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Delineating the role of Toll-Like Receptor 4 activation and its signaling in experimental arthritis

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Chapter 1

Introduction
Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease affecting the articulating joints. It typically starts in the distal joints of hands and feet and manifests in a symmetrical fashion. The disease culminates in a chronic inflammation of these joints leading to severe swelling and destruction of bone and cartilage (Figure 1). This obviously impairs mobility of the joint and limits its function. Patient’s quality of life is further compromised due to pain accompanying the chronic inflammation (1).

Incidence of RA is 1% worldwide, but varies between different populations (2). Also, incidence among women is elevated compared to men, indicating a hormonal influence on disease. This is further supported by the fact that disease activity is reduced during pregnancy, but increases post-partum (3). The actual cause however, has not been found and it is most likely to be multi-factorial, such as genetic predisposition and exposure to detrimental environmental factors (4). Periodontitis has also been described as a risk factor for RA, as periodontitis prevalence is increased among RA patients (5). Recently, however, periodontitis has emerged as a potential cause of RA. Specific bacterial strains that cause periodontitis have unique enzymes that are potentially involved in generating autoantigens (6).

Systemic immune characteristics of rheumatoid arthritis

Immunologically, RA is characterized by the presence of anti-citrullinated proteins antibodies (ACPAs) and rheumatoid factor (RF) (7), which are antibodies against immune globulins. Both these antibodies are directed against autoantigens, the patient’s own proteins, and classify RA as an autoimmune disease. The fact that several auto-antibodies arise in RA indicates an important role of B-cells in the pathogenesis. These cells originate from the bone marrow and migrate to secondary lymphoid organs such as spleen and lymph nodes (8).
take up (auto)antigens and upon stimulation by T helper cells B-cells start to produce antibodies against the autoantigen (8). A critical role of B-cells in RA has been established by treatment of patients with anti-CD20 therapy (9). This treatment depletes the B-cell pool (10) of the patients for up to a year and disease progression is halted (11).

Another cell type of the adaptive immunity, the T-cell, also plays an important role in the pathogenesis of RA and the synovial cellular infiltrate consists largely of T-cells (12). Current treatments, however, have significantly decreased the number of infiltrated T-cells. In addition, T-cell responses against collagen type II (13) and microbial antigens have been reported in RA patients (14). Over recent years many studies have been performed on a T helper subset, the Th17 cell. This particular T helper cell produces the cytokine interleukin 17 (IL-17) and a crucial role for IL-17 has been shown for cartilage destruction, osteoclastogenesis (15) and promoting pro-inflammatory cytokine production by other cells. Again, new treatment strategies have shown that T-cells play a role in RA. Early anti-CD4 biological lacked efficacy (16;17), however biologicals blocking the interaction between antigen presenting cells (APCs) and T-cells were proven to be effective in the treatment of RA (18;19). Additionally, preliminary data suggest IL-17 targeting as an efficacious therapy for rheumatoid arthritis (20;21). These studies have shown that RA is a systemic disease and that the interface between the innate and the adaptive immunity is of crucial importance.

**Histopathology of rheumatoid arthritis**

When the arthritic joint is examined more closely several features are observed in RA patients. The synovium is heavily infiltrated with inflammatory cells, such as T- and B-cells from the adaptive immune system (22) and macrophages from the innate immune system. In fact, macrophage infiltration into the joint is a measure of disease activity (23). Moreover, the inflammatory cell infiltrate develops into secondary lymphoid organs with antibody producing plasma cells, showing that immune activation is present inside the joint (24). In addition, the synovium lining layer increases in thickness during arthritis. Synovium is normally only one cell layer thick, but the synovial fibroblasts in the lining start to proliferate and deposit extracellular matrix and thereby thicken the synovial lining (25). The thickened lining in conjunction with the infiltrated macrophages forms villi due to proliferation and develop into pannus tissue. This tissue covers the bone and cartilage surfaces and breaks down the extracellular matrix surrounding the chondrocytes causing cartilage erosion. Also the bone structures in the joint are subject to destruction (26). Circulating monocytes extravasate into the
joint and under stimulation from the proper growth factors they can form large multinucleated cells that develop into osteoclasts that absorb bone (27).

To supply the increasing demand for nutrients by the active inflammation in the joint new blood vessels develop in the synovium (28;29). This is, however, not an accurately orchestrated event and creates leaky vessels. Therefore, it becomes easier for attracted leukocytes to extravasate to the site of inflammation. Leukocytes are drawn to the site of inflammation by cyto- and chemokines produced by cells in the inflamed joint. Cyto- and chemokines are chemical messengers that attract new inflammatory cells amplifying the ongoing inflammation. Additionally, these inflammatory cells of both the innate and adaptive immunity have close interactions in the inflamed joint (30). These histopathological changes underline the importance of the local events in RA and how those processes contribute to the pathogenesis of RA.

**Toll-Like Receptors**

Since the discovery of Toll-Like Receptors (TLRs) an entire new field of research has opened in immunity and inflammation. TLRs are a family of 13 receptors and belong to the Pattern Recognition Receptors (PRR), together with C-type lectins, NOD-like receptors, and RIG-like helicases. All these receptors recognize patterns in microbial products rather than specific antigens. Therefore, these TLRs detect a broad range of microbial threats to the host. TLRs are type I transmembrane glycoproteins with a small intracellular signaling domain called TIR domain, sharing a high degree of homology with the IL-1 receptor (31). The extra cellular structure of TLR4 is very complex and consists of many leucine-rich repeats that fold into β-sheets, creating the typical horse shoe shape of TLRs (32). With this large extracellular domain TLRs recognize their ligands.

TLR ligands are called Pathogen Associated Molecular Patterns (PAMPs). TLRs are expressed either extracellularly or intracellularly and this also determines the type of ligand they detect (33). The extracellular TLRs (TLR1, 2, 4, 5, and 6) recognize microbial products that are on the surface of invading pathogens. TLR1, 2, and 6 recognize lipopeptides from the cell wall of Gram positive bacteria and TLR4 is activated by lipopolysaccharide present in the cell wall of Gram negative bacteria. TLR5 is a specific TLR to detect flagellin on bacteria. TLRs expressed intracellularly (TLR3, 7, 8, 9) are receptors that sense RNA and DNA from viruses or bacteria and TLR11 detects an intracellular protein from Toxoplasma gondii (34). For the remaining TLRs (TLR10, 12, and 13) no function or ligand has been described yet.
Besides ligands derived from bacteria and viruses many studies have indicated that TLRs can also be activated by endogenous proteins or oligosaccharides. These are named Damage Associated Molecular Patterns (DAMPs), as they are released from damages tissue (35;36). Some endogenous ligands belong to a group of proteins called Alarmins or acute phase proteins, such as S100 proteins and serum amyloid A proteins (37;38). These are produced in large amounts during inflammation at the site of inflammation or systemically by the liver. In addition, DAMPs can be released upon tissue injury and necrosis (39). For instance heat shock proteins which normally reside intracellularly are released from necrotic cells and can subsequently stimulate TLRs. Also breakdown products of extracellular matrix have been shown to stimulate TLRs (40). Fragments of hyaluronan and fibronectin are capable of activating TLR4 and, interestingly, these breakdown products are also present in the inflamed joints of RA patients (41-43).

**Toll-Like receptor activation and intracellular signaling**

TLR activation requires dimerization to induce subsequent signaling (44). TLR2 dimerizes with either TLR1 or TLR6 in order to recognize lipoproteins, whereas other TLRs form a homodimer upon ligand binding (45). TLR4 activation requires a more orchestrated sequence of events (46). LPS binding protein extracts LPS from more complex structures and transfers it to CD14 present on the cell membrane. CD14 transports the LPS molecules to myeloid differentiation factor-2 (MD-2), which forms a hydrophobic pocket for the LPS molecule. MD-2 with the LPS initiates homodimerization of TLR4 and subsequently induces intracellular signaling.

TLR signaling can be divided in two different signaling pathways, MyD88 dependent and MyD88 independent or TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway (Figure 2) (47). The MyD88 pathway is used by all TLRs except TLR3. Upon TLR dimerization myeloid differentiation primary response gene 88 (MyD88) is recruited to the intracellular TIR domain of the TLR protein and binding of MyD88 requires the adaptor protein Mal (48). The formed complex initiates assembly of IL-1R-associated kinase (IRAK) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) complex (49). This subsequently activates Transforming growth factor β–activated kinase 1 (TAK1) and eventually NF-κB, a pro-inflammatory transcription factor (50), which in turn induces expression of various cyto- and chemokines. The MyD88 independent, or TRIF, pathway is utilized by TLR3 and TLR4 (51). To initiate signaling through the TRIF pathway, TLR4 needs to be internalized into endosomes (52). TLR4 requires the adapter molecule TRIF-related adaptor molecule (TRAM) in order to bind TRIF.
(53), whereas TLR3 can directly bind TRIF. This leads to subsequent activation of Receptor-Interacting Protein 1 (RIP1) and TANK-binding kinase 1 (TBK1), which causes late NF-κB activation (54) or interferon regulatory factor (IRF) activation, respectively. The transcription factor IRF3 induces expression of type I interferons (55).

Figure 2. Toll-Like receptor signaling pathways
Upon ligand binding by myeloid differentiation factor-2 (MD-2) at the cell surface TLR4 forms homodimers and initiates signaling. Myeloid differentiation primary response gene (88) (MyD88) binds through Mal to the intracellular domain of TLR4. This is followed by the formation of a protein complex containing IL-1R-associated kinase (IRAK) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). A central triple MAPKinase, transforming growth factor β–activated kinase 1 (TAK1), is subsequently activated and pro-inflammatory gene transcription is induced by NF-κB. When TLR4 is taken up in an endosome TLR4 can initiate TRIF signaling. Binding of TIR-domain-containing adapter-inducing interferon-β (TRIF) to TLR4 requires TRIF-related adaptor molecule (TRAM) and initiates activation of Receptor-Interacting Protein 1 (RIP1) and TANK-binding kinase 1 (TBK1). These signaling molecules activate the transcription factors NF-κB and IRF3 respectively.
**Toll-Like receptors on antigen presenting cells**

TLRs play an important role in controlling the activity of the innate immunity. APCs sense the microbial products with TLRs and this activates the APC, which in turn leads to activation of the adaptive immunity (Figure 3). APCs take up foreign particles or proteins and after intracellular processing they present parts of the particle to T-cells and a specific immune reaction towards the antigen is started (8). This presentation is termed signal 1 in antigen presentation. Signal 2 for the T-cells is given by the APC in co-stimulatory signals expressed on the cell surface, such as CD80 or CD86. The third signal is formed by the cytokines produced by the APCs and has an important role in controlling T-cell differentiation (56;57). TLR stimulation on APCs leads to enhanced antigen presentation, up regulation of co-stimulatory molecules and increased pro-inflammatory cytokine expression and forms therefore a bridge between the innate and adaptive immunity.

**Figure 3**

Dendritic cells (DC) have cell-cell contact with T-cells through MHC-II with antigen (DC) and the T-cell Receptor (1). Additionally, a co-stimulatory signal is given to the T-cell by the DC through engagement of CD80/86 and CD28 (2). The final instructions given by antigen presenting cells (APCs) is mediated by cytokines (3). These proteins create an environment that determines the T-cell subset that is formed.

**Toll-Like Receptors on non-immune cells**

Besides the important role of TLRs on APCs TLRs are also expressed and functional on non-immune cells, including the cells present in the joint such as endothelial cells, chondrocytes and fibroblasts. Stimulation of TLRs on endothelial cells promotes proliferation of the endothelial cells and subsequent angiogenesis.
(58). TLR stimulation on chondrocytes induces matrix metalloproteinase (MMPs) expression that degrades the extracellular matrix of chondrocytes and thereby causes cartilage erosion (59). Fibroblasts have been perceived as simple bystanders during inflammation for a long time. Over recent years, however, it has appeared that fibroblasts are actively involved in joint inflammation and can actually initiate inflammation (60-62). TLR activation on fibroblasts is a potent trigger for cyto- and chemokine production and creates a more inflammatory environment in the joint cavity. In addition, fibroblasts are major contributors to cartilage erosion by producing MMPs during inflammation (63). This illustrates that TLRs are also important on non-immune cells and could play an important role in innate joint inflammation.

Figure 4. Putative inflammatory loop in rheumatoid arthritis
Cells in the joint are activated through TLR stimulation (1). This leads to the production of cyto- and chemokines that will draw inflammatory cells to the joint and initiates joint inflammation. During this inflammatory process the adaptive immunity will be involved by formation of antigen specific antibodies and activation of T-cells (2). This will result in joint destruction and endogenous TLR ligands (DAMPs) are released from this process (3). These DAMPs, and potential exogenous TLR ligands (PAMPs), can restimulate the TLRs on cells in the joint and the infiltrated inflammatory cells causing further aggravation of the arthritis and joint destruction.
**Putative perpetuating cycle of inflammation involving Toll-Like receptors**

Toll-Like receptors appear to play a role in RA. TLRs are potentially involved in sustaining the inflammatory loop in chronic inflammation (Figure 4) (64;65). Either PAMPs or DAMPs trigger TLR activation and initiate an inflammatory response. After the first response the adaptive immunity will be involved and antigen specific T- and B-cells arise, increasing inflammation and joint destruction. During this process TLR stimulating ligands are generated by degradation of extracellular matrix or released from necrotic cells, such as fibronectin (40), heat shock proteins (36), hyaluronic acid fragments (35), and supposedly serum amyloid A proteins (66). These products in turn stimulate TLRs, further enhancing inflammation and increasing joint destruction.

**Toll-Like receptors in rheumatoid arthritis**

An increasing body of evidence indicates a role of TLRs in rheumatoid arthritis. TLR2 and TLR4 have been found in arthritic synovium and their expression is elevated compared to healthy controls (67-69). In addition to that, TLR7 and TLR8 expression on macrophages and monocytes was found to be increased in RA patients (70). Besides increased expression of TLRs in synovium, these receptors are also functional. TLR2 activation increased chemokine expression (71) and stimulation of TLR2 and TLR4 on synovial tissue also shows increased response by producing increased levels of pro-inflammatory cytokines (72), which possibly fuels the inflammation. In addition, TLR2 and TLR4 stimulation induces RANKL expression in synovial fibroblasts, promoting osteoclastogenesis and subsequent bone erosion (73). Finally, spontaneous cytokine release from synovial explants can be decreased by over expressing a TLR signaling inhibitor (74;75) or by blocking TLR2 or TLR4 (76;77).

In addition to the *ex vivo* experiments with patient material, a study performed in patients with recent onset of RA has shown that these patients are TLR4 hyperresponsive (78). Moreover, it was shown that patients with a mutation in TLR4, causing TLR4 hyper-responsiveness, did not respond to single disease-modifying anti rheumatic drugs (DMARD) therapy and required combination DMARD therapy (79). Unfortunately, the exact role of TLRs in the pathogenesis of RA remains to be determined. This will be rapidly clarified when specific TLR inhibitors become available for patient treatment.

**Toll-like receptors in experimental arthritis**

In experimental arthritis models the role of TLRs in the pathogenesis has been extensively studied. The use of gene knock out animals has given a good insight
into the role of TLRs in disease progression. This has revealed that TLR2 is involved in joint inflammation induced by bacterial ligands injected directly into the joint cavity (80). Moreover, joint inflammation induced by intra-articular injections of zymosan is also partially TLR2 mediated (81). However, further investigation revealed that TLR2 deficiency in a systemic T-cell driven arthritis model increased inflammation and arthritis due to lack of proper inhibitory regulatory T-cell development (77). Other TLRs, such as TLR3 and TLR7, are possibly also involved in chronic experimental arthritis involving the adaptive immunity (82-84). Interestingly, TLR expression is regulated by IL-17 (85). This important T-cell derived cytokine in arthritis therefore creates a feed forward loop between IL-17 and TLR expression that can promote IL-17 driven processes.

Toll-Like receptor 4 in experimental arthritis

TLR4 has attracted considerable attention over recent years as a pivotal receptor in inflammation and immune activation. TLR4 has been shown to be particularly important in generating Th17 cells. Lack of TLR4 in the spontaneous IL-1Ra-/- arthritis model showed that TLR4 is involved in aggravating arthritis and promotes the development of Th17 in this T-cell driven arthritis (77). This was confirmed in another autoimmune model of arthritis in which Th17 levels were decreased when TLR4 was (71;86). The importance of TLR4 on APCs in experimental arthritis is further illustrated by animals lacking a natural TLR4 inhibitor (RP105) on APCs, that show increased arthritis and joint pathology (87), TLR4 blockade also showed therapeutic efficacy and reduced joint pathology (88). Taken together, these data suggest an important role of TLR4 in aggravating arthritis by increasing systemic Th17 levels and mediating joint destruction.

Controlling negative feedback on inflammation

Inflammatory signals elicit also negative feedback to limit progression and detrimental effects of inflammation. Besides several factors known to interfere with either receptor ligand interactions or signaling, a newly described pathway has attracted attention. This pathway involves a complex event that can be divided in three parts (89). First, an initial inflammatory trigger via TLRs induces expression of pro-inflammatory cytokines and type I interferons (90). Second, these products subsequently stimulate cells in an auto and paracrine manner inducing receptor expression of the TAM family, consisting of Tyro3, Axl, and Mer (89). And lastly, stimulation of these TAM receptors by their natural ligands Growth arrest specific 6 (Gas6) and Protein S (Pros1) induces expression of suppressor of cytokine signaling (SOCS)1 and 3 (89;91). Both proteins are known to down regulate inflammation by interfering with downstream TLR signaling (92). SOCS1 directly interacts with Mal, blocking TLR2 and TLR4 signaling (93), whereas SOCS3 affects
the TRAF6 and TAK1 complex (94). Both events prevent further downstream signaling and subsequent activation of pro-inflammatory transcription factors, such as NF-κB (95;96). The relevance of SOCS3 in experimental arthritis has been shown before by ectopic overexpression of SOCS3 in APCs, which ameliorated CIA and impaired T-cell development (97).

Therefore the TAM receptors form a potential new therapeutic tool to treat arthritis. Activation of these receptors does not block all pro-inflammatory activity of the immune system, but enhances the negative feedback that is potentially lost in autoinflammatory and autoimmune diseases. This enhancement can possibly halt progression of disease while not interfering with the natural host defense mechanisms.

**Aim and outline of this thesis**

The role of TLRs has been established in experimental animal models of arthritis and more evidence is gathered that TLRs and TLR activation play a role in RA. Further investigation on which cell compartment and where, systemic vs local, TLRs plays a dominant role is necessary for a complete understanding of the role of TLRs in arthritis. The TLR4 signaling pathways pose promising targets and by using gene therapy and knockouts we have studied the role of two TLR4 signaling pathways; the MyD88 dependent and the TRIF pathway. With this understanding novel methods to interfere with TLR4 activation were developed and tested.

To identify in which cell compartment TLR4 plays a dominant role in experimental arthritis bone marrow transplantation was performed to distinguish between bone marrow derived cells and resident cells (chapter 2). Interestingly, TLR4 expression in both compartments was necessary to develop full blown arthritis. This important role of TLR4 expression locally in the joint was confirmed in a study where IL-1 was overexpressed intraarticularly. Compared with wild type animals, TLR4 deficient mice showed reduced joint destruction, indicating a role of TLR4 in the local inflammatory process (chapter 3). Locally TLR4 is involved in a putative inflammatory feedback loop in which endogenous ligands for TLR4 play a crucial role. We have shown that a commonly used putative endogenous ligand, serum amyloid a, has inflammatory capacities due to minor amino acid changes, while its natural counterpart has only marginal effects (chapter 4). TLR4 is commonly regarded as a key receptor in determining the antigen presenting cell (APC) activity. For that reason we attempted to knock down TLR4 on splenic APCs and thereby modulating the adaptive immunity during arthritis.
Remarkably, knock down of TLR4 splenic APCs did not ameliorate experimental arthritis, but caused an unexpected slight increase in arthritis (chapter 5).

Several TLR4 signaling pathways have been studied. TLR4 signaling involves TGF-β activated Kinase 1 (TAK1) activation and results in activating the pro-inflammatory transcription factor NF-κB. Here, we found that TAK1 is crucial in the monocyte and macrophage population and that TAK1 inhibition in these cells ameliorates experimental arthritis (chapter 6). However, TAK1 is not a suitable target to block TLR4 signaling in rheumatoid arthritis synovial fibroblasts (chapter 7). TRIF signaling is largely unstudied and supposedly anti-inflammatory (98). Therefore, we determined the role of this pathway in T-cell dependent flare-up of experimental arthritis. Interestingly, TRIF appears to play a proinflammatory role during this T-cell dependent flare of arthritis (chapter 8).

A new successful approach to block TLR signaling and treat arthritis is described (chapter 9). This new approach is focused on increasing negative feedback on inflammation rather than blocking proinflammatory signals. This study shows that stimulation of a recently discovered family of receptors, the TAM receptors, that regulate TLR signaling can ameliorate arthritis.

Finally, the results of this thesis are summarized and the implications of the results are discussed (chapter 10).
Chapter 1

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Toll-like receptor 4 on bone marrow derived cells as well as on tissue resident cells participates in aggravating auto-immune destructive arthritis

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Abstract

A prominent role of Toll-like receptor 4 (TLR4) in arthritis is emerging. TLR4 is functional on immune cells and stromal cells. The aim was to investigate the involvement of TLR4 on bone marrow (BM) derived and resident cells in arthritis. Reciprocal sex-mismatched BM transplantation was performed between IL-1Ra/-/TLR4+/+ and IL-1Ra/-/TLR4/- double knockout animals in Balb/c background. Arthritis was assessed macroscopically and by histopathology. Immunity was evaluated by splenic cytokine production and flow cytometry on draining lymph node (DLN) cells. Arthritis progression was reduced to similar extent in animals lacking TLR4 on BM-derived, resident cells, or both. Histology revealed that joint inflammation was partially TLR4 dependent on either BM-derived or resident cells. TLR4 plays an additive role on BM-derived and resident cells in promoting cartilage erosion. In contrast, TLR4 was equally important on BM-derived and resident cells in mediating bone erosion. Systemically, TLR4 on both BM-derived and resident cells contributed to IL-17 production by splenic T-cells, whereas in the draining lymph nodes of arthritic joints this was not the case. Interestingly, in DLN the dominant cells producing IL-17 were CD4 negative and cell numbers were determined by TLR4 on the BM-derived cells. TLR4 is necessary on both BM-derived and resident cells for full-blown joint swelling, inflammation, and bone erosion. Furthermore, TLR4 on BM-derived and tissue resident cells show an additive effect in cartilage destruction. Interestingly, TLR4 on BM-derived and tissue resident cells are both required for IL-17 production in spleen, whereas only on BM-derived cells in DLN.
Introduction

Rheumatoid arthritis (RA) is an autoimmune disease culminating in inflamed joints. Chronic inflammation will lead to destruction of bone and cartilage in the joint and severely impair mobility. Although the initiating factor of RA is unknown, several mechanisms have been found to drive inflammation, such as Toll-like receptors (TLR). Expression of TLR2 and TLR4 has been found in synovial tissue of RA patients (1). Moreover, spontaneous cytokine release from synovial explants is partly mediated by TLR4 (2;3).

TLRs comprise a family of 13 different receptors that belong to the pattern recognition receptors and are involved in the innate immunity by recognizing pathogen associated molecular patterns (PAMPs) present on microorganisms. Upon receptor ligation, antigen presenting cells (APCs) upregulate co-stimulatory molecules on the cell surface and release cytokines, which act as second and third signals in T-cell activation. Therefore, TLRs play a crucial role in controlling T-cell differentiation and proliferation and form the bridge between the innate and adaptive immunity.

Besides hematopoietic cells, TLRs are also present and functional on non-immune cells. For instance, TLR function on endothelial cells has been shown to be involved in angiogenesis (4) and upregulation of adhesion molecules (5). Moreover, TLR4 expressed on endothelial cells only was sufficient for neutrophil influx and bacteria clearance (6). In addition, TLRs on chondrocytes has been implicated in mediating its own degradation by induction of matrix degrading enzymes (7). TLR expression and function on synovial fibroblast has been extensively studied and provided evidence that TLRs play a role on fibroblasts in the pathogenesis of RA (8-10).

In addition to recognizing microorganisms, TLRs can also be activated by endogenous ligands. These damage associated molecular patterns (DAMPS) and alarmins are released during inflammation, necrosis, or in response to tissue injury. Several of these DAMPS have been found in the arthritic joint, such as fibronectin (11), heat shock proteins (12), and breakdown products of hyaluronic acid (13). This could lead to an inflammatory loop where the breakdown products of inflammation stimulate TLRs and maintain inflammation.

Previous studies have demonstrated that TLR4 plays an important role in experimental arthritis. Blocking of TLR4 by *B. quintana* LPS decreased arthritis severity and joint pathology in a prophylactic and therapeutic manner (14). Further investigation of a spontaneous arthritis model, the IL-1 receptor antagonist (IL-
1Ra) knock-out mice, showed that lack of TLR4 did not affect disease incidence, but reduced Th17 levels before disease onset and diminished arthritis severity in late stages of disease (15). This is in agreement with recent studies indicating a relevant role of Th17 cells in RA pathogenesis (16). These studies showed that TLR4 is not involved in onset of arthritis, but plays an important role in aggravating experimental arthritis (14;15;17).

TLR4 is expressed on immune and non-immune cells. Therefore, we set out to identify in which cellular compartment TLR4 plays a dominant role in experimental arthritis. To this end, a sex-mismatched reciprocal bone marrow transplantation between IL-1Ra knockout and IL-1Ra/TLR4 double knockout animals in Balb/c genetic background was performed. This enabled to discriminate between bone marrow derived cells, as a major source of APCs, and resident cells in the joint, such as endothelial cells, fibroblasts and chondrocytes. This will further pinpoint the role of TLR4 in arthritis. Here, we show that TLR4 on both BM derived cells and resident cells is necessary to obtain full-blown experimental arthritis. TLR4 is involved in both cellular compartments in mediating joint destruction and IL-17 production by splenic T-cells. In draining lymph nodes, however, TLR4 on BM-derived cells appears to be crucial in generating CD4+ IL-17 producing cells.

Material and Methods

Animals

IL-1Ra/- animals in Balb/c genetic background were kindly provided by M. Nicklin and were generated as previously described (18) and the IL-1Ra/-/TLR4/- were generated as reported before (15). TLR4/- animals were kindly provided by S. Akira (19). Animals were housed in filter top cages with food and water ad libitum. During transplantation, one week before bone marrow transplantation, and until ten days after transplantation, animals were caged in individually ventilated cages. Age matched littermates were used in all experiments. Animal studies were approved by the Institutional Review Board of Radboud University Nijmegen and were performed according the appropriate codes of practice.

Bone marrow transplantation

Age-matched and sex-mismatched bone marrow transplantsations were performed as described previously (20). Ratio female-male recipients was on average 47-53% among groups. Briefly, one week before transplantation mice received ciprofloxacin (80 mg/l) and sucrose (6 g/l) in drinking water, until 10 days after transplantation. Mice were irradiated with 7.5 Gy at four weeks of age.
followed by intra venous injection of ten million bone marrow cells, harvested from femurs and tibias.

**Y-chromosome staining**

Bone marrow cells were flushed from femurs and forced through 70 µm mesh to yield single cell suspension. Cells were taken up in hypotonic 0.57% KCl solution for 8 minutes at 37°C followed by fixation in ice cold methanol-acetic acid. Cells were dropped on glass slides and Y-chromosome was detection using a biotinylated probe (Starfish, Cambio, UK), according to protocol of manufacturer. Briefly, probe was denatured for 10 minutes at 65°C en kept at 37°C for 60 minutes. Slides were dehydrated by serial ethanol washing followed by 1.5 minute denaturing in 70% formamide. Subsequently, slides were quenched in ice cold 70% ethanol for 4 minutes and dehydrated by serial ethanol washing. Probe was incubated overnight on the slides in a humidified chamber at 37°C. The probe was detected with streptavidin-FITC and cells were counterstained with DAPI. For each slide over 100 cells were counted in multiple areas of the slide and positive number of cells was expressed in percentages.

**Clinical evaluation of arthritis**

Development of arthritis was macroscopically scored using an arbitrary scoring system as follows: 0, no redness or swelling; 0.25, slight redness; 0.5 slight redness and swelling; 0.75-1.0, mild redness and swelling; 1.25-1.5, moderate redness and swelling; 1.75-2, severe redness and swelling. Only ankle joints that developed arthritis were evaluated.

**Histology**

Ankle joints were isolated and fixed in 4% paraformaldehyde for minimally 8 days followed by decalcification in 5% formic acid and subsequently embedded in paraffin. Hematoxylin and eosin staining of 7 µm sections were used to determine inflammation (score 0-5) in a blinded manner, at least two section per joint. Safranin O and fast green staining was used to score cartilage destruction, on a scale from 0 to 5 in a blinded manner. At least 4 cartilage surfaces of three sections per joint. Bone erosion was scored on Safranin O stained sections in a blinded manner and at least 10 bone surfaces were scored in at least two sections per joint.

**Cell isolation, purification, stimulation**

At 25 weeks of age mice were sacrificed and cells were isolated. Peritoneal macrophages were isolated by lavage of the peritoneum with ice cold phosphate
buffered saline (PBS). Spleens were forced through 70 µm mesh and after erythrocyte lysis by osmotic shock. Cells (200,000 per well) were stimulated in RPMI 1640 (Invitrogen) with penicillin/streptomycin, pyruvate, and 100 µg/ml LPS. Spleens were forced through 70 µm mesh and after erythrocyte lysis by osmotic shock. CD11b+ cells were isolated using specific MACS CD11b+ beads (Miltenyi, Utecht, The Netherlands). T-cells were subsequently isolated with anti-CD3 MACS beads (Miltenyi, Utecht, The Netherlands). Cells (200,000 per well) were stimulated in RPMI 1640 (Invitrogen) with 10% FCS, penicillin/streptomycin, pyruvate, 50 ng/ml PMA, 1 µg/ml Ionomycin. Draining lymph nodes were extracted from knees and groins and a single cell suspension was obtained by forcing lymph nodes through 70 µm mesh and cultured in RPMI 1640 (Invitrogen) supplemented with 5% FCS penicillin/streptomycin, pyruvate, 50 ng/ml PMA, 1 µg/ml Ionomycin, and Brefeldin A (BD Pharmingen).

