamster ovary (CHO) CD14/human TLR4 (HuTLR4) cell lines. CHO CD14/HuTLR4 cells were activated upon coculturing with serum from patients with inactive RA (n=5) and with serum and synovial fluid from patients with active RA (n=7). In contrast, serum from patients with systemic sclerosis (SSc) (n=5) and systemic lupus erythematosus (SLE) (n=5) failed to induce CHO CD14/HuTLR4 cell activation. As expected, LPS, a potent inducer of TLR4, was highly capable of activating TLR4-expressing cells. Values represent the mean and SEM.

tion of cytokines, we tested whether TLR4 agonists were present in RA serum and synovial fluid. To this end, we used CHO reporter cell lines that were stably transfected with CD14 alone (CHO/CD14), with CD14 and human TLR2 (CHO/CD14/HuTLR2), and with CD14 and human TLR4 (CHO/CD14/HuTLR4). These cells were incubated overnight with 10% serum and/or synovial fluid from RA patients.

Whereas incubation of the TLR4-expressing CHO cells with normal human serum did not result in an up regulation of CD25, incubation of the cells with serum from RA patients with low levels of disease activity (Disease Activity Score <3.0 [41]) (n = 5) resulted in increased expression of CD25 surface expression (18%) (Figure 5). Intriguingly, the serum from RA patients with active disease (n = 7) even further increased the expression of CD25 to 35%, which was similar to that found after incubation with synovial fluid (36%) from the same patients. A comparison with serum samples from patients with SSc (n = 5) and SLE (n = 5) demonstrated that, although at a lower level than in RA, serum from SSc patients (CD25 expression level 11%) also contained TLR4 ligands, whereas SLE serum (1%) did not. In contrast, incubation of the CHO/CD14 and CHO/CD14/HuTLR2 cell lines with the same set of sera did not result in increased CD25 expression, suggesting that the stimulation was TLR4 specific. The addition of polymyxin B in these experiments had no effect on CD25 expression, and results of the Limulus amebocyte lysate assay were negative for all synovial fluid samples measured. Stimulation with LPS, included as a positive control, resulted in a potent activation response, as seen by the increased expression of CD25 (66%).

Discussion

In this study, we present novel data regarding the increased expression of TLR3 and TLR7 in synovial tissue from RA patients compared to that of healthy individuals. Since TLR3 and TLR7 are receptors that recognize single-stranded and double-stranded viral mRNA, these findings shed new light on the possible involvement of viruses in RA pathogenesis. Currently, a large body of evidence points towards the involvement of DNA viruses in the pathogenesis of RA. In the synovial tissue of RA patients, parvovirus B19, Epstein-Barr virus, and cytomegalovirus were found to be frequently present (3,4,7,42-45). Although not fully investigated, it is also very likely that RNA viruses occur in the synovial compartment. In light of this, it is interesting that local TLR3 and TLR7 expression is increased in the synovial tissue of RA patients. Although the mechanisms behind this increased expression of viral recognition molecules have not been elucidated thus far, it is tempting to speculate that the recognition of viral RNA and/or mRNA from the host itself leads to the activation of antigen-presenting cells (APCs) that are abundant in the RA synovial compartment, thereby amplifying the inflammatory reaction.

Moreover, the finding that DC stimulation mediated by TLR3 and TLR7/8 results in a markedly skewed balance towards IL-12 is interesting, since we recently demonstrated that IL-12 is indirectly involved in the up regulation of TLR2 and TLR4, both of which are highly expressed in RA synovial tissue (33). These data suggest that TLR3/7 signaling results in an up regulation of TLR2/4, subsequently leading to a higher sensitivity for endogenous ligands, favoring a vicious inflammatory circle. In addition, we produ-

ced novel data showing that the stimulation of multiple TLR pathways simultaneously results in clear synergistic effects with respect to the production of inflammatory mediators. The combination of TLR4 and TLR7/8 pathways especially resulted in synergistic effects, further substantiating the idea that the combination of endogenous ligands and viral mRNA might contribute to the pro-inflammatory environment and propel a chronic inflammatory response in the synovial compartment.

Interestingly, we found that DCs from RA patients secrete higher levels of TNFα and IL-6 compared to DCs from healthy controls upon triggering with specific TLR2 (LTA) and TLR4 (LPS and EDA) ligands. Since much controversy exists about the specificity of LTA, we also tested other TLR1/TLR2 and TLR2/TLR6 heterodimer-specific ligands (FSL-1 and Pam_3Cys) (46,47). Although the absolute levels of pro-inflammatory cytokines by stimulation with FSL-1 and Pam_3Cys were lower compared to LTA, clear differences between RA patients and healthy controls remained, indicating that this phenomenon is specific for TLR2 and TLR4 pathways. The fact that TLR4 ligands were increased in the serum and synovial fluid from RA patients further substantiates a potential role of TLR4 in the onset and/or perpetuation of this condition. Every reaction that is started has to be terminated; the same is true for the immune response. Therefore, the innate immune system is subjected to sophisticated regulation, and there is increasing evidence that an imbalance between pro- and antiinflammatory pathways might give rise to autoimmunity. Some intracellular adapters (e.g., TIRAP) (48) and inhibitory molecules (e.g., SIGIRR) (49) are involved in TLR2 and TLR4 signaling. However, it is likely that many more, yet unidentified, molecules are implicated. Seen in this light, the increased TLR2 and TLR4 responses found in RA might be caused by an alteration in one of these molecules.

DCs are professional APCs that are unrivaled in their capacity for activating naive and effector T cells. DCs initiate immunity and uphold tolerance, 2 critical processes necessary to successfully combat pathogenic invaders and prevent autoimmunity (16). Autoimmunity is attributed to the breakdown of tolerance when lymphocyte clones fail to discriminate between self and nonself, resulting in destructive responses directed to multiple organs. The mechanisms by which autoimmunity is triggered are not fully understood, but DCs and TLRs are likely to play a role in this process. Recently, Ichikawa and colleagues (30) and Waldner and associates (31) provided a conceptual framework demonstrating that TLRs are key molecules for the breakthrough of tolerance. Our data show that triggering different TLRs at the same time resulted in marked synergy with respect to the production of pro-inflammatory mediators, indicating that simultaneous stimulation of different TLR pathways facilitates an environment in which the breakthrough of tolerance is likely to occur.

Taken together, these data suggest that TLRs are involved in the regulation of inflammatory responses in the synovial compartment. TLRs recognize many exogenous and host-derived molecules; the latter are present in RA serum and synovial fluid. In this light, it could be envisaged that, upon the occurrence of a certain event, a variety of TLR ligands that lead to the activation of DCs are released in the synovial compartment. Perhaps a trivial joint trauma or viral

infection leads to such an event. As a result, cell activation and the subsequent release of pro-inflammatory mediators results in cartilage breakdown and the subsequent release of more endogenous TLR ligands. At the same time, the abundance of pro-inflammatory cytokines leads to an increased surface expression of TLRs and an increased capability to sense and react to TLR ligands. Normally, every pro-inflammatory response should lead to the activation of a counteractive mechanism to restore the immunologic balance. However, in RA, such a mechanism seems to be insufficient, resulting in a vicious circle of cell influx and activation, ending up in total joint destruction.

In conclusion, this is the first study to show that TLR3 and TLR7 expression is increased in RA synovial tissue and that the simultaneous triggering of multiple TLR pathways results in the augmentation of the pro-inflammatory response, favoring a pro-inflammatory environment and the breakthrough of tolerance in RA.

Acknowledgements

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CHAPTER 4

Identification of small heat shock protein B8 (HSP22) as a novel TLR4 ligand and potential involvement in the pathogenesis of rheumatoid arthritis

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Dendritic cells (DCs) are specialized antigen presenting cells that can be activated upon pathogen recognition as well as recognition of endogenous ligands, which are released during inflammation and cell stress. The recognition of exogenous and endogenous ligands depends on TLRs, which are abundantly expressed in synovial tissue from rheumatoid arthritis (RA) patients. Furthermore TLR ligands are found to be present in RA serum and synovial fluid and are significantly increased, compared to serum and synovial fluid from healthy volunteers and patients with systemic sclerosis and systemic lupus erythematosus. Identification of novel endogenous TLR ligands might contribute to the elucidation of the role of TLRs in RA and other autoimmune diseases. In this study, we investigated whether five members of the small heat shock protein (HSP) family were involved in TLR4-mediated DC activation and whether these small HSPs were present in RA synovial tissue. In vitro, monocyte-derived DCs were stimulated with recombinant αA crystallin, αB crystallin, HSP20, HSPB8, and HSP27. Using flow cytometry and multiplex cytokine assays, we showed that both αA crystallin and HSPB8 were able to activate DCs and that this activation was TLR4 dependent. Furthermore, Western blot and immunohistochemistry showed that HSPB8 was abundantly expressed in synovial tissue from patients with RA. With these experiments, we identified sHSP αA crystallin and HSPB8 as two new endogenous TLR4 ligands from which HSPB8 is abundantly expressed in RA synovial tissue. These findings suggest a role for HSPB8 during the inflammatory process in autoimmune diseases such as RA.

Introduction

Dendritic cells (DCs)[3] are professional antigen presenting cells (APC) that regulate the delicate balance between immunity and tolerance. In the absence of inflammation, immature DCs (iDCs) reside in the periphery, highly capable for Ag uptake, which is largely arranged by FcγRs and TLRs. Upon ligand binding, DCs undergo maturation, which coincides with a down-regulation of Ag recognition receptors, up-regulation of MHC and costimulatory molecules, and production of pro-inflammatory mediators such as TNFα and IL-12. To date, a large body of evidence points towards the crucial role for DCs in the onset and perpetuation of arthritis (1, 2, 3, 4).

Many signals are able to induce DC activation, and among the most powerful of these are microbial and viral products, also called pathogen-associated molecular patterns, which are recognized by TLRs. Exogenous TLR ligands include lipoteichoic acid, LPS, CpG motifs of bacterial DNA, and viral RNA (5, 6, 7, 8). More recently, however, many studies addressed the role of endogenous ligands in the TLR-mediated cell activation (9, 10, 11, 12). Intriguingly, such endogenous ligands, including fibronectin, heat shock proteins (HSPs), hyluronic acid, and host-derived RNA, are released upon tissue damage and cell stress, events that are likely to occur during inflammatory conditions. The activation of cells by endogenous components from distressed or injured cells supports the so-called “Danger Model” proposed by Matzinger (13). This theory suggests that the immune system is more concerned with damage than with foreignness and is called into action by alarm signals from injured tissues, rather than by the recognition of nonself. In this light, it is tempting to speculate that an immune response to endogenous components plays a role in the initiation of autoimmune diseases. Accordingly, TLRs are implicated in the breakdown of tolerance

as shown in experimental models of diabetes (14), Crohn's disease (15), and multiple sclerosis (16, 17), and recent research suggested that simultaneous or sequential triggering of different TLR pathways might be the event that sets off autoimmunity (18). Rheumatoid arthritis (RA) is such an autoimmune disease, characterized by chronic inflammation of synovial joints leading to cartilage damage and ultimately total joint destruction. Although the exact pathways leading to the initiation and perpetuation of synovial inflammation remains obscure, recent evidence addressed the potential role of TLRs in arthritis. At first, TLR2, TLR3, TLR4, TLR7, and several ligands were found to be highly expressed in synovial tissue from RA patients, compared to that from healthy donors (3, 4, 19). Second, we have shown that TLR-mediated activation of DCs from RA patients led to significantly higher levels of the key pro-inflammatory cytokines TNFα and IL-6, compared to DCs from their healthy counterparts. Finally, the involvement of various TLRs in the onset and perpetuation of experimental arthritis has been elegantly shown (20, 21, 22).

These latter findings have sparked the research into the role of several endogenous TLR ligands in RA of which HSPs have been studied the most thoroughly thus far. HSPs constitute a family of proteins that carry out housekeeping functions that are important for the survival of eukaryotic cells. More recently, evidence emerged on a role for HSPs during innate and adaptive immune responses (23, 24, 25). HSPs can be classified into several distinct families from which HSP60 (HSP60, HSP65, and GROEL) and HSP70 (HSP72, HSP73, and GRP170) have been the subject of extensive studies with regard to immunogenetic properties. The small HSPs (sHSPs), including αA crystallin, αB crystallin, HSP27, HSPB8 (HSP22), and HSP20, represent another class of HSPs, ranging in size from 12 to 43 kDa (26, 27, 28). In contrast with HSP60 and HSP70, the role of sHSPs in autoimmune diseases have not been subjected to similar research. To characterize the potential role of sHSPs in arthritis, in this study, we investigated the ability of five members of the sHSPs (αA crystallin, αB crystallin, HSPB8, HSP27, and HSP20), to stimulate monocyte-derived DCs and the possible role for TLRs in this process. In this study, we demonstrate, for the first time, that the sHSP αA crystallin and B8 (HSP22) clearly induced DC maturation and cytokine production, which was TLR4 dependent. In addition, HSPB8, but not αA crystallin, was highly expressed in synovial tissue from RA patients, further substantiating a potential role for HSPB8 during the inflammatory process in RA.

Materials & Methods

Patients/study population

For this study, a total of seven RA patients attending the Department of Rheumatology at Radboud University Nijmegen Medical Center and seven healthy volunteers were included. The patients fulfilled the American College of Rheumatology criteria for RA (29), and they all gave their informed consent. Patients using high-dose prednisolone (>10 mg/day) or anti-cytokine therapies (anti-TNFα and/or IL-1Ra) were excluded from this study. For immunohistochemical analysis, synovial biopsies from RA patients (n = 5) were obtained using small needle arthroscopy. On average, 20 biopsy samples were obtained from the medial and lateral suprapattelar pouch on each occasion. The local Medical Ethics Committee approved the study protocol.

Expression and purification of the sHSPs

For the expression of the different sHSPs, pET16b constructs were used containing the coding sequence of human αA crystallin, αB crystallin, HSPB8, HSP27, or rat HSP20. Expression of each protein was induced in Rosetta (DE3)pLysS cells (Novagen) for 3 h at 37°C with 0.4 mM isopropyl -D-1-thiogalactopyranoside. Cells were spun down from four cultures of 450 ml and resuspended in 20 ml of 25 mM Tris-HCl, 2 mM EDTA, and 50 mM glucose) (TEG50), pH 8.0, containing protease inhibitors (one complete protease inhibitor mixture tablet; Roche). The cell suspension was frozen, thawed, and sonicated three times for 30 s. The suspension was cleared by centrifugation, and the supernatant was desalted with a HiTrap 26/10 column (Amersham Biosciences) in 25 mM Tris-HCl and 2 mM EDTA) (TE), pH 7.0. The different sHSPs were purified over a DEAE-Sepharose fast-flow column and Source 15Q column (Amersham Biosciences) at room temperature and a flow rate of 2 ml/min, using a gradient from 0 to 1000 mM NaCl in TE (pH 7.0).

Culture of monocyte-derived DCs

Monocyte-derived DCs were cultured essentially the same as described previously (30). Briefly, PBMC were isolated from heparinized venous blood by using density-gradient centrifugation over Ficoll-Paque (Amersham Biosciences). Low-density cells were collected and washed with citrated PBS 5% FCS, whereafter the cells were allowed to adhere for 1 h at 37°C in RPMI 1640 Dutch modification (Invitrogen Life Technologies) supplemented with 2% human serum (PAA Laboratories) in 25-cm^2 cell culture flasks (Corning). Adherent monocytes were cultured in RPMI 1640 Dutch modification supplemented with 10% FCS and antibiotic-antimycotic (Invitrogen Life Technologies) in the presence of IL-4 (500 U/ml; Schering-Plough) and GM-CSF (800 U/ml; Schering-Plough) for 6 days. Fresh culture medium with the same supplements was added at day 3 where after iDCs were harvested at day 6. The iDCs were resuspended in fresh cytokine-containing culture medium and transferred to culture plates in a concentration of 0.5 x 10^6 cells/ml and stimulated as described in the subsequent section.

Stimulation of monocyte-derived DCs with sHSPs

Monocyte-derived iDCs were stimulated with 1 and 10 µg/ml each of these five members of the sHSP family; αA crystallin, αB crystallin, HSPB8, HSP27, HSP20. LPS (Sigma-Aldrich) stimulation (2 µg/ml) was used as a positive control for inducing DC maturation. To correct for potential endotoxin contamination 10 µg/ml polymyxin B (PMB) was added. Furthermore, the sHSPs in the presence of PMB were also tested for the presence of Gram-negative bacterial endotoxin by the Limulus amebocyte lysate (LAL) test, performed according to the manufacturer's instructions (Multitest LAL Pyrogen Plus; BioWhittaker). Moreover, we also performed experiments with heat-inactivated HSPB8 and αA crystallin. To test the involvement of TLR4 in stimulation with these sHSPs, 100 nM TLR4 antagonist (E5564; provided by the Eisai Research Institute, Andover, MA) was added to the culture (31). After 24 h of culture at 37°C in the presence of 5% CO_2, culture supernatants were collected. Supernatants were stored at –20°C until cytokine measurement was performed.

Table 1. Mean fluoresence intensity of the DC surface molecules CD14, CD83, CD83, CD86, MHCI and MHCII

	CD14	CD80	CD83	CD86	MHCI	DR/DP
Immature DC	6 (1)	21 (8)	21 (7)	165 (53)	152 (40)	462 (111)
LPS	4 (1)	111 (17)	41 (7)	294 (23)	218 (68)	770 (237)
HSPB8	5 (2)	65 (12)	38 (11)	238 (57)	262 (21)	610 (197)

The values shown represent the mean and SEM (between brackets) of all DCs used in the experiments

Stimulation of peritoneal macrophages from TLR4 knock-out mice with αA crystallin and HSPB8

BALB/c TLR4-deficient (TLR4–/–) mice were provided by S. Akira (Osaka University, Osaka, Japan). Peritoneal macrophages from TLR4–/– and wild-type mice (both BALB/cA background) were isolated by peritoneal lavage using ice-cold DMEM/10% FCS. Cells were put into 24-well-plates (Corning) at a concentration of 1 x 10^6 cells/ml. After a 4-day adjustment period, culture medium was changed to one containing 10 µg/ml HSPB8 or control medium. To correct for potential endotoxin contamination, medium contained 10 µg/ml PMB. After 24 h, the culture medium was collected and stored at –20°C until cytokine measurement was performed.

Phenotypical analysis of monocyte-derived DCs

The phenotypical analysis of monocyte-derived DCs was performed using standardized flow cytometry protocols as described previously (30). Briefly, a number of 1 x 10^5 DCs were incubated with mAbs against human CD14 (DakoCytomation), CD80 (BD Biosciences), CD83 (Beckman Coulter), CD86 (BD Pharmingen), MHC class I (MHC-I) (clone W6/32), and MHC-II DR/DP (clone Q1514) for 30 min at 4°C. Cells were then washed and incubated with FITC-conjugated goat anti-mouse IgG (Zymed Laboratories) for 30 min at 4°C in complete darkness. Subsequently, cells were washed and analyzed with a fluorescence-activated cell sorter (FACSCalibur; BD Biosciences) for the proportion of positive cells relative to cells stained with relevant IgG isotypes. Cells were gated according to their forward- and side-scattering patterns. For each marker, 10^4 cells were counted in the gate.

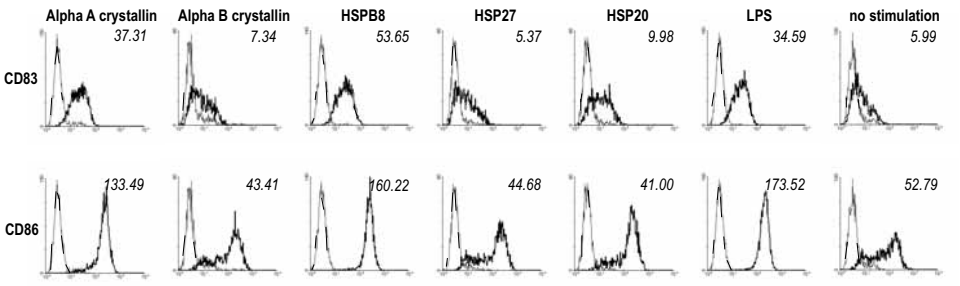

Figure 1. Expression of cell surface markers CD83 and CD86 after stimulation with several small heat-shock proteins. FACS® analysis of the indicated markers (bold line) or isotype controls (thin line) from DC within the life gate. Human monocyte-derived DCs were stimulated with 10 mg/ml αA crystallin, αB crystallin, HSPB8, HSP27, HSP20, 2 mg/ml LPS or medium for 48 hours.

Measurement of cytokines in culture supernatant

Human TNFα, IL-6, IL-10, IL-12p70, and mice IL-6 levels were measured in the supernatants of the DC cultures and the peritoneal macrophage cultures, respectively, using commercially available kits (Bio-Rad) according to the manufacturer's instructions (32). Cytokine levels were measured and analyzed with the Bio-Plex system and its software (Bio-Rad). The sensitivity of the cytokine assay was <5 pg/ml for all cytokines measured.

SDS-PAGE and Western blotting of αA crystallin and HSPB8 in synovial tissue

Synovial tissue samples containing 50 µg of synovium proteins together with 10 ng αA crystallin or HSPB8 were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Hybond; Amersham Biosciences). Membranes were then blocked with 5% nonfat dried milk in TBST (15 mM Tris-HCL (pH 8.0), 150 mM NaCl, 0.1% Tween 20) and probed with primary rabbit anti-HSPB8 serum or rabbit anti- A-serum and secondary swine-anti-rabbit peroxidase-coupled Abs. Immunoreactive bands were visualized by the ECL Western blotting detection kit (Pierce).

