Inflammation Control in Rheumatoid Arthritis

Modulating Toll-like Receptor Responses

Kim Catharina Martina Santegoets

"Inflammation Control in Rheumatoid Arthritis – Modulating Toll-like Receptor Responses"

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Inflammation Control in Rheumatoid Arthritis

Modulating Toll-like Receptor Responses

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(met een samenvatting in het Nederlands)

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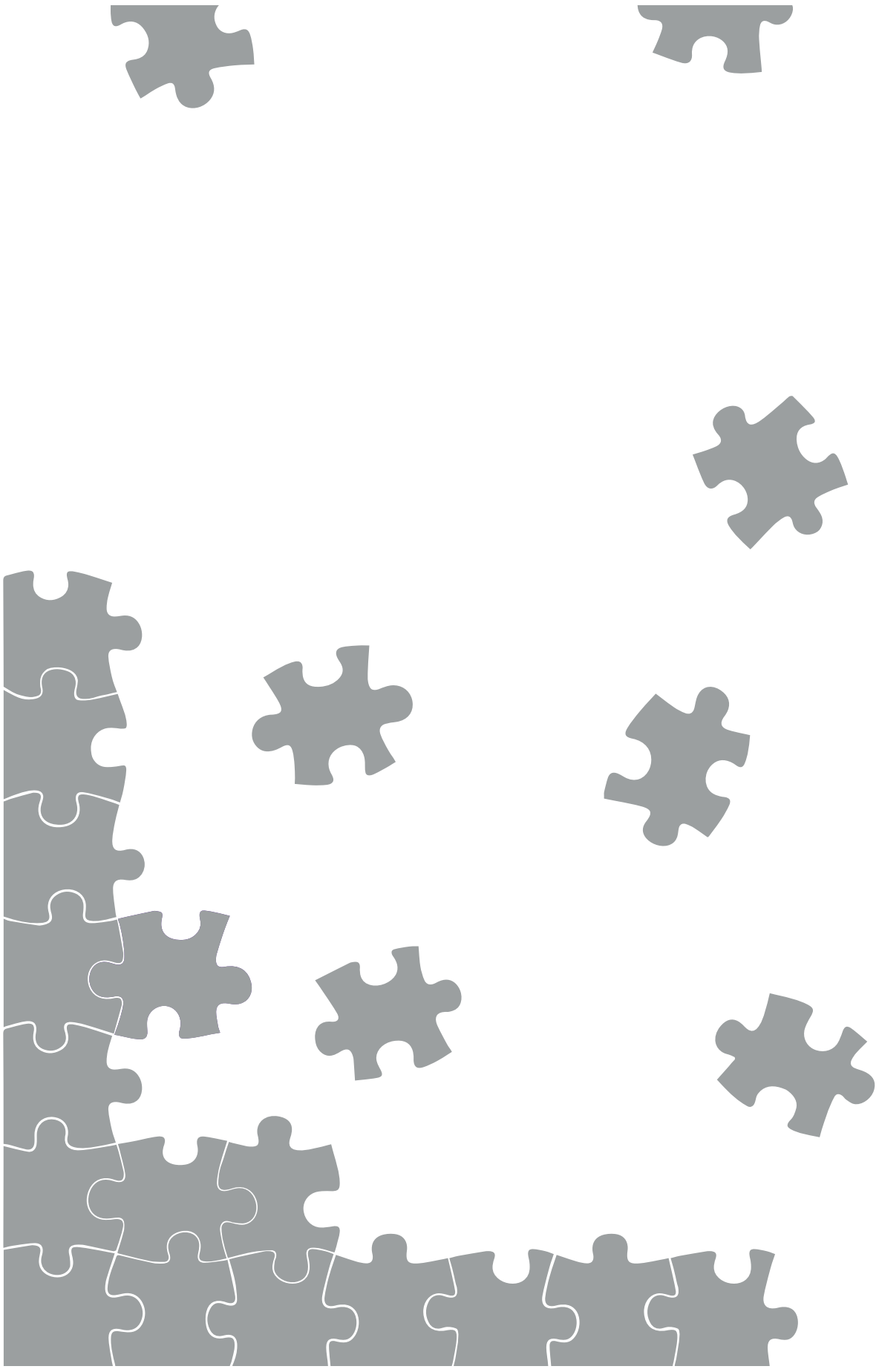

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General Introduction

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It is amazing how our immune system can keep us healthy, at least most of the time. The immune system is a very powerful system that needs to be tightly regulated to respond adequately to invading pathogens, while restoring homeostasis when the threat is cleared. If an immune response is insufficient this can lead to severe/unresolving infections. On the other hand over activation of the immune system can result in severe tissue damage, chronic inflammation or allergic reactions. In addition, the immune system needs to guard the thin line between recognition of 'altered self' in the case of tumor cells and pathogenic recognition of 'self' resulting in autoimmunity. To keep this system in balance close collaboration between many different immune cells is required, which is regulated in time and space. For fine-tuning of these responses, all immune cells express a wide array of receptors, both activating and inhibitory, that together determine the force and direction of the initiated immune response.

The aim of this thesis was to better understand mechanisms involved in the tight regulation of this balance, which we studied in the context of chronic inflammation seen in patients with rheumatoid arthritis (RA).

Rheumatoid arthritis

RA is the most common inflammatory arthritic disease, affecting approximately 1% of the population worldwide, mostly women.[1] The disease is characterized by chronic synovial inflammation (painful and swollen joints), autoantibody production, and eventually cartilage and bone destruction. In addition to arthritis also extra-articular organ involvement can occur, including the skin, eye, heart, lung, renal, nervous and gastrointestinal systems. Luckily, much has changed in the treatment of RA in the last two decades and severe deformations are hardly seen anymore. The aim of treatment nowadays is to reach remission, as measured for example by the disease activity score DAS28 (DAS28 ≤2.6), or a state of minimal disease activity. However RA is a very heterogeneous disease and while methotrexate and biologicals have improved the lives of the majority of the patients, there is still a significant proportion of patients that do not respond to these forms of therapy. This underscores the need for research to better understand what pathological and resolving processes are involved in this disease.

Approximately 70% of the RA patients have autoantibodies against the Fc portion of IgG molecules (Rheumatoid Factor) and/or citrullinated proteins (anti-citrullinated protein antibodies; ACPAs), although also other autoantibodies have been detected.[2;3] Of these autoantibodies ACPAs are almost exclusively found in RA patients and represent the most specific serological marker for RA.[4] The presence of these autoantibodies often precedes clinical onset of the disease and is associated with more aggressive disease course implying a pathogenic role in RA.[5] The most frequent proteins against which ACPAs are produced are fibrinogen, α-enolase and vimentin, which are abundantly present in the RA synovium.[6;7] Citrullination is a post-translational conversion of arginine residues to citrulline by peptidylarginine deiminase enzymes (PADs). Normally PADs only citrullinate intracellular proteins, which are therefore shielded from recognition by the immune system. However, when during synovial inflammation cells may undergo necrosis, they can

release citrullinated proteins and activated PADs, which can start to citrullinate synovial proteins. When these proteins are not degraded properly, they can be taken up by antigen-presenting cells (APCs) together with other danger signals, and presented to T cells which in turn can trigger autoreactive B cells to produce ACPAs.[8]

Whether this latter process occurs is also dependent on genetic predisposition. There is a strong association with certain HLA genes with autoantibody positive RA, mainly within the HLA-DRB1 gene.[9-11] Multiple RA risk alleles within this gene share a conserved amino acid sequence within the epitope binding region and are therefore known as the 'shared epitope' alleles.[12] It has been repeatedly shown that this genotype has a superior capability for presentation of citrullinated peptides.[13-15] In addition, non-HLA genes have been associated with RA susceptibility and disease severity, such as PTPN22, STAT4, IRF5 and Fc gamma receptors[16], all involved in immune signaling. However, the presence of these gene variants alone does not determine if a person gets RA and the concordance in monozygotic twins is only 15-30%.[17;18] While this is markedly increased compared to the risk to develop RA in the general population, this does imply an important role for environmental factors.

One of the environmental factors associated with RA is smoking. Smoking together with the presence of shared epitope alleles synergistically increases one's risk of having ACPA+ RA, which is linked via increased citrullination of proteins in the lungs of smokers.[19-21] Also infectious agents, such as Epstein-Barr virus, proteus species and *Porphyromonas gingivalis*, have been linked to RA, although the mechanism of their involvement in RA remains elusive. Special emphasis has been put on the possible role for *P. gingivalis* in RA, because this bacterium is unique in terms of having a PAD enzyme. *P. gingivalis* PAD can citrullinate both bacterial and human proteins, but differs mechanistically from human PAD possibly increasing immunogenicity of the created citrullinated peptides.[22] In addition, ***P. gingivalis*** also expresses bacterial enolase, with high similarity to human α-enolase (a target of autoantibodies in 40-60% of RA patients), indicating that molecular mimicry between bacterial and human proteins might also play a role.[23-25] *P. gingivalis* is a pathogen involved in chronic periodontitis, an inflammatory disease of the tissue surrounding the teeth (the periodontium). Periodontitis is more common in RA patients compared to the general population.[26-29] This is also interesting from a historical perspective. RA emerged in Europe in the 17th century when trade started with the new world, possibly introducing new pathogens to Europe. This coincided with the import of huge amounts of tobacco and sugar from the Caribbean. The use of which increases the risk for developing periodontitis and/or RA.

Somehow, although the exact triggers are still unknown, synovial inflammation develops in RA patients and turns chronic. In this process many immune cells are involved, illustrated by the different treatment options in RA that target innate cells (blocking of TNFα or IL-6), APC-T cell interaction (abatacept), T cells (methotrexate) or B cells (rituximab). Here we focus on the innate immune system, which acts as a deciding factor for the initiation of immune reactions (DCs) but also as effector cells (macrophage and DCs) in producing inflammatory mediators contributing directly to the inflammatory process.

Macrophages

Macrophages are one of the resident cells present in synovial tissue and their numbers increase in an inflamed joint, where they can make up around 30-40% of the cellular content.[30] They are important producers of inflammatory cytokines and enzymes involved in driving inflammation and joint destruction and their levels correlate with disease activity and joint destruction.[31;32] RA macrophages are mainly associated with 'type 1' like cytokines such as TNFα, IL-6 and IL-1β, and could be sustained by factors like granulocyte-macrophage colony-stimulating factor (GM-CSF), present in the inflamed RA joint.[33-35] GM-CSF and macrophage colony-stimulating factor (M-CSF) are growth factors involved in macrophage differentiation. GM-CSF drives the differentiation of inflammatory macrophages, while M-CSF is involved in macrophage differentiation under more homeostatic conditions. These growth factors can also be used to differentiate pro- and anti-inflammatory macrophages from monocytes *in vitro*.[35]

Synovial macrophages can get activated via pattern-recognition receptors (PRRs) that recognize conserved molecular patterns of pathogens, but also endogenous products produced during inflammation. These PRRs include Toll-like receptors (TLRs), C-type lectins, nucleotide-binding-domain containing receptors (NLRs) and RIG-I-like receptors, of which mainly TLR agonists are found increased in RA. RA macrophages can also get activated by IgG containing immune complexes (ICs), which are recognized via Fc gamma receptors (FcγRs). Furthermore macrophages interact with other immune cells, such as activated T cells, which contributes to synovial TNFα production.[36-38] In addition to producing inflammatory mediators, activated macrophages are a source of collagenases and matrix metalloproteinases (MMPs), involved in cartilage degradation. Removal of synovial macrophages in a murine model for arthritis reduced both inflammation and cartilage damage, supporting the importance of these cells in the pathogenic process in RA.[39;40]

However, next to the inflammatory role described for macrophages in RA, they are also involved in resolution of inflammation. Inflammation resolution is more and more recognized as an active process and multiple models have shown a crucial role for macrophages, including but not limited to their removal of apoptotic neutrophils.[41;42] Mainly M-CSF or 'type 2' macrophages seem to be involved in this process. Macrophages are very plastic cells that can change their phenotype and function, but it remains unclear which triggers are involved in this process and how much this is dependent on phenotype switching or infiltration of new regulatory macrophages.

Dendritic cells

DCs are the professional APCs of the immune system. Immature DCs continuously sample their environment for invading pathogens and express a wide array of receptors to do so. Upon recognition of pathogen associated molecular patterns (PAMPs) by PRRs, immature DCs undergo a maturation process enabling them to present antigens to T cells. During DC maturation, antigen is processed and presented on HLA molecules, co-stimulatory molecules including CD80 and CD86 are up regulated and inflammatory cytokines are

produced. Depending on the combination of PRRs triggered, DCs start producing different cytokines important for the direction of the induced T cell differentiation. DCs also up regulate CCR7 which is involved in DC migration to the lymph nodes where they interact with and instruct T cells. In their immature state DCs are also important for immune tolerance. Presentation of antigens in the absence of co-stimulatory molecules can result in T cell anergy (inactivity) or the induction of regulatory T cells.[43] Therefore DCs are important cells regulating the balance between tolerance and immunity and play a seminal role in autoimmune diseases such as RA.

In human blood we have several types of DCs including myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs can be further subdivided in mDC1, which are potent $CD4^+$ T cell activators, and mDC2 which are better cross-presenters and potently activate $CD8^+$ T cells.[44;45] In RA patients, the numbers of myeloid DCs are decreased in the peripheral blood and increased in the synovial fluid, suggesting selective homing to the joints, where they have an activated phenotype.[46;47] During inflammation, monocytes can be an alternative source for tissue DCs. The $CD1c^+$ inflammatory DCs found in RA synovial fluid are distinct from blood mDC1s and show similarities with in vitro derived monocyte-derived DCs (moDCs) and inflammatory macrophages, suggesting they could be derived from monocytes.[48] Because of this and because levels of DCs in the blood are very low we used moDCs for our in vitro research. These moDCs can be cultured from monocytes in the presence of GM-CSF and IL-4 and have antigen capture and processing as well as T cell activating capacity similar to mDCs.[49]

An important role for DCs in RA pathogenesis is further supported by arthritis models in mice, in which the transfer of antigen-pulsed DCs can induce arthritis and (CD11c) DC depletion can prevent the induction of auto-reactive T and B cells and subsequent arthritis development.[50;51] APCs and mainly the cytokines they produce are also effective therapeutic targets in the treatment of RA nowadays. Most biologicals are directed against cytokines produced by activated APCs, such as TNFα (adalimumab, infliximab and etanercept), IL-6 (tociluzimab) or IL-1 (anakinra). Another effective therapy, CTLA4-Ig (abatacept) binds CD80/CD86 on the surface of APCs and thereby blocks the co-stimulatory signal necessary for T cell activation. In addition to these therapies clinical trials are being performed using tolerogenic DCs to regain tolerance in RA.

T cells

Upon antigen capture and processing DCs travel to the lymph nodes where they instruct T cells. The direction of CD4 T cell differentiation is mainly dependent on the cytokine milieu created by the activated DC, that depends on the kind of pathogen encountered. Antigen presentation to T cells in the presence of IL-12(p70) induces T helper 1 (Th1) cells that produce high levels of interferon γ (IFNγ). This promotes the clearance of viruses and intracellular bacteria by enhancing the activation state of macrophages. A second Th subset, the so-called Th2 cells are induced by IL-4 and mainly produce IL-4, IL-5 and IL-13. These factors help in the class-switching of B cells and promote the clearance of extracellular parasites.[52] A combination of cytokines including TGF-β, IL-6, IL-1 and IL-23

induce and maintain Th17 cells, so called after the main cytokine produced IL-17.[53-55] These cells are mainly involved in neutrophil activation and the clearance of extracellular bacteria and fungi. This system is balanced by the presence of regulatory T cells that mediate immunosuppression. A combination of these different T helper cells is crucial to provide the immune system with the capacity to mount an appropriate defense against different pathogens. However, both Th1 and Th17 cells have also been described as mediators of pathology in chronic inflammatory diseases such as RA. Increased levels of Th1 and/or Th17 cells and their cytokines have been found in the peripheral blood and synovial fluid of RA patients and correlate with disease activity[56-59] Interestingly, IL-17 synergizes with TNFα to further promote activation of synovial fibroblasts and chondrocytes and mediate tissue destruction.[60;61] However despite their suggested pathogenic role, levels of IL-17 producing T cells in the blood and at inflammatory sites are always very low in comparison to other T helper subsets. Furthermore, citrulline-specific auto reactive T cells have been found increased in RA patients and mainly show a Th1 memory phenotype.[62]

In addition to antigen specific T cell activation via APCs, synovial T cells may also get activated by the large amounts of inflammatory cytokines produced locally, such as TNFα, IL-6 or IL-15. This is supported by the fact that RA synovial T cells show many similarities to *in vitro* cytokine activated T cells.[36;37] These cytokine activated T cells (Tck) can interact with monocytes/macrophages and induce an unbalanced inflammatory cytokine response, further contributing to macrophage activation in RA.[36-38]

Cellular recognition of the microenvironment

APCs are specialized cells to sense their environment and detect pathogens and other danger molecules and get activated to induce an immune response. To be able to achieve this APCs express a wide array of receptors, including TLRs, Fc gamma receptors and integrins. These receptors are important to recognize pathogens directly or opsonized by antibodies or complement. However these receptors can also recognize endogenous proteins or autoantibodies present in the RA joint and thereby contribute to RA pathology.

Toll-like receptors

TLRs are pattern-recognition receptors capable of potently activating many different cells. TLRs recognize both endogenous molecules, released upon cell activation/damage (damage-associated molecular patterns; DAMPs), and a wide range of conserved constituents from pathogens (pathogen-associated molecular patterns; PAMPs). At present, 10 TLR subtypes have been identified in humans, each having its specific ligands, cellular localization and expression profiles. TLR2 (as heterodimer with TLR1 or TLR6) and TLR4 are extracellular receptors that are designed to recognize lipid-based structures both from gram-positive and gram-negative bacteria including lipopeptides and lipopolysaccharides (LPS) respectively. Extracellular TLR5 recognizes flagellin, a component of flagellated bacteria. For TLR10, which is believed to originate from the TLR1/TLR6 precursor, no ligand has been described thus far. TLR3, TLR7, TLR8 and TLR9 are intracellular receptors located in the endosomal

compartment and are involved in the recognition of nucleic acids derived from viruses, bacteria and the host. TLR3 is activated by double stranded RNA (dsRNA) and TLR7 and TLR8 by single stranded (ssRNA). DNA and more specifically (bacterial) unmethylated CpG DNA is recognized by TLR9 (Figure 1)(an extended overview is presented in [63]).

Downstream of TLRs, adaptor proteins like MyD88 (all TLRs except TLR3) and TRIF (TLR3 and TLR4) activate protein kinases such as mitogen activated protein kinases (p38, ERK, JNK), ultimately leading to nuclear translocation of transcription factors. These transcription factors including activator protein-1 (AP-1), nuclear factor κB (NF-κB) and members of the interferon regulatory factor (IRF) family (reviewed in [64;65]) induce the expression of various inflammatory cytokines including TNFα, (pro)IL-1β and IL-6, type I IFNs, and chemokines. This system is balanced by the presence of numerous inhibitory proteins and more anti-inflammatory cytokines such as IL-10 that are also induced upon TLR triggering and are involved in a time-dependent termination of initiated cell activation. These inhibitory proteins target the TLR signaling cascade at different levels, including decoy receptors such as single Ig IL-1-related receptor (SIGIRR), TRIF inhibition by SARM, and more downstream inhibitors like TNFα-induced protein 3 (TNFAIP3 (A20)), Toll interacting protein (TOLLIP) and the suppressor of cytokine signaling 1 and 3 (SOCS1, 3).

The TLR system is highly specific in that distinct cellular responses are observed depending on the TLRs involved, with important effects on ensuing inflammatory and adaptive immune responses. Much of this specificity is likely to result from the use of various co-molecules and down-stream adaptor pathways by the various TLRs. For example CD14 and MD-2 function as co-receptors for LPS, fine-tuning cell-type specific effects of TLR4 and influencing the threshold for TLR4 signaling. In the last few years it has also become clear that TLRs not only induce cell activation, but can also modulate inflammatory responses. While simultaneous stimulation of some TLRs results in synergistic induction of cytokine production, TLR2 has been demonstrated to inhibit cytokine production induced by TLR4 or TLR7/8 in DCs with clear effects on T cell responses induced.[66;67] The regulatory role of TLR2 also extends to effects on regulatory T cells.[68;69]

In the RA synovium there is an abundant expression of TLR2, 3, 4, 5, 7 and 9 compared to osteoarthritis patients or healthy controls and TLR stimulation is a strong inducer of chemokine production by synovial fibroblasts.[70-75] The latter is likely to contribute to the accumulation of immune cells in an arthritic joint. Many endogenous TLR ligands have been found in arthritic joints, such as GP96 and SNAPIN, which activate cells via TLR2, small heat shock protein B8 that can activate TLR4, and self-RNA from damaged cells which is likely to stimulate macrophages via TLR3 or TLR7/8.[76-80] Blocking antibodies against these TLRs reduce spontaneous cytokine production by RA synovial tissue cultures, supporting an active involvement of these TLRs in the abundant cytokine production seen in RA.[80-82] This is further supported by mice models for arthritis, which all depend on TLR triggering. Synovial injection with TLR ligands, such as CpG DNA, dsRNA or streptococcal cell wall fragments induces a self-limiting form of arthritis in mice.[83-85] Furthermore the spontaneous arthritis that develops in IL-1 receptor antagonist knockout (IL-1ra$^{-/-}$) mice also depends on an initial microbial trigger and does not develop in germ-free mice. TLRs can induce both immune

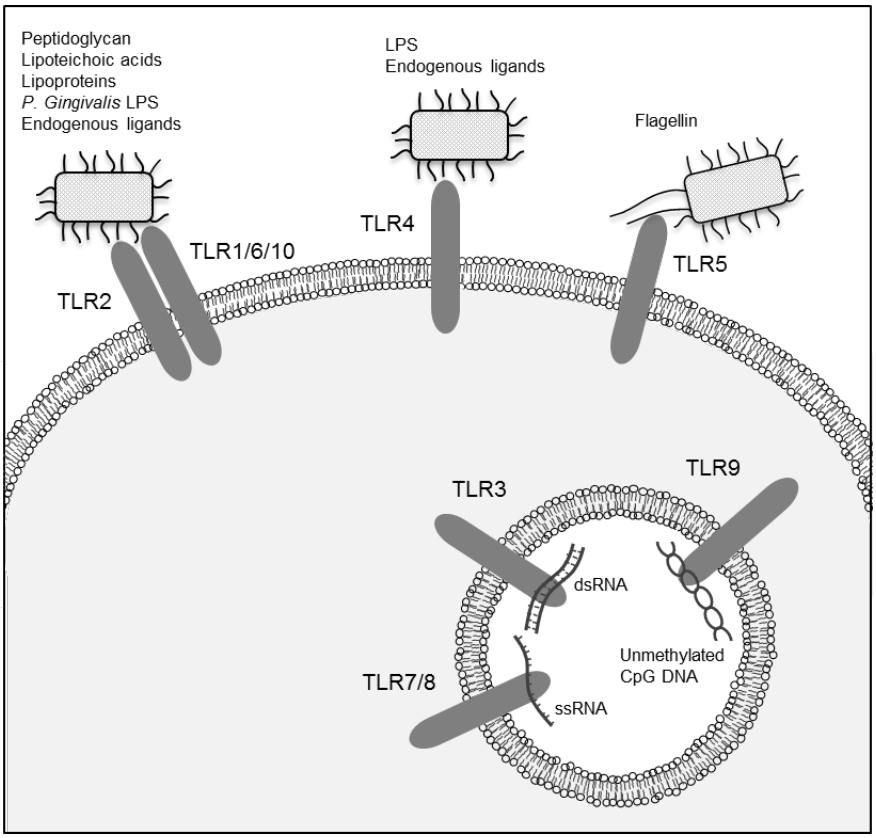

Figure 1. Overview of different TLRs and their ligands

activation and regulation in this model, with TLR4$^{-/-}$ mice being protected against severe arthritis, while TLR2$^{-/-}$ mice developed more severe arthritis.[81]

The question that still remains in RA is what triggers these TLRs on both immune and non-immune cells present in an arthritic joint. It is hypothesized that an initial microbial trigger or minor trauma can induce tissue damage, leading to the release of endogenous TLR ligands, thus creating a vicious circle of inflammation.

Fc gamma receptors

Fc gamma receptors are present on many immune cells, including myeloid cells such as DCs, monocytes and macrophages, and recognize antibodies of the IgG type and IgG containing immune complexes (ICs). The family of FcγRs consists of the high affinity FcγRI, that can bind monomeric IgG, and the low affinity FcRIIa, IIb, IIc, IIIa and IIIb mainly involved in recognition of ICs and IgG coated pathogens. All FcγRs except FcγRIIb are linked to an intracellular immunoreceptor tyrosine-based activation motif (ITAM) and activate cellular responses (Figure 2). Triggering of these activating FcγRs can induce phagocytosis, antigen

presentation, pro-inflammatory cytokine production and antibody-dependent cellular cytotoxicity. FcγRIIb is the only inhibitory FcγR containing an immunoreceptor tyrosine-based inhibition motif (ITIM). As the only inhibitory FcγR, FcγRIIb is an important brake on the immune system by inhibition of cellular activation via the activating FcγRs on many immune cells and inhibition of the B cell receptor. The resulting immune response upon the binding of ICs or IgG coated pathogens thus depends on the balance between the activating and inhibitory FcγRs.

FcγRs are expressed on both innate and adaptive immune cells, although their expression on the adaptive immune cells is more limited compared to innate cells. B cells express only FcγRIIb, which forms an important brake on B cell receptor signaling and antibody production. NK cells mainly express FcγRIII, important for the induction of antibody-dependent cell-mediated cytotoxicity by NK cells. Innate cells on the other hand express a more complex pattern of FcγRs. The balance of activating and inhibitory FcγRs on these cells thus determines their response towards ICs. Research of this balance was hampered by the lack of specific antibodies differentiating between FcγRIIa and FcγRIIb, since their extracellular domains are very much alike. The development of new antibodies specifically recognizing FcγRIIb made it possible to investigate the balance of activating and inhibitory FcγRs on myeloid cells that express a mix of FcγRs.

The importance of FcγR in autoimmune arthritis has mainly been demonstrated in arthritis models. Different FcγR knockout mice have shown that activating FcγRs are essential for induction of arthritis, while deletion of the inhibitory FcγRII (mice lack FcγRIIa)

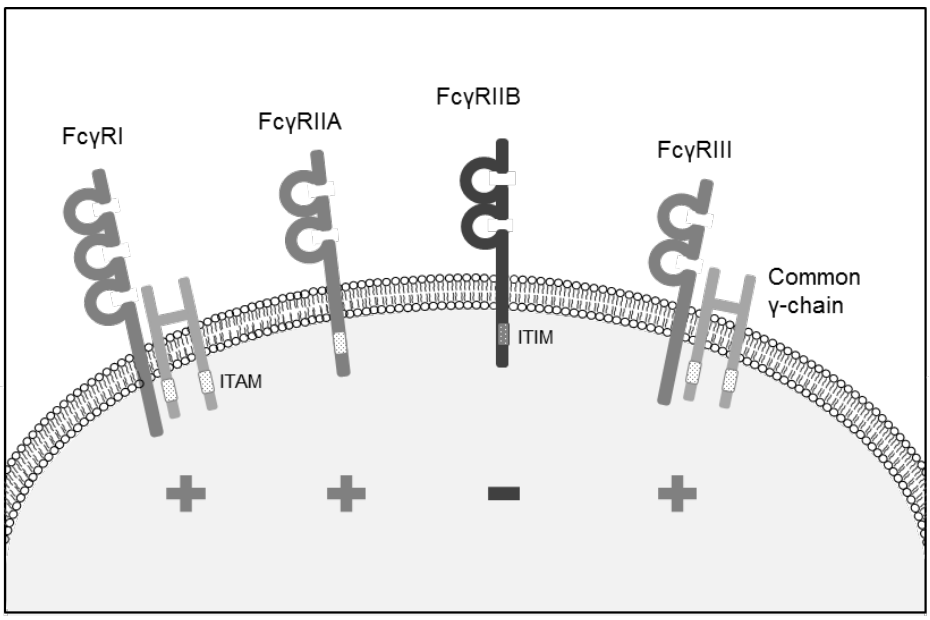

Figure 2. Overview of different FcγRs. FcγRIII is depicted as FcγRIIIa which is expressed on monocytes, macrophages and DCs. Neutrophils express a GPI anchored variant (FcγRIIIb). FcγRIIc is similar to FcγRIIa, but is not expressed in all individuals and therefore not depicted in this figure.

induced arthritis even in non-susceptible mice.[86-90] FcγRIIb not only inhibits activating FcγRs, but is also important for effective clearance of ICs.[91] Furthermore, FcγRIIa transgenic mice are hyper-responsive to pathogenic antibodies and blocking of this receptor in these mice inhibited development and stopped progression of collagen-induced arthritis.[92] In human synovium all FcγRs are highly expressed and correlate with macrophage markers and levels of TNFα and MMP1.[93] Also genetic studies support a role for FcγRs in RA. Natural variants of the activating FcγRIII have been associated with RA susceptibility[94-97] and a functional variant in the inhibitory FcγRIIb is associated with increased radiological joint damage in RA patients.[18]

β2 integrins

Integrins are widely expressed transmembrane adhesion receptors that mediate cell-cell and cell-extracellular matrix adhesion and consist of an α and a β subunit. β2 integrins are specifically expressed on cells of the immune system, mainly myeloid cells, and play an important role in leukocyte extravasation, DC-T cell interaction and homotypic interaction between macrophage-like cells during osteoclast formation. β2 integrins consist of the common β chain CD18, combined with CD11a (LFA-1), CD11b (Mac-1 / Complement receptor (CR) 3), CD11c (CR4) or CD11d. They bind a wide variety of proteins including several ICAMs, fibrinogen and complement components, depending on the α subunit present. In addition to their role in cell adhesion, β2 integrins are also involved in bacterial recognition. CD18 together with CD11b or CD11c can recognize complement coated pathogens and are therefore named CR3 and CR4 respectively. CR3/Mac-1 however has also been described to bind to different bacterial/fungal fragments directly, including LPS, β-glucans and *P. gingivalis* fimbriae. β2 integrins are therefore important sensors for immune cells to respond their extracellular environment, which includes other cells, extracellular maxtrix proteins and sometimes pathogens.

Since leukocyte infiltration of synovial fluid and tissue is a hallmark of inflammatory arthritis it is likely that β2 integrins are somehow involved. And indeed, CD18 knockout mice are almost completely resistant to arthritis development in the K/B x N serum transfer model.[98] A similar dependency on CD11a was found using knockout mice or blocking antibodies, and was characterized by the absence of inflammatory cell infiltrate in the synovium. In contrast, $CD11b^{-/-}$ mice showed a tendency towards more inflammation suggesting a possible anti-inflammatory role for Mac-1/CR3.[98] Lack of or very low levels of β2 integrin expression in humans causes Leukocyte Adhesion Deficiency, which is mainly characterized by severe infections. However, subtle changes in β2 integrin expression and/or function might affect leukocyte extravasation, bacterial recognition or inflammatory behavior of immune cells and could thereby contribute to the inflammatory process in RA or other chronic inflammatory diseases.

All these receptors involved in recognition of the extracellular environment have their own regulatory mechanisms, such as TLR2 inhibition of TLR4 responses, molecules like SARM and A20 that inhibit TLR signaling and the presence of activating and inhibitory FcγRs. In

addition, these receptor systems can interact with each other and thereby modulate the cellular response towards ligands that trigger multiple receptors. For example, RNA or DNA containing immune complexes can be recognized by DCs via FcγRs, delivering the nucleic acids to intracellular endosomes/lysosomes where they can bind to TLR7, 8 or 9, promoting DC activation.[99;100] By receptor cooperation optimal immune responses can be induced towards a wide variety of pathogens, but it can also regulate autoimmune inflammation; with positive or negative effects for the host.

Thesis outline

Myeloid cells play an important role in the regulation of inflammation. Macrophages and DCs are important producers of inflammatory cytokines and DCs initiate the activation of the adaptive immune system. These mechanisms are designed to protect us from infections, but also play a pivotal role in recognition of endogenous danger signals involved in autoimmunity. Next to initiation of inflammation, macrophages and DCs can also have tolerogenic or resolution inducing functions. They express a wide array of receptors recognizing triggers from their environment, including TLRs, FcγRs and integrins. These can all be involved in immune activation, but also need regulatory mechanisms to prevent over activation. In this thesis we aimed to better understand intrinsic pathways for inflammation control and how they function in a chronic inflammatory/autoimmune disease like RA.

In *chapter 2* we aimed to learn more about regulatory mechanisms involved in RA disease control by studying RA patients that were able to control their disease activity without the need of further RA medication. We compared DC phenotype and function of these patients with healthy controls and other RA patients and focused on FcγR expression and their interaction with TLR4. *Chapter 3* further elucidates on the role played by activating and inhibitory FcγRs on pro- and anti-inflammatory macrophages. Here we extended the knowledge about FcγR interaction with TLRs beyond TLR4 and also studied the effect of FcγR triggering on macrophage activation via cytokine activated T cells. Both TLR and cytokine activated T cells appear to play a role in macrophage activation in RA. In *chapter 4* we investigated a possible synergy between these macrophage activation pathways and studied the effect of abatacept (CTLA4-Ig) on T cell mediated macrophage activation.

Much research focuses on the initiation of immune responses, while a proper resolution of inflammation is also essential to prevent chronic inflammatory diseases. *Chapter 5* describes a novel mechanism of how the proinflammatory behavior of macrophages and DCs might become more anti-inflammatory/pro-resolution when many immune cells have infiltrated an inflammatory site. This has a major impact on TLR responses and involves integrin mediated cell-cell contact.

In RA patients the inflammation mostly resides in the synovial joints, where many DCs have been found in the synovial fluid. Therefore we wondered what the effect would be of this synovial fluid environment on DC function and focused on the effect of RA synovial fluid on the activation of DCs by TLR ligands (*Chapter 6)*.

Pathogens such as *P. gingivalis* have been implicated in the pathogenesis of RA.

Research so far has mainly focused on epidemiological associations between *P. gingivalis* and periodontitis with RA. In *chapter 7* we aimed to determine an immunological link between *P. gingivalis* and RA by studying alterations in *P. gingivalis* responses in RA patients compared to healthy controls and patients with psoriatic arthritis.

In *chapter 8* the findings presented in this thesis are summarized and put into a broader perspective.

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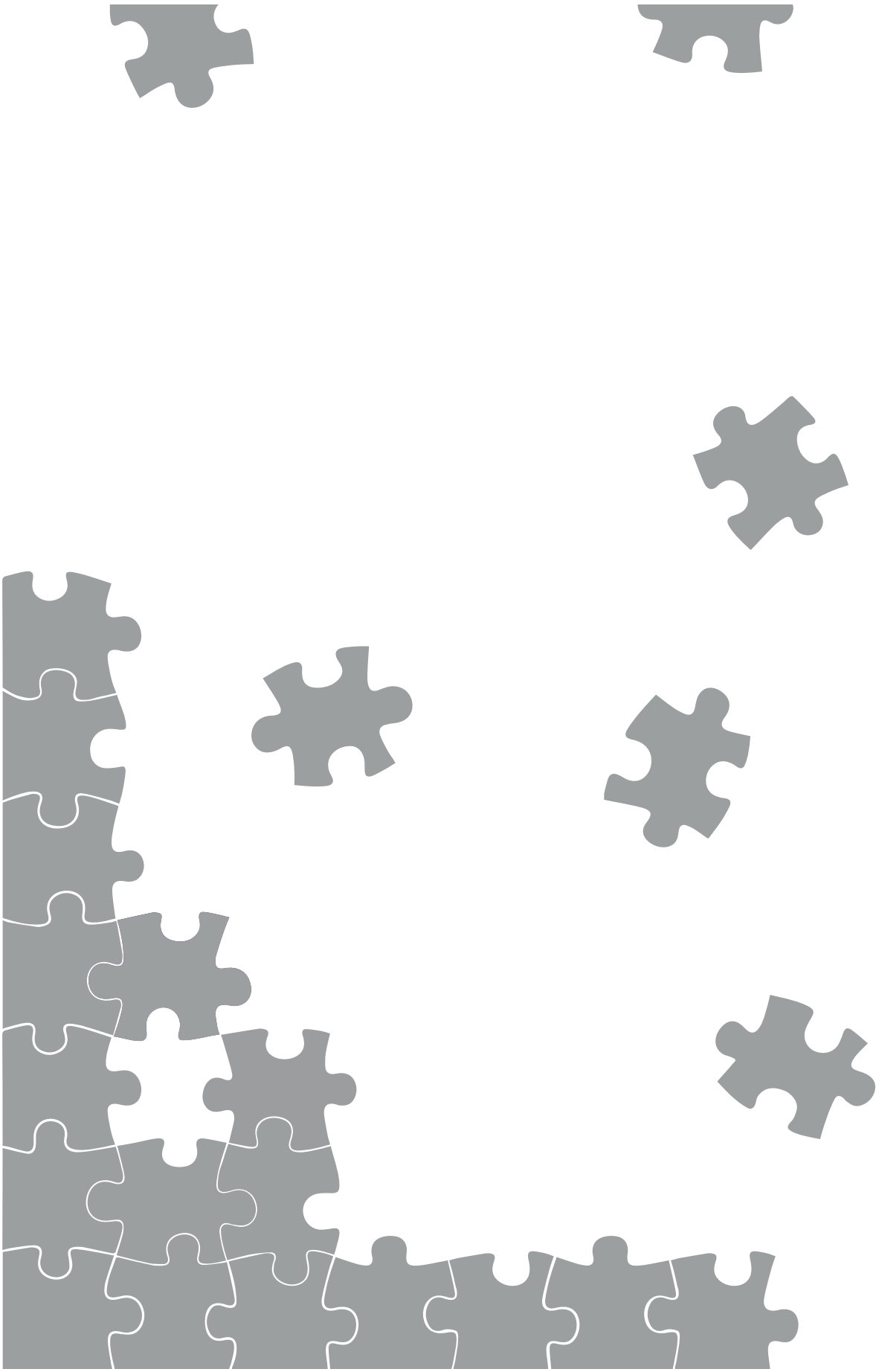

2

The inhibitory FcγIIb receptor dampens TLR4-mediated immune responses and is selectively up-regulated on dendritic cells from rheumatoid arthritis patients with quiescent disease

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Rheumatoid arthritis (RA) is a common autoimmune disease leading to profound disability and premature death. Although a role for Fc gamma receptors (FcγRs) and Toll-like receptors (TLR) is accepted their precise involvement remains to be elucidated. FcγRIIb is an inhibitory Fc receptor important in the maintenance of tolerance. We hypothesized that the inhibitory FcγRIIb inhibits TLR responses on monocyte-derived DCs and serves as a counter-regulatory mechanism to dampen inflammation and we surmised that this mechanism might be defective in RA. The expression of the inhibitory FcγRIIb was found to be significantly higher on DCs from RA patients having low RA disease activity in the absence of treatment with anti-rheumatic drugs. Notably, the expression of activating FcγRs was similarly distributed among all RA patients and healthy controls. Intriguingly, only DCs with a high expression of FcγRIIb were able to inhibit TLR4 mediated secretion of pro-inflammatory cytokines when stimulated with immune complexes. In addition, when these DCs were co-incubated with the combination of a TLR4 agonist and immune complexes a markedly inhibited T cell proliferation was apparent, regulatory T cell development was promoted and T cells were primed to produce high levels of IL-13 compared to stimulation of the DCs with the TLR4 agonist alone. Blocking FcγRIIb with specific antibodies fully abrogated these effects demonstrating the full dependence on the inhibitory FcγRIIb in the induction of these phenomena. This TLR4-FcγRIIb interaction was shown to dependent upon the PI3K and Akt pathway.

Introduction

Rheumatoid arthritis (RA) is a characteristic autoimmune disease typified by polyclonal B cell stimulation and production of autoantibodies targeting the synovial membrane, cartilage and underlying bone. RA is a frequent disorder that affects 1% of the population worldwide thereby representing the most common inflammatory rheumatic condition. Despite a longstanding effort to understand and control the deranged immune process, the crucial events by which T-cell and B-cell tolerance is breached are poorly defined. To date still a substantial part of the patients suffer from severe disability and a decreased life expectancy.[1]

Various cell types are recruited into the inflamed synovium where they are activated culminating in the secretion of a myriad of inflammatory mediators (reviewed in[2]). Numerous pathways could lead to the activation of immune cells in the synovium and accumulating evidence points towards the role of immune complexes (IC) binding to activating Fc gamma receptors (FcγR) and Toll-like receptor (TLR) ligands.[3-8] FcγR are expressed on the cell surface of various hematopoietic cell types. They recognize IgG and IgG containing IC and as a result constitute the link between humoral and cell-mediated immunity.[9] In man, the FcγR system comprises of two opposing families, the activating FcγRs I, IIa and III and the inhibitory FcγRIIb, the balance of which determines the outcome of IC mediated inflammation. Thus far, accumulating evidence that illustrates the vital role of a balanced FcγR system in arthritis originates mainly from analyses of mouse models. For example, deletion of FcγRIIb can result in an aggravation of experimental arthritis[10;11] or lead to a fulminate lupus-like disease[12], whereas deletion of activating FcγR subtypes has the opposite effect.[13] Subsequent studies have demonstrated that the FcγR balance determines biological responses of macrophages and dendritic cells (DCs) upon IC mediated stimulation further identifying the pivotal role of FcγR in the immune response.[14-17]

A role of micro-organisms in RA pathogenesis has been advocated for a long time. The identification of TLRs as receptors for conserved pathogen associated molecular patterns as well as endogenous ligands sparked a revolution of research that constitutes the basis of our current way of thinking regarding the role for TLR in arthritis. First, various research groups have demonstrated that endogenous TLR ligands are abundant in RA patients, both in the circulation as well as in the synovial compartment.[18-20] Second, the expression of various TLR subtypes was clearly increased in the synovial compartment of RA patients[3;4;21] compared to their healthy counterparts and TLR ligands induce an augmented inflammatory response by macrophages and DCs from RA patients.[3;22] Finally, studies in mice revealed that the triggering of TLR aggravates arthritis whereas inhibition of the TLR4 pathways either by genetic knockdown[23] or by addition of TLR antagonists drastically reduced the arthritis incidence and severity.[24]

The ability of the immune system to distinguish self from non-self is central to its diametrically opposed functions; to protect against invading pathogens and, at the same time, maintain non-responsiveness to self. Given the ubiquitous nature of endogenous TLR ligands during life highly regulated counter-regulatory responses must be in place to secure an adequate balance between immunity and tolerance.[25-27] Led by recent work from our

group and others, we postulated that the inhibitory-activation FcγR paradigm might not be the full story in that FcγRIIb might also control TLR4 mediated cell activation.[17;28] This view on TLR4-FcγR cross talk can be carried over to DCs since these cells have been shown to be under tight control by both receptor systems and play a decisive role in the regulation of the balance between immunity and tolerance.[15;16;25;29] The pathways that underlie cross talk between TLR4 and FcγR are currently unknown. It has been reported that, at least some of the mediators implicated in FcγR mediated signaling, are involved in the TLR4 signaling cascade. For example, stimulation of TLR4 results in the recruitment of SHIP to lipid rafts where it is tyrosine phosphorylated and SHIP appears to be a positive regulator of TLR4 activation by enhancing MAPK phosphorylation and decreasing Akt phosphorylation.[30] In turn, PI_3K is known to reverse the effects of SHIP in both the TLR4 and FcγR pathways and in both pathways serine/threonine kinase Akt was shown to play a central role.

Here we demonstrate, for the first time, that the inhibitory FcγRIIb directly inhibits TLR4 mediated cell activation and functions as a counter regulatory mechanism designed to dampen TLR mediated responses. Strikingly, only DCs from RA patients who were able to discontinue their use of disease modifying anti-rheumatic drugs (DMARDs) without the occurrence of subsequent disease flares expressed remarkable high levels of FcγRIIb whereas the expression of activating FcγR was unaltered. Exclusively, DCs from those patients who were able to inhibit TLR4 mediated DC activation and subsequent T cell proliferation but also restored the ability to induce T regulatory capacity by DCs. Collectively, here we show a unique counter-regulatory pathway for TLR4 mediated immune responses that is aberrant in RA underscoring the pivotal role for FcγRIIb in RA and opening novel avenues for therapeutic intervention.