RNA isolation and quantitative PCR analysis

Total RNA was extracted from cells using TRI reagent (Sigma) according to manufacturer’s protocol. Isolated RNA was treated with DNase followed by reverse transcription of 1 µg RNA into cDNA using Moloney murine leukemia virus reverse transcriptase 0.5ug/µl ologo(dT) primers, and 12.5 mM dNTPs (Invitrogen). Quantitative real-time PCR was performed using the StepOnePlus sequence detection system (Applied biosystems, Foster City, CA) PCR was performed in a total reaction volume of 12.5 µl consisting of appropriate cDNA. Five µM of forward and reverse primer and the SYBR green PCR master mix (Applied biosystems). PCR protocol consisted of 2 min at 50°C and 10 min of 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Quantification of PCR signals was achieved by calculating the difference between the cycle threshold value (Ct) of the gene of interest with the Ct value of their reference gene glycerldehyde-3-phophate dehydrogenase (GAPDH) for each sample (delta Ct) and expressed as relative mRNA expression (2^-ΔCt).

Flow cytometric analysis DLN cells

Stimulated DLN cells (4 hours) were stained with anti-mouse CD4-APC (1:200) (BD Pharmingen) or IgG2a-APC controle (BD pharimingen) for 30 minutes at 4°C. Subsequently, cells were stained intracellularly with anti-IL17-FITC and anti-IFNγ-PE or with their controls IgG1-PE and IgG1-FITC, respectively, according to instructions by manufacturer (BD Pharmingen). Stained cells were analyzed using FACScalibur (Becton Dickinson) and analyzed with FlowJo software.
**Cytokine measurements**

IL6, IL-17A, and IFNγ levels in culture supernatants were measured on a Luminex-100 System (Luminex corp.) using a bead-based multiplex immunoassay (Milliplex, Merck Millipore). Data analysis was performed with Bio-Plex Manager software (Bio-Rad Laboratories).

**Statistics**

Statistical differences were determined by one-way ANOVA and appropriate post-test in GraphPad 5.0 software, p values below 0.05 were considered significant.

**Results**

**Complete bone marrow reconstitution and functional TLR4 chimeras**

BM engraftment efficiency was determined by staining BM-derived cells with a Y-chromosome probe at time of sacrifice. Eighty percent of cells stained positive for the Y-chromosome probe in a female recipient of male BM (Figure 1A), whereas no cells contained a Y-chromosome in male recipients that received female bone marrow (Figure 1A-B). Reconstitution with TLR4⁻/⁻ BM abolished the LPS response of peritoneal macrophages by 87% (Figure 1C). Conversely, TLR4⁺/⁺ BM restored the LPS response in peritoneal macrophages of TLR4⁻/⁻ recipients (Figure 1C). These results indicate nearly complete reconstitution of the BM and functional TLR4 chimeras.
TLR4 on BM and resident cells contribute equally to arthritis

Arthritis incidence was monitored after BM transplantation (Figure 2A) and ranged from 50% to 87% between groups. Animals that expressed TLR4 on both resident and BM derived cells showed increased arthritis severity as compared to the animals that lacked TLR4 on either BM derived cells, resident cells, or both cell compartments (Figure 2B). This indicates that TLR4 on BM and resident cells are equally important in the progression of joint inflammation in this spontaneous poly-articular arthritis model.

**Figure 2**

![Figure 2](image)

**Figure 2. Arthritis incidence and severity of TLR4 chimeras**

Arthritis development was evaluated over time by macroscopic scoring of the ankle joints. A) Disease incidence of bone marrow chimeras. B) Arthritis severity of bone marrow chimeras, arbitrary score of positive ankle joints (scale 0-2). Statistics: Data represented as mean + SEM. Two-Way ANOVA, with Bonferroni post test, compared to TLR4+ host/TLR4+ BM (* p< 0.05, ** p<0.01, *** p<0.001). n: TLR4+ host/TLR4+ BM = 9, TLR4+ host/TLR4- BM = 9, TLR4- host/TLR4+ BM = 13, TLR4- host/TLR4+ BM = 6.

TLR4 on BM and resident cells mediates joint pathology

To evaluate the contribution of TLR4 on BM-derived and resident cells to inflammation, and cartilage and bone erosion, ankle joints were evaluated histologically. Only a trend was observed in the inflammation score of ankle joints from animals in all groups in which TLR4 was lacking (Figure 3A). Cartilage erosion, however, was significantly reduced in complete TLR4 knockout animals (Figure 3B). This points towards an additive contribution for TLR4 on both resident and BM derived cells in cartilage degradation, since both chimeras showed an intermediate degree of cartilage erosion. Bone erosion was significantly
TLR4 participates on BM-derived and resident cells in experimental arthritis

diminished when TLR4 was absent on either BM-derived cells, resident cells, or both and this suggests a crucial role of TLR4 expression on BM and resident cells together for bone erosion in experimental arthritis (Figure 3C). Figures 3D-G show representative histopathological images of the influence of TLR4 on either cellular compartment on the severity of joint inflammation and destruction.

**TLR4 controls macrophage IL-6 and T-cell IL-17 production during arthritis**

To determine the effect of TLR4 chimerism on innate immune cell activation during arthritis, splenic CD11b+ macrophages were isolated at 25 weeks of age and stimulated with PMA and ionomycin for imprinted cytokine production. No differences were observed in IL-1β, IL-23, or TGFβ mRNA expression in CD11b+ macrophages. However, Figure 4A shows that TLR4 on BM derived cells is responsible for IL-6 production by CD11b macrophages in the spleen, as a clear trend in IL-6 production can be observed. This suggests that TLR4 is involved in controlling IL-6 production by splenic macrophages. To examine whether this altered APC activity affected adaptive immunity, CD3+ cells from spleen were isolated at 25 weeks of age and IFNγ and IL-17A production was determined on mRNA and protein level. Figure 4B shows that IFNγ was only marginally affected by the absence of TLR4 on BM or resident cells, however a clear trend was observed in complete TLR4 knockout animals. Total lack of TLR4 caused a strong reduction of 78% in IL-17 production by splenic T-cells (Figure 4C). Again, the chimeric animals showed an intermediate level of IL-17 production. This suggests an additive contribution for TLR4 on both resident and BM derived cells in differentiation of splenic T-cells toward the Th17 subset.

**TLR4 on BM-derived cells is responsible for CD4−IL-17A+ cells in draining lymph nodes**

Cells from draining lymph nodes (DLN) were isolated and analyzed for the number of IFNγ and IL-17A producing cells. Similar to the effects on IFNγ production found in the spleen, Th1 levels were slightly decreased in complete TLR4 knockouts in DLN and also CD4+/IFNγ+ cell numbers were marginally decreased when TLR4 was absent on BM derived cells (Figure 5A). In contrast, all chimeras showed a slight decrease in the number of Th17 cells in DLN compared with complete TLR4+/+ animals (Figure 5B). Interestingly, TLR4 appears to be responsible for promoting CD4+/IL-17A producing cells in DLN. Animals in which BM-derived cells lacked TLR4 showed significantly reduced levels of CD4+/IL-17A+ cells in DLN, whereas a TLR4−/− host with TLR4+/+ BM showed an intermediate level of CD4+/IL-17− cells (Figure 5B). This indicates a crucial role of TLR4, in particular on BM-derived cells, in the development of CD4− IL-17A producing cells at the site of inflammation.
Figure 3. Histological analysis of ankle joints
Knee joints were isolated and fixed in paraformaldehyde and subsequently embedded in paraffin. 7 µm sections were stained with hematoxylin/eosin (inflammation) or safranin-o/fast green (cartilage and bone erosion). A) Microscopic score of inflammation (scale 0-5), infiltrate and exudate combined. B) Microscopic score of cartilage erosion (scale 0-5). C) Microscopic score of bone erosion (scale 0-5). Data represented as mean ± SEM. Statistics: One-Way ANOVA with Bonferroni post test (* p<0.05). n: TLR4+ host/TLR4+ BM = 11, TLR4+ host/TLR4- BM = 13, TLR4- host/TLR4 BM = 20, TLR4- host/TLR4+ BM = 12. D-G) Representative histological pictures of safranin O stained sections for determining bone and cartilage erosion. Magnification: 200x. Closed arrow heads: cartilage erosion, open arrow heads: bone erosion. Arbitrary scoring scales used in this experiment; inflammation, most infiltrated synovium obtained a score of 5; cartilage erosion, complete erosion is scored 5; bone erosion, complete breach of bone is scored 5.
Discussion

Previous publications point at a role for TLR4 in sustaining the putative inflammatory loop in the chronic phase of arthritis (21). To further pinpoint the role of TLR4 in experimental arthritis we performed a BM transplantation to discriminate between bone marrow derived and resident cells.

In this study we found that TLR4 activation on both the resident cells and BM-derived cells is important in the chronic phase of experimental arthritis. This revealed a complex interplay between BM-derived cells and resident cells in generating IL-17 producing T-cells and TLR4-mediated joint pathology.

The decrease in arthritis severity in BM chimeras where TLR4 was lacking indicates that TLR4 is involved on both BM derived cells and resident cells in controlling inflammation. Lack of TLR4 on the BM derived inflammatory cells in the inflamed joint possible causes reduced activation by endogenous ligands derived from the destructive inflammation. This reduced activation will subsequently lead to reduced recruitment of other inflammatory cells into the joint. TLR4 on resident cells on the other hand, can also control chemokine production and fibroblasts are likely candidates for production of chemokines upon TLR4 stimulation. Additionally, fibroblasts are also important in cartilage breakdown by forming pannus tissue and producing matrix metallo-proteinases (MMPs). In contrast to inflammation, TLR4 appears to play an additive role on BM derived cells and resident in cartilage breakdown. TLR4 activation is an important proinflammatory signal for fibroblasts (22) and chondrocytes (23) to produce MMPs, but also macrophages contribute to the MMPs present at the site of inflammation (24).

It appears that both the BM cells and the resident cells produce factors crucial in controlling osteoclast activity. Likely candidates are IL-6 by BM cells and TGF-β by resident tissue cells. Both these factors are also involved in development of Th17 (25;26) and this T-cell subset is strongly correlated with bone erosion (27). In accordance, when TLR4 is lacking on BM cells, resident cells, or both a trend towards decreased Th17 levels was observed in DLN of inflamed ankle joints. This is in line with previous studies showing a crucial role of TLR4 in T-cell differentiation (28).

This mechanism is perhaps also accountable for the results found on IL-17 production by splenic T-cells, since the resident cells also appear, surprisingly, to be important in controlling IL-17 production by splenic T-cells on both mRNA and protein level. Several cytokines are important in developing Th17 cells, such as IL-1β, IL-6, IL-23, and TGF-β. Unfortunately, we were not able to determine the levels of cytokines, such as IL-6 and IL-17, directly at the site of inflammation.
which could have given insight in the local process. Nonetheless, this experiment, and other research groups, has shown that IL-6 is derived from TLR4+ BM cells (29). The IL-1β production was found to be dependent on both the BM and resident cells (29-31) and also the TGF-β production was suppressed when TLR4 is absent on BM, resident cells or both (29). Taken together, it appears that both the factors derived from BM cells and from splenic resident cells are crucial for the development of IL-17 producing cells. It has been shown before that splenic stroma cells are involved in maturing DCs in the spleen (32), and TLR4 on these stromal cells could potentially play a role in maturing the DCs. Therefore, the role of TLR4 is not confined to the BM cell compartment in the systemic development of IL-17 producing T-cells.

Figure 4

Spleen were isolated at 25 weeks of age and CD11b+ and CD3+ cells were selected using MACS beads. Cells were stimulated with PMA and ionomycin for 24 hours. A) IL-6 mRNA expression and IL-6 protein production by CD11b+ cells isolated from spleen upon stimulation with PMA/ionomycin. B-C) IFNγ and IL-17A mRNA expression and protein expression by purified splenic CD3+ cells upon stimulation with PMA/ionomycin. Data represented as mean ± SEM. Relative expression: 2^{-dcT}. Group size IL-6 measurements: TLR4+ host/TLR4+ BM (n= 4 mRNA, n=10 protein), TLR4+ host/TLR4- BM (n= 7 mRNA, n=11 protein), TLR4- host/TLR4 BM (n= 3 mRNA, n=13 protein), TLR4- host/TLR4+ BM (n= 6 mRNA, n=11 protein). Group size IFNγ/IL-17A measurements: TLR4+ host/TLR4+ BM (n= 4 mRNA, n=5 protein), TLR4+ host/TLR4- BM (n= 5 mRNA, n=7 protein), TLR4- host/TLR4+ BM (n= 6 mRNA, n=6 protein), TLR4- host/TLR4+ BM (n= 6 mRNA, n=9 protein).
Locally in the DLN we did find an essential role of TLR4 on BM derived cells in generating IL-17 producing T-cells, but not the Th17 cell. The nature of these CD4-IL-17 producing cells in our study remains unclear. Multiple studies have recently shown that also other than CD4+ T-cells can produce IL-17. A subset of CD8+ T-cells, the Tc17 cells, have been described to produce IL-17 and are involved in chemically induced diabetes (33) and psoriatic skin lesions (34). In addition, another T-cell population was found to produce IL-17 but did not express CD4 or CD8. These cells have been found in kidneys of SLE patients (35) and in respiratory infection in mice (36). Cells that are on the border of the innate and adaptive immunity, such as NKT cells, are also capable of producing IL-17. These cells have been shown to produce vast amounts of IL-17 upon CD3 and IL-23 stimulation (37) and in particular the NK1.1- iNKT cells (38). Moreover, the iNKT cells have been shown to be important in experimental arthritis (39). Lastly, the γδ T-cells have been shown to produce IL-17 in response to mycobacterium infection (40) and IL-23 alone is sufficient to induce IL-17 production in these cells. Interestingly, IL-23R+ IL-17 producing γδ T-cells have been found in increased numbers in ankylosing spondylitis patients (41). Moreover, in arthritic mice the Vγ4+ γδ T-cells were particularly important in collagen-induced arthritis (42). Most importantly, unpublished observations showed us that 80% of IL-17 producing cells in the DLN of IL-1Ra-/- animals are γδ-TCR positive. This points towards an important role of this innate T-cell in IL-1Ra-/- animals. Whether the CD4- IL-17 producing cells in our BM chimeras are the γδ T-cells remains to be determined, but in any case it appears that TLR4 is important in controlling the IL-17 production by CD4- cells.

The net effect on clinical outcome of TLR4 absence on BM cells, resident cells, or both was equal in our disease model. In other models a similar phenomenon was found as clinical disease outcome in infective pyelonephritis, abscess formation, was equal in bone marrow chimeras and total TLR4<sup>−/−</sup> animals (43). Moreover, an alcohol induced liver damage model also showed similar liver damage when TLR4 was lacking on the BM, residents cells or both (29). TLR4 probably plays different roles on BM and resident cells, nevertheless the net clinical outcome is identical. On BM cells TLR4 probably controls macrophage and dendritic cell activation and thereby controlling IL-17 production by CD4+ cells in the joint. On resident cells TLR4 is possibly involved in regulating adhesion molecules on endothelium and cytotoxic, and chemokine expression by fibroblasts, which will determine leukocyte migration and extravasation into the joint as well as inducing TGFβ to drive Th17 differentiation. Despite different mechanisms and involvement of TLR4 on different cell types, the net outcome on disease severity is similar.
In summary, we have shown an equally important role of TLR4 on BM cells and resident cells in joint swelling and joint pathology of experimental arthritis. BM cells and resident cells both determine IL-17 production by splenic T-cells. However, BM cells are crucial in the development of CD4- IL-17 producing cells that requires further identification. This study shows that TLR4 inhibition on specific cell types could be used to ameliorate arthritis and potentially decreases side effects.

Figure 5. Flow cytometry of draining lymph node cells
A) Cells from DLN of the ankle joints were isolated at 25 weeks of age, with full-blown arthritis (endpoint) and stained for CD4 (extracellular) and IFNγ (intracellular). B) Cells from DLN of ankle joints stained for CD4 (extracellular) and IL-17A (intracellular). C) Representative FACS-plots of DLN cells gated negative for CD4 of three separate experiments and measurements. Data represented as mean ± SEM. Statistics: Two-Way ANOVA with Bonferroni post test (** p<0.01, *** p<0.001). n: TLR4+ host/TLR4+ BM = 8, TLR4+ host/TLR4- BM = 6, TLR4- host/TLR4- BM = 7, TLR4- host/TLR4+ BM = 6.
References


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Chapter 3

Local interleukin-1 driven joint pathology is dependent on Toll-like receptor 4 activation

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Abstract

Toll-like receptors (TLRs) may contribute to the pathogenesis of chronic inflammatory destructive diseases through recognition of endogenous ligands produced upon inflammation or degeneration of extracellular matrix. The presence of endogenous TLR agonists has been reported in rheumatoid joints. In the present study, we investigated the significance of TLR2 and TLR4 activation by locally-produced endogenous ligands in the severity of joint inflammation and destruction. Local joint pathology independent of systemic immune activation was induced by overexpression of IL-1β and TNF in naïve joints using adenoviral gene transfer. Here we report that at certain doses IL-1-induced local joint inflammation, cartilage proteoglycan depletion and bone erosion is dependent on TLR4 activation, whereas TLR2 activation is not significantly involved. In comparison, TNFα-driven joint pathology seemed to be less dependent on TLR2 and TLR4. The severity of IL-1-induced bone erosion and irreversible cartilage destruction was markedly reduced in TLR4−/− mice, even though the degree of inflammation was similar, suggesting uncoupled processes. Furthermore, the expression of Cathepsin K, a marker for osteoclast activity, induced by IL-1β was dependent on TLR4. Overexpression of IL-1β in the joint as well as ex vivo IL-1 stimulation of patellae provoked the release of endogenous TLR4 agonists capable of inducing TLR4-mediated cytokine production. These data emphasize the potential relevance of TLR4 activation in rheumatoid arthritis, particularly with respect to IL-1 mediated joint pathology.
Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent joint inflammation and concomitant cartilage and bone destruction. Despite intensive research, many features of the immunopathology of RA are yet to be explored. The discovery of Toll-like receptors (TLRs) as essential components of the immune system has introduced new candidates to the field of research on the pathogenesis of arthritis. TLRs are a family of evolutionarily conserved transmembrane receptors, which are expressed by a variety of immune cells, including monocytes, macrophages, dendritic cells, neutrophils, B cells and certain types of T cells; however, non-immune cells such as fibroblasts and chondrocytes also express TLRs (1;2). The major function of TLRs is to recognize pathogen-associated molecular patterns (PAMPs), which are highly conserved in evolution and are shared by many microorganisms. At the same time, TLRs show considerable target specificity. For instance, diacylated and triacylated lipoproteins of Gram-positive bacteria are sensed by TLR2 in co-operation with TLR6 and TLR1, respectively; whereas Lipopolysaccharides (LPS) of Gram-negative bacteria are recognized by TLR4 (2).

Signal transduction through TLRs leads to the activation of several transcription factors among which NF-κB and activator protein 1 (AP-1). Thereby, TLR activation controls the expression of a number of proinflammatory cytokines such as Interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF), chemokines such as IL-8 and macrophage inflammatory protein-1, and matrix metalloproteinases (MMPs) all of which are relevant to the pathogenesis of RA (3;4).

Besides PAMPs, TLRs are capable of recognizing endogenous ligands produced or released upon cell stress, inflammation or degradation of extracellular matrix. In this context, TLR4 can recognize some matrix components such as heparan sulphate and extra domain A (EDA) of fibronectin (5;6), while biglycan, hyaluronan fragments, high mobility group box 1 (HMGB1) and some endogenous heat shock proteins activate both TLR2 and TLR4 (7-9). The presence of endogenous TLR ligands such as fibronectin fragments, HMGB1 and heat-shock proteins has been shown in rheumatoid synovium (10-14). It has been reported that rheumatoid synovial fibroblast-like cells synthesize EDA-containing fibronectin (15). Furthermore, some inflammatory cytokines of high interest in the field of rheumatology such as TNFα, IL-1 and IL-6 can induce the expression of HSP70 in cultured synovial fibroblast-like cells (12). Another endogenous TLR4 ligand, the calcium-binding protein S100A8, has also been found in RA synovial membrane (16;17). Considering the highly inflammatory character of RA and the
accompanying damage to the extracellular matrix, other endogenous TLR ligands are also very likely to be present in arthritic joints.

The idea of the involvement of TLR2 and TLR4 in RA is supported by their enhanced expression in blood and synovial cells of RA patients (18-21). In addition, monocyte-derived dendritic cells and synovial macrophages from RA patients are over-responsive to TLR2 and TLR4 stimulation compared to cells from healthy controls or cells from patients with other forms of inflammatory arthritis (22;23). Factors other than the level of TLR2 and TLR4 expression were suggested to contribute to the increased activation. As TLR-mediated inflammatory responses may induce further tissue damage and promote the generation of more endogenous ligands, it has been hypothesized that TLRs can engender a self-sustaining inflammatory loop responsible for chronic progression of inflammation (24;25). Nevertheless, the contribution of endogenous TLR ligands in the joint to local inflammatory and destructive processes has not thoroughly been studied yet. Therefore, we aimed to examine the involvement of endogenous TLR2 and TLR4 activation in joint inflammation, cartilage destruction and bone erosion in vivo.

It is of interest that exogenous TLR ligands including LPS have extensively been used to aggravate or reactivate arthritis in distinct animal models (26-29); however, the worldwide usage of microbial TLR ligands as adjuvants in arthritis models complicates the study of the contribution of endogenous ligands in arthritic process. In the present study, we used an adenoviral-based cytokine-overexpressing system instead of the commonly-used arthritis models to circumvent the necessity of application of exogenous TLR2 and TLR4 ligands for evoking the immune response. TNFα and IL-1β were the cytokines overexpressed locally in the joints. Years of research has implicated prominent roles of these cytokines in arthritis, and their prolonged overexpression in animal joints mimics the inflammatory and destructive processes observed in RA. Since the model used here does not involve systemic or adaptive immune responses, it enables us to specifically study the role of endogenous TLR2 and TLR4 ligands produced at the site of inflammation in the affected joints.

Materials and methods

Animals

Male C57Bl/6 mice were purchased from Janvier, France. TLR2−/− and TLR4−/− mice in C57Bl/6 background were kindly provided by Prof. S. Akira (Osaka, Japan).
The mice were housed in filter-top cages, and water and food were provided ad libitum. Gender-matched animals (10-12 weeks of age) were used in all experiments. Animal studies were approved by the Institutional Review Board and were performed according to the related codes of practice.

**Adenoviral vectors**

AdIL-1β virus was kindly provided by Dr. C. D. Richards (McMaster University, Ontario, Canada) and was engineered as described previously (30). AdTNFα virus was a kind gift from Dr. J.K. Kolls (Children’s Hospital of Pittsburgh, Pittsburg, USA). Virus construction and production was as reported in previous studies (31). The empty viral vector Ad5del70-3 was used as negative control throughout the studies.

**Induction of arthritis using adenoviral transfer of IL-1β and TNFα genes**

Local joint inflammation and destruction was induced in C57Bl/6 WT, TLR2-/− and TLR4-/− mice (n = 6 mice per group) by intraarticular injection of 6 μl saline containing 3.105 plaque forming units (PFU) AdIL-1β or 1.107 PFU AdTNFα virus. 1.107 PFU of the control virus Ad5del70-3 was injected into the contralateral knee joint. In the following studies, the dose of AdIL-1β was enhanced to 3.106 PFU per joint to enforce IL-1β-induced cartilage destruction. Previous reports have validated this adenoviral delivery system as an effective means of cytokine overexpression in synovial tissue (32;33).

**Preparation of patella washouts and measurement of cytokines**

Patellae with surrounding tissue were isolated after intraarticular injection of the viruses. Patella washouts were prepared by culturing the patellae in PRMI containing 0.1% bovine serum albumin (BSA) for 1 hour at room temperature. Cytokine concentrations were determined using the Bioplex cytokine assays from Bio-Rad (Hercules, CA, USA) following the manufacturer’s instructions.

**Histology**

For histological assessment of arthritis, total knee joints were isolated at day 4 of viral transduction and fixed during 4 days in 4% formaldehyde, then decalcified in 5% formic acid and embedded in paraffin. Tissue sections of 7 μm were stained using the Haematoxylin & Eosin to study the inflammatory cell influx or using the Safranin O staining to determine proteoglycan depletion and cartilage and bone destruction. Histopathological changes were scored on a scale from 0 to 3 by two observers in a blinded manner as described previously (26).
Isolation of RNA from synovial biopsies

Synovial biopsies from knee joints were isolated from lateral and medial sides of patellae using a 3 mm punch (Stiefel, Wachtersbach, Germany). Total RNA was isolated in 1 ml TRIzol reagent (Sigma, St. Louis, MO, USA), then precipitated with isopropanol, washed with 70% ethanol, and dissolved in water. RNA was treated with DNase and subsequently reverse transcribed into complementary DNA using oligo(dT) primers and MMLV reverse transcriptase.

Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) for quantification with SYBR Green and melting curve analysis. Primer sequences (forward and reverse, respectively) were as follows: for GAPDH (house-keeping gene), 5’-GGC-AAA-TTC-AAC-GGC-ACA-3’ (forward) and 5’-GTT-AGT-GGG-GTC-TCG-CTC-TG-3’ (reverse); for Cathepsin K, 5’-GAA-GCA-GTA-TAA-CAG-CAA-GGT-GGA-T-3’ (forward) and 5’-TGT-CTC-CCA-AGT-GGT-TCA-TGG -3’ (reverse). 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 during the last 30 seconds. For all PCRs, SYBR Green Master Mix was used in the reaction. Primer concentrations were 300 nM. The threshold cycle (Ct) value of the gene of interest was corrected for the Ct of the reference gene GAPDH to obtain the ∆Ct, then ∆∆Ct was calculated compared to Addel control of mice with the same genotype.

Immunohistochemistry

The presence of active osteoclasts was evaluated by immunohistochemical staining for Cathepsin K on paraffin sections of the knee joints 4 days after viral transduction. The percentage of polymorphonuclear (PMN) and mononuclear cells (MNCs) was evaluated using staining for NIMP-R14 and F4/80 markers, respectively. Sections were deparaffinized in xylol and rehydrated in serial dilutions of ethanol. Endogenous peroxidase was blocked using 1% hydrogen peroxide for 15 minutes. For NIMP-R14, five minutes treatment with 0.1% trypsin/0.1% CaCl2 (pH 7.8) preceded this step. Sections were incubated with the following antibodies for 1 hour: rabbit anti-mouse Cathepsin K antibodies (200 μg/ml, kind gift of Dr. E. Sakai, Nagasaki, Japan), rat anti-mouse NIMP-R14 (500 μg/ml, kind gift of Dr. M. Strath, London, UK) and rat anti-mouse F4/80 (500 μg/ml, AbD Serotec). Rabbit normal IgG (Santa Cruz Biotechnology) and rat IgG2b (BD Pharmingen) were used as negative controls. Subsequently, sections were incubated with biotinylated swine anti-rabbit or rabbit anti-rat antibodies, followed by peroxidase labeled streptavidin. Color was developed
with diaminobenzidine, and tissues were counterstained with Hematoxylin. Staining was performed on two tissue sections per mouse (n = 5 WT and n = 6 TLR4-/− mice). Cathepsin K positive cells were quantified along the bone surfaces in the patella-femur area using Leica Qwin software (Leica Microsystems, Rijswijk, The Netherlands). Mean number of positive cells per 1000 nm² for each mouse is depicted in the graph. Percentage of NIMP-R14 and F4/80 positive cells was scored arbitrarily in two sections per mouse in a blinded manner.

**Assessment of endogenous TLR4 ligands in patella washouts upon IL-1 overexpression or stimulation**

HEK293 and HEK293-mTLR4/MD2/CD14 were purchased from InvivoGen and cultured according to the manufacturer’s guidelines. For stimulations, 5.10⁴ cells/well were used in flat-bottom 96-wells plates. To validate the HEK293-mTLR4 cellular response, cells were stimulated with hIL-1β and hTNFα both 10 ng/ml (R&D), Pam3Cys (10 μg/ml, ECM Microcollections), Poly I:C (25 μg/ml, InvivoGen), LPS (10, 100 and 1000 ng/ml, Sigma) and Gardiquimod (1 μg/ml) for 24 hours. To assess the induction of TLR4 agonists by IL-1 and TNF, one hour patella washouts obtained upon several days of in vivo Addel or AdIL-1β overexpression, or supernatants of patellae ex vivo cultured with IL-1β or TNFα (10 ng/ml each, n = 6 patellae per group) for 24 hours were added to HEK293 and HEK293-TLR4 cells in a volume ratio of 1:10. Assays were performed in triplicate and human IL-8 was measured in culture supernatants as readout using the Bioplex cytokine assays. Where mentioned, 1 μg/ml TNF blocker Enbrel (Amgen, Thousand Oaks, CA) was added to the cultures in order to inhibit the TLR4-independent TNF-mediated response. HEK293-TLR4 cells were preincubated with 1 μg/ml B. quintana LPS as a TLR4 antagonist (34;35) for 30 minutes to specifically inhibit the TLR4-mediated response.