Immunohistochemical analysis of human synovial biopsies

Synovial tissue samples were embedded in Tissue Tek OCT (Sakura) and snap-frozen in liquid nitrogen. Cryostat sections (5 µm) were mounted on superfrost slides and stored at –80°C until processing. For HSPB8

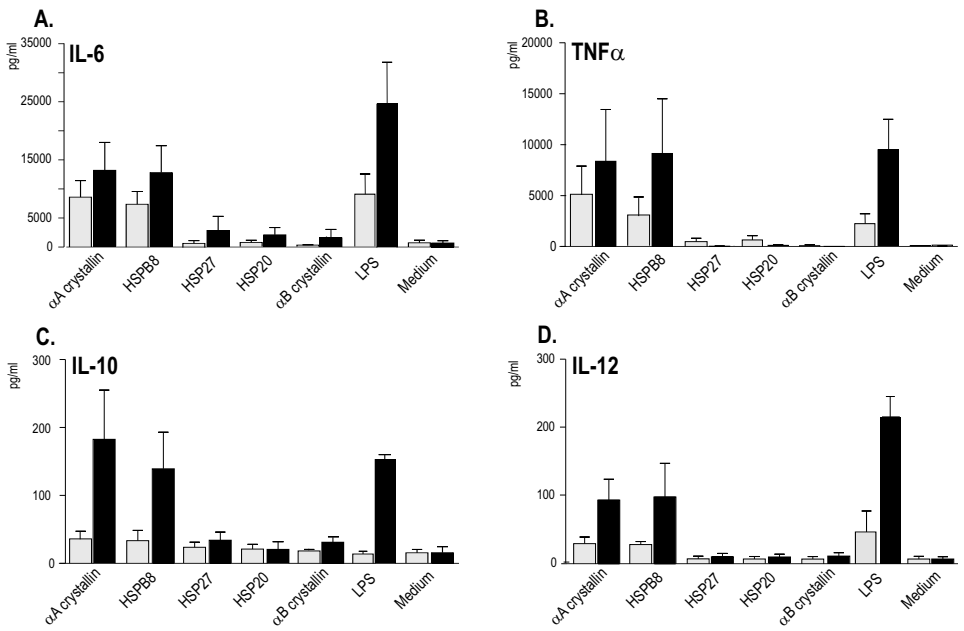

Figure 2. Cytokine production by DCs from RA patients and healthy controls after stimulation with several small heat-shock proteins. Mean production of IL-6 (A), TNFα (B), IL-10 (C) and IL-12p70 (D) by DC from RA patients (black bars) and healthy controls (grey bars) upon stimulation with 10 mg/ml αA crystallin, αB crystallin, HSPB8, HSP27, HSP20, 2 mg/ml LPS or medium for 24 hours (n=7 for αA crystallin and HSPB8, for other small HSP n=3). Error bars represent the SEM. The sensitivity of the cytokine assay was below 5 pg/ml for each measured cytokine.

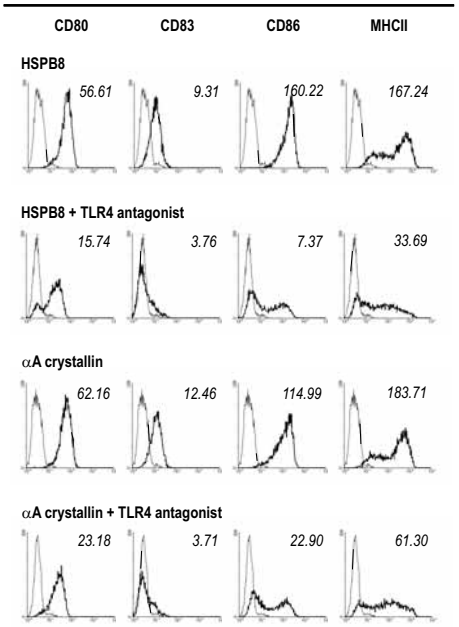

Figure 3. Expression of cell surface markers CD80, CD83, CD86 and MCHII after stimulation with HSPB8 or αA crystallin in the presence and absence of a TLR4 antagonist. FACS® analysis of the indicated markers (bold line) or isotype controls (thin line) from DCs within the life gate. Human monocyte-derived DCs were stimulated with 10 mg/ml HSPB8 or αA crystallin for 48 hours in the presence or absence of a TLR4 antagonist (E5564).

staining, sections were incubated with 2 µg/ml goat anti-human HSPB8 (Abcam) for 60 min. After this, endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 15 min, and the secondary Ab, biotinylated mouse anti-goat IgG (Jackson ImmunoResearch Laboratories), was incubated for 30 min. Vectastain ABC reagent (Vector Laboratories) was incubated for 30 min, developed with diaminobenzidine (Sigma-Aldrich), and counterstained with hematoxylin for 30s.

For αA crystallin staining, sections were incubated with rabbit anti-human αA crystallin serum (50x diluted) for 60 min. After this, endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 15 min and the secondary Ab, biotinylated swine anti-rabbit IgG (Dako), was incubated for 30 min. Subsequently, slides were incubated with streptavidin-HRP (Dako) for 30 min, developed with diaminobenzidine (Sigma-Aldrich), and counterstained with hematoxylin for 30 s.

Results

αA crystallin and HSPB8 selectively induce DC maturation

To study the potency of sHSPs to induce DC maturation, we compared iDC stimulation using 5 different sHSPs (αA crystallin, αB crystallin, HSPB8, HSP27, and HSP20) to TLR4-mediated DC maturation using LPS. The iDCs are characterized by the absence of CD14, low levels of CD83, and intermediate levels of the costimulatory molecules CD80 and CD86 and MHC molecules. LPS-mediated DC maturation is characterized by a clear up-regulation of CD83, the costimulatory molecules CD80 and CD86, and MHC molecules (Table I). In our experiments, stimulation with αA crystallin and HSPB8 induced a clear DC maturation as seen by the absence of CD14 and high mean levels of CD80, CD83, CD86, and MHC-II, which was comparable to LPS-mediated stimulation (CD83 and CD86 expression from a representative healthy individual; Figure 1) In contrast, stimulation with the sHSP αB crystallin, HSP20, and HSP27 did not affect the phenotype of iDCs at all (Figure 1).

In addition, we studied whether differences between RA DCs and DCs from healthy controls exist. The sHSP αA crystallin and HSPB8 induced maturation of RA DCs to an equal extent, compared with that seen in control DCs (data not shown). To correct for potential endotoxin contamination, experiments were performed in the presence of PMB. Only small

differences in FACS results were observed when cultured with or without PMB (data not shown).

Increased cytokine production upon DC stimulation with αA crystallin and HSPB8

DC maturation by TLR ligands is known to be accompanied by an increased production of pro-inflammatory cytokines. Therefore, we investigated whether phenotypic maturation of DCs induced by αA crystallin and HSPB8 coincided with an increased production of the inflammatory mediators TNFα, IL-6, IL-10, and IL-12p70 as seen in LPS-mediated DC maturation (3, 33). As shown in Figure 2, 10 µg/ml αA crystallin and HSPB8 clearly induced secretion of IL-6, TNFα, and to a lesser extent, IL-10 and IL-12p70, and resembled the cytokine pattern induced by LPS stimulation. In contrast with this, but in line with the results of the FACS® analysis, αB crystallin, HSP27, and HSP20 hardly induced cytokines.

Interestingly, although not statistically significant, cytokine levels produced by RA cells

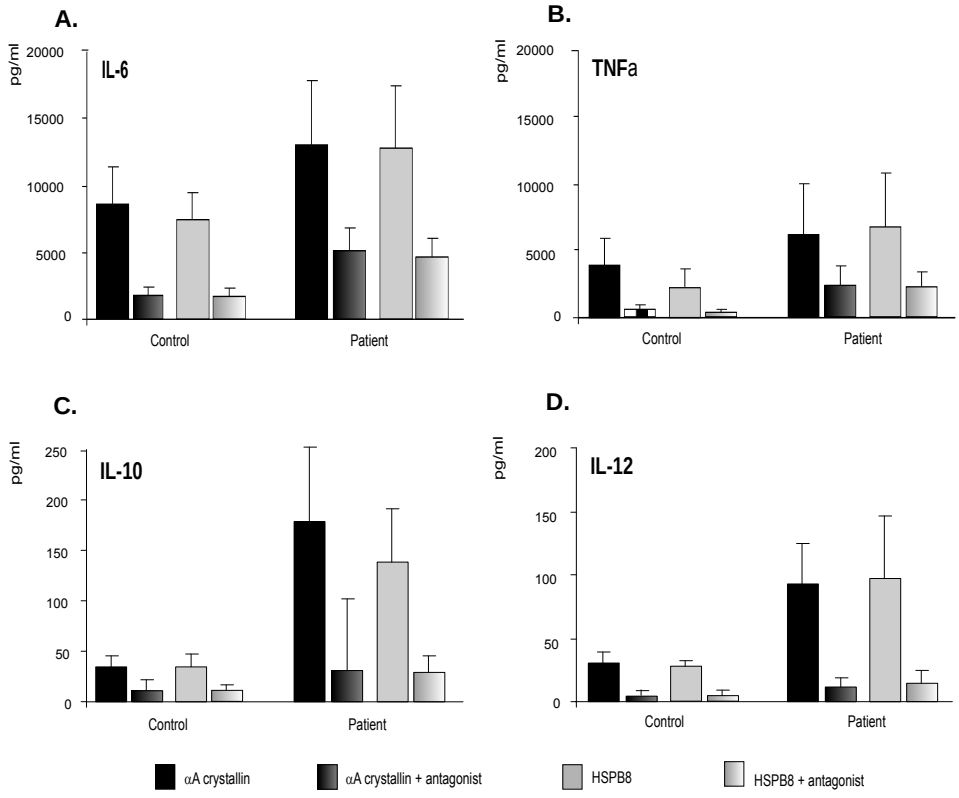

Figure 4. Cytokine production by DCs from RA patients and healthy controls after stimulation with αA crystallin and HSPB8 in the presence or absence of a TLR4 antagonist. Mean production of IL-6 (A), TNFα (B), IL-10 (C), and IL-12p70 (D) by DCs from RA patients and healthy controls upon stimulation with 10 µg/ml αA crystallin or HSPB8 for 24 h (n=5). Cells were cultured in the presence (shaded bars) or absence (filled bars) of 100 nM TLR4 antagonist (E5564). The values shown are corrected for the cytokine production by unstimulated DCs. Error bars represent SEM. The sensitivity of the cytokine assay was <5 pg/ml for each measured cytokine.

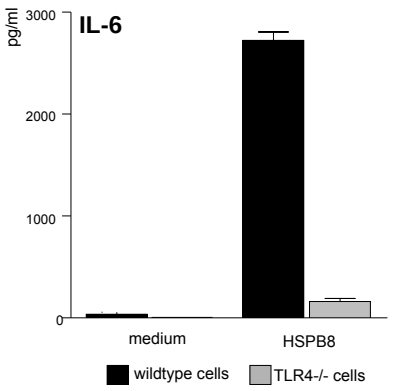

Figure 5. IL-6 production by peritoneal macrophages from TLR4–/– mice and wild-type mice upon stimulation with HSPB8. Mean IL-6 production by peritoneal macrophages from TLR4–/– (n=7) and wild-type (n=7) mice after incubation with 10 µg/ml HSPB8 for 24 h. The sensitivity of the cytokine assay was <5 pg/ml.

were markedly increased, compared to the cytokine levels produced by DCs from healthy controls upon stimulation with αA crystallin and HSPB8 (IL-6 13,000 pg/ml; TNFα 8,500 pg/ml). Although the absolute cytokine levels produced by RA cells were markedly increased, the shape of the TLR-sHSP dose-response curves were similar for RA cells as well as cells from healthy controls. These results exclude that the differences in cytokine production are a result of different shapes of dose-response curves.

As tested by dose-response experiments, both HSPs were able to activate DCs in a concentration as from 50 ng HSP/ml, resulting in production of IL-6 (data not shown).

To correct for potential endotoxin contamination, we performed all the cell stimulation experiments in the presence of PMB; however, addition of PMB only slightly decreased the sHSP-mediated cytokine production, indicating that hardly any endotoxins were present in our sHSP preparations. In addition, we performed experiments with heat-inactivated HSPB8 and αA crystallin, which showed a significant reduction in TNFα production (98% reduction), excluding that endotoxin contamination played a role in our experiments.

αA crystallin- and HSPB8-mediated stimulation of DCs is largely TLR4 dependent

Many studies refer to the potential role of TLR4 in the recognition of HSPs, their role in DC maturation, and the potential implication for TLRs in the pathogenesis of arthritis. Therefore, we studied whether TLR4 was involved in DC activation by HSP αA crystallin and HSPB8. To this aim, we stimulated DCs with αA crystallin and HSPB8 in the presence and absence of a TLR4 antagonist (E5564) (31). With respect to DC maturation, adding a TLR4 antagonist clearly inhibited DC maturation, as seen by a clear decrease in the expression of CD80, CD83, CD86, and MHC-II (Figure 3). In line with this finding, the production of IL-6 (80% decrease), TNFα (85% decrease), IL-10 (70% decrease), and IL-12p70 (80% decrease) by DCs from healthy individuals was markedly reduced by the addition of a TLR4 antagonist (Figure 4). Although somewhat less clear, this effect was demonstrated

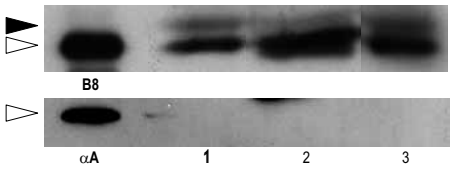

Figure 6. Western blot detection of HSPB8 and αA crystallin in synovial tissue from RA patients. Synovial tissue samples (n= 3) were analyzed for the expression of HSPB8 and αA crystallin (open arrows). As a positive control, rHSPB8 and rαA crystallin were used. The black arrow indicates a modified form of HSPB8.

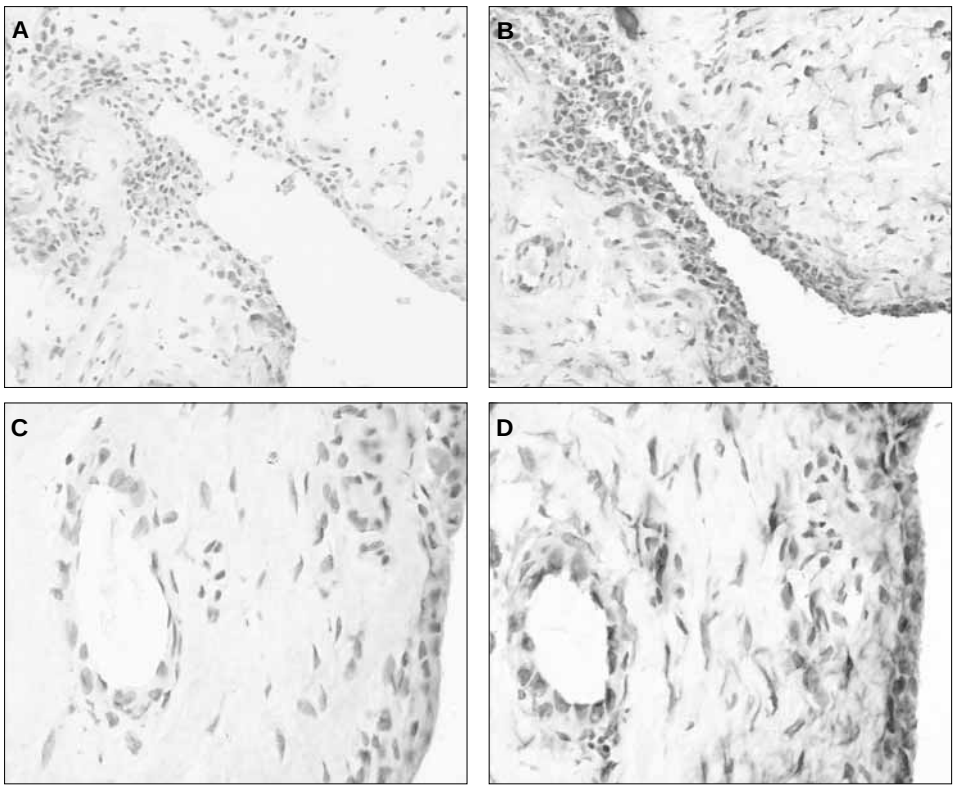

Figure 7. Immunohistochemical detection of HSPB8 in synovial tissue from RA patients. A. Staining of the synovial lining with an isotype control Ab (magnification x200). B. Staining of the synovial lining with a specific Ab against HSPB8 (magnification x200). C. Staining of synovial vascular endothelial cells with an isotype control Ab (magnification x400). D. Staining of synovial vascular endothelial cells with a specific Ab against HSPB8 (magnification x400). All synovial tissue sections were counterstained with hematoxylin.

for DCs from RA patients as well (IL-6, 60% decrease; TNFα, 65% decrease; IL-10, 80% decrease; and IL-12p70, 85% decrease).

TLR4–/– macrophages do not respond to HSPB8 stimulation

To further confirm the TLR4 dependency of HSPB8 stimulation, we stimulated peritoneal macrophages of wild-type and TLR4–/– mice with HSPB8-containing medium or medium alone. In both wild-type macrophages as well as TLR4–/– macrophages, medium did not induce the production of IL-6. As expected, and confirming our previous data, HSPB8 stimulation of wild-type macrophages resulted in a markedly increased production of IL-6 The production of other cytokines (TNFα and IL-10) was below detection level. The almost complete abrogation of IL-6 production by peritoneal macrophages from TLR4–/– mice further confirms the TLR4 dependency of HSPB8-mediated cell activation (Figure 5).

HSPB8 is abundantly expressed in synovial tissue from RA patients

Thus far, the main expression of αA crystallin

and HSPB8 in human tissues was confined to the eye lens, skeletal muscle, heart, and placenta (34). To study the potential role for sHSPs in synovial inflammation, we tested the expression of αA crystallin and HSPB8 in RA synovium using Western blotting and immunohistochemistry techniques.

Interestingly, Western blotting showed clear staining for of HSPB8 in RA synovial tissue, whereas αA crystallin staining was completely absent (Figure 6).

Additional immunohistochemistry showed that the expression of HSPB8 was particularly present in the lining (Figure 7B) and the vascular endothelial (Figure 7D) cells. HSPB8 also was expressed in the sublining, albeit to a lesser extent.

Discussion

Antigen recognition by TLRs results in activation of the innate immune system and leads to the secretion of many inflammatory mediators such as TNFα, IL-6, and several chemokines. Therefore, the innate immune response is likely to play a considerable role both in the initiation and perpetuation of the inflammatory processes during RA. In this study, we demonstrated that two members of the sHSP family, αA crystallin and HSPB8, were able to activate DCs by inducing maturation and cytokine production. Blockade of TLR4, using a selective TLR4 antagonist (E5564) (31), clearly abrogated this effect, demonstrating the TLR4 dependency of this HSP stimulation. In line with this finding, incubation of peritoneal macrophages from TLR4–/– mice, with HSPB8 and αA crystallin, did barely lead to the production of inflammatory cytokines, whereas wild-type mice clearly did show an increased cytokine production. Finally, we provided evidence for a role of HSPB8 in the pathogenesis of RA by demonstrating that HSPB8, but not αA crystallin, was highly present in RA synovial tissue.

Lately, an increasing amount of literature points to a role for TLRs in the recognition of host-derived molecules, such as hyaluronan, fibronectin fragments, HSPs, mRNA from necrotic cells, and mammalian chromatin (9, 10, 11, 12, 35). However, recently it has been shown that the stimulating effect some of these endogenous TLR ligands is caused by endotoxin contamination and not by the ligand itself (36, 37). To exclude the possibility that LPS contamination interfered with our experiments, we performed all the stimulation experiments in the presence and absence of PMB, a natural LPS binding protein. Second, we tested the presence of LPS using the LAL test, which showed to be negative for all five HSP samples tested. Furthermore, experiments with heat-inactivated HSPB8 and αA crystallin showed a significant decrease in TNFα production, excluding the involvement of endotoxins in the TLR4-mediated DC activation. Finally, all five HSPs tested were simultaneously prepared using the same protocols. The fact that DC maturation and activation could be achieved only by incubation with HSPB8 and αA crystallin underscores the fact that an effect of endotoxins could be ruled out.

The ability of HSPB8 and αA crystallin to activate cells using TLR4 is intriguing because we and others found that TLRs are highly expressed in RA synovial tissue (3, 4, 19, 38). We also found that, upon stimulation of TLR2 and TLR4, DCs from RA patients produce significantly higher amounts of pro-inflammatory cytokines, compared to DCs from healthy controls

(3). Additionally, models of experimental arthritis support the role for TLRs in this inflammatory disease (20, 39). Furthermore, the involvement of TLRs in tolerance breakthrough has been demonstrated in experimental models of diabetes and multiple sclerosis (14, 16, 17).

Therefore, our finding that HSPB8 is abundantly expressed in the synovial tissue of RA patients underscores that HSPB8 could act as a TLR4 ligand during RA, thereby amplifying the inflammatory loop. Because recent literature shows that simultaneous stimulation of TLRs might be the event that sets of autoimmunity, it is tempting to speculate that HSPB8, together with other endogenous TLR ligands, which are released in the synovial compartment, are involved in this process (3, 40).

The expression of HSPB8 in human tissues, particularly in skeletal and smooth muscles, heart, and brain, has been described well (34, 41). The finding that HSPB8 also is expressed in synovial tissue is new and could be explained by the fact that synovial tissue is normally not part of the tissue data bank used to screen the tissue distribution of new proteins.

In conclusion, we identified two new TLR4 agonists, HSPB8 and αA crystallin. HSPB8 is highly expressed in RA synovial tissue and is likely to be released during synovial inflammation. The release of this protein in the synovial joint could potentially lead to an ungoing activation of inflammatory cells, thereby amplifying the inflammatory loop of synovial inflammation. Further research is necessary to elucidate the precise role of HSPB8 in the pathogenesis of RA.

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CHAPTER 5

Allograft inflammatory factor 1 (AIF-1) is correlated with TLR4 hyperresponsiveness during rheumatoid arthritis

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Rheumatoid Arthritis (RA) is a chronic autoimmune disease that is characterized by inflammation of the synovial joints, which ultimately leads to joint destruction. Although the exact cause still has to be identified, many evidence addressed a role for Toll-like receptors (TLRs) in the pathogenesis of this disease. Since it has been shown that TLR4 stimulation of dendritic cells (DCs) from RA patients results in much higher production of pro-inflammatory cytokines compared to DCs from healthy individuals, we here tried to identify potential factors that are involved in the TLR4 hyper-responsiveness during RA.

Using array-based gene expression profiling, expression of allograft-inflammatory 1 (AIF-1) was found to be significantly enhanced in DCs from RA patients. This enhanced expression was confirmed using real-time PCR, in DCs as well as in monocytes. Furthermore we found the enhanced expression of all AIF-1 isoforms in cells from RA patients using real-time PCR. In vitro stimulation experiments showed that the expression of total AIF-1 as well as its separate isoforms strongly correlated with the TLR4-induced production of IL-6 and TNFα in cells from RA patients as well as in cells from healthy controls. Therefore it is highly likely that the enhanced expression of AIF-1 determines hyperresponsiveness towards TLR4.

In conclusion, using a gene expression approach, we identified AIF-1 as a potential factor to be involved in the TLR4 hyperresponsiveness in RA, although the exact function of the several AIF-1 isoforms has not been elucidated thus far. Therefore, further investigation into the specific function of the separate AIF-1 isoforms might provide additional evidence with respect to the exact role of AIF-1 in the pathogenesis or chronicity of RA.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, which mainly affects the synovial joints. The synovial inflammation is characterized by a massive infiltration of cells including T-cells, B-cells, macrophages and dendritic cells (DCs), resulting in destruction of the joints. Since DCs are abundantly present at strategic locations in RA synovial tissue (1), increasing evidence addressed a role for DCs in the pathogenesis of RA (1-4).