Materials and Methods

Study population

A total of thirty-two RA patients attending the Department of Rheumatology at the Radboud University Nijmegen Medical Centre and ten healthy controls were included. The patients were selected from our well-documented prospective cohort consisting of more than five hundred RA patients. All patients who were not on DMARD therapy for more than 2 years were selected, this resulted in a total population of 11 patients. By extensive screening outside of this cohort five additional RA patients not on DMARD therapy were found in our outpatient clinic. RA patients on DMARD therapy were all selected from our prospective cohort. All patients fulfilled the American College of Rheumatology criteria for RA at the time of disease diagnosis and gave their informed consent.[31] Patients using biological agents and/or prednisolone were excluded from the study. Before every vena puncture, in order to obtain monocytes for DCs culture, the disease activity of the RA patients was determined. To quantify the disease activity the DAS28 was used. The DAS28 incorporates the number of swollen and tender joints out of 28 joints, the erythrocyte sedimentation rate and a score on the visual analog scale on well-being. The presence of

an erosive disease was scored as positive if on the last X-rays from the feet or hands at least one erosion was present scored by means of the modified Sharp/Van der Heyde method.[32] The local Medical Ethics Committee approved the study protocol.

Culture of monocyte-derived Dendritic Cells

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from heparinized venous blood by using density-gradient centrifugation over Ficoll-Paque (Amersham Bioscience). Monocytes were obtained using CD14 microbeads and MS columns (Miltenyi Biotec). This isolation method results in the isolation of untouched monocytes and circumvents artificial activation by FcγR ligation as occurs during the isolation of monocytes by means of adherence with human serum as used previously.[32] DCs were generated by culturing isolated monocytes in RPMI-1640 Dutch modification (Invitrogen Life Technologies) supplemented with 10% FCS and antibiotic-antimycotic (Invitrogen Life Technologies) in the presence of IL-4 (500 U/ml; Schering-Plough) and GM-CSF (800 U/ml; Schering-Plough) for 6 days in a concentration of $10x10^6$ cells per 10 ml culture medium in 75-cm^2 cell culture flasks (Corning). Fresh culture medium (5 ml) with the same supplements was added at day 3 where after the DCs were harvested at day 6. DCs were resuspended in fresh culture medium in a concentration of $0.5x10^6$ DCs/ml and either transferred to 24 well (1 ml) or 96 well (0.1 ml) culture plates and stimulated as described.

Stimulation of peripheral blood lymphocytes

The peripheral blood lymphocytes that remained after the extraction of the $CD14^+$ cells, as described above, were washed with citrated PBS containing 5% FCS and resuspended in culture medium in a concentration of $1x10^6$ cells/ ml. $1x10^5$ cells were plated per well in a 96 well flat bottom plate in triplo and were stimulated overnight with PMA (50 ng/ml, Sigma) and Ionomycin (1 µg/ml, Sigma). The supernatants were collected for cytokine measurements.

Phenotypical analysis of monocyte-derived DCs

Using standardized flow cytometry protocols as described previously the phenotypical analysis of monocytes and monocyte-derived DCs was performed.[33] The expression of FcγRs was determined on monocytes and monocyte-derived DCs using the antibodies for human FcRI (CD64, clone 10.1, Dako) and FcγRIII (CD16, clone DJ130c, Dako), the FcγRIIb specific Fitc-labeled antibody 2B6 (Macrogenics Inc) and clone IV.3 which preferentially binds to FcγRIIa (Medarex, kindly gifted by Dr. J. Ronnelid).[15] Immature DCs were further characterized by staining with mAbs against human CD14 (Dakocytomation), CD80 (BD Biosciences), CD83 (Beckman Coulter), CD86 (BD Pharmingen), MHCII DR/DP (clone Q1514), ILT3 (R&D systems), ILT4 (R&D systems) and DCIR (BD Biosciences). As secondary antibody FITC-conjugated goat anti-mouse IgG (Zymed Laboratories) was used. DCs matured for 24 hours with LPS in the presence or absence of IC were analyzed for the expression of CD86 and MHCII as classical markers for DC activation. The level of apoptosis of the stimulated DCs was determined by annexin-V staining and with propidium iodide. Cells were analyzed

with a fluorescence-activated cell sorter (FACSCalibur; BD Biosciences) for the proportion of positive cells and the mean fluorescence intensity relative to cells stained with the relevant IgG isotypes.

Dendritic cell stimulation

Day 6 DCs were replated in a concentration of $0.5x10^6$ DCs/ml and either transferred to 24 well (1 ml) or 96 well (100 µl) culture plates. DCs were then put in contact with medium, with heat-aggregated human immunoglobulins (IC, in all experiments used in a concentration of 50 µg/ml), prepared and used as previously described[33], with immune complexes derived from the serum of healthy controls, RA patients or RA synovial fluid (Pegylated-IC, Peg-IC), double-purified LPS or with the combination of IC or Peg-IC and double-purified LPS. The Peg-IC precipitates were purified and washed in a single-step centrifugation procedure as described in [34], briefly 1 ml of phosphate-buffered saline (PBS) containing 5% human serum albumin (HSA) and 2·5% PEG 6000 (PBS–HSA–PEG) was added to 1·5 ml autoclaved Eppendorf tubes. Plastic cylinders made from 5 ml autoclaved pipette tips (by cutting off about 1·5 cm of the tips) were introduced into the Eppendorf tubes containing PBS–HSA–PEG. Hyaluronidase (Sigma-Aldrich, Stockholm, Sweden) treated synovial fluid or sera precipitated overnight were diluted 1 : 3 in RPMI-1640 containing 2·5% PEG 6000 and then placed on top of the PBS–HSA–PEG in the pipette tips. An interface was formed with the less dense, red RMPI-1640 solution on top. The tubes were then centrifuged at 2100 *g*, 4°C for 20 min, whereby the precipitates in the upper 2·5% PEG–RPMI solution were centrifuged down to the bottom of the Eppendorf tube. The remaining PBS–HSA–PEG solution was removed and the precipitated pellet was immediately resolubilized in ice-cold sterile PBS to the original serum volume. The precipitates were totally resolved in PBS leaving no insoluble aggregates. The dissolved PEG precipitates were then placed on ice until used in cell culture experiments.

The used Escherichia coli Lipopolysaccharide (100 ng/ml, Sigma-Aldrich) was double-purified at our lab using the phenol-water extraction method to remove any remaining protein contamination.[35] In experiments using intracellular signaling molecule inhibitors DCs were pre-treated with these inhibitors for 1h at 37°C before adding the stimulants. The following inhibitors were used in the mentioned concentrations: Wortmannin (PI3K inhibitor, 0.1 µM), Akt inhibitor IV (0.1 µM), Akt inhibitor X (0.5 and 5 M), SB203580 (p38 inhibitor, 20 µM), Rottlerin (PKCδ inhibitor, 10 µM), LFM-A13 (Btk inhibitor, 50 µM). All inhibitors were obtained from Calbiochem. Supernatants were collected after 24 hours for cytokine measurements, except for the experiments in which mentioned otherwise. In some experiments DCs were harvested after 24 hours of stimulation with LPS in the presence or absence of IC and subjected to FACS analysis for the determination of the level of CD86 and MHCII expression. To determine the level of intracellular retention of TNFα, DCs were subjected to 5 times repeated freeze (-80°C)-thaw cycles in their supernatants before TNFα measurements. To determine the role of the inhibitory FcγRIIb on the modulation of TLR4 responses by IC, selective ligation of the activating FcγR was performed by blocking FcRIIb. Immature day 6 DCs were washed with PBS and incubated for 30 minutes at 4°C

with the monoclonal antibody 2B6 (5 µg/ml) to selectively block FcγRIIb, DCs were washed with PBS three times, resuspended in culture medium and stimulated with LPS and LPS in combination with IC. IC were added to the DCs 5 minutes in advance of LPS. Preincubation with the mAb IV.3 (5 µg/ml) allowed for the absence of FcγRIIa stimulation upon IC stimulation, while preincubation in the absence of mAb allowed for the shared ligation of activating and inhibitory FcRs. All solutions used in the experiments, except the LPS dilution, were checked for endotoxin contamination by Limulus Amebocyte Lysate assays. None were positive.

Mixed leucocyte reaction

At day 7 matured DCs were harvested from their 24 well plates, washed in PBS and resuspended in a concentration of $1x10^5$ DCs/ml in culture medium. $5x10^3$ DCs were replated in 96 round bottom well plates. $CD3^+CD25^-$ T cells from healthy controls were obtained by negative selection using microbeads against CD14 (M5E2), CD16 (3G8), CD19 (4G7), CD33 (P67.6), CD56 (B159), CD25 (MA251) and CD235 (BD Biosciences, Erembodegem, Belgium) combined with sheep anti-mouse IgG coated magnetic beads (Dynal Biotech, Oslo, Norway) and MS columns (Miltenyi Biotec) after PBMC were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) of buffy-coats obtained from normal healthy donors. This resulted in a $CD3^+CD25^-CD127^+$ T cell purity of >95%. $5x10^4$ $CD3^+$ T cells were added to the DCs in the 96 round bottom well plates. T cell proliferation was monitored at day three during the mixed leucocyte reaction (MLR) by tritiated thymidine incorporation. The cells were pulsed overnight (day 3-day 4) with tritiated thymidine (0.5 µCi) and thymidine incorporation was analyzed by a gas scintillation counter. The tritiated thymidine incorporation is expressed as mean count per 5 minutes and SD of at least quadruplicate measurements. To determine the differentiation profile of the present T cells stimulation assays were performed. To this end, at day six at least quadruplicate wells were incubated with PMA (50 ng/ml, Sigma) and Ionomycin (1 µg/ml, Sigma) for 12 hours before the collection of supernatants.

Determination of the induction of regulatory T cells in MLR

T Cells from the MLR were phenotypically analyzed by five color flowcytometry as described previously.[36] Cells were washed twice with phosphate-buffered saline supplemented with 0.2% bovine serum albumin (Sigma). The following conjugated mAb were used: CD127 (hIL-7R-M21) PE, (BD Biosciences, Erembodegem, Belgium), FoxP3 (PCH101) FITC (Ebioscience, San Diego, CA), CD4(T4) PC7, CD8 (SFCI21Thy2D3) ECD and CD25 (B1.49.9) PC5 (Beckman Coulter). First the $CD4^+CD25^+$ T cells were gated by using anti-CD4 and anti-CD25 antibodies. This subset of $CD4^+CD25^+$ T cells was further characterized by determining the level of expression of FoxP3 and CD127 according to the indicated settings. Isotype matched antibodies were used to define marker settings. Intracellular analysis of FoxP3 was performed after fixation and permeabilization, using Fix and Perm reagent (Ebioscience, San Diego, CA).

Table I. Phenotype and clinical characteristics of RA patients

Subpopulation of RA patients	DMARD(-)	DMARD(+)
Patients (n)	16	16
Age (yrs)	69 ± 9	67 ± 13
Disease duration in yrs; mean (range)	13 (2-33)	9 (2-20)
DMARD use in years; mean (range)	7 (0-22)	8 (2-19)
Rheumatoid factor positivity, n (%)	10 (63%)	11 (69%)
Rheumatoid factor level (mean ± SD)	123 ± 225	317 ± 492
Erosive disease, n (%)	12 (75%)	13 (81%)
DAS at inclusion of study	3.0 ± 1.0	3.7 ± 1.4
Mean DAS over past year	2.9 ± 0.8	3.3 ± 1.2
Number of DMARDs during follow-up	1.8 ± 1.4	2.2 ± 1.1

Measurement of cytokines in culture supernatants

Levels of TNFα, IL-12p70, IL-17, IL-4, IFNγ and IL-13 were measured in the supernatants using commercially available kits (Bio-Rad) according to the manufacturer's instructions. Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad). The sensitivity of the cytokine assay was < 5 pg/ml for all cytokines measured.

Statistical analysis

Differences between groups were analyzed using paired Student's t-tests or the Mann-Whitney U test. Correlations were analyzed using Spearman tests. P values less than 0.05 were considered significant.

Results

Monocyte-derived DCs from RA patients able to halt DMARD use express FcγRIIb at high levels

RA is a tremendously heterogeneous disease in nature characterized by disease flares and remissions as measured by the disease activity score (DAS28) now generally accepted and used worldwide.[37] Next to this, the dependency on Disease Modifying Anti-Rheumatic Drugs (DMARDs) ranges from those perpetually in need of potent immunosuppressive drugs to a subset of patients who are able to discontinue its use. Based on these facts we divided RA patients into four categories using a well-documented prospective cohort of RA patients.[38-40] Accordingly, at the time of study inclusion, RA patients were divided into those having moderate to high disease activity (DAS28 > 3.2) and those having a low disease activity (DAS28 < 3.2) with or without the use of DMARDs. All patients in the current study fulfilled the ACR criteria for rheumatoid arthritis at the time of inclusion in the inception cohort and suffered from a longstanding RA with a mean disease duration of 13 yrs (2-33 yrs) for RA patients not on DMARD therapy and 9 yrs (2-20) for those on DMARD therapy at the

start of the current study. Notably, no significant differences were observed between both groups regarding rheumatoid factor, disease duration or the presence of erosions (Table I). We examined the expression of FcγR subtypes in RA patients using the unique antibodies recently described to discriminate the FcγRIIa and FcγRIIb isoforms.[15;33] Compared with healthy controls, all patients having a moderate to high DAS28 (regardless of DMARD use) or those having a low DAS28 using DMARDs (DMARD(+) RA) displayed a similar expression profile both of activating and inhibitory FcγRs (Figure 1A and B). Strikingly and in sharp contrast, all patients not on DMARD therapy and having a low disease activity (DAS28<3.2) (further designated as DMARD(-) RA) expressed a significantly higher level of FcγRIIb on their monocyte-derived DCs whereas the expression of activating FcγR was not notably different (Figure 1A and B). With respect to various DC markers (CD14, CD80, CD83, CD86, MHCII) as well as the ITIM bearing molecules ILT3, ILT4 and DCIR, which are known to be expressed on "tolerogenic" DCs[41;42] no differences were observed (data not shown). In patients on DMARD therapy no correlation between DAS28 and FcγRIIb expression could be observed whereas this correlation was clearly present in those individuals who did not use anti-rheumatic drugs (Figure 1C). These observations substantiate that the lack of DMARD use alone did not explain the high FcγRIIb expression in RA patients with a self-regulated low disease activity but rather indicates that they constitute a different class of RA patients. Of note, we also evaluated the expression of the FcγR on monocyte-derived DCs from patients suffering from other immune-related diseases such as systemic lupus erythematosus and psoriatic arthritis. None of these patients DCs expressed FcγRIIb to such high levels as observed in RA patients having quiescent disease (data not shown). In order to determine whether the differences in FcRIIb were already present on the progenitor cells of the DCs, monocytes from healthy controls, DMARD(+) and DMARD(-) RA patients were evaluated for their expression of FcγRIIb. No significant differences in the expression of FcγRIIb were found (Figure 1D).

ICs inhibit TLR4-mediated cytokine release on DCs from DMARD(-) RA patients

We surmised that the high expression of FcγRIIb on DCs from DMARD(-) RA patients has clear functional consequences. To ascertain whether this idea holds true, we measured the production of the pro-inflammatory mediators TNF-α and IL-12p70 produced by DCs upon co-incubation of LPS (TLR4 agonist) compared with the combination of LPS and IC. In contrast with DCs from RA patients using DMARDs, which express low FcγRIIb levels (designated as $DC_{low\text{-}Fc\gamma RIIb}$) (increase TNFα 2% ± 2 and IL-12p70 19% ± 4 (mean ± SEM)), DCs from DMARD(-) RA patients, which express high FcγRIIb levels (designated as $DC_{high\text{-}Fc\gamma RIIb}$), clearly inhibited the production of TNFα (-28% ± 2) and IL-12p70 (-43% ± 4) upon stimulation with the combination of LPS and IC compared with that seen upon stimulation with LPS alone (Figure 2A). Importantly, all $DC_{high\text{-}Fc\gamma RIIb}$ were derived from DMARD(-) RA patients (untreated RA patients with a low DAS28) and all DCs from DMARD(-) RA patients were $DC_{high\text{-}Fc\gamma RIIb}$ while all $DC_{low\text{-}Fc\gamma RIIb}$ were derived from DMARD(+) RA patients (RA patients on DMARD therapy with a low DAS28 score). As expected, like $DC_{low\text{-}Fc\gamma RIIb}$ from DMARD(+) RA patients DCs from healthy controls were unable to suppress TLR4 responses by the

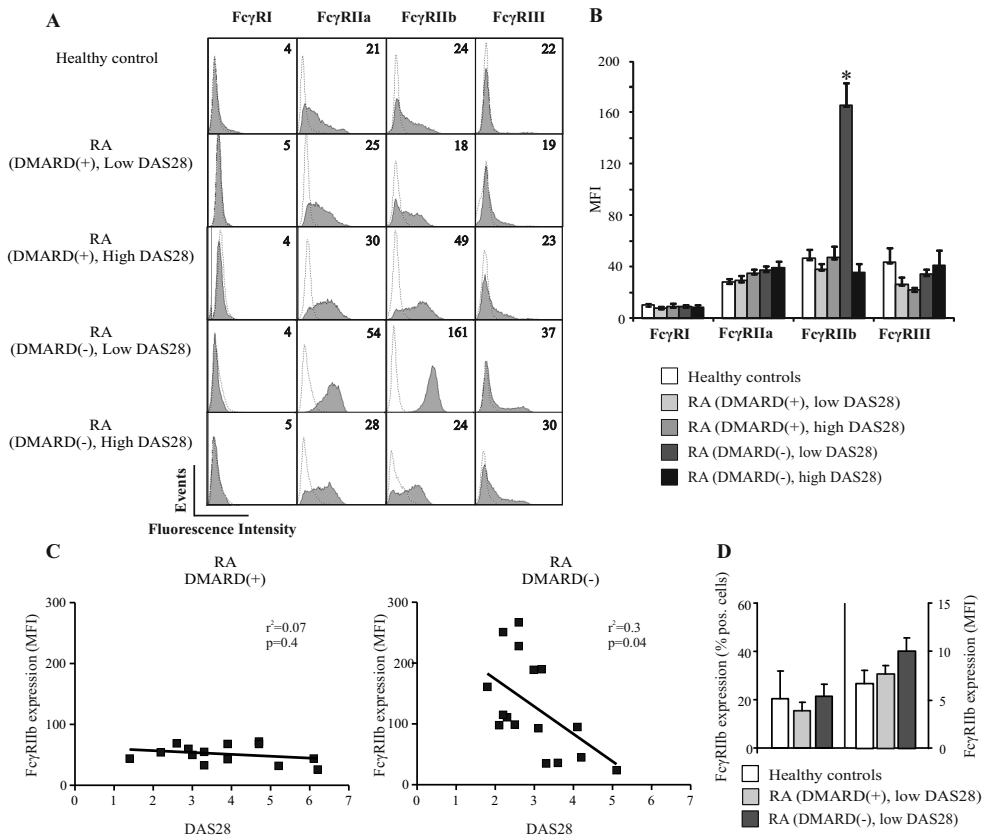

Figure 1. FcγRIIb expression is markedly increased on DCs from RA patients having quiescent disease. (A) Whereas the expression of activating FcγR subtypes (FcγRI, IIa and IIIa) is comparable in all groups (one representative individual from each group is shown), the8.5is increased only on immature DCs from RA patients having low disease activity who had stopped DMARD use at least 2 years ago. Mean fluorescence intensity is presented as measured by flowcytometry. (B) Expression of activating and inhibitory FcγR on DCs from the different RA subgroups (DMARD(+), DAS28>3.2, n=6; DMARD(+), DAS28<3.2, n=10; DMARD(-), DAS28>3.2, n=5; DMARD(-), DAS28<3.2, n=11) and healthy individuals (n=10). Mean and SEM are for combined data from 5-11 independent experiments. * indicates a p-value < 0.001, comparing the DMARD(-), DAS28<3.2 group to the four other groups (C) No correlation is present between FcγRIIb expression on DCs and disease activity in RA patients using DMARDs whereas there is a clear correlation in patients who halted DMARD use. The disease activity score DAS28 was measured at inclusion of the study. (D) Expression of FcγRIIb on monocytes from DMARD(+), DAS28<3.2 (n=5); DMARD(-), DAS28<3.2 (n=5) RA patients and healthy individuals (n=6). Mean and SEM are for combined data from 5 independent experiments.

ligation of IC (data not shown). Unstimulated or IC-stimulated $DC_{high\text{-}Fc\gamma RIIb}$ and $DC_{low\text{-}Fc\gamma RIIb}$ did not release any detectable levels of TNFα or IL-12p70 demonstrable of their immature nature. We next determined the inhibitory capacity of FcγRIIb over a range of LPS doses. The inhibitory effect of IC was potent since it was found to be effective in a wide range of LPS concentrations (10 pg/ml - 1μg/ml, data not shown). The use of heat aggregated human immunoglobulins in our experiments is well controlled but artificial. Therefore we repeated these experiments to ensure that naturally occurring immune complexes exert comparable effects with regard to FcγRIIb mediated TLR inhibition.[34] Pegylated-immune complexes (Peg-IC) isolated from the serum from RA patients inhibited TLR4 responses to comparable levels as observed with IC attesting that circulating immune complexes can tune TLR mediated immune responses via FcγRIIb *in vivo* (Figure 2B). Peg-IC isolated from healthy individuals were able to inhibit TLR4 mediated DC activation to the same extent as those Peg-IC from RA patients again underscoring the importance of FcγRIIb as a check point for tolerance. Interestingly, Peg-IC isolated from the synovial fluid of RA patients were

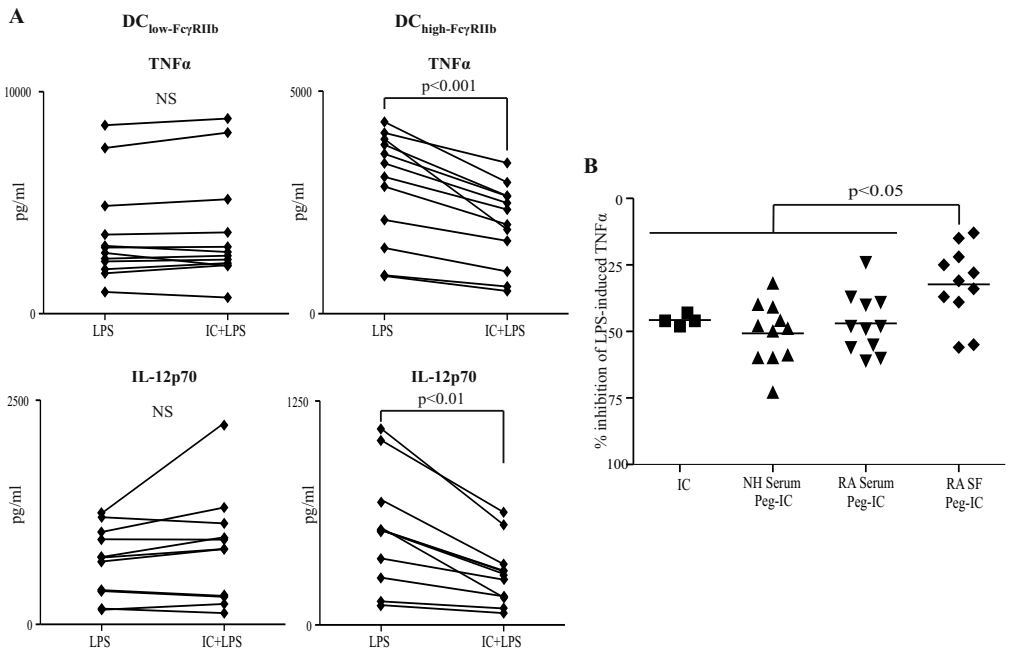

Figure 2. Only TLR4 mediated cytokine production by DCs from DMARD(-) RA patients is inhibited by co-stimulation with IC. (A) Immature DCs from RA patients able to successfully discontinue DMARD therapy, of which all expressed high levels of FcγRIIb (further designated as $DC_{high\text{-}Fc\gamma RIIb}$), markedly inhibit TLR4 mediated secretion of TNFα and IL-12 upon co-culture with IC compared to those stimulated with LPS only. In contrast, DCs from RA patients on DMARDs ($DC_{low\text{-}Fc\gamma RIIb}$) were unable to inhibit TLR4 mediated cytokine production. The results displayed here originated from 10 independent experiments. (B) Immune complexes isolated by PEG precipitation from the serum of healthy controls (n=11) and RA patients (n=11) dampen the release of TNFα to the same extent as do artificial immune complexes. IC isolated from synovial fluid of RA patients (n=11) are less able to inhibit the production of TNFα. Results from $DC_{high\text{-}Fc\gamma RIIb}$ from a representative DMARD(-) RA patient are shown.

less able to inhibit TLR4 responses. To exclude the possibility that intracellular retention of TNFα[43] would explain our results we compared the release of TNF-α upon stimulation of DCs with the combination of IC and LPS before and after repeated freeze-thaw cycles. The inhibitory effect of IC was still clearly present suggesting that retention of TNFα was virtually neglectable (data not shown). To investigate whether the inhibition of TLR4 by FcγRIIb was prolonged or temporal, we stimulated $DC_{high\text{-}FcRIIb}$ with LPS in the presence or absence of IC at baseline and measured the amount of TNFα in the supernatants at several time points. TNF-α production was inhibited to the same extent at all time points (data not shown). Homo-aggregation of FcγRIIb on B cells induces apoptosis. The levels of Annexin V and Propidium Iodide were similar throughout the experiments, which refutes apoptosis as an explanation for our observations (data not shown). Notably, both IC and Peg-IC were found to be negative for LPS contamination using Limulus Amebocyte Lysate assays (data not shown).

FcγRIIb stimulation inhibits TLR4-mediated DC maturation which has clear effects on T cell responses

To further delineate whether the TLR4 dependent phenotypic maturation of DCs was affected by IC, $DC_{high\text{-}FcγRIIb}$ were stimulated with the combination LPS and IC and compared to those stimulated with LPS alone. Subsequent analysis of CD86 and MHCII demonstrated that the addition of IC to LPS halted the phenotypic maturation significantly (Figure 3A). In sharp contrast, IC had no significant effect on $DC_{low\text{-}FcγRIIb}$. Since the expression of such maturation markers exerts direct effects on T cell instruction, we next examined their capacity to induce T-cell proliferation of allogeneic $CD3^+CD25^-CD127^+$ T cells. The potency of $DC_{high\text{-}FcγRIIb}$ to induce T-cell proliferation upon stimulation with the combination of LPS and IC was markedly diminished compared to stimulation with LPS alone ($P < 0.05$) (Figure 3B). As expected, on $DC_{low\text{-}FcγRIIb}$ the addition of IC to LPS had no inhibitory effect. Since the pathogenic role for Th1 cells in rheumatoid arthritis is evident from a wide range of clinical and experimental observations, we extended our result by analysis of T cell cytokines considered to reflect Th1 (IFN-γ) or Th2 (IL-13) status. Exposure of T cells to $DC_{high\text{-}FcγRIIb}$ co-incubated with the combination of LPS and IC strongly increased the potential of T cells to secrete IL-13 compared to $DC_{high\text{-}FcγRIIb}$ incubated with LPS alone, whereas this effect was absent on $DC_{low\text{-}FcγRIIb}$ (Figure 4A). IL-4 was also detectable in the MLR supernatants in a similar pattern as IL-13 however at much lower concentrations (data not shown). Strikingly, T cells directly isolated from the circulation from RA patients with high FcγRIIb levels produce significant higher levels of IL-4 and IL-13 compared to those from patients characterized by DCs with a low FcγRIIb (Figure 4B). Since T cells able to excrete IL-17, designated Th17, have recently been mentioned as mediators of inflammation in experimental arthritis models we measured the IL-17 content in MLR with $CD3^+CD25^-CD127^+$ T cells and $DC_{high\text{-}FcRIIb}$ activated by LPS alone or in combination with IC. No significant differences were observed (data not shown).[44] These observations may suggest that FcγRIIb mediated TLR4 inhibition has clear consequences on the Th1/Th2 axis but does not touch Th17 development. To further explore the modulating effect of

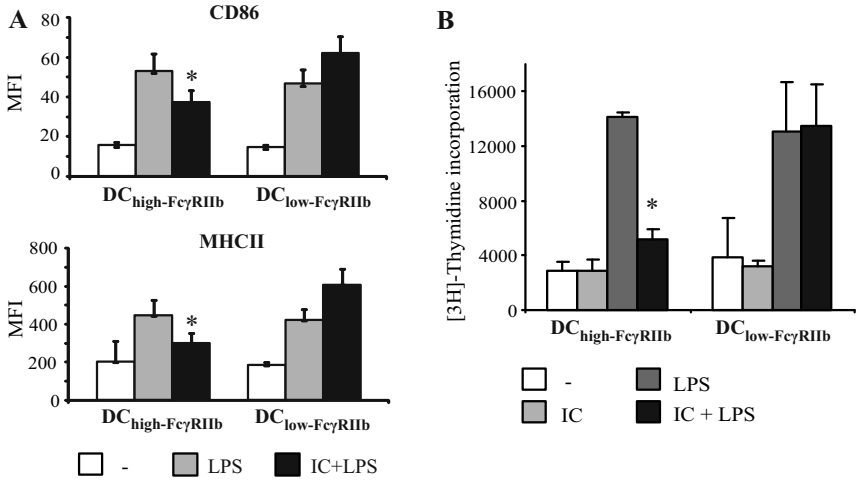

Figure 3. **Co-stimulation of $DC_{high\text{-}Fc\gamma RIIb}$ with IC inhibits TLR4 induced phenotypic maturation and has clear consequences for T cell priming.** (A) DCs were cultured for 24 hrs with medium, with LPS alone or with LPS and IC. A decreased expression of cell surface makers CD86 and MHCII upon stimulation with combination of LPS and immune complexes by DC expressing high levels of FcγRIIb (n=5) is shown. In contrast, DCs that express low levels of FcγRIIb (n=5) did not display an altered expression of these cell surface markers upon co-incubation with IC. Mean and SEM are for combined data from 5 independent experiments. (B) To determine the effect of IC mediated FcγR activation on the ability of $DC_{low\text{-}Fc\gamma RIIb}$ and $DC_{high\text{-}Fc\gamma RIIb}$ to induce T cell proliferation $CD3^+CD25^-$ T cells were stimulated for 3 days with DCs. The DCs were activated before the MLR with the described ligands for 24 hours and were subsequently extensively washed. Incubation of $DC_{high\text{-}Fc\gamma RIIb}$ with the combination of LPS and IC markedly inhibited T cell proliferation measured by 3H-Thymidine incorporation at day 3 compared to those stimulated with LPS alone. In sharp contrast, the addition of IC to LPS did not have any effect on T cell proliferation induced by $DC_{low\text{-}Fc\gamma RIIb}$. For these experiments 5 RA patients of each group were tested in 4 independent experiments. * indicates a p-value < 0.05

FcγRIIb signaling on the ability of DCs to influence T cell differentiation, we next studied the capacity of $DC_{high\text{-}Fc\gamma RIIb}$ and $DC_{low\text{-}FcRIIb}$ to induce regulatory T cells (Treg). To this end, $CD3^+CD25^-CD127^+$ T cells were stimulated with $DC_{high\text{-}Fc\gamma RIIb}$ or $DC_{low\text{-}FcRIIb}$ activated with medium, IC, LPS or the combination of IC and LPS and were analyzed by flowcytometry after 6 days of co-culture. Newly induced $CD4^+CD25^+FoxP3^+CD127^-$ T cells were present in all the cultures. However, the relative presence of $CD4^+CD25^+FoxP3^+CD127^-$ T cells was significantly diminished by the prior activation of the DCs with LPS. Interestingly, the inhibitory effect of TLR4 on the relative presence of Treg observed by the addition of LPS was completely abolished by the addition of IC to $DC_{high\text{-}Fc\gamma RIIb}$. The addition of IC did not have any effect when looking at $DC_{low\text{-}Fc\gamma RIIb}$ (Figure 4C and D). Collectively, these data indicate that the FcγRIIb mediated inhibition of TLR4 responses has clear functional consequences for DC mediated T cell instruction and might explain the higher number of Th2 cells found in vivo in DMARD(-) RA patients.[45]

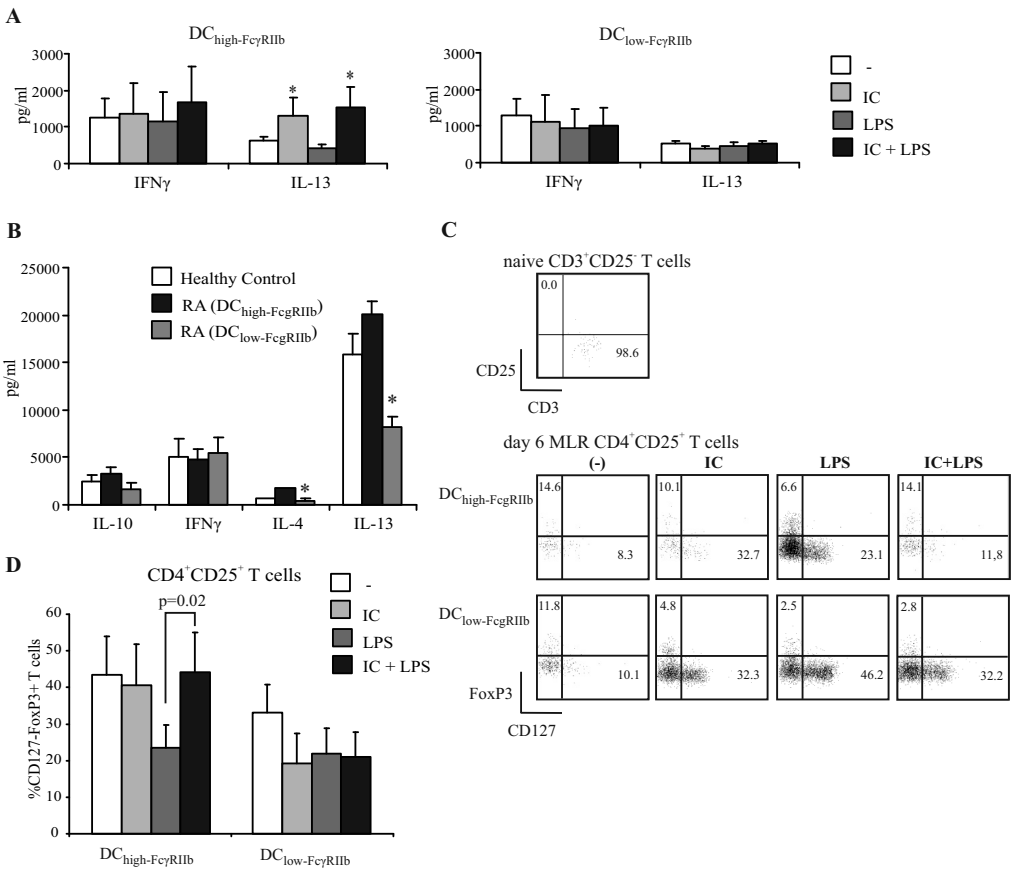

Figure 4. $DC_{high\text{-}Fc\gamma RIIb}$ co-stimulated with IC promote the differentiation of Th2 cells and the $CD4^+CD25^+FoxP3^+CD127^-$ T-cell population. (A) At day five during MLR T cells were exposed to PMA (50 ng/ml) and ionomycin (1 µg/ml), supernatants were collected after 12h for measurement of the released levels of IFNγ and IL-13. T cells incubated with $DC_{high\text{-}Fc\gamma RIIb}$ that were stimulated with IC with or without LPS released increased levels of IL-13 compared to those cultured with $DC_{high\text{-}Fc\gamma RIIb}$ stimulated with medium or LPS alone. In contrast, co-stimulation of $DC_{low\text{-}Fc\gamma RIIb}$ with IC did not sort an effect on IL-13 secretion. In both groups no effect could be observed by the addition of IC on the production of IFN-γ. Data are mean ± SEM and represent 4 RA patients from each group in four independent experiments. (B) Peripheral blood lymphocytes (PBL) from healthy controls and RA patients without and with DMARD therapy were obtained and stimulated overnight with PMA and ionomycin. The cytokines released in the supernatants were measured by luminex. PBL from RA patients who had successfully stopped DMARD use secreted significantly more IL-13 and IL-4 compared to those obtained from DMARD(+) RA patients while IL-10 demonstrated a similar trend. In line with our data originating from the MLR experiments, IFNγ levels were similar between groups. Bars are means ± SEM of 4 healthy controls and RA patients in each group. (C,D) $CD3^+CD25^-$ $CD127^+$ T cells were stimulated for 6 days with DCs from RA patients with a low DAS28 and either treated with DMARDs or not. After overnight incubation with medium alone, the presence of $CD4^+CD25^+FoxP3^+CD127^-$ regulatory T cells was determined. The decreased relative presence of T regulatory cells due to the pre-stimulation of $DC_{high\text{-}Fc\gamma RIIb}$ with LPS was completely reversed by the addition of IC to the DC cultures. In clear contrast, the addition of IC to LPS had no effect on T regulatory cell development when $DC_{low\text{-}Fc\gamma RIIb}$ were used. The percentage of $CD127^-$ $FoxP3^+$ regulatory T cells as part of the $CD4^+CD25^+$ subgroup of T cells is shown from a representative MLR in panel c. The combined results from three independent experiments ± SEM are shown in panel (D). First the $CD4^+CD25^+$ T cells were gated by using anti-CD4 and anti-CD25 antibodies. This subset of $CD4^+CD25^+$ T cells was further characterized by determining the level of expression of FoxP3 and CD127. Isotype matched antibodies were used to define marker settings. * indicates a p-value < 0.05.

The effect of IC on TLR4 signaling release is FcγRIIb dependent

There is a remarkably clear correlation (R^2 = 0.89, P = 0.001) between the level of FcγRIIb expression and the potential to inhibit TNF secretion upon LPS + IC stimulation by DCs in RA patients (Figure 5A). This strongly suggests that the level of FcγRIIb expression on DCs determines the level of inhibition of TLR4 responses instrumented by the addition of IC. By using a blocking antibody against FcγRIIb we confirmed that the inhibitory effect of IC on TLR4 signaling in $DC_{high\text{-}Fc\gamma RIIb}$ is fully dependent on FcγRIIb (Figure 5B).[15;16] In contrast, blocking FcγRIIa had no effect on IC mediated dampening of TLR4 dependent TNF-α

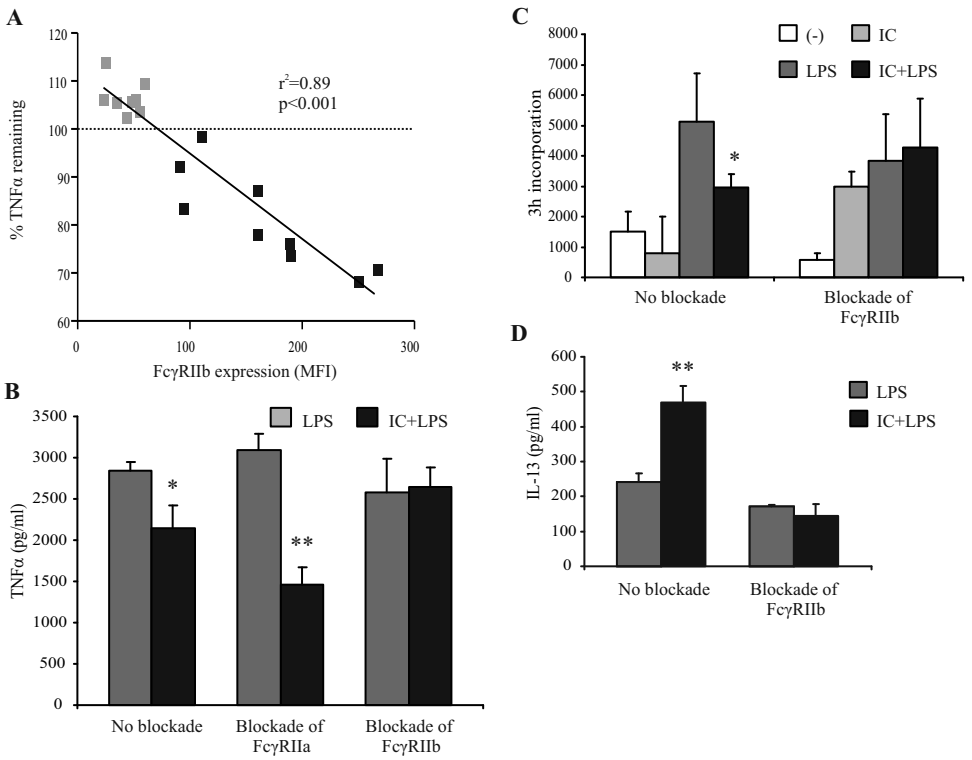

Figure 5. The modification of the TLR4 response by IC is solely mediated by the inhibitory FcγRIIb. (A) The capacity of IC to inhibit TLR4 mediated TNFα secretion is highly correlated with the expression of the inhibitory FcγRIIb on DCs. Grey are patients on DMARDs (n=8) and black are patients who had stopped DMARD therapy (n=9). (B) The inhibitory effect on the LPS-induced release of TNFα by IC on $DC_{high\text{-}Fc\gamma RIIb}$ could be completely reversed by the blockade of FcγRIIb. DCs with a high expression of FcγRIIb were cultured for 6 days, on day 6 the DCs were washed and the activating FcγRIIa and the inhibitory FcγRIIb were blocked with their respective antibodies. The DCs were subsequently plated in 24 wells plates, exposed to IC and after 5 minutes LPS was added to the wells. Supernatants were obtained 24 hours later. Data are representative of four individual experiments with in total five RA patients with similar results. Bars represent mean and SD of triplicate wells. (C) The inhibition of $CD3^+CD25^-$ T cell proliferation induced by LPS-activated $DC_{high\text{-}Fc\gamma RIIb}$ by the addition of IC (measured by 3H thymidine incorporation) was completely abolished by the prior blockade of FcγRIIb. Bars are mean ± SD representative of 3 independent experiments with $DC_{high\text{-}Fc\gamma RIIb}$ from 3 RA patients. (D) As for T cell differentiation, the increased secretion of IL-13 by T cells in MLR with $DC_{high\text{-}Fc\gamma RIIb}$ was abolished upon blockade of FcγRIIb. Mean and SD are representative for data from 3 independent experiments with $DC_{high\text{-}Fc\gamma RIIb}$ from 3 RA patients. For all figures * indicates a p-value < 0.05, ** indicates P –value <0.01.

secretion. The expression of co-stimulatory molecules (data not shown), inhibition of T cell proliferation (Figure 5C) and increased production of IL-13 by T-cells (Figure 5D) mediated by the addition of IC to $DC_{high\text{-}Fc\gamma RIIb}$ were fully abrogated by blocking FcγRIIb implying that the inhibitory FcRIIb was solely responsible for the inhibitory effect of IC on TLR4 immune responses.

The inhibitory effect of FcγRIIb is mediated via the PI_3K/Akt pathway

To further dissect the cross talk between FcγRIIb and TLR, we first explored the effect of inhibiting PI3K and Akt in our system since these are intricately involved in FcγR and TLR signaling. Inhibition of the PI3K/Akt pathway, with three separate PI_3K or Akt inhibitors, led to an increased release of TNFα upon LPS stimulation of DCs (Figure 6A). Extensive dose-response curves were performed to obtain the levels at which the inhibitors had their optimal effect (data not shown). In addition we found that both the PI_3K inhibitor as well as the Akt inhibitors fully abrogated the inhibitory effect of FcγRIIb on LPS mediated TNFα and IL-12p70 production by DCs (Figure 6B and 6C). Inhibition of the MAP kinase p38, PKCδ and Btk that also have been advocated to play a part in the signaling of either TLR or FcγR did not have any effect on the FcγRIIb inhibitory potential (Figure 6D and E).

Discussion

The present study establishes a novel role of the inhibitory FcγRIIb providing a general checkpoint of our immune system tuning TLR4 mediated immune activation. The ability of the immune system to distinguish self from non-self is seminal to the ability to protect the host from the detrimental effects of invading pathogens. The mechanisms that orchestrate these properties operate at discrete checkpoints involving central and peripheral tolerance. Given the complexity of these processes it is not surprising that central tolerance is frequently incomplete. Therefore, inhibitory signaling has emerged as a critical feature of peripheral tolerance. The role of TLR in the initiation of immunity is extensively shown. However, the counter-regulatory mechanisms that control this inflammation and thus should prohibit chronic inflammation and uphold tolerance are not yet identified. The FcγRIIb mediated inhibitory capacity on TLR signaling that we show here provides a novel mechanism on how the immune system exploits FcγRIIb as a counter-regulatory mechanism to limit inflammation in order to prohibit exaggerated damage to host tissues.