**Statistical analysis**

Group measures are expressed as the mean + SEM. Statistical significance was assessed using the Mann Whitney U test performed on GraphPad Prism 4.0 software (GraphPad software Inc., USA). P values lower than 0.05 were considered significant.
Results

Local cytokine production upon adenoviral gene transfer of IL-1β and TNFα

Local IL-1β and TNFα production was determined after adenoviral gene transfer of these cytokines into the naïve joints of C57Bl/6 WT mice. Intraarticular injection of AdIL-1β or AdTNFα virus in the wild-type mice resulted in the production of high levels of the respective cytokine detectable in the patella washouts one day after gene transfer (Figure 1). Concentrations of both cytokines decreased in time, with low levels being still detectable at day 11 (153.1 ± 20.8 pg/ml IL-1β for AdIL-1 and 120.0 ± 29.8 TNFα for AdTNF). Comparison of the local cytokine concentration in WT, TLR2-/- and TLR4-/- mice revealed that TLR2 and TLR4 gene deficiency did not affect the viral transduction or cytokine production, as the cytokine expression was similar in the three groups (Figure 1). Furthermore, intraarticular injection of a similar dose of the control virus Ad5del70-3 into the joint did not induce detectable levels of IL-1β or TNFα (data not shown).

Joint pathology upon adenoviral overexpression of IL-1β and TNFα

Prolonged expression of IL-1β or TNFα in the knee joints of wild-type mice induced pathological changes in the joint resembling those observed in RA. These included joint inflammation, i.e. synovial hyperplasia and inflammatory cell influx, bone erosion and depletion of matrix proteoglycans (PG) in articular cartilage (Figure 2). The Ad5del70-3 virus used as negative control throughout the experiments sporadically induced very low degree of synovial inflammation, but was unable to cause any sign of cartilage or bone damage in WT or TLR-/- animals (Figure 2A).
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The severity of synovial inflammation, PG depletion and bone erosion in the WT mice was similar for IL-1 and TNF overexpression at the virus doses chosen here. Neither AdIL-1β nor AdTNFα induced erosion of cartilage surface at these doses.

Although IL-1β production upon its adenoviral overexpression was similar in WT and TLR-/- mice (Figure 1), the severity of joint inflammation was significantly reduced in TLR4-/- mice compared to WT mice at the AdIL-1β dose used here (Figure 2B). Immunohistochemical staining for NIMP-R14 and F4/80, markers for polymorphonuclear (PMN) and mononuclear cells (MNCs), respectively, revealed the presence of PMNs and relatively lower numbers of MNCs (Table 1). Despite significant reduction in the extent of synovial inflammation in TLR4-/- mice (Figure 2B), the composition of exudate as well as infiltrate cells remained unchanged (Table 1), suggesting a general effect on various cell types.

In addition to synovial inflammation, joints of TLR4-/- mice exhibited also less severe PG depletion and bone erosion compared to WT mice (Figures 2C and D). TLR2-/- animals tended to have reduced joint inflammation and bone erosion; however, this reduction did not reach statistical significance (Figures 2B and D). Proteoglycan loss in the cartilage of TLR2-/- mice was not affected as well (Figure 2C).

In comparison with IL-1, TNFa-induced joint inflammation remained unaffected in the knockout mice, and PG depletion and bone erosion seemed less dependent on TLR2 and TLR4, as no significant differences were found between WT and TLR-/- mice (Figures 2E-G). Representative images of the joint inflammation and damage upon IL-1β overexpression in WT mice in comparison with TLR2-/- and TLR4-/- mice are shown in Figure 3.

Table 1. TLR4 deficiency does not affect the composition of inflammatory cells in the joint despite significant reduction in the extent of inflammation.

<table>
<thead>
<tr>
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<th>Infiltrate</th>
<th>Exudate</th>
<th>Synovial inflammation</th>
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<tr>
<td></td>
<td>NIMP-R14+ (%)</td>
<td>F4/80+ (%)</td>
<td>NIMP-R14+ (%)</td>
</tr>
<tr>
<td>WT</td>
<td>1.13 ± 0.18</td>
<td>30.0 ± 4.4</td>
<td>18.6 ± 2.6</td>
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<tr>
<td>TLR2-/-</td>
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<td>34.2 ± 5.2</td>
<td>20.0 ± 1.8</td>
</tr>
<tr>
<td>TLR4-/-</td>
<td>0.65 ± 0.09*</td>
<td>33.3 ± 3.6</td>
<td>20.8 ± 1.5</td>
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</table>

In addition to synovial inflammation, joints of TLR4-/- mice exhibited also less severe PG depletion and bone erosion compared to WT mice (Figures 2C and D). TLR2-/- animals tended to have reduced joint inflammation and bone erosion; however, this reduction did not reach statistical significance (Figures 2B and D). Proteoglycan loss in the cartilage of TLR2-/- mice was not affected as well (Figure 2C).
Figure 2. TLR4 dependency of local IL-1-induced joint pathology
A: Minor histological changes at day 4 of intraarticular injection of the Addel control virus (1x10^7 PFU per joint). Figures B-G show the histological scores of arthritis in WT (black bars), TLR2-/- (grey bars) and TLR4-/- (white bars) mice at day 4 of intraarticular overexpression of AdIL-1β (3.5x10^5 PFU per joint, B-D) or AdTNFα (1x10^7 PFU per joint, E-G). Synovial inflammation (B and E) was scored on Hematoxylin and Eosin-stained tissue sections. Proteoglycan depletion (C and F) and bone erosion (D and G) were scored on Safranin O-stained sections. Each parameter was scored on a 0-3-point scale in a blinded manner. Values are the mean ± SEM of 6 mice per group. n.s. = not significant, n.d. = not detectable. * = P < 0.05 compared to WT by Mann-Whitney U test.
**IL-1-mediated cartilage destruction partially depends on TLR4 activation**

Considering the more striking TLR4 dependency of IL-1-driven joint pathology, further studies focused on IL-1β-induced arthritis. IL-1 inhibits chondrocyte PG and collagen synthesis at low concentrations; however, at high concentrations it also stimulates the synthesis of matrix degenerating proteases (36). Therefore, we enhanced the dose of AdIL-1β to enforce cartilage destruction. Joint inflammation upon intraarticular injection of the enhanced dose of AdIL-1β was increased to near maximum score and was similar in WT, TLR2-/- and TLR4-/- animals (Figure 4A). At this dose, a marked erosion of articular cartilage was observed in the WT mice (Figures 4B and C). Interestingly, the severity of cartilage destruction was clearly diminished in TLR4-/- mice compared to WT animals despite similar degree of joint inflammation (Figures 4B and C). TLR4-/- mice remained significantly protected at later time point (day 11 of virus injection), when severe erosion of articular cartilage was apparent (data not shown). TLR2-/- animals had a similar extent of cartilage erosion as the WT mice, again emphasizing the specific TLR4 dependency of IL-1-mediated cartilage destruction.

**Severe bone erosion and osteoclast formation upon IL-1β overexpression is dependent on TLR4**

Prolonged presence of high doses of IL-1β caused severe bone erosion in patella as well as femur of WT mice (Figure 5A). Although TLR2-/- mice had similar degree of bone erosion as the WT mice, TLR4-/- animals expressed substantially less bone erosion (Figure 5A). Remarkably, a large number of multinucleated cells with osteoclast-like morphology were observed along the outer bone surfaces as well as in the intratrabecular space in HE-stained tissue sections of the WT mice. Therefore, we examined the expression of the osteoclast marker Cathepsin K using quantitative PCR and immunohistochemistry, and compared WT and TLR4-/- mice in this respect. Expression of Cathepsin K mRNA in synovial tissue of WT mice was upregulated by IL-1 overexpression compared to Adel control (Figure 5B). While TLR2-/- mice showed similar upregulation, TLR4-/- mice had approximately 40% lower expression (Fig. 5B). On immunohistochemistry, Cathepsin K protein was highly expressed in osteoclast-like cells adjacent to the bone surface in WT mice (Figures 5C and D). Quantitative analysis revealed that, consistent with PCR data, Cathepsin K protein expression was significantly reduced in TLR4-/- knee joints compared to the WT joints (Figure 5C). A representative picture of the multinucleated Cathepsin K-expressing cells is shown in Figure 5D.
IL-1, but not TNF, induces release of endogenous TLR4 agonists from patella.

In vivo data indicated the involvement of TLR4 activation in the severity of joint inflammation and destruction upon overexpression of IL-1β. To assess the capability of IL-1β and TNFα to induce endogenous TLR4 agonists, we used HEK293 cells stably expressing components of the murine TLR4 complex (TLR4, MD2 and CD14). Validation of the cytokine response of this cell line to a range of cytokines and TLR ligands revealed that HEK-TLR4 cells produce IL-8 in a dose-dependent manner upon stimulation with LPS (Figure 6A). While no response to TLR2 and TLR7 ligands (Pam3Cys and Gardiquimod, respectively) were observed, low response to TLR3 stimulation (Poly I:C) was detected. Importantly, HEK-TLR4 cells did not respond to IL-1β itself, while being sensitive to TNFα (Figure 6A).

Incubation of HEK-TLR4 cells with patella washouts from AdIL-1β-transduced WT joints resulted in robust TLR4 activation and high cytokine production, an effect not found upon stimulation with washouts from the Addel-transduced
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joints (Figure 6B). This indicates that AdIL-1β overexpression in the joint induces the release of endogenous TLR4 ligands from patellae and the surrounding synovial tissue. Interestingly, these effects could be mimicked ex vivo when naive WT patellae were stimulated with recombinant IL-1β. Patellae with minimal surrounding tissue were incubated with IL-1 or TNF for 24 hours and the culture supernatants were used to detect TLR4 agonists using the HEK-TLR4 cell line. Since the latter cells respond to TNFα and TNFα might be present in the supernatants, high concentrations of the TNF blocker Enbrel, revealed to completely inhibit TNFα effects in the same assay, were added to inhibit non-specific responses. As expected, supernatants of IL-1-stimulated patellae induced TLR4 activation; however, supernatants from TNF-stimulated patellae did not (Figure 6C). The negative control cell line HEK293 did not respond to any of these stimuli, excluding any non-TLR4 mediated response of the cells (Figure 6C). The cytokine response of HEK-TLR4 cells to IL-1-stimulated patella supernatants was abolished in the presence of TLR4 antagonist, confirming the TLR4 specificity of the response (Figure 6D).

Figure 4

Data show joint pathology at day 4 of high AdIL-1β overexpression (3.5x10^6 PFU per joint). Synovial inflammation (A) was scored on Hematoxylin and Eosin-stained, and cartilage destruction (B) was scored on Safranin O-stained sections on a 0-3-point scale. Values are the mean ± SEM of at least 5 mice per group. n.s. = not significant. ** = P < 0.01 compared to WT by Mann-Whitney U test. C: Representative images of Safranin O-stained tissue sections showing reduced joint pathology in TLR4-/- mice compared to WT and TLR2-/- mice. Open and stealth arrows indicate cartilage destruction and bone erosion, respectively. Original magnification x100.
Figure 5. TLR4 dependency of local IL-1-induced bone erosion

(A) and Cathepsin K expression (B-D) despite similar synovial inflammation at day 4 of high AdIL-1β overexpression (3.5x10^6 PFU per joint). Cathepsin K mRNA expression (B) was measured by quantitative real-time PCR. The threshold cycle (Ct) value of Cathepsin K was corrected for the Ct of the reference gene GAPDH to obtain the ΔCt, then ΔΔCt was calculated compared to the Addel control. Cathepsin K protein expression (C) was detected by immunohistochemistry and quantified using Leica Qwin software. Values in A (n ≥ 5) and B (n = 4) are the mean ± SEM. The horizontal bars in C represent the mean. n.s. = not significant. * = P<0.05 compared to WT by Mann-Whitney U test. D: Representative images of immunohistochemical staining of Cathepsin K, the osteoclast marker involved in bone resorption. Positive cells at the outer surface of the bone, mineralized cartilage and the intratrabecular space are depicted in the figure. Original magnification x200. P = patella; F = femur.
Discussion

We have recently demonstrated the involvement of TLR4 activation in two chronic destructive models of arthritis, i.e. collagen-induced and spontaneous IL-1rn-/- arthritis, in which the adaptive immune response represents a central part of the pathogenesis (34;37). The dominant role of TLR4 in the established phase of arthritis rather than the onset suggested the contribution of endogenous rather than exogenous TLR4 agonists in arthritic process. In the present study, we addressed the question whether locally produced endogenous TLR2 or TLR4 ligands contribute to the severity of inflammatory and destructive processes in the joint. Here we used sustained local overexpression of IL-1β and TNFα as model cytokines for RA and excluded any effect of TLR2 or TLR4 gene deficiency on the viral transduction and the induced cytokine production (Figure 1). The failure to distinguish between the immunomodulatory roles of TLRs and their sole innate activation by local endogenous ligands has been overcome in this model, as no systemic and adaptive immune activation is involved.

Although both IL-1β and TNFα are produced in high concentrations by inflamed RA synovium and similarly stimulate the production of other inflammatory mediators such as IL-6, IL-8 and PGE2 (38-40), they exhibit specific characteristics as well. IL-1 inhibits chondrocyte anabolic functions and mediates breakdown of proteoglycans in cartilage (36). Furthermore, it promotes the production of nitric oxide and tissue destructive enzymes, and the activation of osteoclasts and bone resorption (39). TNFα is mainly involved in synovial inflammation through activation of endothelial cells and amplification of chemokines; however, it also contributes to osteoclast differentiation and activation via upregulation of RANKL expression on mesenchymal cells and T cells (41). Several animal models of arthritis support a central role for IL-1 in driving cartilage destruction, as opposed to the role of TNFα particularly in joint inflammation (42;43). In this study, higher concentration of AdTNFa virus was used compared to AdIL-1b virus in order to achieve similar joint inflammation and destruction and permit an equitable comparison of the two cytokines. Comparable degree of synovial inflammation and tissue damage induced by IL-1 and TNF would allow the production and release of similar amounts of endogenous TLR ligands in case both cytokines would possess this capability.

Histological examination of the joints revealed TLR4 dependency of IL-1-induced local joint pathology. TLR4-/- animals were protected against multiple pathological effects mediated by IL-1 including synovial inflammation, cartilage proteoglycan depletion and bone damage (Figures 2 and 3), whereas the effects of TNF seemed less dependent on TLR4. Indeed, the subsequent ex vivo assays confirmed that
IL-1β was capable of inducing the release of endogenous TLR4 agonists from patella, whereas TNFα was not (Figure 6). The difference between IL-1 and TNF in this respect might have resulted from differential regulation of matrix degradating enzymes or differential induction of intracellularly expressed endogenous ligands; however, further studies in this respect are warranted. Involvement of TLR4 in systemic TNF-driven arthritis models such as TNF transgenic model requires further investigation as well, as other immune processes might be involved there. Furthermore, a role for TLR4 seems plausible in later phases of TNF transgenic arthritis model where the disease pathogenesis becomes IL-1-dependent (44).
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The presence of endogenous TLR2 ligands in the tested conditions cannot be excluded and might explain the tendency of reduced joint pathology in TLR2-/− animals. Nevertheless, TLR2 deficiency did not exert any considerable influence on local joint pathology in this model where adaptive immunity is not involved. Of high relevance, IL-1-driven cartilage and bone destruction was still TLR4 dependent under the condition of similar degree of inflammation, as occurred when AdIL-1 dose was enhanced (Figures 4 and 5). This indicates that the role of TLR4 in inflammation may be uncoupled from its role in joint destruction and suggests that reduced cartilage and bone destruction in TLR4-/− mice does not necessarily rely on diminished inflammation.

Given the poor regenerative capacity of cartilage and conceding the central role of IL-1 in cartilage destruction, the substantial role of TLR4 in IL-1-mediated cartilage destruction is of crucial importance. Besides enzymes released from synovial cells and the consequent breakdown of cartilage matrix, breakdown may have resulted from the direct TLR4 activation of chondrocytes and the induction of MMPs from the latter. A direct effect of TLR4 activation on chondrocyte anabolic function, e.g. collagen type II and aggrecan synthesis, has been reported before (1). In addition, TLR4 activation of primary osteoarthritic chondrocytes strongly induces MMP and NO release from these cells (45). In vivo evidence supporting the contribution of TLR4 to MMP-mediated cartilage destruction comes from our previous findings indicating TLR4-dependent expression of the MMP-specific aggrecan neoepitope VDIPEN in murine arthritic joints (46). Involvement of TLR4 in cartilage destruction makes it an intriguing candidate to target in combination with TNF in order to provide protection against cartilage destruction, an area where TNF blockers seem to fail (47).

A role for TLR4 in driving Cathepsin K expression and the concomitant bone erosion (Figure 5) is in line with a previous report on promotion of osteoclastogenesis in monocyte cultures by TLR4 stimulation of the co-cultured fibroblast-like synoviocytes (48). Moreover, this observation is consistent with recent findings in another in vivo model of arthritis in which TLR4 activation was found to be partially responsible for Cathepsin K expression in the joint (46).

The present data point toward a role for TLR4 in the “danger model” of immunity and hence, may have implications for other inflammatory and tissue-destructive diseases beyond RA. Evidence from non-infectious disease conditions such as myocardial and hepatic ischemia-reperfusion injury and non-bacterial lung injury supports the involvement of TLR4 activating self molecules in “sterile” inflammation (49-52). A role for TLR4 in atherosclerosis, where it might interact with endogenous ligands in atherosclerotic plaque, has also been indicated (53).
In the context of RA, several reports support the presence of TLR4 agonists in RA synovial fluid and serum, and indicate that activation of TLR4 by endogenous ligands partially defines the inflammatory character of RA synovial tissue (23;37). Indeed, the spontaneous production of proinflammatory cytokines and some MMPs by RA synovial membrane cells can be inhibited by overexpression of dominant-negative forms of MyD88 and Mal, two essential adaptor molecules in signaling through TLR2 and TLR4 (54).

Endogenous TLR4 agonists may either be derived from the inflammatory or necrotic cells, or become released upon degradation of the extracellular matrix (ECM). Detection of endogenous TLR4 agonists in supernatants of patellae ex vivo cultured with IL-1β where inflammatory cells are absent (Figure 6) suggests resident components of the joint such as ECM as one of the sources of TLR4 agonists. Arthritic joints most presumably contain multiple TLR4 ligands, some of which might have greater clinical impact than others. For instance, concentration of EDA+ fibronectin in RA synovial fluid, but not plasma, is revealed to be a valuable predictor of radiographic joint destruction in RA patients (55). An important marker of inflammation, serum amyloid A (SAA) 3, has recently been reported to activate TLR4 using a higher affinity for the TLR4/MD2 receptor complex than the classical TLR4 ligand of microbial origin lipid A (56). Importantly, the human homologues of SAA3, i.e. SAA1 and SAA2, are upregulated in RA synovium, induced by IL-1β, and contribute to the production of MMPs by primary chondrocytes (57), hence representing good candidates to activate TLR4. The exact source and nature of endogenous TLR4 agonists in our system remain, however, to be determined. The relative contribution of various ligands and the insight in the mechanisms of TLR4 activation in RA will provide opportunities to develop novel RA-specific therapeutic interventions without interfering with innate immune function in anti-microbial defense.

Acknowledgements

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References


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Chapter 4

Is the SAA we use really SAA?

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Rheumatoid arthritis affects articulating joints resulting in destruction of bone and cartilage. The chronic inflammation is maintained by a putative inflammatory feedback loop involving Toll-Like Receptors and endogenous proteins, such as serum amyloid A (SAA) (1). Here, we would like to address a peculiar observation that the recombinant Apo-SAA protein used may not reflect the endogenous SAA protein.

Many pro-inflammatory properties have been described for the recombinant Apo-SAA protein (PeproTech), used by Connolly et al. and many other research groups, such as IL-23p19 induction(2), inflammasome activation (3), angiogenesis(4) and fibroblast migration(5). The recombinant Apo-SAA commonly used has two amino acids substituted when compared with the endogenous Apo-SAA1. These modifications are on position 61 and 72 of the recombinant proteins. Amino acid 61 in Apo-SAA1 is an aspartic acid and the Apo-SAA contains a asparagine (D61N) and amino acid 72 is a histidine in A-SAA1 and an arginine in A-SAA (H72R). Both these substitutions are present in a natural isoform of human SAA2 (Apo-SAA2β).

We directly compared both commercially available recombinant Apo-SAA proteins for their pro-inflammatory properties. Figure 1A shows the effect of Apo-SAA1 and Apo-SAA on NF-κB activation in NIH 3T3 murine fibroblast reporter cells. The recombinant endogenous Apo-SAA1 only slightly activated NF-κB at high concentrations (1.5 fold), whereas the modified recombinant Apo-SAA increases NF-κB activation 5.2 fold at a concentration of 50 µg/ml. This shows that Apo-SAA1 is less potent in stimulating murine fibroblasts, whereas the modified Apo-SAA is a potent NF-κB activator. To rule out species differences recombinant Apo-SAA1 and recombinant Apo-SAA were used to stimulate rheumatoid arthritis synovial fibroblasts (RASF), known to produce and respond to Apo-SAA proteins. Figure 1B shows slight IL-8 mRNA regulation by endogenous recombinant Apo-SAA1. In contrast, recombinant modified Apo-SAA induces IL-8 mRNA in a dose dependent manner. Thus, Apo-SAA1 and Apo-SAA also have different potencies on human cells.

Possible explanations for this profound effect on biological activity could involve the isoelectric point (pl) of these proteins. Both mutations increase the pl from < 7.2 up to 7.2, resulting a shift from a negatively charged protein towards a neutral protein (Protein Calculator v3.3, www.scripps.edu) Additionally, tertiary protein structure is affected by these substitutions (I-TASSER software; Center for Computational Medicine and Bioinformatics University of Michigan) and could potentially lead to altered receptor binding and activation. Although this is based on theoretical calculations, it might give us an explanation how minor amino acid substitutions affect biological activity of proteins and peptides.
In our hands recombinant Apo-SAA1 and Apo-SAA have different biological activities. Apparently, substitution of two amino acids can increase the pro-inflammatory activity of Apo-SAA1, at least with regard to NF-κB activation and IL-8 mRNA induction. In addition, we are not the first questioning the pro-inflammatory effects of SAA proteins. Björkman et al., reported that freshly isolated Apo-SAA1 from arthritis patients lacked biological activity when directly compared with Apo-SAA on neutrophils (6). Whether these differential effects of recombinant Apo-SAA1 and Apo-SAA would also be found on more complex tissues, such as synovial explants, remains to be determined. We therefore recommend to include the endogenous Apo-SAA1 protein, either recombinant or isolated, in future studies to exclude misrepresentation of Apo-SAA1 protein activity. Additionally, this is an example that we should take care in using minimally modified proteins in biological assays.
Chapter 4

References


Chapter 5

The role of Toll-Like Receptor 4 on splenic antigen presenting cells in experimental arthritis

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Rheumatoid arthritis (RA) is an autoimmune disease characterized by joint destruction due to chronic inflammation. Antigen presenting cells (APCs) play an important role in RA and infiltrate the synovium of joints. Toll-like receptors (TLRs), also expressed by APCs, play a role in inflammation and TLR4 is particularly important for the aggravation of arthritis. Therefore, we investigated the role of TLR4 on APCs during arthritis. Mice with collagen-induced arthritis (CIA) were injected intravenously before onset of disease with lentivirus encoding short hairpin RNA against TLR4 (shTLR4) to knock down TLR4 gene expression on splenic APCs. Disease development was monitored macroscopically and knee joints were analyzed for histopathology. Draining lymph nodes were analyzed for T-cell subsets by flow cytometry and inflamed synovium was analyzed for cyto- and chemokine mRNA expression. Unexpectedly, shTLR4 treatment led to a slight increase in incidence, macroscopic swelling and bone erosion. Analysis of T helper subsets revealed only marginal effects of shTLR4 in early stages of disease, which dissipated over time. shTLR4 did appear to be effective, since IFNγ producing cells were significantly decreased in DLN of shTLR4 treated mice, especially the number of CD4- cells producing IFNγ. Analysis of draining lymph nodes showed that chemokine production was increased, possibly reflecting the increase in swelling and inflammatory cell influx into inflamed knee joints. In conclusion, lentivirus encoding a short hairpin RNA against TLR4 targeting APCs before disease induction was not effective in ameliorating experimental arthritis. Targeting of TLR4 only on APCs might not be suitable to treat arthritis.
Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation, synovial hyperplasia, and cartilage and bone destruction in multiple joints causing pain, discomfort and impairment of mobility (1). The mechanisms of initiation and advancement of inflammation in RA are still unknown. The discovery of Toll-like receptors (TLRs) as essential components of the innate immune system has introduced new candidates implicated in the development and progression of RA.

TLRs are part of the family pattern recognition receptors (PRRs) present on several immune cells, including macrophages, dendritic cells, B-, and T cells (2). TLRs are involved in the recognition of conserved pathogen-associated molecular patterns (PAMPs). TLRs can also recognize danger-associated molecular patterns (DAMPs) released during inflammation or necrosis (3).

One of the TLRs that appear to be important for the progression of RA is TLR4. Expression of TLR4 in synovial tissue of RA patients is increased when compared to OA patients and healthy controls (4). The presence of endogenous TLR4 ligands, such as fibronectin, has also been demonstrated in synovium of RA patients (5). TLR4 also plays an important role in experimental arthritis. Systemic blocking of TLR4 in mice after induction of collagen-induced arthritis in mice resulted in decreased arthritis severity, breaking an inflammatory loop (6). Additionally, IL-1Ra/TLR4 double-deficient mice were protected from severe disease and Th17 levels were reduced by 50% (7). This confirms the role of TLR4 in generating aggressive and destructive Th17 cells. Further studies have suggested a role for TLR4 both locally in the joint and systemically in generation of IL-17 producing T cells (8).

Moreover, synovial tissue of RA patients is heavily infiltrated by macrophages and this infiltration is strongly correlated with the disease severity (10). TLR4 activation on macrophages isolated from RA synovium showed an increased response when compared to macrophages from other inflammatory disorders (9). Due to the increased expression and responsiveness it is conceivable that TLR4 is also involved in the inflammatory process in RA.

Taking together all the evidence that point at TLR4 as a key receptor in development of RA and that APCs are key players in RA, we investigated the role of TLR4 activation on APCs. We aimed to knock down TLR4 gene expression by intravenous injecting lentivirus, specifically targeting splenic APCs (11;12). The lentivirus encoded a short hairpin RNA complementary to TLR4 mRNA, thereby inducing TLR4 mRNA degradation. We hypothesized that knocking down TLR4 on
APCs would lead to a reduction of both clinical and histological characteristics of CIA, via inhibition of Th17 differentiation.

Unexpectedly, shTLR4 treatment caused a trend in increased swelling and inflammatory cell influx. Additionally, a trend towards increased cytokine expression was observed in synovium of shTLR4 treated mice and this was accompanied by an increase in chemokine production in DLN and synovium. shTLR4 appeared to be functional in vivo since it decreased the number of IFNγ producing cells in DLN. This decrease was accompanied by an increase in chemokine production and an increased trend in swelling and cellular influx.

**Materials and methods**

**Animals**

Male DBA1/J mice aged 10-12 weeks (Janvier, Elavage, France) were housed in individually ventilated cages and fed a standard diet with freely available food and water. All in vivo studies complied with national legislation and were approved by local authorities for the care and use of animals with related codes of practice.

**Cell culture**

The Human Embryonic Kidney 293T (HEK293T) cell line was used for the production of lentivirus. The 3T3 NFκB luciferase fibroblast reporter cell line was used in functional assays with the produced lentivirus. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco - Invitrogen, Paisley, United Kingdom) with 10% fetal calf serum (FCS), P/S or gentamycin, 1% pyruvate and during lentivirus production, 0.01mM cholesterol (Sigma, Steinheim, Germany) was added. Cells were cultured at 37°C and 5% CO₂.

**Production of lentivirus**

The transfer vector for the lentivirus coding the short hairpin RNA against TLR4 (shTLR4) and the short hairpin control (shCon; scrambled), both kindly gifted by P. Quax (Leiden University Medical Center). Plasmid DNA was isolated with the use of maxi-prep according to manufacturer’s protocol (Qiagen, Hilden, Germany). The resulting transfer vector (75.6 μg) was added to 56.35 μg MDL, 26.6 μg VSV-G and 18.9 μg RSV-REV and an alcohol precipitation was performed. Hereafter, the pellet was dissolved in aqua dest and used for CaPO₄ transfection of HEK293T cells. Medium of day 1 and 2 after transfection was kept in 4°C and purified with a 20% sucrose cushion using Discovery 100 Sorvall centrifuge. Pellets were taken up in phosphate buffered saline (PBS) and stored at -80°C. Concentration of the
lentivirus was determined by performing p24<sup>GAG</sup> ELISA according to manufacturer’s protocol (Innogenetics, Gent, Belgium).

**Reporter assay**

3T3 NFκB luciferase fibroblast cells were plated in 96 wells plate with 30,000 cells/well in 50 μl medium. Cells were transfected with 200 ng p24<sup>GAG</sup>/30,000 cells in regular medium complemented with 8 μg/ml polybrene (Sigma). At day 6, cells were stimulated for 6 hours with lipopolysaccharide (LPS; 100 ng/ml) or Pam3CysSerLys4 (P3C; 100 ng/ml). Hereafter, cells were lysed, Bright-Glo (Promega, Leiden, The Netherlands) was added and luminescence was measured using LUMIstar OPTIMA (BMG LABTECH, Cary, USA).

**Bone marrow derived dendritic cells**

Femurs and tibias of DBA1/J mice were flushed and bone marrow was harvested. After preparing a single cell suspension cells were plated (250,000 cells per cm<sup>2</sup>) with 833 ng p24GAG equivalent of shControl or shTLR4 and 20 ng/ml GM-SCF. Medium was changed on day 4 and day 8.