Many external stimuli are able to induce DC activation, and among the most powerfull of these are microbial and viral products such as lipoteichoic acid, LPS, and viral RNA. These components, also called pathogen-associated molecular patterns, can be recognized by pattern recognition receptors (PRR), including Toll-like receptors (TLRs) (1;5-8). More recently, however, many studies provided evidence for TLR-mediated immune activation by “self molecules”, including heat-shock proteins, fibronectin, hyaluronic acid and host derived RNA (9-12). These so called endogenous ligands can be released upon tissue damage and cell stress, events that are likely to occur during inflammatory conditions. In vivo evidence for TLR-mediated signaling that was induced by endogenous ligands comes from studies of acute tissue injury, as in trauma or hemorrhagic shock (13;14). The possible involvement of TLRs in the pathogenesis of RA has been studied intensively in the last few years, resulting in an increasing body of evidence. First, TLRs are abundantly expressed in RA synovial tissue and this expression is markedly increased compared to synovial tissue from patients with osteoarthritis and healthy controls (15;16). Moreover, the endogenous TLR ligand heat-shock protein B8 (HSPB8) was expressed in synovial tissue. In vitro

studies have demonstrated that TLRs are under strong regulation of several cytokines, such as TNFα, IL-1β and IFNγ, which play key roles in RA (15;17-19). The involvement of TLRs in the pathogenesis of RA was also recently underscored by some clinical research, as treatment of RA using anti-TNFα therapy resulted in significantly decreased levels of TLR4 (20) and inhibition of the TLR4 signaling pathway in RA patients using Chaperonin 10 (heat shock protein 10, XToll™) was shown to be well tolerated and efficacious (21). Recently we demonstrated that upon stimulation of TLR2 and TLR4, DCs from RA patients produced much higher cytokine levels compared to DCs from healthy controls, whereas the cytokine responses upon stimulation of other TLRs did not differ (16). Interestingly, the expression levels of TLR2 and TLR4 were not significantly different between cells from RA patients and healthy volunteers, excluding that the enhanced cytokine production resulted from increased TLR expression. The fact that TLR2 as well as TLR4 stimulation showed an enhanced cytokine production by RA cells, also excludes a role for CD14 in this process. The purpose of this study was to investigate the factors that are differentially expressed at basal level in DCs from RA patients compared to healthy controls, which might be associated with enhanced responses upon TLR4 stimulation. Therefore, we performed gene expression profiling and subsequent real-time PCR. We found that basal mRNA expression of allograft inflammatory factor I (AIF-1) was significantly increased in DCs from RA patients and this expression correlated with the cytokine levels produced upon TLR4 stimulation.

Materials & methods

Patients / study population

A total of 17 RA patients and 17 healthy volunteers were included in this study. RA patients were attending the Radboud University Nijmegen Medical Centre, fulfilled the American College of Rheumatology criteria for RA (22) and all gave informed consent. Patients using high dose prednisolone (>10mg/day) or anti-cytokine therapies (anti-TNFα and/or IL-1Ra) were excluded from this study. The local Medical Ethics Committee approved the study protocol.

Isolation, generation and stimulation of monocytes and monocyte-derived DCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood using density gradient centrifugation over Ficoll-Paque (Amersham Biosciences, Roosendaal, The Netherlands). Low-density cells were collected and washed with citrated PBS 5% FCS. For monocytes, $CD14^+$ cell fraction was isolated using MACS® cell separation, according to the manufacturer's instructions. Briefly, PBMC were incubated with anti-human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 minutes, subsequently cells were washed and $CD14^+$ fraction was separated from the $CD14^-$ fraction over a MACS® MS separation column (Miltenyi Biotec, Bergisch Gladbach, Germany).

For monocyte-derived DCs, PBMCs were allowed to adhere for 1 hour at 37°C in RPMI-1640 Dutch Modification (Invitrogen Corporation, Carlsbad, CA, USA supplemented with 2% human serum (PAA Laboratories, Pasching, Austria) in 25cm^2 cell culture flasks (Corning Incorporated, NY, USA). Adherent monocytes were cultured

in RPMI-1640 Dutch modification supplemented with 10% fetal calf serum (FCS) and antibiotic-antimycotic (Life Technologies) in the presence of IL-4 (500 U/ml, R&D Systems, Minneapolis, MN, USA) and GM-CSF (800 U/ml, R&D Systems, Minneapolis, MN, USA) for 6 days. Fresh culture medium with the same supplements was added at day 3 where after immature DCs were harvested at day 6.

Monocytes and immature DCs, in a concentration of $0.5x10^6$ cells/ml were stimulated with 1mg/ml LPS. Subsequently, culture supernatants were removed and 1ml TRIzol reagent (Sigma St. Louis, MO, USA) was added to the cells and stored at –20°C until RNA isolation was performed.

RNA isolation

For array-based gene expression profiling RNA from 5 RA patients and 5 healthy controls was isolated from unstimulated DCs using RNAeasy (Qiagen Benelux, Venlo), according to the manufacturers instruction. RNA was quantified by RiboGreen assay (Molecular Probes Europe BV, Leiden) adjusted for a crystal 96-well microtiterplate (Thermo Labsystems, Vantaa) using FluoStar Galaxy (BMG Labtechnologies, Toronto). RNA integrity was assessed on 1% SeaKem® gold agarose gel. All samples passed the quality control, and were subjected to the subsequent labeling and hybridization. RNA samples from 3 representative RA patients and 2 representative healthy controls were used for array-based gene expression profiling.

For real-time PCR analysis, total RNA from 12 RA patients and 12 healthy controls was extracted in 1 ml of TRIzol reagent, an improved single-step RNA isolation method based on the method described by Chomczynski et al. (23).

Oligonucleotide array analysis.

One microgram of total RNA was used as starting material for cDNA preparation. Generation of biotinylated cRNA target and subsequent hybridization, washing and staining of HG_U133 Plus2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA) were performed according to Affymetrix Expression Analysis Technical Manual for one-cycle amplification (24). The arrays were then scanned using a laser scanner GeneChip®Scanner (Affymetrix) according to the manufacturers instruction and analyzed by using Affymetrix Gene Chip Operating Software (GCOS) version 1.4. Normalization, model-fitting, and filtering were performed using DNA-Chip Analyzer (dChip) version 1.3 (Harvard) (25). Subsequently, pair-wise comparisons were performed by dChip applying the criteria: >2 fold change and $p<0.05$ by t-statistics. Functional cluster analysis was performed by dChip incorporating gene ontology (GO) terms for biological process, molecular function, cellular localization and protein domain (http://www.geneontology.org) as well as pathways collection from GenMAPP (http://www.genmapp.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.ad.jp/kegg). $P<0.001$ was considered statistically significant.

Real-time PCR

Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C, with data collection in the last 30 seconds. All PCR were performed with SYBR Green Master mix (Applied Biosystems), 10ng cDNA, and primer concentration of 300 nmol/L in a total volume

Table 1. Oligonucleotide primers for quantitatiave PCR analysis

cDNA	FORWARD PRIMER	REVERSE PRIMER
hGAPDH	ATC TTC TTT TGC GTC GCC AG	TTC CCC ATG GTG TCT GAG C
hAIF-1 total	GGA AAA AGC GAG AGA AAA GGA AA	AAT CAG GGC AAC TCA GAG ATA GCT
hAIF-1 isoform 1	CTC AGT CCC CTG CTG AAA ACC	GAG AGC AGA AGG GAT AAG CGC
hAIF-1 isoform 2	TGG TGT GCA GGC CTA AGA AGA	CTT CCG CAT CCC CAT TCT C
hAIF-1 isoform 3	GGA GAG CCT GCA GAC AGA GG	GGC TCA TAG CTC AGC AGA TGC

Software package Primer Express Version 2.0 (applied Biosystems) was used to select appropriate primer sets. All sequences are presented in the 5->3' direction

of 25 µl. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the gene of interest of each sample with the Ct values of the reference gene GAPDH (δCt). Relative expression is calculated as $2^{-\delta Ct}$x10,000. Primers sequences for gene expression analysis for hGAPDH total hAIF-1 and the AIF-1 isoforms are depicted in Table I.

Measurement of cytokines in culture supernatant

TNFα and IL-6 levels were measured in the supernatant of the cell cultures, using commercially available kits (Bio-Rad Laboratories, Hercules, USA) according to the manufacturer's instructions (26). Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad Laboratories). Data analysis was done with Bio-Plex Manager software (Bio-Rad Laboratories).

Statistical analysis

Statistical analysis for array-based gene expression was performed using dChip. The t-statistic is computed as (mean1 – mean2) / sqrt(SE(mean1)2 + SE(mean2) 2), and its p-value is computed based on the t-distribution, and the degree of freedom is set according to Welch modified two-sample t-test (25).
Differences in AIF-1 mRNA expression, determined by real-time PCR, and LPS-induced cytokine production, were calculated using Student's T-test or Mann-Whitney U test as appropriate. P values were two sided and the level of significance was set at P<0,05. Cor-

Table 2. Overview microarray analysis results (functional clusters)

FUNCTIONAL CLUSTERS	NUMBER IN LIST	NUMBER ON ARRAY	P VALUE
Gene Ontology			
Immune response	9	974	0.000031
Complement activation, classical pathway	3	58	0.000125
innate immune response	3	108	0.000782
Protein domain			
Complement C1q protein	3	33	0.000045
Pathway			
Complement Activation Classical	3	29	0.000034

The enrichmnts in GO/protein domain/pathway are assesed by using dCHIP. P-values were calculated base on the number of genes belonging to GO/protein domain/pathwas withing the selected group of genes (RA vs. healthy controls).

Table 3. Gene list belonging to the functional clusters

GENE NAME	FUNCTIONAL CLUSTERS (FOLD CHANGE)		
	IMMUNE RESPONSE	COMPLEMENT ACTIVATION	INNATE IMMUNE RESPONSE
Complement component 1, Q subcomponent, beta polypeptide	5	5	5
Myeloid cell nuclear differentiation antigen	3		
Immunoglobulin lambda joining 3	-4		
Membrane-spanning 4-domains, subfamily A, member 1	-4		
Secreted and transmembrane 1	5		
Immunoglobulin lambda locus	-4		
Immunoglobulin heavy locus	-4		
Complement component 1, Q subcomponent, alpha polypeptide	5	5	5
Complement component 1, Q subcomponent, gamma polypeptide	3	3	3

relation between the real-time PCR values and cytokine levels were evaluated by Pearson's linear regression model and $p<0.05$ was considered statistically significant.

Results

Microarray analysis of DCs derived from RA patients.

The RNA isolated from DCs derived from 3 RA patients (RA) and 2 healthy controls was used for the array-based gene expression study. All RNA samples were of sufficient quality and integrity as determined by RNA gel, and were subsequently analyzed by using human genome array U130 plus 2.0. (Affymetrix, Santa Clara) as described in the materials and methods section. After normalization, model-based expression fitting and filtering by using dChip, total filtered genes were further analyzed and clustered. We have identified 104 differentially expressed genes; 33 up regulated genes (subcluster 1) and 71 down regulated genes (subcluster 2) between DCs derived from RA and healthy controls by applying criteria of more than 2 fold change and statistical significance ($p<0.05$; Figure 1). Remarkably, in this group of genes statistically significant enrichment of genes involved in immune response, complement activation,

Table 4. RA genes with highest fold change difference compared to healthy controls

		FOLD CHANGE	ACCESSION	GENE SYMBOL
1.	C-type lectin domain family 12, member A	11.	NM_138337	CLEC12A
2.	Epithelial membrane protein 1	8.	NM_001423	EMP1
3.	Allograft inflammatory factor 1	8.	AF299327	AIF-1
4.	Purinergic receptor P2Y, G-protein coupled, 12	8.	AA810452	P2RY12
5.	C-type lectin superfamily 4, member G	8.	BC039679	CLEC4G
6.	Membrane-spanning 4-domains, subfamily A, member 6A	7.	AA045175	MS4A6A
7.	Secreted and transmembrane 1	5.	BF939675	SECTM1
8.	Complement component 1, Q subcomponent, alpha polypeptide	5.	NM_015991	C1QA
9.	cAMP responsive element binding protein 5	5.	AI819043	CREB5
10.	Complement component 1, Q subcomponent, beta polypeptide	5.	NM_000491	C1QB
11.	MOB1, Mps one binder kinase activator-like 2B	5.	AI692878	MOBKL2B
12.	Ras-induced senescence 1	4.	BF_062629	TMEM158
13.	Myeloid cell nuclear differentiation antigen	3.	NM_002432	MNDA
14.	Complement component 1, Q subcomponent, gamma polypeptide	3.	AI184968	C1QC
15.	Reticulon 1	3.	NM_021136.	RTN1.
16.	Chromosome 9 open reading frame 28	3.	BC000122	FAM125B
17.	EST	3.	AA047234	
18.	Carboxypeptidase, vitellogenic-like	3.	NM_031311	CPVL
19.	Potassium voltage-gated chanel, Isk-related family, member 3	3.	AI692703	KCNE3
20.	Calpain 2, (m/II) large subunit	3.	M23254	CAPN2

and innate immune response were found. In agreement with gene function, protein domain of complement c1q protein as well as pathway signature of classical complement activation were found. These results are summarized in Table 2. There was a large overlap among the genes belonging to these biological processes, and three subcomponents of complement component 1q (C1q) were the common element complying these gene categories (Table 3). These results demonstrate that DCs from RA patients have a gene expression signature very different from healthy controls (Figure 1 box), although they can mature and differentiate similar to DCs from healthy controls. The most pronounced signature is the activation of classical complement pathway.

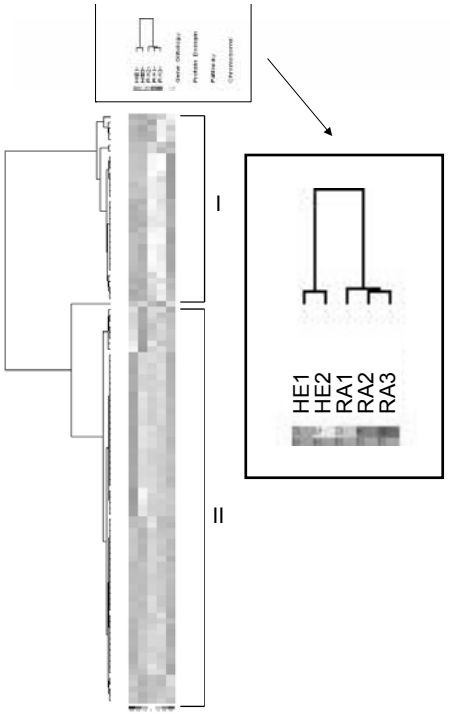

Figure 1. Hierarchical cluster analysis of differentially expressed genes. RNA were isolated from DCs derived from three RA patients (RA) and two healthy donors (HE). The individual samples were coded by donor origin and the number. The gene expression profiling was performed as described in Materials and Methods. Hierarchical cluster analysis was performed on genes (vertical dendrogram) and samples (horizontal dendrogram) according to the algorithm described in Materials and Method. The magnified view in the box shows sample information in color blocks (1st row :individual sample color code, 2nd row: blue for HE, green for RA). The colors represent relative expression level. Red: up regulation, Blue: down regulation, White: avarage.

We then analyzed the genes with highest fold change difference compared to healthy controls. Table 4 enlists the top 20 genes, in which the redundant genes are eliminated. The values derived from different probe sets against the same gene were very similar, demonstrating the accuracy of analysis. We identified allograft inflammatory factor 1 (AIF-1) as one of the genes with the highest magnitude of differential expression. Interestingly, AIF-1 is located at chromosomal position 6p21, a region that is associated with various autoimmune disorders, including RA (40).

Real-time PCR analysis of AIF-1 expression

At least three alternatively spliced variants of AIF-1 are described thus far, which might exert different functions. Since array-based microarray analysis did not discriminate between the separate isoforms, we determined the expression of the alternatively spliced AIF-1 variants using real-time PCR. As shown in Figure 2A, the expression of total AIF-1 was significantly enhanced in DCs from RA patients (relative expression healthy controls 2906 vs. RA 17900; p value = 0.04), which was a comparable to the results of the microarray analysis. The separate AIF-1 isoforms also showed clearly enhanced expression in DCs from RA patients (Figure 2B – 2D), however this difference was not statistically significant for all of the AIF-1

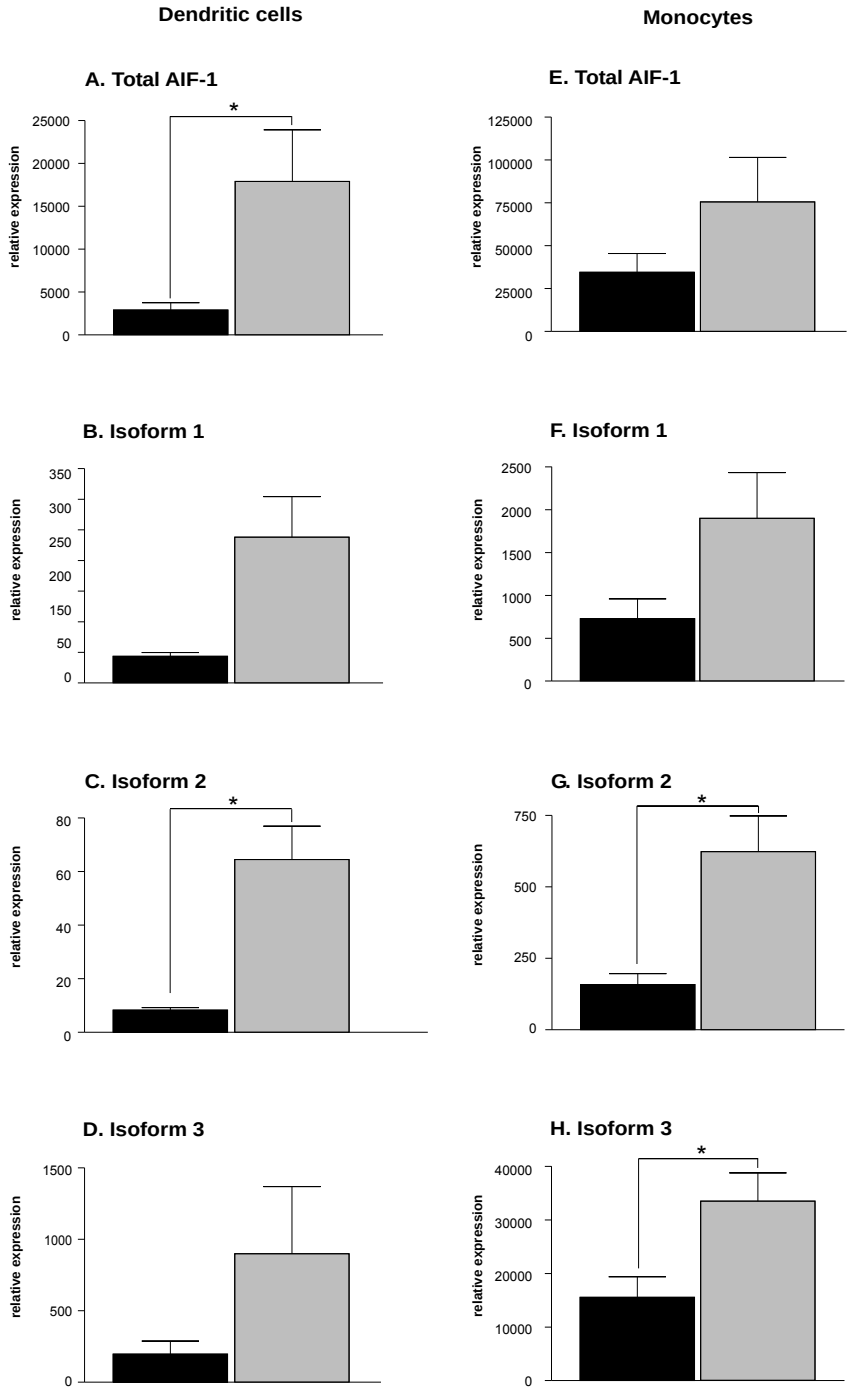

Figure 2. Relative mRNA expression ($2^{-\delta CT}$ x 10,000) of the total AIF-1 expression (A) and the separate isoforms, isoform 1 (B), isoform 2 (C) and isoform 3 (D) on dendritic cells and on monocytes (E-H) from healthy controls (N = 5) and RA patients (N = 5). Error bars represent SEM.

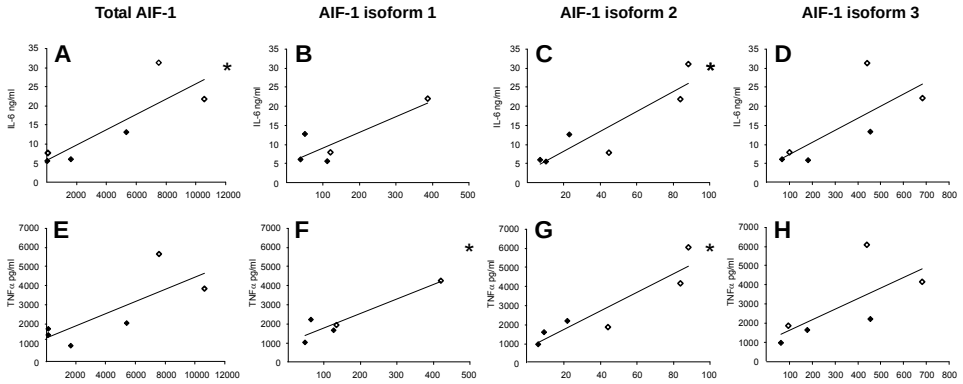

Figure 3. Correlation between relative mRNA expression of total AIF-1 and its separate isoforms and TLR-induced production of IL-6 and TNFα in dendritic cells. In this figure, values of healthy individuals (filled checks) as well as RA patients (open checks) are depicted. *P<0.05, statistically significant.

isoforms (relative expression isoform 1 healthy controls 44 vs. RA 238; NS, isoform 2 healthy controls 8 vs. RA 65; p value = 0.01, isoform 3 healthy controls 197 vs. RA 899; NS). In addition, we determined the AIF-1 expression on LPS-stimulated (mature) DC. Here we found that although the expression of AIF-1 on DCs was clearly down-regulated by LPS, expression on mature DCs from RA patients was still significantly enhanced compared to mature DCs from healthy individuals.