In arthritis, it has been irrefutably shown that the balance between activating and inhibitory FcγR is seminal in controlling both susceptibility to and the severity of the disease.[5;10;11;17;34;46-49] Here we demonstrate that the expression of FcγRIIb was insufficient to inhibit TLR4 mediated immune activation in all but RA patients able to suppress disease activity in the absence of anti-rheumatic drugs, suggesting that the high FcγRIIb expression observed in these latter patients might underlie their state of disease “remission”. Further evidence for the existence of such deranged FcγRIIb system in RA comes from the use of intravenous immunoglobulins (IVIG) that have been applied successfully to treat a variety

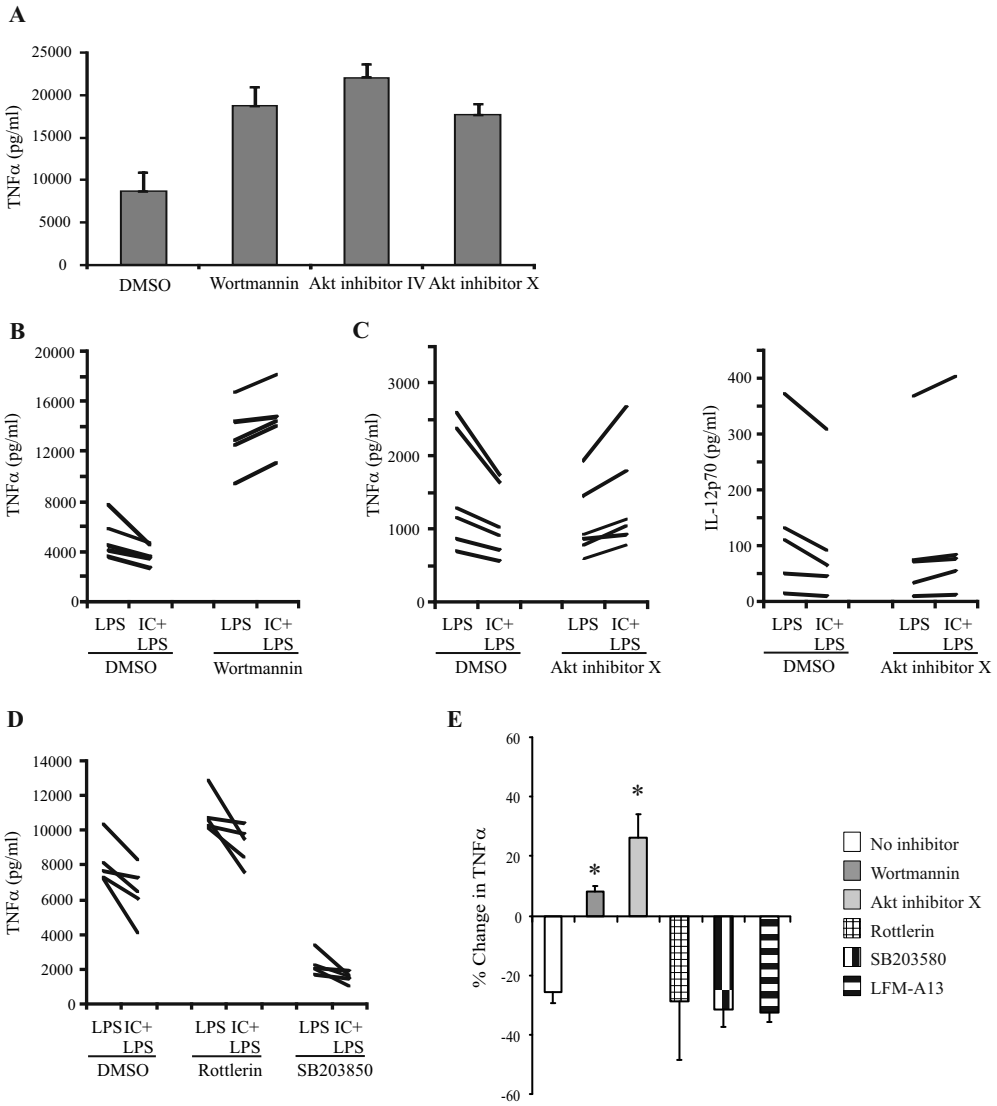

Figure 6. The PI3K/Akt pathway is crucial in the inhibition of TLR4 signalling by FcγRIIb. (A) DC from a healthy control were stimulated with LPS in the presence of the PI3K inhibitor Wortmannin (0.1 μM) or in the presence of the Akt inhibitors IV (0.1 μM) or X (0.5 μM). Data are mean and SD of duplicate wells representative for data from 3 independent experiments. (B-D) LPS-induced TNFα secretion with and without co-stimulation of $DC_{high\text{-}FcγRIIb}$ by IC in the absence or presence of inhibitors for PI3K (Wortmannin, 0.1 μM), Akt (Akt inhibitor X, 5 μM), PKCδ (Rottlerin, 10 μM) and p38 MAP kinase (SB203580, 20 μM). Results are from separate $DC_{high\text{-}FcγRIIb}$ from 5-6 DMARD(-) RA patients in each figure. (E) The percentage change in LPS-induced TNFα secretion with and without co-stimulation of $DC_{high\text{-}FcγRIIb}$ by IC was measured by luminex technology in the absence or presence of inhibitors for PI3K, Akt, PKCδ, p38 and Btk (LFM-A13, 50 μM). Results are the mean and SEM of 3-6 separate experiments with $DC_{high\text{-}FcγRIIb}$ of 3-6 DMARD(-) RA patients. * indicates a p-value < 0.05 compared to $DC_{high\text{-}FcγRIIb}$ stimulated in the absence of an inhibitor.

of immune-related diseases such as immune thrombocytopenic purpura, SLE, Kawasaki disease and Guillain-Barre syndrome. Whereas the mechanisms that exert the effects of IVIG remain enigmatic, binding to and up regulation of the inhibitory FcγRIIb is advocated ([50] reviewed in [51]). In this line it is remarkable that the administration of IVIG to RA patients has been disappointing.[52;53] Recently, Kaneko et al. provided evidence for the role of sialylation of IC as another mechanism underlying the distinct effect of IC observed during inflammation and health.[54] Our data underscores the existence of such mechanism since pegylated IC obtained from the synovial fluid, thus originating from a more inflammatory environment, exerted significant less inhibitory capacity compared to pegylated IC obtained from the peripheral blood of RA patients. However, the fact that these former IC are still able to inhibit TLR4 responses indicates that the inhibitory capacity of the FcγRIIb system is superior over the level of sialylation.

The precise pathways underlying FcγRIIb mediated inhibition of ITAM are extremely complex and not completely elucidated let alone the mechanisms by which it might inhibit other immune receptors such as TLR4. Prime suspects are PI3K and it's counteracting opponent SHIP. Whereas PI3K is known to increase PIP3 levels and thereby the level of phosphorylated Akt SHIP degrades PIP3 and reduces the level of Akt phosphorylation. Inhibiting Akt or PI3K led to an increase in TLR4 induced cytokine production by DCs in our experimental set-up. Based on our observations we propose a mechanism centered on the PI_3K/Akt-SHIP balance by which FcRIIb might inhibit TLR4 signaling. In our conceptual framework FcγRIIb activation leads to the recruitment and phosphorylation of SHIP to the cell membrane decreasing the level of SHIP available for enhancement of the TLR4 pathway. Increased levels of phosphorylated Akt might thus arise due to a shifted balance towards PI_3K, instead of SHIP, closely associated with the TLR4 signaling cascade, leading to decreased LPS mediated DC activation. The likely massive presence of phosphorylated SHIP near FcγRIIb in $DC_{high\text{-}FcγRIIb}$ activated by IC thus potentially increases the PI_3K/Akt inhibitory signal leading to a decrease in TLR4 mediated cytokine production. Most likely however, this proposition is still an oversimplified scheme of the signaling events taking place. SHIP for example has been found to bind to adaptor molecules belonging to the family of Dok proteins which were first identified as substrates for the p210(bcr/abl) oncoprotein and are implicated in inhibitory signaling.[55-57] The function of the Dok proteins, which are phosphorylated by Lyn, has been linked to the facilitation or sustainment of the activation of SHIP and the inhibition of the ras pathway.[58;59] In addition, ligation of TLR4 by LPS has been shown to rapidly induce the phosphorylation and adaptor function of Dok proteins and the absence of Dok proteins resulted in the elevated activation of Erk and hyperproduction of TNFα.[60] Whether this inhibitory effect of the Dok proteins on TLR4 signalling is linked to their role as adaptor molecules needs further investigation. It does however demonstrate that the events taking place intracellularly in our experiments are far more intricate than we have tried to contemplate. The changes in intracellular TLR4 signaling induced by FcγRIIb leads to a decreased release of the pro-inflammatory cytokines TNFα, IL-6 and IL-12p70 as well as an increased capability of the DCs to induce Tregs and Th2 cells. These changes are seen as important events in the resolution of Th1 mediated inflammation. Intriguingly,

the absence of SHIP in mice results in the increased presence of alternatively activated macrophages as well as a spontaneous allergic inflammation in the lungs characterized by elevated levels of the Th2 cytokines IL-13 and IL-4.[61] These findings make it even more tempting to speculate that FcγRIIb signaling is designed to induce alternatively activated DCs capable of dampening inflammation.

DCs from patients having quiescent disease display a more tolerogenic phenotype as compared to those from RA patients having active disease, which is witnessed by the lower level of cytokine secretion, higher levels of Foxp3 expressing T cells and higher production of Th2 cytokines by T cells primed by these DCs. These observations are in keeping with that of Dhodapkar et al. demonstrating that FcγRIIb is crucial to keep DCs in immature state in steady state conditions.[16;52] As we show that healthy individuals had circulating IC, which exerted an inhibitory capacity comparable to that seen by RA IC, these findings suggest that circulating immune complexes orchestrate the $DC_{high\text{-}FcγRIIb}$ tolerogenic phenotype by a constant down tuning of TLR mediated immune activation. As anticipated, healthy individuals did not express FcγRIIb at high levels potentially mirroring their steady state situation. These data further substantiate that FcγRIIb is designed to tune immune responses, restoring steady state conditions. Presumably, at some stage of immune activation, pathways must be turned on that lead to a clear shift in FcγR expression toward the inhibitory subtype. The pathways that regulate the expression of FcγR remain obscure. Although, IL-13/IL-4 with and without the combination of IL-10 has been demonstrated to skew the FcγR balance toward the inhibitory subtype, whereas IFN-γ strongly shifts this balance toward activating FcγR. Although, we have previously shown that the regulatory capacity of IL-13 is lost in RA patients, the high levels of FcγRIIb observed in DMARD (-) RA patients could not be explained by IL-13 or IL-10 since blocking these mediators during culture of DCs from RA patients with quiescent disease did not show any effect ([63] data not shown). In addition, the expression of FcγRIIb seen in these patients reached such extra-ordinary high levels that they could not be reached by the addition of these mediators to DCs from healthy controls. Thus, these data indicate that other mechanisms must be responsible for the regulation of FcγRIIb expression. The identification of such pathways is likely to result in the delineation of the processes underlying the deranged up-regulation of FcγRIIb that was found to be specific for RA patients. This, in turn, would significantly contribute to the development of therapeutic targets that are specifically designed to act on the defective pathway that underlies the chronic course of RA, a current unmet need in the treatment of this condition.

Here we show for the first time that, taken together, most RA patients have a deranged expression of the inhibitory FcγRIIb, rendering them incapable of controlling TLR-mediated immune activation. Our data strongly suggest that the up-regulation of FcγRIIb expression opens novel therapeutic avenues for the treatment of RA and other autoimmune conditions where TLR signaling is implicated.

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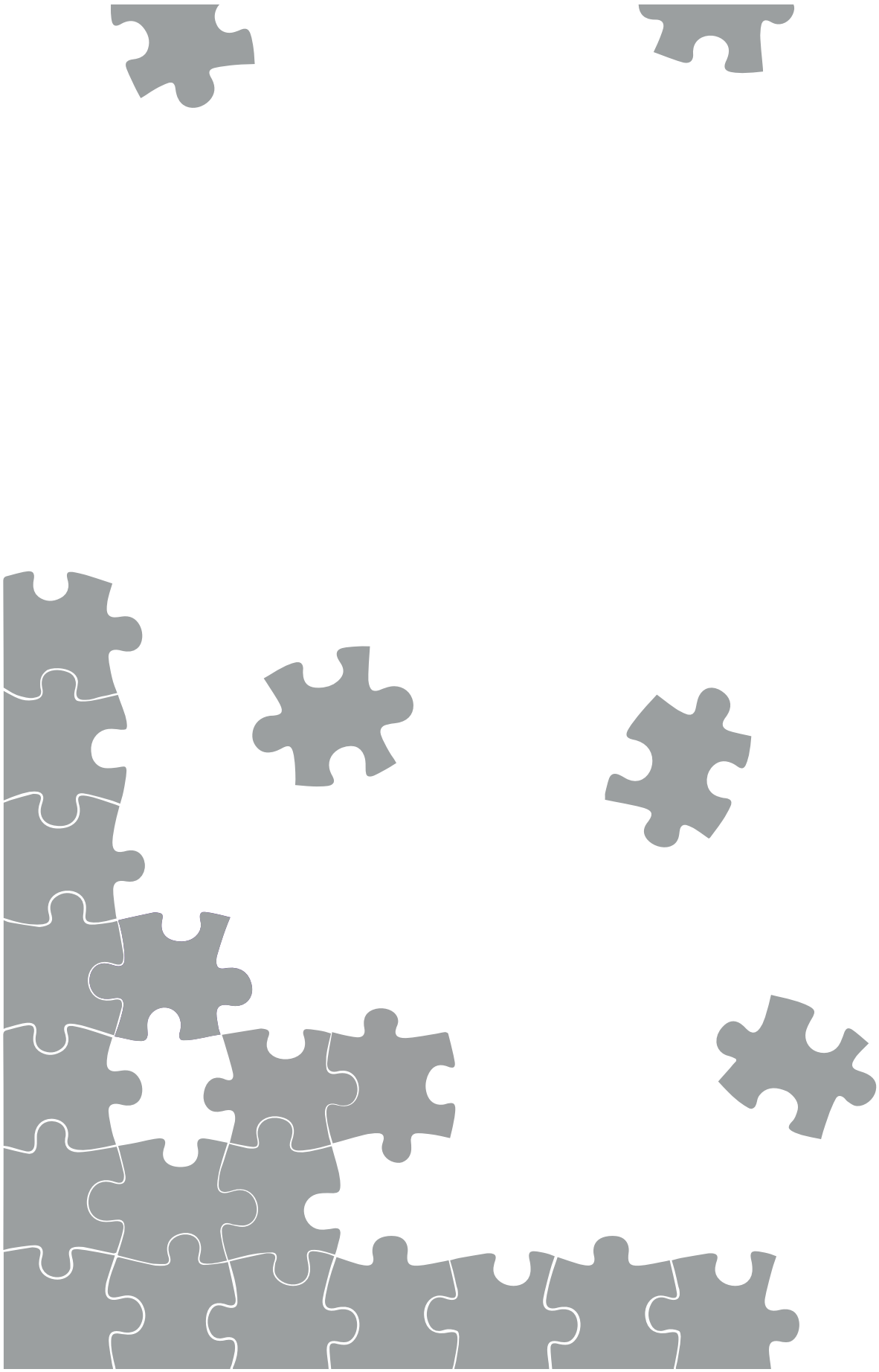

3

Fc gamma receptor IIb on GM-CSF macrophages controls immune complex mediated inhibition of inflammatory signals

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3

In rheumatoid arthritis (RA) macrophages play a major role in amplifying synovial inflammation. Important activating signals are those induced by Toll-like receptor (TLR) ligands and by activated T cells. The balance between activating and inhibitory Fc gamma receptors (FcγRs) on macrophages might be crucial in modulating these inflammatory responses. The purpose of this study was to determine FcγR expression on pro- and anti-inflammatory macrophages (gmMϕ and mMϕ, respectively) and identify functional consequences on immune complex uptake and macrophage activation. Human monocytes were isolated and differentiated into gmMϕ and mMϕ. A full FcγR characterization of both macrophage subtypes was performed and uptake of fluorescent immune complexes (ICs) was determined. FcγRIIb isoforms were determined by qPCR. Macrophages were stimulated via different TLRs or cytokine activated T cells in the presence or absence of ICs and cytokine production was determined. Blocking studies were performed to look into the pathways involved. mMϕ expressed high levels of the activating FcγRIIa and FcγRIII and low levels of the inhibitory FcγRIIb, while the FcγR balance on gmMϕ was shifted towards the inhibitory FcγRIIb. This was accompanied by a clear increase in FcγRIIb1 mRNA expression in gmMϕ. This resulted in higher IC uptake by mMϕ compared to gmMϕ. Furthermore, FcγR-mediated stimulation of gmMϕ inhibited TLR2, 3, 4 and 7/8 mediated cytokine production via FcγRIIb and PI3K signaling. In addition, gmMϕ but not mMϕ produced TNFα upon co-culture with cytokine activated T cells, which was reduced by IC binding to FcγRIIb. The latter was dependent on PI3K signaling and COX2.FcγR expression patterns on gmMϕ and mMϕ are significantly different, which translates in clear functional differences further substantiating FcγRIIb as an interesting target for inflammation control in RA and other autoimmune/ inflammatory diseases.

Introduction

One of the major pathways underlying the pathogenesis of rheumatoid arthritis (RA) is the aberrant production of inflammatory cytokines by macrophages. In the arthritic joint, macrophages are one of the main effector cells present and their levels correlate with disease activity and joint destruction.[1;2] Their levels are mainly associated with inflammatory cytokines such as TNFα and interleukin (IL) 1β, and could be sustained by factors like granulocyte-macrophage colony-stimulating factor (GM-CSF), present in the RA synovial joint.[3-5] Multiple pathways are proposed to play a role in macrophage activation in RA. One mechanism inducing cytokine production by RA macrophages is the triggering of Toll-like receptors (TLRs). Many endogenous TLR ligands have been found in an arthritic joint, such as GP96 and SNAPIN, which activate cells via TLR2, small heat shock protein B8 that can activate TLR4, and self-RNA from damaged cells which is likely to stimulate macrophages via TLR3 or TLR7/8.[6-10] Blocking antibodies against these TLRs reduce spontaneous cytokine production by RA synovial tissue cultures, confirming they are not only present in the arthritic joint but also contribute to the abundant cytokine production seen in RA.[10-12]

Another pathway mediating synovial macrophage activation is by direct interaction with activated T cells. Cytokine activated T cells resemble RA synovial T cells in their contact-dependent effector function and activation phenotype.[13;14] These cells can be cultured from peripheral blood lymphocytes in the presence of IL-2, IL-6 and TNFα (cytokine activated T cells, Tck) and induce an unbalanced, inflammatory cytokine response from monocytes.[14]

Another component present in many RA patients are auto-antibodies. These can form immune complexes (IC) and especially when deposited in tissues they can activate macrophages. Soluble ICs can have cell activating but also inhibitory effects, as is emphasized by IVIg treatment.[15] An important deciding factor for the cellular response to ICs is the balance of activating and inhibitory Fc gamma receptors (FcγRs).

The FcγR system consists of the activating FcγRI, FcγRIIa and FcγRIII that trigger cell activation via an immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic domain and the inhibitory FcγRIIb that signals via an immunoreceptor tyrosine-based inhibition motif (ITIM).[16] As the only inhibitory FcγR, FcγRIIb is an important brake on the immune system by inhibition of cell activation via the activating FcγRs on a wide array of cells and inhibition of the B cell receptor. FcγRIIb has two major isoforms, namely FcγRIIb1 and FcγRIIb2, which differ in their capabilities to mediate endocytosis and in their distribution on immune cells.[17-20] FcγRIIb1 predominates in B cells, while FcγRIIb2 is the major isoform in myeloid cells. We and others have previously shown that IC binding to FcγRIIb can also inhibit TLR4 signaling.[21;22] In our previous report, only RA patients that could control their disease activity without the need of anti-rheumatic drugs had high FcγRIIb levels on their dendritic cells (DC) and were capable of this inhibition.[21] This supports an important regulatory role for FcγRIIb in controlling inflammation in RA.

Since proinflammatory macrophages are important in the pathogenic process in RA and there is no data on the expression and function of the inhibitory FcγR on such macrophages we aimed to delineate the expression of FcγR receptors on homeostatic M-CSF macrophages (mMϕ) and inflammatory GM-CSF macrophages (gmMϕ). We

determined the complete FcγR balance on gmMϕ and mMϕ and tested whether functional differences were attributed to this. We mainly focused on combined FcγR triggering with macrophage activation via a range of TLRs implicated in RA pathology or activated T cells and found that FcγRIIb was able to dampen both TLR and Tck induced TNFα production when this inhibitory FcγRIIb was highly expressed.

Materials and methods

Ethics statement

The study protocol was approved by the medical ethical committee of the Radboud university medical center (Nijmegen, the Netherlands) and the University Medical Center Utrecht (Utrecht, the Netherlands) and all healthy volunteers gave their written informed consent. All experiments were performed in accordance with the Helsinki Declaration.

Culture of monocyte-derived gmMφ and mMφ and Tck cells

Peripheral blood mononuclear cells were isolated from venous blood of healthy volunteers using density-gradient centrifugation over Ficoll (GE Healthcare, Uppsala, Sweden). Monocytes and CD4+ T cells were obtained using CD14 and CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). gmMϕ and mMϕ were generated by culturing monocytes in the presence of GM-CSF (800 U/ml; R&D Minneapolis, Minnesota, USA) or macrophage colony-stimulating factor (M-CSF, 25 ng/ml; R&D) for 6 days. Macrophages were cultured in 6 well plates (Corning, New York, USA) with $1{,}0x10^6$ cells per well in 2 ml medium (RPMI-1640 Dutch modification (Gibco Life Technologies, Grand Island, New York, USA)) supplemented with 10% FCS, antibiotic-antimycotic and L-glutamine (Gibco Life Technologies). Culture medium with the same supplements (1 ml) was added at day 3 and the cells were harvested at day 6. In parallel, autologous CD4+ T cells were cultured in complete medium with recombinant human IL-2 (25 ng/ml), IL-6 (100 ng/ml) and TNFα (25 ng/ml) at $2x10^6$ cells/ml for 6 days (all from R&D).

Phenotypical analysis

Using standardized flow cytometry protocols as described previously gmMϕ and mMϕ were phenotyped using antibodies against CD14, CD163 (both BD Biosciences, Franklin Lakes, New Jersey, USA) and MHC-II DR/DP (clone Q1514).[23] FcγR expression was determined with antibodies against FcγRI (CD64, PE labeled, clone 10.1; Dako, Glostrup, Denmark), FcγRIII (CD16, PE labeled, clone DJ130c; Dako), clone IV.3 which preferentially binds to FcγRIIa (StemCell Technologies, Vancouver, Canada) and the FcγRIIb specific antibody 2B6 (Alexa488 labeled; MacroGenics, Rockville, Maryland, USA). Expression of unlabeled markers was visualized via a FITC labeled goat-anti-mouse secondary antibody. Cell fluorescence was measured on a FACS Calibur (BD) and analyzed using Flowjo software for the mean fluorescence intensity (MFI) and the proportion of positive cells relative to cells stained with the appropriate IgG isotypes.

RNA isolation and qPCR

Total RNA was extracted in 0.5 ml of TRI-reagent and treated with DNase to remove genomic DNA before being reverse-transcribed into cDNA. qPCR was performed on a Quantstudio 12K Flex (Life Technologies) with SYBR Select Master Mix (Life Technologies), 7.5 ng cDNA and a primer concentration of 0.5 µM in a total volume of 15 µl. qPCR signals were quantified by comparing the cycle threshold value (Ct) of the gene of interest of each sample with the Ct value of the reference gene GAPDH (ΔCt). Results were deployed as relative expression ($2^{-\Delta Ct}$). The following primers were used: GAPDH forward ATGGGGAAGGTGAAGGTCG, reverse GGGGTCATTGATGGCAACAATA; FcγRIIb1 forward GGATTTCAGCTCTCCCAGGAT, reverse CGGTTCTGGTCATCAGGCTC; FcγRIIb2 forward AAAGCGGATTTCAGCCAATC, reverse CAAGACAATGGAGACTAAATACGGT.

Phagocytosis and binding assay

Phagocytosis assays were performed with fluorescently labeled ICs, prepared as previously described.[24] Macrophages were incubated with FITC-labeled ICs (50 µg/ml) for 30 min at 4°C and 37°C to determine binding and uptake, respectively. Unattached ICs were washed away before determining binding and uptake by flow cytometry. To determine IC uptake extracellular attached FITC-IC was quenched by adding trypan blue (1/40 diluted in PBS, Sigma-Aldrich) to the samples just before determining the IC uptake by flow cytometry.

Stimulation of monocyte-derived macrophages

At day 6 macrophages were harvested and plated in a concentration of $0.5x10^6$ cells/ml in 96 well culture plates (100 µl). Immune complexes used in this study were prepared by heating human IgG (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS at 63 °C for 30 minutes (heat-aggregated immune complexes (IC)), as previously described[25] and were used in a concentration of 50µg/ml. Macrophages were stimulated or not with ICs for 15-30 minutes before the addition of TLR agonists for 20 hours. The following concentrations of TLR agonists were used: Pam3CSK4 (5 µg/ml, EMC Microcollections, Tübingen, Germany), Poly(I:C) (25 µg/ml, Invivogen, San Diego, California, USA), LPS (100 ng/ml, E. coli 0111:B4, Sigma-Aldrich) and R848 (2 µg/ml, Invivogen) for TLR2/1, 3, 4 and 7/8 respectively. The LPS was double-purified to remove any contaminating proteins as described previously.[26] Macrophages were also co-cultured with cytokine-activated T cells for 20 hours in a 1:5 ratio in the presence or absence of IC (50µg/ml) prestimulation for 1 h.

FcγRIIb blocking was performed by 30 min incubation of mϕ-1 with 10 µg/ml 2B6 antibody (MacroGenics) or an isotype control at 4°C before stimulation with ICs and LPS or Tck. In other experiments gmMϕ were treated with the PI3K inhibitors Wortmannin (0.1 µM; Calbiochem, San Diego, California, USA) or LY294002 (10 µM; Calbiochem) or COX2 inhibitor I (20µM; Calbiochem) for 1 h at 37°C before stimulation.

Measurement of cytokines in culture supernatants

Levels of IL-10 and TNFα were measured in the supernatants using commercially available kits (Millipore, Billerica, Massachusetts, USA) according to the manufacturer's instructions.

Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad, Hercules, California, USA).

Statistical analysis

Differences were analyzed using paired Student's t-tests. P values less than 0.05 were considered significant.

3

Results

gmMφ express high levels of the inhibitory FcγRIIb, while mMφ express higher levels of the activating FcγRIIa and FcγRIII

Monocytes were cultured into pro- and anti-inflammatory gmMϕ and mMϕ in the presence of either GM-CSF or M-CSF. To confirm the phenotype of our gmMϕ and mMϕ we first analyzed their expression of CD14, CD163 and MHC-II. In line with literature the expression of CD14 and CD163 was higher on mMϕ, while MHC-II was increased on gmMϕ (Figure 1A).[21] We further evaluated the expression of activating and inhibitory FcγRs. The monomeric IgG receptor FcγRI was similarly expressed in gmMϕ and mMϕ, while FcγRIII expression was highly increased on mMϕ compared to gmMϕ (regarding both MFI and percentage of positive cells, Figure 1B). Investigating the activating and inhibiting subtype of FcγRII separately, we observed a marked difference between the gmMϕ en mMϕ. Whereas the activating FcγRIIa is expressed higher on mMϕ, expression of the inhibitory FcγRIIb was increased on gmMϕ (Figure 1B). More specifically, the FcγRIIb/FcγRIIa ratio was 1.56 for gmMϕ and 0.48 for mMϕ. Thus, gmMϕ display an FcγR balance favored towards the inhibitory subtype whereas the opposite was found on mMϕ. FcγR expression was also determined on gmMϕ and mMϕ from some RA patients, which showed a similar FcγR distribution compared to healthy controls (data not shown). In vivo in situations in which GM-CSF is produced most likely also a basal level of M-CSF will be present. To determine the effect of the combination of both growth factors on macrophage development we also cultured macrophages with GM-CSF and M-CSF. This resulted in a phenotype similar to gmMϕ, suggesting GM-CSF is dominant over M-CSF, at least regarding FcγR expression (data not shown).

We further aimed to differentiate between the two major FcγRIIb isoforms, FcγRIIb1 and FcγRIIb2. Since the extracellular domain of these isoforms is the same we used qPCR to determine the expression of these variants in mMϕ and gmMϕ. Using isoform specific primers we found that FcγRIIb2 expression was similar in both macrophage subtypes, while FcγRIIb1 expression was significantly increased in gmMϕ compared to mMϕ (Figure 1C). gmMϕ thus have an increased expression of the FcγRIIb variant usually more predominant in B cells which is less capable of mediating endocytosis.

The capacity to take up ICs is an important function of macrophages. To evaluate the functionality of the altered aforementioned FcγR balance, we investigated whether the gmMϕ and mMϕ display a different binding and uptake capacity of ICs. mMϕ show a significantly increased potential for both binding and uptake of ICs compared to gmMϕ

(Figure 1D). This is fitting with the enhanced expression of FcγRIIa and FcγRIII on mMϕ, which have a higher affinity for most IgG isotypes compared to FcγRIIb[27] and the increased expression of the non-endocytosing FcγRIIb1 on gmMϕ.

ICs inhibit TLR induced cytokine production by gmMφ but not by mMφ.

To further evaluate the functional consequences of the differential FcγR expression on gmMϕ and mMϕ and to test whether ICs can also inhibit TLR4 signaling in human macrophages that express high FcγRIIb levels, gmMϕ and mMϕ were stimulated with TLR

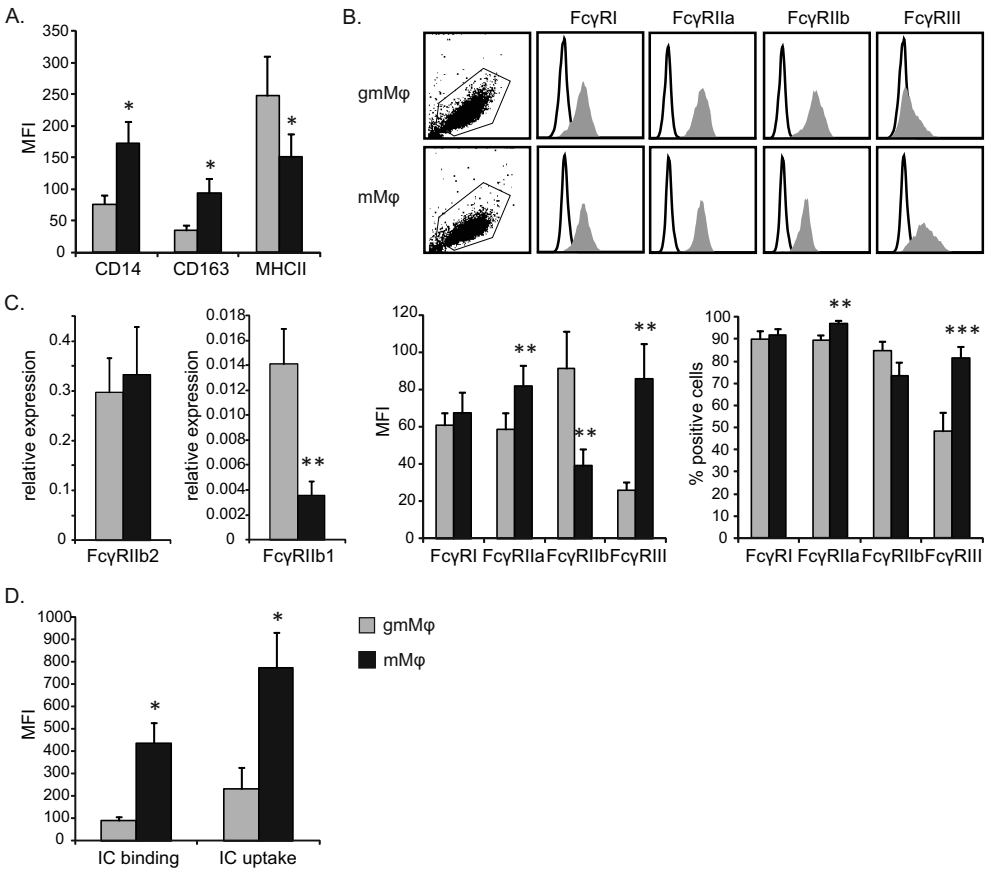

Figure 1. gmMϕ express high FcγRIIb levels, while mMϕ express more FcγRIIa and FcγRIII. Monocytes were cultured for 6 days with GM-CSF or M-CSF into gmMϕ and mMϕ respectively. Expression of CD14, CD163 and MHC-II (A) and all FcγRs (B) was determined by flow cytometry. (B) For FcγR expression, representative FACS plots are shown together with bar graphs showing mean (and SEM of) MFI and percentage of positive cells from 11 donors. Histograms show isotype control (thin line) and FcγR specific antibody (solid grey). (C) mRNA expression of FcγRIIb1 and FcγRIIb2 were determined by qPCR and plotted as relative expression compared to GAPDH. Bars are mean and SEM of 7 donors. (D) gmMϕ and mMϕ were incubated with FITC-labeled ICs (50 µg/ml) for 30 min at 4°C for binding and 37°C for uptake. IC uptake was determined in the presence of trypan blue. Bars are mean and SEM from 4 donors. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to mϕ-1.

ligands in combination with ICs. mMϕ and gmMϕ were first stimulated with ICs alone to determine the effect of differential FcγR expression on these cells on IC induced cytokine production. In mMϕ ICs induced significant but low levels of TNFα and IL-10 production, while there was no clear cytokine induction observed in gmMϕ (Figure 2A). Upon TLR 4 stimulation with LPS gmMϕ produced high levels of TNFα and low levels of IL-10, while mMϕ were marked by their relatively high IL-10 production and low production of TNFα which corroborates the literature (Figure 2B).[5;28] After co-stimulation with ICs, gmMϕ were able to significantly attenuate TNFα production compared to those stimulated with LPS alone, while IL-10 production was relatively unaffected (Figure 2B). In contrast, but in line with our observations on FcγR expression, the addition of ICs to LPS did not result in inhibition of TLR4 mediated cytokine production in mMϕ. In fact, mMϕ produced significantly more IL-10 after co-stimulation with ICs.

To determine if this inhibitory pathway can also affect cytokine induction by other TLR ligands, similar experiments were performed with specific ligands for TLR2/1 (Pam3CSK4), TLR3 (Poly(I:C)) and TLR7/8 (R848). These experiments learned that the inhibitory effect of ICs on TLR signaling by gmMϕ is not limited to TLR4, but also extends to TLR2/1, TLR3 and TLR7/8 (Figure 2C), further substantiating the pivotal role of the FcγR balance in the regulation of cell activation. Again, IL-10 production by gmMϕ was not clearly affected by the presence of ICs and the inhibitory effect on TNFα production was not present in mMϕ (data not shown). To determine if ICs could also affect TLR induced cytokine production after TLR stimulation has already occurred, we performed a time course with addition of ICs from 2 hours prior to Pam3CSK4 till 2 hours after Pam3CSK stimulation. ICs were able to significantly modulate TNFα production during the whole time range, without significantly affecting IL-10 (data not shown). ICs can thus modulate TLR induced cytokine production before and after TLR triggering.

Immune complexes can inhibit gmMφ activation by activated T cells

Another important activator of RA synovial macrophages are cytokine activated T cells. We therefore evaluated cytokine production in co-cultures of Tck with gmMϕ or mMϕ. Tck induced a synergistic production of TNFα when co-cultured with gmMϕ (Figure 3A), while IL-10 is almost absent, resulting in an unbalanced proinflammatory response. The TNFα production by mMϕ after co-culture with Tck is much lower and not significantly different from mMϕ alone (Figure 3A). In contrast to mMϕ stimulation by TLR ligands, in co-culture with Tck also IL-10 production remained low. Thus, Tck mainly stimulate gmMϕ. To determine if ICs could inhibit Tck induced TNFα production when macrophages express high FcγRIIb levels, gmMϕ were stimulated with ICs and Tck. IC co-stimulation reduced the TNFα release by gmMϕ with approximately 50% upon Tck stimulation (Figure 3B). ICs can thus modulate both TLR and T cell induced gmMϕ activation.

The inhibitory effect of ICs is mediated via FcγRIIb and the PI3K pathway

To confirm whether the high FcγRIIb expression on gmMϕ was indeed responsible for the inhibitory effect of ICs on TLR and Tck induced signaling in these cells, we used a blocking

antibody against FcγRIIb. Blocking of FcγRIIb fully abrogated the inhibitory effect of ICs on both TLR and Tck induced TNFα production (Figure 4A and B).

In DCs our group has previously shown that the PI3K/Akt pathway is involved in the crosstalk between FcγRIIb and TLR4.[21] To determine if this pathway is also involved in the FcγRIIb effect on macrophages, we blocked PI3K signaling before stimulation of gmMϕ with ICs and LPS. The IC mediated inhibition of LPS induced TNFα production was abrogated in the presence of Wortmannin or LY294002, confirming the role of the PI3K pathway in FcγRIIb mediated TLR4 signaling inhibition in gmMϕ (Figure 4C and data not shown). Mice studies pointed towards an additional role for prostaglandin E2 in the inhibitory actions of FcγRIIb on TLR4 signaling.[22] To test this in the human setting we performed our experiments

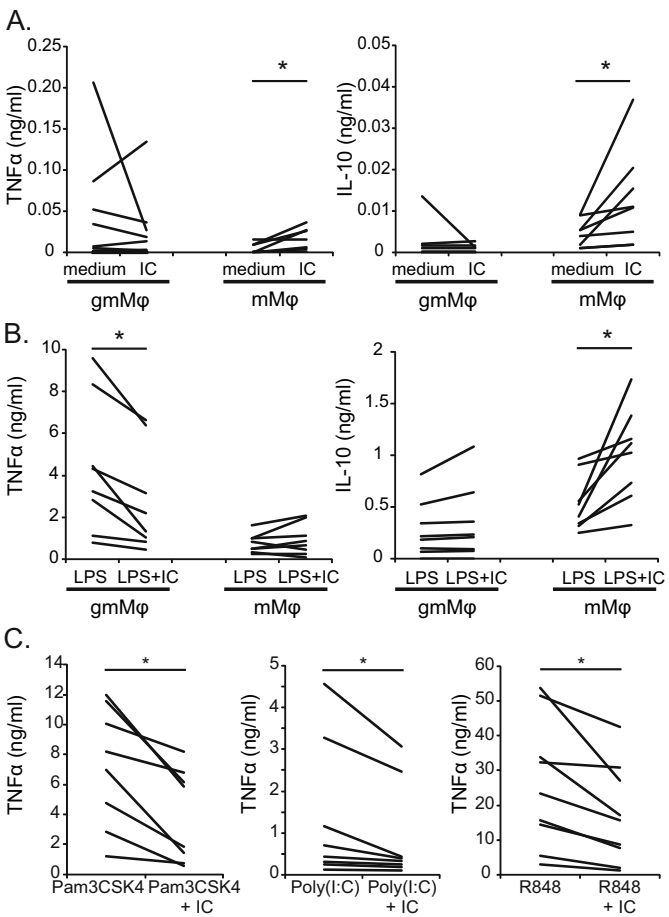

Figure 2. Immune complexes can inhibit TLR2, 3, 4 and 7/8 induced cytokine production in gmMϕ. gmMϕ and mMϕ were stimulated with ICs (50 μg/ml) (A), LPS (100 ng/ml) or LPS + ICs (B) and TNFα and IL-10 were measured in culture supernatants after 20 hours. (C) gmMϕ were stimulated with Pam3CSK4 (5 μg/ml), Poly(I:C) (25 μg/ml) or R848 (2 μg/ml) in the presence or absence of ICs. After 20 hours, supernatants were collected and analyzed for TNFα levels. Figure shows data of at least 7 donors for all stimulations. * $P < 0.05$ difference with and without IC.

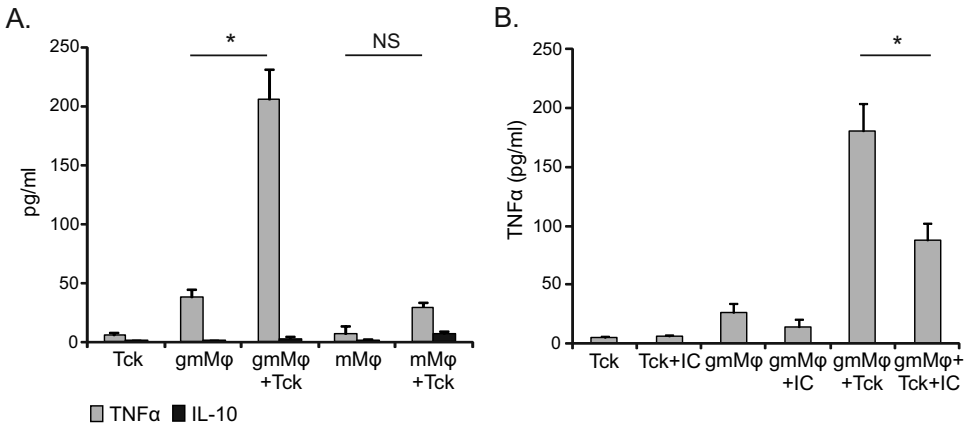

Figure 3. IC can inhibit T cell mediated macrophage activation in gmMϕ. gmMϕ or mMϕ and Tck were cultured from the same donor. (A) At day 6 the macrophages and Tcks were harvested, washed and cultured together in a ratio of 1:5. TNFα and IL-10 were measured in the supernatant after 20 hours. Bars are mean and SEM from 3 independent experiments. (B) gmMϕ were cultured alone or in a 1:5 ratio with Tck in the presence or absence of ICs (50 μg/ml) for 20 hours before collecting the supernatant. Bars are mean and SEM from 6 independent experiments. * $P < 0.05$, NS is not significant.

in the presence of a COX2 inhibitor. This did not affect the IC mediated inhibition of cytokine production by gmMϕ upon TLR4 stimulation (Figure 4C). To test if the same mechanism was involved in IC mediated blocking of other TLRs we tested FcγRIIb blocking and PI3K inhibition also for ICs in combination with TLR2/1 stimulation and this gave similar results as shown for TLR4 (data not shown).

To further determine if similar pathways are involved in inhibition of Tck induced macrophage activation we performed the Tck experiments in the presence of Wortmannin or a COX2 inhibitor. As for TLR activation a functioning PI3K pathway was necessary for the inhibitory effect of ICs, however IC mediated blocking of gmMϕ activation via Tck also appeared to be dependent on prostaglandin production, as is exemplified by the lack of IC mediated inhibition in the presence of a COX2 inhibitor (Figure 4D). IC mediated inhibition of gmMϕ TNFα production via both pathways is mediated via binding to FcγRIIb and involves the PI3K pathway. Prostaglandins are necessary for the effect of IC when combined with Tck, but not for TLR mediated cell activation.

Discussion

The present study shows that gmMϕ have a relatively high expression of FcγRIIb compared to the activating FcγRs, while this balance is shifted towards the activating FcγRs on mMϕ. gmMϕ secrete large amounts of TNFα upon stimulations relevant in RA, such as TLR ligands and cytokine activated T cells. Under these conditions inhibitory immune receptors, such as FcγRIIb, are crucial to counter-regulate the induced inflammatory responses to prevent excessive tissue damage. We show that the switched balance towards the inhibitory FcγRIIb

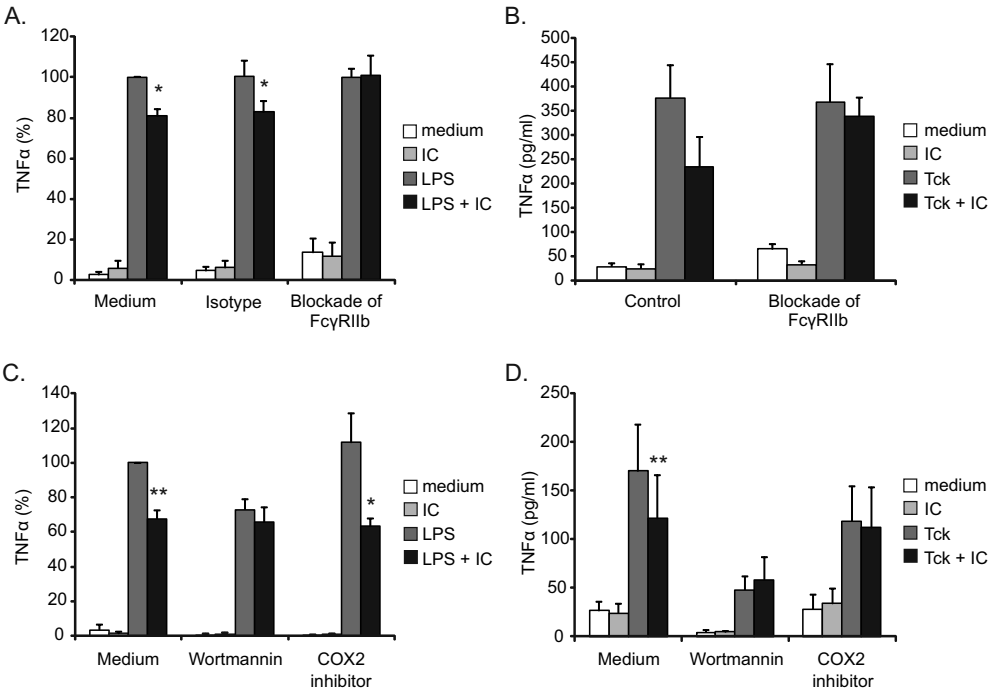

Figure 4. TLR and Tck inhibition by IC is mediated via FcγRIIb and PI3K. gmMϕ were pre-incubated for 30 minutes with the FcγRIIb specific blocking antibody 2B6 (10 μg/ml) or an isotype control before stimulation with IC (50 μg/ml) and LPS (100 ng/ml) (A) or Tck (B). gmMϕ were pre-incubated with Wortmannin (0.1 μM) or a Cox2 inhibitor (20 μM) for 1 hr before stimulation with IC and LPS (C) or Tck (D). 20 hour supernatants were collected to analyze TNFα levels. In the graphs showing TLR stimulation (A and C) the percentage of TNFα production is plotted with the LPS only stimulation set at 100%. In the Tck graphs (B and D) absolute values are shown. All graphs show the mean and SEM of at least 3 experiments. * $P < 0.05$

on gmMϕ is functionally relevant and can inhibit TNFα secretion from these cells induced by either TLRs or Tck in the presence of soluble ICs. This way it could function as a natural brake in an attempt to prevent excessive cytokine production and inflammation in RA.