**Induction of CIA**

Bovine type II collagen was dissolved in 0.05M acetic acid to a concentration of 2 mg/ml and was emulsified in equal volumes of Freund’s complete adjuvant (2 mg/ml of Mycobacterium tuberculosis strain H37Ra) (Difco, Detroit, USA). Mice were immunized intradermally at the base of the tail with 100 μl of emulsion (50 μg of bovine type II collagen). After 18 days, 40 μg lentivirus, encoding shTLR4, shCon, TAK1/K63W or GFP (n=11), dissolved in 200 μl PBS was injected intravenously through the tail vain per animal. After 3 days, mice were given an intraperitoneal booster injection of 100 μg of type II collagen dissolved in PBS on day 21. Two independent observers monitored clinical signs of arthritis in paws and ankle joints, macroscopically scored using an arbitrary scoring system until the end of the experiment. Cumulative scoring based on redness, swelling, and, in later stages, ankylosis was as follows: 0=no changes; 0.25=1-2 toes red or swollen; 0.5=3-5 toes red or swollen; 0.5= swollen ankle; 0.5=swollen footpad; 0.5=severe swelling and ankylosis (redness, excessive edema and deformation), with a maximal score of 2 per paw.

**Isolation, culture and stimulation of murine cells**

Draining lymph nodes (DLN) were mashed and plated, 300,000 cells/well were cultured in RPMI, 5% FCS, 1% pyruvate, gentamycin and stimulated either with PMA (50 ng/ml; Sigma), ionomycin (1 μg/ml; Sigma) and golgi-plug Brefeldin A
(1 µl/ml; BD Biosciences - Pharmingen, Breda, The Netherlands) for 4 hours for fluorescence-activated cell sorting (FACS) staining or with PMA/ionomycin (same concentrations) for 24 hours for RNA isolation.

**Antibodies and FACS analyses**

Th1 and Th17 cells in DLN were stained using anti-CD4-APC (1:200), then washed with PBS and fixed in Cytofix/Cytoperm paraformaldehyde containing saponin (BD Biosciences – Pharmingen). Subsequently, cells were washed with ICS buffer (PBS, 1% bovine serum albumin (BSA), 2% FCS, 0.1% saponin) and cells were stained with IL-17-PE and IFN-FITC. All antibodies were purchased from BD Biosciences – Pharmingen. Cells were analyzed using FACSCalibur flow cytometer (BD Biosciences – Pharmingen) and analyzed with FlowJo software.

**RNA isolation**

Synovium and liver samples were disrupted using the MagNaLyser (Roche, Woerden, The Netherlands). Total RNA was extracted from tissue homogenates and cells with TRizol (Sigma), precipitated with isopropanol and washed with 70% ethanol and dissolved in water. To restrict DNA contamination, all samples were treated with DNase and enzyme was inactivated with EDTA. RNA was reverse transcribed into cDNA using oligo-dT primers and reverse transcriptase.

**Quantitative PCR**

The quantitative PCR (qPCR) was performed using the StepOnePlus sequence detection system (Applied biosystems, Foster City, USA) in a 96-wells plate. In every well, 5 µl SYBR Green Master Mix (Applied biosystems) and 1 µl forward and reverse primer (both 5 µM) were added. The genes investigated are shown per experiment. cDNA was diluted 1:10. The threshold cycle of the gene of interest was corrected for the reference gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) to obtain the ΔCt. Data expressed as relative expression (2-dCt).

**Histology**

Whole knee joints were isolated at day 30 and fixed in phosphate buffered 4% formaldehyde for 21 days followed by decalcification with 5% formic acid and embedded paraffin wax. Tissue sections of 7µm were stained with hematoxylin (Merck, Darmstadt, Germany) and eosin (Merck) (H&E) to study inflammatory cell influx, or with safranin-O (BHD chemicals, Poole, UK) and fast green (BHD chemicals) to determine bone and cartilage erosion. Three sections per knee were scored on a scale from 0-3 in a blinded manner.
**Immunohistochemistry**

Expression of GFP was evaluated in paraffin sections of the knee joints. Sections were incubated in citric buffer for 2 hours for antigen retrieval. Endogenous peroxidase was blocked using 3% H₂O₂ for 15 minutes. Tissue sections were incubated with rabbit anti-GFP antibodies or rabbit normal IgG (both 1:800; Cell Signaling, Boston, USA) overnight at 4°C followed by incubation with goat anti-rabbit antibodies for 30 minutes. Colour was developed with diaminobenzidine and sections were counterstained with hematoxylin.

**Measurement of cytokines**

IL-6 and KC concentrations in serum were measured on a Luminex-100 System (Millipore, Billerica, USA) using a magnetic bead-based multiplex immunoassay (Millipore). Data analysis was performed with Bio-Plex Manager software (Bio-Rad Hercules, Veenendaal, The Netherlands).

**Statistical analysis**

All data are expressed as mean ± SEM. Statistical significance was determined using the Student’s unpaired t-test or Mann-Whitney U test, as shown per experiment, using GraphPad Prism 4.0 software (GraphPad Software, San Diego, USA). p-values less than 0.05 were considered statistically significant.

**Results**

**Short hairpin RNA against TLR4 is functional in vitro**

The hairpin against TLR4 was validated in vitro using murine NF-κB luciferase reporter NIH3T3 fibroblasts. Figure 1A shows that five days after transduction the NF-κB activation in 3T3 fibroblasts was decreased by shTLR4 after LPS stimulation. Moreover, NF-κB activation by Pam3Cys through TLR2 was not decreased, indicating TLR4 specific targeting by shTLR4. To evaluate the effectiveness of shTLR4 in target cells, antigen presenting cells, bone marrow derived macrophages (BMDM) were generated. Figure 1B shows that shTLR4 treatment reduced TLR4 messenger RNA (mRNA) expression. shTLR4 treatment also resulted in a reduced LPS response, indicated by decreased production of IL-6 upon LPS stimulation (Figure 1B). A non significant increase of IL-6 mRNA expression was observed in cells treated with shTLR4 after Pam3Cys stimulation. Taken together, these data indicate that shTLR4 effectively reduces TLR4 mRNA expression and specifically reduces proinflammatory signaling and cytokine expression upon LPS stimulation.
No reduction of clinical and histological characteristics of CIA with shTLR4

To study the role of TLR4 on APCs during collagen-induced arthritis shTLR4 lentivirus was injected 5 days before onset of disease. As seen in Figure 2A, a trend towards increased disease incidence and disease severity was observed in the mice treated with shTLR4 when compared with shCon-treated group. On histology, no significant differences were found in bone erosion, cartilage erosion and a minor trend in cellular influx (Figure 2B). These data point at a trend towards increased inflammation when TLR4 expression was knocked down in APCs.

Increased cytokine expression in synovium of shTLR4 treated mice

To study expression levels of cytokines in synovium that mediate joint pathology, mRNA was isolated from synovium. In line with the macroscopic scores, a trend of increase in expression of inflammatory cytokines IL-1β and tumour necrosis factor α (TNF-α) in synovium with shTLR4 were observed (Figure 3), but not for IL-6.
The role of Toll-Like Receptor 4 on splenic antigen presenting cells in experimental arthritis

Increased chemokine expression in DLN and synovium

To study if chemokine expression could explain the increased swelling in shTLR4 treated animals, production of interferon gamma-induced protein 10 (IP-10) and regulated and normal T cell expressed and secreted (RANTES) were measured in DLN. Figure 4 shows that a minor trend was observed in both IP-10 and RANTES expression. Further analysis of inflamed synovium showed an increased expression of IP-10, RANTED and KC mRNA. This increase in chemokine production could explain the trend towards increased arthritis severity of shTLR4 treated animals.

Figure 2. Clinical and histological characteristics of collagen-induced arthritis with shTLR4
Individual mice received 40µg of lentivirus intravenously encoding either a short hairpin RNA against TLR4 (shTLR4) or a short hairpin control (shCon) on day 18 of CIA. (A) Incidence of and severity of arthritis (n=7) (scale 0-2 each hind paw). (B) Inflammation, cartilage erosion, and bone erosion. Knee joints (n=8) were isolated, fixed and embedded in paraffin. Tissue sections were stained with hematoxylin/eosin or safranin-O/fast green. Three sections per knee were scored on a scale from 0-3 in a blinded manner. Data presented as mean±SEM.

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Reduction of IFNγ production in DLN by TLR4 knockdown

It has previously been described that activation of TLR4 is involved in T helper cell development and especially Th17 cells. Therefore, we investigated the frequency of Th1 and Th17 cells both in DLN and spleen on day 23 and 30 of CIA. In spleens we did not observe any differences in Th1 and Th17 levels on both days (data not shown). Figure 5A shows that Th1 levels were marginally higher in shTLR4 treated animals and a clear trend towards decreased Th17 levels was observed in DLN of shTLR4 treated mice on day 23. This indicates a functional effect of shTLR4. However, this trend was lost at day 30 of CIA (Figure 5B). Interestingly, the number of IFNγ producing cells was significantly reduced at day 30 of CIA (Figure 5C), which was due to decreased CD4-IFNγ+ cells in DLN. These results indicate that treatment with shTLR4 does not affect T helper subsets but does affect immunity by decreasing the number of IFNγ producing cells in DLN.

Figure 3

**Figure 3. Expression levels of inflammatory cytokines in synovium with shTLR4**

Synovium was isolated from mice treated with lentivirus intravenously, encoding either shTLR4 or shCon (n=6). mRNA was extracted and analyzed for expression of IL-1β, IL-6, and TNFα. All data are presented as mean ±SEM.

Discussion

In this study we attempted to ameliorate experimental arthritis by targeting TLR4 on splenic APCs with systemic delivery of lentivirus encoding a short hairpin RNA against TLR4. We hypothesized that reduced TLR4 activation on APCs would lead to reduced T-cell activation and subsequent decreased arthritis severity. However, we found a trend towards increased ankle joint swelling. Histological analysis showed an trend towards increased bone erosion in knee joints of mice with collagen-induced arthritis.
The role of Toll-Like Receptor 4 on splenic antigen presenting cells in experimental arthritis

APCs were targeted by injecting lentivirus intravenously. This delivery route of lentivirus preferentially targets macrophages in spleen (12) and also dendritic cells (13). Moreover, overexpressing a TAK1 mutant does ameliorate collagen-induced arthritis (14). Therefore, this approach was used to target TLR4 expression on APCs with lentiviral mediated overexpression of a short hairpin against TLR4 (shTLR4). Effectiveness of shTLR4 was shown in vitro where NF-κB activation was significantly reduced after LPS stimulation by shTLR4 overexpression. In the target cells, APCs, shTLR4 was also proven to be effective in decreasing the proinflammatory reaction elicited by LPS, showing its effectiveness in achieving TLR4 knockdown. In support, shTLR4 was effective in vivo in another inflammatory model, where it was shown to reduce TLR4 expression and decrease vein graft disease (15).
Despite functional shTLR4 we did not observe decreased arthritis severity in animals treated with lentivirus overexpressing shTLR4. In fact, a trend towards increased incidence, swelling and cellular influx was observed. Unfortunately, we could not directly assess TLR4 protein expression and therefore direct evidence of TLR4 knockdown should be gathered in future experiments. However, a significant decrease in IFNγ producing cells in draining lymph nodes was detected and it therefore appears that shTLR4 was functional. This decrease in IFNγ producing cells was mainly in the CD4+ population, while leaving Th1 cell numbers unaffected. These CD4+ cells could be NK cells, NKT cells, CD8+ cytotoxic lymphocytes or macrophages. Low levels of TLR4 expression has been...
detected on NK cells (16), but whether these cells are targeted by intravenously injected lentivirus remains unclear. IFNγ production by NKT cells also involves TLR4 activation on dendritic cells (17). Additionally, TLR4 on APCs is involved in generating IFNγ producing CD8+ T-cells and reduced TLR4 expression could lead to diminished levels of CD8+ IFNγ producing T-cells. Lastly, macrophages also produce IFNγ (18) and direct targeting of TLR4 on macrophages could decrease IFNγ production due to decreased activation.

The observed decrease in IFNγ production by DLN cells could partly account for the trend towards increased bone erosion. IFNγ has been widely regarded as a proinflammatory cytokine, but several reports point to a regulatory role of IFNγ (19). Moreover, lack of IFNγ leads to increased severity of CIA and antigen-induced arthritis (20;21). Additionally, TLR4 knockouts showed reduced IFNγ production in inflamed joints during antibody induced arthritis (22). Therefore, a decrease in IFNγ production could lead to increased arthritis severity. IFNγ is known to inhibit Th17 development (19), which in turn is involved in bone erosion (23). However, further studies are necessary to confirm the number of Th17 cells in the inflamed joint after shTLR4 treatment.

RANTES is known to attract Th17 cells (24) and we did observe an increase of RANTES mRNA expression in inflamed synovium of shTLR4 treated mice. Additionally, an increase in IP-10 and KC mRNA expression was found, providing a possible explanation for the increased swelling in shTLR4 treated mice. The mechanism how TLR4 knockdown in splenic APCs results in a local increase of chemokine production remains unclear. Unpublished observations in our lab indicate a possibly different role of TLR4 on macrophages and DCs. Where knockdown of TLR4 on macrophages reduces inflammatory cytokine production upon LPS stimulation, an increase in MHCII transactivator Ciita expression as well as MHC-II itself is found in DCs. Since shTLR4 was administered prior to the booster injection with bovine collagen type II, increased MCHII expression and activation could result in enhanced antigen presentation, T-cell activation and subsequent increased production of chemokines, such as T-cell derived RANTES. However, more studies are required to dissect the role of TLR4 on different types of APCs in activating T-cells.

Additionally, TLR4 is expressed and functional on other cells in addition to APCs and a role of TLR4 on these cells cannot be neglected. TLR4 is also expressed and functional on other hematopoietic cells, such as B-cells and activated T-cells (2). In addition, the contribution of TLR4 on non-hematopoietic cells, such as synovial fibroblasts, appears to play a role in the pathogenesis of arthritis. A previous report has indeed shown a role of TLR4 on local resident cells in experimental
arthritis (8). Therefore, we question whether TLR4 targeting on APCs is a suitable therapeutic approach for arthritis. Further studies are needed to identify the exact role of TLR4 on APCs during inflammation and arthritis.

Several other reports indicate a regulatory role of TLR4 during infection and inflammation. LPS treated pDCs have anti-inflammatory effects during chronic kidney disease by inducing Treg cells (25). Moreover, B. pertussis in acute lung infection induced IL-10 production through TLR4 in macrophages and DCs resulting in IL-10 secreting T-cells and subsequently decreases inflammation. Additionally, TLR4 defective mice showed increased numbers of neutrophils 14 days post infection and lymphocytes 21 days post infection in lavage fluid of infected lungs. Additionally, at these later time points IL-1β, IL-6 and MIP-1α were increased in TLR4 deficient mice (26). Imado et al., suggested that TLR4 signaling is necessary for the induction of tissue protective factors and protects against graft-versus-host disease. However, excessive signaling is detrimental and TLR4 stimulation is a delicate balance between tissue protection and inflammation (27). Lastly, in an animal model for MS a protective role for TLR4 was found, where TLR4 knockout animals showed increased pathology due to increased IL-6 and IL-23 production resulting in increased Th17 levels (28). Again, a delicate role for TLR4 was determined, since immunization did not involve a booster injection overriding the potential regulatory role of TLR4.

In conclusion, collagen-induced arthritis could not be treated with specifically targeting TLR4 on splenic APCs by lentiviral mediated overexpression of shTLR4. In fact, arthritis severity showed a trend towards increased severity, most likely due to increased chemokine expression in inflamed joints. Direct evidence of TLR4 knockdown was not observed and should be confirmed in further studies. Additionally, different strategies to inhibit TLR4 on APCs should be explored, such as TLR4 inhibitors in lipoplexes or lentiviral mediated ectopic overexpression of TLR4 inhibitors should be attempted (29;30). This will rule out any possible off-target effects of short RNA strands or activation of RNA sensing receptors (31). Moreover, TLR4 appears to have more effects than merely proinflammatory and its role on different subsets of APCs warrants further investigation.

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Chapter 6

Intravenous delivery of HIV-based lentiviral vectors preferentially transduces F4/80+ and Ly-6C+ cells in spleen, important target cells in autoimmune arthritis

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Abstract

Antigen presenting cells (APCs) play an important role in arthritis and APC specific gene therapeutic targeting will enable intracellular modulation of cell activity. Viral mediated overexpression is a potent approach to achieve adequate transgene expression levels and lentivirus (LV) is useful for sustained expression in target cells. Therefore, we studied the feasibility of lentiviral mediated targeting of APCs in experimental arthritis. Third generation VSV-G pseudotyped self-inactivating (SIN)-LV were injected intravenously and spleen cells were analyzed with flow cytometry for green fluorescent protein (GFP) transgene expression and cell surface markers. Collagen-induced arthritis (CIA) was induced by immunization with bovine collagen type II in complete Freund’s adjuvant. Effect on inflammation was monitored macroscopically and T-cell subsets in spleen were analyzed by flow cytometry. Synovium from arthritic knee joints were analyzed for proinflammatory cytokine expression. Lentiviruses injected via the tail vein preferentially infected the spleen and transduction peaks at day 10. A dose escalating study showed that 8% of all spleen cells were targeted and further analysis showed that predominantly Ly6C+ and F4/80+ cells in spleen were targeted by the LV. To study the feasibility of blocking TAK1-dependent pathways by this approach, a catlytically inactive mutant of TAK1 (TAK1-K63W) was overexpressed during CIA. LV-TAK1-K63W significantly reduced incidence and arthritis severity macroscopically. Further histological analysis showed a significant decrease in bone erosion in LV-TAK1-K63W treated animals. Moreover, systemic Th17 levels were decreased by LV-TAK1-K63W treatment in addition to diminished IL-6 and KC production in inflamed synovium. In conclusion, systemically delivered LV efficiently targets monocytes and macrophages in spleen that are involved in autoimmune arthritis. Moreover, this study confirms efficacy of TAK1 targeting in arthritis. This approach may provide a valuable tool in targeting splenic APCs, to unravel their role in autoimmune arthritis and to identify and validate APC specific therapeutic targets.
Introduction

Inflammatory diseases, such as rheumatoid arthritis, are characterized by infiltration of leukocytes into the inflamed tissue consisting of various immune cells from both the innate and adaptive immune system. Macrophages in arthritis have been extensively studied and play an important role in maintaining inflammation and joint destruction (1;2). Moreover, a general approach of macrophage specific targeting with lipoplexes ameliorates experimental arthritis (3;4).

Specifically modulating macrophage activity during inflammatory conditions is a suitable approach to limit systemic side effects of treatment. In addition, gene therapy can be a powerful technique to achieve intracellular modulation of protein expression or signaling. Effective gene therapeutic treatment of collagen-induced arthritis has been achieved by ectopic overexpression of suppressor of cytokine signaling 3 (SOCS3) in splenic antigen presenting cells (APCs) (5). In addition, knock down of TNF receptor I by overexpression of a short hairpin was also effective in ameliorating experimental arthritis (6). Both studies used adenoviral vectors, ensuring a high, but transient transgene overexpression. Over recent years, HIV based lentiviral vectors have been optimized regarding safety and production and proved to be a valuable tool in order to get long term transgene expression.

Multiple studies have evaluated the feasibility of intravenous injection of lentivirus. These studies shown that the spleen is one of the organs predominantly targeted by lentivirus injected intravenously (7). Further analysis on localization on GFP expression in the spleen showed large fluorescent clusters overlapping the marginal zone (8). Cell surface markers revealed that mostly MHC-II positive cells were targeted by lentivirus (8;9), indicating preferential targeting of APCs by lentivirus.

Specific targeting of APCs by lentivirus makes it possible to study the role of these APCs in inflammatory conditions. In addition, specific interference with gene expression can possibly identify new therapeutic targets in APCs. To validate macrophage targeting a known potent therapeutic target was used, TGF-beta activated kinase 1 (TAK1). This MAP kinase is a key signaling protein as it is used by many pro-inflammatory signals. Toll-Like receptor and cytokine receptor activates intracellular signaling which converges to TAK1 and subsequently activate AP-1 and NF-κB. Therefore, TAK1 poses a compelling target to block pro-inflammatory
signaling. Moreover, TAK1 has been shown to be important in autoimmune arthritis (3) and is a suitable target to validate macrophage specific targeting by lentiviral vectors.

In this study, we validated intravenous lentivirus injections into DBA1/J mice by determining expression kinetics, a dose escalating study, and analysis of targeted cells. This has shown that predominantly splenic F4/80+ and Ly-6C+ cells are targeted by this approach. To evaluate the gene therapeutic potential of LV-mediated targeting of splenic APCs, a TAK1 kinase negative mutant (TAK1-K63W) was over expressed during collagen-induced arthritis. TAK1-K63W decreased knee joint inflammation macroscopically and systemic Th17 levels in the spleen were reduced. In addition, bone erosion was diminished by TAK1-K63W and pro-inflammatory cytokine expression in synovial tissue was decreased. This study shows that intravenous delivery of lentiviral vectors enables evaluation of therapeutic targets in splenic monocyte/macrophage-like cells that are important cells in autoimmune diseases.

**Material and Methods**

*Ethics Statement and mice*

All in vivo studies complied with national legislation and were approved by local authorities (Animal Ethics Committee, Radboud University Nijmegen. Permit number: 2009-177) for the care and use of animals with related codes of practice. Male DBA/1 mice aged 10-12 weeks (Janvier, Elavage, France) were housed in top-filter cages and fed a standard diet with freely available food and water.

*Induction and monitoring of collagen-induced arthritis*

Bovine type II collagen was dissolved in 0.05M acetic acid to a concentration of 2 mg/ml and was emulsified in equal volumes of Freund’s complete adjuvant (2mg/ml of Mycobacterium tuberculosis strain H37Ra) (Difco, Detroit, MI). Mice were immunized intradermally at the base of the tail with 100ul of emulsion (50ug of bovine type II collagen). Subsequently, mice were given an intra-peritoneal booster injection of 100ug of type II collagen dissolved in phosphate buffered saline (PBS) on day 21. Two independent observers monitored clinical signs of arthritis in paws and ankle joints, macroscopically. Cumulative scoring based on redness, swelling, and, in later stages, ankylosis was as follows: 0=no changes; 0.25=1-2 toes red or swollen; 0.5=3-5 toes red or swollen; 0.5= swollen ankle; 0.5=swollen footpad; 0.5=severe swelling and ankylosis, with a maximal score of 2 per paw.
**Plasmids**

For generation of recombinant lentiviral vectors we used of the third-generation self-inactivating transfer vector pRLL-cPPT-PGK-mcs-PRE-SIN (PGK-empty) containing the human phosphoglyceratekinase (PGK) promoter (kind gift from J. Seppen, AMC Liver Center, Amsterdam, The Netherlands). For cloning we used cloned Pfu DNA polymerase (Stratagene, La Jolla, CA) and T4 DNA Ligase (New England Biolabs, Ipswich, MA). All generated constructs were verified by sequencing. The cDNA sequences of a kinase-inactive mutant of human TAK1 (K63W) were PCR cloned from pEGFP-C1-TAK1-K63W into NheI/NsiI sites of PGK-SIN using the following primers: RV 5’-ATGCATTCATGAAGTGCCTTGTCAG-3’, FW 5’-GCTAGCGCCACCATGTCGACGCCCTCCGCCGCC-3’ (non-tagged, Kozak sequence for enhanced translation introduced).

**Lentiviral vector production**

Packaging of VSV-G pseudotyped recombinant lentiviruses was performed by transient transfection of 293T cells. One day prior to transfection, 293T cells were seeded in a T75 flask at 1x10^5 cells/cm^2 in DMEM supplemented with 10% FCS, 1 mM pyruvate, 40 µg/ml gentamicin and 0.01 mM water-soluble cholesterol (Sigma). Cells were co-transfected with 19 µg transfer vector, 14 µg gag/pol packaging plasmid (pMDL-g/p-RRE), 4.7 µg rev expression plasmid (RSV-REV) and 6.7 µg VSV-G expression plasmid (pHIT-G) by calcium phosphate precipitation. Transfections were performed in 6 ml DMEM without antibiotics and cholesterol and cultured for 16 hours. Thereafter medium was replaced with fully supplemented DMEM and supernatant harvested after 24 and 48 hours. Cell debris was removed by centrifugation at 1500 rpm for 5 minutes at 4 °C, followed by passage through a 0.45 µm pore polyvinylidene fluoride Durapore filter (Millipore, Bedford, MA, USA). For concentration by ultracentrifugation 28 ml supernatant was overlaid on 4 ml 20% sucrose solution and centrifuged at 25,000 rpm for four hours in a Surespin 630 rotor (Thermo Fisher Scientific, Waltham, MA). Pelleted viruses were resuspended in sterile PBS and stored at -80 °C. Viral titers were determined by assaying p24 values with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Abbott Diagnostics, Hoofddorp, the Netherlands) and expressed as ng p24/µl.

**In vivo imaging**

**In vivo** bioluminescent imaging was performed on an IVIS Lumina system (Caliper Life Sciences, Hopkinton, MA, USA), 10 minutes after intraperitoneal injection of 150 mg/kg D-Luciferine (Caliper Life Sciences) dissolved in PBS. Mice were anesthetized with isoflurane/oxygen, placed on their back into the light tight
chamber and imaged for 4 minutes with a sensitive CCD camera. Images taken were quantified using the Living Image 3.0 software (Caliper Life Sciences). Luciferase activity is presented in photons emitted per second per square cm.

**RNA isolation and quantitative PCR analysis**

Spleen, synovium, and liver samples were disrupted using the MagNaLyser (Roche). Total RNA was extracted from the tissue homogenates and from cells using TRI reagent (Sigma) according to manufacturer’s protocol. Isolated RNA was treated with DNAse followed by reverse transcription of 1µg RNA into cDNA using Moloney murine leukemia virus reverse transcriptase 0.5µg/µl oligo(dT) primers, and 12.5mM dNTPSs (Invitrogen). Quantitative real-time PCR was performed using the StepOnePlus sequence detection system (Applied biosystems, Foster City, CA). PCR was performed in a total reaction volume of 12.5 µl consisting of appropriate cDNA. Five µM of forward and reverse primer and the SYBR green PCR master mix (Applied biosystems). PCR protocol consisted of 2 min at 50°C and 10 min of 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Quantification of PCR signals was achieved by calculating the difference between the cycle threshold value (Ct) of the gene of interest with the Ct value of their reference gene glycerldehyde-3-phosphate dehydrogenase (GAPDH) for each sample (delta Ct).

**Histology**

Spleens were isolated and fixed in phosphate buffered 4% paraformaldehyde and embedded in paraffin wax. Whole knee joints were dissected and fixed in phosphate buffered 4% paraformaldehyde followed by decalcification with 5% formic acid, and embedded in paraffin wax. Serial tissue sections (7µm) were stained with Safranin O (BDH chemicals, Poole, UK) and counterstained with fast green (BHD Chemicals) or with hematoxylin / eosin (Merck, Germany) and eosin (Merck, Germany) (H&E). Serial sections were scored for histopathologic changes on a 0-3 scale, by 2 independent observers in a blinded manner. Joint inflammation was determined by the presence of synovial cell infiltrates and inflammatory cell exudates. Connective tissue destruction was determined by cartilage and bone erosion.

**FACS analysis**

Spleens from mice were mashed, filtered and erythrocytes were removed by osmotic shock and directly stained for GFP expression and CD3 (1:200 eBioscience), CD19 (1:50 BD Pharmingen), F4/80 (1:400, Biolegend), and Ly-6C (1:800, Biolegend) cell surface markers. For T-cell subset analysis in arthritic
mice CD3+ cells were isolated from spleen using the Pan T cell Isolation Kit (Miltenyi Biotec, Germany) according to manufacturer instructions. Purified CD3+ cells were stimulated in RPMI 1640 (Invitrogen) supplemented with 10% FCS penicillin/streptomycin and pyruvate with 50ng/ml PMA, 1ul/ml/10^6 cells Brefeldin A (BD Pharmingen) and 1ug/ml ionomycin. After 4 hours of stimulation cells were stained with anti-mouse CD4-APC (1:200) (BD pharmingen) or IgG2a-APC controle (BD pharmingen) for 30 minutes at 4°C. Subsequently, cells were stained intracellular with anti-IL17-FITC and anti-IFNg-PE or with their controls respectively IgG1-PE and IgG1-FITC according to instructions by manufacturer (BD Pharmingen). Stained cells were analyzed using FACScalibur (Becton Dickinson) and analyzed with FlowJow software.

Bone marrow derived dendritic cells

Femurs and tibia’s of DBA1/J mice were flushed and bone marrow was harvested. After preparing a single cell suspension cells were plated (250,000 c ells per cm²) with 833 ng p24GAG equivalent and 20 ng/ml GM-SCF. Medium was changed on day 4 and day 8.

Statistics

Statistical differences were determined by one-way ANVA or student’s T-test (two sided) and GraphPad 5.0 software. P values below 0.05 were considered significant.

Results and Discussion

Expression kinetics is dependent on route of administration

Although previous studies have shown efficient transduction of spleen cells by intravenously delivered lentivirus, there are considerable differences between Balb/c and C57Bl/6 mice(10). Therefore the kinetics of lentiviral mediated transgene expression needs to be determined in DBA1/J mice, an autoimmune prone strain used for the CIA model. We injected 10 µg p24GAG equivalent (approximately 5x10^8 viral particles) of lentivirus encoding luciferase intravenously via the retro orbital sinus and luciferase activity was determined by in vivo imaging. Figure 1A shows that maximal luciferase activity was reached at day 7 after virus injection. Organ distribution of the luciferase activity is shown in Figure 1B and strikingly a substantial level of luciferase activity was found around the throat of the animals. These are possibly the submandibular lymph nodes draining the eye, but the precise localization was not identified. To avoid targeting
Figure 1. Transgene expression kinetics.
Every animal received 10 µg of p24GAG equivalent of lentivirus encoding firefly luciferase intravenously. Luciferase activity was determined after injection of luciferine using biophotonic imaging with the IVIS Lumina. Region of interest was placed over abdomen of mice. Kinetics of transgene expression (A) and organ distribution of transduction (B) after lentivirus injection via the retro orbital sinus, n=6. B). Comparison between retro orbital sinus and tail vein injection (n=2 in both groups) for abdominal expression (C) and localization of transgene expression in tail vein injected animals (D).