To examine whether the difference in AIF-1 expression between healthy controls and RA patients was specific for DCs or whether this was already present on monocytes, we determined the expression of total AIF-1 and the separate AIF-1 isoforms on monocytes also (Figure 2E– 2H). Although not statistically significant, just as in DCs, the expression of total AIF-1 was enhanced in monocytes from RA patients (relative expression healthy controls 34560 vs. RA 75580; NS). Furthermore, the expression of the AIF-1

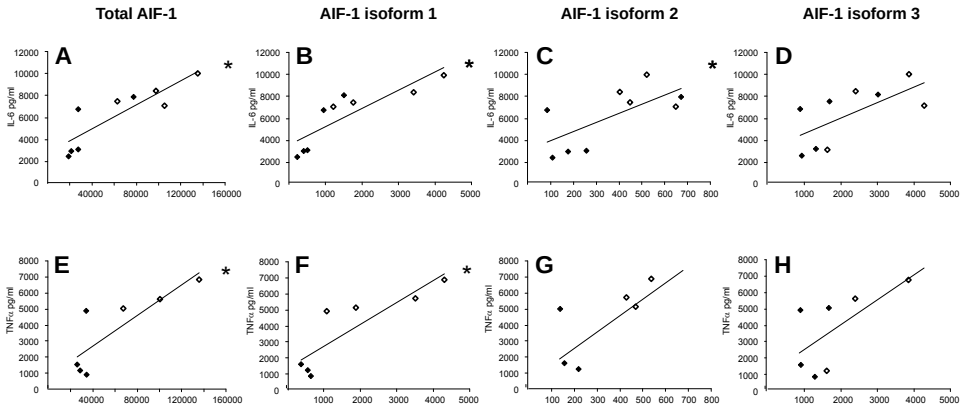

Figure 4. Correlation between relative mRNA expression of total AIF-1 and its separate isoforms and TLR-induced production of IL-6 and TNFα in monocytes. In this figure, values of healthy individuals (filled checks) as well as RA patients (open checks) are depicted. *P<0.05, statistically significant.

isoforms was enhanced in RA patients compared to healthy controls as well (relative expression isoform 1 healthy controls 729 vs. RA 1890; NS, isoform 2 healthy controls 158 vs. RA 623; p value = 0.02, isoform 3 healthy controls 15550 vs. RA 33450; p value = 0.03).

Correlation of AIF-1 expression and TLR4-induced cytokine production

Since DCs from RA patients express higher levels of AIF-1 mRNA and it is known that cells from RA patients produce higher levels of IL-6 and TNFα upon TLR4-mediated stimulation, we investigated whether the basal AIF-1 expression on immature DCs was correlated with the TLR4-induced cytokine production. As shown in Figure 3, the expression of total AIF-1 in DCs is strongly correlated with the TLR4-induced production of IL-6 (pearson r = 0.84, p value = 0.04) and to a lesser extent with TNFα (pearson r = 0.77, NS). Furthermore, the separate AIF-1 isoforms were also correlated with the production of IL-6 (isoform 1: pearson r = 0.85, NS; isoform 2: pearson r = 0.90, p value 0.04; isoform 3: pearson r = 0.74, NS) and TNFα (isoform 1: pearson r = 0.93, p value = 0.02; isoform 2: pearson r = 0.92, p value 0.01; isoform 3: pearson r = 0.70, NS).

To check whether the correlation between AIF-1 expression and TLR4-induced cytokine production was specific for DCs we determined the correlation between total AIF-1 and the separate AIF-1 isoforms TLR4-induced IL-6 and TNFα production on monocytes as well. As shown in Figure 4, the expression of total AIF-1 in DCs is strongly correlated with the TLR4-induced production of IL-6 (pearson r = 0.86, p value = 0.003) and TNFα (pearson r = 0.83, p value = 0.02). Furthermore, the separate AIF-1 isoforms were also correlated with the production of IL-6 (isoform 1: pearson r = 0.86, p value = 0.003; isoform 2: pearson r = 0.67, p value 0.04; isoform 3: pearson r = 0.66, NS) and TNFα (isoform 1: pearson r = 0.87, p value = 0.01; isoform 2: pearson r = 0.66, NS; isoform 3: pearson r = 0.66, NS).

Discussion

In the present study, using an array-based gene expression approach and real-time PCR, we tried to identify factors that were differentially expressed in cells from RA patients compared to healthy controls. Strikingly, among the 20 genes with the highest fold upregulation in RA patients we found 5 genes which all are involved in the innate immunity; two C-type lectins and three complement components. We also indentified the AIF-1 gene among the 20 genes with highest fold upregulation. Interestingly, AIF-1 is located at chromosomal position 6p21, a region that is associated with various autoimmune disorders, including RA (40).

In this study we demonstrated that AIF-1 expression in monocytes and DCs from RA patients was strongly enhanced compared to monocytes and DCs from healthy controls. Furthermore, the expression of total AIF-1 as well as its separate isoforms strongly correlated with the TLR4-induced cytokine production on DCs as well as on monocytes.

AIF-1 (allograft inflammatory factor 1) was first identified in rat cardiac allografts undergoing chronic rejection (27). The expression was confined to macrophages and neutrophils and was strongly regulated by the T-cell derived cytokine IFNγ. Ever since, AIF-1 expression was studied in several pathological conditions such as autoimmune encephalomyelitis, diabetes and cerebral infarction (28-30). Overexpression of AIF-1 augmen-

ted lipopolysaccharide-induced production of IL-6, IL-10 and IL-12 in a mouse macrophage cell line, suggesting a role for AIF-1 in inflammatory processes (31). However, the exact role of AIF-1 in inflammation and immune regulation is still not fully understood.

There are several alternatively spiced variants of AIF-1 and at least 3 isoforms encoded by different exons have been described (32). AIF-1 transcript 2 and 3 have a specific domain, whereas AIF-isoform 1 is just a common fragment of AIF-1 isoform 2 and isoform 3 (33).

Recently, it was demonstrated that AIF-1 protein was markedly increased in affected skin from patients with systemic sclerosis compared to normal skin and that the AIF-1 positive cells include T-cells, macrophages and endothelial cells (33). It is very interesting that we demonstrated here that the expression of all AIF-1 isoforms was increased in monocytes and DCs from RA patients, which is, like systemic sclerosis, classified as a rheumatic autoimmune disease as well.

The fact that the increased AIF-1 expression was not specific for DCs, but also seen in freshly isolated monocytes, suggests that its expression was under regulation of systemic inflammatory factors. For example, IL-1β and TNFα, which are increased in serum of RA patients and are considered as key cytokines in the pathogenesis of RA (34-36), are both known to promote the expression of AIF-1 in macrophage cell lines (37). Therefore it is quite conceivable that AIF-1 expression in RA patients is enhanced due to increased serum levels of IL-1β and TNFα. In this context, it would be of great interest to investigate whether anti-TNFα therapy, which is now successfully used in the clinic to treat RA, would decrease the expression of AIF-1 on peripheral blood monocytes in turn. However, no research has been conducted to investigate this subject thus far.

Previously, we described that DCs from RA patients produce much higher amounts of cytokines upon stimulation of TLR4 (16). In the present study we found that the expression of total AIF-1 as well as some of the separate isoforms was strongly correlated with the TLR4-induced production of IL-6 and TNFα. These findings suggest that AIF-1 is involved in the TLR4 hyperresponsiveness during rheumatoid arthritis. There are some indications that AIF-1 isoform 2 and isoform 3 could have opposite effects on cell activation as it was reported that overexpression of isoform 3 caused proliferation of vascular smooth muscle cells (VSMC), whereas overexpression of isoform 2 inhibited proliferation (38). However, our study does not indicate different functions of AIF-1 isoform 2 and isoform 3 thusfar. Further studies into the function of the AIF-1 isoforms are required to elucidate the exact role of the AIF-1 isoforms.

Very recently, an independent article that did not use an array-based gene expression approach, also suggested a role for AIF-1 in the pathogenesis of RA (39). Here, expression of AIF-1 was found to be higher in synovial tissue and synovial fluid from RA patients compared to patients with osteoarthritis (OA) and AIF-1 levels in synovial fluid correlated with the IL-6 concentration. These results seem to be in line with our findings, although this study did not address a role for the separate AIF-1 isoforms. These results further indicate the potential involvement of AIF-1 in the pathogenesis or chronicity of RA.

In conclusion, using an array-based gene expression analysis, we identified AIF-1 as a potential factor to be involved in the TLR4 hyperresponsiveness in RA. We showed en-

hanced expression of total AIF-1 and its separate isoforms in monocytes and DCs from RA patients. The strong correlation between AIF-1 and TLR4-mediated cytokine production, further substantiates a role for AIF-1 in this process. However, further investigation into the specific function of the separate AIF-1 isoforms might provide additional evidence with respect to the exact role of AIF-1 in the TLR4 hyperresponsiveness as well as the pathogenesis of RA.

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CHAPTER 6

Synovial inflammation during rheumatoid arthritis; a role for IFNα

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Objective. To investigate whether the synovial expression of TLR3 and TLR7 was associated with the expression of the pro-inflammatory cytokines IFNα, TNFα, IL-1β, IL-12, IL-17 and IL-18 and to study whether the expression of these receptors and cytokines were related to each other in vitro.
Methods. Using immunohistochemistry, TLR3, TLR7 IFNα, TNFα, IL-1β, IL-12, IL-17 and IL-18 expression was determined on synovial specimens from 34 RA patients with active disease. In vitro, monocytes, monocyte-derived dendritic cells and RA synovial fibroblasts were stimulated via TLR3 and TLR7 and IL-1β production was measured by real-time PCR and Luminex bead array technology. TLR3 and TLR7 mRNA expression upon IFNα, IL-1β and IL-18 stimulated cells was assessed using real-time PCR. Functional experiments were performed by IFNα pre-incubation and subsequent TLR3 and TLR7 stimulation.
Results. Synovial TLR3 and TLR7 expression was associated with the presence of IFNα, IL-1β and IL-18, but not with TNFα, IL-12 and IL-17. High amounts of TLR3 and TLR7-induced IL-1β mRNA were produced, however no biologically active IL-1β was released. Whereas both IL-1β and IL-18 were not able to regulate TLR expression, IFNα incubation led to significantly enhanced TLR3 and TLR7 mRNA expression levels, which was functional in terms of TNFα and IL-6 production.
Conclusion. In this study we showed that the expression of TLR3 and TLR7 was associated with IL-1β, IL-18 and IFNα in RA synovial tissue. Furthermore, we demonstrated a role for IFNα in the in vitro regulation of TLR3 and TLR7. These results suggest the involvement of anti-viral immune responses in RA and IFNα as a key player in chronic inflammation

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, which is characterized by chronic inflammation of the synovial joints. Although the cause of RA is still unknown, the role of viruses has been brought forward many times in the past as a factor implicated in the pathogenesis of RA. For example, cytomegalovirus (1), Epstein-Barr virus (2) and parvovirus B19 (3) are all frequently present in synovial tissue and synovial fluid from RA patients. Furthermore it was demonstrated that dsRNA, has clear arthritogenic properties (4), a finding that further substantiated the potential role of viruses in RA.

Although type I interferons (IFNα/IFNβ) are considered as products of the antiviral immune response, the precise pathways that underlie such a response is complex and not elucidated to a full extent. However, it is generally accepted that the first inflammatory response against virus infections is mediated by so-called pattern recognition receptors (PRR), from which the Toll-like receptor family (TLR) has been studied most intensively. While TLR2 and TLR4 were identified to recognize ligands from bacterial origin, TLR3, TLR7 and TLR9 serve as receptors for viral nucleic acids and therefore would have a key role in antiviral immunity (5-8) (9;10), by effective production of type I IFNs. Production of type I IFNs by TLR-mediated immune activation requires activation of IRF-3 and IRF-7 (11); TRIF appeared to be the key adaptor molecule that enables TLR3 to trigger production of type I IFNs, whereas TLR7 and TLR9 induce production of type I IFNs TRIF-independently, via MYD88 (12). In addition, to induce an appropriate antiviral response, production of type I IFNs was also found to be involved in the immune response against ligands from bacterial origin, as autocrine production of type I IFNs upon TLR4-mediated lipopolysaccharide (LPS) stimulation was

required for optimal secretion of IL-12 and TNFα by dendritic cells (DCs) (13). The role of type I IFNs in RA was recently elegantly shown by Pouw-Kraan and collegues, who demonstrated that RA patients had a significantly elevated expression of genes which were regulated by type I IFNs compared to healthy individuals (14).

Recently, it was demonstrated that TLR3 and TLR7 are abundantly expressed in synovial tissue from RA patients (15) and that RA synovial fibroblasts could be activated by endogenous RNA released from necrotic RA synovial fluid cells via TLR3 (16). TLR7, on the other hand, can be activated by highly conserved RNA sequences, which are for example present in sera from patients with systemic lupus erythematosus (17;18). Formerly, it has already been demonstrated that host-derived heat-shock proteins, fibronectin and hyaluronic acid, which are likely to be abundant in the RA synovial compartment, are able to induce an immune response via TLR4 (19-21). The finding that tissue-damage-induced release of endogenous factors can stimulate an immune response against self antigens, could lead to the development of autoimmune diseases, such as RA.

Besides type I IFNs, other cytokines have been studied for their role in the pathogenesis of RA. IL-1β, for example, was one of the first cytokines of interest in the field of RA, because of its biological role in joint destruction, shown in models of experimental arthritis, followed by TNFα, which acts strongly synergistic with IL-1β (22;23). In particular for TNFα, its role in RA has been further emphasized by the success of anti-TNFα therapy in RA patients. IL-12 and IL-18, known as Th1-directing cytokines, are alone or in combination strong inducers of IFNγ and have shown to be important in the development of arthritis in murine models (24;25). Furthermore, both cytokines are present in synovial tissue from RA patients and cause strong upregulation of TLR2 and TLR4, mainly driven by production of IFNγ (26). A more recent cytokine of interest in the area of RA is IL-17, a strong inducer of IL-1β and TNFα, which has shown to be involved in joint inflammation during experimental arthritis (27;28). In the upcoming years, phase III clinical trials will establish whether these cytokines will be useful as therapeutic targets in RA.

The complex mechanisms that underlie the pathogenesis of RA and the involvement of various cytokines in these processes raised the question if these cytokines played a role in the expression and regulation of specific TLRs during the disease. Therefore we investigated whether the synovial expression of TLR3 and TLR7 was associated with the degree of inflammation and the expression of the pro-inflammatory cytokines IFNα, TNFα, IL-1β, IL-12, IL-17 and IL-18. Furthermore we studied whether these cytokines were able to regulate TLR3 and TLR7 expression in cells which are present in RA synovial tissue.

Methods

Patients / study population

For immunohistochemistry, synovial biopsies from RA patients (n=34) were obtained, using small needle arthroscopy, from the medial and lateral suprapatellar pouch on each occasion. For in vitro experiments, heparinized venous blood was collected from 5 RA patients and 12 healthy volunteers. Patients were attending the department of Rheumatology of the Radboud University Nijmegen Medical Centre, fulfilled the American College of Rheumatology criteria for

RA (29) and they all gave their informed consent. Patients using high dose prednisolone (>10mg/day) or anti-cytokine therapies (anti-TNFα and/or IL-1Ra) were excluded from this study. The local Medical Ethics Committee approved the study protocol.

Immunohistochemical staining of synovial biopsies

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. Staining of IL-1β, IL-12, IL-17, IL-18 and TNFα was performed as described previously (30). For TLR3, TLR7 and IFNα staining, sections were incubated for 60 minutes with antibodies against human TLR3 (T-17), human TLR7 (V-20) or IFNα (FL-198), which were all obtained from Santa Cruz, California, USA. After this, endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 15 minutes and subsequently the appropriate biotinylated secondary antibody (mouse anti-goat: Jackson ImmunoResearch, West Grove, PA, USA / swine anti-rabbit: DakoCytomation, Glostrup, Denmark) was incubated for 30 minutes. For TLR3 and TLR7 staining, Vectastain® ABC (Vector Laboratories, Burlingame, CA, USA) reagent was incubated for 30 minutes, developed with DAB (Sigma, St. Louis, MO, USA), and counterstained with hematoxilin for 30 seconds. For IFNα staining, sections were incubated with streptavidin-PO (DakoCytomation, Glostrup, Denmark), developed with DAB (Sigma, St. Louis, MO, USA), and counterstained with hematoxilin for 30 seconds.

Staining was semi quantitatively scored on a 5-point scale (scores 0-4) at 200x magnification; a score of 0 represented no or minimal staining, score 1 stands for 10%-20% positive cells, 2 for 30%-40%, 3 for 50%-60%, and a score of 4 represented staining of a more that 60% of the cells.

Isolation and culturing of monocytes and monocyte-derived DCs

PBMCs were isolated from heparinized venous blood using density gradient centrifugation over Ficoll-Paque (Amersham Biosciences, Roosendaal, The Netherlands). Low-density cells were collected and washed with citrated PBS 5% FCS. For monocytes, $CD14^+$ cell fraction was isolated using MACS® cell separation, according to the manufacturer's instructions. Briefly, PBMCs were incubated with anti-human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 minutes, subsequently cells were washed and $CD14^+$ fraction was separated from the $CD14^-$ fraction over a MACS® MS separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$ cell fraction was eluted from the column, washed again and resuspended in a concentration of $0{,}5x10^6$ cells/ml in RPMI 1640 Dutch Modification (Invitrogen Corporation, Carlsbad, CA, USA), supplemented with 10% FCS, plated in 6-well plates and O/N cultured at 37°C and 5% CO_2.

For monocyte-derived dendritic cells, PBMCs were allowed to adhere for 1 hour at 37°C in RPMI-1640 Dutch Modification (Invitrogen Corporation, Carlsbad, CA, USA supplemented with 2% human serum (PAA Laboratories, Pasching, Austria) in $25cm^2$ cell culture flasks (Corning Incorporated, NY, USA). Adherent monocytes were cultured in RPMI-1640 Dutch modification supplemented with 10% fetal calf serum (FCS) and antibiotic-antimycotic (Life Technologies) in the presence of IL-4 (500 U/ml, R&D systems, Minneapolis, MN, USA) and GM-CSF (800 U/ml, R&D systems, Minneapolis, MN, USA) for 6 days. Fresh culture medium with the same supplements was added at day 3 where after immature DCs were harvested at day 6. Immature DCs were resuspended

Table 1. Oligonucleotide primers for quantitatiave PCR analysis

cDNA	FORWARD PRIMER	REVERSE PRIMER
hGAPDH	ATC TTC TTT TGC GTC GCC AG	TTC CCC ATG GTG TCT GAG C
hTLR3	AGA GTT GTC ATC GAA TCA AAT TAA AGA G	CAT TGT TCA GAA AGA GGC CAA AT
hTLR7	TGC CAT CAA GAA AGT TGA TGC T	GGA ATG TAG AGG TCT GGT TGA AGA G
hIL-1β	AAT CTG TAC CTG TCC TGC GTG TT	TGG GTA ATT TTT GGG ATC TAC ACT CT
hIFNβ	ATG ACC AAC AAG TGT CTC CTC C	GCT CAT GGA AAG AGC TGT AGT G

Software package Primer Express Version 2.0 (applied Biosystems) was used to identify appropriate primer sets. All sequences are presented in the 5->3' direction

in fresh cytokine-containing culture medium, transferred to 6-well culture plates (Corning Incorporated, NY, USA) in a concentration of $0,5´10^6$ cells/ml and incubated at 37°C and 5% CO_2 for 24 hours.

Isolation and culturing of RA synovial fibroblasts

Immediately after surgery, the synovial tissue was minced and digested with Dispase at 37°C for 60 minutes. After washing, the cells were grown in Dulbecco's minimum essential medium (Gibco Invitrogen, Basel, Switzerland) supplemented with 10% FCS, 50 IU/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 0.2% Fungizone (all from Gibco Invitrogen). Cell cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO_2. For the experiments, cultured synovial fibroblasts, between passages 4 and 8, were grown in 12-well culture plates (6×10^4 RA synovial fibroblasts/well) and O/N cultured at 37°C and 5% CO_2.

Stimulation of monocytes, monocyte-derived dendritic cells and RA synovial fibroblasts

To study TLR mRNA expression upon cytokine stimulation, monocytes (which express TLR7), monocyte-derived DCs and RA synovial fibroblasts (which both express TLR3) were, after a 24 hour resting period, stimulated for 8 hours with 20 ng/ml TNFα, IL-1β, IL-12, IL-17, IL-18 and 100 IU/ml IFNα (all R&D systems, Minneapolis, MN, USA). Subsequently, culture supernatants were removed and 1ml TRIzol reagent (Sigma St. Louis, MO, USA) was added to the cells and stored at –20°C until RNA isolation was performed.

To study functional upregulation of TLRs, monocytes, monocyte-derived DCs and RA synovial fibroblasts were, after an O/N resting period, stimulated IFNα with (R&D systems, Minneapolis, MN, USA) for 24 hours and subsequently stimulated with the TLR3 and TLR7 agonists poly(I:C) and loxoribin respectively (25mg/ml; both Invivogen, San Diego, USA) or medium. After another 24 hours, culture supernatants were collected and stored at -20°C until cytokine measurement was performed.

RNA isolation and real-time PCR

Total RNA was extracted in 1ml of TRIzol reagent (Sigma St. Louis, MO, USA), an improved single-step RNA isolation method based on the method described by Chomczynski et al (31). Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). PCR conditions were as followed: 2 min at 50°C and 10 min

at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, with data collection in the last 30 seconds. All PCRs were performed with SYBR Green Master mix (Applied Biosystems), 10 ng cDNA, and primer concentration of 300 nmol/L in a total volume of 25 µl. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the gene of interest of each sample with the Ct values of the reference gene GAPDH. Primers sequences for gene expression analysis for hGAPDH, hTLR3, hTLR7 and IL-1β are depicted in Table 1.

Measurement of cytokines in culture supernatants

TNFα, IL-1β and IL-6 levels were measured in the supernatants of the cell cultures, using commercially available kits (Bio-Rad Laboratories, Hercules, USA) according to the manufacturer's instructions (32). Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad Laboratories). Data analysis was done with Bio-Plex Manager software (Bio-Rad Laboratories). IFNα production was analyzed with murine monoclonal capture and HRP-conjugated anti-IFNα antibodies (Bender MedSystems, Vienna, Austria) using standard ELISA procedures.

Statistical analysis

Correlations of the expression of TLRs and cytokines in human synovial biopsies were calculated using the Pearson's linear regression model. Differences in mRNA expression and cytokine production, upon cell stimulation with cytokines and TLR agonists, were calculated using Mann-Whitney U test. P values were two sided and the level of significance was set at $P<0,05$.