The important regulatory role of FcγRIIb is extensively shown in animal models for autoimmunity (Reviewed in [29]). In this context it was shown that the transfer of RA but not healthy control serum can induce arthritis in FcγRIIb$^{-/-}$, but not in normal B6 mice.[30] This was caused by the IgG portion supporting a pathogenic role for IgG (auto) antibodies from RA patients and an important regulatory role for FcγRIIb. This model bypasses the effect of B cells because human IgG is passively transferred and it thus shows that FcγRIIb expression on other effector cells, including macrophages and DCs, is crucial to prevent autoimmune inflammation.

We demonstrated for the first time that FcγRIIb can inhibit cytokine induction by a wide range of TLRs, of which ligands have been found in the arthritic joint, including TLR2, TLR3, TLR4 and TLR7/8. In addition, FcγRIIb can also inhibit macrophage TNFα production induced by activated T cells. This way FcγRIIb can actively control two important

stimulatory pathways for macrophages in RA. Inhibitor studies taught us that normal PI3K signaling is necessary for FcγRIIb inhibition of both TLR and Tck induced cytokine release, while prostaglandins are only involved in the latter. Prostaglandins were postulated as an essential signaling molecule in FcγRIIb mediated inhibition of TLR4 in mouse macrophages.[22] However, in our human experimental setting prostaglandins are dispensable for FcγRIIb mediated inhibition of TLRs. In our cultures prestimulation of gmMϕ with ICs for only 15-30 minutes or up to 2 hours after TLR stimulation was enough to get inhibition, while in mouse macrophages the dependency on prostaglandins was demonstrated after 24 hour prestimulation with ICs.[22] So prostaglandins are not necessary for the direct inhibition of TLR4 signaling by FcγRIIb in humans, but might have additional inhibitory effects at later time points. This would be in line with the dependency of Tck inhibition on prostaglandin production, since the induction of TNFα in this setting is described to be much slower (peaks at 24 hrs.) compared to TLR stimulated TNFα induction (peaks at 4-8 hrs.).[31] However, much is still unknown about the pathways involved in macrophage activation upon interaction with Tcks. CD69, CD18 and CD49d on the Tck were shown to be involved in the induction of TNFα by monocytes upon Tck co-culture.[14] On monocytes/macrophages ICAM-1 and VCAM-1 might be involved as binding partners for CD18 and CD49d, respectively. Thus far, no direct interactions are known between these molecules and FcγR signaling. Hence, our work justifies more research focused at deciphering potential mechanisms involved in FcγRIIb inhibition on T cell mediated macrophage activation.

Interestingly, the increased membrane FcγRIIb expression on gmMϕ coincides with an increased expression of FcγRIIb1 on mRNA level. This suggests that FcγRIIb1 expression on gmMϕ could play a role in the inhibitory effects of ICs on these cells. Although the FcγRIIb isoforms have been repeatedly shown to have differential endocytosis potential[17-19], not much is known about possible differences in inhibitory signaling. It has been described that FcγRIIb1 is differently phosphorylated in B cells compared to FcγRIIb2 and might have additional inhibitory functions[19;32], but this has not been repeated by another group in macrophage cell lines[33]. Whether this could have functional implications for macrophage responses towards ICs needs to be further investigated.

Some groups have tried to identify the macrophage phenotype or the FcγR expression on macrophages from RA synovial tissue. FcγRII overall, FcγRIIb in particular and FcγRIII were all increased in RA synovium and correlated with the amount of macrophages present.[34;35] Looking at in vitro markers for gmMϕ and mMϕ it remains difficult to fully characterize the macrophages from the synovial tissue since they express markers representing both phenotypes.[36;37] Supported by our data the expression of FcγRIIb could be an additional marker for gmMϕ while FcγRIII marks mMϕ macrophages. The ratio between these two FcγRs could be a good discriminator between these macrophage subsets. The high expression of activating FcγRs by mMϕ, which are mainly described for their controlling/homeostatic functions, and the high expression of the inhibitory FcγRIIb on the more inflammatory gmMϕ might seem contradictory. However, because of the easily activated phenotype of gmMϕ regulatory mechanisms including those via FcγRIIb are crucial to prevent excessive inflammation and tissue damage. In addition, the capacity of mMϕ to

remove IC is facilitated by the high expression of FcγRs, preventing accumulation of IC and thereby preventing unwanted inflammatory responses. Next to that we show that small ICs in combination with TLR stimulation mainly increase IL-10 production in mMϕ and no major induction of TNFα was observed.

The in vivo situation is not as black and white as shown by this in vitro model, but knowledge about functional characteristics of these macrophage subsets combined with more detailed phenotyping of local macrophages, including differentiation between FcγRIIa and FcγRIIb in different diseases might give clues about the pathogenic processes going on in vivo. This could possibly give leads for therapeutic options to increase FcγRIIb expression on macrophages even further to induce a more inhibitory phenotype.

Conclusions

gmMϕ and mMϕ are characterized by a different FcγR balance, with high FcγRIIa and FcγRIII levels on mMϕ and increased FcγRIIb expression on pro-inflammatory gmMϕ. The relatively high FcγRIIb expression on gmMϕ makes these cells sensitive to IC mediated inhibition of proinflammatory cytokine release upon stimulation by TLR ligands and Tck, which can be an important feedback mechanism to prevent excessive inflammation. This shows that FcγRIIb mediated cell inhibition is not restricted to ITAM containing receptors or TLR4, but can broadly regulate immune responses. Specific targeting of FcγRIIb might therefore open novel therapeutic avenues for RA and other chronic immune mediated inflammatory disorders.

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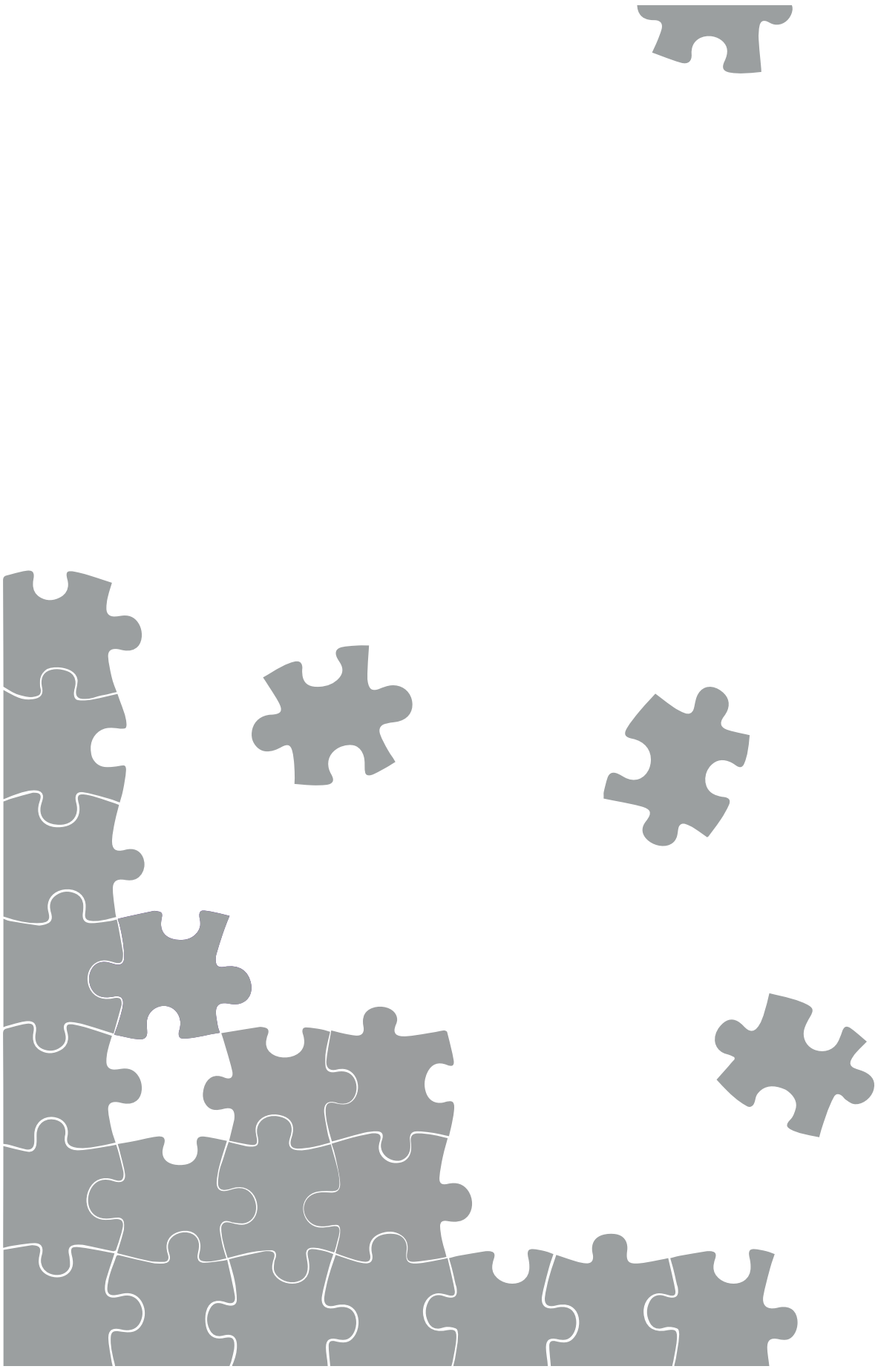

4

Abatacept modulates proinflammatory macrophage responses upon cytokine-activated T cell and Toll-like receptor ligand stimulation

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We investigated whether abatacept might reduce proinflammatory cytokine production by macrophages upon contact with cytokine activated T cells and/or stimulation with TLR ligands. Macrophages and cytokine-stimulated T cells (Tck) were added together in the presence of abatacept or a control Ig, with or without TLR ligands. The production of cytokines was determined by luminex. Abatacept reduced Tck-induced production of TNFα by macrophages. Tck and TLR ligands synergistically induced the production of proinflammatory cytokines by macrophages, especially IL-12p70. The production of IL-12p70 coincided with the production of IFNγ, which were both reduced in the presence of abatacept. Tck induce the production of TNFα by macrophages and facilitate the highly increased production of proinflammatory cytokines in the presence of TLR ligands. Abatacept was shown to potently suppress these pathways suggesting that its role may extend beyond antigen specific T cell mediated effector function.

Introduction

One of the major pathways underlying the pathological process in rheumatoid arthritis (RA) is the aberrant production of proinflammatory cytokines by macrophages. RA synovium is highly enriched with infiltrating $CD68^{+}CD163^{-}$ macrophages, and their decrease upon treatment is correlated with changes in disease activity.[1] Monocyte-derived $CD163^{-}$ macrophages differentiated in vitro in the presence of granulocyte-macrophage colony-stimulating factor (mϕ-1) exhibit the same characteristics as these infiltrating macrophages.[2] One crucial pathway mediating synovial macrophage activation is that driven by a direct interaction with activated T cells. In particular, cytokine-activated T cells recapitulate the functional properties of synovial T cells.[4] This depends on cell-cell contact between memory $CD4^{+}CD45RO^{+}$ T cells and macrophages via CD69, CD18 and CD49d and was independent of the production of soluble mediators.[3] $CD4^{+}CD45RO^{+}$ T cells are the main infiltrating cells in the pannus, underlining their pivotal role in the pathogenesis of RA. Brennan *et al*[4] demonstrated that human peripheral blood lymphocytes when cultured in the presence of interleukin (IL) 2, IL-6 and tumour necrosis factor α (TNF) (cytokine-stimulated T cells (Tck)), display the same characteristics as activated synovial T cells.

Another mechanism whereby cytokine production is induced in macrophages is the ligation of Toll-like receptors (TLRs). Many endogenous ligands for TLRs have been found in the arthritic joint, such as small heat shock protein B8 which activates TLR4 and self-RNA and self-DNA, which more than likely activate cells in the joint via TLR3 and TLR8, respectively.[5-8] Combinations of TLR ligands with T-cell-derived signals such as CD40 ligand or interferon γ (IFNγ) have been demonstrated to be potent in the induction of proinflammatory cytokine production by macrophages. Since Tck strongly resemble the T cells present in synovitis, it is of importance to know whether these cells are indeed capable of displaying synergistic effects with TLR ligands, thereby contributing to the deranged cytokine profile found in the RA synovial cavity.[9]

Abatacept (CTLA4 with an Fc tail) is a new agent in the armamentarium against RA. It blocks the instruction of T cells by antigen-presenting cells such as dendritic cells (DCs) and macrophages by binding to CD80 and CD86 on the antigen-presenting cells, thereby preventing binding to CD28 on the T cell, which is necessary for full T cell activation. We recently demonstrated in a murine system that abatacept also modulates follicular helper T cell maturation.[10] Memory $CD4^{+}CD45RO^{+}$ T cells possess significant intracellular reservoirs of CTLA4, which, upon stimulation, are transported readily to the cell surface. Activation of mouse DCs via CD80/86 by CTLA4-expressing T cells resulted in the production of IFNγ by the DCs.[11] It was recently suggested that CTLA4-Ig, via a direct effect on macrophages, reduces the ability of macrophages to produce cytokines upon the stimulation with concanavalin-A-activated Jurkat T cells.[12] We therefore investigated whether abatacept could influence the RA disease course by diminishing the proinflammatory cytokine production by macrophages induced upon contact with cytokine-activated T cells and TLR ligands.

Materials and Methods

Culture of monocyte-derived type I macrophages and Tck

Peripheral blood mononuclear cells were isolated from buffy coats from healthy blood donors by using density-gradient centrifugation over Histopaque (GE Healthcare, UK). The local Medical Ethics Committee approved the study protocol. Monocytes and $CD4^+$ T cells were obtained using CD14 or CD4 microbeads, respectively, and AutoMACS (Miltenyi Biotec). Mϕ-1 were generated by culturing isolated monocytes in the presence of GM-CSF (800 U/ml) for 6 days. Mϕ-1 were cultured in six-wells plates at a concentration of $5x10^5$ in 2 ml of culture medium (RPMI 1640, 10% fetal calf serum, penicillin/streptomycin and L-glutamine; Gibco, Grand Island, New York, USA). Fresh culture medium (1 ml) with the same supplements was added at day 3 after which the mϕ-1 were harvested at day 6. In parallel, $CD4^+$ T cells from the same donor were cultured in complete medium with recombinant human IL-2 (25 ng/ml), IL-6 (100 ng/ml) and TNFα (25 ng/ml) (all from R&D: Minneapolis, Minnesota, USA) at a density of $2x10^6$/ml for 6 days, after which they were also harvested.

Reagents

Abatacept was provided by Bristol-Myers Squibb. Chi L6 is a chimeric fusion protein consisting of the V region of murine L6 antigen, combined with a human IgG1 C region; it was used as a control fusion protein (control Ig) in these studies (Bristol-Myers Squibb: Uxbridge, UK). The proteins were used in a final concentration of 20 μg/ml.

Stimulation of monocyte-derived mφ-1

Harvested day 6 mϕ-1 were washed, counted and plated in a concentration of $5x10^4$ cells/well in 96-well culture plates. For cell contact assays after extensive washing, $2x10^5$ cytokine-activated T cells were added to the mϕ-1 in the absence or in the presence of abatacept or the control protein in a concentration of 20 μg/ml. In some experiments, Tck fixed in 4% paraformaldehyde were used. After 24 h, the supernatants were collected for analysis of cytokine levels. For the TLR stimulation, TLR agonists were added to mϕ-1 either in the presence or in the absence of Tck and/or abatacept/control protein and, after 24 h, the supernatants were harvested. Lipopolysaccharide (LPS) was used at a concentration of 1 ng/ml (*Escherichia coli* 0111:B4; Sigma-Aldrich: St Louis, MO, USA) and R848 was used in a concentration of 1 μg/ml (InvivoGen: San Diego, CA, USA).

Measurement of cytokines in culture supernatants

Levels of IFNγ, TNFα, IL-12p70, IL-6, IL-10, IL-13 and IL-17 in the supernatants were measured using commercially available kits (BioSource International, Camarillo, CA, USA) according to the manufacturer's instructions. Cytokine levels were measured and analysed with the Bio-Plex system (Bio-Rad: Hemel Hempstead, UK).

Statistical analysis

Differences were analysed with Students t tests to compare two stimulations. p Values less than 0.05 were considered significant.

Results

After 6 days of differentiation syngeneic mϕ-1 and Tck cells were harvested, counted and replated together in 96-wells plates for cell contact assays. Mϕ-1 readily produced TNFα upon exposure to Tck, while IL-6 and IL-10 were virtually not released (Figure 1A and data not shown). This effect was at least partly mediated by cell-cell contact, as paraformaldehyde fixed Tck, which are incapable of secreting cytokines, displayed stimulatory capacity, albeit lower than that observed in live cell cocultures (Figure 1B). Non-cytokine-stimulated $CD4^+$ T cells did not induce TNFα production by mϕ-1 (data not shown). Next, we evaluated whether abatacept might interfere with the production of TNFα by mϕ-1 upon contact with Tck. Blocking of CD80/86 by abatacept resulted in a markedly decreased production of TNFα, uncovering a novel manner by which abatacept might interfere in the RA disease

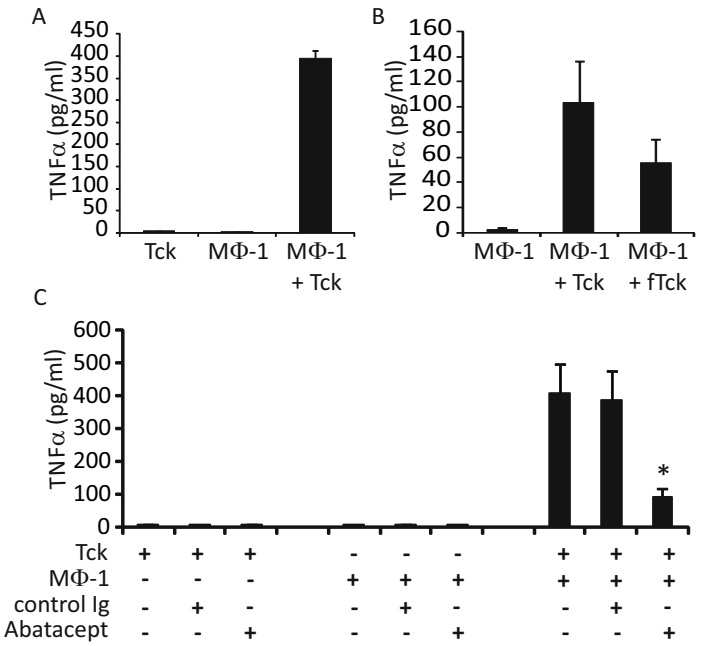

Figure 1. Mϕ-1 readily produce tumour necrosis factor α (TNFα) upon exposure to cytokine-stimulated T cells (Tck), which are inhibited by abatacept. (A) Mϕ-1 were stimulated with Tck for 24 h, after which the supernatants were measured for the presence of TNFα. Data are from triplicate wells and are representative of five individual experiments with similar results. Bars are mean and SD. (B) Mϕ-1 were stimulated with Tck or Tck fixed in paraformaldehyde for 24 h, after which the supernatants were measured for the presence of TNFα. Data are from triplicate wells and are representative of three individual experiments with similar results. Bars are mean and SD. (C) Mϕ-1 were stimulated with Tck with medium, a control Ig (20 μg/ml) or abatacept (20 μg/ml) for 24 h, after which the supernatants were measured for the presence of TNFα. Data are from five individual experiments. Bars are mean and SEM. * $p<0.05$ compared to control Ig.

process (Figure 1C). To additionally study the potential effect of Tck on TLR mediated stimulation of mϕ-1, we used ligands for TLR4 and TLR7/8, together with Tck, to determine whether they would cooperate in the production of proinflammatory cytokines by mϕ-1. Synergistic release of TNFα, especially of IL-6, was observed when mϕ-1 were exposed to a TLR4 or TLR7/8 ligand together with Tck, while non-cytokine-stimulated $CD4^+$ T cells did not induce any effect (Figure 2A,B and data not shown). Abatacept did not significantly reduce the production of TNFα or IL-6 under these circumstances (data not shown).

The IL-12p70/IFNγ axis appears to play an important role in the pathogenesis of RA[13;14]; therefore, we aimed to determine whether abatacept might additionally modulate this pathway. Stimulation of mϕ-1 with either TLR ligands or Tck alone induced the production of marginal levels of IL-12p70 and IFNγ. However, stimulation with either TLR4 or TLR7/8 ligands, in combination with Tck resulted in clear release of IL-12p70 and IFNγ (Figure 3A,B and data not shown). We additionally measured IL-13 and IL-17 (distinct Th2 and Th17 cytokines, respectively) and found that these cytokines were not produced (IL-17) or were produced at very low levels (IL-13) (data not shown). Blockade of CD80/86 by abatacept resulted in a significant reduction in the production of IL-12p70 as well as IFNγ (Figure 3C,D). Abatacept had no effect on cytokine production induced by TLR ligands alone, providing evidence that the effect of abatacept is not due to inhibitory signalling

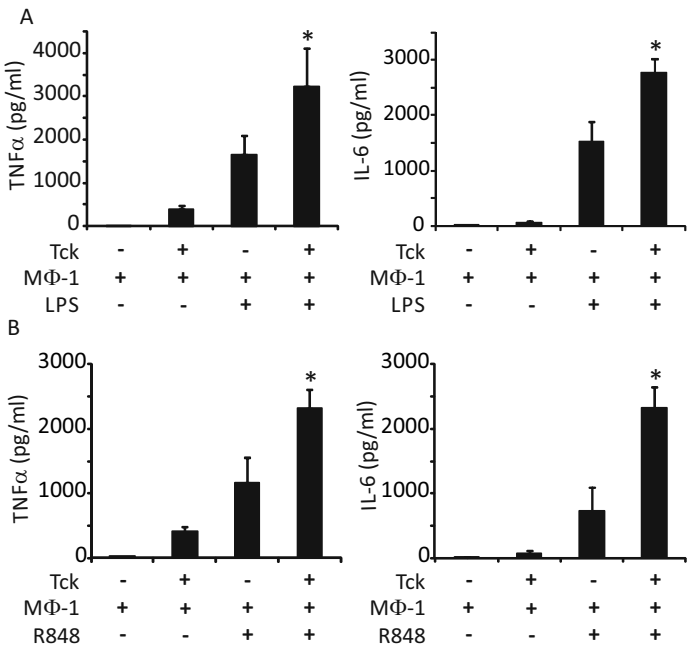

Figure 2. The combination of cytokine-stimulated T cells (Tck) and Toll-like receptor (TLR) ligands induces a clearly increased production of tumour necrosis factor α (TNFα) and interleukin (IL) 6 by mϕ-1. Mϕ-1 were unstimulated or stimulated with Tck or lipopolysaccharide (A), R848 (B) or a combination of both. After 24 h, the supernatants were collected and measured for their TNFα (left panels) and IL-6 (right panels) content. Data are from five individual experiments. Bars are mean and SEM. * $p<0.05$ compared to stimulation with TLR ligands alone.

through CD80/86 but was due to the blockade of Tck/mϕ-1 interactions (data not shown).

Discussion

Abatacept is a valuable new drug in the treatment of RA, but its mode of action remains to be fully elucidated. We provide evidence that, in addition to inhibiting T cell activation and the reduction of migration of T cells into B cell follicles[10], abatacept inhibits the Tck-induced production of proinflammatory cytokines by inflammatory $CD68^{+}CD163^{-}$ macrophages.

Various pathways have been implicated in the pathogenesis of the dysregulated proinflammatory cytokine environment in RA. We demonstrate herein that the combination of TLR ligands and cytokine activated T cells results in the synergistic release of TNFα and IL-6 and importantly licenses macrophages for the production of IL-12p70.

In a recent study it was demonstrated that abatacept reduces inflammation of the synovium without disrupting cellular constituents in patients unresponsive to anti-TNFα therapy. Interestingly especially the expression of IFNγ was reduced by 52%.[14] We provide a mechanistic explanation for this observation. We observed that abatacept reduces the production of IL-12p70 and IFNγ by mϕ-1/Tck cocultures activated by a TLR ligand by

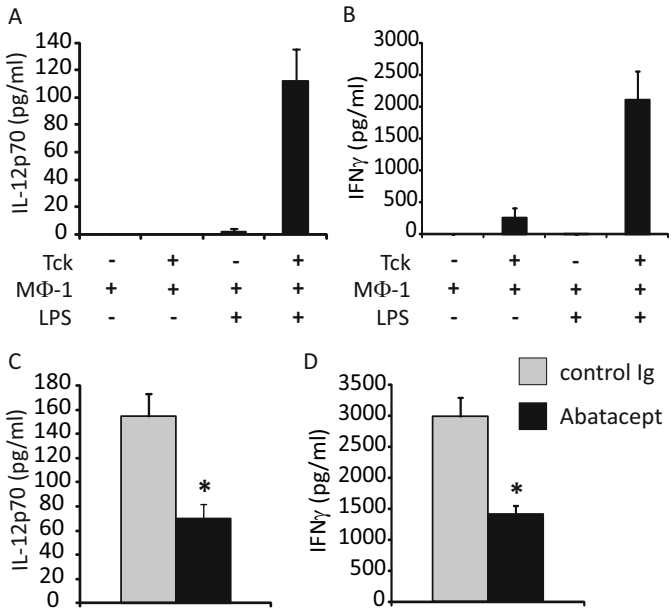

Figure 3. The combination of cytokine-stimulated T cells (Tck) and Toll-like receptor ligands licenses Tck/mϕ-1 cocultures to produce interleukin (IL) 12p70 and interferon γ (IFNγ), dampened by the presence of abatacept. Mϕ-1 were unstimulated or stimulated with Tck, lipopolysaccharide (LPS) or a combination of both. After 24 h, the supernatants were collected and measured for their IL-12p70 (A) and IFNγ (B) content. Data are from four individual experiments. Bars are mean and SEM. Mϕ-1 were stimulated with Tck and LPS in the presence of the control Ig (grey bars) or abatacept (black bars). After 24 h, the supernatants were collected and measured for their IL-12p70 (C) and IFNγ (D) content. Data are from four individual experiments. Bars are mean and SEM. * $p<0.05$ compared to control Ig.

approximately 50%, whereas the production of TNFα and IL-6 was unaffected. Th1 cells and natural killer T cells are capable of producing copious amounts of IFNγ and IL-12p70 is crucial in both the differentiation of Th1 cells and the induction of IFNγ production by natural killer T cells.[15-17] The IL-12p70/IFNγ axis was recently demonstrated, using the K/BxN serum transfer model and IL-12p35$^{-/-}$ mice, to promote antibody-induced joint inflammation underscoring the role played by IL-12p70 in arthritis.[13] The production of IFNγ by macrophage/Tck cocultures upon activation with TLR ligands might explain our results regarding the highly increased release of IL-12p70. IFN is known to potently increase the release of IL-12p70 by macrophages upon TLR stimulation, while IL-12p70 might, in its turn, be important in the induction of IFNγ by Tck. This might lead to a vicious circle increasing the production of both IL-12p70 and IFNγ, which can be prevented by the presence of abatacept.

The question remains how abatacept is capable of inhibiting the release of cytokines by proinflammatory macrophages upon the activation by Tck. Abatacept might directly activate certain pathways in macrophages via CD80/86, resulting in the inhibition of cytokine release upon Tck stimulation, as was suggested by Cutolo *et al.*[12] They demonstrated, using a concanavalin-A-activated Jurkat T cell cell line, that abatacept was capable of reducing the (immunocytochemically) detectable levels of IL-6 and TNFα in RA synovial tissue macrophages, underscoring the clinical relevance of the results described in this paper. A direct effect on the macrophages by abatacept would, however, be a rather specific inhibitory effect since no difference in cytokine release was observed upon TLR stimulation. Activation of CD80/86 by CTLA4-expressing T cells was demonstrated to induce IFNγ production by mouse DCs.[11] It thus appears likely that the activation of CD80/86 by CD28 or CTLA4 expressed by the Tck plays a role in the cytokine production by the macrophages, which is inhibited by abatacept. In addition, steric hindrance might play a role by inhibiting full contact in the immunological synaps between necessary components leading to full instruction of the macrophages by Tck. Whereas we did not add an antibody that specifically binds a receptor known to play a role in this process, Brennan *et al*[4] did use a blocking antibody against the β2 integrin lymphocyte function-associated antigen 1 in experiments with Tck and macrophages. This had no effect on the level of TNFα produced, substantiating that a role for steric hindrance might be minimal.

Another point of interest was that we observed that stimulation with Tck, together with TLR ligands, hampered the ability of abatacept to interfere in the production of TNFα and IL-6. This appears to implicate that TLR signalling is capable of overruling some of the proinflammatory signalling cascades induced by Tck in mϕ-1, leading to full-blown cytokine release despite the presence of abatacept.

In conclusion, we demonstrated that abatacept is capable of interfering with the production of TNFα by mϕ-1 induced by the contact with cytokine-activated T cells. Moreover, in the presence of TLR ligands, which are present and plentiful in the arthritic joint, activated T cell/mϕ-1 cocultures produced highly increased levels of TNFα, IL-6 and even IL-12p70 and IFNγ. Abatacept was shown to potently interfere in the production of IL-12p70 and IFNγ under these circumstances.

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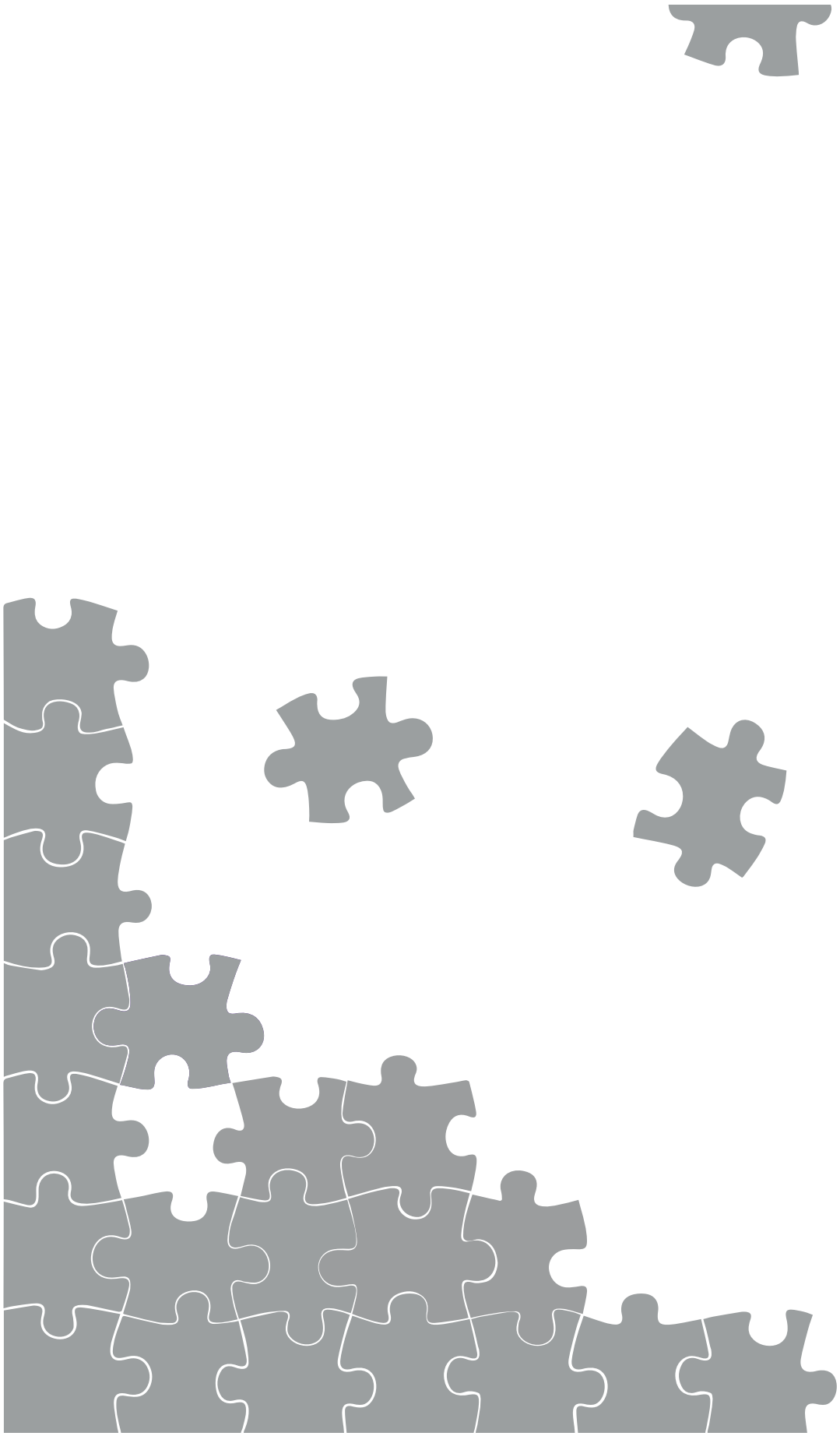

5

Cellular interaction via Mac-1 controls the proinflammatory function of macrophages

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Submitted for publication

5

Macrophages are important mediators of inflammation but also crucial for the resolution phase. What induces this shift in function is still not fully understood. We sought to analyze whether the accumulation of macrophages itself could induce a phenotype shift from proinflammatory to more anti-inflammatory macrophages aimed at dampening the immune response. Human macrophages and dendritic cells (DCs) were cultured from monocytes in different cell densities and effects on TLR induced cell activation and phagocytosis were determined. Several inhibitors were used to determine the pathways involved. In addition, macrophages were isolated at different time points during murine wound healing and the TLR induced cytokine production was associated with macrophage numbers. Increased cell-cell contact led to GM-CSF macrophages producing lower levels of TNFα, IL-1β and IL-6, while IL-10 and VEGF were increased upon TLR stimulation. This was supported by an in vivo wound healing model in which increased amounts of F4/80+ macrophages at day 4 and 6 after wounding were associated with decreased TNFα and IL-1β production upon TLR stimulation ex vivo. Further analysis showed that this functional shift depended on Mac-1 mediated cell-cell contact, signaling via p38 MAP kinase, activation of COX2 and was characterized by increased SOCS3 expression. Similarly, proinflammatory cytokine production by DCs as well as their ability to induce T-cell proliferation and Th1 polarization was restricted by cell-cell contact. Concluding, Mac-1 mediated cellular interaction during macrophage (and DC) differentiation serves as an inhibitory mechanism designed to dampen immune responses, placing Mac-1 central in the regulation of inflammation.

Introduction

Upon entering an inflammatory site monocytes differentiate into effector cells, such as macrophages and dendritic cells (DCs), with distinct phenotypes and roles in inflammation and immunity. Through the production of a vast array of mediators these subsets of immune cells control the outcome of the inflammatory process.[1] In this light, it is well appreciated that macrophages are important mediators of inflammation but are also crucial for resolution of inflammation, mediating tissue homeostasis and wound healing.[2;3] How these cells can have such varied functions and if the same cells switch from one phenotype into the other or that new macrophage subsets migrate during an immune process is still unclear. A better understanding of this process may provide tools to dampen inflammation in diverse autoimmune/inflammatory diseases such as rheumatoid arthritis and psoriasis in which there is a skewed balance towards proinflammatory cytokines. Alternatively, in cancer this could give leads on how suppressive tumor-associated macrophages can be modified, improving anti-tumor responses.

Macrophage differentiation and function is partially mediated by the presence of growth factors such as M-CSF and GM-CSF.[4-6] M-CSF is constitutively expressed during steady state conditions and is thought to regulate the development and maintenance of tissue macrophages.[7] In addition, these macrophages might have a role in suppression of inflammation due to their preferential secretion of IL-10, their suppressive effect on the development of IFNγ producing T cells and their ability to induce regulatory T cells.[8;9] Whereas under steady state conditions GM-CSF is barely present, locally at sites of inflammation GM-CSF is clearly detectable, where it can prime myeloid cells for the release of proinflammatory cytokines.[10;11] In correspondence with their *in vivo* distribution, human monocyte-derived macrophages differentiated in the presence of GM-CSF (gmMϕ) produce high levels of the proinflammatory cytokines TNFα, IL-6 and IL-1β whereas M-CSF differentiated macrophages (mMϕ) mainly produce IL-10 and trophic factors such as TGFβ and VEGF upon activation.[1;8]

Macrophages can get activated via Toll-like receptors (TLRs) or other pattern recognition receptors. This activation needs to be tightly controlled to enable macrophages to rapidly mount responses to inciting pathogens but avoid excessive tissue damage by an over activated immune reaction. While a panoply of negative regulators of TLR responses are known (e.g. IL-10, A20, SOCS, and SIGIRR)[12;13], the mechanisms regulating these factors in macrophages and DCs infiltrating an inflammatory lesion are largely unknown.[14] Different signaling receptors have been shown to inhibit TLR responses in macrophages and/or DCs, including Fc gamma receptor IIb and β2 integrins.[15;16] β2 integrins are leukocyte specific heterodimers consisting of a common β subunit CD18, combined with CD11a (LFA-1), CD11b (Mac-1), CD11c or CD11d.[17] They bind a wide variety of proteins including fibrinogen, C3bi, and several ICAMs depending on the alpha unit present. β2-integrins are known to play an important role in cell-matrix and cell-cell contact, facilitating monocyte extravasation into tissues and homotypic aggregation of macrophages involved in osteoclast and multinucleated giant cell formation.[18-20] They are important sensors for a cell's microenvironment including interaction with neighboring cells.

Since monocytes that enter a site of inflammation become proinflammatory macrophages under the influence of locally produced GM-CSF we hypothesized that there should be a negative feedback mechanism restricting the proinflammatory phenotype when sufficient amounts of macrophages have infiltrated a site of inflammation. In this study we show that human gmMϕ differentiated in increasing cell densities down regulate their proinflammatory phenotype and gain more characteristics of alternatively activated/ resolution macrophages. Increased macrophage numbers during wound healing were also directly associated with reduced inflammatory cytokine production by these macrophages. This shift was dependent on cell-cell contact via β2-integrin Mac-1 and COX2-dependent lipid mediator production and is characterized by increased expression of the inhibitory molecule SOCS3. These observations could imply a novel and important feedback mechanism seminal to the resolution of inflammation.

Materials and Methods

Culture of monocyte-derived macrophages and DCs

All experiments were performed in accordance with the Helsinki Declaration and approved by the local Medical Ethics Committees of the Radboud UMC and the UMC Utrecht. All donors gave written informed consent. PBMCs from healthy volunteers were isolated by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare), after which monocytes were obtained using CD14 MACS beads (Miltenyi Biotec). Monocyte-derived macrophages were generated by culturing isolated monocytes in the presence of GM-CSF (800 U/ml, R&D) or M-CSF (25 ng/ml, R&D) for 6 days, and are referred to as gmMϕ and mMϕ respectively.[21] Monocyte-derived DCs were differentiated in the presence of IL-4 (500 U/ml, R&D) and GM-CSF (800 U/ml). Macrophages/DCs were differentiated in 6- or 24-well plates (Corning) with the number of cells per well as indicated in 2 ml of culture medium (RPMI-1640 Dutch modification (Invitrogen) supplemented with 10% FCS, antibiotic-antimycotic and L-glutamine (Invitrogen)). Fresh culture medium (1 ml) with the same supplements was added at day 3. In blocking experiments antibodies against LFA-1 (clone L15, 5 μg/ml), Mac-1 (clone Bear-1, 5 μg/ml), CD18 (clone L19, 5 μg/ml), CD11c (Leaf purified clone Bu15, 10 g/ml, Biolegend), IL-6 receptor (10 μg/ml, R&D) or a mouse IgG_1 isotype control (eBioscience) were added at the beginning and day 3 of gmMϕ differentiation. The following inhibitors were used during macrophage differentiation: SB203850 (p38 MAP kinase inhibitor, 2 μM) and COX2 inhibitor I (10 μM) (both Calbiochem). Inhibitors were titrated and the used concentrations did not affect cell survival. To determine the effect of TGFβ1 on macrophage differentiation, gmMϕ were differentiated in 6-well plates in a density of $0.5x10^6$ or $2x10^6$ cells per well with or without 5 ng/ml recombinant human TGFβ1 (Invitrogen).

Stimulation of macrophages and DCs

Harvested day 6 macrophages and DCs were washed, counted and plated in a concentration of $0.5x10^6$ cells/ml in 24-well (1 ml) or 96-well (100 μl) culture plates. Cells were stimulated

with TLR or dectin-1 agonists for 18-24 hours. The following concentrations were used: Pam3CSK4 (5 μg/ml, EMC Microcollections), LPS (100 ng/ml, *E. coli* 0111:B4, Sigma) and R848 (2 μg/ml, InvivoGen) for TLR 2, 4 and 7/8 respectively and Curdlan (50 μg/ml, Wako) for dectin-1 stimulation. The LPS was double-purified to remove any contaminating proteins.[22] Desiccated *Mycobacterium tuberculosis* (H37Ra, Difco) was used in a concentration of 100 μg/ml.

Flow cytometry

Using standardized flow cytometry protocols as described previously[23], phenotypic analysis of macrophages and DCs was performed. Monoclonal antibodies against CD163, TLR2, TLR4, CD14, CD80, CD86 (all BD Biosciences), MHC-II DR/DP (clone Q1514), CD18 (clone L19) CD11a (clone L15) and CD11b (clone Bear1) were used. Expression was visualized via a FITC labeled goat-anti-mouse secondary antibody. Live cells were analyzed for the proportion of positive cells and the mean fluorescence intensity relative to cells stained with the appropriate IgG isotypes.

Measurement of cytokines and prostaglandin E2

Levels of human IL-10, TNFα, IL-12p70, IL-6, IL-1β, VEGF, IFNγ, IL-13 and IL-17A were measured in supernatants using commercially available kits (Bio-Rad) according to the manufacturer's instructions. Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad). Sensitivity of the cytokine assay was < 5 pg/ml for all cytokines measured. Mouse IL-1β, IL-6 and TNFα were measured using Milliplex kits (Millipore) according to the manufacturer's instructions. Prostaglandin E2 was measured using a PGE_2 elisa kit (Enzo Life Sciences) according to the manufacturer's instructions.

Phagocytosis assays

Phagocytosis assays were performed with labeled beads (2 μM carboxylate modified FluoSpheres, nile red 535/575nm, Invitrogen) or *Escherichia coli* (K-12 strain, Alexa Fluor 488 BioParticles, Invitrogen). Macrophages were left on ice or were incubated at 37°C for 15, 30 or 60 minutes with 10 beads per cell or 50 μg/ml labeled *E. coli.* Hereafter the cells were washed and analyzed by flow cytometry.

RNA isolation and real-time PCR

Total RNA was extracted in TRIzol reagent and treated with DNase before reverse transcription into cDNA. Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems) as described previously[24] and results are depicted as relative expression using reference gene GAPDH. Primer sequences for human *SOCS1, SOCS3, TOLLIP, SIGIRR, A20, COX2 and GAPDH* can be found in Table I.