This particular site and achieve more efficient delivery of LV to the spleen the retro orbital sinus injection site was compared with tail vein injections. Figure 1C shows that the expression kinetics of lentivirus injected via tail vein in spleen and liver are different from injection via retro orbital sinus. Maximal luciferase activity peaks at day 10 and expression levels of luciferase were elevated compared to retro orbital sinus injections. Tail vein injection also showed more luciferase activity in the liver and spleen area (Figure 1B and 1D). Unexpectedly, luciferase activity was also detected around the knee joint. This is most likely to originate from the bone marrow, as these cells are also transduced by lentivirus injected intravenously (7;10;11). Additionally, these results were also observed with retro orbita plexus injections (data not shown). It appears that injection of lentivirus via the tail vein favors transgene expression levels and location and all subsequent experiments were performed with tail vein injections. Injection directly into a
Intravenous delivery of lentivirus preferentially targets splenic APCs

vein is potentially preferable over retro orbital sinus as the distribution of the lentivirus throughout the body is better than into a sinus, which has a slow blood flow.

*Dose escalating study of lentivirus*

After establishing the delivery route and transgene expression kinetics the optimal dose was determined by injecting increasing doses of lentivirus encoding GFP. Figure 2A shows that increasing doses of lentivirus also resulted in increasing percentage of GFP positive cells in spleen with 7.4% of total spleen cells were GFP positive when injected with 40 µg of p24GAG equivalent of lentivirus. Analysis of the GFP mRNA expression showed an increase in GFP mRNA with increasing dose of lentivirus, except at 10 µg lentivirus due to large variation. In addition, GFP mRNA expression showed a 12 fold increase in spleen compared to liver samples (Figure 2B). Together with the luciferase activity measurements shown in Figure 1D we concluded that lentivirus efficiently transduces cells in the spleen. Although maximal expression of GFP was not reached we used 40 µg of p24GAG LV per animal in subsequent as a safe dose showing no side effects. GFP is known to elicit a specific immune response (12), but no increase in splenic cell number was observed in the dose escalating study (data not shown).

**Figure 2**

*Figure 2. Dose finding of intravenous lentivirus injection.*
Increasing doses of lentivirus encoding GFP were injected via the tail vein and GFP expression was analyzed 10 days after injection. A) Total spleen cells were analyzed by flow cytometry for GFP expression. Statistics: one-way ANOVA with Bonferonni post test compared to PBS injected animals, n=4 per group. ** p<0.01, *** p<0.001. B) GFP mRNA expression in liver and spleen samples. Relative expression = 2^{-ΔΔCt}. Data represented as mean±SEM.
Figure 3. Localization splenic GFP expression and identification of transduced cell types by cell surface marker analysis.

A) Increasing doses of lentivirus encoding GFP were injected via the tail vein and GFP expression was analyzed by immunohistochemistry ten days after virus injection. Representative histological images of experiment (magnification 200x), dotted line outlines white pulpa. W = white pulpa, R = red pulpa. Arrow heads indicate GFP positive cells. B) Flow cytometric analysis of GFP expressing cells 4 and 7 days after virus injection. Data represented as average (±SEM) GFP positive cells in gate indicated on x-axis (n=4). C) Representative plots of cell marker and GFP FACS analysis.
Macrophages and monocytes are preferentially targeted by lentivirus

Immunohistochemistry was done to determine the location of GFP expression in the spleen. Figure 3A shows that with increasing dose of lentivirus also more GFP protein is detected. GFP expression was detected around the white pulpa near the marginal zone, in line with other reports (8;13). In a separate experiment it was determined which cells are targeted by lentivirus in the spleen, leukocytes were isolated from spleen and GFP expression was determined in combination with cell surface markers (Figure 3B). This confirmed that lentivirus only marginally targets T- and B-cells, since the IHC showed that there was no GFP expression in the white pulpa of the spleen. In contrast, it appears that lentivirus preferentially targets F4/80+ and Ly-6C+ cells as 30-40% of cell populations were GFP positive. The GFP expression in F4/80+ cells remains stable for up to ten days after virus injection (data not shown). This preferential targeting of the APC population has also been described by others (8;9). The monocyte population shows a sharp drop off in GFP expression between day 4 and 7 after virus injection. This probably reflects migration or differentiation of Ly-6C+ monocytes, whereas the F4/80 expressing macrophages present in the red pulpa reside in the spleen (14). The antigen presenting cell (APC) population in the spleen consists of many different subtypes of dendritic cells and macrophages. Here, we show a preferential targeting of the macrophage and monocyte population, but identification of the exact APC subpopulation remains to be determined.

Lentivirus targets cells that play an important role during arthritis.

Next, we evaluated if the targeted cells also play a functional role in inflammatory diseases. Therefore mice immunized with bovine collagen type II were injected with lentivirus encoding a kinase negative mutant of TAK1 (TAK1-K63W) to decrease inflammatory signaling by Toll-Like Receptors and cytokines. This target was identified as an important signaling molecule in experimental arthritis and provides a suitable target to study the efficacy of intravenous injection of lentivirus (3). Figure 4A shows that disease incidence is decreased in animals injected with lentivirus overexpressing the negative TAK1 mutant. In addition, macroscopic swelling of the knee joints was significantly decreased at time of sacrifice (Figure 4B). Further histological analysis (Figure 4C-E) showed only a trend towards decreased inflammation and cartilage erosion. Bone erosion, however, was significantly decreased in mice treated with TAK1-K63W. This is in line with the altered Th17 population, which plays an important role in osteoclast activity and bone erosion (15). In addition, Ly-6C positive cells are precursors of osteoclasts (16) and inhibition of pro-inflammatory signaling on these cells possibly also inhibits osteoclast formation. A strong trend is found towards decreased levels
Figure 4. Effect of splenic TAK1 targeting on arthritis incidence and knee joint swelling.
A) Arthritis incidence was monitored over time macroscopically. B) Swelling of knee joints macroscopically at time of sacrifice (day 30 of CIA). Data represented as mean±SEM. Statistics: macroscopic scoring: Student’s t-test, n=7 per group. ** p<0.01. C-E) Knee joints were isolated and evaluated for pathohistological features. C) Inflammation scores determined on HE stained sections. D) Cartilage erosion scores determined on safranin-O stained sections. E) Bone erosion scores determined on safranin-O stained sections. F-G) Representative histological images for bone erosion of knee joints. Original magnification 100x. Bone erosion indicated with arrow head. P=patella, F=femur. Statistics: histology: Student’s t-test, n=8 for GFP, n=6 for TAK1-K63W. * p<0.05.
of cell influx into the joint and Swirski and colleagues have described a pool of cells in the spleen that migrate towards the site of inflammation (17). These cells were CD11b and Ly-6C positive and we have found Ly-6C+ cells expressing GFP after lentivirus administration (Figure 3B). Additionally, Ly6C-high expressing monocytes are attracted to the site of inflammation from the bone marrow in a kidney injury model (18). Taken together, lentivirus injected intravenously could possibly target this pool of migrating monocytes in the spleen.

*Local inflammation decreased upon lentiviral targeting of macrophages and monocytes*

From the inflamed joints synovium was extracted and mRNA analysis showed that proinflammatory cytokine mRNA expression was decreased when mice were treated with lentivirus encoding the TAK1 mutant (Figure 5). Although not significant, IL-1β production shows a trend towards diminished production in the inflamed synovium and might underly the trend in decreased cartilage erosion. The decrease in IL-6 production also supports the diminished levels of Th17, as IL-6 is a crucial factor for Th17 development (19). Moreover, RANK and

Figure 5

![Graphs showing cytokine expression in synovium](image)

**Figure 5. Proinflammatory cytokine expression in synovium of arthritic animals.** Synovium was isolated from arthritic animals and disrupted. Total mRNA was isolated and analyzed for A) cytokines (IL-1β, IL-6, KC) and B) RANK, and RANKL mRNA expression. Data represented as mean±SEM. Relative expression = 2^{-ΔCt}. Statistics: Student’s t-test, n=6. * p<0.05.
RANK ligand, important for osteoclast formation and function are decreased in synovium of inflamed knee joints (Figure 6B) (20). Together with altered systemic Th17 levels, this possibly causes the decrease in bone erosion. Additionally, production of the chemokine KC is significantly reduced and possibly explains the clear trend in reduced inflammation observed on histology. This shows that targeting the macrophages and monocytes in the spleen and liver can have distal effects on the inflamed tissue and shows the importance of this cell population in experimental arthritis.

**Figure 6**

A) Spleen cells were isolated and stimulated with PMA, ionomycin, and Brefeldin A for 4 hours and subsequently stained for CD4, IFNγ, and IL-17. Percent positive cells from CD4 gate is shown. B) Representative FACS analysis plots. IL-17 and IFNγ positive cells in CD4+ gate shown. Data represented as mean±SEM. Statistics: Student’s t-test, two tailed, n=7. * p<0.05.

**Lentivirus targets cells controlling the adaptive immunity**

To study if the targeted cells are involved in controlling the adaptive immunity, T-cell subsets were determined in the spleen. At a systemic level, the TAK1 mutant had only marginally effects on Th1 development in the spleen. However, Th17 cell population was significantly decreased in the spleen by overexpression of the TAK1 mutant (Figure 6A). This suggests that lentivirus delivered intravenously also targets cells that are involved in controlling the adaptive immunity. This is in
line with previous studies that showed transduction of MHC-II+ cells in spleen (9) and targeting of dendritic cell precursors and influence T-cell proliferation (21). In addition, systemic lentivirus delivery has been used to induce tolerance in collagen-induced arthritis and showed clear potential in affecting T-cell function, with decreased proliferation (11).

**Bone marrow derived DCs express reduced levels of IL-12 and IL-23**

To evaluate how TAK1-K63W overexpression interferes with activation of the adaptive immunity we generated bone marrow derived DCs transduced with LV-GFP or LV-TAK1-K63W. These DCs were analyzed for IL-12p35, IL-12p40, and IL-23p19 mRNA expression and showed diminished levels of IL-12p35 and IL-23p40 and an increase in the IL-12p40 subunit. These decreased levels could cause the decrease in Th1 and Th17 development during the CIA. Additionally, the increase in IL-12p40 could enhance the inhibition on Th1 and Th17 development, since the IL-12p40 subunit can antagonize the function of IL-12 (22) and IL-23 (23). These data show an important role for TAK1 in DCs in controlling the adaptive immunity.

**Figure 7**

![Graph](image)

**Figure 7. IL-12 and IL-23 mRNA expression decreased in bone marrow derived DCs**
Bone marrow cells were harvested and transduced with either LV-GFP or LV-TAK1-K63W and subsequently differentiated into dendritic cells. After ten day differentiation period, mRNA was collected and analyzed for IL-12p35, IL-12p40, and IL-23p19. Data represented as mean±SEM, Relative expression = 2^ΔΔCt, n=2.

TAK1 as therapeutic target in arthritis has been established by Courties and colleagues (3), in which TAK1 expression was knocked down using RNA interference and ameliorated collagen-induced arthritis. That study also showed altered T-cell subsets upon TAK1 targeting in APCs, but also showed a more efficacious therapy on arthritis severity. In the present study we overexpressed a negative mutant of TAK1 which is a competitive mutant and complete blockade of TAK1 cannot be achieved. In addition, lentivirus tropism is possibly more selective than lipoplexes. Nevertheless, we find matching results with TAK1 targeting in collagen-induced arthritis.
Interestingly, a novel role for TAK1 has been described recently (24;25). Both studies have investigated the role of TAK1 in the production of pro-inflammatory cytokines and, remarkably, TAK1 negatively regulates cytokine production in myeloid cells. In these studies lipopolysaccharide was used as stimulus, which is a very potent activator of neutrophils and macrophages. In addition, cytokine production was only determined over 24 hours. In contrast, collagen-induced arthritis involves a more complex process than a single stimulation and the clinical features of collagen-induced arthritis were studied for 12 days on our study.

In summary, this study shows an effective method to target a monocyte and macrophage population in spleen that is involved in inflammation and immunity. Blocking almost all pro-inflammatory signals, by overexpression of a TAK1 mutant, disrupts progression of experimental arthritis and impairs T-helper cell development. Injection of lentivirus intravenously provides a straightforward tool to study potential therapeutic targets in monocytes and macrophages during inflammatory conditions. Additionally, this study confirms the efficacy of TAK1 targeting in arthritis and emphasizes the role of this crucial MAP kinase in monocytes and macrophages during autoimmune arthritis.

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Intravenous delivery of lentivirus preferentially targets splenic APCs

References


monocytes are selectively recruited to injured kidney and differentiate into functionally distinct populations. J Immunol 183: 6733-6743.


Toll-like Receptor 4 Signalling is Specifically TAK1-independent in Synovial Fibroblasts

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Abstract

Activated synovial fibroblasts are key players in the pathogenesis of rheumatoid arthritis (RA) by driving inflammation and joint destruction. Numerous molecules including cytokines and toll-like receptor (TLR) ligands induce pro-inflammatory signalling and gene expression through a hierarchical network of kinases. Upstream mitogen-activated protein kinase kinase kinases (MAP3Ks) represent an attractive target for RA treatment. In this study we sought to determine the role of the MAP3K transforming growth factor-β activated kinase 1 (TAK1) in cytokine and TLR-mediated signalling. TAK1 activity was inhibited using either a small molecule inhibitor or lentivirally-overexpressed kinase-inactive TAK1-K63W mutant in murine embryonic and human dermal and synovial fibroblasts. Fibroblasts were stimulated with IL-1, TNF, TLR2 or TLR4 agonists and responses were evaluated using transcriptional reporters, Western blotting and analysis of gene expression of collagenases (MMP3,13), cytokines (IL-1β,-6) and chemokines (IL-8, MCP-1). TAK1 inhibition abrogated cytokine- and TLR-induced NF-κB and Saa3-promoter reporter activation in murine and human dermal fibroblasts. In synovial fibroblasts, TAK1 regulated IL-1 and TNF-α mediated NF-κB, but not Saa3-promoter reporter activation. Inducible mRNA expression of cytokines, collagenases and chemokines, except MCP-1, was TAK1-dependent for IL-1, TNF and TLR2 signalling. Unexpectedly, TLR4-mediated NF-κB reporter activation and inducible mRNA expression was fully TAK1-independent. Accordingly, NF-κB p65 and p38 MAPK phosphorylation was unaffected by TAK1 inhibition. In general, TAK1 crucially regulates IL-1 and TNF signalling in fibroblasts. Interestingly, TLR4 signalling is specifically TAK1-independent in synovial fibroblasts. Consequently, therapeutic TAK1 inhibition in arthropaties may not dampen the damage-associated molecular pattern-mediated TLR4 activation of synovial fibroblasts.
Introduction

Rheumatoid arthritis (RA) is a systemic chronic autoimmune disease that mainly affects the synovial joints that ultimately leads to joint destruction. Prior to and during joint inflammation the synovial lining tissue, consisting of synovial fibroblasts and macrophages, becomes activated and hyperplastic resulting in invasion and degradation of adjacent cartilage and bone. In both experimental and human arthritis, synovial fibroblasts have been identified as cells that actively drive inflammation and joint destruction (1, 2). Moreover, transmigration of activated RA synovial fibroblasts has been implicated in mediating the spreading of destructive arthritis to unaffected joints (3). Due to their key role in RA pathogenesis, synovial fibroblasts are major target cells for treatment of disease.

Synovial fibroblasts are potently activated by cytokines, such as IL-1 and TNF (4-6), and toll-like receptor (TLR) ligands (7-9). TLRs 2 and 4 are predominantly expressed in synovial fibroblasts and their expression is increased in RA patients (8, 10). Although TLRs are primarily activated by exogenous pathogens, they also recognize endogenous damage-associated molecular patterns (DAMPs) that are abundantly present in arthritic joints (7, 11). In experimental arthritis, it has been demonstrated that TLR4 activation promotes the onset and severity of disease (12). Moreover, DAMP-mediated activation of TLR4 specifically in synovium has been found to be crucially involved in joint destruction (13).

After ligation of their respective receptors, expression and secretion of pro-inflammatory mediators including cytokines, chemokines and matrix metalloproteinases (MMPs) is induced through multiple signalling cascades including nuclear factor-κB (NF-κB) and the mitogen-activate protein kinase (MAPK) families p38, c-Jun-N-terminal kinase (JNK), and extracellular-signal regulated kinase (ERK) (reviewed in (14)). These kinases are regulated through phosphorylation by their upstream kinases IkB kinase (IKK) and MAPK kinase (MAPKK), respectively. In turn, MAPKK kinases (MAP3Ks) control the activation of IKK and MAPKK and are activated through interactions with receptor-associated proteins, such as IL-1R-associated kinases (IRAKs) and TNFR-associated factors (TRAFs). The MAP3K family comprises numerous members of which MEK kinase-1, -2 (MEKK1,-2) and transforming growth factor-β activated kinase 1 (TAK1) are most abundantly expressed in RA synovial fibroblasts (15).

TAK1 has been identified as the key regulator of IL-1, TNF and TLR-induced activation of NF-κB and MAPK pathways in mice (16-18). Inhibition of TAK1 catalytic activity prevented chemical-induced inflammation in mice (19). However, studies using cell-type specific TAK1-deficient mice have also revealed that TAK1...
Chapter 7

is a crucial regulator of homeostasis in cartilage, skin, epithelium and liver (20-23). Therefore, insight into the cell-specific regulation of signalling pathways by TAK1 is indispensable for developing a treatment based on its inhibition.

Until now, it has been shown that TAK1 regulates the IL-1-induced JNK pathway and activator protein-1 (AP-1) transcription factor in synovial fibroblasts (24). Here, we investigated the role of TAK1 in TNF-, TLR2-, and TLR4-mediated signal transduction and induction of pro-inflammatory gene expression in murine embryonic and primary human dermal and synovial fibroblasts. The present study reveals that TLR4 signalling is specifically TAK1-independent in synovial fibroblasts.

Materials and methods

Patients and samples

Synovial tissue was obtained from RA (n=4) and osteoarthritis (OA) (n=2) patients undergoing open joint replacement surgery or arthroscopic synovectomy at the Clinic of Orthopedics, Waldkrankenhaus “Rudolf Elle”, Eisenberg, Germany (kindly provided by R.W. Kinne, University of Jena). Informed patient consent was obtained and the study was approved by the Ethics Committee of University Hospital Jena. RA and OA patients were classified according to the ACR criteria. Clinical characteristics of patients are described in Huber et al. (25). Synovial fibroblasts were purified from synovial tissue as previously published (26). Briefly, the tissue samples were minced, digested with trypsin/collagenase p (Sigma, St. Louis, MO), and the resulting single cell suspension was cultured for seven days. Non-adherent cells were removed by medium exchange. Fibroblasts were obtained by negative isolation using Dynabeads M-450 CD14 MACS purification. Human dermal fibroblasts were obtained from skin biopsies of healthy volunteers at the Radboud University Nijmegen Medical Centre (kind gift from J. Schalkwijk, Department of Dermatology).

Cell culture

Mouse embryonic fibroblasts (NIH-3T3) were maintained in DMEM supplemented with 1 mM pyruvate, 40 μg/ml gentamicin, and 5 or 10% fetal calf serum (FCS), respectively. Stable transcriptional reporter cell line NIH-3T3-5xNF-κB-luciferase was described before (27). Human dermal and synovial fibroblasts were cultivated in DMEM supplemented with 1mM pyruvate, 80 μg/ml gentamicin, and 10% FCS. Cells were kept at 37 °C in a humid atmosphere containing 5% CO2.
Plasmids

For generation of recombinant lentiviral vectors we used of the third-generation self-inactivating transfer vector pRLL-cPPT-PGK-mcs-PRE-SIN (PGK-empty) containing the human phosphoglycerate kinase (PGK) promoter (kind gift from J. Seppen, AMC Liver Center, Amsterdam, The Netherlands). For cloning we used cloned Pfu DNA polymerase (Stratagene, La Jolla, CA) and T4 DNA Ligase (New England Biolabs, Ipswich, MA). All generated constructs were verified by sequencing. A lentiviral transcriptional luciferase reporter containing four tandemly-arranged NF-κB binding sites (pTRH2-NF-κB-Luc) was purchased from System Biosciences (Mountain View, CA). Construction of the Saa3-promoter luciferase reporter is described in previously (27). The cDNA sequences of a non- and EGFP-tagged kinase-inactive mutant of TAK1 (K63W) were PCR cloned from pEGFP-C1-TAK1-K63W (kind gift from M. Kracht, Rudolf-Buchheim-Institute for Pharmacology, Giessen, Germany) into Nhel/NsiI sites of PGK-empty using the following primers: RV 5’-ATGCATTCATGAAGTGCCTTGTCAG-3’, FW 5’-GCTAGCGCCACCATGTCGACGCCTCCGCCGCC-3’ (non-tagged, Kozak sequence for enhanced translation introduced), and FW 5’-GCTGGTTAGTGAACCGTCAG-3’ (EGFP-tagged).

Lentiviral vector production

Packaging of VSV-G pseudotyped recombinant lentiviruses was performed by transient transfection of 293T cells. One day prior to transfection, 293T cells were seeded in a T75 flask at 1x105 cells/cm2 in DMEM supplemented with 10% FCS, 1 mM pyruvate, 40 μg/ml gentamicin and 0.01 mM water-soluble cholesterol (Sigma). Cells were co-transfected with 19 μg transfer vector, 14 μg gag/pol packaging plasmid (pMDL-g/p-RRE), 4.7 μg rev expression plasmid (RSV-REV) and 6.7 μg VSV-G expression plasmid (pHIT-G) by calcium phosphate precipitation. Transfections were performed in 6 ml DMEM without antibiotics and cholesterol and proceeded for 16 hours. Thereafter medium was replaced with fully supplemented DMEM and supernatant harvested after 24 and 48 hours. Cell debris was removed by centrifugation at 1500 rpm for 5 minutes at 4 °C, followed by passage through a 0.45 μm pore polyvinylidene fluoride Durapore filter (Millipore, Bedford, MA, USA). For concentration by ultracentrifugation 28 ml supernatant was overlaid on 4 ml 20% sucrose solution and centrifuged at 25.000 rpm for four hours in a Surespin 630 rotor (Thermo Fisher Scientific, Waltham, MA). Pelleted viruses were resuspended in sterile PBS and stored at -80 °C. Viral titers were determined by assaying p24gag values with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Abbott Diagnostics, Hoofddorp, the Netherlands) and expressed as ng p24gag/μl.
**Luciferase measurements**

For in vitro reporter studies, cells were seeded at 5x104 cells per well in a Krystal 2000 96-wells plate (Thermo Labsystems, Brussels, Belgium) and transduced for 4 hours with 25 ng p24gag equivalents lentivirus in 50 μl medium supplemented with 8 μg/ml polybrene (Sigma). Transduction efficiency was 80-100% and comparable between donors (data not shown). Consecutively, cells were serum starved (1% FCS) for two days, pre-incubated for one hour with indicated concentrations TAK1 inhibitor (11,12-dihydro-5Z-7-oxozaenol, AnalytiCon Discovery, Potsdam, Germany) or dimethyl sulfoxide (DMSO) as vehicle control and stimulated with recombinant murine or human IL-1β (1 ng/ml, R&D Systems Europe, Oxford, UK), TNFa (10 ng/ml, Abcam, Cambridge, UK), E. Coli LPS (1 mg/ml, Sigma), Pam3Cys (1 μg/ml, EMC Microcollections, Tübingen, Germany) for indicated hours. Cells were lysed in ice-cold lysis buffer (0.5% NP-40, 1 mM DTT, 1 mM EDTA, 5 mM MgCl2, 100 mM KCl, 10 mM Tris-HCl pH 7.5, 1x protease inhibitor cocktail (Roche, Mannheim, Germany)). Luciferase activity was quantified using the Bright-Glo luciferase assay system (Promega, Madison, WI, USA) by adding an equal volume of Bright-Glo to the cell lysate. Luminescence was quantified in a luminometer (Lumistar, BMG, Offenburg, Germany), expressed as relative light units (RLU) and normalized to total protein content of the cell extracts.

**Western blot analysis**

Synovial fibroblasts were seeded at 1x106 cells per well in 6-wells plates and serum starved (1% FCS) for forty eight hours. Cells were pre-incubated for one hour with 500 nM TAK1 inhibitor or DMSO, followed by stimulation with human IL-1β (1 ng/ml) or LPS (1 μg/ml) for 5 and 30 minutes, as indicated. Lysis was performed in 10 mM TrisHCl (pH 7.05), 50 mM NaCl, 30 mM sodium pyrophosphate, 1% Triton X-100, 2 mM Na3V04, 50 mM NaF, 20 mM β-glycerophosphate, 1 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM PMSF, 1 μM microcystin. Cell lysates were subjected to SDS-PAGE on 8% gels and western blotting was performed as described (28). Proteins of interest were detected by antibodies against phospho(S536)-p65 (Cell Signaling, Danvers, MA, USA), p65 (C-20, Santa Cruz, CA, USA ), phospho (TY180/2)-p38 (Invitrogen) and p38 (29).

**RNA isolation**

Cells were seeded at 90% confluency in 24-wells plates and serum starved (1% FCS) for forty eight hours. Consecutively, cells were pre-incubated with TAK1 inhibitor or DMSO for one hour and stimulated as mentioned above. Thereafter, cells were washed in ice-cold PBS and total RNA was extracted using TRI reagent (Sigma). Isolated RNA samples were treated with RNase-free DNase I (Qiagen,
Venlo, the Netherlands) for 15 minutes. Synthesis of cDNA was accomplished by reverse transcription PCR using an oligo(dT) primer and Moloney murine leukemia virus Reverse Transcriptase (Invitrogen).

**Quantitative PCR**

QPCR was performed using SYBR Green PCR Master mix and the ABI 7000 Prism Sequence Detection system (Applied Biosystems Inc., Foster City, CA) according to the manufacturer’s instructions. Primers were designed over exon-exon junctions in Primer Express (Applied Biosystems Inc.) and used at 300 nM in the PCR reaction. PCR conditions were as follows: 2 minutes at 50 ºC and 10 minutes at 95 ºC, followed by 40 cycles of 15 seconds at 95 ºC and one minute at 60 ºC. Gene expression (cycle threshold, Ct) values were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene (ΔCt = Ctgene − CtGAPDH). Primers sequences are listed in Supplementary Data.

**Statistics**

Data are represented as means±SEM and significant differences were calculated using a one-way analysis of variance (ANOVA) or Repeated Measures ANOVA followed by Bonferroni’s Multiple Comparison test (GraphPad Prism, San Diego, CA, USA), where appropriate. P-values of less than 0.05 were regarded significant.

**Figure 1**

*Figure 1. TAK1-dependent NF-kB activation in murine fibroblasts. TAK1 inhibition by an inhibitor and LV-TAK1-K63W*

(a): NIH-3T3-5xNF-kB-luciferase cells were either transduced with indicated p24gag equivalents LV-K63W (triangles) and control lentivirus (PGK-empty) or pre-treated with indicated concentrations inhibitor (squares) and vehicle. Two days post-transduction or one hour after pre-treatment, respectively, cells were stimulated with IL-1β (1 ng/ml) for 6 hours. (b): NIH-3T3-5xNF-kB-luciferase fibroblasts were pre-treated with 500 nM inhibitor or vehicle and, stimulated for 6 hours with murine IL-1β (1 ng/ml), TNFα(10 ng/ml), LPS (TLR4, 1 μg/ml) and Pam3Cys (TLR2, 1 μg/ml). (a and b) NF-kB activation was measured by luciferase assay and data are expressed as the percentage of activity compared to controls (mean±SEM, n=3-5). (c) Induction of Saa3 mRNA expression by IL-1β TNFα, LPS (TLR4) and Pam3Cys (TLR2) in NIH-3T3 stably transduced with LV-TAK1-K63W or PGK-empty. Data are represented as fold induction (mean±SEM, n=3) over untreated cells. * P<0.05, *** P<0.001 by ANOVA.
Results

TAK1 regulates cytokine- and TLR-induced NF-κB activation in murine fibroblasts

TAK1 has been identified as the crucial mediator of IL-1- and TNF-induced activation of NF-κB, JNK and p38 MAPK signal transduction pathways in NIH-3T3 fibroblasts (29). Using a 3T3 NF-κB reporter cell line, we investigated TAK1-inhibitory efficiency of a small molecule inhibitor (19) and lentivirally-overexpressed kinase-inactive TAK1-K63W mutant (LV-K63W) (Figure 1A). Maximal inhibition of IL-1β-induced NF-κB activity was achieved at 500 nM inhibitor (66.2±8.6%) or 150 ng p24gag equivalents LV-K63W per 5x10^4 cells (65.4±6.2%), respectively. There were no significant differences in maximal inhibition between inhibitor and LV-K63W, and aforementioned concentrations were applied throughout following experiments. Next, we analysed TAK1-mediated regulation of TLR-induced signal transduction (Figure 1B). Treatment of fibroblasts with inhibitor resulted in a significant reduction in NF-κB activation (~60%, P < 0.05) induced by TLR2 (Pam3Cys) and TLR4 (LPS) agonists. As a control, IL-1- and TNF-induced NF-κB activation were also potently suppressed by inhibitor. Finally, we evaluated the effect of TAK1 inhibition on the inducible expression of serum amyloid A3 (Saa3), which has been identified as a strictly TAK1-dependent TNF target gene (29) (Figure 1C). Stable transduction with LV-K63W also completely prevented induction of Saa3 expression by IL-1β and LPS. These results confirm the crucial role of TAK1 in mediating cytokine- and TLR-mediated signal transduction in murine fibroblasts.