Results

Synovial TLR3/7 expression and association with IL-1β and IL-18, and IFNα

Synovial biopsies from 24 active RA patients were used to detect TLR3, TLR7 and IFNα expression. Previously, these biopsies were also used to detect the presence of TNFα, IL-1β, IL-12, IL-17 and IL-18 (30). Since we are interested in the regulation of TLR3 and TLR7 in RA synovial tissue, we examined whether the expression of these TLRs was associated with the expression levels of

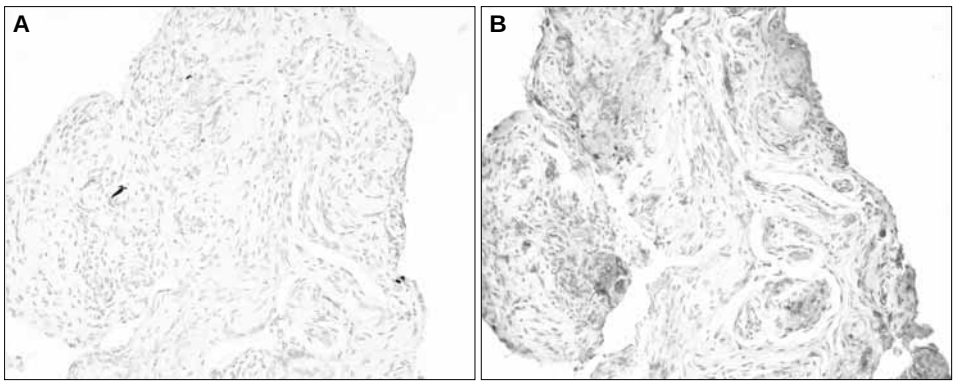

Figure 1. Staining of synovial tissue with an isotpe control antibody (A) or with a specific antibody against IFNα. Synovial tissue sections were counterstained with hematoxylin, magnification 200x.

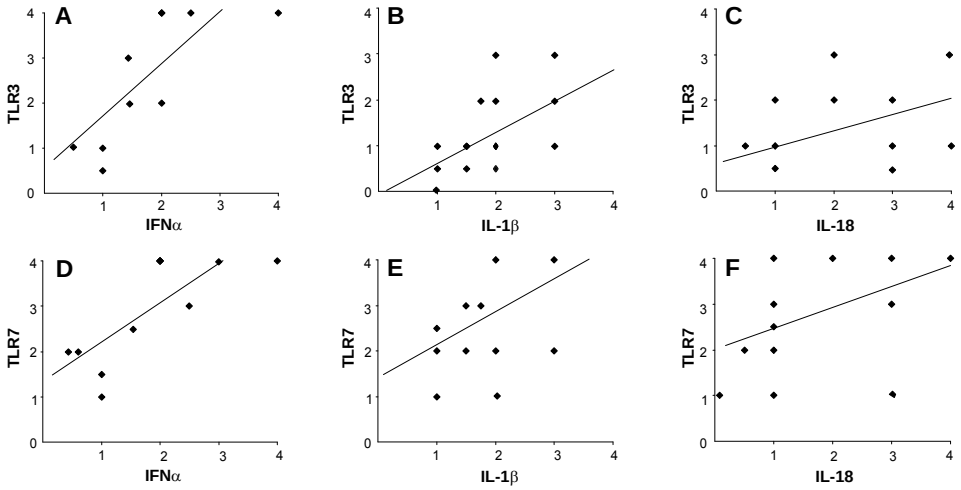

Figure 2. Correlation between TLR3/7 expression and IFNα, IL-1β, and IL-18 in synovial biopsies from RA patients. Expression of TLR and cytokines were semi quantitatively scored on a 5-point scale. Expression of TLR3 was associated with the expression of IFNα (A), IL-1β (B) and IL-18 (C). Expression of TLR7 was also associated with the expression of IFNα, (D) IL-1β (E) and IL-18 (F).

IFNα, TNFα, IL-1β, IL-12, IL-17 and IL-18.

IFNα, which is known as a mediator produced upon TLR3 and TLR7 stimulation, was highly expressed in the lining as well as the sublining from RA synovial biopsies (Figure 1). Its expression was strongly associated with the expression of TLR3 and TLR7 in the lining (Figure 2A and 2D) as well as the sublining (TLR3 lining Pearson r = 0.76, P=0.02 / TLR3 sublining Pearson r = 0.77, P=0.01 / TLR7 lining Pearson r = 0.70, P=0.04 / TLR7 sublining Pearson r = 0.84, P=0.01).

Moreover, both IL-1β and IL-18 were associated with the expression of TLR3 in the lining (Figure 2B and 2C) (IL-1 lining Pearson r = 0.54, P=0.02 / IL-18 lining Pearson r = 0.48, P=0.04), although the correlation was much weaker than the correlation between IFNα and TLR3. In addition, IL-18 expression was also associated with the expression of TLR7 in the lining (Pearson r = 0.48, P=0.04) and although not statistically significant, the same trend was observed for the level of IL-1β and the TLR7 expression in the lining (Figure 2E and 2F). In contrast, nor IL1β or IL-18 were associated with either TLR3 or TLR7 expression in the sublining. Neither TNFα, nor IL-12, nor IL-17 was correlated with the TLR expression levels.

Cytokine production by monocytes, monocyte-derived dendritic cells and synovial fibroblasts

As we demonstrated that the synovial expression of TLR3/7 was associated with the expression of IFNα, IL-1β and IL-18, we further investigated the relation between IL-1β and TLR3/TLR7 expression. It is generally accepted that stimulation of TLR3 or TLR7 leads to the production of type I IFNs. In line with this, in our experiments we found that TLR3 or TLR7 stimulation resulted in clearly enhanced mRNA expression levels of IFNβ, and low levels of IFNα protein could be detected in culture supernatants (data not shown). In contrast, much less is known about IL-1β production induced by TLR3 or TLR7 ac-

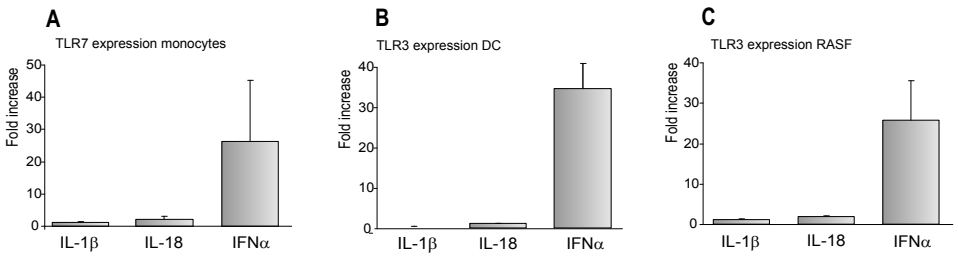

Figure 3. IL-1β, IL-18 and IFNα-induced TLR3/7 mRNA expression by monocytes (A), monocyte-derived DC (B) and RA synovial fibroblasts (RASF; C). Cells were incubated with 20ng/ml IL-1β or IL-18, or 100 IU/ml IFNα for 8 hours and subsequently TLR mRNA expression was determined by real-time PCR.

tivation. In order to investigate this potential relation between TLR3/TLR7 and IL-1β we stimulated TLR3-expressing monocyte-derived DCs and synovial fibroblasts with poly(IC) and TLR7-expressing monocytes with loxoribine. After 8 hours of stimulation we analyzed the IL-1β mRNA expression using real-time PCR. All cell types investigated showed markedly increased expression of IL-1β mRNA upon stimulation of either TLR3 or TLR7 (data not shown). Subsequently, we determined culture supernatant levels of IL-1β after 24 hours of TLR3 or TLR7 stimulation. In contrast with what is known about TLR4-mediated monocyte stimulation, neither TLR7-induced stimulation of monocytes nor TLR3-induced stimulation of monocyte-derived DCs or RA synovial fibroblasts resulted in secretion of IL-1β.

TLR regulation by cytokines

We demonstrated that TLR3/TLR7 stimulation could result in the production of IFNα, but not in the production of IL-1β, at least not on protein level. Therefore, we investigated whether the synovial association between TLR3/TLR7 and IFNα, IL-1β and IL-18 could be caused as IFNα, IL-1β and IL-18 regulate the expression of TLR3/TLR7. For this, we cultured monocytes (TLR7-expressing cells), monocyte-derived dendritic cells (TLR3-expressing cells) and RA synovial fibroblasts (TLR3 expressing cells) in the presence of IFNα, IL-1β and IL-18. After 8 hours of incubation, TLR3 and TLR7 mRNA expression was determined by real-time PCR. Interestingly, IFNα did significantly enhance the TLR3 mRNA expression on RA synovial fibroblasts and monocyte-derived DCs. In addition TLR7 mRNA expression on mono-

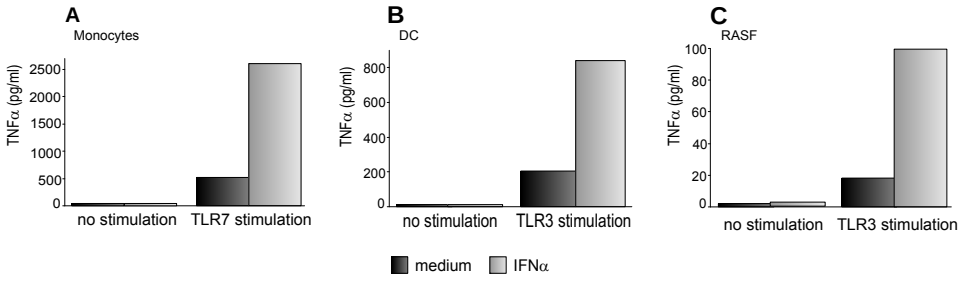

Figure 4. TLR3/TLR7-induced TNFα production by monocytes (A), monocyte-derived DC (B) and RA synovial fibroblasts (C) in the presence (gray bars) or absence (black bars) of IFNα. Graphs represent TNFα levels of one representative individual.

cytes, was also strongly increased upon incubation with IFNα (Figure 3). The increased IFNα-induced TLR expression was equal between RA patients and healthy volunteers and was specific for TLR3 and TLR7, since the expression of TLR2 as well as TLR4 was not regulated upon IFNα incubation (data not shown). In contrast to IFNα, IL-1β and IL-18 did not lead to up regulation of TLR3 or TLR7 mRNA. In addition we tested whether other RA-associated cytokines, which were not associated with TLR3/TLR7 in RA synovial tissue, were able to regulate the expression of TLR3 and TLR7. However, none of the cytokines investigated, namely IL-12, IL-17 and TNFα, did lead to up regulation of TLR3 or TLR7 mRNA.

Functional effects of IFNα on TLR-mediated cytokine production

As we demonstrated that expression of TLR3 and TLR7 was strongly up regulated by IFNα, we also wanted to know whether this enhanced TLR expression was functional, in terms of increased TLR-mediated cytokine production. For this we incubated TLR7-expressing monocytes and TLR3-expressing monocyte-derived DCs and RA synovial fibroblasts with IFNα or medium for 16 hours and subsequently stimulated TLR3 or TLR7 with poly(IC) and loxoribine as appropriate. As expected TLR3 stimulation on monocytes, as well as TLR7 stimulation on monocyte-derived DCs did not lead to any cytokine production, because these receptors were not expressed on protein level by these cells, even after incubation of IFNα. In contrast, TLR7 stimulation on monocytes and TLR3 stimulation on monocyte-derived DCs and RA synovial fibroblasts led to production of IL-6 (data not shown) and TNFα, which was significantly enhanced when the cells were preincubated with IFNα, compared to cells that were stimulated with TLR3/7 ligands in the absence of IFNα preincubation (Figure 4).

Discussion

In this study we demonstrated that TLR3 and TLR7 in synovial tissue from RA patients were associated with the presence of IFNα, IL-1β and IL-18. IFNα in turn, was able to induce upregulation of TLR3 and TLR7, whereas IL-1β and IL-18 were not. Pre-incubation of monocytes, monocyte-derived DCs and RA synovial fibroblasts with IFNα, resulted in enhanced cell activation in terms of cytokine production upon TLR3 and TLR7 stimulation.

Type I IFNs are the key cytokines that regulates the innate immune response against viruses (33). Type I IFNs are released upon transcription of IRF-3 or IRF-7 which can be induced following TLR3- or TLR7-mediated cell activation. TLR3 and TLR7 are highly expressed in synovial tissue from RA patients (15) and TLR3 and TLR7 ligands, such as cytomegalovirus, Epstein-Barr virus and parvovirus B19 have been demonstrated in the synovial joints from RA patients also (1-3).

Besides being produced after TLR3 and TLR7 activation, type I IFNs are able to increase the cellular expression of TLR3 and TLR7 in turn. Therefore type I IFNs might act as key regulators in maintaining the inflammation processes during RA. Very recently, it was demonstrated that a type I IFN signature was present in RA patients, as large-scale expression profiling by microarray analysis showed markedly elevated expression of IFN type I regulated genes in pheripheral blood cells of RA patients (14). Type I IFNs are known to inhibit TNFα and IL-1β production, whereas it enhances

the production of IL-10 and IL-1Ra (34) and therefore quite promising in suppressing arthritis activity. Although IFNβ therapy was shown to be effective in animal models of collagen-induced arthritis (CIA), several clinical trials of IFNβ in patients with RA have not shown to be effective so far (35-37). The fact that type I IFNs type I strongly up regulate TLR3 and TLR7, which in turn are continuously stimulated by ligands present in the synovial joints, might in fact maintain the inflammatory processes and therefore lead to IFNβ therapy failure. However, the actual role for TLRs in the failure of IFNβ therapy in RA should be subject of further studies in the future.

Identification of endogenous TLR agonists is of great interest in terms of autoimmune disorders. In particular for TLR4 several endogenous agonist have been described thus far. For example hyaluronan fragments, heparan sulfate, fibronectin and (small) heat-shock proteins (19-21;38) can all be released in RA joints as a result of inflammation-induced tissue injury and cell stress. In addition, Brentano et al described an endogenous ligand for TLR3, as endogenous RNA, released from necrotic synovial fluid cells, was able to TLR3-mediated activation of RA synovial fibroblasts (16). Highly conserved RNA sequences within small nuclear ribonucleoprotein particles are able to activate immune cells via TLR7 and could act as an endogenous auto antigen in systemic lupus erythematosus (17;18). TLR-mediated immune responses in the synovial joints might result in the release of endogenous TLR ligands, originating from cells under stress or tissue damage. Therefore is not unlikely that a self sustaining loop of TLR-activation and generation of new endogenous TLR ligands might lead to a chronic inflammatory process as occurs during RA.

Expression of TLR3 and TLR7 in RA synovial tissue was, besides the association with IFNα, also associated with the expression of IL-1β and IL-18. Furthermore, stimulation of TLR3 and TLR7 resulted in markedly enhanced expression of IL-1β and IL-18 mRNA, although no active IL-1β protein could be detected in the culture supernatants. It is known that production of the active IL-1β and IL-18 proteins requires cleavage of the proIL-1β and proIL-18 molecules by activation of caspase-1. A lack of caspase-1 activating capacity of TLR3 and TLR7 might explain why those TLRs do not directly induce active IL-1β and IL-18, even though high levels of IL-1β and IL-18 mRNA were present.

It is generally believed that bacterial lipopolysaccharides are activators of caspase-1 through stimulation of TLR4 (39). Recently it was described that cells from RA patients showed enhanced activity, in terms of cytokine production, upon TLR4-mediated stimulation compared to cells from healthy controls (15). Since TLR4 stimulation could induce the production of type I IFNs by the TRIF-IRF3-mediated pathway (40), we speculate that the enhanced TLR4-mediated cell activation in RA patients, leads to enhanced levels of type I FNs, which in turn results in up regulation of TLR3 and TLR7. So TLR4 seems to be able to induce IFNα-mediated TLR3 and TLR7 upregulation as well as production of active IL-1β and IL-18. In this light, it is conceivable that TLR3 and TLR7 do not directly relate to IL-1β and IL-18, but that correlation of these TLRs and IL-1β/IL-18 in RA synovial tissue results from the fact that they are both regulated after TLR4 activation.

Lately, it has been demonstrated that stimulation of several different TLRs at the same

time, leads to synergistically induced levels of pro-inflammatory mediators (15;41). Preliminary experiments of our group showed that synergistically induced cytokine levels, produced upon co-stimulation of TLR3 and TLR4, were even more enhanced when cells were preincubated with IFNα (unpublished results). In this light, it is tempting to speculate that the presence of type I IFNs in the synovial joint of RA patients, at least partly, contributed to the breakthrough of tolerance and the development of autoimmunity.

In contrast to our findings, Severa and colleagues recently demonstrated that priming of immature DCs, that usually do not express TLR7, with exogenous IFNβ induced a functionally active TLR7. These differences in experimental outcome might be explained by dissimilarities in the experimental setup. For example, Severa et al., cultured monocyte-dendritic cells in the absence of IL-4 and GM-CSF for 20 hours before incubation with IFNβ. In the past, we have seen that monocyte derived dendritic cells, which were not fully matured, show some sort of re-differentiation, resulting in monocyte-like cells (unpublished results). Since monocytes, in contrast to monocyte-derived dendritic cells, do express TLR7, this might explain why Severa and colleagues demonstrate IFN-induced TLR7 regulation.

In conclusion, in this study we showed that the expression of TLR3 and TLR7 was associated with IFNα, IL-1β and IL-18 in RA synovial tissue. Furthermore, we demonstrated the involvement of IFNα in the regulation of TLR3/TLR7 in vitro, a mechanism that might underlie the associated synovial expression. These results suggest the involvement of anti-viral immuneresponses in RA and type I interferons as a key player in chronic inflammation.

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CHAPTER 7

Consequences of the Asp299Gly Toll-like receptor 4 polymorphism in rheumatoid arthritis

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Objective. To investigate whether the genetic TLR4 variant, Asp299Gly, has consequences with respect to TLR4 mRNA and protein expression. In addition, cytokine production upon stimulation of RA mononuclear cells with exogenous and endogenous TLR4 ligands was studied.
Methods. PBMCs from 28 RA patients carrying (AG; N=14) or not carrying (AA; N=14) the genetic TLR4 variant, Asp299Gly, were incubated with LPS (exogenous TLR4 ligand), HSPB8 (endogenous TLR4 ligand) or medium. After 24 hours of incubation, levels of IL-6, TNFα and IL-10 were determined in the culture supernatants. Baseline TLR4 expression was determined by real-time PCR and flowcytometry.
Results. Baseline TLR4 mRNA and protein expression were not significantly different in cells from RA patients carrying the genetic AG TLR4 variant, compared to the patients who were homozygous for the wild type allele. In contrast, the relative expression of TLR4-bearing $CD14^+$ cells in PBMCs from patients with the Asp299Gly TLR4 variant was significantly higher than in PBMCs from patients without the polymorphism. Upon exogenous TLR4 stimulation production of IL-6 and TNFα and IL-10 were clearly decreased (61%, 66% and 56% decrease respectively) by PBMCs from patients carrying the TLR4 variant, just as the production of IL-6, TNFα and IL-10 (54%, 69% and 41% decrease respectively) upon endogenous TLR4 stimulation.
Conclusion. These experiments showed that, while TLR4 expression was not reduced in PBMCs from RA patients carrying the TLR4 variant, exogenous as well as endogenous TLR4 stimulation did lead to impaired pro- and anti-inflammatory cytokine production by these cells.

Introduction

The innate immune system provides an essential defense mechanism against microbial pathogens. Microbial components, known as pathogen-associated molecular patterns, can be recognized by Toll-like receptors (TLRs), which belong to the family of pattern-recognition receptors. Nowadays, 11 TLRs have been identified in humans; they are expressed by numerous cell types and tissues, where they detect pathogens leading to the induction of an immune response. Besides being involved in the recognition of microbial pathogens, recent research shows that TLRs are also implicated in the recognition of "self" molecules such as fibronectin, heat-shock proteins, hyaluronic acid and host derived RNA [1-4]. These so called endogenous ligands are released upon tissue damage and cell stress, events that are likely to occur during inflammatory conditions, including rheumatoid arthritis (RA).

RA is an inflammatory autoimmune disease, which is characterized by a massive infiltration of inflammatory cells into the joint resulting in chronic inflammation, eventually leading to destruction of the synovial joints. Although the exact mechanisms that are responsible for the initiation and perpetuation of the disease remain obscure, recent evidence addressed a role for TLRs. TLRs are significantly higher expressed in synovial tissue from RA patients compared to patients with osteoarthritis (OA) and healthy controls [5-7]. Furthermore, monocyte-derived dendritic cells from RA patients produce much higher amounts of the pro-inflammatory cytokines IL-6 and TNFα upon TLR2- and TLR4-mediated stimulation than cells from healthy controls, whereas cytokine production upon TLR3 and TLR7 stimulation is not enhanced [6]. The role of TLRs in RA has recently been underscored by the finding that HSPB8, identified as an endogenous ligand for TLR4, is abundantly expressed in

RA synovial tissue [8]. Finally, the involvement of TLRs in arthritis has been demonstrated in several animal models of experimental arthritis [9, 10] and very recently in a double-blind ramdomised trial [11].

Several years ago, Arbour et al described two common co-segregating missense mutations (Asp299Gly and Thr399Ile) affecting the extracellular domain of TLR4, thatwere associated with hyporesponsiveness to bacterial lipopolysaccharide (LPS). In addition, transfection of THP-1 cells with either wild-type or mutant alleles of TLR4 demonstrated that cells transfected with the Asp299Gly allele showed a decreased response to LPS, whereas cells transfected with the Thr399Ille allele did not [12]. Ever since, many studies were performed that investigated the potential association of the TLR4 polymorphisms with the susceptibility to infectious diseases, such as respiratory virus infections and septic shock [13, 14], and non-infectious disorders, such as Crohn's disease, acute allograft rejection and asthma [15-17]. Moreover, research was performed to investigate the potential association between the TLR4 polymorphisms and the susceptibility to and severity of RA. Whereas two studies described that the Asp299Gly variant in the TLR4 gene was not associated with susceptibility to RA, our group found that this variant did associate with decreased susceptibility, but not with disease severity [18-20]. The diverse results of these studies might result from the limited size and clinical characteristics of the study population, which makes it hard to draw definite conclusions.

Following the results described by Arbour et al, several groups conducted research to investigate the functional consequences of the Asp299Gly variant in individuals during health and disease, with respect to cytokine production upon exogenous TLR stimulation [21-25]. However, due to conflicting results these studies had led to controversy regarding this issue. Since TLR4 seems to be involved in the pathogenesis of RA, and might be a promising therapeutic target for the future, further research into the role of TLR4 in cell activation seems warranted. Here we investigated the potential differences between mononuclear cells (PBMCs) from RA patients carrying and not carrying the genetic Asp299Gly TLR4 variant with respect to TLR4 mRNA expression, TLR4 protein expression and cytokine production upon TLR stimulation with exogenous as well as endogenous TLR4 agonists. We demonstrate that, cytokine production by cells from patients with the genetic TLR4 variant was significantly diminished upon exogenous as well as endogenous TLR4 stimulation, despite the fact that the TLR4 expression was unaffected.