Autologous MLR

$CD4^+CD45RA^+$ naïve T cells were obtained by negative CD4 selection and subsequent CD45RO depletion (Miltenyi Biotec). $50x10^3$ autologous $CD4^+CD45RA^+$ T cells were added

Table I. Primer sequences for qPCR analysis

Primers	Forward	Reverse
GAPDH	ATCTTCTTTTGCGTCGCCAG	TTCCCCATGGTGTCTGAGC
SOCS1	CCCTGGTTGTTGTAGCAGCTT	TTGTGCAAAGATACTGGGTATATGTAAA
SOCS3	TCGGACCAGCGCCACTT	CACTGGATGCGCAGGTTCT
TOLLIP	CGACTGAACATCACGGTGGTA	CAGGGACTCCGGGATGGT
SIGIRR	TGAAAGACGGGCTTCCATTG	TTCAGTGCTGGTCACGTTGAC
A20	AAGAAACTCAACTGGTGTCGAGAA	TGCCGTCACCGTTCGTT
COX2	CCAGTATAAGTGCGATTGTACCC	TCAAAAATTCCGGTGTTGAGCA

to $5x10^3$ DCs in 96-well round bottom plates. The leukocyte mixture was stimulated with R848 (2 µg/ml) or left unstimulated. Proliferation was analyzed at day 3 by flow cytometric staining for EdU (5-ethynyl-2'-deoxyuridine) incorporation (Invitrogen). At day 6 of the co-culture, the cells were incubated with PMA (50 ng/ml, Sigma) and ionomycin (1 µg/ml, Sigma) for 16 hours before the collection of supernatants.

In vivo wound healing model

Mice (129Sv x C57Bl/6 mixed background, 10-12 weeks old) acclimatized for 7 days before experimental start. All experiments and protocols were reviewed and approved by the Radboud University Nijmegen animal experimentation committee and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals. The mice were maintained in groups on a 12-hr light/dark cycle and SPF-conditions with unrestricted access to food and water. Four circular full-thickness excisional wounds were generated in the shaved back skin of anesthetized mice (n=6, groups consisting of 3 males and 3 females (D2,4) and 2 males and 4 females (D6)) using sterile 4-mm biopsy punches (Kai Medical). The wounds were left undressed, and mice were sacrificed 2, 4, and 6 days after wounding. Wound tissue was collected using 6-mm biopsy punches, allowing normal skin to be included. As additional controls, non-wounded back skin was collected at day 0 (n=17) and at the experimental end points (n=18).

Immunohistochemistry and semi-quantitative scoring

Isolated skin tissue was fixed for 24 hours in 4% paraformaldehyde and further processed for routine paraffin embedding. Paraffin sections (5 µm) of wounds were stained essentially as described previously incubating primary antibody rat anti-F4/80 (1:350; Sigma) overnight at 4°C.[25] After washing, sections were incubated with a biotin-conjugated donkey anti-rat secondary antibody (Jackson ImmunoResearch) for 1 hr and developed using ABC peroxidase (Vector Laboratories) and diaminobenzidine detection reagents (DAB; Sigma). The staining was enhanced with 0.5% $CuSO_4$ in 0.9% NaCl, and sections were counterstained with hematoxylin. As negative control, the primary antibody was omitted. Light microscopic images were captured using a Zeiss Imager Z1 microscope with an AxioCam MRc5 digital camera under the control of AxioVision rel. 4.8 software (Carl Zeiss). The percentage of

F4/80 positive cells was evaluated by a semi-quantitative scoring system. Extent was scored as: 0, ≤ 5%; 1, 6-25%; 2, 26-50%; 3, > 50%. Scoring was done three times by a blinded investigator.

Macrophage isolation from wound tissue

Three 6-mm wound biopsies were used from each mouse for macrophage isolation. The wound biopsies were washed in PBS with antibiotics and digested for approximately 2,5 hours at room temperature with 0.27% collagenase type XI (Sigma) and 0.01% DNaseI (Sigma) in RPMI medium containing antibiotics. The cell suspension was filtered over a 70 µm cell strainer and digestion was stopped by washing the cells with RPMI containing 10% FCS. Cells were resuspended in PBS containing 0.5% BSA and were stained with a F4/80-PE antibody (clone BM8, Biolegend) and 7-AAD (Biolegend) for flow sorting on a FACSAriaII (BD Biosciences). For the gating strategy, live leukocytes were selected based on FSC-A vs SSC-A, followed by exclusion of 7-AAD positive cells. Doublets were excluded using SSC-W vs SSC-H and FSC-W vs FSC-H gating. Macrophages were identified as F4/80 positive cells. $F4/80^{+}/7\text{-}AAD^{-}$ macrophages were plated in 96-well plates and stimulated with 1 µg/ml R848.

Statistical analysis

Statistical analysis was performed using GraphPad software. Differences between groups were analyzed using the Mann-Whitney U test and when appropriate a paired students T test was used. Semi-quantitative scores were analyzed using non-parametric Kruskall-Wallis with Dunn's posthoc test. Differences were considered significant when $p < 0.05$.

Results

Cell density affects the inflammatory function of gmMφ

In order to test our hypothesis, we started by culturing monocytes for 6 days in the presence of GM-CSF or M-CSF to yield pro- and anti-inflammatory macrophages, respectively, and analyzed their phenotype. In accordance with the literature the expression of CD163, CD14 and TLR2 was higher on mMϕ while the expression of CD86 and MHCII was increased on gmMϕ (Figure 1A).[26] The expression of TLR4 and dectin-1 did not differ. Next we differentiated mMϕ and gmMϕ in increasing cell concentrations. After differentiation, the macrophages were harvested, counted and plated in equal cell numbers before stimulation with LPS. In line with the literature gmMϕ produced much higher levels of TNFα, IL-1β and IL-6 compared to mMϕ. Strikingly, culturing gmMϕ in increasing concentrations, mimicking an inflammatory setting with a high influx of monocytes/macrophages, resulted in reduced production of TNFα, IL-1β and IL-6 while the contrary was observed for IL-10 and VEGF after stimulation with LPS (Figure 1B). This effect was dependent on cell concentration and was less pronounced in the already less inflammatory mMϕ. Similar effects were noted upon stimulation of TLR2, endosomal TLR7/8 or with complete *M. tuberculosis* bacteria (Figure 1C). To exclude differences caused by the amount of GM-CSF or medium per cell, the same

amount of macrophages were differentiated in equal volumes in a 6- or 24-well plate. This confirmed that reduction in surface area and thereby increasing cell density is sufficient to increase IL-10 and reduce TNFα production upon subsequent TLR stimulation (Figure 1D and data not shown). Cell survival was unaffected underscoring the idea that this effect is solely explained by an increased cell density and not due to selection or phagocytosis of apoptotic cells (Figure 1E).

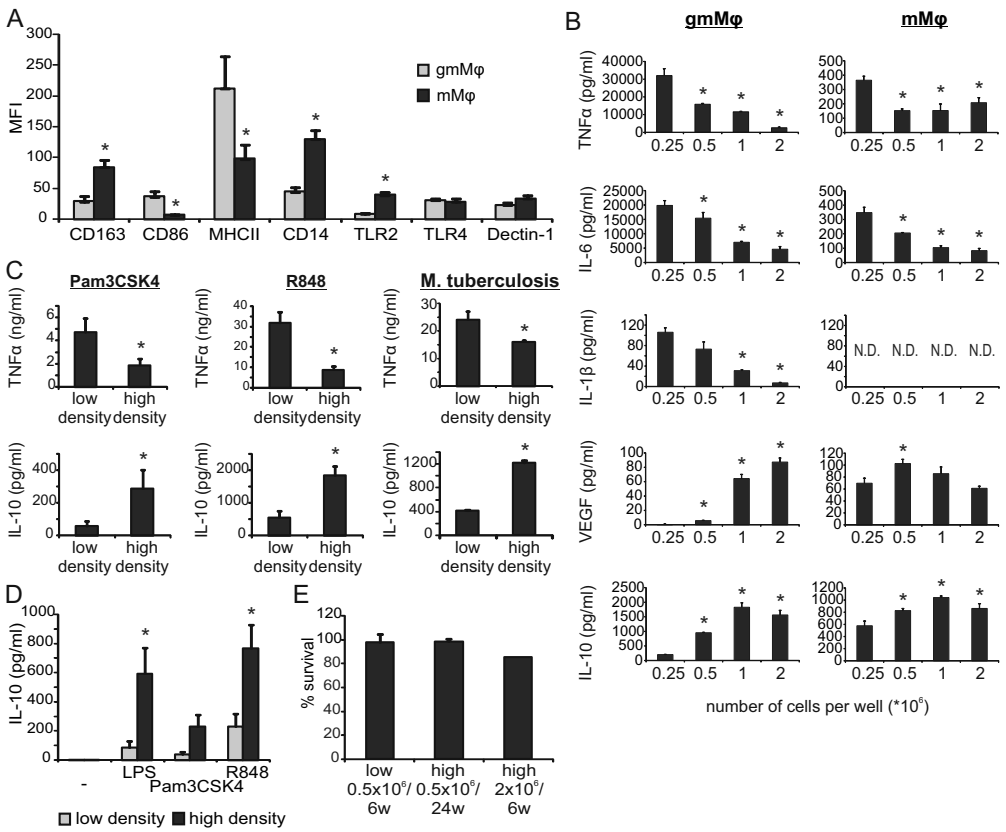

Figure 1. Differentiation of monocytes into gmMϕ in increasing density induces less inflammatory macrophages. (A) Monocytes were differentiated into gmMϕ and mMϕ and expression of several markers was determined by flow cytometry. (B) gmMϕ and mMϕ were differentiated in increasing cell concentrations. Day 6 macrophages were harvested, plated in equal cell numbers and stimulated with LPS for 24 hours before cytokine measurement in the supernatant. Results are presented as mean and SD of three replicates representative of three independent experiments. N.D. is not detectable. (C) gmMϕ were differentiated with $0.5x10^6$ (low density) or $2.0x10^6$ (high density) cells/well in 6-well plates in equal volumes of culture medium. gmMϕ were plated in equal cell numbers and stimulated with Pam3CSK4, R848 or desiccated M. Tuberculosis. (D) $0.5x10^6$ gmMϕ were differentiated in a 6- (low density) or a 24-well plate well (high density) and subsequently stimulated with LPS, Pam3CSK4 or R848. (E) gmMϕ differentiated in different cell concentrations were counted and the amount of cells was compared to the amount of monocytes at the start of the culture (% survival). (A, C, D, E) Bars represent mean and SEM of at least three independent experiments. * $p < 0.05$

We further evaluated whether gmMϕ differentiated in higher densities would gain more features of so-called 'alternatively activated' macrophages such as an increased capacity to phagocytose particles. Indeed, increasing macrophage density boosted their ability to phagocytose labelled beads as well as labelled *E. coli* considerably, whilst the expression of pivotal antigen uptake receptors remained unchanged (Figure 2A-C). As described for 'alternatively activated' macrophages[27], high density gmMϕ, in contrast to those differentiated at a low density, responded vigorously to dectin-1 ligation, a pattern recognition receptor important in the recognition of *Candida spp.*[28], by releasing massive amounts of TNFα and IL-6 as well as IL-10 (Figure 2D). Collectively, these results implicate a major impact of cell density on the behavior of inflammatory macrophages.

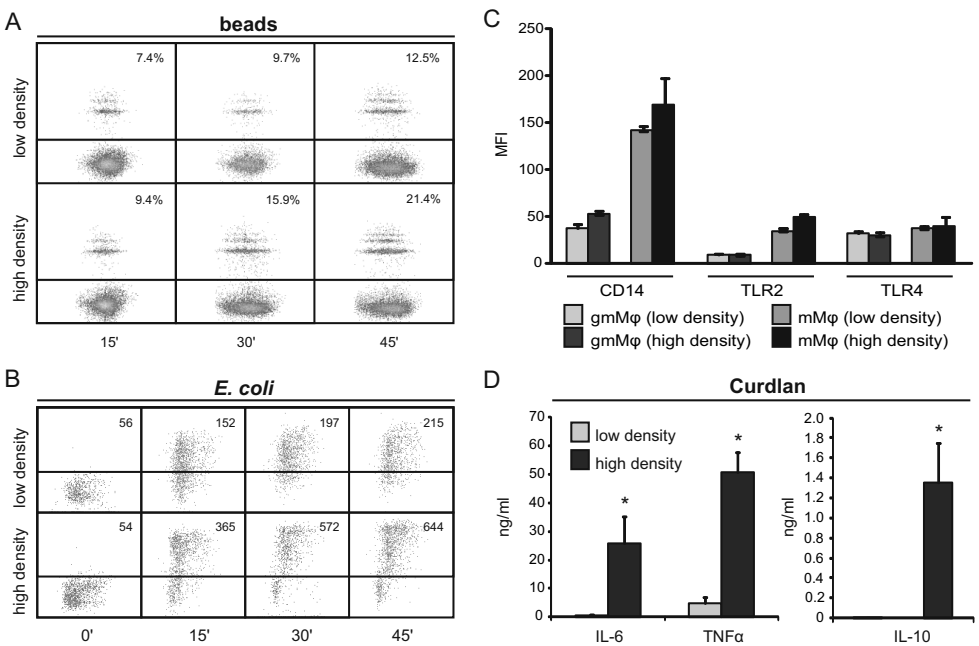

Figure 2. High density gmMϕ display characteristics of alternatively activated macrophages. gmMϕ were differentiated in low ($0.5x10^6$) or high density ($2x10^6$, 6-well plate) after which they were analyzed for their ability to phagocytose labeled beads (A) or labeled E. coli (B) by flow cytometry. Numbers are percentage positive cells (A) and MFI (B). Data are representative for three independent experiments. (C) Expression of CD14, TLR2 and TLR4 was determined on low and high density gmMϕ and mMϕ. (D) Low and high density gmMϕ were stimulated with Curdlan and TNFα, IL-6 and IL-10 levels were determined. Bars are mean and SEM of four independent experiments, * $p < 0.05$ compared to low density gmMϕ.

Increased macrophage density in vivo is associated with decreased cytokine production ex vivo

To determine if this mechanism could play a role *in vivo* we studied macrophages in a murine excisional wound healing model, because there is a clear influx of macrophages in a 'semi natural' setting.[29] Mice were wounded on the back and wound tissue was collected after 2, 4 and 6 days. Immunohistochemistry showed a clear influx of F4/80$^+$ macrophages at day 4 and 6 after wounding (Figure 3A, B). F4/80$^+$ macrophages were isolated from the wounds and stimulated with TLR7/8 ligand R848 to determine cytokine responses 2, 4 and 6 days after wounding. Both TNFα and IL-1β were significantly decreased at day 4 and/or day 6, corresponding with the highest numbers/density of F4/80$^+$ cells (Figure 3C). The same trend was observed for IL-6 production. Correlation of F4/80 scores with cytokine production by the isolated macrophages, independent of time after wounding, showed a direct negative correlation for both IL-1β and IL-6 with macrophage numbers (Figure 3D). This model supports *in vivo* relevance for our findings that increased macrophage numbers are associated with decreased inflammatory cytokine production.

The shift towards less inflammatory macrophages is dependent on 2 integrin Mac-1 and signaling via MAP kinase p38

To determine what signaling pathways could underlie this density-mediated shift in macrophage behavior we went back to our human system and looked at β2 integrins. We first evaluated CD18, CD11a and CD11b expression on monocytes, gmMϕ and mMϕ and noted an increased expression of CD18 and CD11b on gmMϕ, independent of cell density (Figure 4A). Blocking the common β chain CD18 during gmMϕ differentiation under high-density circumstances reduced cellular interaction of the macrophages in culture (Figure 4B). CD18 blocking also prevented the increased IL-10 and decreased TNFα production upon TLR stimulation in high density gmMϕ, while cytokine production in low density gmMϕ was unaffected (Figure 4C). Blocking of individual β2 integrins by antibodies directed against CD11a or CD11b showed that only blocking of CD18 and CD11b could prevent the cytokine shift seen at high cell densities, pointing towards Mac-1 as the receptor involved in this effect (Figure 4D). Blocking of CD11c was also tested in this system and did not affect cytokine production, similar to CD11a blocking (data not shown). Adding excess IgG to block Fc gamma receptors during antibody treatment did not affect the results. CD18 or CD11b blocking of high density macrophages not during the differentiation but at the time of stimulation with LPS did not have the same effects on IL-10 and TNFα production (data not shown). Taken together, our observations show a pivotal role of Mac-1 in controlling cell function in inflammatory conditions where high cell densities are likely.

Next we evaluated the expression of intracellular inhibitors of TLR signaling pathways that might be involved including SOCS1, SOCS3, TOLLIP, SIGIRR and A20. Of these, SOCS3 was significantly up regulated when gmMϕ were differentiated in high densities (Figure 5A), an effect which was prevented by Mac-1 blocking (Figure 5B). MAP kinase p38 has been implicated in the induction of IL-10 and SOCS3 expression after integrin triggering.[30]

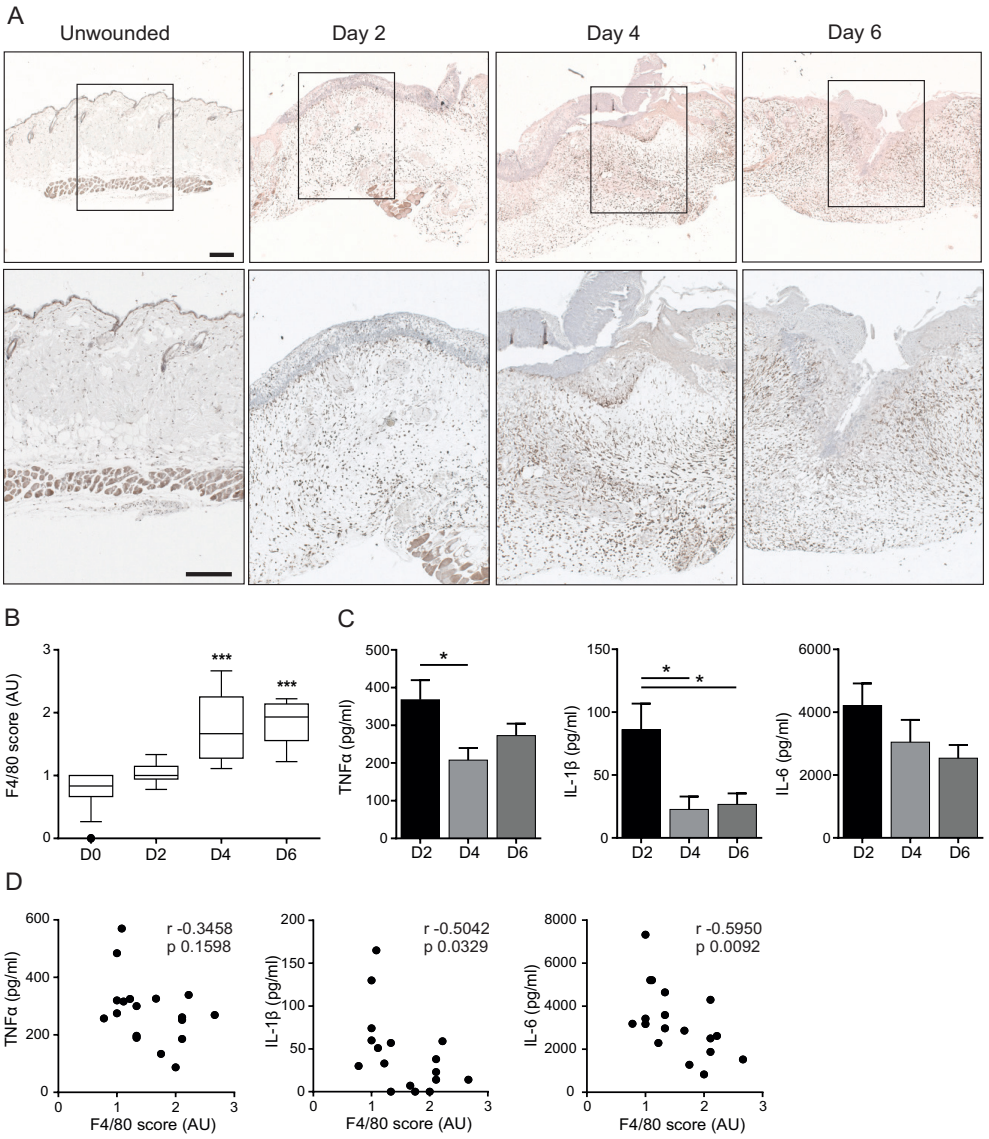

Figure 3. Increased macrophage infiltration in wound tissue is associated with decreased cytokine production by infiltrated macrophages. (A, B) Infiltration of macrophages is shown 2, 4 and 6 days after wounding and compared to unwounded control skin. (A) Representative pictures of skin and wound sections stained for F4/80 (Zeiss Imager Z1 microscope with an AxioCam MRc5 digital camera under the control of AxioVision rel. 4.8 software, magnification 50x and 100x). Rulers represent 200 µm. (B) Mean F4/80 scores of in triplicate scored sections of 6 mice in each group (and 17 control skin sections, D0) were shown in a box-and-whisker plot with 10-90 percentiles. *** $p < 0.001$ compared to D0. Macrophages were isolated from the wound biopsies and stimulated with R848 for 20 hours. (C) TNFα, IL-1β and IL-6 levels were determined in the supernatant. * $p < 0.05$ between indicated time points. (D) Individual F4/80 scores were correlated with TNFα, IL-1β and IL-6 production after stimulation of isolated macrophages.

To establish the involvement of p38, SB203580 was used to block p38 during gmMϕ differentiation. This prevented the basal induction of SOCS3 and the increased IL-10 production after TLR stimulation (Figure 5C,D), while leaving TNFα unaffected. Signaling via Mac-1 thus increases basal SOCS3 levels and TLR induced IL-10 production via p38.

The shift towards more anti-inflammatory gmMφ can be transferred by a soluble factor and is COX2 dependent

To determine if increased cell-cell contact induced the release of soluble factors that contribute to the change of macrophage function upon increased cell density, we cultured macrophages in a trans-well culture system. Increasing gmMϕ density in the bottom well of a trans-well system also induced a more anti-inflammatory function of macrophages in the upper well of which the density was not changed (Figure 6A), indicating the involvement of

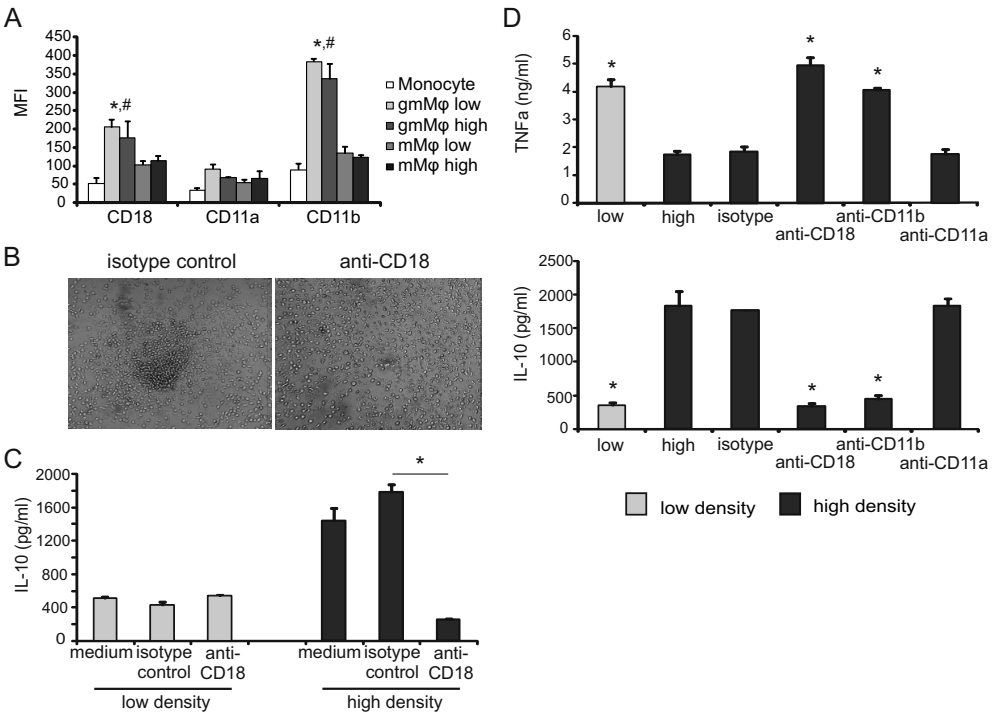

Figure 4. Mac-1 blocking prevented the differentiation of gmMϕ towards less inflammatory macrophages. (A) Expression levels of CD18, CD11a and CD11b were determined by flow cytometry on monocytes and low and high density macrophages ($0.5x10^6$ and $2x10^6$ in 6-well plates). Bars are mean and SEM of three experiments. * $p < 0.05$ compared to monocytes, # $p < 0.05$ compared to mMϕ. Monocytes were cultured into gmMϕ at low and high density in the presence of blocking antibodies against CD18 or an isotype control antibody (both 5 μg/ml). (B) At day 3 photographs were taken of the high density cultures at a magnification of 100x. (C) At day 6 macrophages were plated in equal cell numbers and stimulated with LPS for cytokine measurement. (D) Low and high density gmMϕ, and high density gmMϕ differentiated in the presence of an isotype control antibody or blocking antibodies against CD18, CD11b or CD11a were stimulated with LPS. (C, D) Bars are mean and SD of triplicate wells representative of three experiments.

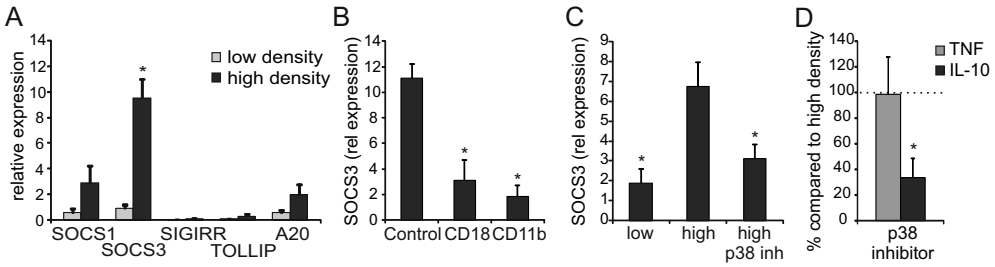

Figure 5. High density macrophages show a Mac-1 and p38 dependent up regulation of SOCS3. (A) Low and high density gmMϕ were analyzed for mRNA expression of *SOCS1, SOCS3, SIGIRR, TOLLIP* and *A20.* Expression is relative to *GAPDH* expression (x100). Data are presented as mean and SEM of four independent experiments. * $p < 0.05$ compared to low density. (B) *SOCS3* expression was determined in high density gmMϕ differentiated in the presence of blocking antibodies against CD18 or CD11b or an isotype control. * $p < 0.05$ compared to control. (C) *SOCS3* mRNA expression was also determined in the presence of p38 inhibitor SB203580 (2 μM). Data are presented as mean and SEM of three independent experiments. * $p < 0.05$ compared to high density gmMϕ. (D) High density gmMϕ were differentiated in the presence of 2 μM SB203580 and stimulated with LPS. Cytokine production is depicted as a percentage compared to high density culture alone. Bars are mean and SEM of five experiments.* $p < 0.05$ compared to high density gmMϕ.

a soluble factor. IL-6 and IL-10 have been implicated in the induction of anti-inflammatory macrophages, and we found that high density gmMϕ spontaneously produced marginal levels of IL-6 while IL-10 production was undetectable (Supplemental Figure 1A). To investigate a possible role for IL-6, we blocked IL-6 receptor during differentiation which had no effect on cytokine production (Supplemental Figure 1B). Another interesting candidate would be prostaglandins[31] and we found that the expression of COX2, an enzyme involved in prostaglandin production, was increased in high density macrophages (Figure 6B). To test if this pathway was involved, we used a COX2 inhibitor during gmMϕ differentiation to prevent prostaglandin production. Dose-dependent inhibition of COX2 led to the reversal of the anti-inflammatory phenotype of high density gmMϕ (Figure 6C,D). As a possible mediator, PGE_2 levels were measured in culture supernatant but these were low and not different between low and high density cultures (Supplemental Figure 1C). This points towards a possible role for other prostaglandins, such as PGD_2 and/or 15-deoxy$\Delta^{12\text{-}14}PGJ_2$ which have been described to be involved in resolution of inflammation.[32]

TGFβ1 prevents the development of more anti-inflammatory gmMφ

Since TGFβ1 is often found in high levels during chronic inflammation we tested gmMϕ differentiation in the presence of TGFβ1. TGFβ1 prevented the density dependent induction of IL-10 and reduction of TNFα upon TLR activation (Figure 7A). Also the induction of SOCS3 expression was absent in the presence of TGFβ1 (Figure 7B). In line with the increased TNFα (and IL-10) production upon dectin-1 stimulation by Curdlan in high density gmMϕ, addition of TGFβ1 prevented this increased response and reduced it to levels found in low density gmMϕ (Figure 7C and data not shown). TGFβ1 can thus have clear effects on gmMϕ increasing their responsiveness towards TLR ligands, while reducing their cytokine

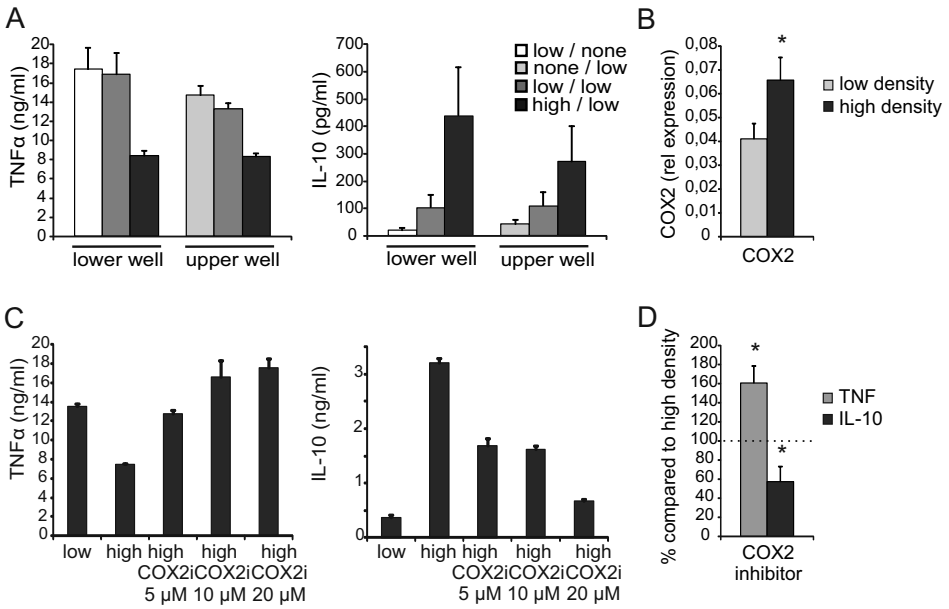

Figure 6. The less inflammatory gmMϕ phenotype induced by cell-cell contact is carried over by a soluble factor and can be halted by inhibiting COX2. (A) gmMϕ were differentiated in a transwell system with no cells (none /), $0.5x10^6$ (low /) or $2x10^6$ (high /) cells in the lower well and no cells (/ none) or $0.5x10^6$ (/ low) in the insert. At day 6 the gmMϕ were harvested, replated separately and stimulated with LPS, to determine TNFα (left panel) and IL-10 (right panel). (B) *COX2* expression was determined by qPCR in low and high density gmMϕ. mRNA expression is shown relative to *GAPDH* (x100). Bars are mean and SEM of 6 different donors. (C) Low and high density gmMϕ were differentiated in the absence or presence of increasing amounts of COX2 inhibitor after which they were harvested, washed and stimulated with LPS. Bars are mean and SD of duplicate wells representative of three experiments. (D) High density gmMϕ were differentiated in the presence of 10 μM COX2 inhibitor and stimulated with LPS. Cytokine production is depicted as a percentage compared to high density culture without an inhibitor. Bars are mean and SEM of five experiments.* $p < 0.05$ compared to high density gmMϕ.

response upon dectin-1 ligation. This indicates that the presence of TGFβ1 might support the continuation of inflammation in the presence of (endogenous) TLR ligands.

The inflammatory phenotype of DCs is also controlled by cell-cell contact

We next sought evidence for a similar role for cell density in the regulation of monocyte-derived DCs, since these also expressed high levels of CD11b and CD18 (Figure 8A). As with gmMϕ, DCs differentiated in high densities produced increased levels of IL-10 and decreased levels of TNFα and IL-12p70 after TLR stimulation (Figure 8B-D). Since DCs are the professional antigen-presenting cells of the immune system we wondered whether DC density would affect T cell proliferation and polarization upon TLR activation. High density DCs induced less T cell proliferation compared to low density DCs and T cell polarization was altered, with most pronounced effects on IFNγ production (Figure 8E, F). This demonstrates that the DCs become less capable of inducing strong Th1 responses when differentiated in the close proximity of neighboring cells.

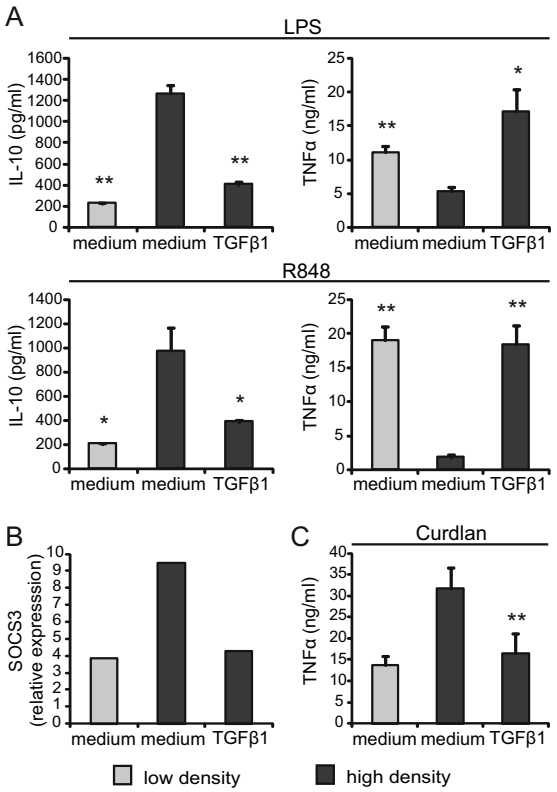

Figure 7. The less inflammatory gmMϕ phenotype at high density is inhibited by TGFβ1. $0.5x10^6$ (low density) or $2x10^6$ (high density) gmMϕ were differentiated in 6-well plates and in addition high density gmMϕ were differentiated in the presence of TGFβ1 (5 ng/ml). (A) Day 6 gmMϕ were harvested, washed and stimulated with LPS or R848 and IL-10 and TNF were measured. Bars are mean and SD of triplicate wells, representative of four independent experiments. (B) In similar experiments the expression of *SOCS3* was determined by qPCR. Expression is relative to GAPDH expression (x100). Data are representative of three independent experiments. (C) Day 6 gmMϕ were also stimulated with Curdlan. Bars are mean and SEM of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared to high density gmMϕ.

Discussion

In an inflammatory lesion monocytes are one of the first cells to appear, where they differentiate into macrophages or DCs. These macrophages and DCs are highly capable of producing proinflammatory cytokines when they encounter inflammatory stimuli. This is essential to clear an infection, but can be damaging to the host if this process is not tightly controlled. The invoked immune response can lead to tissue damage culminating in the release of endogenous ligands recognized by TLRs, which in turn will activate macrophages and DCs to produce more inflammatory cytokines starting a vicious cycle of damage and inflammatory mediator release. We provide evidence for a model in which cell-cell contact caused by the influx of inflammatory cells itself is able to dampen

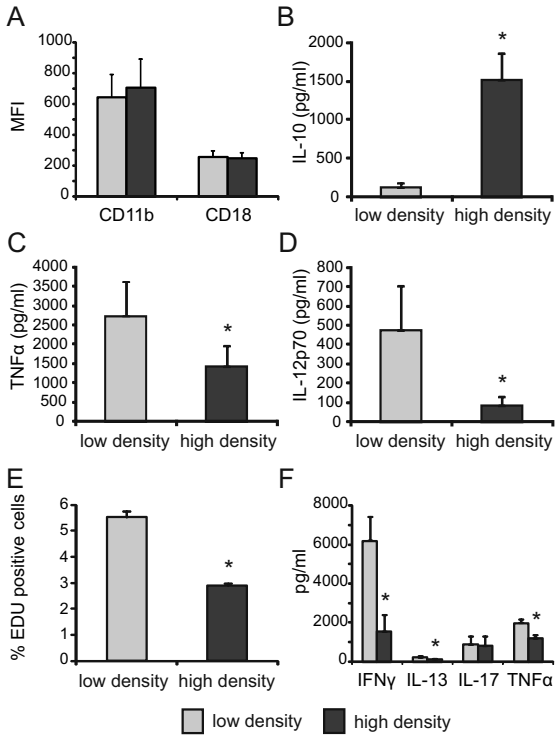

Figure 8. DCs are subject to down modulation of their proinflammatory phenotype when differentiated at a higher density. MoDCs were differentiated in 6-well plates with either $0.5x10^6$ (low density) or $2x10^6$ (high density) cells per well. (A) CD18 and CD11b expression was determined by flow cytometry. (B-D) MoDCs were stimulated with R848 and the levels of IL-10, TNFα and IL-12p70 were measured in the supernatant. (E, F) Low and high density DCs were cultured 1:10 with autologous $CD4^+CD45RA^+$ T cells. R848 was added and the DCs-T cell coculture was subsequently cultured for 3 days before the addition of EdU to determine proliferation rates (E) or for 6 days before stimulation with PMA and ionomycin (F). (E) EdU incorporation was measured by flow cytometry. (F) After 16 hours of PMA and ionomycin stimulation, supernatants were collected and the levels of IFNγ, IL-13, IL-17 and TNFα were measured. Data are presented as mean and SEM of at least three independent experiments. * $p < 0.05$ compared to low density DCs.

this self-perpetuating loop. Cell-cell contact via Mac-1 potently reduced the release of proinflammatory cytokines by stimulated gmMϕ, while IL-10 production was increased. The dampening of inflammatory cytokine production upon increased cell numbers was found in human *in vitro* experiments with macrophages and DCs as well as in murine wound healing macrophages. Further characterization of the pathways involved identified a p38 dependent up regulation of SOCS3 expression and a dependence of COX2 in the cytokine shift seen upon differentiation at high density. Our results extend on findings from Wang *et al* describing that β2 integrin stimulation with fibrinogen interfered with TLR signaling resulting in increased IL-10 and decreased TNFα and IL-6 levels and others that showed enhanced TLR responses in $CD11b^{-/-}$ mice.[16;33] These findings highlight the importance of Mac-1 in inflammation control by, among others, modulating cytokine production by

macrophages, thereby restoring immune homeostasis.

The importance of β2 integrins in inflammation control is supported by studies in CD18 hypomorphic mice. These mice developed an aggravated carditis and demonstrated a tendency towards an increased arthritis severity after infection with *Borrelia burgdorferi.*[34] This was marked by a massively increased infiltration of macrophages in the heart and an increased production of MCP-1. It thus appears that in mice with low levels of β2 integrins the resolution phase of the immune response is suboptimal resulting in the aggravated innate immune response observed. The intricate involvement of β2 integrins in inflammation was also demonstrated by the increased severity of a psoriasis model in CD18 hypomorphic mice previously demonstrated to crucially depend on activated macrophages.[35;36]

COX2 activity has previously been shown to be involved in both the induction and resolution phase of inflammation.[32;37;38] COX2 is responsible for the production of prostaglandins from arachidonic acid. These include PGE_2 which can have both pro- and anti-inflammatory effects and PGD_2 and PGJ_2 which can contribute to the resolution phase of inflammation. In addition, the production of these prostaglandins can also trigger the production of other pro-resolution mediators such as lipoxins and resolvins. These lipid mediators could play an important role in our model, in which the shift towards less inflammatory macrophages was dependent on COX2 activity. Fitting with our data and that of others, COX2 inhibition by NSAIDs can thus prevent the induction of inflammation but also hamper resolution.[37] Alternatively, they can increase the effect of immunotherapy in cancer and reduce the presence of tumor-associated suppressive macrophages.[39] This underscores the importance of the described feedback pathway and the therapeutic possibilities of modulating this pathway in chronic inflammation and cancer.

In wound healing, macrophages have been shown to be crucial for normal healing[40;41] and a decreased macrophage influx in diabetic ulcers was found to result in a non-resolving inflammation, which could be prevented by MCP-1 treatment restoring macrophage influx.[42] Wound macrophages have a complex phenotype with mixed marker expression, but they are mainly proinflammatory early after wounding and more alternatively activated in later stages.[43] Daley *et al* have shown that this phenotype shift is independent of IL-4 and IL-13, but coincides with a clear increase in monocyte/macrophage numbers[29], suggesting this may be dependent on increased cell-cell contact, as proposed here.

The role of TGFβ in inflammation is complex. While its anti-inflammatory functions are apparent from literature[44], its proinflammatory effects are less clear although it was demonstrated that the inhibition of one of its receptors during LPS stimulation decreased TNFα production by gmMϕ.[45] Animal models of arthritis also support this dual role of TGFβ, since systemic administration of TGFβ to mice prevents collagen induced arthritis, while its local administration to the joints induces synovitis and aggravates disease.[46;47] The beneficial systemic effects of TGFβ are most likely caused by its effects on T cell tolerance, while the detrimental effects of local TGFβ administration could at least partially be explained by preventing the anti-inflammatory phenotype shift of macrophages at the site of inflammation. Thereby the presence of TGFβ could prevent the resolution of inflammation when large amounts of monocytes have entered a site of inflammation,

promoting chronic inflammation.

Altogether, we demonstrate that the proinflammatory phenotype of macrophages is tightly controlled by cell-cell contact via Mac-1. This phenotypic shift was dependent on p38 and COX2 and was transferable by a soluble factor, implicating the release of lipid mediators as part of this process. This feedback mechanism stimulates the resolution of inflammation and the restoration of tissue homeostasis. Efforts to restore or prevent the naturally existing capacity to limit immune responses would be an important step towards patient tailored medicine in a wide variety of diseases.