Figure 2

**Figure 2. TAK1-dependent NF-κB and Saa3-promoter activation in human dermal fibroblasts.** Dermal fibroblasts were co-transduced with 150 ng LV-TAK1-K63W / PGK-empty and 25 ng LV-Saa3/ NF-κB-Luc, serum-starved for two days and thereafter stimulated with human IL-1β (1 ng/ml), TNFa (10 ng/ml), LPS (TLR4, 1 μg/ml) for 6 hours. NF-κB (a) and Saa3-promoter (b) activation were determined using a luciferase assay. Data are expressed as the percentage of activity compared to PGK-empty (mean±SEM, n=4). Induction of IL-1β, IL-6 and IL-8 mRNA expression by IL-1β was determined by RT-PCR (c). Data are represented as fold reduction compared to PGK-empty (2-ΔΔCt, ΔΔCt= ΔCt K63W- ΔCt control). Statistical differences were determined using ANOVA. ** P<0.01, *** P<0.001
Toll-like Receptor 4 Signalling is Specifically TAK1-independent in Synovial Fibroblasts

TAK1-dependent signalling in primary human dermal fibroblasts

Since activated fibroblasts play an important role in the pathogenesis of chronic inflammatory diseases (1, 30), inhibition of inflammatory signal transduction represents a straightforward treatment strategy. Therefore, we assessed the contribution of TAK1 to pro-inflammatory signal transduction in primary human dermal and synovial fibroblasts. Overexpression of TAK1-K63W in dermal fibroblasts completely abrogated IL-1β-induced NF-κB activation (Figure 2A). Also NF-κB activation through TNF-α (66.2±2.5%) and TLR4- (68.5±4.6%) was strongly reduced. Since SAA3 is a pseudogene in humans and TAK1 directly regulates the transcriptional activity of the Saa3 promoter (29), we additionally used a Saa3-promoter luciferase reporter for studying the effect of TAK1 inhibition in human fibroblasts (Figure 2B). TAK1 crucially regulated IL-1, TNF- and TLR4-mediated activation of Saa3-promoter reporter as revealed by an approximately eighty percent reduction of luciferase activity. Reduction of NF-κB and Saa3-promoter activation through TAK1 inhibition correlated with a suppression of IL-1β, IL-6 and IL-8 gene expression (Figure 2C). These data identify TAK1 as a pivotal regulator of pro-inflammatory signalling in dermal fibroblasts.

Cytokine- and TLR-mediated induction of transcriptional reporters and pro-inflammatory genes in synovial fibroblasts

In order to identify TAK1-dependent signalling pathways in synovial fibroblasts, we first validated whether cytokines and TLR ligands induced NF-κB and Saa3-promoter transcriptional reporters (Figure 3A,B) and pro-inflammatory gene expression (Figure 3C-F). We found strong upregulation of both reporter activities upon IL-1, TNF and TLR4 stimulation, whereas TLR2 stimulation only led to marginal induction. Accordingly, expression of NF-κB target genes IL-1β, IL-6 and IL-8 was strongly upregulated upon cytokine and TLR stimulation. Additionally, we detected significantly induced expression of AP-1 (MMP3/13) (31) and interferon regulatory factor-3 (MCP-1) (32) transcription factor target genes.

TLR4-mediated signalling is TAK1-independent in synovial fibroblasts

Next, we repeated experiments as described above in the presence of TAK1 inhibitor. Consistent with our previous results in murine and human dermal fibroblasts, IL-1 (47.9±7.9%) and TNF-mediated (48.4±6.2) NF-κB activation was significantly reduced through TAK1 inhibition (Figure 4A). In contrast, induction of Saa3-promoter activation was unaffected by treatment with TAK1 inhibitor (Figure 4B). Surprisingly, both NF-κB and Saa3-promoter activation through TLR4 triggering demonstrated TAK1-independent, as indicated by a complete lack of inhibition of transcriptional reporters upon inhibitor treatment. These
Figure 3

**Figure 3. Induction of transcriptional reporters and pro-inflammatory gene expression in synovial fibroblasts**

Fibroblasts were transduced with 25 ng LV-Saa3/NF-κB-Luc, serum-starved for two days and thereafter stimulated with indicated ligands for 6 hours. NF-κB (a) and Saa3-promoter (b) activation were determined using a luciferase assay. Data are represented as fold induction over basal levels (mean±SEM, n=4). Induction of pro-inflammatory gene expression was determined by RT-PCR (c-f). Synovial fibroblasts were serum-starved for two days, pre-incubated for one hour with vehicle, and either left untreated or stimulated for 6 hours with IL-1β (1 ng/ml, c), TNFα (10 ng/ml, d), LPS (TLR4, 1 μg/ml, e), and Pam3Cys (TLR2, 1 μg/ml, f). Data are represented as fold induction (2-ΔΔCt) compared to untreated cells (means±SEM, six donors). Statistical differences were determined using ANOVA. *P<0.05, ** P<0.01, *** P<0.001.
Toll-like Receptor 4 Signalling is Specifically TAK1-independent in Synovial Fibroblasts

Results prompted us to investigate phosphorylation of components of the NF-κB and MAPK pathway by Western blotting (Figure 4C,D). Stimulation of synovial fibroblasts with IL-1 and LPS clearly induced phosphorylation of p38 MAPK and the p65 subunit of NF-κB. In the presence of TAK1 inhibitor, IL-1β-induced phosphorylation of both substrates was considerably reduced. Corroborating the data above, TAK1 inhibition failed to affect phosphorylation induced by LPS.

To conclude, we analysed the effect of TAK1 inhibition on the induction of pro-inflammatory genes by cytokines (Figure 5A,B) and TLR agonists (Figure 5C,D). Indeed, induction of NF-κB target genes IL-1β, IL-6 and IL-8 through IL-1 and TNF was significantly reduced by TAK1 inhibitor treatment. Corresponding with the established regulation of JNK-AP-1 pathway by TAK1 (24), IL-1 and TNF-induced MMP3 expression was also clearly reduced. The IRF3-target gene MCP-1 demonstrated TAK1-independent for all applied stimuli. Whereas TAK1 inhibition resulted in suppression of TLR2-induced IL-1β, IL-6 and MMP3 expression, TLR4-induced gene expression was completely unaffected by inhibitor treatment. Together these data reveal TAK1-independent TLR4 signal transduction that is specific for synovial fibroblasts.

Figure 4. TAK1-independent TLR4 signalling and Saa3-promoter activation in synovial fibroblasts
Fibroblasts were transduced with 25 ng LV-Saa3/NF-κB-Luc and consecutively serum-starved for two days, pre-treated with 500 nM inhibitor or vehicle for one hour, and either left untreated or stimulated with human IL-1β (1 ng/ml), TNFα (10 ng/ml), LPS (TLR4, 1 mg/ml) for 6 hours. NF-κB (a) and Saa3-promoter (b) activation were measured using a luciferase assay and data are represented as relative activity (mean+SEM, four donors, quadruplicate measurements) compared to untreated cells. Western blot analysis of NF-κB p65 and p38 MAPK phosphorylation (c and d). Fibroblasts were serum-starved for two days, pre-treated with 500 nM inhibitor or vehicle for one hour, and stimulated with human IL-1β (c) and LPS (d) for indicated time points. A representative picture of two donors is shown. Statistical differences were determined using ANOVA. *** P < 0.001.
Discussion

Given the multitude of activating molecules and redundancy and complexity of MAPK signalling (33, 34), upstream MAP3Ks are of particular interest as therapeutic targets as they potentially couple multiple receptors to downstream signalling pathways. In this study, we investigated the role of the MAP3K TAK1 in mediating pro-inflammatory signalling in several types of fibroblasts and revealed specific TAK1-independent TLR4 signalling in human synovial fibroblasts.

Although there is substantial evidence that IL-1 signals through both TAK1 and MEKK3 (35, 36) information on a physiological role of these two kinases in synovial fibroblasts is limited. In two studies, analysis of MAP3K expression on mRNA and protein level in synovial fibroblasts revealed abundant expression of TAK1 and
trace amounts of MEKK3 (15). Knock down of MEKK3 did not affect IL-1β-induced MAPK activation and TAK1 was identified as the crucial mediator in JNK, but not ERK or p38 MAPK pathways. siRNA-mediated suppression of TAK1 did not significantly inhibit NF-κB nuclear translocation and DNA binding, but suppressed IL-6, a typical NF-κB target gene (24). Hence, our results, using transcriptional reporter systems confirm that a significant portion of NF-κB activation through IL-1R and TNFR is mediated by TAK1.

Unexpectedly, we discovered that TAK1 plays no role in TLR4-induced NF-κB activation and pro-inflammatory gene expression in synovial fibroblasts. Several studies have emphasized a central role for TAK1 in LPS/TLR4-mediated NF-κB activation in murine macrophages, embryonic fibroblasts, B-cells, and human HEK293 cells (16, 17, 37, 38). In addition, we have revealed that TAK1 regulates NF-κB in murine NIH-3T3 fibroblasts and primary human dermal fibroblasts. TLR4 activates signal transduction through TRIF-(TIR-domain-containing adapter-inducing interferon-β) and MyD88-dependent pathways. Upon ligand binding MyD88 is recruited, which subsequently leads to the recruitment and phosphorylation of IRAK proteins, which then interact with TRAF6. The activated complex activates downstream IKK and MAPKKs through an interaction with TAK1. The TRIF-dependent pathway activates the interferon regulatory factor-3 (IRF3) pathway through TANK binding kinase-1 (TBK1). NF-κB and MAPK pathways are activated through interaction with receptor interacting protein-1 (RIP1), which consecutively interacts with TRAF6 and TAK1 (39). Maximal induction of inflammatory cytokines such as TNFα and IL-6 is dependent on the activation of both TRIF and MyD88 pathways (40). Based on these studies, we expect TAK1-dependent regulation of MyD88- and TRIF-dependent NF-κB and MAPK activation, but not the IRF3 pathway. The latter was confirmed by our RT-PCR analysis results, which indicated indeed that regulation of the IRF3 target gene MCP-1 (32) was not inhibited by treatment of synovial fibroblasts with TAK1 inhibitor. Inhibition of IL-1β-induced NF-κB activation by inhibitor treatment suggested a MyD88-TAK1-IKK pathway in synovial fibroblasts. Based on the latter, and our observations that TAK1 does not mediate TLR4 signal transduction in synovial fibroblasts, at least two mechanism might underlie these remarkable results.

First, the TLR4-MyD88-IKK/MAPK pathway can be regulated by a MAP3K other than TAK1. Recent investigations have unravelled a TAK1-independent and MEKK3-dependent mechanism for TLR8-mediated IKK and JNK activation (41), and a similar pathway was discovered for IL-1 mediated NF-κB activation (42). As in our study, NF-κB activity is not completely inhibited by the TAK1 mutant or the TAK1 inhibitor, and knowledge on regulation of MEKK3 in synovial fibroblasts is very limited. Therefore, MEKK3 is a likely candidate that accounts for TAK1-
independent signalling in synovial fibroblasts. Thus, a divergence of IL-1R/TLR MyD88-dependent pathways into IL-1R-TAK1- and TLR4-MEKK3 routes could be a possibility.

Second, TLR4 signalling through the MyD88-dependent pathway might be less dominant or even ablated in synovial fibroblasts. A possible mechanism for such phenomenon has been described for LPS-tolerance induction in human monocytes and dendritic cells (43, 44). Upon restimulation with a TLR4 ligand tolerized cells show decreased TLR4-MyD88 complex formation, which results in impaired IRAK-1 phosphorylation (45). Moreover, it has been shown that IRAK-M is upregulated in RA synovial fibroblasts, which acts as a negative regulator of the MyD88-dependent pathway, and is associated with tolerance induction (46, 47). If this were to take place in synovial fibroblasts, we would expect predominant signal transduction through TRIF. However, in the case of pre-dominant TRIF signalling we would still expect activation of the TRIF-TRAF6-TAK1 pathway, unless TAK1 can be bypassed for NF-κB and MAPK signalling in this route.

Circumventing TAK1 in TLR4 mediated NF-κB activation can be achieved by TANK, which was shown to slightly induce NF-κB transcription after overexpression (48). Another pathway without involvement of TAK1 mediated NF-κB activation has been discovered in IL-1 signalling (49). In this pathway, TRAF6 is associated with p62, which activates atypical protein kinase C and this subsequently leads to NF-κB activation.

Not only TLR4 signalling, but also cytokine-induced Saa3-promoter activation was specifically TAK1-independent in synovial fibroblasts. This promoter is synergistically activated through cooperation of NF-κB and CAAT/enhancer-binding protein (C/EBP) transcription factors (50, 51). Since IL-1/TNF-induced NF-κB activation proved to be TAK1-dependent, the lack of Saa3-promoter activation upon TAK1 inhibition could point towards differential regulation of C/EBP transcription factors in synovial versus dermal fibroblasts.

In conclusion, using a previously characterized dominant-negative mutant or small molecule inhibitor of TAK1, we have found a more restricted role for this MAP3K in mediating pro-inflammatory signalling in synovial fibroblasts. This phenomenon proved independent from disease as it was observed both in RA and OA patients. In the light of the recently established role of TLR4 in experimental arthritis (12, 13, 52), insight into TLR4 signalling in synovial fibroblasts is of particular interest for understanding pathogenesis and treatment. The underlying mechanism for TAK1-independent regulation of TLR4 signalling, potentially TLR4 tolerance or involvement of an alternative MAP3K, remains to be addressed in future research.
Acknowledgements

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Chapter 7

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Toll-like Receptor 4 Signalling is Specifically TAK1-independent in Synovial Fibroblasts


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Chapter 8

TLR4 and TRIF mediated chemokine production controls Th17 accumulation

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Abstract

TLR4 activation increases Th17 development and plays a role in autoimmunity. TLR4 uses two distinct signaling pathways, the proinflammatory MyD88 pathway and the TRIF pathway. The putative anti-inflammatory TRIF pathway is less well studied in arthritis. Therefore, the role of TLR4 and TRIF signaling in T-cell dependent flare-up of experimental arthritis was studied. Antigen-induced arthritis (AIA) was induced in wild type, TRIF−/− and TLR4−/− (C57Bl/6). Three weeks after primary arthritis, smoldering inflammation was flared. T helper subsets, cytokine and chemokine production were determined 4 hours and 4 days after flare-up. Joint swelling and pathology were determined four days after flare-up. TLR4 deficiency decreased Th17 levels by 44% in the inflamed synovium immediately after flare-up, whereas TRIF deficiency did not affect Th17 levels. IL-6 production, however, was increased in TRIF−/− animals. Four days after flare-up, both TRIF−/− and TLR4−/− showed reduced numbers of Th17 in draining lymph nodes, 50 and 58% respectively. Additionally, a trend in reduced IP-10 and RANTES production was observed in draining lymph nodes of TRIF−/−, possibly explaining the reduced swelling and inflammation in TRIF knock out animals. TLR4 is important in Th17 cell numbers immediately after flare-up inflammation and during resolution of inflammation. TRIF signaling, however, appears to control Th17 cell numbers only during the resolution phase. The chemokine RANTES appears to be an important factor in Th17 accumulation. Moreover, TRIF signaling is involved in swelling and inflammatory cell influx into the inflamed joint, for which IP-10 is potentially an important chemokine.
Introduction

Rheumatoid arthritis is a disease with unknown etiology affecting the articulating joints. The chronic inflammation results in destruction of the joint causing pain and immobility. Toll-Like Receptors (TLRs) have emerged over recent years as important players in inflammatory and autoimmune diseases, such as arthritis. TLRs bridge the innate and adaptive immunity by determining antigen presenting cell (APC) activity and thereby controlling T-cell activation (1).

TLR4 has been extensively studied in experimental arthritis models (2-4), were it was found that TLR4 has been found to have an aggravating role in established arthritis. TLR4 is a unique TLR in the fact that it uses two signaling pathways, the MyD88 dependent and a MyD88 independent pathway (5). The latter uses TIR-domain-containing adapter-inducing interferon-β (TRIF), resulting in NF-κB and interferon regulatory factor 3 (IRF3) activation respectively (6). The MyD88 pathway is shared with nearly all TLRs, making it subject of many studies. The TRIF pathway is only shared with TLR3 and is a crucial pathway during viral infections (7), because it induces type I IFN production. Although important, it is less well studied and its role in inflammatory conditions should be evaluated.

TRIF signaling results in activation of IRF3 and subsequent production of IFNα and IFNβ (6), which in turn can induce expression of suppressor of cytokine signaling (SOCS) proteins (8). These proteins have anti-inflammatory capacities by blocking downstream signaling of TLRs and cytokines (9). Moreover, TRIF signaling induces IL-27 production that controls Th17 development (10). Type I IFNs also have direct effect on the adaptive immunity as they are involved in isotype switching of antibodies (11) and control T-cell activation directly (12). Therefore, TRIF signaling and the accompanied type I IFN production play a complex role during inflammation and possibly also in arthritis.

In this study we investigated the role of TLR4 and TRIF in antigen-induced arthritis (AIA). A primary inflammation was flared-up after three weeks where it develops in a T-cell dependent local inflammation. Considering the anti-inflammatory effects of TRIF and type I IFNs we anticipated an increase in inflammation and joint destruction. However, we found a proinflammatory role of TRIF signaling in this T-cell dependent flare-up of arthritis, as Th17 numbers subsided more rapidly and swelling and cellular infiltrate were reduced in TRIF knockout animals. Additionally, TLR4 was important in Th17 cell numbers in the arthritic joint, but played a minor role in joint destruction.
Material and Methods

Mice

C57BL/6 wild type, TLR4 knock outs (kind gift from Akira, Tokyo, Japan), and TRIF knock outs (kind gift from Bruce Beutler, The Scripps Research Institute, US) mice were housed in individually ventilated cages and fed a standard diet with freely available food and water. Animal studies were approved by the Institutional Review Board of Radboud University Nijmegen and were performed according the appropriate codes of practice.

Antigen induced arthritis

Mice were immunized with 100 µg of mBSA (Sigma), emulsified in 100 µl Freund’s complete adjuvant (Difco Laboratories, Detroit, MI< USA). Injections were divided over both flanks and footpads of the forelegs. Heat Killed Bordetella pertussis (RIVM, Bilthoven, The Netherlands) was administered intra peritoneally as an additional adjuvant. Two subcutaneous booster injections with in total 50 µg mBSA/Freund’s complete adjuvant were given in the neck region 1 week after the initial immunization. Three weeks after these injections, primary AIA was induced by injecting 60 µg mBSA in 6 µl of phosphate buffered saline into the right knee joint, resulting in chronic arthritis. At week three of arthritis, 2 µg of mBSA was injected intraarticularly into the arthritic joint to induce a flare-up of the smoldering inflammation.

Joint swelling

Joint swelling was assessed by measuring the accumulation of $^{99}$mTc in the inflamed joint due to increased blood flow and edema. For this, 0.74 MBq of $^{99}$mTc in 200 µl of saline was injected subcutaneously. After several minutes of distribution throughout the body, gamma radiation in the knee joints was measured externally. Swelling was expressed as the ratio of gamma counts in the right (inflamed) knee joint to gamma counts in the left (control) knee joint. Ratios higher than 1.1 were considered to represent joint swelling.

Histology

Knee joints were isolated and fixed in 4% paraformaldehyde for minimally 8 days followed by decalcification in 5% formic acid and subsequently embedded in paraffin. Haematoxylin and eosin staining of 7 µm sections were used to determine inflammation (score 0-5) and safranin-O and fast green staining was used to score bone erosion (score 0-5) and cartilage destruction (score 0-5).
**RNA isolation and quantitative PCR analysis**

Synovium was disrupted with MagnaLyze (Roche) and total RNA was extracted using TRI reagent (Sigma) according to manufacturer’s protocol. Isolated RNA was treated with DNase followed by reverse transcription of 1 ug RNA into cDNA using Moloney murine leukemia virus reverse transcriptase 0.5ug/ul ologo(dT) primers, and 12.5mM dNTPSs (Invitrogen). Quantitative real-time PCR was performed using the StepOnePlus sequence detection system (Applied biosystems, Foster City, CA) PCR was performed in a total reaction volume of 12.5 µl consisting of appropriate cDNA. Five µM of forward and reverse primer and the SYBR green PCR master mix (Applied biosystems). PCR protocol consisted of 2 min at 50°C and 10 min of 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Quantification of PCR signals was achieved by calculating the difference between the cycle threshold value (Ct) of the gene of interest with the Ct value of their reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each sample (delta Ct) and expressed as relative mRNA expression (2^(-delta Ct)).

**Synovium cell isolation**

Procedure has been used as described before (13). Briefly, knee joint synovium was dissected, and synovial biopsy specimens were incubated with enzymatic digestion buffers (Liberase; Roche) for 30 minutes at 37°C. A 70-µm nylon cell strainer (BD Falcon) was then used to process the digested tissue. The cell preparation was collected in RPMI with 10% fetal calf serum (FCS). To isolate the single mononuclear cells, Lympholyte-M (Cederlane) was used, according to the manufacturer’s protocol. All cells were cultured in RPMI 1640 (Gibco-Invitrogen) supplemented with 10% FCS.

**Flow cytometric analysis spleen and draining lymph nodes (DLN) cells**

DLNs were disrupted by a 70 µm mesh and cells were stimulated in RPMI 1640 (Invitrogen) supplemented with 5% FCS penicillin/streptomycin, pyruvate, 50ng/ml PMA, 1 ug/ml Ionomycin, and Brefeldin A (BD Pharmingen). After 4 hours of stimulation cells were stained with anti-mouse CD4-APC (1:200) (BD pharmingen) or IgG2a-APC controle (BD pharmingen) for 30 minutes at 4°C. Subsequently, cells were stained intracellularly with anti-IL17-FITC and anti-IFNg-PE or with their controls respectively IgG1-PE and IgG1-FITC according to instructions by manufacturer (BD Pharmingen). Stained cells were analyzed using FACScalibur (Becton Dickinson) and analyzed with FlowJow software.
Cytokine measurements

IL6, KC, IL-β, TNF-α, IL-17A, and IFNγ levels in patella wash outs were measured on a Luminex-100 System (Luminex corp.) using a bead-based multiplex immunoassay (Milliplex, Merck Millipore). Data analysis was performed with Bio-Plex Manager software (Bio-Rad Laboratories).

Statistics

Statistical differences were calculated using GraphPas 5.0 software with one-way ANOVA analysis. TLR4 and TRIF knockouts were compared to wild type animals. P values under 0.05 were considered significant.

Figure 1

**Figure 1.** T helper subsets in inguinal lymph nodes and synovium after primary arthritis induction

Seven days after arthritis induction with 60 µg mBSA intraarticularly cells from inguinal lymph nodes (A) and synovium (B) were isolated and stained for extracellular CD4 and intracellular IFNγ and IL-17A. Data represented as mean ± SEM, DLN n=6, synovium n = 3.
Results

**TRIF and TLR4 are not involved in primary antigen-induced arthritis**

To evaluate the role of TRIF signaling and TLR4 activation in AIA TRIF and TLR4 knockouts were immunized and arthritis was locally induced. No differences were found on joint pathology (data not shown), indicating that TRIF and TLR4 play a minor role in primary AIA. To study the effects of TRIF and TLR4 on T-cell skewing we analyzed the Th1 and Th17 cells in DLN and synovium of the affected joints. Figure 1 shows that neither TRIF signaling nor TLR4 is involved in regulating Th1 and Th17 cell numbers in DLN. In synovium, however, a non-significant increase in Th1 and decrease of Th17 was observed in TRIF knockouts. This shifts the T-cell balance towards Th1 in TRIF/- compared to wild types and TLR4/-.

Figure 2. Cytokine release from synovium 4 hours after flare-up

Four hours after flare-up injection with 2µg mBSA patella and surrounding synovial tissue was extracted and incubated in RPMI medium for 1 hour. IL-6, IL-1β, TNF-α, IP-10, and RANTES were measured with a bead-based multiplex immunoassay. Data represented as mean ± SEM, statistics: one-way ANOVA, with Bonferroni post test (TRIF/- and TLR4/- compared to WT), n=6, * p<0.05.
TRIF regulates IL-6 production and TLR4 determines chemokine production during flare-up

To study the role of TRIF signaling and TLR4 activation in flare-up of smoldering arthritis a non-arthritic dose of antigen was injected three weeks after the primary arthritis. The flare-up resulted in vast amounts of IL-6 produced in synovium (Figure 2). TRIF signaling appears to be an important inhibitor of IL-6 production, since TRIF knockout animals showed significant elevated production of IL-6. In addition, TNFα and IL-1β showed a clear trend towards increased production in TRIF knockouts. This indicates that TRIF signaling inhibits the cytokine burst immediately after reactivation of inflammation, most likely through upregulation of suppressor of cytokine signaling (9). Interestingly, production of chemokines IP-10 and RANTES was not increased in TRIF knockouts. These chemokines are regulated differently than the NF-κB dependent genes that are increased. Cytokine expression in TLR4 knockouts was only marginally affected. The TRIF dependent chemokines IP-10 and RANTES, however, show a clear trend towards decreased production in the synovium of arthritic joints of TLR4−/− mice.

Figure 3. Thelper subset analysis and cytokine release from synovium 4 hours after flare injection

Four hours after flare-up injection with 2 µg mBSA patella and surrounding synovial tissue was extracted and incubated in RPMI medium for 1 hour. (A) After incubation synovial tissue was minced and digested for 45 minutes with Liberase enzyme cocktail. Inflammatory cells were subsequently isolated with Lympholyte-M and stimulated with PMA and ionomycin for 4 hours. Cells were stained for extracellular CD4 and intracellular IFNγ and IL-17A. (B) IFNγ and IL-17 were measured with a bead-based multiplex immunoassay. Data represented as mean ± SEM, statistics: one-way ANOVA, with Bonferroni post test (TRIF−/− and TLR4−/−compared to WT), n=6, * p<0.05.
TLR4, but not TRIF, deficiency decreases Th17 cell numbers in synovium immediately after flare-up

RANTES is a chemokine for Th17 cells (14), whereas IP-10 is capable of attracting Th1 cells (15). Therefore we determined Th1 and Th17 cells in the inflamed joints. Additionally, we determined IL-17 and IFNγ production in synovium of arthritic knee joints. Figure 3 shows that TRIF deficiency did not affect the Th1 and Th17 cell numbers nor the production of IFNγ. A minor increase of IL-17 production was observed, possibly due to increased in IL-6 and IL-1β production. Taken together, it appears that TRIF does not play a role in T-cell accumulation at the site of inflammation immediately after flare-up. In contrast, TLR4 does control the number of Th17 cells in the inflamed joint, since TLR4 knockouts showed reduced Th17 cells and reduced IL-17 production. Therefore, it appears that TLR4 mediated of IP-10 and RANTES expression could be important factors in controlling the accumulation of T-cells, and more specifically Th17 cells, at the site of inflammation.

TRIF and TLR4 maintain Th17 levels in later phase of arthritis

To study the role of TRIF and TLR4 in controlling Th1 and Th17 levels during the resolution of arthritis, DLN and synovium of affected joints were isolated for Th1 and Th17 analysis. TLR4 knockouts show a slight decrease in Th1 levels in synovium and DLN, but again significant diminished levels of Th17 cells in DLN (Figure 4A and B). These results confirm the role of TLR4 in controlling the adaptive immunity and in particular Th17. In contrast to the earlier time point, we do find a decrease in Th17 cells in DLN of TRIF knockouts during the resolution phase. This might indicate that TRIF signaling plays a role in prolonging arthritis.

To study whether IP-10 and RANTES are also involved in determining the T-cell levels during the resolution phase, we determined their production in synovium. Figure 4C shows marginal effects on IP-10 production, possibly linked to the slight changed in Th1 levels observed. RANTES expression is non-significantly decreased in TRIF and TLR4 knockouts and this could explain the marginal effects on Th17 in synovium (Figure 4B). Since IP-10 production has considerably dropped and also RANTES levels are diminished, it is conceivable that both chemokines play a minor role in subsiding inflammation.
Chapter 8

TRIF regulates swelling and inflammatory cell influx

Swelling of knee joints was measured to study the effects of TRIF and TLR4 on inflammation. Both TRIF and TLR4 decreased joint swelling (Figure 5A), albeit TLR4 not significantly. The inflammatory cell influx into inflamed knee joints was examined on histology (Figure 5B), in which both TRIF and TLR4 appear to play a role, although TLR4 failed to reach statistical significance. Both the decreased joint swelling and cell influx could be caused by the decreased chemokine production in TRIF and TLR4 knockouts, due to diminished recruitment of inflammatory cells. Despite decreased number of Th17 cells and slightly reduced swelling and inflammatory influx in TLR4 knockouts, no improvements was observed on cartilage and bone degradation. TRIF signaling, however, appears to play a minor role in cartilage and bone destruction, since a trend is observed in decreased cartilage and bone erosion.

Figure 4

Figure 4. T helper subsets in inguinal lymph nodes, and synovium 4 days after flare-up

Cells from inguinal lymph nodes (A) and synovium (B) were isolated and stimulated with PMA, ionomycin, and golgi-plug for 4 hours. Cells were stained for extracellular CD4 and intracellular IFNγ and IL-17A. (C) Synovial production of IP-10 and RANTES was measured with a bead-based multiplex immunoassay. Data represented as mean ± SEM, statistics: one-way ANOVA, with Bonferonni post test (TRIF-/- and TLR4-/-compared to WT), n=6 for spleen and inguinal lymph nodes, n=3 for synovium, * p<0.05.