Materials & Methods

Patients / study population

A total of 14 RA patients heterozygous for the Asp299Gly TLR4 variant (AG) and 14 RA patients without this variant (AA), attending the Radboud University Nijmegen Medical Centre were included in this study. Patients fulfilled the American College of Rheumatology criteria for RA [26] and gave informed consent. Patients using high dose prednisolone (>10mg/day) or anti-cytokine therapies (anti-TNFα and/or IL-1Ra) were excluded from this study. Demographic and clinical data, such as age, gender, mean DAS28 from the last 6 months of disease follow up, rheumatoid factor, age at the onset of the disease, duration of the disease and

Table 1: Oligonucleotide primers for real-time PCR analysis.

cDNA	FORWARD PRIMER	REVERSE PRIMER
hGAPDH	ATC TTC TTT TGC GTCGCC AG	TTC CCC ATG GTG TCT GAG C
hTLR4	GGC ATG CCT GTG CTG AGT T	CTG CTA CAA CAG ATA CTA CAA GCA CAC T

Software package Primer Express Version 2.0 (Applied Biosystems) was used to identify appropriate primer sets. All sequences are presented in the 5->3' direction.

DMARD use, were determined to characterize the groups of patients with and without the variant. The local Medical Ethics Committee approved the study protocol. Since only 0.1% of the RA patients is homozygous for the Asp299Gly TLR4 variant, no patients with the GG phenotype were included in this study.

Genotyping of the TLR4 Asp299Gly variant

Genomic DNA was isolated from whole blood using salt extraction [27]. Genotyping of the TLR4 Asp299Gly polymorphism (869A>G; rs4986790) was based on restriction fragment length polymorphism (RFLP) analysis. PCR amplification of TLR4 gene was performed in 50µl reaction volume containing 50ng genomic DNA, 0.4 pmol of each primer (forward 5'-ATACTTAGACTACTACCTCCATG-3' reverse 5'-AGCCTTTTGAGAGATTTGAGT-3'), 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 0.01% (w/v) gelatin, 0.001% (v/v) Triton X-100, 0.35 mM dNTPs and 2 U Taq DNA polymerase (Invitrogen, Breda, The Netherlands). PCR conditions were 5 min denaturation at 94°C, followed by 37 cycles (94 C for 1 min, 48 C for 1 min, and 72 C for 1 min) and a final extension at 72°C for 10 min. The product was purified on Multiscreen™ PCR plates (Millipore, Billerica, MA, USA) and genotyped by RFLP analysis using NcoI (Invitrogen, Breda, The Netherlands). RFLP analysis was validated by using several sequenced control samples of each genotype (AA, AG and GG). Digested products were separated on a 4% agarose gel resulting in the following fragments: homozygotes common variant (AA; 195 bp), heterozygote (AG; 195 + 172 + 23 bp). Since the Asp299Gly TLR4 variant shows strong linkage disequilibrium with the Thr399Ile polymorphism, all patients with the genetic variant selected for this study carry both TLR4 polymorphisms.

Generation and culture of PBMCs

PBMCs were isolated from heparinized venous blood using density gradient centrifugation over Ficoll-Paque (Amersham Biosciences, Roosendaal, The Netherlands). Low-density cells were collected and washed with citrated PBS 5% FCS, where after the cells were resuspended in a concentration of $0,5x10^6$ cells/ml in RPMI 1640 Dutch Modification (Invitrogen Corporation, Carlsbad, CA, USA), supplemented with 10% FCS, plated in 6-well plates and cultured by 37°C and 5% CO_2 for 16 hours. Subsequently, 200ng/ml LPS (exogenous TLR4 agonist; Sigma, St. Louis, MO, USA) or 10mg/ml HSPB8 (endogenous TLR4 agonist; kindly provided by W. Boelens, Dept. of Biochemistry, Nijmegen Center for Molecular Life Sciences, the Netherlands) was added to the culture. After 24 hours of stimulation culture supernatants were collected and stored at -20°C until cytokine measurement was performed.

RNA isolation and real-time PCR

Total RNA was extracted in 1 ml of TRIzol reagent, an improved single-step RNA isolation method based on the method described by Chomczynski et al [28]. Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with data collection in the last 30 seconds. All PCRs were performed with SYBR Green Master mix (Applied Biosystems), 10ng cDNA, and primer concentration of 300nmol/L in a total volume of 25µl. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the gene of interest of each sample with the Ct values of the reference gene GAPDH (ΔCt). Primer sequences for gene expression analysis for hGAPDH and hTLR4 are depicted in Table 1.

Flowcytometry

Flowcytometry was performed to determine the cell surface expression of TLR4 on PBMCs. First, $1x10^5$ PBMCs were incubated with antibodies against human CD14 (DakoCytomation, Glostrup, Denmark), CD3 (Becton-Dickinson, San Jose, USA), TLR4 (HTA125; Santa Cruz, California, USA) and the appropriate (isotype) control antibodies for 30 minutes at 4°C. Cells were then washed and subsequently incubated with the appropriate goat anti-mouse fluorescein isothiocyanate-conjugated IgG (both from Zymed Laboratories, South San Francisco, USA) in complete darkness. Cells were then washed again, fixed in PBS 1% paraformaldehyde (Sigma, St. Louis, MO, USA) and analyzed with a fluorescence activated cell sorter (FACSCalibur, Becton-Dickinson, San Jose, USA) for the mean fluorescence intensity (MFI) relative to cells stained with relevant IgG isotypes.

Measurement of cytokines in culture supernatants

TNFα, IL-6 and IL-10 levels were measured in the supernatants of the PBMC cultures, using commercially available kits (Bio-Rad Laboratories, Hercules, USA) according to the manufacturer's instructions [29]. Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad Laboratories). Data analysis was done with Bio-Plex Manager software (Bio-Rad Laboratories).

Statistical analysis

Differences in TLR expression (mRNA and protein) and cytokine production were analyzed using the Mann-Whitney U test. Differences in demographic and clinical characteristics were calculated using Student t-test, Mann-Whitney U test or Chi-square test as appropriate. P values were two sided and the level of significance was set at $P<0.05$.

Results

Patient characterization

A total of 14 RA patients with the Asp299Gly TLR4 variant (AG) and 14 RA patients without this variant (AA), were included in this study. No significant differences in demographic and clinical disease characteristics between patients with different genotypes were observed (Table 2).

TLR4 expression in cells from RA patients with the Asp299Gly TLR4 variant was not affected

To investigate whether the Asp299Gly TLR4

Table 2: Demographic and clinical characteristics of RA patients with (AG) and without (AA) genetic TLR4 variant.

CHARACTERISTIC	A/G (N=14)	A/A (N=14)	*P*
Age (years)	66 (SD = 10)	63 (SD = 11)	NS
% women	55%	62%	NS
Mean DAS28	3.3 (SD = 1.3)	3.2 (SD = 1.3)	NS
% RF positive	78%	87%	NS
Age at onset (years)	54.3 (SD = 8.7)	55.7 (SD = 12.3)	NS
Disease duration (years)	11.8 (SD = 6.3)	10.0 (SD = 5.6)	NS
% patients using DMARD*	89%	78%	NS

* None of the RA patients was using more than 1 DMARD at the same time. NS: not significant.

variant has an effect on the expression of this receptor on PBMCs from RA patients, we determined TLR4 mRNA expression levels in freshly isolated PBMCs. As expected, TLR4 mRNA was present in PBMCs from RA patients. Real-time PCR analysis showed that TLR4 mRNA expression levels were not statistically different between cells from RA patients with the Asp299Gly variant (ΔCt ± SEM: TLR4 = 4.2 ± 0.3) compared to patients without the variant (TLR4 = 4.5 ± 0.3) (Figure 1). Even 24hr after TLR stimulation, the expression of TLR4 was not different between the two groups of RA patients (data not shown), excluding a secondary effect on TLR expression of the stimuli used.

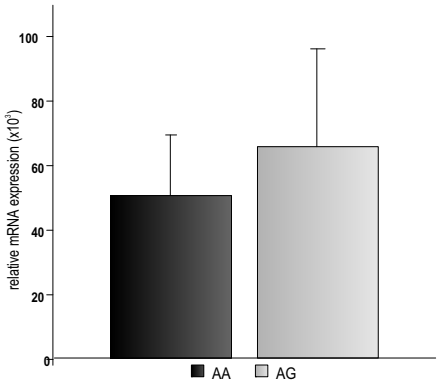

Figure 1. Relative mRNA expression ($2^{-\delta Ct}$) of human TLR4 on freshly isolated PBMCs from RA patients (N=9) with the Asp299Gly TLR4 variant (gray bars) and patients (N=9) without the polymorphism (black bars). Error bars represent the SEM.

To confirm our real-time PCR results of TLR4 expression, we performed FACS analysis to study the cell surface expression of TLR4 on the $CD14^+$ and $CD3^+$ cell fraction of PBMCs. First, TLR4 was present on the $CD14^+$ cell fraction. In line with the real-time PCR results, there was no statistically significant difference in protein expression levels between cells from RA patients with the Asp299Gly variant (MFI ± SEM: TLR4 = 16.7 ± 3.4) compared to patients without the variant (TLR4 = 13.9 ± 3.5) (Figure 2). TLR4 expression on the $CD3^+$ fraction of PBMCs was not detectable using FACS analysis.

Since TLR4 expression was only detectable on the $CD14^+$ fraction of PBMCs, we also investigated whether the Asp299Gly TLR4 variant has an effect on the amount of $CD14^+$ cells in PBMCs from RA patients.

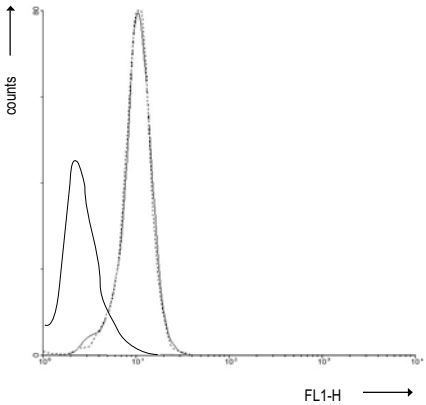

Figure 2. FACS analysis for human TLR4. CD14 expressing cells, within life gate, from one representative RA patient with the Asp299Gly TLR4 variant (bold line) and one representative patient without the polymorphism (dotted line) are shown. The thin grey peak represents the appropriate isotype control.

FACS analysis showed that the percentage of $CD14^+$ cells in PBMCs from patients with the Asp299Gly variant was significantly higher than in PBMCs from patients without the variant (mean ± SEM: 7.5% ± 1.0 vs. 14.4% ± 2.8) (Figure 3).

Decreased cytokine production upon TLR4 stimulation by PBMCs from patients with the TLR4 variant

To investigate the functional consequences of the Asp299Gly variant in the TLR4 gene, we stimulated PBMCs from RA patients with and without the variant with an exogenous (LPS) as well as endogenous (HSPB8) TLR4 agonist for 24 hours and subsequently analyzed the presence of IL-6, TNFα and IL-10 in the culture supernatants. As we demonstrated that the amount of TLR4-bearing $CD14^+$ cells in PBMCs from the RA patients was different between patients with and without the Asp299Gly TLR4 variant, we corrected the levels of IL-6, TNFα and IL-10 for the amount of $CD14^+$ cells in the wells. We found that cytokine production upon TLR4 mediated stimulation of PBMCs from RA patients with the TLR4 variant was significantly decreased compared to RA patients without the variant. As depicted in Figure 4, levels of the pro-inflammatory cytokine IL-6 were 61% decreased upon stimulation with the exogenous TLR4 agonist LPS (mean ± SEM AA: 7684pg/ml ± 1324pg/ml; AG: 3012pg/ml ± 454pg/ml), whereas stimulation with the endogenous TLR4 agonist HSPB8 resulted in a 54% decrease in cytokine production by cells from patients with the TLR4 variant (AA: 4376pg/ml ± 916pg/ml; AG: 2022pg/ml ± 512pg/ml). Production of the pro-inflammatory cytokine TNFα by cells from patients with the TLR4 variant was also significantly decreased; 66% decrease upon LPS stimulation (AA: 5790pg/ml ± 1592pg/ml; AG: 1987pg/ml ± 330pg/ml) and 69% decrease upon HSPB8 stimulation (AA: 4268pg/ml ± 1191pg/ml; AG: 1338pg/ml ± 386pg/ml). Also, levels of the Th2 directing cytokine IL-10 were significantly decreased upon TLR4 stimulation of PBMCs from RA patients with

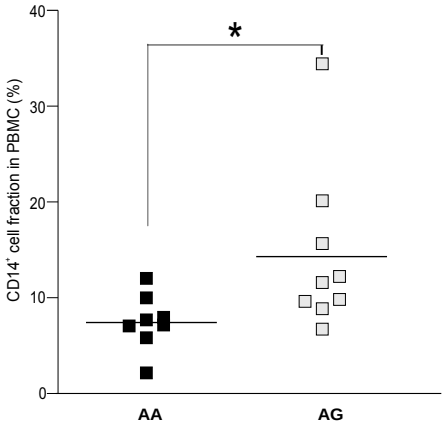

Figure 3. Percentage of $CD14^+$ cells in PBMCs from RA patients with the Asp299Gly TLR4 variant (gray squares) and RA patients without the polymorphism (black squares). The horizontal line represents the median; * $P < 0.05$.

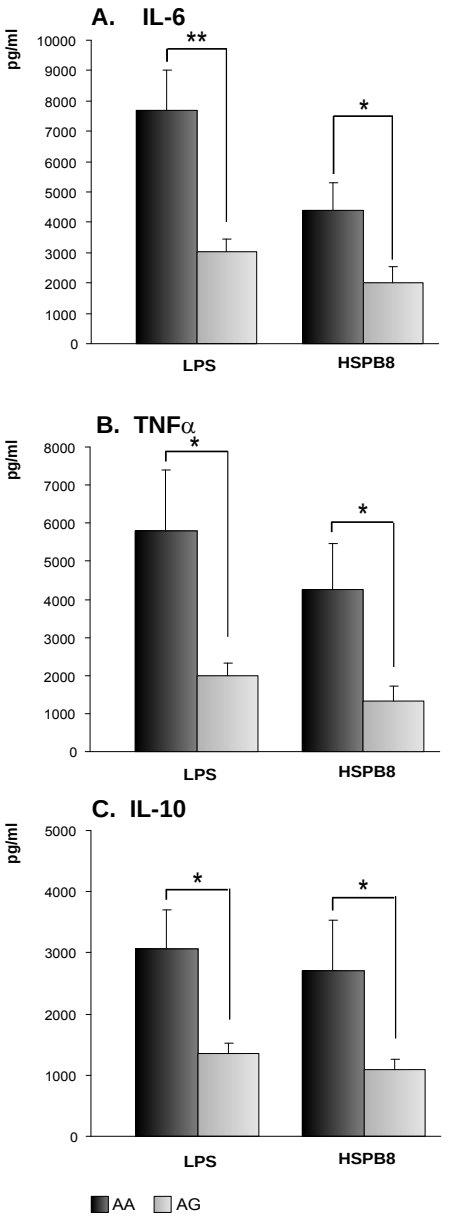

Figure 4. Mean cytokine production by PBMCs from RA patients (N=14) with the Asp299Gly variant and RA patients (N=14) without the polymorphism after stimulation with LPS (200ng/ml) and HSPB8 (10mg/ml) for 24 h. Error bars represent the SEM. The sensitivity of the cytokine assay was <5 pg/ml for each measured cytokine; * P < 0.05; ** P< 0.01.

the variant, (56% decrease upon LPS stimulation; AA: 3070pg/ml ± 638pg/ml; AG: 1360pg/ml ± 162pg/ml and 41% decrease upon HSPB8 stimulation; AA: 1834pg/ml ± 185pg/ml; AG: 1090pg/ml ± 168pg/ml).

Discussion

In the present study, we demonstrated that the Asp299Gly TLR4 polymorphism had no influence on the mRNA and protein expression of TLR4, but in contrast cell stimulation via this receptor with either exogenous or endogenous TLR4 agonists led to a clearly decreased secretion of IL-6, TNFα and IL-10. In the last few years, several groups already investigated the functional consequences of the Asp299Gly TLR4 polymorphism in health and disease, resulting in conflicting conclusions. For example, Schippers et al and Rittersma et al found that the TLR4 variant had no functional consequences in terms of cytokine release upon TLR4 stimulation of whole blood from patients undergoing elective cardiopulmonary bypass surgery and patients who underwent successful elective stent placement respectively [23,24]. In contrast, Arbour et al described that the TLR4 variant was associated with hyporesponsiveness to LPS and that THP-1 cells transfected with this mutant allele showed a decreased response to LPS [12]. Furthermore, Kinane et al demonstrated that epithelial cells from subjects heterozygous for the Asp299Gly TLR4 polymorphism were functionally hypo-responsive, evidenced by mRNA and protein response profiles of pro-inflammatory cytokines and chemokines [25]. Interestingly, all studies mentioned above, which concluded that the Asp299Gly TLR4 polymorphism did not lead to impaired cytokine responses upon in vitro LPS challenge, did not correct for

the amount of monocytes. In 2002, Sabroe et al demonstrated that neutrophils, which are abundantly present in whole blood, are hardly involved in TLR4-induced cytokine production, although they do express low levels of TLR4 [30]. However, indirectly, these neutrophils are likely to be activated by inflammatory mediators from TLR4-stimulated monocytes, leading to the production of pro-inflammatory cytokines such as TNFα and IL-6 [31]. Therefore, it is conceivable that cytokine production by indirectly activated neutrophils might veil the effects of the Asp299Gly polymorphism on TLR4-bearing mononuclear cells, explaining the discrepancy between the results of various groups who studied the functional consequences of the TLR4 variant. Furthermore, we demonstrated that the relative amount of $C14^+$ cells in PBMCs from patients with the Asp299Gly variant was significantly higher than in PBMCs from patients without the variant, a phenomenon that might veil the effects of the Asp299Gly polymorphism in RA patients as well. Based on these findings, in our study we chose to correct cytokine responses for the amount of, TLR4-bearing, $CD14^+$ cells. Very recently, Rallabhandi et al described that HEK293T cells transfected with the polymorphic TLR4 variant showed decreased sensitivity to Gram-negative LPS and two structurally unrelated TLR4 agonists, compared to cells transfected with the wild type TLR4, under conditions of comparable TLR4 surface expression [32]. These results are in line with our findings and further emphasize the functionality of genetic TLR4 variants.

The finding that the relative amount of $CD14^+$ cells in PBMCs from RA patients with the Asp299Gly variant was significantly higher than in PBMCs from patients without the variant is very interesting and should be subjected to further research into RA and other (autoimmune) diseases. Recently, Ferwerda and colleagues investigated whether the Asp299Gly TLR4 polymorphism was associated with the development of active tuberculosis in HIV-infected patients [33]. Interestingly, they showed an increased prevalence of the Asp299Gly variant in all subgroups of patients having low T-cell counts, suggesting an effect of the Asp299Gly variant on the relative amount of $CD4^+$ T-cells as well.

In terms of autoimmunity it is very interesting that cells from RA patients are known to produce much higher levels of cytokines upon TLR2 and TLR4 stimulation than cells from healthy controls [6]. During RA, tissue damage and cell stress leads to the release of endogenous TLR agonists, including fibronectin, heat-shock proteins, hyaluronic acid and host-derived RNA into the inflamed joint. It is tempting to speculate that these tissue-damage-induced endogenous components could risk the initiation of autoimmunity. This reasoning supports the so called 'danger theory' which suggests that the immune system is more concerned with damage than with foreignness and is called into action by alarm signals from injured tissues rather than by the recognition of non-self [34]. The role of TLRs in RA is recently further underscored by the finding that inhibition of the TLR4 signaling pathway using Chaperonin 10 (heat shock protein 10, XToll™), seemed to be efficacious in treatment of the symptoms of rheumatoid arthritis [11].

Presence of the Asp299Gly TLR4 variant in patients with RA results in less serious immune responses upon TLR4 stimulation using either exogenous or endogenous agonists, but as we showed here, these patients have significantly higher levels of $CD14^+$

cells. This phenomenon could possibly be the reason that, although the Asp299Gly TLR4 polymorphism is associated with an impaired immune response, RA patients do not show remarkable effects of the polymorphism in terms of disease severity [19]. Furthermore, especially cells from RA patients with active disease, release such high levels of pro-inflammatory cytokines that even in the presence of the genetic TLR4 variant, TLR4 stimulation results in enhanced cytokine production compared to healthy individuals. Moreover, Chaperonin 10, which seems to be a promising treatment for RA, leads to markedly decreased levels of LPS-induced TNFα, whereas in contrast production of the anti-inflammatory IL-10 rapidly increases [35]. From this, one might conclude that the balance between Th1 and Th2 cytokines is evenly important in the pathogenesis of RA than the absolute pro- and anti-inflammatory cytokine levels present. Effective treatment of RA using anti-cytokine therapies such as anti-TNFα and IL-1Ra might also be a result of altered Th1/Th2 balances caused by selectively diminished levels of TNFα and IL-1 respectively. In this light, it is plausible that the Asp299Gly TLR4 polymorphism is not associated with disease severity, as this polymorphism affects the IL-10 production as well.

In conclusion, here we provide evidence that the presence of the Asp299Gly TLR4 variant has a clear impact on the functional level despite the fact that the TLR4 expression level is unaffected. However, since the Asp299Gly polymorphism also affects relative expression of TLR4-bearing, $CD14^+$ cells and since the polymorphism only diminishes absolute cytokine levels but does not interfere in the balance between Th1 and Th2-directing cytokines, this might explain why presence of the polymorphism does not affect the disease severity.

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CHAPTER 8

Summary and final considerations

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Toll-like receptors in rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease, which mainly affects the synovial joints with a preference for the small joints of the hands and feet. Synovial inflammation is characterized by massive influx of T cells, B cells, fibroblast-like synoviocytes, macrophages, and dendritic cells (DC) in the synovial tissue. Although the exact pathways leading to the initiation and perpetuation of synovial inflammation remains obscure, recent evidence addressed the potential role of Toll-like receptors (TLRs) in arthritis.