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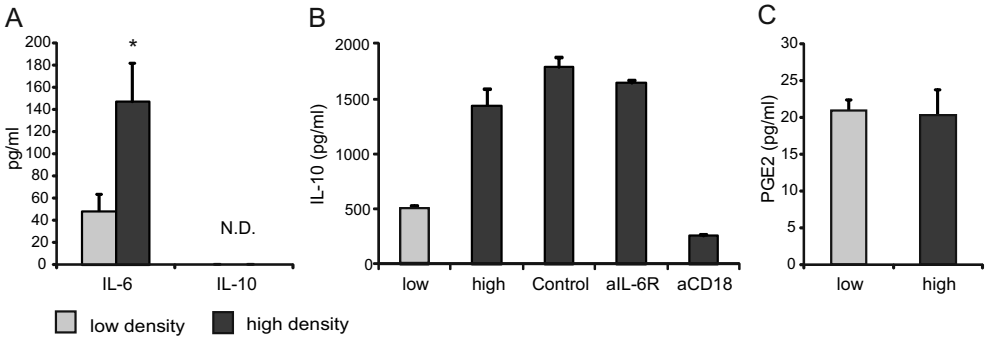
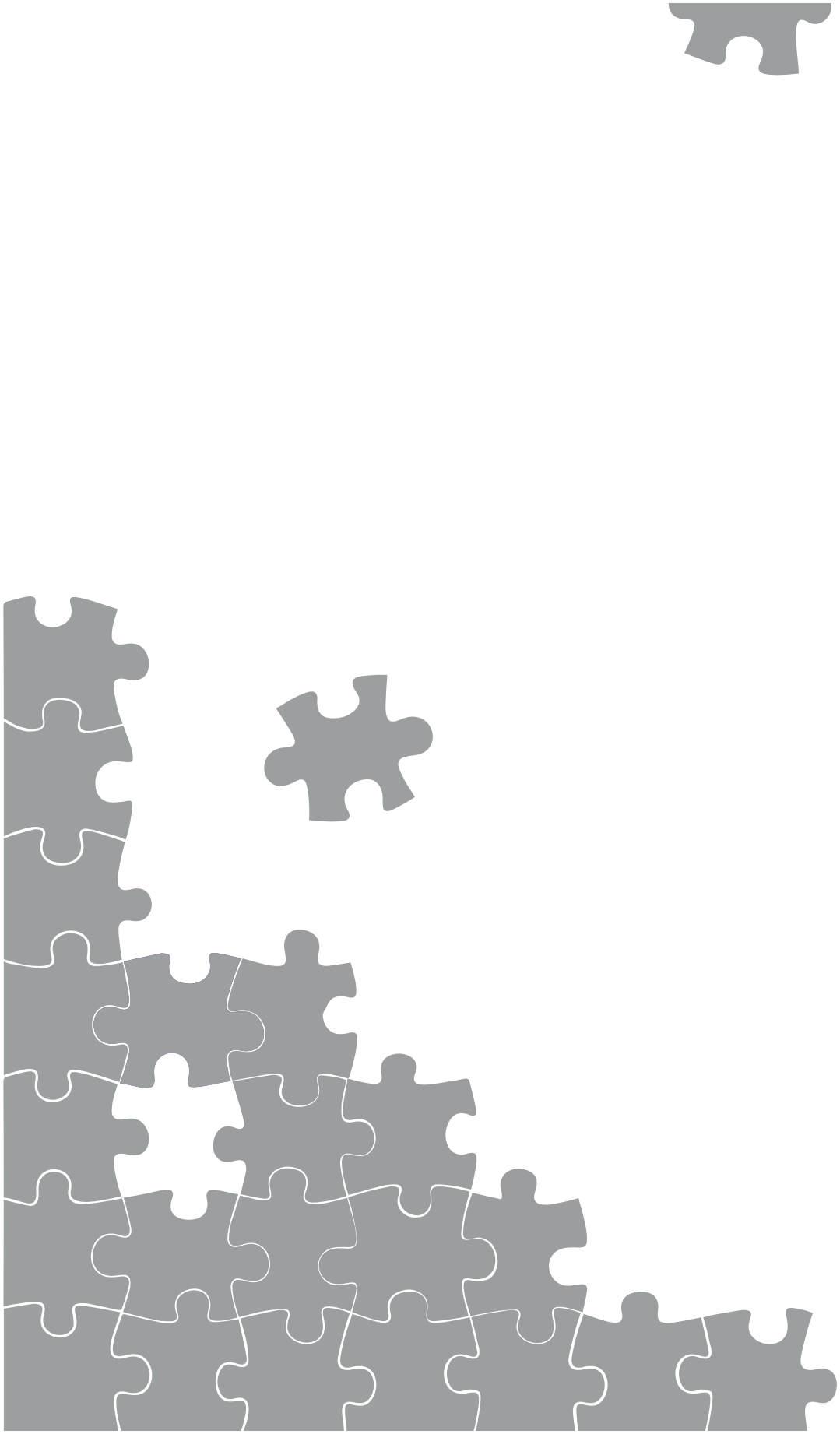


Supplementary figure 1. Spontaneous production of soluble mediators. (A) Il-6 and IL-10 release was measured in low and high density gmMϕ in the absence of TLR stimulation. Bars are mean and SEM of 8 donors. (B) gmMϕ were cultured at high density in the presence of medium, isotype matched control antibody or blocking antibodies against IL-6R or CD18. After their differentiation they were stimulated with LPS and the release of IL-10 was measured. Bars are mean and SD of duplicate wells representative of three experiments. (C) PGE2 was measured in day 6 culture supernatant of gmMϕ differentiated in low and high density. Bars are mean and SEM of 3 independent experiments. * $p < 0.05$ compared to low density.



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Immune modulating effects of a synovial fluid microenvironment on TLR induced cytokine production by dendritic cells

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Rheumatoid arthritis (RA) is a common autoimmune disease mainly affecting the joints. It involves many different immune cells including dendritic cells (DCs), which regulate the balance between tolerance and immunity and are able to produce large amounts of inflammatory cytokines. Since immune processes important for RA are mainly located within the synovial cavity, we aimed to determine the effect of a synovial fluid (SF) containing environment on DC function. Monocyte-derived DCs were cultured in the presence of 10% RA SF in combination with different Toll-like receptor (TLR) stimuli. Cytokine production, DC maturation and effects on T cell polarization were analyzed. SF did not induce inflammatory cytokine production by DCs, but appeared to have strong inhibitory effects on TLR induced TNFα, IL-12, IL-1β, IL-6 and MCP-1 release. In contrast, the more anti-inflammatory cytokines IL-10 and IL-27 were not affected by the presence of SF. SF-exposed DCs did fully up regulate MHC and co-stimulatory molecules upon TLR stimulation, but T cell polarization was affected resulting in an increased Th2 differentiation. The inhibitory components in RA SF were heat-stable and also present in non-RA SF. A role for IL-10, TGFβ or hyaluronic acid was excluded. The inhibitory effect of SF could be reproduced by a fatty acid enriched lipid fraction isolated from RA SF. A SF containing environment thus has strong immune modulating effects on DCs with a protective role for lipid mediators.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation and subsequent damage to cartilage and bone, resulting in pain, swelling and loss of function of the joints. Normal synovial joints contain a thin layer of synovial fluid (SF) to lubricate the joint and to supply nutrients to the avascular articular cartilage. SF consists of a blood plasma ultra-filtrate with locally produced products, such as hyaluronic acid and lubricin, produced by synoviocytes lining the synovium. In RA, the synovial cavity is infiltrated with large amounts of immune cells, including T cells, B cells, neutrophils, macrophages and dendritic cells (DCs) often accompanied by an increased volume of SF.

Myeloid DCs are found increased in RA SF[1;2], where they play a critical role in the balance between tolerance and immunity.[3;4] As professional antigen-presenting cells they link the innate and the adaptive immune system, instructing T cell differentiation. In addition, DCs can produce inflammatory cytokines directly involved in the pathogenesis of RA, such as TNFα, IL-6 and IL-1β. DCs can get activated by pathogen or damage associated molecular patterns, immune complexes or cytokines. For the recognition of pathogen-derived or damage associated molecules DCs are equipped with a wide range of pattern recognition receptors, including Toll-like receptors (TLRs), C-type lectins, and nucleotide-binding oligomerization domain (NOD) proteins. Data from animal studies and *in vitro* experiments with human synovial tissue point to an important role for TLRs in the initiation and maintenance of synovial inflammation in RA (Reviewed in [5]). TLRs recognize both exogenous ligands from bacteria or viruses, such as lipopolysaccharide (LPS), lipopeptides and nucleic acids[6], and endogenous ligands, such as hyaluronic acid, fibronectin, heat-shock proteins and host mRNA.[7-11] These endogenous ligands are produced upon tissue damage and cell stress, processes likely to occur in the inflamed synovium. Hiterherto, it is unknown how immune cells are driven to orchestrate chronic immune responses or even breakthrough of tolerance in the synovial compartment. The purpose of this research was to investigate how SF would drive or affect such responses. RA SF contains high levels of inflammatory cytokines, chemokines and metalloproteinases that are important mediators of inflammation and damage. Consistent with this, joint aspiration whereby the excessive amount of SF (and SF resident immune cells) is removed has a beneficial effect on disease progression.[12] On the other hand also immune modulating effects have been described for RA SF. Cell-free RA SF and supernatants from TNFα stimulated fibroblasts have been shown to inhibit interferon responses in macrophages[13;14] It has also been shown that T cells isolated from RA SF respond poorly to antigens and that SF suppressed T cell proliferation *in vitro.*[15;16] However, other reports suggest an increased responsiveness of SF T cells on autologous recall antigens.[16]

We aimed to further elucidate the effects of the SF containing environment on DC activation taking place in the synovial joint. To study this we used monocyte-derived DCs and stimulated these cells with different TLR ligands in the presence of cell-fee RA SF. SF had a clear immune modulating effect on TLR stimulated DCs, not affecting DC maturation,

but potently reducing inflammatory cytokine production, resulting in Th2 skewing when cultured with naïve CD4 T cells. This inhibitory effect of SF could be reproduced by a fatty acid enriched lipid fraction isolated from RA SF, implicating anti-inflammatory/pro-resolution lipid mediators in RA SF as protective factors reducing DC cytokine production.

Methods

All experiments were performed in accordance with the Helsinki Declaration and approved by the local Medical Ethics Committee of the Radboud UMC and the UMC Utrecht.

Patients

SF was obtained from 20 RA patients attending the department of Rheumatology in the Radboud university medical center (Nijmegen) or the University Medical Center Utrecht. All patients fulfilled the American College of Rheumatology criteria for RA at the time of diagnosis and gave their informed consent. The SF was aspirated from the patients' joint as part of standard care and leftover material was used for this study. In addition blood was drawn from healthy donors and 5 RA patients. Patients using biological agents and/or high dose prednisolone were excluded from the study. In most experiments a pool of RA SF was used consisting of 7-12 different SFs. In addition to RA SF also SF was used from 2 psoriatic arthritis (PsA) patients, 2 osteoarthritis (OA) patients, 2 gout patients and 2 pseudogout patients (all from patients attending the department of Rheumatology in the Radboud university medical center, Nijmegen).

Culture and stimulation of monocyte-derived DCs

Monocytes were purified from heparinized blood by density-gradient centrifugation over Ficoll (Amersham Bioscience) followed by CD14 selection using anti-CD14 magnetic beads (Miltenyi Biotec). Isolated monocytes were cultured for 6 days in the presence of GM-CSF (800 U/ml; R&D) and IL-4 (500 U/ml; R&D) to generate monocyte-derived DCs (moDCs). Fresh culture medium with the same growth factors was added at day 3. Day 6 moDCs were harvested, washed and stimulated with TLR or dectin-1 agonists for 20-24 hours in the presence or absence of 10% pooled cell-free RA SF (unless otherwise specified). The following TLR agonists were used: Pam3CSK4 (5 µg/ml, EMC Microcollections), Poly(I:C) (25 µg/ml, InvivoGen), LPS (100 ng/ml, *E. coli* 0111:B4, Sigma) and R848 (2 µg/ml, InvivoGen) for TLR 2/1, 3, 4 and 7/8 respectively. The LPS was double-purified to remove any contaminating proteins.[17] Dectin-1 was stimulated by Curdlan (50 µg/ml, Wako).

Neutralizing TGFβ antibodies (kindly gifted by Dr. R. Lafyatis, Boston University, Boston, USA) and IL-10R blocking antibodies (both R&D) were used at a concentration of 10 µg/ml and were added to the DCs 30 minutes before SF addition.

Mixed leukocyte reactions (MLR)

At day 7, LPS and R848 stimulated moDCs with our without 10% SF were harvested, washed and resuspended in fresh culture medium in a concentration of $0.1x10^6$ cells/ml

to be replated ($5x10^3$/well) in round bottom 96-well plates. CD4+ T cells were obtained from another donor by negative selection using microbeads (Miltenyi) and MS columns. CD4+CD45RA+ naive T cells were separated from the CD4+CD45RO+ T cells by positive selection for CD45RO. CD4+CD45RA+ naive T cells ($50x10^3$) were co-cultured with the DCs for 7 days. At day 6, the cells were incubated with PMA (50 ng/ml; Sigma) and ionomycin (1 μg/ml; Sigma) overnight before collection of the supernatants.

Human embryonic kidney 293/TLR4-MD2-CD14 cell activation assay

Human embryonic kidney 293 (HEK293) cells stably expressing TLR4, MD2 and CD14 were obtained from Invivogen and cultured according to the manufacturer's instructions. Stimulations were performed when the cells reached confluence in flat bottom 96 well plates. HEK293/TLR4-MD2-CD14 were stimulated with medium or LPS (100 ng/ml or 1 μg/ml) in the presence of 10% pooled RA SF or medium. After 20 hours the supernatants were collected and IL-8 was measured by luminex.

Cytokine measurements

Levels of human IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17, TNFα, interferon (IFN)γ and MCP-1 were measured in culture supernatants using commercially available kits (Bio-Rad) according to the manufacturer's instructions. Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad). Sensitivity of the cytokine assay was < 5 pg/ml for all cytokines measured. For some experiments TNFα was measured using PeliKine Compact ELISA kits (Sanquin Reagents) according to the manufacturer's instructions.

RNA isolation and qPCR

Total RNA was extracted in 1 ml of TRI-reagent and treated with DNase to remove genomic DNA before being reverse-transcribed into cDNA. Quantitative real-time PCR was performed with SYBR Green Master mix and a ABI/Prism 7000 sequence detection system (both Applied Biosystems). Expression was normalized to the expression of GAPDH and deployed as relative expression. Primer sequences are shown in Table 1.

Flow cytometry

Immature DCs or DCs maturated by LPS and R848 in the presence or absence of SF were stained with mAbs against human CD80 (BD Biosciences), CD83 (Beckman Coulter), CD86 (BD Pharmingen), MHC-I (clone W6/32) and MHC-II (clone Q1514). Expression was visualized using FITC-conjugated goat anti-mouse IgG (Zymed Laboratories). The level of dead cells was determined by staining with propidium iodide. Cells were analyzed with a FACSCalibur (BD Biosciences) for the proportion of positive cells and the mean fluorescence intensity relative to cells stained with the relevant IgG isotypes.

Measurement of metabolic activity

Metabolic activity of DCs was measured by a XTT assay (Roche Diagnostics). Briefly, DCs ($50x10^3$ cells/well) were stimulated with LPS and R848 with or without SF in 96 well culture

plates and incubated for 20 h. Then, 75 µl of the XTT test solution, prepared by mixing 1 ml of the XTT labeling reagent and 20 µl electron coupling reagent, was added to each well. After 4 h incubation at 37°C, the absorbance was measured on an ELISA reader (Tecan Sunrise) at a test wave length of 450 nm and a reference wavelength of 595 nm.

Lipid extraction

400 µl RA SF was acidified by adding 200 µl HCOO-NH_4 (pH 3.3) after which 1 ml ethyl acetate was added. This was mixed at room temperature for 30 minutes before centrifuging 5 min at 15.000g. The water layer was frozen in a -60°C alcohol bath after which the upper layer was transferred to a new vial. The water layer was thawed and new ethyl acetate was added to do a second round of lipid isolation to increase efficiency. The two lipid containing upper layers were pooled and dried under a N_2-flush. The dried lipids were stored at -80 and dissolved in complete culture medium in a concentration similar to that in SF. The samples were sonicated for 10 minutes to improve their solubility.

Statistical analysis

Statistical analysis was performed using GraphPad software. Differences were analyzed with a paired or unpaired students T test depending on the comparison. Differences were considered significant when $p < 0.05$.

Results

Synovial fluid inhibits TLR induced TNFα and IL-12 production by moDCs

To determine the effect of RA SF on DC activation we first cultured monocyte-derived DCs (moDC) from healthy donors and added varying concentrations of pooled cell-free RA SF (1, 2, 5, 10 & 20%) for 20 hours, and determined cytokine induction. SF alone did not induce TNFα, IL-12p70 or IL-10 production by DCs (data not shown). To evaluate whether SF could influence DC activation by TLRs we stimulated the cells with LPS (TLR4 ligand) and R848 (TLR7/8 ligand) in combination with varying concentrations of SF. Counter intuitively, increasing amounts of RA SF dose dependently decreased TNFα and IL-12p70 production, while the release of IL-10 was unaffected (Figure 1A). As the addition of 10% SF already gave almost maximal inhibition this concentration was used throughout the following experiments. To assess whether the observed inhibitory effect mediated by SF on moDCs was specific for certain stimuli, we next stimulated moDCs with a broad range of TLR ligands. In line with previous observations, 10% SF inhibited TNFα induction by TLR1/2 (Pam3CSK4), TLR3 (Poly(I:C)), TLR4 and TLR7/8 stimulation (Figure 1B). IL-12p70 release was also reduced by SF when combined with strong IL-12p70 inducing stimuli such as LPS, R848 or a combination of Poly(I:C) and Pam3CSK4 (Figure 1B and data not shown). IL-10 production on the other hand was unaffected. SF thus dampens inflammatory cytokine release upon TLR stimulation of moDCs. To determine if this inhibitory effect extends beyond TLR stimulation, moDCs were also stimulated via dectin-1 by curdlan in combination with SF, which led to similar results (Figure 1B). The inhibitory effects of

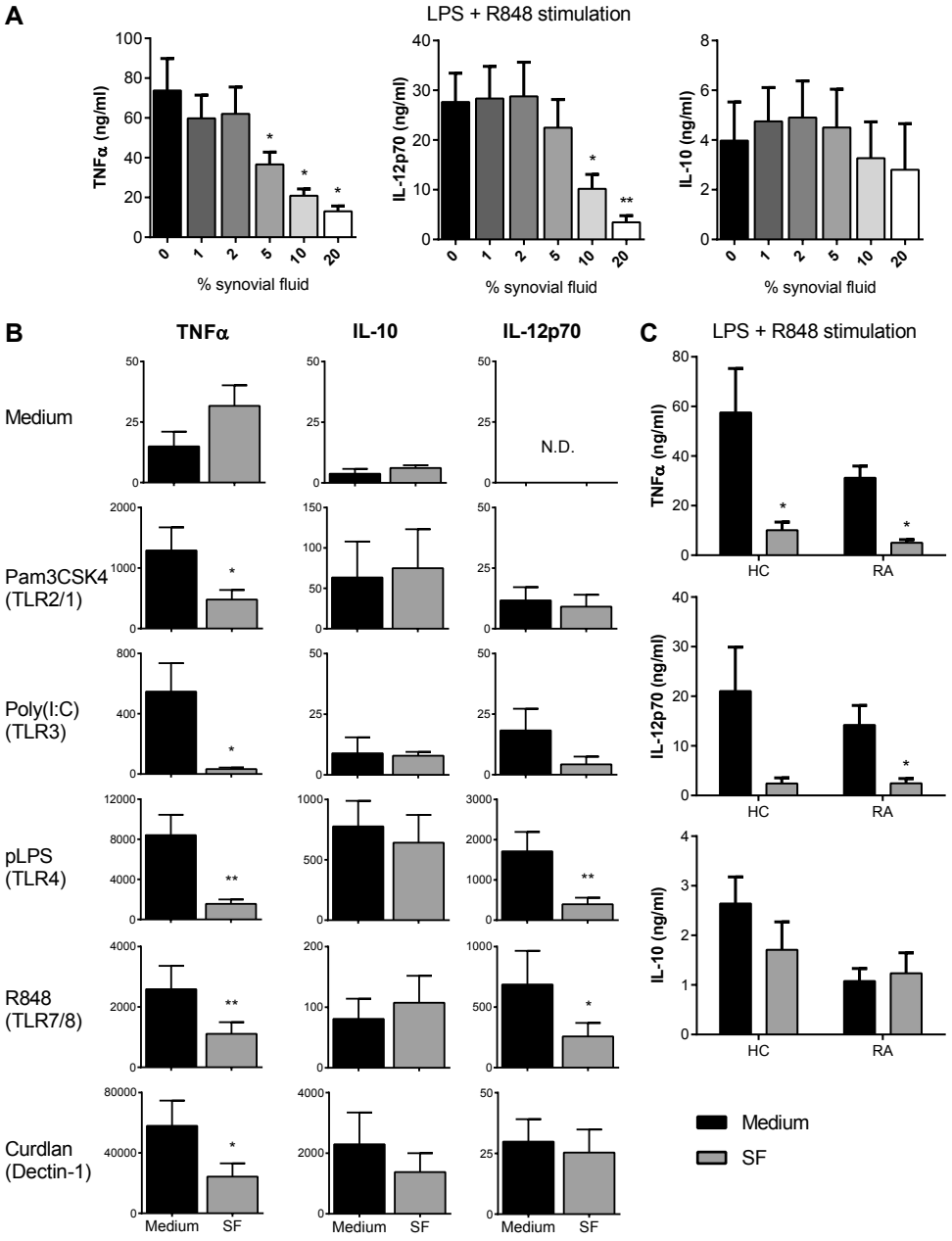

Figure 1. RA SF inhibits TLR induced inflammatory cytokine production by DCs. (A) MoDCs of 5 donors were cultured with 1-20% pooled cell-free RA SF combined with LPS and R848 stimulation. Cytokine production was measured in the supernatant after 20 hrs. (B) MoDCs were stimulated with different TLR ligands or curdlan in combination with 10% pooled RA SF. At least 6 donors were included for each stimulation (C) Healthy donors (n=4) and RA patients (n=5) were stimulated in parallel with 10% SF combined with LPS and R848. Bars are mean and SEM. * $p<0.05$, ** $p<0.01$ compared to control without 10% RA SF.

SF on TLR induced cytokine production were robustly replicated throughout multiple independent experiments including 5 RA patients (Figure 1C).

The addition of SF to DC cultures did not negatively affect cell survival, as showed by propidium iodide staining, or a metabolic activity measurement (Figure 2A,B), excluding the possibility that cell death caused the observed reduction in cytokine production. SF even increased DC metabolic activity, indicating an active process induced by SF. To assess whether the high viscosity of SF could cause the observed effects, we reduced SF viscosity by sonication or hyaluronidase treatment. These alterations did not affect the effect of SF suggesting that steric hindering prohibiting TLR ligand binding could not explain the inhibitory effect of SF. (Figure 2C,D). To further substantiate that SF mediated inhibition of TLR mediated activation is an active process rather than inhibition of ligand-receptor binding, we investigated whether the SF effect was time-dependent. Interestingly, the inhibitory effect of SF was clear even when added up to 4 hours after TLR stimulation (Figure 2E), although the inhibitory activity was maximal when added within 2 hours from the TLR stimulation. Although SF could be added up to 2-4 hours after TLR stimulation and still have an inhibitory effect, it had to be present during TLR stimulation, because only pre-incubation of the cells in SF for 4 hours was ineffective (Figure 2F).

Synovial fluid has a broad effect on cytokine production and affects the IFN pathway

To better understand the effects sorted by SF we investigated the production/secretion of a wider panel of inflammatory mediators. IL-1β, IL-6 and MCP-1 (CCL2) secretion (Figure 3A) as well as the mRNA expression of different IL-12 family members (IL-12p35, IL-12p40 and IL-23p19)(Figure 3B) were significantly reduced upon TLR triggering in the presence of SF. SF thus does not only affect IL-12 secretion, but already prevents the induction of its mRNA expression early on. In sharp contrast, IL-27p28 and EBI3 levels, together forming IL-27, were not altered by the presence of SF upon stimulation with TLR4/7/8 agonists, similar to what is seen for IL-10 (Figure 3B).

To follow up on data from Gordon *et al.* who showed that RA SF blocked the interferon pathway upon macrophage stimulation with TNFα[13], we assessed the effect of SF on the IFN pathway upon TLR stimulation, which is critically involved in the induction of IL-12.[18] The combination of LPS and R848 induced IFNβ and IFNλ1 but not IFNα mRNA expression after 4 hours which was almost fully abrogated in the presence of SF (Figure 3C and data not shown). In addition, the expression of the transcription factors IRF 1, 7 and 8, which are involved in the induction of IFNs and IL-12, was also inhibited by SF (Figure 3C). The inhibitory effect of SF on TLR induced cytokine production however is not limited to the abrogation of the type 1 IFN amplification loop, since it also reduced TLR2 induced cytokine production and TLR2 triggering does not induce IFN production.[19] Stable levels of IL-10 and IL-27 induction upon TLR stimulation in a SF containing environment shows that TLR induced cytokine production is not totally prevented but shifted towards more immune-regulating cytokines.

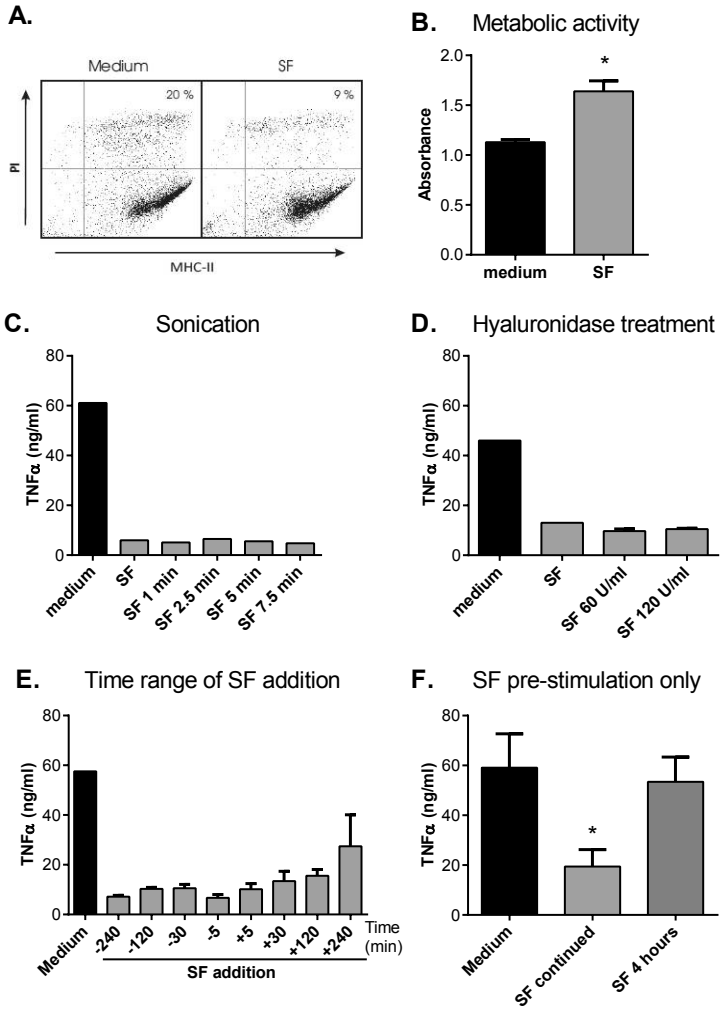

Figure 2. Reduced cytokine production is not caused by cell death or SF viscosity. (A) DCs were stimulated with LPS and R848 in the presence or absence of 10% SF for 20 h. Cell death was determined by double staining for MHC-II and propidium iodide (PI). The dot-plots are representative for 3 independent experiments. (B) DCs were stimulated similar to described in A. After 20 h, XTT test solution was added to the DCs and the absorbance was measured after 4 h incubation at 37 °C. The relative absorbance as depicted in the figure is the absorbance measured at the test wave length of 450 nm minus the absorbance at the reference wave length of 595 nm, measured in triplo. (C) SF was sonicated for 1-7.5 minutes reducing its viscosity. The sonicated SF was then used in combination with LPS+R848 stimulation. (D) Pooled RA SF was treated with hyaluronidase (60 U/ml or 120 U/ml) for 30 minutes at 37°C, before addition to DCs and costimulation with LPS+R848. (E) SF was added to LPS+R848 stimulated cells from 4 hours before till 4 hours after TLR stimulation. The mean and SD of two donors are shown. (F) moDCs were pre-treated with SF for 4 hours and SF was washed away before stimulation with LPS+R848 (n=4). * $p<0.05$ compared to control without 10% RA SF.

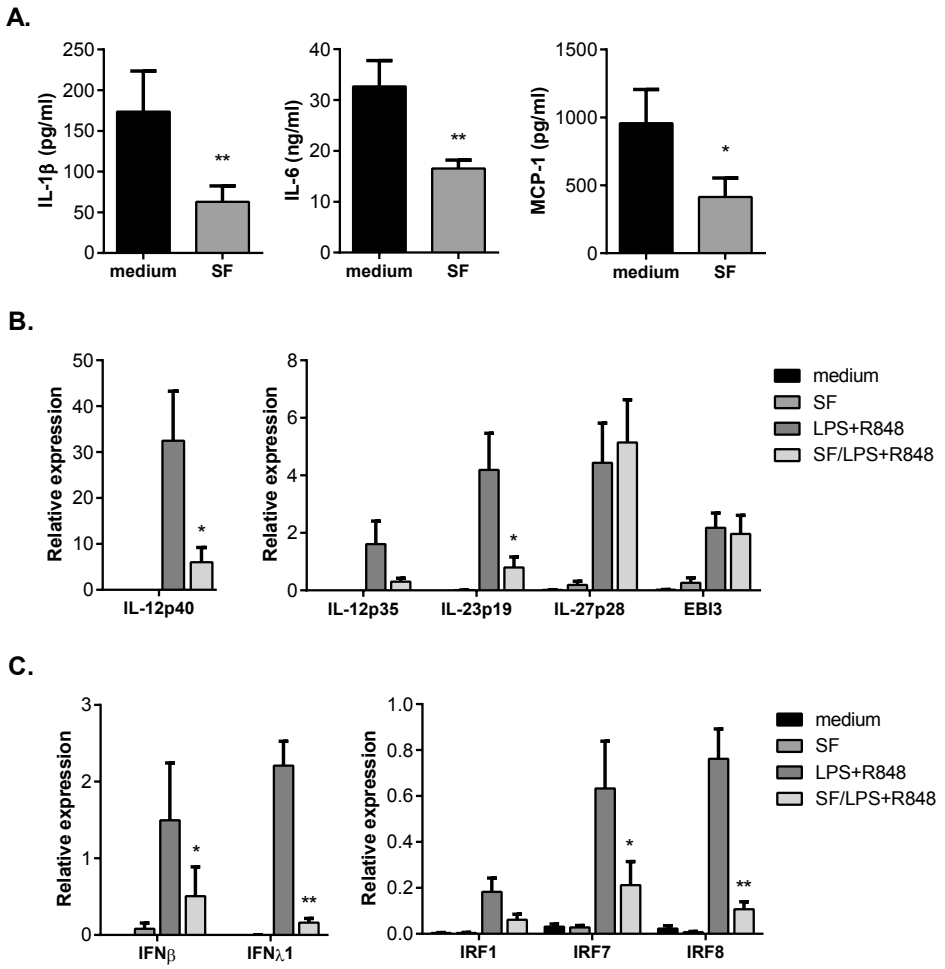

Figure 3. Production of multiple cytokines and IFNs is affected by RA SF. All cells were stimulated with a combination of LPS and R848, in presence of absence of 10% SF. (A) IL-1β, IL-6 (both n=10) and MCP-1 (n=5) release in the supernatant was determined by luminex. (B) qPCR was performed to determine the mRNA expression of all IL-12 family subunits 4 hours after DC stimulation with LPS+R848 in the presence or absence of 10% SF. (C) IFNβ, IFNλ1, IRF1, IRF7 and IRF8 mRNA was also determined 4 hours after DC stimulation. mRNA expression levels are normalized to GAPDH expression and depicted as relative expression. Bars are mean and SEM of 4-7 experiments. * $p<0.05$, ** $p<0.01$ compared to control without 10% RA SF.

Addition of synovial fluid induces a semi-mature phenotype that affects T cell polarization

Normally, upon activation by so-called danger signals, DC undergo a maturation process that is typified by an increased expression of cell surface markers adept for antigen presentation (MHC molecules, CD80, CD86). As shown in figure 4A, SF does not induce DC maturation itself. However, in contrast to the effects seen on cytokine production, the presence of SF

did not alter the ability of TLR ligands to induce DC maturation implying that the addition of SF leads to so-called "semi-mature" DCs which display a matured phenotype but decreased cytokine production compared to fully matured DCs (Figure 4A). To assess whether this SF induced semi-mature DC phenotype affected T cell instruction we performed an allogeneic MLR. Despite the markedly reduced IL-12p70 production by DCs stimulated in the presence of SF, the effect on IFNγ release was minimal, with a consequent but only marginal decrease in IFNγ release (not significant) (Figure 4B). However, SF-exposed DCs did induce more IL-13 production by differentiated T cells (P=0.022), representative for a polarization towards Th2 cells (Figure 4B). The characteristic Th17 cytokine IL-17 showed a trend towards decreased production in the presence of SF-exposed DCs (P=0.052). SF thus, most likely via altered cytokine production by DCs, also affects T cell polarization promoting Th2 responses and possibly reducing Th17 induction.

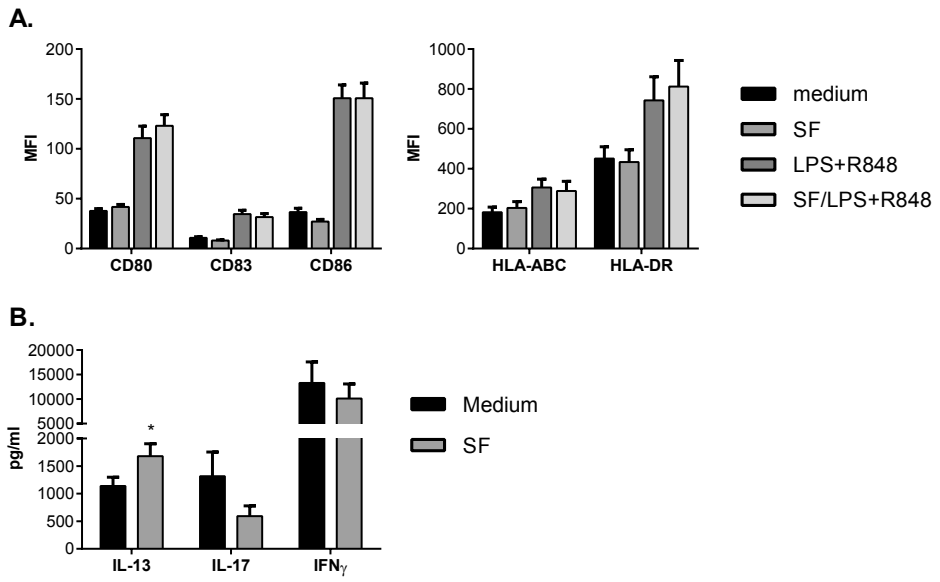

Figure 4. SF does not affect TLR induced DC maturation, but does affect T cell polarization. (A) DCs were stimulated with LPS and R848 and maturation markers were measured by flow cytometry after 20 hours of stimulation in the presence or absence of 10% SF. Bars represent mean and SEM of 7 donors. (B) MoDCs were stimulated with LPS+R848 in the presence or absence of 10% SF. After 20 hours the cells were harvested, washed and cocultured with allogeneic CD4+CD45RA+ naïve T cells (DC:T cell ratio 1:10) for 7 days. At the end of day 6 PMA and ionomycin was added and supernatant was taken after overnight stimulation. Bars are mean and SEM of 10 donors. * $p<0.05$ compared to control without 10% RA SF.

The inhibitory effects of SF are caused by a heat stable fraction, including lipids

In an attempt to identify the inhibitory factor(s) in RA SF, the SF was heat inactivated at 56°C for 30 minutes which had no effect on the inhibitory effect (Figure 5A), excluding heat sensitive proteins such as complement factors as the inhibitory factor. To gain more insight into the factors that might be involved, RA SF was compared to SF from patients with other rheumatologic conditions. This revealed that the inhibitory factor present in RA SF was not specific for RA being also present in other sources of SF tested (Figure 5B). It therefore seems to be a general SF factor. Hyaluronic acid was already excluded since hyaluronidase treatment to reduce SF viscosity had no effect on the SF inhibition (Figure 2D). Similarly, antibodies neutralizing TGFβ or blocking the IL-10 receptor did not affect the inhibitory effect of SF (data not shown). Additional experiments with HEK392 cells stably expressing TLR4, MD2 and CD14 confirm that the presence of SF does not prevent binding of LPS to TLR4 (Figure 5C). The fact that LPS induced IL-8 production in these cells was unaffected by the presence of SF further suggests that the effect of SF was dependent on receptors or signaling pathways that are present in moDCs but not in HEK cells. 10% SF on its own did induce some IL-8 production in these cells, but this was only minor compared to the levels upon LPS stimulation.

RA SF has been shown by others to include several fatty acid derived pro-resolving lipids, including maresins, resolvins and lipoxin A4 (LXA_4)[20], that could be involved in the inhibitory effects of SF we observed. LXA4 for example has been shown to be able to inhibit both the NF-κB and the IFN pathway.[21-23] Interestingly, formyl peptide receptors including the LXA_4 (and resolving D1) receptor ALX/FPR2, are not expressed in HEK293 cells.[24;25] To determine if this group of fatty acid derived lipid mediators could be involved in the inhibitory effects of SF we observed, we performed an ethyl acetate based lipid extraction on RA SF. This fatty acid enriched lipid fraction, brought back to its original concentration, was able to inhibit LPS induced TNFα production to a similar level as seen with complete synovial fluid (Figure 5D). This supports the presence of potent anti-inflammatory/pro-resolution lipids in RA SF, which are able to reduce TLR induced inflammatory cytokine production.

Discussion

Although RA SF is known to contain many inflammatory mediators, cell-free SF appears to have an anti-inflammatory effect on TLR or dectin-1 stimulated DCs. The presence of SF reduced the capacity of DCs to produce inflammatory cytokines, such as TNFα, IL-12, IL-1β and IL-6 upon stimulation with different TLR ligands. The induction of IL-10 protein and IL-27 mRNA subunits, two cytokines mainly known for their anti-inflammatory functions, was not affected by the presence of SF, supporting active immune modulation and not only dampening of cytokine production. SF-exposed DCs did up regulate maturation markers to a full extend upon TLR triggering, but T cell instruction was altered and shifted towards a possibly protective Th2 phenotype. SF thus contains anti-inflammatory factors, including lipids, that can play a protective role in the joint, limiting inflammatory responses of DCs

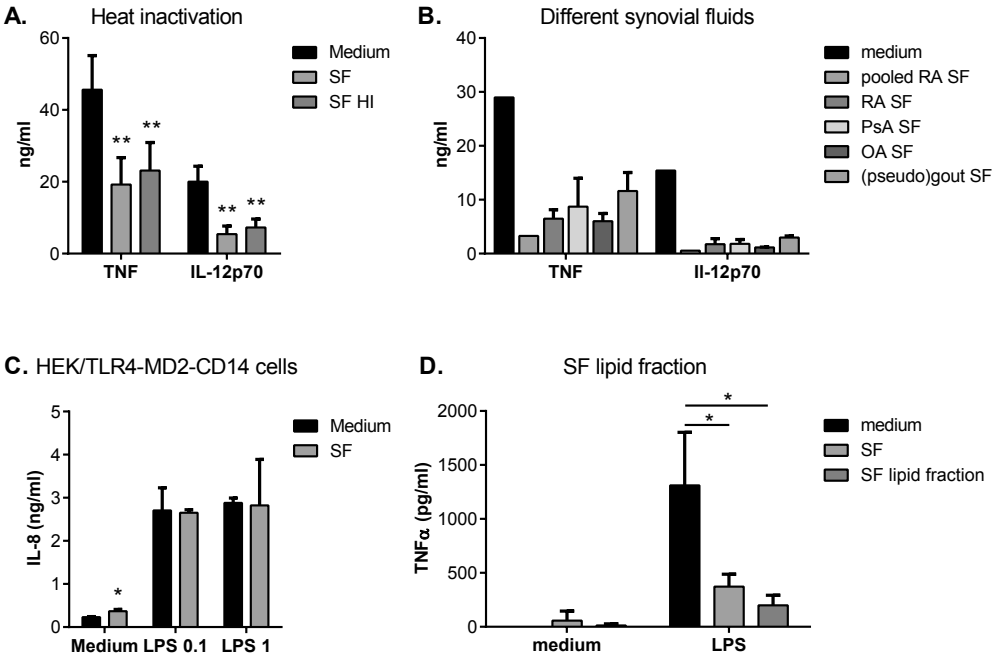

Figure 5. The inhibitory component in SF is heat stable, not specific for RA and includes lipids. moDCs were stimulated with LPS+R848 in the presence or absence of 10% SF for panel A and B. (A) SF was used untreated or after incubation at 56°C for 30 minutes (HI) (n=7). (B) In addition to pooled RA SF, also synovial fluids from separate RA patients (n=3), PsA patients (n=2), OA patients (n=2) and (pseudo)gout patient (n=4) were included. (C) HEK/TLR4-MD2-CD14 cells were stimulated with LPS, 10% pooled RA SF or a combination of these. IL-8 production was measured in triplo. (D) A lipid fraction was isolated from 4 RA SFs by an ethyl acetate extraction. MoDCs were stimulated with LPS, 10% SF (from 4 RA patients), SF lipids (isolated from SF of the same RA patients) or a combination of these and TNFα production was measured by elisa. Bars represent combined data of 4 different SFs and matched SF lipids. Data are representative for 3 moDC donors. * p<0.05, ** p<0.01 compared to control without 10% SF.

upon pathogen or host derived danger signals that trigger these cells via TLRs or dectin-1.

Synovial fluid is a very complex fluid containing many different factors and a wide variety of immune cells. RA SF contains MMPs that have destructive effects on the cartilage, chemokines such as MCP-1 attracting immune cells from the circulation and many different (pro- and anti-inflammatory) cytokines and lipids interacting with immune cells and fibroblasts. The mixture of factors present in SF can therefore have different effects on different cell types and also influence the disease process by attracting new immune cells to the joint. We therefore cannot say that SF has an anti-inflammatory function in general, but it does contain factors that limit further inflammatory responses by DCs and possibly other monocyte-derived cells such as macrophages, as was suggested by others.[13] We only looked at a fraction of the processes going on in an inflamed joint, but this can help to identify factors present in the SF that can influence inflammation in a positive way that might be interesting tools for treatment to induce resolution and end chronic inflammation

going on in RA.

Despite the inhibitory effects of SF during TLR stimulation of DCs, the presence of SF is not able to prevent chronic inflammation in RA patients. This could implicate that (endogenous) TLR ligands present in the RA SF are not a major driver of the inflammatory process, that they mainly induce inflammation via other cell types, or that the inhibitory function we observe in vitro is not sufficient or dysfunctional in vivo. The experiments presented here were all performed with moDCs, which were used as a model system for myeloid DCs and inflammatory monocyte-derived DCs found in the RA joint [26]. We confirmed the inhibitory effect of SF on TLR induced cytokine production by moDCs cultured from RA patients, but to determine if DCs in the RA joint might be insensitive to this inhibition, cross-over experiments have to be performed with mDCs isolated from matched peripheral blood and synovial fluid, cultured in their own or each other's microenvironment. With these experiments we will be able to identify if RA synovial mDCs respond similar to SF in their environment compared to blood mDCs or that these cells have become insensitive to inhibitory factors present in the SF. Only limited data is available comparing TLR induced cytokine production by mDCs isolated from the peripheral blood or SF, which mainly pointed towards an increased IL-10 production by mDCs from SF while TNFα was similar to that from blood mDCs.[1] However, it remains yet to be investigated how these cells would respond in a SF containing environment.

The inhibitory fraction of RA SF was found to be heat-resistant, not affected by sonication or hyaluronidase treatment, and unlikely to be a RA specific mediator since similar effects were seen with OA, PsA and (pseudo)gout SF. Fatty acid derived lipids like prostaglandins, leukotrienes, lipoxins, resolvins and protectins are important lipid mediators involved in the regulation of inflammatory processes from initiation to resolution. These short distance signaling molecules are produced in the joint and are found in the synovial fluid.[20;27] Although some of these lipids have been found increased in RA SF compared to OA SF, there is a large overlap between levels found in OA SF and in RA SF.[27] The lipid containing fraction of RA SF showed an inhibitory effect on LPS induced cytokine production, which was at least as strong as seen with complete RA SF. This clearly shows the anti-inflammatory/pro-resolution potential of this lipid fraction, but additional experiments including lipid exclusion from RA SF are necessary to determine if this fraction is the sole inhibitory component of SF on TLR stimulation or if other factors are also involved. Then, the next step would be to further characterize which lipids are responsible for this inhibitory effect. LXA_4 would be an interesting candidate. It has been found in RA and OA SF, can regulate inflammatory cytokine production and the influx of inflammatory cells and is necessary for resolution of collagen-induced arthritis in mice.[20;28] But also omega-3 fatty acid derived products such as resolvins and protectins could have similar protective effects and increased dietary intake of omega-3 fatty acids from fish oils seems to be beneficial for RA patients and have protective effects in murine arthritis models.[29-31]

In conclusion, a SF containing environment dampens inflammatory cytokine production by DCs upon TLR stimulation and directs T cell differentiation towards a more protective Th2

phenotype. This could be part of a natural feedback mechanism that is aimed to dampen inflammatory responses and protect the joint integrity, in which lipid mediators seem to play an important role. Further characterization of the exact mediators involved in this immune modulatory effect from SF could provide new therapeutic opportunities.