TRIF regulates swelling and inflammatory cell influx

Swelling of knee joints was measured to study the effects of TRIF and TLR4 on inflammation. Both TRIF and TLR4 decreased joint swelling (Figure 5A), albeit TLR4 not significantly. The inflammatory cell influx into inflamed knee joints was examined on histology (Figure 5B), in which both TRIF and TLR4 appear to play a role, although TLR4 failed to reach statistical significance. Both the decreased joint swelling and cell influx could be caused by the decreased chemokine production in TRIF and TLR4 knockouts, due to diminished recruitment of inflammatory cells. Despite decreased number of Th17 cells and slightly reduced swelling and inflammatory influx in TLR4 knockouts, no improvements was observed on cartilage and bone degradation. TRIF signaling, however, appears to play a minor role in cartilage and bone destruction, since a trend is observed in decreased cartilage and bone erosion.
Discussion

In the present study the role of TRIF and TLR4 in T-cell dependent flare-up of arthritis was investigated. We show that TLR4 is important in accumulation of IL-17 producing CD4+ T-cells directly after injection of antigen initiating inflammation. The chemokines IP-10 and RANTES potentially play a crucial role in the attraction of T cells. TRIF signaling, however, is involved in prolonging arthritis, since TRIF knockouts show reduced levels of Th17 during the resolution phase of arthritis. In accordance, joint swelling and inflammation in TRIF knockout animals was decreased compared with wild type animals.

Figure 5

TLRs bridge the innate and adaptive immunity by instructing T-cell development (1). The role of TLR4 has been extensively studied in Th17 differentiation and development and in this study we confirm a role of TLR4 in controlling Th17 cell numbers at the site of inflammation. Additionally, TLR4 plays an aggravating role in experimental arthritis (2). However, in this model we observe only a minor role
of TLR4 in joint swelling. The effects of TLR4 on joint destruction was negligible however, which is likely due to very limited joint destruction generating little TLR4 stimulating DAMPs and causing an inferior role of TLR4. This is in contrast with previous findings (16) where TLR4 knockouts were completely protected against joint destruction during primary AIA. Their immunization protocol differs greatly from our experimental design. Midwood et al., initiated primary AIA 7 days after immunization, whereas we allowed an immunization period of 28 days before initiating primary arthritis. This difference has a significant impact on the process involved, the protocol used by Midwood et al will not involve a strong adaptive immunity response. Therefore, the role of TLR4 could be entirely different in both protocols.

The role of TRIF signaling in inflammation is less well studied and here we describe an unexpected result where TRIF signaling decreases Th17 cell numbers at the site of inflammation during the resolution phase of arthritis. This is in contrast with previous reports in other experimental models of arthritis and multiple sclerosis that show an increase of Th17 development in TRIF knockout animals (10;17). First, TRIF played a protective role an experimental arthritis model induced by repeated local injections with streptococcal cell wall fragments (SCW). This model, however, is based on strong TLR2/NOD2 stimulation that does not signal through TRIF. Addiotionally, it has been shown that this model is less dependent on FcγReceptor triggering (22) and this could imply a minor role of B-cells and antibody formation, which is crucial for the AIA. Second, Guo et al. showed an anti-inflammatory effect of TRIF signaling by induction of IL-27, which limits Th17 development. In contrast to our study, Guo et al have studied the onset and pathology of experimental autoimmune encephalomyelitis, whereas we studied the role of TRIF during flare-up of a smoldering arthritis. This potentially could have consequences for the IL-27-Th17 axis, making it of less importance during disease rather than the onset of disease. This requires further study however. Additionally, TLR4 mediated TRIF signaling in dendritic cells (DCs) is important for production of IL-23 (18), an important survival factor for Th17 cells (19). Moreover, TRIF signaling in DCs is crucial for optimal DC maturation and subsequent T-cell activation (20). TRIF signaling appears to play a proinflammatory role in a T-cell mediated flare-up of arthritis due to a possible less important role of IL-27 and potentially reduced levels of IL-23 produced by DCs.

Accumulation and migration of leukocytes is directed by chemokines. In this study we have determined the of RANTES (CXCL5) in synovium samples of inflamed joints. It appears that TLR4 controls chemokine production immediately after flare-up and this could be linked to the changes in Th1 and Th17. RANTES is involved in recruitment of Th17 cells towards tumors (14). In TRIF knockouts
no change in Th17 cells was found immediately after flare-up, neither was there a decrease in RANTES production. During the resolution phase however, a decrease of Th17 cells in DLN coincidently a decrease in TRIF dependent RANTES production in synovium was observed. Therefore, TLR4 and TRIF mediated production of RANTES appears to dictate Th17 cell accumulation during T-cell dependent flare-up of experimental arthritis. Additionally, we have studied IP-10 (CCL10) production in inflamed synovium. Production of IP-10 was less affected in knockout animals and indeed we found that Th1 cell accumulation was only slightly affected, since IP-10 chemokine attracts Th1 cells (15).

Both IP-10 and RANTES are IRF3 dependent genes, but TLR mediated TRIF activation does not exclusively regulate IRF3. RIG-I and MDA-5 receptors are also involved in controlling IRF3 activation in a TRIF independent manner (21). This redundancy in the IRF3 control probably causes only a minor decrease in IRF3 responsive genes in TLR4 and TRIF knockouts. Therefore, the production of IRF3 genes is only partially reduced and leads to partial reduction of T-cell attraction to the inflammatory site. Moreover, the difference between TLR4 and TRIF signaling caused a discrepancy in timing of chemokine production and T-cell accumulation. This complex system needs further study to elucidate this interesting difference in timing of TLR4 and TRIF mediated T-cell accumulation at the inflammatory site.

In summary, we show that TRIF signaling is involved in swelling and cellular infiltrate of T-cell dependent flare-up of experimental arthritis, while playing a minor role in joint destruction. However, both TLR4 and TRIF signaling are important in controlling Th17 cell numbers, likely through induction of RANTES production.

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References

Chapter 9

Therapeutic Efficacy of Tyro3, Axl, and MerTK Agonists in Collagen-Induced Arthritis

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Abstract

Hyperactivation of innate immunity by Toll-Like Receptors (TLR) can contribute to the development of autoinflammatory or autoimmune diseases. This study evaluated TAM receptor activation, physiological negative regulators of TLRs, by their agonists Growth arrest specific 6 (Gas6) and Protein S (Pros1) to prevent collagen-induced arthritis. Adenoviruses overexpressing Gas6 and Pros1 were injected intravenously (i.v.) or intra-articularly (i.a.) into mice during collagen-induced arthritis. Splenic T-helper subsets of intravenously injected mice were studied by flow cytometry and knee joints of mice injected i.v. and i.a. were assessed histologically. Synovium of i.a injected mice was evaluated for cytokine and suppressor of cytokine signaling (SOCS) expression. Pros1 significantly reduced ankle joint swelling when overexpressed systemically. Further analysis of knee joints revealed a moderate reduction of joint pathology and a significant reduction of splenic T-helper 1 cells when Pros1 was overexpressed systemically. Local Gas6 overexpression decreased joint inflammation and joint pathology. Pros1 treatment showed a similar trend of protection. Consistently, Gas6 and Pros1 reduced cytokine production in synovium. Moreover, IL-12 and IL-23 mRNA levels were reduced by Gas6 and Pros1 with a corresponding decrease in IFNγ and IL-17 production. TAM ligand overexpression was associated with an increase in SOCS3, which likely contributed to the amelioration of arthritis. We provide the first evidence that TAM receptor stimulation by Gas6 and Pros1 can be used to ameliorate arthritis when applied systemically or locally. TAM receptor stimulation limits proinflammatory signaling and the adaptive immunity. This pathway provides a novel strategy to combat rheumatoid arthritis.
Introduction

Rheumatoid arthritis is an auto-immune disease manifesting in articulating joints causing destruction of cartilage and bone. The cause of this disease is still unknown and treatment has focused on down regulating inflammation by blocking downstream signaling or neutralizing harmful cytokines. Although successful in the clinic, these therapies have substantial side effects and a high rate of non-responders among patients. Natural negative feedback mechanisms can potentially be used therapeutically to halt progression of the inflammatory process and initiate recovery. This approach could possibly limit side effects as the body’s own self-regulating responses are enhanced rather than uncontrolled and systemic blocking of cytokines, important in host defense.

One such controlling system of inflammation is that of the TAM receptors. Tyro3, Axl, and MerTK comprise a family of tyrosine kinase receptors and have been implicated in the negative regulation of inflammation. The regulatory role of TAM receptors in inflammation was found in triple knockout mice for the TAM receptors as these animals showed excessive lymphocyte proliferation and autoimmunity (1). Moreover, proinflammatory cytokine expression by macrophages is inhibited upon Gas6 treatment (2). Two ligands are described for the TAM receptor family, Gas6 and Pros1 (3). Both these ligands bind to phosphatidylserine on cell membranes and subsequently stimulate TAM receptors (4).

Gas6 has been shown to regulate Toll-Like Receptor (TLR) signaling in dendritic cells via activation of the Axl receptor (5). Stimulation of cells via the Axl receptor in conjunction with IFNAR leads to upregulation of suppressor of cytokine signaling (SOCS) proteins 1 and 3 (6;7), inhibitors of inflammation. SOCS1 blocks intracellular signaling e.g. NF-κB activation since SOCS1 can directly inhibit Mal, an adapter molecule for TLR2 and TLR4 (8). TLRs have also been implicated in maintaining the chronic inflammatory loop in RA synovium (9;10). and TLR2 and TLR4 play an important role in arthritis (11;12). SOCS3 also prevents binding of TRAF6 to TAK1, a key signaling molecule in e.g. TLR, IL-1 receptor and TNF receptor signaling (13;14). The protective role of SOCS proteins in experimental inflammatory mouse models has been shown by ectopic overexpression of SOCS3 in collagen-induced arthritis (15). This resulted in altered splenic T helper cell responses towards antigens and ameliorated arthritis.

Taking into account that inflammation can be resolved by SOCS3 in CIA, we set out to determine if overexpression of Gas6 or Pros1 can ameliorate experimental arthritis. Here, we report for the first time to our knowledge that TAM stimulation can ameliorate arthritis. Systemic overexpression of Pros1 decreases arthritis...
severity and is capable of reducing splenic Th1 cell numbers. Gas6 and Pros1 are both also capable of decreasing arthritis when overexpressed intra-articularly as joint pathology and synovial proinflammatory cytokine production were significantly reduced in the inflamed joint.

**Material and Methods**

**Mice**

Male DBA/1 mice aged 10-12 weeks (Janvier, Elavage, France) were housed in filter-top cages and fed a standard diet with freely available food and water. All in vivo studies complied with national legislation and were approved by local authorities for the care and use of animals with related codes of practice.

**Cloning strategy**

The constructs pCDNA6AmGas6 and pCDNA6AmProS were cloned with KpnI and XbaI in the pShuttle vector behind the cytomegalovirus promoter (CMV). The pShuttleCMVmGas6 and pShuttleCMVmProS were cloned into the E1 deleted region of the adeno-5 virus backbone pAdEasyI.

**Construction of adenoviral vectors**

Viral vectors were E1A,B and E3 deleted and were produced according to the method described by (16). The purified recombinant adenoviral vector DNA was transfected into N52E6 viral packaging cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Virus was purified using two CsCl gradient centrifugations and stored in small aliquots at -80°C. The viral titer of the purified viral vectors was determined in human embryonic retinoblastoma 911 indicator cells by immunohistochemical detection of viral capsid protein, 20 hours after transfection.

**Induction of CIA**

Induction of collagen-induced arthritis has been described before (17). Briefly, bovine type II collagen was dissolved in 0.05M acetic acid to a concentration of 2 mg/ml and was emulsified in equal volumes of Freund’s complete adjuvant (2mg/ml of Mycobacterium tuberculosis strain H37Ra) (Difco, Detroit, MI) Mice were immunized intradermally at the base of the tail with 100µl of emulsion (50µg of bovine type II collagen). Subsequently, mice were given an intra-peritoneal booster injection of 100µg of type II collagen dissolved in phosphate buffered saline (PBS) on day 21. One day after the booster injection, immunized mice
were injected intravenously with 3x10^8 focus-forming units (FFU); for intra-articular injection into both knees with 1x10^7 FFU Ad5.Gas6 or Ad5.ProS or Ad5.Luciferase. Two independent observers monitored clinical signs of arthritis in paws and ankle joints, macroscopically. Cumulative scoring based on redness, swelling, and, in later stages, ankylosis was as follows: 0=no changes; 0.25=1-2 toes red or swollen; 0.5=3-5 toes red or swollen; 0.5=swollen ankle; 0.5=swollen footpad; 0.5=severe swelling and ankylosis (redness, excessive edema and deformation), with a maximal score of 2 per paw.

**Histological analysis**

Whole knee joints were dissected and fixed in phosphate buffered 4% paraformaldehyde followed by decalcification with 5% formic acid, and embedded in paraffin wax. Serial tissue sections (7μm) were stained with safranin O (BDH chemicals, Poole, UK) and counterstained with fast green (BHD Chemicals) or with hematoxylin / eosin (Merck, Germany) and eosin (Merck, Germany) (H&E). Serial sections were scored for histopathologic changes on a 0-3 scale, by 2 independent observers in a blinded manner. Joint inflammation was determined by the presence of synovial cell infiltrates and inflammatory cell exudates. Connective tissue destruction was determined by the depletion of cartilage proteoglycan (loss of safranin O staining of the non-calcified upper cartilage layer) and by cartilage and bone erosion.

**RNA isolation and quantitative PCR analysis**

Synovium and liver samples were disrupted using the MagNaLyser (Roche). Total RNA was extracted from the tissue homogenates and from cells using TRI reagent (Sigma) according to manufacturer’s protocol. Isolated RNA was treated with DNase followed by reverse transcription of 1μg RNA into cDNA using Moloney murine leukemia virus reverse transcriptase 0.5μg/μl oligo(dT) primers, and 12.5mM dNTPSs (Invitrogen). Quantitative real-time PCR was performed using the StepOnePlus sequence detection system (Applied biosystems, Foster City, CA) PCR was performed in a total reaction volume of 12.5 μl consisting of appropriate cDNA. Five μM of forward and reverse primer and the sYBR green PCR master mix (Applied biosystems). PCR protocol consisted of 2 min at 50°C and 10 min of 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Quantification of PCR signals was achieved by calculating the difference between the cycle threshold value (Ct) of the gene of interest with the Ct value of their reference gene glyceroldehyde-3-phosphate dehydrogenase (GAPDH) for each sample (delta Ct).
**Immunohistochemistry**

Local expression of SOCS3 was evaluated on paraffin sections of the knee joints. Sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked by 1% hydrogen peroxide for 15 minutes. Tissue sections were overnight incubated with anti-SOCS3 (Abbiotec, Emelca), followed by incubation with biotinylated goat α-rabbit. Peroxidase labeled streptavidine (Vectastain, Brunschwig) and diaminobenzidine were used for staining. Haematoxylin was used as counterstaining. Sections were arbitrarily scored on a scale from 0 to 3 on at least 2 sections per joint.

**Assessment of anti-collagen antibody titers**

Concentrations of anti-bovine CII IgG1 and IgG2a antibodies were determined using ELISA. Briefly, 96-wells plates were coated with 0.1 µg of bovine type II collagen. Non-specific binding sites were blocked by a 5% solution of milk powder. Serial dilutions of mice sera were added followed by incubation with isotype-specific goat anti-mouse antibodies (peroxidase labeled) and 5-aminosalicylic acid as substrate. Absorbance was measured at 450 nm.

**Flow cytometric analysis of splenic T-cells**

Spleens from mice were mashed, filtered and erythrocytes were removed by osmotic shock. After washing CD3+ cells were isolated using the Pan T cell Isolation Kit (Miltenyi Biotec, Germany) according to manufacturer’s instructions. Purified CD3+ cells were stimulated in RPMI 1640 (Invitrogen) supplemented with 10% FCS penicillin/streptomycin and pyruvate with 50ng/ml PMA, 1µl/ml/10^6 cells Brefeldin A (BD Pharmingen) and 1µg/ml lonomycin. After 4 hours of stimulation cells were stained with anti-mouse CD4-APC (1:200) (BD Pharmingen) or IgG2a-APC control (BD Pharmingen) for 30 minutes at 4°C. Subsequently, cells were stained intracellularly with anti-IL17-FITC and anti-IFNγ-PE or with their controls respectively IgG1-PE and IgG1-FITC according to instructions by manufacturer (BD Pharmingen). Stained cells were analyzed using FACScalibur (Becton Dickinson) and analyzed with FlowJow software.

**Fluorescent imaging**

CIA mice received an i.v. injection with activatable ProSense 680 NIR-fluorescent probe (150 µl, 2nmol) (PerkinElmer, Bedford USA) at day 28 of CIA. Probe becomes activated upon enzymatic cleavage by cathepsins (B,K,L,S) present in arthritic joints. Cathepsin activity is a measure of inflammation and especially
macrophage infiltration. At the time of imaging (24 hours post i.v. injection), mice were anesthetized with 2.5% isoflurane/oxygen, knee joints were shaved and placed on their back inside the light-tight chamber and imaged with the In Vivo Imaging System (IVIS) Lumina (Caliper Life Sciences), using the Cy5.5 filter. The collected data were analyzed using Living Image 3.0 (Caliper Life Sciences). Two-dimensional regions of interest (ROI) were drawn around the knee and ankle joints and fluorescent signal intensity was measured corrected for background and auto fluorescence signal.

**Measurement serum levels IL-6 and KC**

IL-6 and KC levels in serum were measured on a Luminex-100 System (Luminex corp.) using a magnetic bead-based multiplex immunoassay (Milliplex, Merck Millipore). Data analysis was performed with Bio-Plex Manager software (Bio-Rad Laboratories).

**Statistics**

All data is represented as mean ± SEM and analyzed with GraphPad 5.0 software. Statistical significance was determined by either 1-Way ANOVA or Two-Way ANOVA with Bonferroni post test, comparing Ad-Gas6 and Ad-Pros1 groups with Ad-Luciferase.

**Results**

**Systemic overexpression of Gas6 and Pros1 moderately reduces arthritis**

Adenoviruses expressing Luciferase, Gas6, or Pros1 were administered intravenously to mice immunized with bovine collagen type II. As shown in Figure 1 overexpression of either Gas6 or Pros1 did not affect arthritis incidence. However, arthritis severity was slightly reduced 36 days post immunization when Gas6 was overexpressed. Moreover, Pros1 treatment resulted in a significant decrease in arthritis severity. In addition to scoring the macroscopic swelling and redness of the joints, knee joints were isolated to enable detailed examination on the effects of TAM activation on cell influx, bone and cartilage. This revealed a trend in decreased inflammation, cartilage erosion, and bone erosion when Gas6 or Pros1 were overexpressed systemically (Figure 1B-C). These data point towards a protective role of TAM activation in experimental arthritis.
Figure 1. Effect of systemically overexpressed Gas6 or Pros1 on arthritis.
Mice with CIA intravenously injected with adenovirus encoding for either luciferase, Gas6, or Pros1 after immunization and arthritis development was monitored over time. (A) Arthritis incidence and severity of hind paws. Severity was determined macroscopically; mean of the cumulative score of ankle and wrist joints per animal. Statistics: two-way ANOVA with Bonferroni post test, compared to Ad-Luciferase (* p<0.05). (B) Histological analysis of knee joints for inflammation (HE stained sections), bone and cartilage erosion (safranin O stained sections). Data represented as mean ± SEM, n=9 per group. (C) Representative safranin-O stained sections indicating cartilage erosion (closed arrows) and bone erosions (open arrows). Scale bar = 100 µm. P = patella, F = femur.
**Systemically overexpressed Gas6 and Pros1 suppress the proinflammatory immune response**

To study the effects on macrophage activity, serum was taken and evaluated for circulating cytokine levels. The TLR-inducible IL-6 and KC were detected in serum and Gas6 and Pros1 overexpression reduced circulating IL-6 levels in serum significantly by 59% and 78%, respectively. In addition, Pros1 caused a 68% decline in circulating KC levels (Figures 2A), potentially explaining a decrease in inflammatory cell influx into the inflamed joints. Moreover, serum IL-6 and KC levels significantly correlated with macroscopic arthritis scores (IL-6 correlation: $R^2 = 0.41$, p value 0.001. KC correlation: $R^2 = 0.33$, p value 0.004). This indicates that Gas6 and Pros1 decreased systemically produced cytokines during inflammatory conditions and possibly control antigen presenting cell (APC) activation and function. To study the effect of TAM ligand overexpression systemically on B-cells the antibody titers against bovine collagen type II were determined (Figure 2B). Both Gas6 and Pros1 did not have an effect on collagen type II specific IgG1 or IgG2a antibody titers. This suggests that TAM ligands did not influence B-cell function. To further analyze the effects of Gas6 and Pros1 overexpression on the adaptive immunity, splenic CD3$^+$ cells were isolated and T cell differentiation was determined. TAM receptor stimulation significantly reduced Th1 levels, whereas Th17 levels were unaffected by either treatment (Figure 2C). In accordance, mRNA expression level of T-bet was decreased significantly, whereas RORγT expression was unchanged (Figure 2D). This indicates that TAM activation has a clear effect on T-cell immunity by diminishing the development of Th1 cells, resulting in a reduction of arthritis.

**Local overexpression of TAM ligands decreases inflammation and joint pathology**

Gas6 and Pros1 show clear effects on Th1 development, but failed to ameliorate inflammation and joint pathology significantly. To study the effect of Gas6 and Pros1 directly at the inflammatory site, adenoviruses were injected intra-articularly in both knee joints before onset of CIA. During arthritis development the inflammation was measured with the ProSense probe at day 29 (Figure 3A), and TAM activation significantly reduced inflammation in the treated knee joints. Further analysis of inflammation, cartilage, and bone destruction revealed that TAM activation is beneficial for halting joint destruction (Figure 3B-C). Inflammation of the non-treated (ankle) joints was unaltered by either treatment (data not shown) indicating that TAM activation occurred only locally in the knee joint. This indicates that TAM activation directly at the site of inflammation can be applied to treat inflammatory diseases.
Figure 2. Serum cytokines, antibody titers, and splenic Th1 and Th17 analysis.

(A) Serum was harvested at time of sacrifice (day 36 of CIA) and serum levels of IL-6 and KC were measured by Luminex. (B) Anti-bovine collagen type II antibodies in serum measured by ELISA. O.D. = Optical density. (C) Spleens from CIA mice were isolated at time of sacrifice (day 36 of CIA) and CD3+ cells were isolated by negative MACS selection. T-cells were subsequently stimulated with PMA and ionomycin for 4 hours and stained for CD4, IFNγ, and IL-17 and percentage of positive cells were determined by flow cytometry. (C) Percentages of splenic Th1 (CD4+/IFNγ+) and Th17 (CD4+/IL-17+) cells, gated on CD4+ cells. (D) mRNA expression analysis of transcription factors Tbet (Th1) and RORγT (Th17). Relative expression=2-ΔΔCt. Statistics: one-way ANOVA with Bonferroni post test, compared to Ad-Luciferase (* p<0.05, ** p<0.01, *** p<0.001). Data represented as mean ± SEM, n=9 per group.
Figure 3. Effects of local overexpressed Gas6 and Pros1 on joint inflammation and histology.

CIA mice were injected intra-articularly into the left and right knee joint with adenovirus encoding for either Luciferase, Gas6, or Pros1 and arthritis development was monitored over time. (A) Knee inflammation on day 29 of CIA measured with a cathepsin inducible fluorescent probe (ProSense) and knee swelling at time of sacrifice (day 31 of CIA) determined macroscopically (scale 0-2). (B) Histological analysis of knee joints for inflammation (HE stained sections), bone and cartilage erosion (safranin O stained sections). Statistics: one-way ANOVA with Bonferroni post test, compared to Ad-Luciferase (** p<0.01, *** p<0.001). Data represented as mean±SEM. ProSense measurement n=20 per group, knee swelling n=10 per group, histology n=14 per group. (C) Representative safranin-O stained sections indicating cartilage erosion (closed arrows) and bone erosions (open arrows). Scale bar = 100 µm. P = patella, F = femur.
Messenger RNA expression analysis of synovium showed that both Gas6 and Pros1 mRNA were upregulated two days after virus injection (data not shown). Further analysis revealed that both Gas6 and Pros1 reduced matrix metalloproteinase (MMP) expression in synovium (Figure 4A-C). Gas6 and Pros1 significantly reduced MMP13 mRNA expression, whereas MMP14 and MMP9 expression were diminished significantly by overexpressing Gas6 or Pros1 respectively. Altogether, these data show that local TAM activation directly in inflamed joints decreases joint destruction by reduced MMP expression.

**Gas6 and Pros1 decrease cytokine production in synovium**

To study the effects of TAM activation on local cytokine production before clinical manifestation was observed, synovium was isolated at day 24 of CIA. Interestingly, TNFα production was detected before clinical manifestation and was significantly inhibited 87% and 62% by Pros1 and Gas6, respectively. IL-1β and IL-6 were only marginally produced on day 24, but were markedly induced when synovitis occurred (Figures 5A). Gas6 and Pros1 decreased IL-1β production at day 31 of CIA by the inflamed synovium by 65% and 78% respectively. In addition, IL-6 production returned to near basal expression levels by overexpression of Gas6 and Pros1 as IL-6 mRNA expression was significantly reduced by 74% and 92%
respectively. The anti-inflammatory effects of Gas6 and Pros1 were also observed in production of T-cell activating cytokines IL-12 and IL-23. Figure 5B shows that overexpression of Gas6 and Pros1 caused a decline in IL-12 and IL-23 production in synovium resulting in reduced IFNγ and IL-17 levels in the synovium (Figure 5C). In addition, Figure 5D shows that T-cell transcription factors mRNA expression of T-bet and RORγT, responsible for Th1 and Th17 development respectively, was significantly reduced by Gas6 and Pros1. These cytokine expression profiles support the findings of reduced joint pathology, since IL-1 and IL-17 are key factors in cartilage and bone destruction. These data show that local TAM activation by Gas6 and Pros1 reduce proinflammatory cytokine production in inflamed synovium. This probably led to subsequently hampered T-cell activation and proliferation at the site of inflammation.

**SOCS1 mediated anti-inflammatory effects of Gas6 and Pros1**

To unravel the inhibitory mechanism of TAM receptor stimulation, mRNA expression of SOCS1 and SOCS3 was evaluated (Figure 6A). SOCS1 mRNA expression was upregulated 2.3 fold in synovium of mice injected with Gas6 or Pros1 virus, whereas control animals showed a slight down regulation. In contrast, SOCS3 mRNA regulation was marginally affected by Gas6 overexpression and even slightly downregulated by Pros1 overexpression. Since this is in contrast with previous results (18), we determined SOCS3 protein levels by immunohistochemistry. Figure 6B shows representative images of the SOCS3 staining and a clear trend is observed in upregulation of SOCS3 protein by Gas6 and Pros1 (Figure 6C). This suggests that SOCS1 and SOCS3 mediate the anti-inflammatory effects of TAM activation by Gas6 and Pros1.

**Discussion**

A novel inhibitory pathway on TLR and cytokine signaling by TAM receptor activation has been exploited in this study to inhibit experimental arthritis. Here, we show that enhancing the negative feedback on inflammation by TAM receptor activation can be used to treat arthritis in a prophylactic setting. Systemic overexpression of Pros1 affected the T-cell immune response by decreasing Th1 and ameliorated experimental arthritis moderately. Intra-articular overexpression of Gas6 and Pros1 reduced proinflammatory cytokine production in synovium, which was likely to be mediated by SOCS1 and SOCS3. Gas6 also significantly decreased joint destruction when overexpressed in the inflamed joint. We show for the first time that TAM receptor activation by Gas6 and Pros1 in vivo ameliorates arthritis. This puts the TAM pathway forward as a new therapeutic pathway to be exploited to treat arthritis.
Figure 5. Cytokine expression in synovium of locally treated CIA mice.
Synovium was isolated two days after virus injection (day 24 of CIA) and at time of sacrifice (day 31 of CIA) and mRNA was isolated. (A) mRNA expression of TNFα, IL-1β, and IL-6 was determined by qPCR. Statistics: two-way ANOVA with Bonferroni post test, compared to Ad-Luciferase (*** p<0.001). Data represented as mean±SEM, n=4 on day 24 and n=6 on day 31. mRNA expression of cytokines (B-C) and transcription factors (D) at day 31 of CIA. Statistics: one-way ANOVA with Bonferroni post test, compared to Ad-Luciferase (* p<0.05, ** p<0.01). Data represented as mean ± SEM, n=6 per group.
In our study Pros1 decreased splenic Th1 cells by 40% while leaving Th17 levels unaffected. This is in accordance with previous studies in Axl and MerTK double knockout animals. Naïve splenic CD4+ T cells from double knockout mice show a remarkable increase in IFNγ production when stimulated with anti-CD3 and anti-CD28 and no change in IL-17 production. In addition, immunized double knockout mice show increased Th1 development and normal Th17 levels in spleen and DLN (19). In animals that lack the MerTK receptor in the diabetes prone NOD background, a strong Th1 response was observed when β-cells underwent apoptosis (20). Combined with our data, it appears that TAM activation on APCs primarily affects Th1 response in vivo while not influencing Th17 response. Since circulating IL-6 levels were significantly decreased by Gas6 or Pros1 overexpression in our study an effect on Th17 could be expected. However, previous studies have shown that Gas6 can regulate TGF-β expression. Clauser et al. (21) showed that increased Gas6 secretion from carotid plaques correlates with increased TGF-β secretion. In addition, Gas6 knockout animals produce less TGF-β upon induction of liver damage (22). If Gas6, and perhaps Pros1, increase TGF-β levels this could compensate for the reduced IL-6 levels and leaving Th17 levels unaffected.