Toll-like receptors belong to the family of pattern recognition receptors (PRRs), and are constitutively expressed by numerous cell types and tissues (1-6). TLRs can be activated upon recognition of microbial and viral products, such as lipoteichoic acid (LTA), lipopolysaccharide (LPS), CpG motifs of bacterial DNA and viral RNA (7-10). More recently however, many studies addressed the role of endogenous ligands in the TLR-mediated cell activation (11-14). Intriguingly, such endogenous ligands, including fibronectin, heat-shock proteins, hyaluronic acid and host derived RNA, are released upon tissue damage and cell stress, events that are likely to occur during inflammatory conditions. The activation of cells by endogenous components from distressed or injured cells supports the so-called 'Danger Model', proposed by Matzinger et al. (15). This theory suggests that the immune system is more concerned with damage than with foreignness, and is called into action by alarm signals from injured tissues, rather than by the recognition of non-self.

In terms of autoimmunity, it is very interesting that endogenous components released upon tissue damage and cell stress could bring an immune response. This thesis postulated evidence that further substantiates a role for TLRs in the initiation and the chronicity of RA.

TLR expression

First it was shown that TLR2, TLR3, TLR4 and TLR7 were all expressed in RA synovial tissue and that the expression was clearly enhanced compared to synovial tissue from patients suffering from osteoarthritis (OA) or healthy controls (chapter 2 and chapter 3). Furthermore, elevated levels of endogenous TLR4 ligands were described to be present in serum and synovial fluid from RA patients compared to serum from patients with systemic sclerosis and systemic lupus erythematosus (chapter 3). In addition, a new endogenous TLR4 ligand, the so-called heat-shock protein B8 (HSPB8), was described and this protein was demonstrated to be present in synovial tissue from RA patients as well (chapter 4). Although both TLRs and TLR ligands are present in RA synovial tissue, at this moment it is unclear whether they indeed lead to chronic inflammation of the innate immune response. In this thesis, it was hypothesized that exogenous as well as endogenous TLR ligands, which are both shown to be present in the synovial joints of RA patients (16-20), induce a first immune response mediated via TLRs. This leads to the production of pro-inflammatory mediators such as cytokines, chemokines and metalloproteinases. These inflammatory mediators can, in turn, cause damage to the tissue, leading to the release of endogenous factors such as heat-shock proteins, fibronectin and hyaluronan, resulting in a self-sustaining loop of TLR activation and TLR-mediated tissue damage (Figure 1). Furthermore, another important factor in sustaining the inflammatory processes during RA might be that simultaneous stimula-

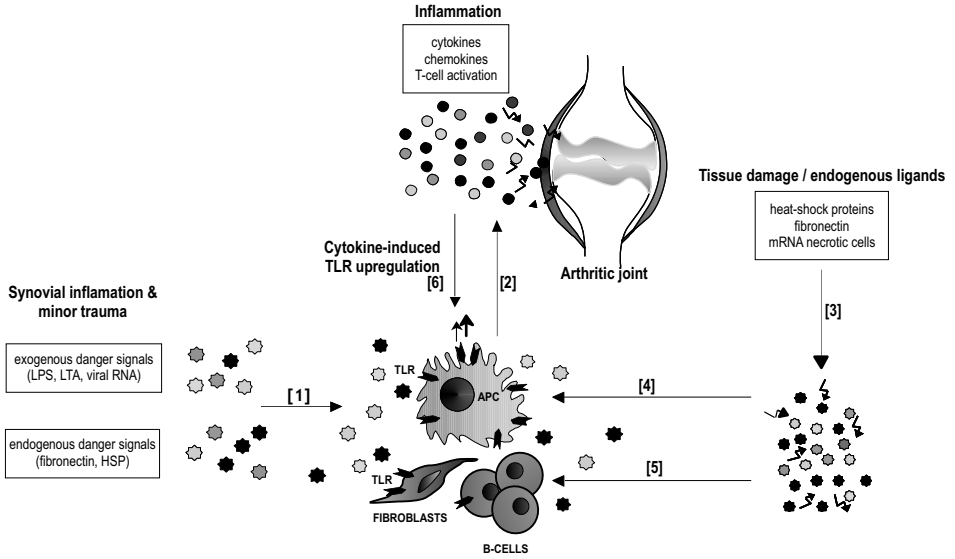

Figure 1. Schematic overview of self-sustaining loop of TLR activation and TLR-mediated tissue damage as a conceptual framework for the chronic inflammation during RA. This figure shows that exogenous as well as endogenous TLR ligands, which are both shown to be present in the synovial joints of RA patients, induce a first immune response mediated via TLR [1]. Single TLR stimulation leads to the production of pro-inflammatory mediators such as cytokines, chemokines and metalloproteinases, however simultaneous stimulation of several TLR results in a synergistically induced immune response characterized by extremely high levels of pro-inflammtory mediators [2]. These inflammatory mediators can, in turn, cause damage to the tissue, leading to the release of endogenous TLR ligands such as heat-shock proteins, fibronectin and hyaluronan [3], resulting in a self-sustaining loop [4] of TLR activation and TLR-mediated tissue damage. Furthermore, endogenous TLR stimulation of B-lymphocytes, for example by stimulation with self-DNA, also results in the production of rheumatoid factor [5]. In addition, the inflammatory mediators, mainly cytokines, are able to induce TLR up regulation [6], which could result in a more serious immune response as well.

tion of specific TLRs results in a synergistic production of pro-inflammatory mediators (chapter 3). It is generally believed that simultaneous TLR stimulation acts as a sort of “combinatorial code’ by which DC discriminate pathogens resulting in a more effective immune response. On the other hand, unwanted release of endogenous ligands, due to tissue damage and cell stress, could synergistically induce an immune response as well, favouring an environment in which breakthrough of tolerance is likely to occur. This idea was underscored by Waldner and collegues, who demonstrated that experimental autoimmune encephalomyelitis, which is an experimental model of multiple sclerosis, was determined, at least partly, by the endogenous activation state of antigen-presenting cells, such as DC (21).

TLR regulation

Besides direct stimulation of TLRs in the synovial joint, TLR-induced immune responses can also be regulated by cytokines such as TNFα, IL-12 and IL-18. TNFα, which was one of the first cytokines of interest in the field of RA (22;23), is able to up regulate expression of TLR4 in vitro (3). In line with this, it was demonstrated that anti-TNFα therapy in RA patients clearly down-modu-

lated the increased expression of TLR2 and TLR4 (24). IL-12 and IL-18, known as Th1-directing cytokines, are alone or in combination strong inducers of IFNγ and have shown to be important in the development of arthritis in murine models (25;26). Furthermore, both cytokines are present in synovial tissue from RA patients and cause strong up regulation of TLR2 and TLR4, mainly driven by production of IFNγ (chapter 2). For TLR3 and TLR7, it was demonstrated that IFNα strongly upregulates expression, which was functional in terms of cytokine production, since subsequent stimulation resulted in enhanced production of inflammatory mediators (chapter 6).

TLR stimulation

In vitro stimulation of the TLRs that were highly expressed in RA synovial tissue revealed interesting differences between TLRs recognizing pathogens from bacterial or viral origin (chapter 3). Monocyte-derived DC from RA patients produced highly elevated levels of the cytokines TNFα, IL-6 and IL-10 upon stimulation with exogenous and endogenous TLR2 and TLR4 ligands, whereas stimulation of TLR3 and TLR7 did not lead to enhanced cytokine production. Using microarray analysis, allograft-inflammatory factor 1 (AIF-1) was identified as a factor that might be involved in the enhanced TLR2/4-mediated immune response in RA patients (chapter 5), since expression of AIF-1 was significantly enhanced in RA patients and correlated with the TLR4-induced cytokine levels. Finally, the Asp299Gly polymorphism in the TLR4 gene of RA patients was shown to be functional since stimulation with exogenous and endogenous TLR4 ligands resulted in clearly diminished cytokine production, despite the fact that the TLR4 expression was unaffected (chapter 7).

TLR4 as the most crucial TLR in the pathogenesis of rheumatoid arthritis

In this thesis we mainly focussed on TLR2, TLR3, TLR4 and TLR7, which could be divided into two categories based on their differential subcellular localization. Whereas TLR2 and TLR4 are present on the plasma membrane, TLR3 and TLR7 reside mainly within the endosomal/lysosomal compartment. The different location of the TLRs relates to the nature of the ligands that are recognized; TLRs at the cell surface mainly respond to extracellular pathogens, while intracellular TLRs are triggered by viral nucleic acids, following the internalization of a virus. This intracellular localization avoids the recognition of, for example, extracellular endogenous mRNA released from necrotic cells, therefore preventing the induction of an immune response against self-antigens.
Nowadays, many endogenous ligands for TLR4 have been identified, such as heat-shock proteins, fibronectin and hyaluronic acid (12-14). In physiological conditions these ligands are present within a cell or embedded in the extracellular matrix and therefore are not able to activate TLR4. However, these endogenous ligands could be released upon tissue damage and cell stress, events that constantly occur during RA. Based on the fact that most endogenous TLR ligands identified can be recognized by TLR4 and the fact that these ligands are very likely to be present in the RA synovial joints, at this moment TLR4 is considered as the most crucial TLR in the pathogenesis of RA. In line with this, it was recently demonstrated that a naturally occurring TLR4 antagonist was able to suppress the clinical and histological characteristics of arthritis as demonstrated in several models of experimental arthritis (27). Although the exact mechanism of this TLR4 antagonist in RA suppresion has not

been identified yet, it is quite conceivable that blocking of TLR4 stimuli in an arthritic environment prevents simultaneous stimulation of TLR4 with one or more other TLRs, resulting in a clear reduction of the clinical and histological characteristics of arthritis.

Non-TLR pathogen recognition receptors

Nod-like receptors

Although the role of TLRs in the innate immune response is well established nowadays, other members of the PRR family are supposed to be involved in the host response to pathogens as well. Where TLRs are membrane-bound proteins, expressed on the cellular membrane or in the endosomal compartment, Nod-like receptors (NLRs) are considered as the cytoplasmic counterparts of TLRs. Mammals have more than 20 NOD family members, including NOD1 (CARD4) and NOD2 (CARD15), which are both able to detect distinct substructures from bacterial peptidoglycan (28;29). Another member of the NLR family, NALP3, is involved in the activation of caspase-1, resulting in the secretion of IL-1β and IL-18 upon bacteria-induced triggers (30). Interestingly, it was demonstrated that NALP3 was also capable of caspase-1 activation in response to uric acid (31), which is released by necrotic cells. In terms of arthritic disorders it is interesting that a disturbed uric acid metabolism could cause gout, where monosodium urate crystals are deposited on the articular cartilage of the joints and in the particular tissue-like tendons. These results indicate that, just as for TLRs, NLRs are able to detect endogenous danger signals, although the evidence is very limited thus far.

Lectins

Besides TLRs and NLRs, also lectins are involved in the direct recognition of specific pathogens. While the main function of TLRs is to induce the production of pro-inflammatory cytokines and T-cell activation upon pathogen recognition, lectins are involved in antigen internalization and degradation in order to enhance antigen processing and presentation (54). Lectins are able to recognize carbohydrate structures and currently there is no evidence that they can discriminate between self and non-self. Most of these carbohydrate-binding proteins belong to the C-type lectin superfamily, because of their calcium (Ca^{2+}) dependency. There are some indications that lectins participate in the prevention of autoimmunity as Galectin-1 had a potent suppressive effect on the induction of experimental autoimmune encephalomyelitis (33). On the other hand, a number of lectins have been identified as auto-antigens, including Galectin-3 which is associated with Crohn's disease and cerebellar soluble lectins, which are associated with multiple sclerosis (34;35). In rheumatoid arthritis, lectins have been put forward as a possible contributing pathogenetic factor also. For example, the C-type lectin DC-SIGN was strongly upregulated in the synovial joints of RA patients compared to synovial expression in patients with osteoarthritis or healthy controls (55). A lot of independent studies, investigating the possible involvement of mannan-binding lectin (MBL) in RA, showed that low MBL concentrations were correlated with a poor prognosis, but were not correlated with disease susceptibility (37).

RNA helicases

With respect to the recognition of single stranded and double stranded RNA, besides TLR3, TLR7 and TLR8, the so-called RNA helicases, such as RIG-I (retinoic acid inducible gene-I), are involved. Whereas

TLRs are activated by the recognition of nucleic acids in the endosomal compartments, RIG-I detects viral RNA within the cytoplasm, which also leads to activation of NF-κB and IRF-3 and subsequently secretion of type-I IFNs. Recently, it was demonstrated that RA patients had a significantly elevated expression of genes which were regulated by type I IFNs compared to healthy individuals (38). Therefore it is quite conceivable that RIG-I plays a role in RA as well, although no data confirming this hypothesis have been published thus far.

All together, besides TLRs, other non-TLR members of the PRR family, such as NLRs, lectins and RNA helicases, are involved in pathogen recognition as well. The co-existence of these receptors and their diverse cellular localization, suggests that the antigen recognition is a complex network of interacting receptors and that the outcome of the immune response will depend on the type of receptors involved (Figure 2). In this thesis it was described that co-stimulation of two or more TLRs at the same time, results in synergistically enhanced cytokine levels (chapter 3). Regarding the close functional similarity between TLRs and the other PRRs, it is likely that also synergistic effects could be induced by co-stimulation of one TLR and one or more other members of the PRR family. Few data into the synergistic effects between TLRs and non-TLRs have turned up so far, mainly about TLRs and NLRs (39;40), however the exact interaction between TLRs and non-TLRs should be subjected to further research in the future.

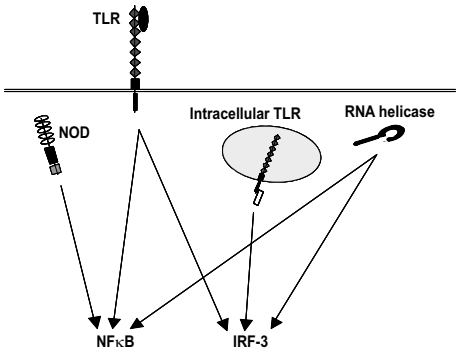

Figure 2. The co-existence of TLR and non-TLR pathogen recognition receptors and their diverse cellular localization, suggests that the antigen recognition is a complex network of interacting receptors and that the outcome of the immune response will depend on the type of receptors involved.

TLRs as a therapeutic target in rheumatoid arthritis

Chaperonin 10

As an increasing body of evidence now underscores a role for TLRs in the pathogenesis of rheumatoid arthritis, these data could provide opportunities for new therapeutic approaches to modulate the innate immunity. Very recently, chaperonin 10, also known as heat-shock protein 10 (HSP10) and Xtoll, was tested with respect to safety and clinical efficacy in patients with rheumatoid arthritis. Primary results of this small, randomised trial suggested that HSP10 is well tolerated and efficacious in, at least, short-term treatment of the symptoms of rheumatoid arthritis (41). HSP10 was originally identified as a factor present in sera during early pregnancy and has shown to be immunosuppressive in experimental autoimmune encephalomyelitis, delayed type hypersensitivity, and allograft rejection models (42-44). Later on, it was demonstrated that purified recombinant human HSP10 incubated with human peripheral blood mononuclear cells reduced LPS-induced NF-κB activation and secretion of TNFα, RANTES and IL-6 (45). Interestingly, reduced secretion of TNFα, RANTES and

IL-6 went together with an increased production of IL-10, which is known as a potent immunosuppressive cytokine able to inhibit TLR4 signaling (46). It is thought that HSP10 may inhibit pro-inflammatory responses by interacting with extracellular HSP60, since anti-HSP60 antibodies prevented and mimicked HSP10 inhibitory activity (45). In this light, HSP10 could be a promising target for the treatment of rheumatoid arthritis since, in addition to current anti-TNFα treatment, effective removal of TNFα is accompanied by the induction of an anti-inflammatory immune response accomplished by IL-10. Nowadays, anti-TNFα therapy is successfully used to treat a substantial number of patients with various indications including rheumatoid arthritis, junvenile rheumatoid arthritis, inflammatory bowel disease, psoriatic arthritis and ankylosing spondylitis. However, this therapy is associated with rare but severe adverse events such as serious infections, lymphoma, or chronic heart failure (47;48). Since HSP10 does only suppress TNFα levels, but does not completely remove all the TNFα from the circulation, the risk of serious side effects might be reduced. Nonetheless, at this moment only a small randomised trial, with substantial limitations of the study design, prove the efficacy of TLR blockade in the treatment of RA. Therefore, supplementary trials for HSP10 and other TLR inhibitors should be performed to further investigate the safety and efficacy of these promising therapeutic agents.

Soluble decoy receptors

The inhibition of TLR activation can also be achieved in several other ways, for example by suppressing the interaction between TLRs and its specific ligands using soluble decoy receptors. It is known that a constant amount of soluble TLR2 is released by human blood monocytes and that several soluble TLR2 isoforms are naturally present in human milk and plasma (49). Although no soluble TLR4 has been described in humans thus far, multiple TLR4 mRNAs have been detected, indicating that soluble TLR4 might functionally exist in humans. In mice it was already demonstrated that soluble TLR4 significantly inhibited the LPS-mediated TNFα production and NF-κB activation (50), however no experiments have been performed in models of experimental arthritis thus far. Another way to block the TLR – TLR ligand interaction is by using monoclonal antibodies against specific TLRs. Although this technique might work perfectly for the extracellular TLRs, preventing ligation to intracellular TLRs might even be more challenging. Recently, it was demonstrated that a naturally occurring TLR4 antagonist was able to suppress the clinical and histological characteristics of arthritis as demonstrated in several models of experimental arthritis (27), suggesting TLR4 blockade as a promising strategy for the treatment of RA.

Inhibition of TLR signaling molecules

Furthermore, interference with the TLR signaling cascade could be an attractive strategy to suppress TLR-induced immune activation, for example by the inhibition of activating components or activation of inhibitory components. For example inhibition of the adaptor molecule Mal, which provides signaling specificity for TLR2 and TLR4, might suppress the enhanced TLR4 response during RA, without interfering in other biologically important pathways, such as IL-1 signaling. Recently, it was shown that soluble ST2 could effectively suppress murine collagen-induced arthritis, as demonstrated by reduced RA pathology and decreased serum levels of IL-6, TNFα and IL-12 (51).

Though it is known that the other member of the ST2 family, ST2L, suppressed TLR signaling by sequestration of MyD88 and Mal (52), the mechanism of action of soluble ST2 is not fully elucidated yet. However, it is demonstrated that soluble ST2 significantly down regulated the mRNA expression of TLR4 in LPS-activated macrophages in vitro (53), a mechanism that could be effective in the suppression of TLR-induced immune responses as well.

TLR downregulation

Besides soluble ST2, the expression of TLRs can also be downregulated by anti-inflammatory cytokines, especially TGFβ and IL-10, or by degradation of TLRs through ubiquitylation, for example by TRIAD3A. Down modulation of TLR expression would decrease the sensibility for exogenous as well as endogenous TLR ligands and might therefore be a strategy for the treatment of RA as well. However, in our experience (unpublished results) even very low levels of TLR expression can lead to full cell activation upon triggering with TLR specific ligands, suggesting that this strategy might only be successful when full ablation of TLR expression can be achieved.

All together, many approaches, with respect to TLRs, are conceivable as therapeutic strategies for RA, although none of these approaches have been tested extensively so far.

Conclusion

In summary, in this thesis we provided substantial data that demonstrated a potential role for TLRs in the pathogenesis of RA. However it cannot be excluded that other, more recently described, non-TLRs contribute to these processes as well, as it is very likely that these PRRs act via very complex interactions with each other to regulate the delicate balance between immunity and tolerance. Furthermore, based on the fact that most endogenous TLR ligands identified can be recognized by TLR4 and the fact that these ligands are very likely to be present in the RA synovial joints, at this moment TLR4 is considered as the most crucial TLR in the pathogenesis of RA. Although preventing TLR stimulation, using HSP10, seems to be a successful target for the treatment of RA, other strategies to reduce or abolish TLR-induced immune responses should be subjected to further investigation henceforth.

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NEDERLANDSE SAMENVATTING

Nederlandse samenvatting

Reumatoïde artritis (RA), in de volksmond reuma genoemd, is een chronische, auto-immuun aandoening die gekenmerkt wordt door gewrichtsontstekingen die uiteindelijk kunnen leiden tot schade aan het kraakbeen en aan het bot. Deze gewrichtsontstekingen worden gekenmerkt door verdikking van de slijmvliezen die de gewrichtsholten bekleden (synovium) en grote aantallen ontstekingscellen zoals T-cellen, B-cellen, fibroblast-achtige synoviocyten, macrofagen en dendritische cellen. De exacte oorzaak van het ontstaan van RA is nog onbekend, maar recent onderzoek wijst erop dat de zgn. Toll-like receptors (TLRs) hierbij betrokken zijn.

TLRs behoren tot de familie van 'pattern recognition receptors' en komen voor op en in verscheidene celtypen en weefsels. Vandaag de dag zijn er 11 verschillende TLRs beschreven en deze TLRs worden geactiveerd na herkenning van microbiële en virale producten (exogene liganden), zoals 'lipoteichoic acid' (LTA), 'lipopolisaccharide' (LPS), bacterieel DNA en viraal RNA. Recentelijk is echter ook aangetoond dat lichaamseigen (endogene) liganden in staat zijn om TLRs te activeren, enkele voorbeelden hiervan zijn fibronectine, heat-shock eiwitten, hyaluronzuren en endogeen RNA. Tijdens ontstekingsprocessen, zoals bij RA, vindt weefselschade en celstress plaats. Hierdoor komen deze endogene producten vrij komen wat kan leiden tot een ontstekingsreactie. Dit verschijnsel is, in het bijzonder bij auto-immuun aandoeningen zoals RA, erg interessant.

In dit proefschrift wordt de rol van TLRs in het ontstaan en de chroniciteit van RA beschreven. In hoofdstuk 2 en hoofdstuk 3 wordt beschreven dat TLR2, TLR3, TLR4 en TLR7 voorkomen (expressie) in synoviaal weefsel van RA patiënten en dat deze sterk verhoogd aanwezig zijn in vergelijking met synoviaal weefsel van patiënten met osteoartritis (artrose) en gezonde vrijwilligers. Daarnaast zijn verhoogde spiegels van endogene TLR4 liganden aangetoond in het serum en synoviaal vocht van RA patiënten in vergelijking met patiënten die lijden aan sclerodermie of systemische lupus erythematodes (hoofdstuk 3). Bovendien wordt een nieuwe endogene TLR4 ligand, het zgn. heat-shock eiwit B8 (HSPB8) beschreven; dit eiwit komt ook voor in synoviaal weefsel van RA patiënten (hoofdstuk 4).

Hoewel zowel TLRs en TLR liganden aanwezig zijn in synoviaal weefsel van RA patiënten is het op dit moment onduidelijk of zij ook daadwerkelijk kunnen leiden tot een chronische ontsteking. In dit proefschrift is aangenomen dat zowel exogene als endogene TLR liganden, welke beide aanwezig zijn in de synoviale gewrichten van RA patiënten, een afweerreactie kunnen veroorzaken via TLR activatie. Dit leidt tot de productie van ontstekingsmediatoren zoals cytokines, chemokines en methalloproteinases en deze mediatoren veroorzaken op hun beurt weefselschade. Hierdoor komen endogene liganden, zoals heat-shock eiwitten, fibronectine en hyaluronzuren vrij waardoor er een vicieuze cirkel ontstaat van TLR activatie en TLR-gemedieerde weefselschade.