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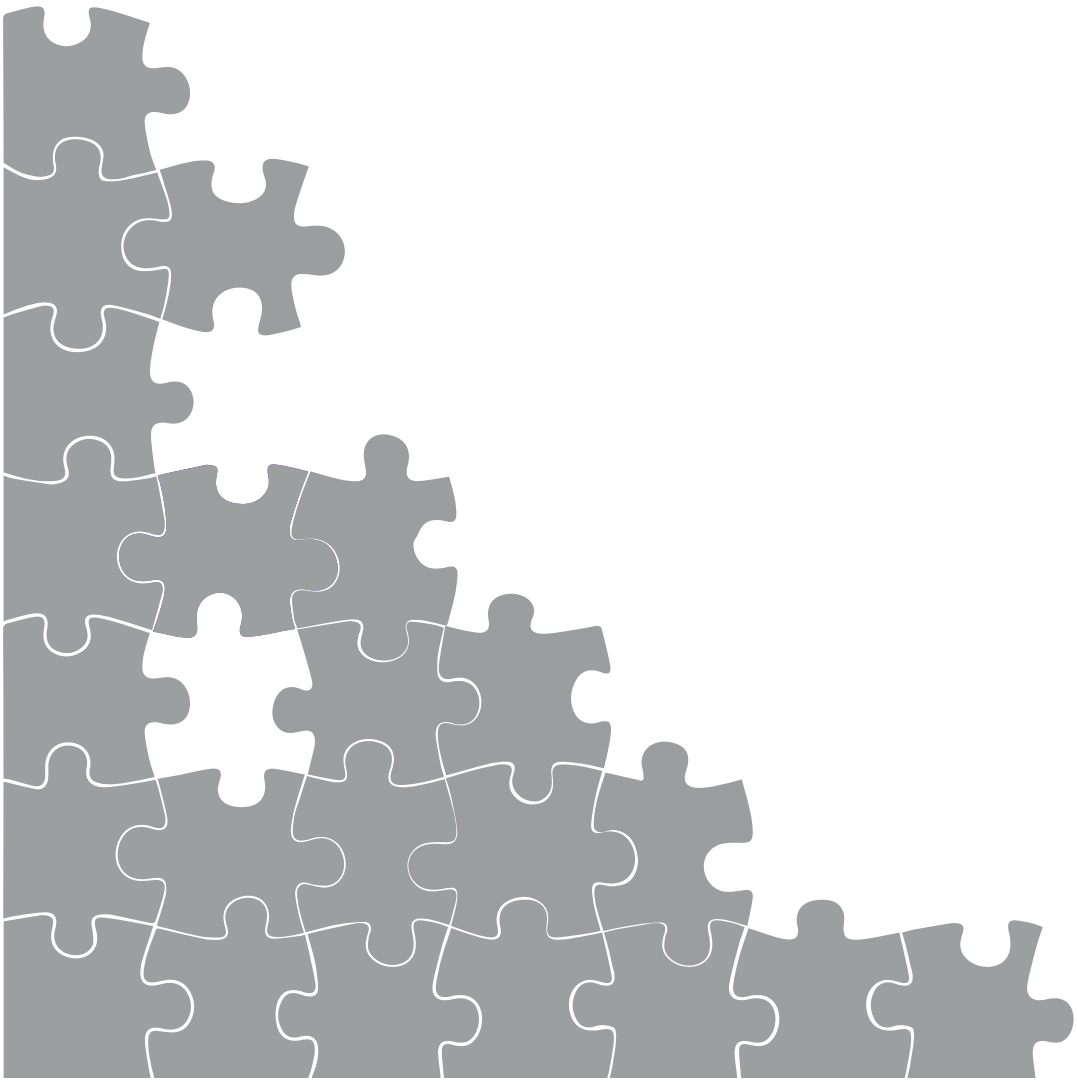

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Impaired response towards *Porphyromonas gingivalis* by dendritic cells and PBMCs from rheumatoid arthritis patients

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Submitted for publication

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The prevalence of periodontitis is increased in rheumatoid arthritis (RA) patients and the severity of periodontitis can affect the level of arthritis. *Porphyromonas gingivalis* is one of the main bacteria involved in periodontitis. Our aim was to determine if there are differences in the innate immune response against *P. gingivalis* between healthy controls and RA patients. Monocyte-derived dendritic cells (DCs) from healthy controls, RA and psoriatic arthritis patients were stimulated with *P. gingivalis*, a range of other bacteria and TLR agonists. Cytokine production was determined and blocking studies were performed to determine which receptors were involved in differential recognition of *P. gingivalis*. Effects on T cell cytokines were also determined in PBMC cultures. Upon stimulation with *P. gingivalis* RA DCs produced less TNFα, IL-12p70 and IL-6 as compared to healthy control DCs, an observation that was not present in patients with psoriatic arthritis nor upon stimulation with other bacteria. In addition, *P. gingivalis* mediated activation of RA PBMCs showed a clear effect on T cell differentiation. From the various possibly underlying mechanisms investigated, only blockade of Mac-1/complement receptor 3 abolished the difference between RA patients and healthy controls, suggesting the involvement of Mac-1 in this process. In conclusion, immune cells from RA patients display a reduced response to *P. gingivalis* which has functional consequences for the immune response. This may result in prolonged survival of *P. gingivalis* possibly driving autoantibody formation and a self-perpetuating loop of chronic inflammation. The possible role of Mac-1 therein warrants further investigation.

Introduction

Rheumatoid arthritis (RA) and chronic periodontitis are two chronic inflammatory diseases resulting in destruction of the synovial joint or the tissue surrounding the teeth (the periodontium), respectively. Several studies clearly demonstrated an epidemiological association between these two diseases.[1-6] Both diseases share some common susceptibility factors including genetic factors and environmental factors like smoking.[7;8] This raises the question whether periodontitis is a risk factor for the development of RA, whether RA predisposes to periodontal disease or whether both diseases present more often together because of common risk factors or a similar disease mechanism. Increased prevalence of periodontitis is already seen in early non-treated RA patients, suggesting periodontitis could be present before RA onset and the increased prevalence of periodontitis seen in RA patients is not a consequence of RA treatment.[4;5] Several small studies indicated that extensive tooth cleaning and oral hygiene instruction to treat periodontitis can also positively affect RA disease activity.[9-11]

Chronic periodontitis is caused by a complex biofilm containing mainly gram-negative bacteria, often including *Porphyromonas gingivalis*. This bacterium is specifically interesting in the context of RA because it is the only prokaryotic organism expressing a peptidylarginine deiminase (PAD)[12], which is able to citrullinate bacterial and human proteins.[13] Anti-citrullinated protein antibodies (ACPAs) are the most sensitive and specific auto-antibodies found in RA patients.[14;15] These antibodies can often be found several years before the clinical onset of RA and are associated with disease progression.[16] This led to the hypothesis that *P. gingivalis* might be involved in the development of ACPAs and possibly RA in a susceptible host. Associations between *P. gingivalis* antibodies and ACPA levels in RA patients support this hypothesis.[17;18]

Prompted by these observations we set forth to investigate a possible immunological link between RA and *P. gingivalis*. Hence, we focused on the innate immune response towards *P. gingivalis* in RA patients versus healthy controls, because this is the first line of defense against bacteria like *P. gingivalis*. Dendritic cells (DCs) are important sentinels of the immune system sampling the micro-environment for potential microbial antigens and initiating an adept immune response when necessary. They are found to co-localize with *P. gingivalis* proteins in the oral mucosa of patients with periodontitis.[19] Furthermore, DNA of *P. gingivalis* has been found in myeloid DCs from the peripheral blood in 72% of orally colonized periodontitis patients.[19] These DCs could be involved in the dissemination of bacteria to distal sites like atherosclerotic plaques or the synovium, where multiple researchers have found DNA from *P. gingivalis* (and other (oral) bacteria) in patients with RA.[20-22]

We found that the immune response to *P. gingivalis* in RA patients is markedly altered which might lead to an increased oral bacterial burden, thereby possibly driving the self-perpetuating loop of chronic inflammation.

Materials and Methods

Study population

In total 35 RA patients and 9 patients with psoriatic arthritis (PsA) attending the Department of Rheumatology in the Radboud university medical center or the University Medical Center Utrecht were included. All patients fulfilled the American College of Rheumatology criteria for RA or the CASPAR criteria for PsA at the time of diagnosis and gave their informed consent. Patient characteristics are shown in Table 1. Patients using biological agents and high dose prednisolone were excluded from the study. In addition 27 healthy controls were included. The mean age of the healthy controls was 37 (range 22-61) and 65% was female. Experiments were performed in accordance with the Helsinki Declaration and approved by the local Medical Ethics Committees of the Radboud UMC and the UMC Utrecht.

Table I. Patient characteristics

	RA	**PsA**
Patients, n	35	9
Age in yr; mean (range)	64 (43-88)	57 (23-77)
% Female	69	33
Disease duration (yr); mean (range)	8.1 (0-26)	8.3 (1-45)
Methotrexate use, n (%)	23 (66)	7 (78)
Sulfasalazine use, n (%)	10 (29)	2 (22)
Hydroxychloroquine, n (%)	7 (20)	0 (0)
Prednisolon, n (%)	3 (9)	1 (11)
Leflunomide, n (%)	1 (3)	0 (0)
No DMARD/prednisolone, n (%)	4 (11)	2 (22)
ESR, mean (range)	12.4 (2-33)	8 (2-18)
CRP ≥5, n (%)	11 (31)	5 (56)
RF positivity, n (%)	24 (69)	1 (17) §
ACPA positivity, n (%)	20 (67) *	NA
Erosions, n (%)	21 (60)	3 (33)
DAS28, mean (range)	2.8 (1.1-6.0)	NA
TJC, mean (range)	3.5 (0-18)	5.2 (0-11)
SJC, mean (range)	2.5 (0-8)	4.4 (0-11)

DMARD, disease modifying anti-arthritic drug; ESR, erythrocyte sedimentation rate; CRP, C reactive protein; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibody; DAS28, Disease activity score of 28 joints, including ESR; TJC, tender joint count; SJC, swollen joint count; NA, not applicable. * ACPA status was unknown for 5 RA patients. § RF status was unknown for 3 PsA patients.

Cell isolation and culture of monocyte-derived DCs and macrophages

PBMCs were isolated from heparinized venous blood using density-gradient centrifugation over Ficoll-Paque (Amersham Bioscience). Subsequently, monocytes were obtained using CD14 microbeads and MS columns (Miltenyi Biotec). Isolated monocytes were cultured for 6 days in the presence of GM-CSF (800 U/ml; R&D) and IL-4 (500 U/ml; R&D) to generate monocyte-derived DCs (moDCs) or with GM-CSF (800 U/ml) only to generate macrophages. Fresh culture medium with the same growth factors was added at day 3.

Cell stimulation

Day 6 moDCs or macrophages were harvested, plated in 96 well culture plates (25.000 cells/ well) and stimulated with a range of TLR agonists or heat-killed bacteria for 18 hrs. The following TLR agonists and heat-killed bacteria were used: LPS (100 ng/ml, *Escherichia coli* 0111:B4, Sigma-Aldrich), Pam3CSK4 (5 µg/ml, EMC Microcollections), FSL-1 (1µg/ml, EMC Microcollections), *P. gingivalis* (ATCC 33277, $1x10^7$/ml, Invivogen), *Proteus mirabilis* (ATCC 142723, $1x10^7$/ml), *Salmonella typhimurium* (ATCC 13311, $1x10^7$/ml), *Shigella sonnei* (ATCC 11060, $1x10^6$/ml), *Klebsiella pneumoniae* (ATCC 13883, $1x10^7$/ml), *E. coli* ($1x10^7$/ml), *Streptococcus mutans* ($1x10^7$/ml), *Prevotella intermedia* (ATCC 9336, concentration unknown) and purified *P. gingivalis* LPS (1 µg/ml, Invivogen). *E. coli* LPS was double-purified to remove any contaminating proteins [23]. All bacteria except *P. gingivalis*, *S. mutans* and *E. coli* were cultured in the Department of Medical Microbiology. The bacteria were heat-killed by incubation at 60 °C for 1 hour. The *E. coli* was a kind gift from J. Butcher (Institute of Infection and Immunity, Glasgow). CXCR4 and Mac-1 were blocked by adding antibodies against CXCR4 (Clone 12G5, functional grade, 10 µg/ml, eBioscience) or CD18 (Clone L19, 5 µg/ml) 30 min before *P. gingivalis* stimulation. An isotype control was used in equal concentrations. PBMCs ($0.5x10^6$ cells/well) were also directly stimulated with *P. gingivalis* or *P. intermedia* for 7 days to determine effects on T cell differentiation.

Flow cytometry

Using standardized flow cytometry protocols as described previously[24] CD18 and CD11b expression was determined on moDCs from RA patients and healthy controls. Clone L19 (anti-CD18) and bear-1 (anti-CD11b) were a kind gift from A. Cambi (Tumor Immunology, Nijmegen). Expression was visualized via a FITC labeled goat-anti mouse secondary antibody. Cells were analyzed using Flowjo for the mean fluorescence intensity relative to cells stained with the appropriate IgG isotypes.

RNA isolation and real-time PCR

Total RNA was extracted in 0.5 ml of TRI-reagent and treated with DNase to remove genomic DNA before being reverse-transcribed into cDNA. TLR1, 2, 4 and 6 and CXCR4 mRNA was quantified by quantitative real-time PCR as previously described [25]. qPCR signals were quantified by comparing the cycle threshold value (Ct) of the gene of interest of each sample with the Ct value of the reference gene GAPDH (ΔCt) and were deployed as relative expression ($2^{-\Delta Ct}$). The following primers were used: GAPDH Forward ATCTTCTTTTGCGTCGCCAG, reverse TTCCCCATGGTGTCTGAGC; TLR1 forward GCATCTTCCATTTTGCCATT, reverse GAACGTGGATGAGACCGTTT; TLR2 forward GAATCCTCCATTCAGGCTTCTCT, reverse GCCCTGAGGGAATGGAGTTTA; TLR4 forward GGCATGCCTGTGCTGAGTT, reverse CTGCTACAACAGATACTACAAGCACACT; TLR6 forward GGCCGAAACTGGTTTATTGA, reverse GGAGTGATGATGGGAGGAGA; CXCR4 forward ATGAAGGAACCCTGTTTCCGT, reverse AGATGATGGAGTAGATGGTGGG.

Measurement of cytokines in culture supernatants

Levels of TNFα, IL-6, IL-10, IL-12p70, IL-13, IL-17 and interferon-γ (IFNγ) were measured in cell-free supernatants using commercially available kits according to the manufacturer's instructions (Milliplex, Millipore). Milliplex kits were measured on a Luminex 200 and the data was analyzed using Bio-Plex Manager software (BioRad).

Statistical analysis

Differences between groups were analyzed using a Mann-Whitney U test. A paired Student's t-test was used to compare differences upon stimulation within the same donor. P values less than 0.05 were considered significant.

Results

Decreased cytokine production by RA immune cells upon stimulation with P. gingivalis

Monocyte-derived DCs were cultured from 31 RA patients and 23 healthy controls and were stimulated with heat-killed *P. gingivalis*. DCs from RA patients secreted significantly less TNFα (P = 0.0001) and IL-12p70 (P = 0.0093) after stimulation with *P. gingivalis* (Figure 1A and B). Those patients with decreased TNFα levels also showed decreased levels of IL-6 compared to their healthy counterparts (P = 0.0474) (Figure 1C). In contrast, IL-10 levels were not significantly different between RA patients and healthy controls (Figure 1D).

To investigate whether our results correlate with RA disease phenotype, we stratified our data accordingly. TNFα, IL-6, IL-10 and IL-12p70 levels were similarly distributed throughout all groups investigated suggesting that the observation of a decreased DC response in RA patients was a disease rather than a phenotype specific phenomenon (Figure 2A and data not shown). The disease specificity for RA was further substantiated by the finding that DCs from patients with psoriatic arthritis secreted similar TNFα, IL-6, IL-10 and IL-12p70 levels upon *P. gingivalis* stimulation compared to healthy counterparts (Figure 2B and data not shown).

We next tested whether the altered response to *P. gingivalis* was limited to DCs. To this aim, we stimulated RA macrophages with *P. gingivalis* and observed a similar decrease in TNFα production (P = 0.0286) (Figure 2C). In addition, freshly isolated PBMCs from RA patients were stimulated with *P. gingivalis* for 7 days to determine effects on T cell cytokines. Fitting with the decreased IL-12 and TNFα production by RA DCs[26;27], RA PBMCs stimulated with *P. gingivalis* produced markedly less IFNγ (P = 0.0085) compared to those from controls (Figure 2D). This reduction was specific for IFNγ since IL-10 and IL-13 production was relatively similar between patients and controls and IL-17 was similar or even slightly increased in RA patients (P=0.078). Overall, these data indicate that also T cell differentiation is affected by altered *P. gingivalis* recognition in RA patients.

The altered cytokine response to P. gingivalis in RA is species specific.

To test the species specificity of the decreased cytokine induction by *P. gingivalis*, we included a multitude of other bacteria that have been implicated in arthritis development

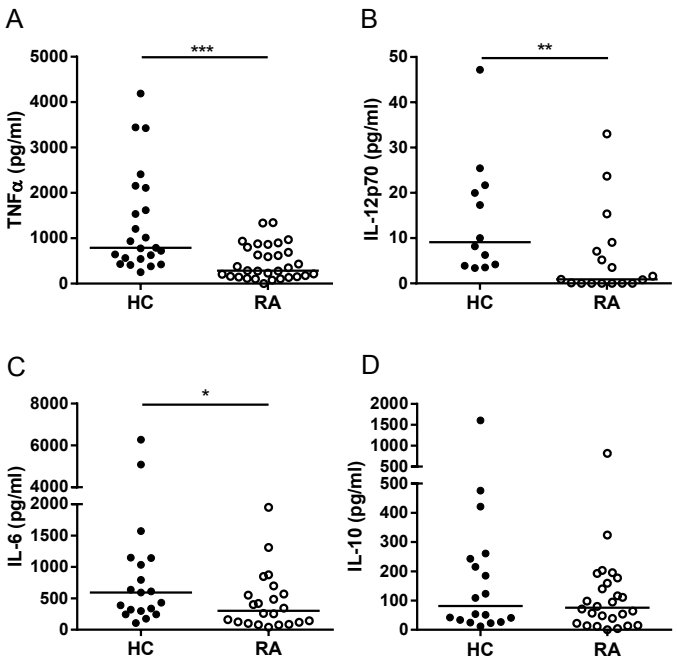

Figure 1. *P. gingivalis* induced cytokine production was decreased in DCs from RA patients. MoDCs from healthy controls and RA patients were stimulated with heat-killed *P. gingivalis* ($1x10^7$/ml) for 18 hrs and TNFα (A), IL-12p70 (B), IL-6 (C) and IL-10 (D) were measured in the supernatant. Experiments, always including healthy controls and RA patients in parallel, in which IL-12p70 could not be detected in any of the donors were excluded from the analysis (B). Panel C shows the IL-6 levels of the RA patients that produced less TNFα compared to their healthy counterparts (5 patients excluded). Horizontal lines in the graphs represent the median. * p<0.05, ** p<0.01, *** p<0.001

including *P. mirabilis, S. typhimurium, S. sonnei, K. pneumoniae,* and *E. coli* and the oral bacteria *P. intermedia* and *S. mutans.*[28;29] In contrast to *P. gingivalis,* none of these bacteria induced similar differences between RA patients and healthy controls (Figure 3A and data not shown). In addition, healthy control and RA PBMCs were also stimulated with the periodontal pathogen *P. intermedia*. Here we could not observe differences in T cell cytokine production as observed with *P. gingivalis* (Figure 2D and 3B). These data suggest that the decreased cytokine response towards *P. gingivalis* in RA patients is not only disease but also species specific.

The altered cytokine response is not caused by altered TLR expression and/or function

The main TLRs thought to be involved in recognition of *P. gingivalis* by immune cells are TLR2 and TLR4. TLR2 functions as a heterodimer with either TLR1 or TLR6 and both combinations have been described to bind *P. gingivalis.*[30] In order to increase our understanding of the downstream pathways that may underlie our observed *P. gingivalis* specific effects we first determined the expression of TLR1, 2, 4 and 6 on moDCs by qPCR. The expression of

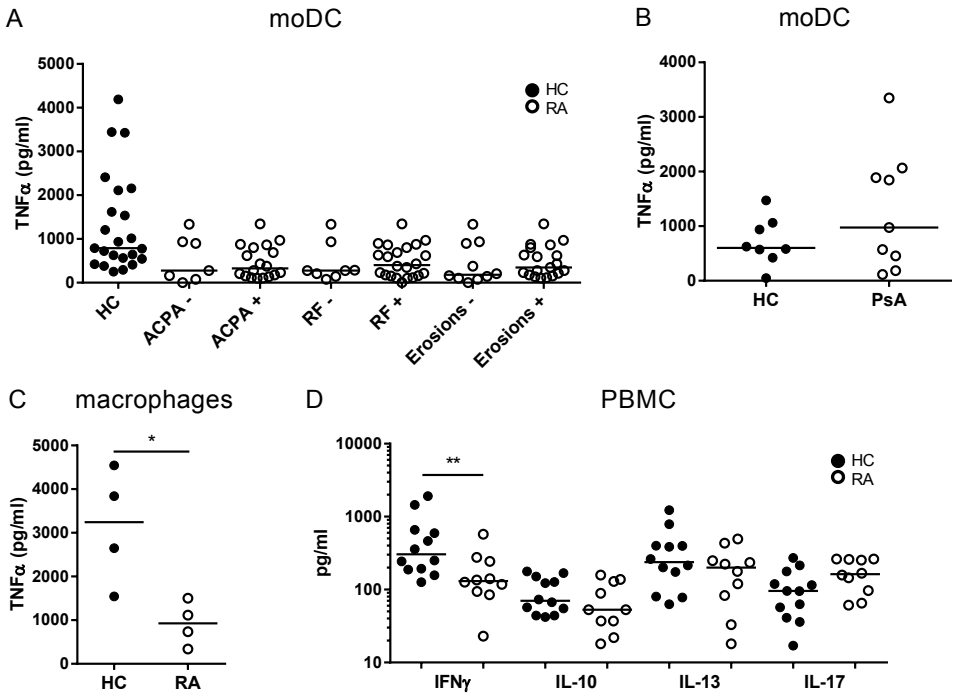

Figure 2. Immune cells from RA but not PsA patients respond less to *P. gingivalis*. *P. gingivalis* induced DC TNFα levels were stratified based on the presence of autoantibodies or erosions (A). DCs from PsA patients and healthy controls were stimulated with *P. gingivalis* ($1x10^7$/ml) for 18 hrs and TNFα production was determined (B). Similar experiments were performed with RA and healthy control macrophages (C). PBMCs from 10 RA patients and 12 HCs were stimulated with ***P. gingivalis*** for 7 days to determine IFNγ, IL-10, IL-13 and IL-17 (D). Lines in the graphs represent the median. * p<0.05, ** p<0.01

these receptors was similar between DCs from RA patients and healthy controls (Figure 4A). To exclude a functional impairment of these TLRs, DCs were stimulated with ligands for TLR2/1, TLR2/6 and TLR4. Again, no differences were observed between DCs from RA patients and controls (Figure 4B and C). Two important immunogenic parts of *P. gingivalis* are its fimbriae and a LPS structure. The LPS is different from other bacteria in that it binds mainly to TLR2 and can either stimulate or inhibit TLR4 signaling.[31] Stimulation with *P. gingivalis* LPS alone induced similar cytokine production in RA patients and healthy controls (Figure 4D), indicating that recognition of other *P. gingivalis* parts, such as its fimbriae, is responsible for the different cytokine response in RA patients.

Blocking of Mac-1 attenuated the difference between RA patients and healthy controls

As shown by several studies, binding of *P. gingivalis* fimbriae to either CXCR4 or Mac-1 can affect TLR2 induced cytokine production.[32-34] Binding of *P. gingivalis* to CXCR4 is described to suppress TLR2 signaling[32] and increased CXCR4 expression has been found on RA immune

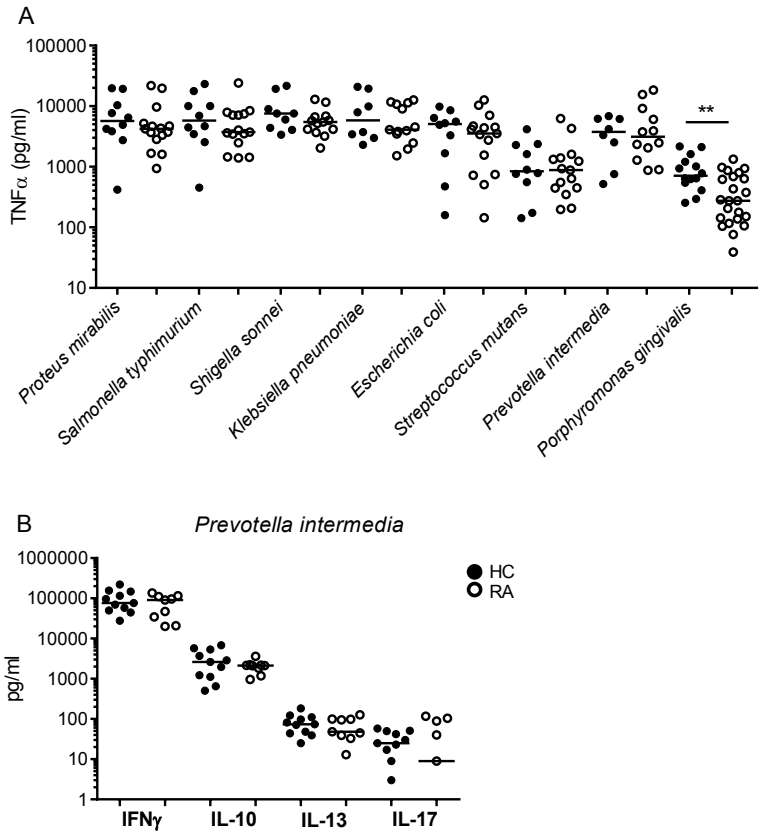

Figure 3. DCs and PBMCs from RA patients do not respond differentially to a range of other bacteria. DCs from RA patients and HCs were stimulated with multiple bacteria and TNFα production was measured in the culture supernatant after 18 hrs (A). At least 8 HCs and 12 RA patients were stimulated for all bacteria. PBMCs were stimulated with *P. intermedia* for 7 days to determine IFNγ, IL-10, IL-13 and IL-17 production (9 RA patients and 11 HCs) (B). IL-17 was undetectable in 4 RA patients and one HC. Lines in the graphs represent the median. ** p<0.01

cells.[35] Therefore we determined CXCR4 expression in moDCs, which was similar between RA patients and healthy controls (Figure 5A). In addition, blocking of CXCR4 did not affect *P. gingivalis* mediated cytokine production suggesting that CXCR4 is not the underlying cause of the diminished cytokine production in RA (Figure 5B).

The other candidate tested was Mac-1 (alternative name, CR3), which has a complex interaction with TLR2 signaling. Binding of *P. gingivalis* to TLR2 can activate Mac-1 and subsequent binding to Mac-1 by *P. gingivalis* fimbriae contributes to the induction of inflammatory cytokines including TNFα.[33;34] Both heterodimers of Mac-1, CD18 and CD11b, were similarly expressed on DCs from RA patients and healthy controls (Figure 5C). Since Mac-1 function does not always correlate with expression, we stimulated RA and healthy control DCs with *P. gingivalis* in the presence of a CD18 blocking antibody. When Mac-1

was blocked, TNFα production upon *P. gingivalis* stimulation was significantly decreased in healthy controls but not in RA patients resulting in similar cytokine production in patients and controls, suggesting that the Mac-1/CD18 pathway is responsible for the altered activation of RA DCs by *P. gingivalis* (Figure 5D).

Discussion

We demonstrate that DCs and macrophages from RA patients produce less inflammatory cytokines upon contact with *P. gingivalis*. This decreased response was both bacterium as well as disease specific. In PBMC cultures the induction of IFNγ, an important activator of the antimicrobial activity of macrophages, was also reduced in RA patients. In this way the impaired response of RA DCs and macrophages to *P. gingivalis* could directly and/or indirectly affect bacterial clearance. This is supported by a mouse study showing an inverse relationship between proinflammatory potential of *P. gingivalis* subtypes and the induction

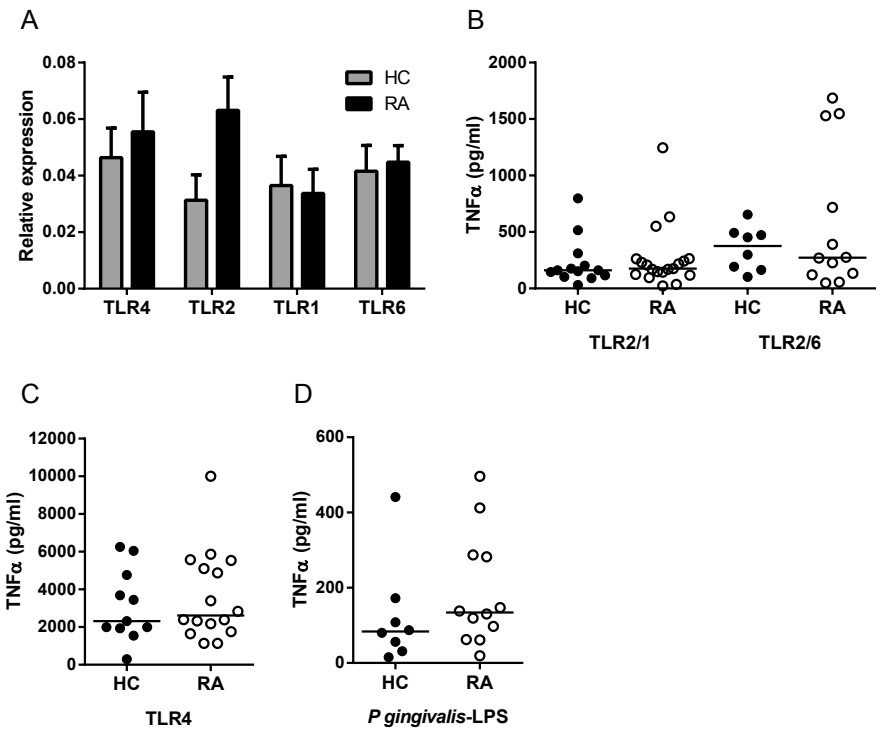

Figure 4. TLR2 or TLR4 expression and function were not different between RA patients and HCs. MoDC TLR4, TLR2, TLR1 and TLR6 expression was determined by qPCR in 9 HCs and 12 RA patients (A). Bars represent mean and SEM. TNFα production was determined in supernatant from DCs stimulated with TLR2/1 ligand Pam3CSK4 (5 μg/ml), TLR2/6 ligand FSL-1 (1 μg/ml) (B) and TLR4 ligand LPS (100 ng/ml) (C) or purified *P. gingivalis* LPS (1 μg/ml) (D). Lines in the graphs represent the median.

of experimental periodontitis.[36] It is tempting to speculate that this decreased cytokine production by RA immune cells could result in impaired clearance and prolonged presence of *P. gingivalis* in RA patients culminating in intra-oral persistence of *P. gingivalis*, which in turn might lead to citrullination of bacterial and human proteins and possibly ACPA development in genetically predisposed individuals.[37;38] This hypothesis is supported by a recent paper showing that periodontal treatment in patients with RA and periodontitis decreased antibody levels to both *P. gingivalis* and citrullinated proteins and decreased RA disease activity.[39] The lack of correlation between cytokine production and clinical phenotype in our patients suggests a generally decreased response towards *P. gingivalis* independent of current disease activity, which might have been present before disease onset. Further studies with healthy controls at risk for developing RA might provide more insight into potential underlying mechanisms.

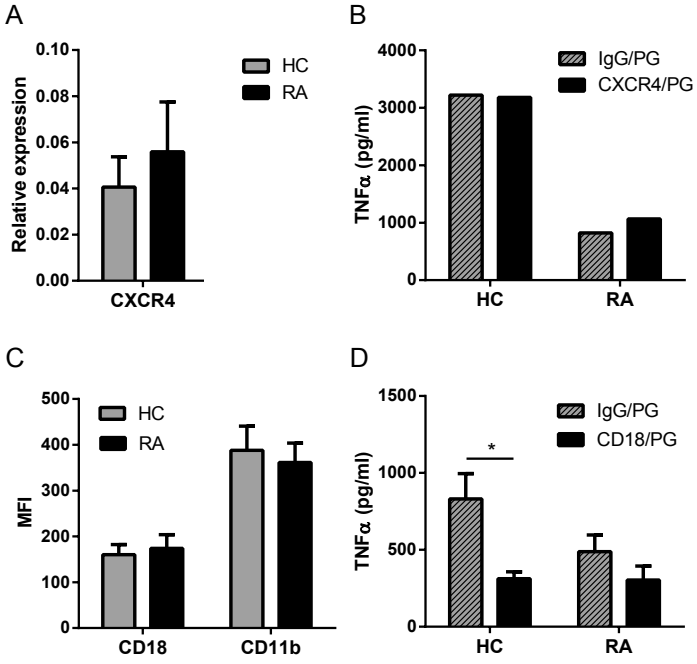

Figure 5. The difference between RA patients and HCs upon *P. gingivalis* stimulation is CD18 dependent. CXCR4 expression in DCs was determined in 9 HCs and 13 RA patients by qPCR (A). DCs were incubated with an anti-CXCR4 antibody (10 μg/ml) or an isotype control (10 μg/ml) for 30 minutes before stimulation with *P. gingivalis* (PG). TNFα production was measured after 18 hrs (B). A representative experiment from 3 independent experiments is shown. CD18 and CD11b expression on DCs from 8 HCs and 8 RA patients was determined by flow cytometry (C). DCs from 4 HCs and 6 RA patients (from three independent experiments) were incubated with anti-CD18 antibody L19 (5 μg/ml) or an isotype control (5 μg/ml) for 30 minutes before stimulation with *P. gingivalis*. TNFα levels were measured in the supernatant after 18 hrs (D). Bars represent mean and SEM. * P<0.05

Alternatively, a reduced immune response towards *P. gingivalis* might also result in a prolonged low level of systemic inflammation, which could increase the risk of developing further systemic disease including arthritis or atherosclerosis. Increased survival of *P. gingivalis* in DCs could also facilitate the extravasation of the bacterium or bacterial fragments to other sites including the joint, a site where *P. gingivalis* DNA has been found.[19-21] It remains difficult to predict the exact consequences of these changes in immune mediators. Sufficient immune activation is necessary to eliminate pathogens like *P. gingivalis* and *P. intermedia* in periodontitis, while an over activation of the immune system is thought to be responsible for eventual tissue damage in both periodontitis and RA. Further studies are needed to enhance our understanding of the potential consequences of our observations.

Some possible limitations of our study should be taken into account when interpreting our results. First, information on periodontal status is lacking. This is an unavoidable consequence of the healthcare setup in the Netherlands where two different specialties see patients with either RA or periodontal diseases. Secondly, information regarding smoking history of our healthy controls and RA/PsA patients is incomplete. However, we were able to compare the *P. gingivalis* induced cytokine response of 7 RA patients that were smoking at RA diagnosis with 7 RA patients that never smoked. This analysis did not show any differences, with a median TNFα production of 275 pg/ml (Interquartile range (IQR) 177-432 pg/ml) in smokers compared to 232 pg/ml (IQR 116-897 pg/ml) in patients that never smoked, minimizing the chance that smoking is a confounding factor. In addition, the age of our healthy donors is lower compared to our RA patients. However, we could not find a correlation between age and *P. gingivalis* induced cytokine production by DCs in our healthy donors or RA patients. Lastly, medication use in RA and PsA patients was rather similar (Table I) and *P. gingivalis* induced cytokine production was not influenced by stratification for treatment strategies excluding a medication effect in our study.

This study supports a role for Mac-1 in the reduced TNFα production by RA DCs upon contact with *P. gingivalis*. Binding of *P. gingivalis* fimbriae to Mac-1 has been described to induce bacterial uptake and proinflammatory cytokine production such as TNFα, while it inhibits IL-12p70 production in monocytes and mouse macrophages via ERK1/2 signaling.[33;34;40] However, multiple studies demonstrated differential regulation of IL-12p70 production in dendritic cells, in which ERK1/2 signaling can induce IL-12p70 production rather than inhibiting its production.[41-43] This would fit with our findings showing a reduced TNFα and IL-12p70 production in DCs from RA patients. Interestingly, early onset periodontal disease is often seen in patients with a leukocyte adhesion deficiency (LAD).[44] This is a very rare disease in which patients have a mutation leading to almost absent expression and/or function of CD18, resulting in recurrent infections. Neutrophil dysfunction is described as one of the main causes of disease in these patients. However, impaired recognition and clearance of bacteria such as *P. gingivalis* by macrophages and DCs could also contribute to the development of periodontitis in these patients. Although this falls beyond the scope of this work, our data justifies further research into the downstream molecular circuitry underlying the altered Mac-1 function in RA.

Collectively, our data support an immunological link between *P. gingivalis* (associated with periodontitis) and RA. RA DCs produced less inflammatory cytokines upon contact with *P. gingivalis*, which was accompanied by a decreased IFNγ production in full PBMC cultures. This could result in impaired clearance and prolonged presence of *P. gingivalis* in RA patients which in turn might, via citrullination or by inducing a low level of systemic inflammation, contribute to the perpetuation and/or severity of RA.

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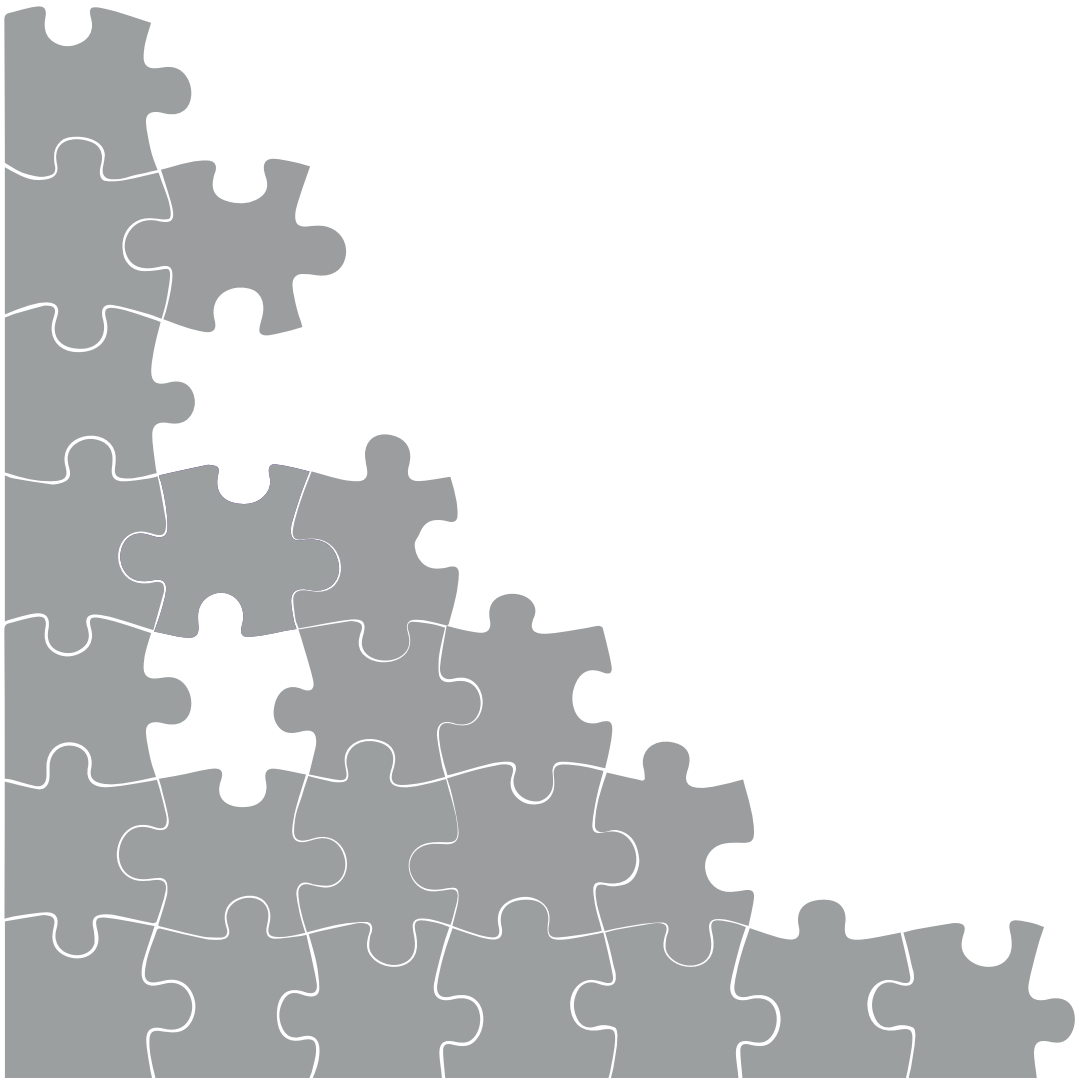

8

General Discussion

8

Rheumatoid arthritis (RA) is a chronic disabling disease mainly affecting the joints. The exact cause of RA remains to be elucidated but it is clear that the immune system plays an important role, together with stromal cells such as fibroblasts. An inflamed RA joint contains increased levels of many immune cells including T cells, B cells, neutrophils, macrophages and dendritic cells (DCs). Inflammatory cytokines, such as tumor necrosis factor-α (TNFα), interleukin-6 (IL-6) and IL-1, have been identified as crucial mediators of inflammation and cartilage destruction and have been proven useful therapeutic targets. Macrophages and DCs are important producers of these inflammatory cytokines in the joint. As antigen-presenting cells (APCs) especially DCs are important for maintaining tolerance (in their immature state) or activating adaptive immune responses (as mature DCs). DCs and macrophages express a wide array of receptors on the cell membrane, able to recognize a diverse array of danger signals from their environment, thereby allowing these cells to initiate a fitting immune response. Examples of these are Toll-like receptors (TLRs), which are important drivers of inflammatory cytokine production in the synovium[1-3] In addition, these myeloid derived cells are equipped with Fc gamma receptors (FcγRs) that bind (autoantibody containing) immune complexes and adhesion molecules like integrins that regulate cellular interaction with the extracellular matrix and other (immune and non-immune) cells.

By understanding how the immune system gets activated and which pathways are in place to regulate/down tune this initial immune activation we might get new tools to more specifically target immune activation in RA, limiting side effects such as an increased risk of serious infections.

DC/macrophage activation via TLRs and interference by FcγRIIb

During RA many endogenous TLR ligands can be found in the joint, including self-RNA/DNA from necrotic cells, heat-shock proteins and S100 proteins. In addition, the recognition of pathogen derived molecular patterns via TLRs might also be involved as a trigger of inflammation in RA patients. The initiated immune response, when not properly controlled, can result in the release of more danger signals and damage products that in turn further activate infiltrated immune cells via TLRs or other pattern recognition receptors, creating a vicious loop of inflammation. In addition, for TLR ligand containing immune complexes (ICs), such as ICs containing citrullinated fibrinogen, FcγRs and TLRs have been shown to cooperate for cellular activation.[4] Whether the recognition of ICs by myeloid cells results in cellular activation or not depends on the FcγRs involved. Most myeloid cells express both activating and inhibitory FcγRs, the combination of which sets the threshold for cellular activation. In RA and other autoimmune diseases, combined triggering of TLR and FcγR pathways are likely and previous data pointed towards a possible FcγR dysregulation in RA patients. Therefore we aimed to further explore FcγR expression and function on DCs from RA patients.

In the clinic we noticed some patients that initially had high disease activity, but over time reached remission without further need of disease-modifying antirheumatic drugs (DMARDs). We hypothesized that these patients might have specific regulatory immune

mechanisms suppressing their disease activity, thereby maintaining disease remission. To look at this, we compared these patients with other RA patients and healthy individuals. Studying their FcγR expression on monocyte-derived DCs (moDCs), we found a highly increased expression of the inhibitory FcγRIIb only on the patients that were in drug-free disease remission (*Chapter 2*). This specific up regulation of an immune regulating receptor in these patients suggests this could well be involved in controlling inflammation. Recently, others have also found an increased FcγRIIb expression on monocytes from RA patients with low disease activity, although these patients were still using medication.[5] Led by earlier work we checked the possible FcγRIIb regulation of TLR4 responses.[6;7] We found that only DCs from patients with very high levels of FcγRIIb dampened TLR4 mediated cell activation upon concomitant IC treatment, resulting in decreased production of inflammatory cytokines like TNFα and IL-12 and decreased DC maturation. These tolerogenic effects could be blocked in the presence of a FcγRIIb blocking antibody, confirming this was indeed caused by FcγRIIb signaling. To further pinpoint where the FcγRIIb and TLR4 pathways interact with each other, multiple kinase inhibitors were used showing the dependence of PI3K and AKT signaling. Increased SHIP phosphorylation limited to high-FcγRIIb DCs upon TLR4 and IC co-stimulation further supported the involvement of the SHIP-PI3K axis in FcγRIIb mediated TLR4 regulation (unpublished data).

Since DCs are the professional APCs of the immune system and we found clear differences in cytokine production and DC maturation upon FcγRIIb co-stimulation, this was likely to also affect T cell responses. Although no differences were observed for interferon-γ (IFNγ) and IL-17, IL-13 production was clearly increased upon simultaneous stimulation of FcγRs and TLR4. This points towards a Th2 shift, which is thought to be protective in RA. In addition, TLR4 triggering prevents the induction of new regulatory CD4+CD25+FoxP3+CD127- T cells, which is abolished by the addition of ICs to high FcγRIIb expressing DCs. High FcγRIIb expression could thus well be an important mechanism responsible for disease control in a specific group of RA patients who were able to maintain in remission upon withdrawal of immunosuppressive drugs.