Gas6 and Pros1 appear to have differential effects depending on local or systemic overexpression. When overexpressed systemically, Pros1 seems slightly more efficacious than Gas6 and locally the reverse effect has been observed. But in fact, no significant differences between Gas6 and Pros1 were found on arthritis. The trends observed between Gas6 and Pros1 could be attributable to different target cells. Systemic overexpressed TAM ligands will affect systemic adaptive immunity by APC activity modulation in the spleen, which was also observed in our study. At the site of inflammation on the other hand, TAM ligands are expressed and secreted into the joint cavity affecting all the cells present, such as infiltrated macrophages, T-cells, and the synovial lining. Fibroblasts in the synovial lining are active contributors to the inflammation (23) and the effects of TAM ligands and TAM receptor expression on synovial fibroblasts is unknown and warrants further investigation.

The anti-inflammatory effects of TAM receptors has been reported to be mediated by SOCS1 and SOCS3 (24;25). Rothlin et al. found that stimulation of the Axl receptor in conjunction with the IFNARI lead to an upregulation of SOCS1 and SOCS3 in dendritic cells, which interfere with intracellular signaling and NF-κB activation. The effects of local Gas6 or Pros1 overexpression appear to be mediated via SOCS1 and SOCS3. Overexpression resulted in upregulation of SOCS1 expression during arthritis, whereas control animals showed a slight downregulation of SOCS1. The pivotal role of SOCS1 in controlling inflammation
has been shown in macrophages from SOCS1 conditional knockout animals, in which TNF-α and IL-6 expression was down regulated upon LPS challenge (26). In our study we also observed a decrease in proinflammatory cytokine production in synovium by overexpressing Gas6 or Pros1 in the joint cavity. In contrast to SOCS1 up regulation, little regulation of SOCS3 mRNA by TAM receptor activation was found. However, immunohistological staining revealed a trend towards increased SOCS3 protein after Gas6 or Pros1 overexpression. SOCS3 mRNA levels are partly controlled by TNF-α (27) and IL-6 (28), of which we found significant differences at day 24 and day 31 of CIA respectively. Therefore, mRNA expression at time of sacrifice could deviate from protein levels. In addition, cytokine signaling has been suggested to prevent SOCS3 turnover (29). The increase in SOCS1 and SOCS3 are also in line with previous studies (30), showing the involvement of SOCS1 and SOCS3 in TAM mediated downregulation of inflammation. Taken together, a significant increase in SOCS1 mRNA in synovium and a clear trend in increased SOCS3 protein could partly account for the anti-inflammatory effects observed by Gas6 and Pros1.

**Figure 6**

*Figure 6. SOCS proteins are up regulated by Gas6 and Pros1.*
mRNA was isolated from synovium isolated two days after virus injection (day 24 of CIA) and at time of sacrifice (day 31 of CIA). (A) Fold increase in mRNA expression of SOCS1 and SOCS3 on day 31 of CIA compared to day 24 of CIA. Statistics: one-way ANOVA with Bonferroni post test, compared to Ad-Luciferase (* p<0.05, *** p<0.001). Data represented as mean±SEM, n=4 on day 24 and n=6 on day 31. (B) Representative images of immunohistochemistry staining of SOCS3 in intraarticular injected knee joints. (closed arrows). Scale bar = 100 µm. P = patella, F = femur. (C) Arbitrary score of SOCS3 staining (scale 0-3). Data represented as mean±SEM, n=14 per group.
Another possible mechanism by which Gas6 and Pros1 exert their anti-inflammatory effects is by inducing phagocytosis. Gas6 and Pros1 can opsonize apoptotic cells by binding to phosphatidylinerine displayed on apoptotic cells. It has been shown before that joint inflammation can be reduced by prophylactic injection of apoptotic cells directly into the joint (31). Clearance of apoptotic leukocytes by lining macrophages decreases their chemotactic activity and thereby limits inflammation. MerTK is predominantly involved in phagocytosis (32) and plays a role in inflammation as well. It has been shown that MerTK downregulates TNF-α production upon LPS stimulation (33) and MerTK is also involved in LPS induced lung injury (34). Both Gas6 and Pros1 are ligands for the MerTK receptor and could therefore increase TAM signaling, via apoptotic cells or direct stimulation of the MerTK receptor on macrophages. However, the exact role of exogenous Gas6 and Pros1 in mediating phagocytosis of apoptotic cells to facilitate resolution of joint inflammation needs further investigation.

Axl and Gas6 have been implicated in maintaining the abnormal vasculature in RA (35) and thereby contributing to inflammation. Here, we show that the net effect of increasing TAM signaling is beneficial for experimental arthritis. TAM ligands could potentially induce SOCS1 and SOCS3 expression in human RA synovium and thereby decreasing inflammation. With decreasing inflammation also the process of angiogenesis will halt and TAM stimulation by Gas6 or Pros1 could potentially treat RA by controlling inflammation irrespective of its putative effect on angiogenesis.

In summary, we provide the first evidence that enhancing natural negative feedback on inflammation by TAM stimulation is efficacious to treat inflammatory arthritis. TAM receptors and their ligands Gas6 and Pros1 offer many possibilities and options to fine tune the negative feedback on inflammation to resolve auto-inflammatory and autoimmune diseases.

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References

Chapter 10

Summary and final considerations
Summary

Toll-Like receptors have emerged as the pivotal factors in controlling the immune system. They are involved in activating the innate immunity and control the adaptive immunity. Moreover, a role for TLRs has been described for several autoimmune diseases, such as rheumatoid arthritis. An important role for TLRs has also been established in experimental arthritis animal models. Especially TLR4 has been extensively studied using TLR4 knockout animals and TLR4 antagonists. These studies revealed an aggravating role of TLR4 in the chronic phase of arthritis, while leaving incidence unaffected.

In this thesis, we set out to evaluate the role of TLR4 on different cell populations and to gain insight into the signaling pathways of TLR4. This enables us to pinpoint the role of TLR4 in arthritis and allows us to design more specific therapies. First, we investigated the contribution of TLR4 on resident cells and bone marrow derived cells. Therefore, bone marrow transplantation was performed in order to make bone marrow chimeras in which TLR4 was absent on either bone marrow derived cells or resident cells (chapter 2). Arthritis development was monitored over time and we observed that animals lacking TLR4 on either the bone marrow derived cells, resident cells, or both developed reduced swelling and diminished joint pathology. Further analysis showed that both the bone marrow cells and resident cells are involved in generating Th17 cells in spleen, whereas the bone marrow derived cells are responsible for CD4+ IL-17 producing cells locally in the lymph node. This unexpectedly indicated that TLR4 is necessary on both bone marrow derived cells and local resident cells to continue the self-stimulated inflammatory loop and achieve full blown arthritis. Although both chimeras showed an equal clinical outcome, the underlying mechanisms might differ considerably. The resident cells, such as chondrocytes, tissue macrophages and fibroblasts also express TLR4 and can be activated during inflammatory processes. Upon TLR4 activation fibroblasts produce cyto- and chemokines as well as matrix degrading enzymes, contributing to joint destruction. The breakdown of extracellular matrix from cartilage, release of alarmins and intracellular proteins during arthritis provide potential endogenous ligands stimulating TLR4.

To evaluate the role of TLR4 stimulating endogenous ligands in the joint cavity a local arthritis was induced by IL-1β overexpression (chapter 3). IL-1β is known to induce joint destruction and this will generate DAMPs potentially capable of stimulating TLR4. TLR4 knockout animals developed reduced joint pathology, indicating a pivotal role of TLR4 activated by endogenous ligands during inflammation and thereby mediating joint destruction locally in the joint.
Many studies have been performed identifying potential endogenous ligands, such as fibronectin, heat shock proteins and serum amyloid A proteins. This research has been hampered by LPS contamination and probably the use of incorrect proteins. We, and others, have shown that a slightly modified serum amyloid A (SAA) recombinant protein can have profound proinflammatory capacities. However, when the non-modified natural form of SAA1 was used in the same assays, we observed only marginal proinflammatory activity (chapter 4). This implies that the search for endogenous ligands of TLRs is not only hampered by contamination, but that we should be cautious in using recombinant modified proteins.

Being Pattern Recognition Receptors it is tempting to view TLRs as immune receptors involved on immune cells and more specifically the antigen presenting cells (APCs). These cells sense the environment and mount an immune response when encountering pathogens carrying the ligands for TLRs. In this classical view of TLRs, we delineated the role of TLR4 on bone marrow derived cells further by specifically knocking down TLR4 expression on APCs in spleen (chapter 5). By intravenous delivery of a lentivirus we were able to target splenic APCs. The lentivirus encoded a short hairpin against TLR4, knocking down TLR4 expression. Despite lack of direct evidence that TLR4 expression was decreased in this experiment, we did find functional effects, since the IFNγ production by CD4+ cells was diminished in draining lymph nodes. Unexpectedly, a slight increase in inflammation and swelling was found in animals treated with the hairpin against TLR4. These results could possibly be explained by a lack of negative feedback on the APC induced by TLR4 activation and thereby enhancing the APC activity under this condition.

**Targeting TLR4 signaling**

Besides the location where TLR4 should be inhibited or which cell should be targeted several ways to inhibit TLR4 signaling have been studied. TLR4 signaling can be divided in MyD88-dependent and TRIF-dependent pathways, resulting in activation of NF-κB and IRF3 respectively. Therefore, both pathways have been studied to evaluate their role in experimental arthritis and their value as a therapeutic tool.

MyD88 signaling after TLR4 activation induces many proinflammatory cytokines and also MMPs. This makes the MyD88 signaling an interesting target and MyD88 knockouts show strongly reduced arthritis (1). To block MyD88 signaling a kinase negative mutant of TGF-beta activated kinase-1 (TAK1) was overexpressed. Overexpression of the mutant was achieved cell specifically by intravenous
delivery of lentivirus (chapter 6). Here, we showed that blocking TAK1 signaling results in ameliorated arthritis, reduced Th17 development, and diminished bone erosion. This reduction might be mediated by targeting the osteoclast precursors and a reduction in the osteoclast forming factors RANK and RANKL in synovium. This indicates that inhibiting MyD88 mediated signaling by blocking TAK1 in splenic APCs is a potential efficacious treatment of arthritis. This triple MAP kinase, however, is used by many proinflammatory signaling pathways and is therefore not TLR(4) specific, but the capability of inhibiting several pathways might explain its effectiveness in treating inflammatory conditions. To evaluate the efficacy of TAK1 inhibition locally, the role of TAK1 in rheumatoid arthritis synovial fibroblasts was studied (chapter 7). Surprisingly, TLR4 signaling was TAK1 independent in these cells. TAK1 signaling can be circumvented by TLR4 by using MEKK3 resulting in NF-κB activation and subsequent production of proinflammatory mediaters. Considering the role of endogenous TLR4 ligands in the inflamed joint, TAK1 inhibition in rheumatoid arthritis synovial fibroblasts might not be a suitable method to treat arthritis. Taken together, TAK1 signaling in APCs poses as an interesting target for treatment of arthritis.

The second signaling pathway used by TLR4 is the TRIF pathway, a putative anti-inflammatory pathway. This pathway induces type I IFNs that subsequently induce the suppressor of cytokine signaling (SOCS) proteins (2). Additionally, TRIF signaling induces IL-27, which limits Th17 development (3). Because of its putative anti-destructive properties, we investigated the role of TRIF signaling during T-cell dependent flare of arthritis (chapter 8). After a primary arthritis the smoldering arthritis is flared using a sub arthritic dose of antigen. This results in rapid accumulation of inflammatory cells into the inflamed joint. TLR4 plays a crucial role in this process by inducing IP-10 and RANTES that draw T-cells to the site of inflammation, whereas TRIF signaling did not play a role. TRIF signaling, however, does appear to play a role in the resolution of the inflammation. Th17 levels subsided more rapidly in TRIF knockouts during later phase of arthritis. Again, decreased expression of TRIF/IRF3-induced chemokines appeared to mediate T-cell migration during the resolution phase of arthritis. These results are in contrast with previous reports indicating anti-inflammatory properties of the TRIF pathway on arthritic bone erosion during SCW-induced chronic arthritis and in an experimental model of multiple sclerosis (3;4). In our experiment we studied the recruitment of T-cells to the site of inflammation rather than onset of disease. Therefore, the role of TRIF in autoimmune processes is multifaceted and the chemokines might play a more important role than type I IFNs or IL-27 in certain conditions.
Enhancing natural negative regulatory feedback

TLR4 signaling is physiologically down regulated by several intracellular inhibitors, such as SOCS1 and SOCS3 (5). These proteins interfere with assembly of signaling protein interaction, inhibit kinase activity, and can induce proteasomal degradation. These mechanisms prevent activation and subsequent downstream proinflammatory signaling (2). Overexpressing SOCS3 has been proven to ameliorate arthritis and modulate T-cells (6). We have found a novel tool to upregulate expression of SOCS1 and SOCS3 through activation of TAM receptors by their natural ligands Gas6 and Pros1 (Chapter 9). Systemic overexpression of Pros1 reduced Th1 levels in spleen and this moderately ameliorated arthritis. Gas6
and Pros1 were more effective in decreasing inflammation when overexpressed locally. This decrease was accompanied by the upregulation of both SOCS1 and SOCS3, pointing to the effectiveness of TAM activation in inhibiting inflammation. This breaks the inflammatory loops and prevents progression of arthritis sustained by endogenous ligands stimulating TLRs. This strategy possibly limits side effects as we have seen a potential increase in inflammation after specific deletion of TLR4 on APCs. By temporarily increasing the natural feedback on inflammation we can possibly circumvent side effects such as a dysfunctional immune system against invading pathogens.

**Future perspectives**

Rheumatoid arthritis treatment is dominated by the use of biologicals, such as various anti-TNF therapeutics and rituximab. These treatments are efficacious in the majority of patients, but a third of the patients are non-responders to current treatment. Moreover, treatment with biologicals pose a major economic burden as these therapeutics are expensive to produce. Therefore, small molecule therapeutics could supplement the current treatment of rheumatoid arthritis cost-effectively and possibly treat current non-responders.

The small molecule TAK-242 has been tested as a specific TLR4 inhibitor in a clinical trial for sepsis and the researchers concluded that it was ineffective in sepsis treatment (7). However, numerous issues have been raised concerning the experimental set up, therefore the efficacy of TAK-242 has not been ruled out (8). Nonetheless, the clinical trial showed that TAK-242 was well tolerated by patients, which makes it a potential treatment for rheumatoid arthritis. Besides a potential new therapeutic agent, this also provides a valuable tool to assess if TLR4 plays a role in rheumatoid arthritis. Although evidence is mounting that TLR4 is playing a role in rheumatoid arthritis, TLR4 inhibition in rheumatoid arthritis has been performed on small scale using recombinant proteins of chaperonin 10 (9). Despite the fact that TAK-242 treatment will not be cell or site specific, it could be an easy and valuable tool to further study the effects of TLR4 blocking in rheumatoid arthritis on a larger scale. The issue of cell specificity could be overcome by sophisticated delivery and release methods, such as antibody directed lipoplexes (10). This could achieve better and specific targeting of TLR4 and thereby limiting side-effects. In addition to TAK-242 several LPS variants have been described to inhibit TLR4 activation, such as ultra pure LPS extracted from Bartonella quintanta (11). However, extensive use of these inhibitors is hampered by complicated culture, production, and purification procedures. Taken together, TAK-242 appears to the most promising tool to assess the role of TLR4 in RA and
is possibly an interesting therapeutic for systemic inhibition of TLR4 in RA patients in the future.

Since we observed an important role of TLR4 on resident cells locally in the joint, inhibiting TLR4 at the site of inflammation could also be beneficial. Local application of TAK-242 would be difficult as it diffuses rapidly, like most small molecules. Therefore protein based TLR4 inhibitors or antibodies against TLR4 would be preferred. Unfortunately, antibody production against TLR4 is complicated to achieve, resulting in poor titers. Only few have been successful and its inhibitory effect was partially based on FcyRIIA (17). Therefore, we have made several attempts to produce protein based TLR4 inhibitors based on TLR4 itself. TLR4 makes use of MD-2 and a natural splice variant scavenges LPS and subsequently prevents TLR4 triggering (12). Additionally, a soluble form of TLR4 can sequester TLR4 ligands and prevent signaling. This soluble TLR4 is naturally occurring as a small peptide (13;14), but in order to potentially broaden ligand binding we also attempted to produce the complete extra cellular domain of TLR4. Unfortunately, both proteins were difficult to overexpress as recombinant proteins. The complex protein structure of TLR4 makes it very difficult to obtain sufficient expression levels. In a last attempt a fusion construct was used (14), but expression proved to be difficult yet again. Since TLR4 is a problematic transgene, other natural inhibitors could overcome this issue. Membrane bound inhibitors, such as RP105 (15) and ST2 (16), could be used locally. When overexpressed, using an adenoviral vector, these molecules could provide a valuable tool to identify the role of TLR4 on synovial fibroblasts. Gene therapy for rheumatoid arthritis should be focused on a stable integrating vector with a disease inducible promoter. When the targeted cells are exposed to inflammatory stimuli transgene expression is induced and inhibition occurs when necessary. Additionally, the inhibition will remain mainly in the joint cavity limiting side effects in combating invading pathogens.

Enhancing natural negative feedback on TLR signaling locally by TAM stimulation provides a new and promising mechanism to treat arthritis. To circumvent inhibition of only one receptor on the targeted cells the secreted Gas6 and Pros1 proteins affect all the cells in the joint. The use of a disease inducible promoter regulating transgene expression will help to fine tune treatment. Gas6 and Pros1 stimulate the TAM receptor system inducing negative feedback on inflammation by upregulation of SOCS proteins, which inhibit proinflammatory signaling (5). This could potentially break the inflammatory loop in the joint space and halt progression of the clinical symptoms and induce recovery.
References


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**Nederlandse samenvatting**

Reumatoïde artritis is een chronische aandoening waarbij de gewrichten ontstoken zijn. Deze continue ontsteking leidt, na verloop van tijd, tot beschadiging van kraakbeen en bot. Dit brengt de kenmerkende vergroeiingen van gewrichten teweeg die met reumatoïde artritis gepaard gaan. De oorzaak van deze chronische ontstkingen is tot op heden onduidelijk en lijkt een combinatie te zijn van genetische aanleg en omgevingsfactoren. In de afgelopen jaren is er veel onderzoek gedaan naar de mechanismen die de gewrichtsontsteking in stand houdt. Het is gebleken dat de zogenaamde Toll-Like Receptoren (TLRs), belangrijk zijn bij de activatie van met name het aangeboren immuunsysteem. Ook is gebleken dat deze receptoren een cruciale rol spelen bij de verbinding tussen het aangeboren en het verworven immuunsysteem. Toll-Like Receptor 4 (TLR4) blijkt een uitermate belangrijke rol te spelen in het activeren van immuun cellen die behoren tot de aangeboren immuinitéit, zoals de macrofaag.

De rol van TLR4 in experimentele artritis is bestudeerd aan de hand van genetische knock-out muizen, die het TLR4 gen missen, en het blokkeren van TLR4 activatie. Hieruit is gebleken dat TLR4 een versterkende rol speelt in experimentele artritis. Dieren waarin het gen ontbrak, lieten een duidelijke verlaging zien van de ontsteking en hadden minder schade in de gewrichten. Na deze sterke aanwijzing dat TLR4 een belangrijke rol speelt in artritis, rees de vraag op welke cellen TLR4 een belangrijke rol speelt. Middels een beenmerg transplantatie (hoofdstuk 2) tussen genetische knock-outs en normale dieren werd het onderscheid gemaakt tussen de cellen afkomstig uit het beenmerg, voornamelijk de bloedcellen, en de lichaams cellen. Hieruit is gebleken dat TLR4 op zowel de beenmergcellen als de lichaamscellen een cruciale rol speelt, doordat in dieren waarin TLR4 ontbrak op de bloedcellen, lichaamscellen of beide celtypen een verlaagde ontsteking te zien was en er minder schade in het gewricht was. Verdere analyse van het verworven immuunsysteem liet zien dat TLR4 op de bloedcellen een belangrijke rol speelt bij het ontstaan van IL-17 producerende T-cellen. Deze soort T-cellen zijn erg destructief tijdens de ontsteking.

Om de rol van TLR4 op de lichaamscellen verder te bestuderen, is een belangrijk pro-inflammatoire cytokine, interleukine 1 (IL-1), tot overexpressie gebracht in de gewrichtsholte (hoofdstuk 3). Uit dit experiment bleek dat TLR4 betrokken is bij gewrichtsschade, want wanneer TLR4 ontbrak, ontstond er minder schade. Omdat IL-1 niet direct TLR4 kan activeren, moeten er tijdens de ontsteking TLR4 activerende producten ontstaan. Dit zijn zogenaamde endogene liganden die ontstaan tijdens een actieve ontsteking en weefselschade. Deze producten, of endogene liganden, activeren TLR4 waardoor de ontsteking sterker wordt.
De stimulerende endogene liganden is grondig onderzocht. Een veel gebruikt model eiwit hiervoor is het recombinant serum amyloid A1. Dit eiwit blijkt een ontstekingsbevorderende werking te hebben. Echter, dit recombinante eiwit is iets gemodificeerd en gebruik van het correcte lichaamseigen eiwit blijkt niet pro-inflammatoire te zijn (hoofdstuk 4). Daarmee staat de rol van het lichaamseigen serum amyloyd A1 als belangrijk endogeen ligand ter discussie.

Door dat TLRs tot expressie komen op immuun cellen, en in het bijzonder antigeen presenterende cellen (APCs), is het voor de hand liggend om TLRs te zien als immuun receptoren. Deze APCs scannen het lichaam op infiltrerende pathogene microben en initiëren een immuunrespons wanneer nodig. De rol van TLR4 op APCs is nader bestudeerd door de expressie van TLR4 op APCs te verlagen in de milt (hoofdstuk 5). Door een lentivirus intraveneus toe te dienen, worden de APCs in de milt getransduceerd en middels RNA interference kon de TLR4 expressie worden verlaagd in de getransduceerde cellen. Ondanks gebrek aan direct bewijs in de behandelde dieren dat de TLR4 expressie daadwerkelijk was verlaagd, zijn er wel functionele effecten gevonden. In de drainerende lymfeknopen werd minder van het cytokine interferon γ geproduceerd door CD4 negatieve cellen en was er een trend in verlaagde Th17 aantallen. Desondanks leek de artritis macroscopisch te zijn toegenomen. Deze resultaten kunnen wijzen op een gebrek aan negatieve terugkoppeling welke geïnduceerd wordt na activatie van TLR4 en daardoor wordt de activiteit van de APCs juist versterkt en de artritis ernstiger.

**TLR4 signalering als doelwit**

Naast de locatie en cel waar TLR4 geremd zou moeten worden om artritis te behandelen, is ook bestudeerd hoe TLR4 optimaal geremd kan worden. Hiervoor is er gekeken naar de intracellulaire signalering. Deze kan voor TLR4 verdeeld worden in twee routes; de MyD88 afhankelijke route en de TRIF afhankelijke route, welke respectievelijk NF-κB en IRF3 activeren. Beide signaal routes zijn bestudeerd om te bepalen of het therapeutische doelwitten zouden kunnen zijn.

MyD88 signalering na TLR4 activatie induceert veel cytokine en MMP productie, welke betrokken zijn bij ontsteking en weefselafbraak. Dit maakt de MyD88 route een interessant doelwit. Bovendien laten dieren waarbij MyD88 ontbreekt een sterk verlaagde artritis zien. Om MyD88 signalering te blokkeren, is er een kinase negatieve mutant van TAK1 gebruikt, een kinase dat actief is in de MyD88 signaleringsroute. De kinase negatieve TAK1 mutant werd door middel van intraveneus toedienen van lentivirus tot overexpressie gebracht (hoofdstuk 6) en dit resulteerde in verlaagde artritis, verminderde Th-17 ontwikkeling en minder botschade. De verminderde botschade werd mogelijk bereikt door het direct
transduceren van de voorlopers van bot afbrekende cellen, de osteoclasten. Ook de groefactor en receptor, RANKL en RANK, die cruciaal zijn voor osteoclastogenese kwamen minder tot expressie in het synovium van de behandelde dieren. Hieruit bleek dus dat het stoppen van de MyD88 route door het blokkeren van TAK1 een mogelijk effectieve behandelstrategie zou kunnen zijn. Echter, TAK1 wordt niet enkel door TLRs, of TLR4 in het bijzonder, gebruikt en is zelfs een van de belangrijkste kinase eiwitten in vele ontstekings-signaleringsroutes. Dit zou overigens wel de effectieve anti-inflammatoire eigenschappen van deze behandeling kunnen verklaren. Aanvullend werd in vitro bestudeerd of het selectief remmen van TAK1 in synoviale fibroblasten ook effectief was (hoofdstuk 7). Het bleek dat TLR4 in staat was NFκB te activeren zonder TAK1 daarbij te betrekken. Hetgeen suggereert dat andere kinases een dominantere rol spelen. Wanneer de TLR4 stimulerende rol van endogene liganden in overweging wordt genomen, is TAK1 remming in de synoviale fibroblast geen geschikte strategie. In APCs, echter, is TAK1 wel een interessant doelwit voor de behandeling van artritis.

De tweede signaal route die door TLR4 wordt gebruikt is de TRIF route, een veronderstelde anti-inflammatoire route. Activatie van deze signaal route induceert type I interferon dat vervolgens resulteert in ‘suppressor of cytokine signaling’ (SOCS) eiwit productie. Ook induceert de TRIF route de aanmaak van IL-27, dat de ontwikkeling van Th17 cellen remt. Deze signaal route is bestudeerd in een T-cel afhankelijke opvlamming van artritis (hoofdstuk 8). Na een primaire ontstekingsfase is de sluimerende artritis aangewakkerd door een lage dosis antigeen. Dit zorgt voor een snelle ophoping van ontstekingscellen in het ontstoken gewricht. TLR4 speelt een cruciale rol in dit proces door het induceren van IP-10 en RANTES chemokines die T-cellen aantrekken naar de plek van ontsteking. Echter, TRIF signalering bleek geen rol te spelen in dit proces. TRIF lijkt wel een rol te spelen tijdens het verdwijnen van de ontsteking, omdat de Th17 cel populatie verlaagd was in de latere fase van artritis in dieren waarin geen TRIF aanwezig was. De oorzaak hiervan kan een verlaagde expressie zijn van chemokines. Deze resultaten zijn in contrast met andere studies die een duidelijk anti-inflammatoire effect hebben laten zien van de TRIF signaal route op bot erosie in bacteriële cel wand artritis en in een experimenteel model van multiple sclerose. In dit experiment hebben we echter de rekrutering van T-cellen bestudeerd en niet het begin van ontsteking. Daarom zou het zo kunnen zijn dat TRIF signalering een andere rol speelt bij de begin van de ontsteking dan bij het aantrekken van T-cellen.
Versterken van natuurlijke negatieve terugkoppeling

De TLR4 signaleringsroutes worden in fysiologische condities geremd door verschillende intracellulaire remmers, zoals SOCS1 en SOCS3. Deze eiwitten interfereren met de samenstelling van signaleringseiwit complexen, remmen de kinase activiteit van de signaal eiwitten en induceren afbraak van deze eiwitten. Deze mechanismen voorkomen de activatie van pro-inflammatoire signalering. Het is reeds aangetoond dat overexpressie van SOCS3 artritis kan verlagen en T-cel response kan moduleren. Hier beschrijven we een nieuwe strategie om
SOCS1 en SOCS3 expressie te induceren door activatie van TAM receptoren door de natuurlijke liganden Gas6 en Pros1 (*hoofdstuk 9*). Systemische overexpressie van Pros1 reduceerde Th1 cel aantallen in de milt en dit verlaagde de artritis in lichte mate. Gas6 en Pros1 waren effectiever in het verlagen van de ontsteking wanneer deze lokaal tot overexpressie werden gebracht. Deze overexpressie ging gepaard met een verhoogde expressie van zowel SOCS1 als SOCS3, wat wijst op de effectiviteit van TAM receptor activatie om de ontsteking te remmen. Dit breekt de ontstekingscascade en voorkomt progressie van artritis die door endogene liganden wordt onderhouden. Deze strategie limiteert mogelijk ook neveneffecten, zoals de gevonden verhoging van ontsteking die geobserveerd werd na specifieke verlaging van de TLR4 expressie op APCs. Door tijdelijk de natuurlijke negatieve terugkoppeling op de ontsteking te verhogen is het wellicht mogelijk om neveneffecten te voorkomen, zoals een dysfunctioneel immuun systeem tegen pathogenen.
Curriculum vitae

Ben van den Brand was born on May 14th 1983 in Tilburg, The Netherlands. After graduating from secondary school and one year of Laboratory School (Hogeschool Brabant, Etten-Leur) Ben started studying Biomedical Sciences at the Radboud University, Nijmegen, The Netherlands, in 2002. During his studies Ben gained substantial international experiences with internships in Sweden, Canada and Australia. Ben received his Master of Science degree in June 2008.

In May 2008 Ben started his PhD at the department of Rheumatology Research & Advanced Therapeutics at the Radboud University Nijmegen Medical Centre, The Netherlands. In this PhD project Ben has explored the feasibility of Toll-Like Receptors as therapeutic target in rheumatoid arthritis. Besides developing research qualities Ben also acquired experience with teaching Biomedical Science and Medicine students at both BSc and MSc level.

Ben has continued his professional life in education and has started teaching at Saxion Hogescholen, Enschede, The Netherlands. There, Ben teaches both practical and theoretical courses concerning biology and laboratory work.
Appendices

Publications


Chapter 11

Dankwoord

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