Een andere belangrijke factor in het in stand houden van de ontsteking bij RA, is de bevinding dat gelijktijdige (simultane) stimulatie van verschillende TLRs leidt tot een sterkere productie

van ontstekingsmediatoren dan elk van de afzonderlijke TLRs samen zouden kunnen bereiken (hoofdstuk 3). Dit noemt men een synergistisch effect. Het vrijkomen van ongewenste endogene liganden als gevolg van weefselschade en celstress zou ook kunnen leiden tot een synergistische productie van ontstekingsmediatoren en hierdoor is het niet onwaarschijnlijk dat de tolerantie wordt doorbroken en een auto-immuun aandoening, zoals RA, kan ontstaan.

Naast de directe activatie van TLRs door lichaamsvreemde en lichaamseigen liganden in de synoviale gewrichten, spelen ook verscheidene ontstekingsmediatoren, zoals cytokines, een rol bij TLR-gemedieerde ontstekingsreacties. Zo kunnen de cytokines IL-12 en IL-18, die beide voorkomen in het synoviaal weefsel van RA patiënten, leiden tot een sterk verhoogde expressie van TLR2 en TLR4, wat wordt aangestuurd door het cytokine IFNγ (hoofdstuk 2). Voor TLR3 en TLR7 is het aangetoond dat IFNα voor een sterke verhoogde expressie kan zorgen, wat leidt tot een sterk verhoogde productie van ontstekingsmediatoren na stimulatie van deze TLRs (hoofdstuk 6).

In hoofdstuk 3 is aangetoond dat dendritische cellen van RA patiënten aanmerkelijk hogere spiegels van de cytokines TNFα, IL-6 en IL-10 produceren na stimulatie van TLR2 of TLR4, terwijl stimulatie van TLR3 of TLR7 niet resulteert in een verhoogde cytokineproductie. Mogelijk is ‘allograft inflammatory factor 1’ (AIF-1) betrokken bij deze verhoogde immuunreactie bij RA patiënten, omdat deze factor verhoogd is in RA patiënten en correleert met de TLR4-gemedieerde cytokineproductie (hoofdstuk 5). Tenslotte, wordt in hoofdstuk 7 beschreven dat een defect in het TLR4 gen bij RA patiënten leidt tot sterk verminderde cytokineproductie na stimulatie met zowel exogene als endogene liganden, terwijl de expressie van TLR4 niet verandert.

Samengevat, dit proefschrift beschrijft een aanzienlijke hoeveelheid data die een rol voor TLRs in het ontstaan en de chroniciteit van RA aantonen. Gebaseerd op deze feiten lijken TLRs een veelbelovend target voor het ontwikkelen van therapieën voor de behandeling van RA patiënten in de toekomst.

DANKWOORD
CURRICULUM VITAE
LIST OF PUBLICATIONS

Dankwoord

Dit is het dan! Mijn proefschrift is af! En aangezien je zo'n proefschrift natuurlijk niet alleen schrijft, wil ik in dit laatste deel van mijn proefschrift van de gelegenheid gebruik maken om iedereen te bedanken die de afgelopen jaren een bijdrage heeft geleverd aan het tot stand komen van dit boekje.

Allereerst gaat mijn dank uit naar Tim, mijn copromotor. Tim, graag wil ik je bedanken voor alle wetenschappelijke ideeën en bijbehorende discussies die verwerkt zijn in dit proefschrift. Ik vond het erg fijn dat je me de afgelopen jaren de vrijheid hebt gegeven mijn eigen weg te zoeken in het onderzoek; dat zal niet makkelijk geweest zijn voor zo'n enthousiaste en gedreven onderzoeker als jij! Bedankt dat je altijd tijd voor me wilde maken, waar ter wereld je ook was.

Vervolgens wil ik mijn beide promotoren, Wim en Piet, bedanken voor de mogelijkheid mee te werken aan deze bijzondere onderzoekslijn, een samenwerking tussen lab en kliniek. Bedankt voor jullie vertrouwen in deze nieuwe onderzoekslijn en in mij als jonge onderzoeker.

En dan natuurlijk Leo. Zeker in de eerste jaren van mijn promotieonderzoek was je mijn vraagbaak op het lab, ik mocht altijd bij je binnenlopen. Met veel plezier denk ik terug aan onze discussies over proefdieren, ik zal nu eenmaal nooit een 'muizendokter' worden. Bedankt ook voor de gezelligheid tijdens de congressen die we samen bezocht hebben in de afgelopen jaren.

Het onderzoek, beschreven in dit proefschrift, had nooit uitgevoerd kunnen worden zonder mijn fantastische collega's binnen onze 'klinische groep'. Twan, mijn maatje, samen met Audrey heb je me wegwijs gemaakt op het lab, waardoor ik me snel thuis voelde in ons (kleine) groepje. Bedankt voor de gezelligheid, je kritische instelling, je geduld en begrip in de vier jaar die we hebben samengewerkt. Ondanks dat je niet in TLRs gelooft (dat zeg je tenminste), geloof je wel in mij en dat is toch het belangrijkste. Ik wens je alle succes met de laatste loodjes van je promotieonderzoek en je opleiding tot reumatoloog, ik weet zeker dat het je allemaal gaat lukken!

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Verder zou ik graag de werkgroepleiders, Fons en de beide Peters, en de post-docs, Arjen, Nozomi, Henk en Isabel, willen bedanken voor de prettige samenwerking en de nuttige discussies op z'n tijd. Marije, ik vergeet steeds dat je inmiddels ook bij de post-docs hoort. Je was mijn steun en toeverlaat tijdens de voorbereidingen voor mijn promotie en het lay-outen van mijn proefschrift. Bedankt voor al je geduld.

Ook wil ik de (oud)analisten van de afdeling, Miranda, Onno, Elly, Fieke, Cora, Monique, Astrid en natuurlijk onze secretaresse Marianne bedanken voor de prettige samenwerking. Annet, wat hebben we toch een gezellige tijd gehad toen we samen U-tje 4 deelden. Birgitte, bedankt voor alle ondersteuning bij m'n immuno's en Liduine bedankt dat je, in drukke periodes, altijd bereid was me wat RNA-werk uit handen te nemen.

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Voordat ik van de gelegenheid gebruik wil maken om mijn familie en vrienden te bedanken wil ik heel veel dank uitspreken voor de gezonde vrijwilligers en de RA patiënten die de afgelopen jaren belangeloos hebben meegewerkt aan mijn onderzoek. Zonder jullie bereidwilligheid om bloed te geven zou er van dit onderzoek niets terecht zijn gekomen.

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Lieve Ellen, het heeft veel voor mij betekend dat je altijd zo trots was op je ‘grote’ zus. Het afgelopen jaar is voor ons allebei erg druk en spannend geweest. Gelukkig konden we altijd ons verhaal aan elkaar kwijt en hielpen we elkaar te relativeren en te motiveren. Je bent een fantastische zus en ik ben blij dat je mijn paranimf wilt zijn!

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En het laatste woord blijft voor Marco. Ik weet dat je het niet nodig vindt dat ik je op deze manier bedank. Je vindt het allemaal zo vanzelfsprekend wat je voor me doet. Daarom zal ik het kort houden: You’re simply the best!!

Curriculum Vitae

Mieke Roelofs werd geboren op 10 maart 1980 te Nijmegen. In 1998 behaalde zij haar VWO diploma aan het Kruisheren Kollege te Uden en begon datzelfde jaar aan de studie Biomedische Gezondheidswetenschappen (tegenwoordig Biomedische Wetenschappen) aan de Radboud Universiteit Nijmegen. Zij liep een korte stage, in het kader van het bijvak Immunologie, op de afdeling Interne Geneeskunde van het UMC St. Radboud onder leiding van prof. dr. Bart-Jan Kullberg. In het kader van haar afstudeerrichting Pathobiologie liep zij stage op de afdeling 'Vaccine Technology & Immunology' en de afdeling 'Virology' bij Intervet International B.V. onder leiding van dr. Harrie Glansbeek en dr. Saskia van de Zande. In september 2002 studeerde zij af in de richting Pathobiologie en zij begon in december 2002 als junior onderzoeker op de afdeling Reumatologie van het UMC St. Radboud. Haar onderzoek naar de rol van Toll-like receptoren in de pathogenese van reumatoïde artritis werd uitgevoerd onder leiding van dr. Timothy Radstake en dr. Leo Joosten, en onder supervisie van prof. dr. Wim van den Berg en prof. dr. Piet van Riel. De resultaten van dat promotieonderzoek staan beschreven in dit proefschrift. In het voorjaar van 2005 won zij tijdens de European Workshop for Rheumatology Research de Young Investigators Award. Vanaf december 2006 heeft zij op dezelfde afdeling onderzoek gedaan naar Toll-like receptor gerelateerde effecten van anti-TNFα therapie bij RA patienten. Dit onderzoek heeft zij mede kunnen uitvoeren door een research grant die zij voor dit project heeft ontvangen van de stichting 'de drie lichten'. Sinds oktober 2007 is zij werkzaam binnen het clinical trial team van Numico Research B.V.

List of publications

Radstake TR, **Roelofs MF**, Jenniskens YM, Oppers-Walgreen B, van Riel PL, Barrera P, Joosten LA, van den Berg WB. Expression of toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-gamma. *Arthritis Rheum. 2004 Dec;50(12):3856-65.*

Radstake TR, Nabbe KC, Wenink MH, **Roelofs MF**, Oosterlaar A, van Lieshout AW, Barrera P, van Lent PL, van den Berg WB. Dendritic cells from patients with rheumatoid arthritis lack the interleukin 13 mediated increase of Fc gamma RII expression, which has clear functional consequences. *Ann Rheum Dis. 2005 Dec;64(12):1737-43.*

Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, van Lieshout AW, Sprong T, van den Hoogen FH, van den Berg WB, Radstake TR. The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis Rheum. 2005 Aug;52(8):2313-22.*

Roelofs MF, Boelens WC, Joosten LA, Abdollahi-Roodsaz S, Geurts J, Wunderink LU, Schreurs BW, van den Berg WB, Radstake TR. Identification of small heat shock protein B8 (HSP22) as a novel TLR4 ligand and potential involvement in the pathogenesis of rheumatoid arthritis. *J Immunol. 2006 Jun 1;176(11):7021-7.*

van Lieshout AW, van der Voort R, le Blanc LM, **Roelofs MF**, Schreurs BW, van Riel PL, Adema GJ, Radstake TR. Novel insights in the regulation of CCL18 secretion by monocytes and dendritic cells via cytokines, toll-like receptors and rheumatoid synovial fluid. *BMC Immunol. 2006 Sep 19;7:23.*

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Abdollahi-Roodsaz S, Joosten LA, Koenders MI, DevesaGiner I, **Roelofs MF**, Radstake TR, Heuvelmans-Jacobs M, Akira S, Nicklin MJ, Ribeiro Dias F, van den Berg WB. Differential roles of TLR2 and TLR4 during experimental arthritis mediated by skewed T cell balance. *Accepted for publication in JCI.*

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Roelofs MF, Abdollahi-Roodsaz S, Joosten LA, van den Berg WB, Radstake TR. The orchestra of Toll-like receptors and their role in autoinflammation and autoimmunity. *Conditionally accepted for publication in Arthritis Rheum.*

Roelofs MF, Wenink MH, Toonen EJ, Coenen MJ, Joosten LA, van den Berg WB, van Riel PL, Radstake TR. Consequences of the Asp299Gly Toll-like receptor 4 polymorphism in rheumatoid arthritis. *Manuscript submitted.*

Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, Wenink MH, Oppers-Walgreen B, van den Berg WB and Radstake TR. Synovial inflammation during rheumatoid arthritis; a role for IFNα. *Manuscript in preparation.*

Roelofs MF, del Galdo F, Wenink MH, Takahashi N, Bendtzen K, Frier LB, van den Berg WB, Radstake TR. Allograft inflammatory factor 1 (AIF-1) expression is correlated with TLR4-induced hyperresponsiveness during rheumatoid arthritis. *Manuscript in preparation.*

COLOUR FIGURES (PART 1)
CHAPTER 1 - CHAPTER 4

Chapter 2, Figure 1.

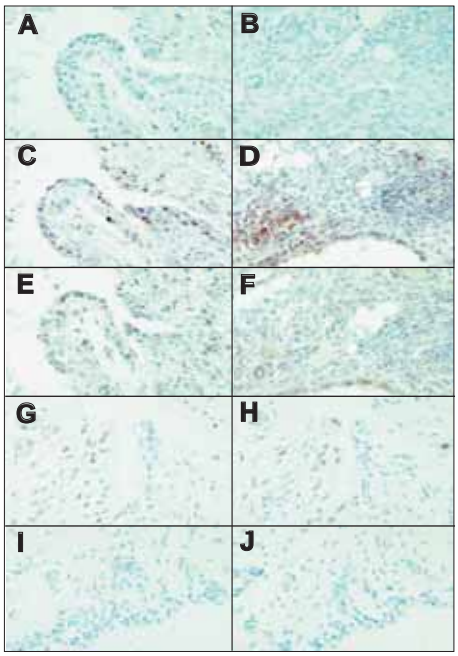

Figure 1. Immunohistochemical detection of Toll-like receptor 2 (TLR2) and TLR4 in synovial biopsy tissues from patients with rheumatoid arthritis (RA), osteoarthritis (OA), and healthy individuals. As a control, staining was performed with nonrelevant isotype control antibody (A and B). Expression of TLR2 (C and D) and TLR4 (E and F) was examined in RA synovial tissue, as compared with TLR2 and TLR4 expression in synovial tissue from patients with OA (G and H) and healthy individuals (I and J). Hematoxylin counterstained; original magnification 400×.

Chapter 3, Figure 1.

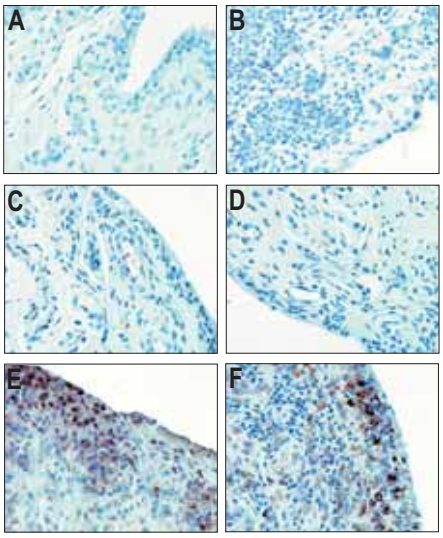

Figure 1. Immunohistochemical detection of TLR3 (left panel) and TLR7 (right panel) in synovial biopsy samples from RA patients and healthy controls. Top panel shows staining with an isotype control antibody. C, E. TLR3 expression in synovial tissue from healthy individuals and RA patients respectively. D, F. TLR7 expression in synovial tissue from healthy individuals and RA patients respectively.All tissues were counterstained with hematoxylin (original magnification × 400).

Chapter 4, Figure 7.

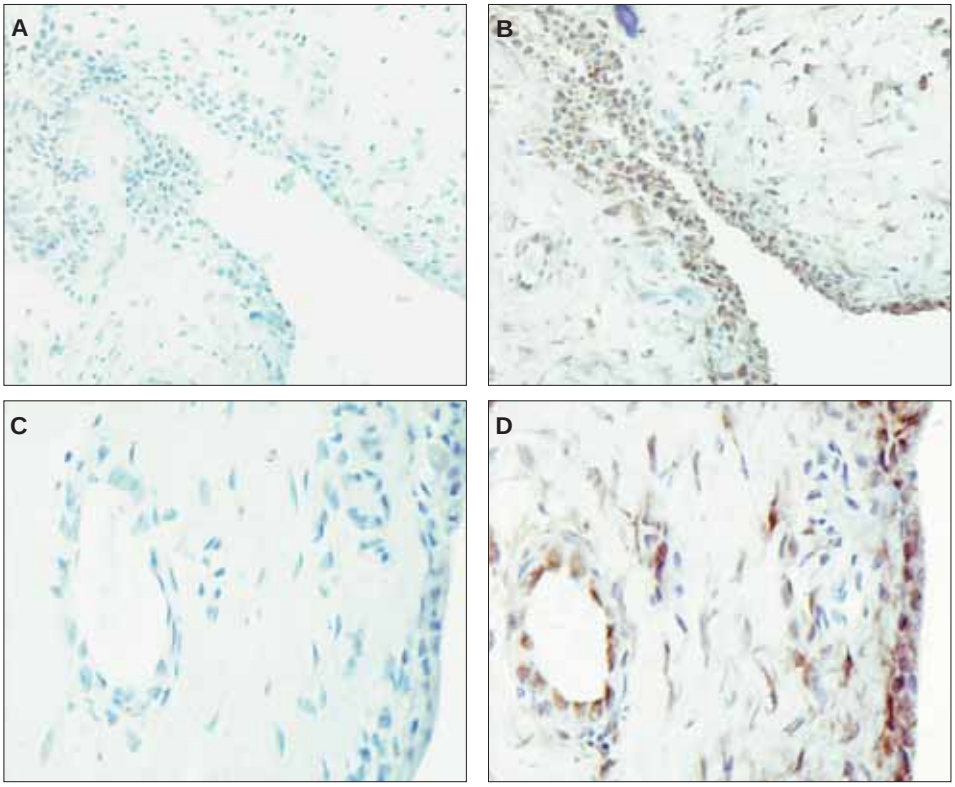

Figure 7. Immunohistochemical detection of HSPB8 in synovial tissue from RA patients. A. Staining of the synovial lining with an isotype control Ab (magnification x200). B. Staining of the synovial lining with a specific Ab against HSPB8 (magnification x200). C. Staining of synovial vascular endothelial cells with an isotype control Ab (magnification x400). D. Staining of synovial vascular endothelial cells with a specific Ab against HSPB8 (magnification x400). All synovial tissue sections were counterstained with hematoxylin.

COLOUR FIGURES (PART 2)
CHAPTER 5 - CHAPTER 8

Chapter 5, Figure 1.

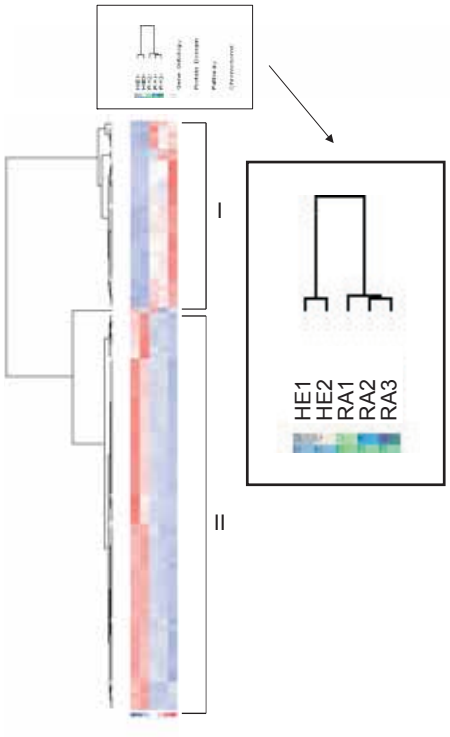

Figure 1. Hierarchical cluster analysis. RNA were isolated from DCs derived from three RA patients (RA) and two healthy donors (HE). The individual samples were coded by donor origin and the number. The gene expression profiling was performed as described in Materials and Methods. Hierarchical cluster analysis was performed on genes (vertical dendrogram) and samples (horizontal dendrogram) according to the algorithm described in Materials and Method. The magnified view in the box shows sample information in color blocks (1st row :individual sample color code, 2nd row: blue for HE, green for RA. The colors represent relative expression level. Red: up regulation, Blue: down regulation, White: avarage.

Chapter 6, Figure 1.

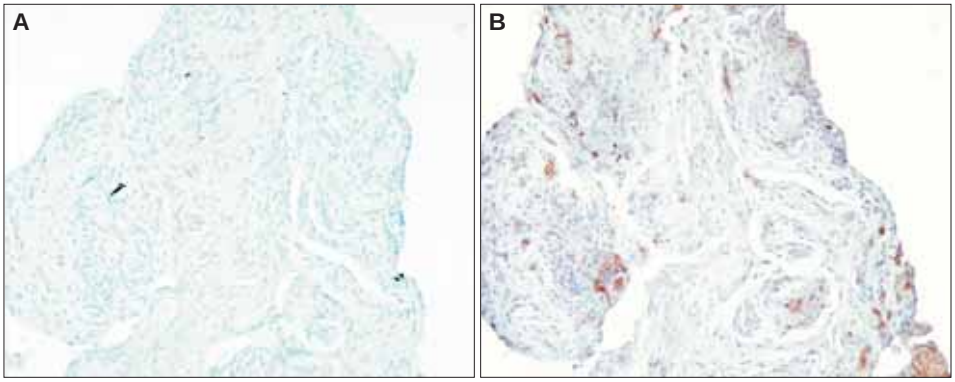

Figure 1. Staining of synovial tissue with an isotpe control antibody (A) or with a specific antibody against IFNa. Synovial tissue sections were counterstained with hematoxylin, magnification 200x.

Abbreviations used in this thesis

APC	Antigen Presenting Cell(s)
cDNA	complementary DNA
DC(s)	Dendritic Cell(s)
FACS	Fluorescence Activated Cell Sorter
FcγR	Fcγ Receptor
FCS	Fetal Calf Serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte / Macrophage Colony Stimulating Factor
HE	Heamatoxylin / Eosin
IFNα	Interferon α
IFNβ	Interferon β
IFNγ	Interferon γ
Ig	Immunoglobulin(s)
IL-1β	Interleukin-1β
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-18	Interleukin-18
IL-4	Interleukin-4
IL-6	Interleukin-6
LPS	Lipopolysaccharide
(m)Ab	(monoclonal) Antibody
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Comples
MMP	Matrix Methalloproteinase
mRNA	messenger RNA
MyD88	Myeloid Differentiation Factor 88
NFκB	Nuclear Factor-kB
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell(s)
PBS	Phosphate Buffered Saline
PMN	Polymorphonuclear Neutrophil(s)
PRR	Pathogen Recognition Receptor
RA	Rheumatoid Arthritis
(RT-)PCR	(Reverse Transcriptase) Polymerase Chain Reaction
Th1	T helper-1
Th2	T helper-2
TLR(s)	Toll-Like Receptor(s)
TNFα	Tumour Necrosis Factor a
WT	Wild-type

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