Because the numbers of RA patients with low disease activity without the use of DMARDs or biologicals are limited we started looking for another myeloid cell type that might have a high FcγRIIb expression to further investigate a possible interaction with other TLRs and other cellular activation mechanisms. Macrophages also play an important role the RA disease process and therefore we investigated the expression of FcγRs on monocyte-derived macrophages that were differentiated towards pro- or anti-inflammatory macrophages, in the presence of GM-CSF (gmMϕ) or M-CSF (mMϕ) respectively. Studying FcγR expression on these macrophages, we found that gmMϕ in contrast to mMϕ expressed high levels of FcγRIIb, both in healthy controls and in RA patients (*Chapter 3*). We confirmed that high levels of FcγRIIb on gmMϕ, similar to DCs, could reduce TLR4 induced cytokine production. We further found similar effects combining IC triggering with TLR2 and TLR7/8 stimulation. Similar to DCs from remission patients FcγRIIb mediated inhibition of TLR responses in gmMϕ was PI3K dependent. Monocyte-derived cells in homeostasis, either DCs or

mMϕ only express low levels of FcγRIIb, while GM-CSF-like macrophages that arise in an inflammatory setting express increased levels of FcγRIIb, thereby increasing their threshold for activation upon exposure to ICs and sensitizing these cells to IC mediated inhibition of TLR responses. We believe this is a normal mechanism activated upon inflammation, which seems to be dysfunctional in most RA patients. This regulatory mechanism is mainly of interest for autoimmune diseases, but the presence of small ICs in serum of healthy donors also supports a role for this mechanism in healthy individuals.

T cell mediated macrophage activation and interference by FcγRIIb and abatacept

In addition to TLR activation of macrophages by either pathogens or endogenous danger signals, another source of synovial macrophage activation are activated T cells. Similar to blocking of TLRs in synovial tissue, also removing T cells from synovial tissue explants reduces inflammatory cytokine production including the central cytokine in RA, TNFα.[1-3;8] Synovial T cells mainly consist of memory T cells that mediate this activation via cell-contact dependent mechanisms involving CD69, CD18 and CD49d.[9] Cytokine stimulated T cells (Tck) display similar characteristics as activated synovial T cells and both induce the release of TNFα but not IL-10 from monocytes, thereby attributing to an unbalanced proinflammatory cytokine environment. We found that mainly proinflammatory gmMϕ were easily activated by Tcks, inducing unbalanced TNFα release (*Chapter 3*). Since we have shown a high FcγRIIb expression on these cells, triggering of which was able to regulate TLR mediated cell activation, we wondered if this might also affect Tck induced cytokine production. And indeed, similar to TLR inhibition by FcγRIIb, ICs also potently reduced Tck induced TNFα production. This was again depended on FcγRIIb and the SHIP-PI3K axis but also involved prostaglandin production, shown by dependency on COX2 enzyme activity. FcγRIIb regulation of the immune system thus is not limited to regulation of the activating FcγRs and B cell activation, but also extends to regulation of TLR and T cell induced macrophage/DC activation (Figure 1). FcγRIIb thus appears to be a central mediator of immune tolerance on different cell types and can modulate different mechanisms of cellular activation.

Since synovial macrophages can get activated via TLRs and by activated T cells an interaction between these two modes of macrophage activation might induce the excessive immune activation seen in the joints of RA patients. Stimulation of macrophages with a combination of Tcks and TLR ligands (TLR4 or TLR7/8 stimulation) showed that the presence of Tcks synergistically increased TNFα and IL-6 production upon TLR stimulation (*Chapter 4*). In addition, while macrophages stimulated by Tck or TLR agonists alone hardly produce any IL-12 (a cytokine mainly produced by DC and involved in Th1 priming), a combination of these stimuli licenses macrophages to produce IL-12. This thus results in a pro-Th1 environment in the synovium of RA patients.

Abatacept (CTLA4 with an Fc tail) is an effective treatment in RA patients. It blocks the instruction of T cells by APCs such as DCs an macrophages by binding to CD80 and CD86 on the surface of the APC. This prevents binding to CD28 on the T cell, necessary for

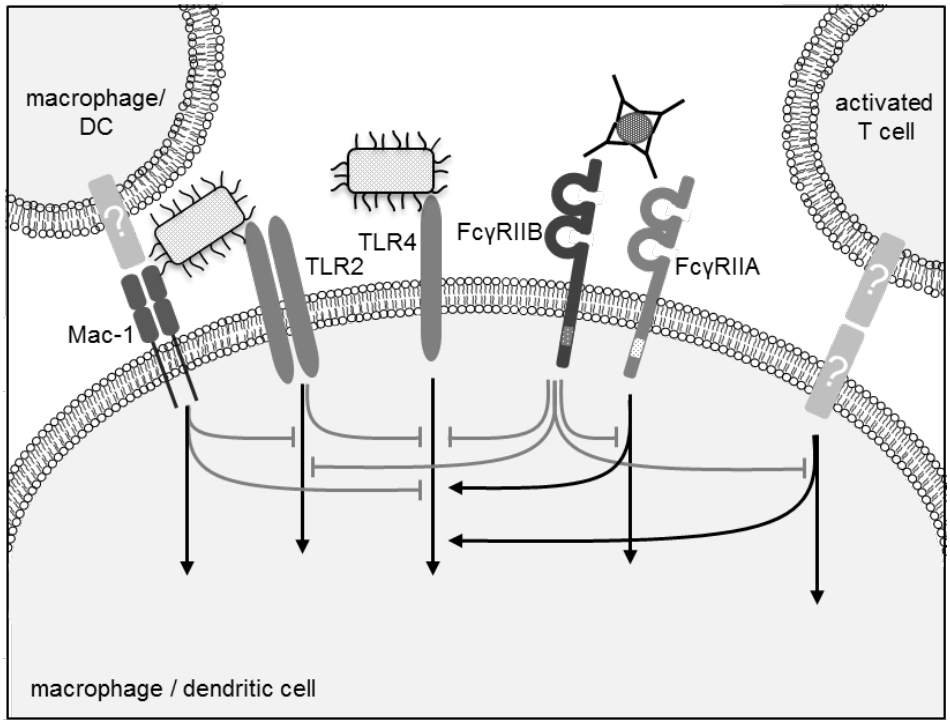

Figure 1. Interactions between different signaling pathways. ⟶ indicates cellular activation pathways, while ⟞ indicates an inhibitory pathway. TLR4 is depicted as a representative TLR and pathways described for this TLR have also been shown for TLR7/8, which is located in endosomes. FcγRIIa is depicted as a representative for the activating FcγRs.

full T cell activation. Next to effects on T cell activation it has been shown that abatacept could also modulate macrophages itself, resulting in reduced cytokine production.[10] We therefore investigated if abatacept could, in addition to affecting APC mediated T cell activation, also affect T cell mediated APC activation (*Chapter 4*). And indeed addition of abatacept to macrophage Tck co-cultures almost completely prevented TNFα release. In addition, abatacept was able to modulate IL-12 and IFNγ production in macrophage-Tck co-cultures. This shows that abatacept, next to direct inhibition of T cell activation, also affects macrophage cytokine production including TNFα and the Th1 inducing cytokine IL-12, which are likely pathogenic in RA patients. The effect of abatacept on macrophages seems to be specific for Tck mediated cell activation, since it cannot inhibit TLR induced cytokine production in our hands. This shows additional functions of abatacept that likely attribute to its effectiveness in vivo. Recent studies also show effects of abatacept on other CD80/CD86 expressing cells, including B cells and osteoclasts, and thereby abatacept seems to target many different cells/processes involved in RA pathogenesis.[11-13]

In line with the work presented in this thesis, it would also be interesting to study if

FcγRIIb stimulation can modulate simultaneous TLR/Tck induced cytokine induction or that this synergistic inflammatory response overrules the inhibitory effects of FcγRIIb. Although abatacept itself is an antibody, the Fc tail has been modified to prevent complement and Fc receptor binding.[14] Therefore effects of abatacept are most likely caused by the antigen recognition domain of abatacept and not by FcγR crosslinking by its Fc tail.

Intrinsic regulation of macrophage activation

When monocytes enter a site of inflammation they turn into inflammatory macrophages or DCs under the influence of factors such as GM-CSF. This primes these cells to actively participate in the immune response to clear a threat. However at a certain point these cells have to be regulated to prevent over activation or chronic inflammation resulting in unwanted tissue damage. Several mechanism are known to dampen inflammation, such as the production of IL-10, the induction of TLR inhibitory molecules such as A20, SIGIRR and SOCS proteins or the induction of lipid mediators such as certain prostaglandins, lipoxins and resolvins. However, it is not completely clear what triggers these resolution factors during inflammation.

Macrophages are known for their plasticity, and function both in the initiation of inflammation and in the resolution phase. This can partially be regulated via the influx of new regulatory macrophages, but likely also macrophages already present and involved in the inflammatory process actively participate in inflammation resolution. Since inflammation is characterized by a massive influx of immune cells, we hypothesized that this influx of cells itself might trigger a regulatory mechanism preventing over production of inflammatory mediators. To study this we cultured macrophages in increasing cell density and evaluated their response to subsequent TLR stimulation. Intriguingly, we found a density dependent down regulation of inflammatory cytokines, together with an increase in anti-inflammatory IL-10 production (*Chapter 5*). At the same time their phagocytic capacity increased, as well as their response towards dectin-1 ligands, characteristics of alternatively activated macrophages.

This correlation between increasing cell numbers and decreasing inflammatory cytokine production upon TLR activation was also seen *in vivo* in a mouse model of wound healing. At day 4 and 6 of wound healing the amount of infiltrated macrophages was clearly increased, while their ex vivo capacity to produce inflammatory cytokines upon TLR stimulation was decreased. Also independent of time after wounding, there was a direct negative correlation between the amount of macrophages infiltrated and the ex vivo production of IL-1β and IL-6 upon TLR stimulation.

This mechanism revealed to be dependent on mac-1 mediated cell-cell interaction. This induced a p38 dependent up regulation of SOCS3 and IL-10. In addition, soluble factors were produced that could transfer the anti-inflammatory effect to other cells. The increased IL-10/TNF ratio induced by increased cell density was dependent on COX2, implicating the production of lipid metabolites such as prostaglandins in this shift. Further studies are necessary to find out which lipid metabolites are involved, but prostaglandin (PG) D_2,PGJ_2 or lipoxins are definitely interesting candidates because of their known anti-inflammatory/

resolution functions.[15] In line with these results non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit COX2 were shown to increase the spontaneous release of TNFα while decreasing IL-10 release from synovial membrane cultures[16], further supporting a role for this mechanism in vivo in humans.

Although high density gmMϕ start to functionally act like mMϕ, producing less TNFα and more IL-10 upon TLR stimulation and increasing their phagocytic capacity, they do not up regulate typical mMϕ markers such as CD14 and CD163, or change levels of CD18 and CD11b. As shown in chapter 3 we observed clear differences between gmMϕ and mMϕ regarding FcγR expression. We also checked these markers on low and high density cultured gmMϕ and while FcγRIIb expression was clearly lower on mMϕ compared to gmMϕ, high density gmMϕ even further up regulated FcγRIIb expression (on both mRNA and protein level, Figure 2). The expression of the activating FcγRs was less affected by cell density. These membrane markers suggest that high density gmMϕ do not turn into mMϕ-like cells, but become a separate regulatory macrophage subset, characterized by high FcγRIIb expression. Again this supports a role for FcγRIIb in controlling inflammation or as a marker of more tolerogenic inflammation-related macrophages, which can be applicable to many inflammatory diseases.

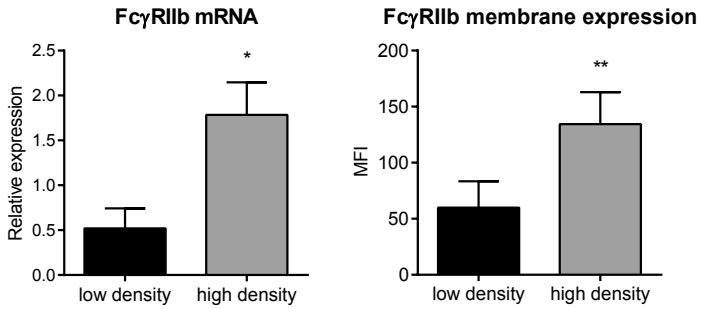

Figure 2. More tolerant high density macrophages have an increased expression of the inhibitory FcγRIIb, measured both by qPCR (n=6) and flow cytometry (n=10). * p<0.05 and ** p<0.01 compared to low density macrophages.

Effects of synovial fluid on DC activation by TLR agonists

Inflammation in RA mainly resides in the joints where high numbers of immune cells are found within the synovial fluid. Although RA synovial fluid contains many inflammatory cytokines and MMPs produced by immune cells and fibroblasts, it likely also contains anti-inflammatory factors that counteract these processes and prevent tissue damage. Not much is known about the effects of this synovial fluid containing environment on immune cell function and therefore we investigated the effect of synovial fluid on DCs in combination with TLR stimulation. Cell-free RA synovial fluid on its own did not induce DC cytokine

production or maturation. The presence of synovial fluid during TLR stimulation however markedly reduced the production of TNFα, IL-12, IL-1β, IL-6 and interferons, without affecting the up regulation of maturation markers or production of more anti-inflammatory IL-10 and IL-27. In addition to direct effects on DCs, synovial fluid exposed DCs induced stronger Th2 responses, while Th1 and Th17 responses seemed slightly reduces. The down regulation of inflammatory cytokine production by DCs could be recapitulated by a fatty acid enriched lipid fraction isolated from RA synovial fluid, suggesting the involvement of proresolution mediators such as lipoxins, resolvins or protectins. Mice knockout for ALX, the receptor for lipoxin A_4 (LXA_4) and resolving D_1, showed an exacerbated and prolonged arthritogenic response upon K/BxN serum induced arthritis.[17] This supports a role for these lipid mediators in dampening inflammatory responses in arthritis, that might be not enough to halt disease activity in RA patients, but could well be protective from even an more aggressive disease course and further structural damage. The eventual effects of synovial fluid in an arthritic joint depends on a complex mix of pro- and anti-inflammatory mediators and the immune cells that reside in it, but the identification of natural anti-inflammatory/ proresolution mediators might improve the treatment of RA. Often after synovial fluid is aspirated to relieve pain and swelling of an arthritic joint, glucocorticoids are left behind to further dampen residual inflammation. However more endogenous mediators identified from the synovial fluid itself might be a potent alternative with fewer side effects compared to glucocorticoids, especially when repeated injections are necessary.

Porphyromonas gingivalis and RA

Microbial triggering has long been speculated to be involved in the pathogenesis of RA, but whether bacteria or viruses indeed play a role still remains unclear. Bacterial DNA has repeatedly been found in inflamed RA joints[18-20], but bacteria are not cultivatable from RA synovial fluid. This indicates that a possible role for bacteria in RA might be more linked to bacterial fragments that end up in the joint and can activate immune reactions than an active infection in the joint itself.

One of the bacteria that has been implicated in RA development is *Porphyromonas gingivalis*. This bacterium is directly involved in the development of periodontitis, a chronic inflammation of the tissue surrounding the teeth (the periodontium). The incidence of this disease is more common in RA patients compared to the general population and interestingly the treatment of periodontitis can also positively affect RA disease activity.[21-27] In addition, *P. gingivalis* is the only bacterium known that can citrullinate bacterial and human proteins, linking it to the specific autoantibodies found in RA patients directed against citrullinated proteins. So far, most research has focused on epidemiological associations between *P. gingivalis* and RA or the presence of *P. gingivalis* antibodies in RA patients. We were interested to see if *P. gingivalis* might interact differently with the immune system of RA patients compared to healthy individuals. We primarily focused on moDCs and found that moDCs from RA patients produced much less inflammatory cytokines upon contact with heat killed *P. gingivalis*, which was most clear for TNFα (*Chapter 7*). A whole range of other bacteria were also included and confirmed specificity of this reduced response for

P. gingivalis. In addition we included a group of patients with psoriatic arthritis as a non-RA diseased control group. These patients did not show an altered response towards *P. gingivalis*, supporting specificity for RA. In addition to effects on DCs and macrophages also T cell differentiation was affected. In line with a decreased TNFα and IL-12 production by RA DCs, PBMC from RA patients showed a clear reduction in IFNγ release. The combined impaired response by DCs and macrophages together with a decreased Th1 response, which normally activates macrophage/DC antibacterial function, could result in impaired clearance of *P. gingivalis* in RA patients.

This impaired response towards *P. gingivalis* was not caused by altered TLR expression or function. Blocking of mac-1 (CR3), which has been described to cooperate with TLR2 for inflammatory cytokine production upon *P. gingivalis* stimulation, however, abolished the difference in TNFα production between RA patients and healthy controls (*Chapter 6)*. This implies a differential function of mac-1 in *P. gingivalis* recognition in RA patients. Mac-1 has multiple binding sites that can trigger differential intracellular signaling events. Which binding pocket is used for *P. gingivalis* is not known, but is probably different from complement coated bacteria since there is no indication of impaired anti-bacterial responses in general in RA patients.

Overall, we see a decreased immune response towards *P. gingivalis* in RA patients. This could result in reduced clearance of *P. gingivalis* and therefore a prolonged presence of these bacteria in RA patients. As a consequence *P. gingivalis* might induce abundant production of citrullinated proteins, increasing the pressure to develop anti-citrullinated protein antibodies (ACPAs). In addition, inflammation originating in the gingiva due to accumulation of pathogenic *P. gingivalis* can result in a low level of systemic inflammation, increasing the risk to develop arthritis or atherosclerosis. However, this would not implicate *P. gingivalis* or its PAMPs as a causative trigger inducing a vicious circle of inflammation in RA, since there is a reduced immune activation. In line with the synergistic effects of TLR ligands and activated T cells seen in chapter 4, we also included Tcks in co-culture with macrophages or DCs stimulated with *P. gingivalis*. This combination of immune cells, likely responsible for a substantial part of the inflammatory cytokines produced in the RA joint, synergistically increased TNFα and IL-6 production upon *P. gingivalis* stimulation (Figure 3). These experiments have only been performed with healthy donors so far and should be repeated with RA patients to see if this combination might cause clear immune activation or this is also decreased in RA patients. This will learn if the immune response to *P. gingivalis* is merely decreased in RA patients likely resulting in reduced bacterial clearance, or might still be highly stimulatory in the (inflamed) joint.

Conclusion

The importance of TLRs in stimulation of the immune system in RA and other chronic inflammatory diseases has been well established. In this thesis we provide new insights into possible regulating mechanisms that could be involved in inflammation control. FcγRs play an important role in autoimmune diseases such as RA and FcγRIIb can dampen TLR induced proinflammatory cytokine production by DCs and inflammatory macrophages. This

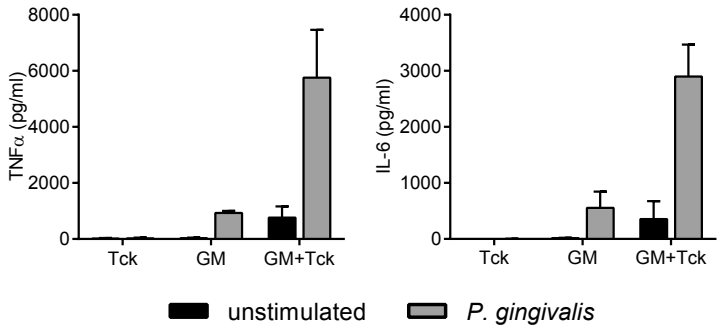

Figure 3. Inflammatory cytokine production by gmMϕ-Tck co-cultures stimulated with *P. gingivalis* (mean and SD of 2 healthy donors)

results in the induction of Th2 and regulatory T cells, counter regulating pathogenic Th1/Th17 responses. Another regulatory mechanism to prevent chronic inflammation is the inhibitory effect of accumulation of macrophages/DCs at a site of inflammation, decreasing their proinflammatory potential and inducing a more regulatory cell function. In addition to reduced production of inflammatory cytokines, T cell proliferation was reduced mainly resulting in decreased Th1 responses. Along with TLRs also activated T cells are involved in macrophage activation in RA, which synergistically increased inflammatory cytokine production by macrophages. Both FcγRIIb and abatacept were able to modulate T cell induced macrophage activation. In addition to reducing TNF/IL-6 production upon T cell mediated activation, abatacept was also able to block the induced IL-12/IFNγ axis when TLRs and activated T cells were combined. To control synovial inflammation SF seems to have the inherent capability to dampen inflammatory responses and promote Th2 over Th1/Th17 polarization, likely induced by proresolution lipid mediators. Although it is probably beneficial to dampen overt immune activation upon endogenous TLR ligands that are produced in excess during chronic inflammation, it remains critical to respond sufficiently to bacterial invasion. We show that DCs (and PBMCs) from RA patients have a decreased cytokine response towards the periodontal bacterium *P. gingivalis*, potentially leading to reduced bacterial clearance. This could be involved in RA by increasing the pressure for ACPA production or by inducing a chronic systemic inflammatory milieu that can affect joint inflammation as well.

We present new regulatory mechanisms to control TLR induced inflammation (Figure 1), but somehow these regulatory mechanisms either do not work properly or are insufficient to prevent chronic inflammation from happening in some patients. More research is necessary to determine why these regulatory mechanisms fail to prevent chronic inflammation in RA and other inflammatory diseases and how they might be used in our advantage.

Future perspectives

DCs and macrophages play an important role in inflammatory responses, both as effector cells producing inflammatory mediators and as APCs instructing T cell responses. However, in addition to their role of inducing inflammatory responses upon triggering by pathogens or other danger signals, they are also critical for maintaining or returning to a state of homeostasis. This dual role has been supported by animal models showing that DCs are crucial for the breach of self-tolerance in arthritis but ablation of DCs under steady-state conditions can also induce spontaneous autoimmunity [28;29]. Similarly, macrophages are accepted as important inflammatory cells in diseases such as RA, but also play important roles in resolution of inflammation.[30;31] The regulation of pro- or anti-inflammatory functions of these cells thus plays a crucial role in regulating normal immune responses, thereby being interesting candidates to study in diseases in which inflammatory responses are dysregulated. The regulatory potential of these cells is exemplified by the use of DCs as therapies to induce specific immunity against malignant cells or tolerogenic DCs as possible therapies for autoimmune diseases.[32-34] The first clinical trials are being performed with these manipulated DCs, which is a very interesting development providing promising therapeutic options. However because these cells are characterized by their plasticity and heterogeneity, caution is warranted regarding their stability over time in vivo.

Focusing on immune regulatory mechanisms that might prevent the development of chronic inflammatory diseases or control disease activity in diseased individuals, RA patients who manage to gain DMARD-free disease remission are a unique patient group to study to identify new regulatory pathways. As an example of this we found FcγRIIb uniquely increased on DCs from these RA patients. Triggering of this receptor can have many immune modulating effects, including inhibition of B cell receptor signaling, inhibition of activating FcγRs and the newly identified inhibitory effects on TLR and T cell mediated APC activation and subsequent T cell responses presented in this thesis (*chapter 2 and 3*). The fact that this receptor is specifically increased on DCs from RA patients who reach DMARD-free disease remission, implies an important role for this receptor in controlling inflammation in RA, likely in part due to the described mechanisms. FcγRIIb modulation could therefore be an interesting target for therapeutic intervention in RA and more knowledge about how the expression and function of this receptor are regulated is warranted. It is interesting that also tolerogenic DCs currently under investigation for treatment of RA express high levels of this receptor (unpublished data in collaboration with C. Hilkens).

With the increased implementation of biologicals in the treatment of RA patients and the recognition of a 'window of opportunity' early in the disease process, in which fast and aggressive treatment can halt further disease progression, induction of disease remission seems a feasible goal in many RA patients.[35-39] It seems that some patients can even discontinue anti-TNF treatment when they reached a sustained low disease activity upon combined DMARD and anti-TNF treatment.[40] Multiple studies are currently being performed to determine if and in what way anti-TNF discontinuation can best be performed, including the POEET study, a nationwide study in the Netherlands. Following up from our

initial data regarding increased FcγRIIb expression on RA patients in drug-free remission, we are currently investigating within the POEET study if FcγRIIb expression on multiple immune cells including DC and monocyte subsets could be used as a biomarker to predict which patients would have a high chance of a sustained low disease activity after cessation of anti-TNF therapy. We will also investigate other immune regulatory mechanisms that might play a role in these patients, such as regulatory T cells or the induction of IL-10 production by effector T cells.

However, we shouldn't forget that there are also RA patients on the other side of the spectrum, that do not respond to any of the current treatment options. These patients likely have different underlying activation pathways or a combination of pathways that can not sufficiently be treated with current options. It is important to further characterize which immune or stromal cells are mostly activated in these patients. Identification of activation pathways induced in these cells or resolution pathways that might be underdeveloped may lead to new therapeutic targets for these patients. Expected heterogeneity within this patient group however might be a challenging hurdle to take.

The work presented in this thesis in *chapter 5*, showing an intrinsic regulatory mechanism in macrophages to become less inflammatory upon increased cell-cell interaction, increased our interest in prostaglandins and other lipid mediators and their role in inflammation. It initially seemed contra intuitive that COX2 inhibition, which is a characteristic of NSAIDs widely used in inflammatory conditions such as RA, would prevent a shift towards more anti-inflammatory macrophages. COX2 is mainly known as the enzyme producing prostaglandin E2 (PGE_2), which is produced during the initiation of inflammation and attributes to vasodilation, swelling and pain. However, COX2 also has anti-inflammatory functions by producing PGD_2, and its breakdown products PGJ_2, which have direct anti-inflammatory properties and together with PGE_2 induce a shift in lipid mediator production from prostaglandins and leukotrienes towards lipoxins including LXA_4 which have strong proresolution/anti-inflammatory capabilities [15;41]. This is supported by animal studies showing that COX2 inhibition prevents or delays resolution of inflammation and could explain the ability of NSAIDs to ameliorate pain and tenderness but not prevent disease progression in RA [42;43]. In addition to prostaglandins and lipoxins that are derived from arachidonic acid also resolvins, protectins and maresins derived from the omega-3 fatty acids eicosapentaenoic acid (EPA) or docoshexaenoic acid (DHA) have been attributed similar proresolving functions. These proresolving mediators limit the influx of neutrophils, promote macrophage clearance of apoptotic neutrophils and pathogens, prevent inflammatory cytokine production by these cells and protect from fibrosis. In addition, both lipoxins and resolvins have been shown to reduce TLR4 induced NF-κB nuclear translocation and cytokine production.[44;45] Some of these lipid mediators have already been detected in RA synovial fluid[46] and a lipid fraction likely containing these proresolving lipids mediates strong suppression of inflammatory cytokine production by DCs, as shown in *chapter 6*. This supports a role for these mediators in RA, but also suggests that the intrinsic production of these mediators is not sufficient to break the inflammatory loop.

Promoting the induction of these proresolving lipids or using stable analogs of these would give interesting opportunities to further promote resolution and hopefully halt chronic inflammation. In line with this, a recent study demonstrated a clear additive effect of EPA/DHA in the treatment of RA patients in a treat-to-target set-up with synthetic DMARDS resulting in less failure of treatment and increased disease remission.[47]

Drugs widely used in the clinics today including aspirin and steroids have been shown to modulate these pathways and promote resolution.[15] Aspirin has the unique capacity to not only inhibit COX enzymes and thereby reduce the production of (inflammatory) prostaglandins and leukotrienes, but also acetylates COX2 resulting in the production of aspirin-triggered lipoxin.[48] This has similar functions to LXA_4 and thereby aspirin differs from other NSAIDs by not only inhibiting inflammation but also promoting resolution. In a similar way aspirin can induce the production of stable analogs of resolvins and protectins and thereby promote beneficial effects from increased dietary intake of omega-3 fatty acids.[49;50] Glucocorticoids might also promote resolution by inducing the production of annexin-1 peptide, which binds to the same receptor as LXA_4 and can mediate similar proresolution effects.[51] Although low dose aspirin seems to be well tolerated and is already used by many adults at risk for cardiovascular disease[52], long-term glucocorticoid use is not wanted because of increased risk of its side effects upon long-term use. The development of chemically stable mimetics of proresolution lipid mediators therefore is a very promising development that likely results in more targeted induction of resolution mechanisms, without side effects of drugs like aspirin and glucocorticoids such as (gastrointestinal) bleeding, osteoporosis and increased risk of infections. However, several limitations have to be overcome before these mimetics could be adopted for clinical use. First, timing during the inflammatory response might be critical, since its natural production is tightly regulated during inflammatory responses and not much is known about this regulation in humans. In addition, most research up to now has mainly been performed in models of acute inflammation. Therefore research in more chronic disease models is needed to establish a role for these mediators as treatment options for chronic inflammatory diseases in humans. This promotes the idea that the ideal therapy for RA and other chronic inflammatory diseases should not only dampen inflammation but also activate resolution.

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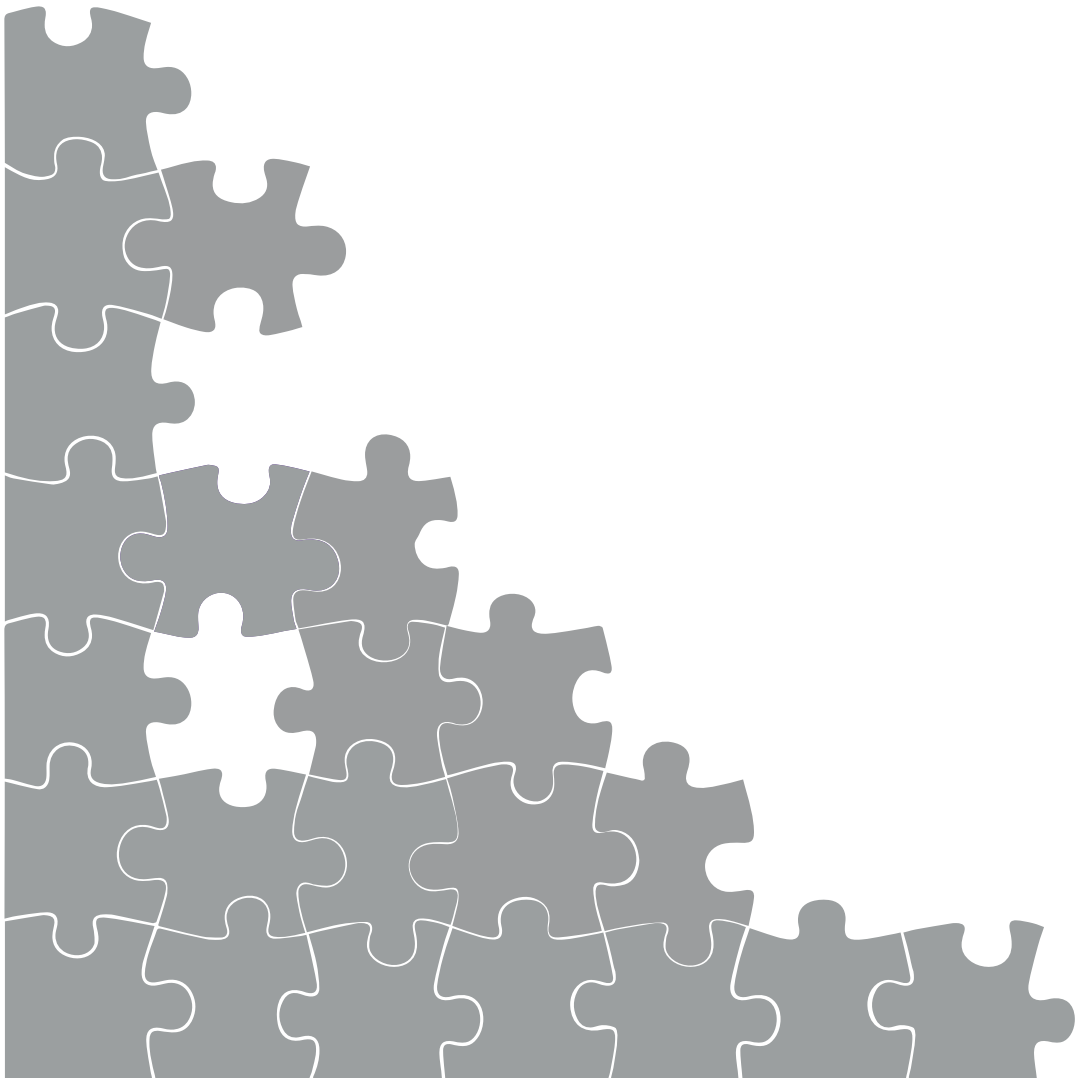

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Nederlandse samenvatting

Het immuunsysteem is erg belangrijk in de afweer tegen bedreigingen van buitenaf zoals bacteriën, virussen en schimmels en zorgt er daarnaast voor dat schade aan het lichaam zelf herkend en opgeruimd wordt. Hierbij zijn verschillende immuuncellen betrokken die allen gespecialiseerde functies hebben en met elkaar samenwerken om ons gezond te houden. Er kan echter ook iets mis gaan waardoor lichaamseigen stoffen herkend worden als een bedreiging en er een immuunrespons tegen ontstaat, zoals het geval is bij autoimmuunziekten zoals reumatoïde artritis (RA). Bij RA worden bestanddelen uit het gewricht door het immuunsysteem gezien als een bedreiging waardoor er een ontsteking ontstaat in het gewricht. In dit proefschrift hebben we onderzoek gedaan naar de activatie van dendritische cellen (DCs) en macrofagen via zogenaamde Toll-like receptoren. DCs zijn gespecialiseerd in het herkennen van bacteriën, virussen en schimmels en activeren andere immuuncellen zoals T- en B-cellen zodat de bedreiging beperkt wordt. Daarnaast zijn DCs ook erg belangrijk voor het behouden van tolerantie ten opzichte van lichaamseigen stoffen en spelen daarom een cruciale rol bij het voorkomen van autoimmuunziekten. Macrofagen bevatten net als DCs veel receptoren gericht op het herkennen van bedreigingen. Ze zijn in staat om grote hoeveelheden ontstekingsmoleculen te produceren en zo bij te dragen aan het ontstaan van en het in stand houden van de ontsteking. Er zijn echter veel verschillende soorten macrofagen en naast een rol in het ontstaan van een ontsteking zijn er ook macrofagen die met name gericht zijn op het onderdrukken van ontstekingsprocessen.

Er zijn 10 verschillende Toll-like receptoren die allen specifieke onderdelen van bacteriën, virussen of schimmels kunnen herkennen en cellen zoals macrofagen en DCs activeren om stoffen uit te scheiden die belangrijk zijn voor de afweerreactie van het lichaam. Naast pathogenen kunnen Toll-like receptoren ook stoffen herkennen die vrijkomen bij schade aan het lichaam zelf, zodat dit opgeruimd kan worden. Wanneer er echter veel van dit soort ontstekingsstoffen vrijkomen kan er een vicieuze cirkel ontstaan waardoor een blijvende (chronische) ontsteking ontstaat. In het gewricht van RA patiënten ontstaan veel schadeproducten, die via binding aan Toll-like receptoren bij kunnen dragen aan het ontstaan en op gang houden van de ontsteking. Om dit onder controle te houden zijn er meerdere mechanismen die de activatie van cellen via Toll-like receptoren kunnen onderdrukken.

In hoofdstuk 2 en 3 beschrijven we onze bevinding dat Fc gamma receptor IIb (FcγRIIb) in staat is om Toll-like receptor responsen in DCs en macrofagen te onderdrukken. Fc gamma receptoren herkennen antilichamen die zelf weer aan onderdelen van pathogenen binden of aan onderdelen van het lichaam zelf in het geval van autoantilichamen, die vaak bij autoimmuunziekten gevonden worden zoals reumafactor en anti-ccp antilichamen. De meeste Fc gamma receptoren activeren cellen, maar FcγRIIb is uniek in zijn remmende functie. Deze receptor hebben we specifiek verhoogd gevonden op DCs van RA patiënten die, na in het begin van de ziekte behandeld te zijn geweest met immuun-onderdrukkende middelen, uiteindelijk zonder medicijnen kunnen. DCs van deze patiënten produceren daardoor minder ontstekingsstoffen en ontwikkelen een ander soort T cellen (T helper 2 en regulatoire T cellen) die bij kunnen dragen aan het remmen van het onstekingsproces.

Andere RA patiënten missen deze sterke verhoging van FcγRIIb en kunnen daardoor mogelijk minder goed de ontsteking zelf onder controle houden. In die context zou het verhogen van FcγRIIb mogelijk een interessant therapeutisch target kunnen zijn in RA.

Wanneer we naar deze Fc gamma receptoren kijken op macrofagen (hoofdstuk 3) zien we dat de verdeling van deze receptoren nogal verschilt tussen pro- en anti-inflammatoire macrofagen. De remmende FcγRIIb komt vooral hoog tot expressie op pro-inflammatoire macrofagen en kan op deze cellen een belangrijke regulerende rol spelen. Naast het remmen van de productie van ontstekingsstoffen na Toll-like receptor stimulatie kan triggering van deze receptor ook een remmend effect hebben op macrofaag activatie door geactiveerde T cellen, een andere belangrijke manier van macrofaag activatie in het gewricht van RA patiënten.

Medicatie die laatste jaren veel gebruikt wordt voor behandeling van RA zijn de zogenaamde 'biologicals' die gemaakt zijn om op specifieke plaatsen in het immuunsysteem in te grijpen. De meest bekende zijn de anti-TNFα middelen die dit eiwit, dat een centrale rol speelt in het onstekingsproces bij RA, kunnen neutraliseren. Andere medicijnen zoals abatacept zijn er op gericht om de communicatie tussen DCs en T cellen te beperken. Dit medicijn remt T cel activatie, maar er is minder bekend over de mogelijke effecten op DCs en macrofagen. Gezien de rol van geactiveerde T cellen voor macrofaag activatie in RA waren we geïnteresseerd in mogelijke effecten van abatacept hierop. Het blijkt dat abatacept een duidelijk remmend effect heeft op de productie van onder andere TNFα door macrofagen na interactie met geactiveerde T cellen (Hoofdstuk 4). Dit is waarschijnlijk ook een belangrijk onderdeel van het werkingsmechanisme van abatacept in RA.

Tijdens een ontsteking worden er veel immuuncellen aangetrokken naar de plaats van ontsteking. Onze hypothese was dat deze toename in immuuncellen mogelijk ook een regulerend effect zou kunnen hebben op het gedrag van deze cellen (Hoofdstuk 5). Om de grote hoeveelheid cellen op de plaats van ontsteking na te bootsen in het lab hebben we macrofagen gekweekt met verschillende aantallen bij elkaar. Het bleek dat macrofagen die met veel andere macrofagen waren gekweekt per cel minder ontstekingsstoffen maken na stimulatie. Hetzelfde zagen we ook terug in een muismodel tijdens de wondgenezing, waarbij een duidelijke toename aan macrofagen zichtbaar was in de wond. De interactie tussen macrofagen via bepaalde receptoren, integrines genaamd, en stoffen die ze maken als reactie op dit contact zorgen ervoor dat deze macrofagen veranderen van erg ontstekingsgericht naar meer ontstekingsremmend. Meerdere moleculen spelen een rol bij deze verandering van macrofaagfunctie, waaronder ook COX2. Dit molecuul en gerelateerde moleculen zijn betrokken bij de vorming van specifieke vetachtige stoffen die een belangrijke rol spelen in de regulatie van het immuunsysteem. Veelgebruikte pijnstillers bij RA, de NSAIDs (non-steroidal anti-inflammatory drugs), werken door onderdrukking van dit molecuul, waardoor pijn en activatie van het immuunsysteem wordt voorkomen. Deze medicijnen kunnen echter ook het beëindigen van een ontsteking voorkomen. Recent heeft men gezien dat deze middelen kunnen zorgen voor een verhoogde productie van ontstekingsstoffen zoals TNFα in RA gewrichtsweefsel. Onze resultaten laten zien dat dit wel eens zou kunnen komen doordat de ontwikkeling van ontstekingsremmende macrofagen

wordt voorkomen.

Ontstekingsremmende vetten zijn ook beschreven in het vocht in de gewrichten (het synoviaalvocht) van RA patiënten. Het synoviaalvocht als geheel, maar ook de hieruit geïsoleerde vetten zijn in staat om de productie van ontstekingsstoffen door Toll-like receptor stimulatie van DCs te onderdrukken (Hoofdstuk 6). Dit speelt waarschijnlijk ook een rol in het RA gewricht, maar is blijkbaar onvoldoende om de ontsteking te stoppen. Dit geeft ons echter wel indicaties over mogelijke therapeutische aangrijpingspunten om deze natuurlijke remming van de ontsteking te kunnen versterken.

Tenslotte hebben we onderzoek gedaan naar een bacterie die als mogelijke link wordt genoemd met het verhoogd voorkomen van parodontitis (een ontsteking van het weefsel rondom de tanden) bij RA patiënten (Hoofdstuk 7). Deze bacterie, *Porphyromonas gingivalis*, zou mogelijk een rol kunnen spelen bij het ontstaan van autoantilichamen die specifiek zijn voor RA, de anti-ccp antilichamen. Wij hebben gekeken of DCs van RA patiënten mogelijk anders reageren op deze bacterie en het blijkt dat DCs van RA patiënten minder ontstekingsstoffen maken wanneer zij in contact komen met deze bacterie. Dit in tegenstelling tot patiënten met een andere vorm van gewrichtsontsteking artritis psoriatica, die vergelijkbaar reageren als gezonde donoren. Naast de verschillen in reactie van DCs zagen we dat ook andere immuuncellen een verminderde respons op deze bacterie bij patiënten met RA vertonen. Mogelijk kan deze bacterie hierdoor minder goed worden opgeruimd en daarom voor meer problemen zorgen bij patiënten met RA en bijdragen aan het ziekteproces.

Met dit onderzoek hebben we meerdere nieuwe mechanismen gevonden die betrokken zijn bij het onderdrukken van macrofaag en DC activatie door Toll-like receptoren of geactiveerde T cellen. Dit speelt waarschijnlijk een belangrijke rol bij de ontsteking in RA maar ook in andere chronische ontstekingsziekten. Daarnaast laten we voor het eerst een intrinsiek defect zien in cellen van RA patiënten in de herkenning van *P. gingivalis*, een bacterie die gelinkt is aan het ziekteproces in RA.

List of abbreviations

ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatism
APC	antigen-presenting cell
CD	cluster of differentiation
COX2	cyclo-oxygenase 2
CR	Complement receptor
CRP	C-reactive protein
DAMP	damage associated molecular pattern
DAS28	disease activity score for 28 joints
DC	dendritic cell
$DC_{low\ FcγRIIb}$	DC expressing a low Fc gamma receptor IIb level
$DC_{high\ FcγRIIb}$	DC expressing a high Fc gamma receptor IIb level
DHA	docoshexanoic acid
DMARD	disease modifying anti-rheumatic drug
DMARD(-) RA	RA patients not on DMARD therapy
DMARD(+) RA	RA patients on DMARD therapy
dsRNA	double-stranded ribonucleic acid
EPA	eicosapentaenoic acid
Erk	extracellular signal-regulated kinase
ESR	erythrocyte sedimentation rate
FACS	fluorescence-activated cell sorter
FcγR	Fc gamma receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glycerylaldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage-colony stimulating factor
gmMϕ	GM-CSF differentiated macrophage (also mϕ-1)
HAGGs	heat-aggregated gamma globulins
HEK293	human embryonic kidney 293 cells
HLA	human leucocyte antigen
HSA	human serum albumin
HSP	heat shock proteins
IC	immune complex
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
IRAK	interleukin-1 receptor associated kinase
IRF	interferon regulatory factor
IVIG	intravenous immunoglobulin
LPS	lipopolysaccharide
LXA4	lipoxin A4
MACS	magnetic bead activated cell sorter
(m)Ab	(monoclonal) antibody
MAPK	mitogen-activated protein kinase
M-CSF	macrophage-colony stimulating factor
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
mMϕ	M-CSF differentiated macrophage (also mϕ-2)
MTX	methotrexate

MyD88	myeloid differentiation factor 88
mϕ-1	GM-CSF differentiated macrophage (also gmMϕ)
mϕ-2	M-CSF differentiated macrophage (also mMϕ)
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	nucleotide-binding oligomerization domain receptors
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
Pam3CSK4	palmitoyl-3-Cys-Ser-(Lys)4
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
Peg-IC	immune complexes isolated by PEG precipitation from serum or synovial fluid
PI3K	phosphoinositide-3-kinase
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKCδ	protein kinase C delta
pLPS	purified LPS
PRR	pattern recognition receptor
PsA	psoriatic arthritis
qPCR	quantitative polymerase chain reaction
RA	rheumatoid arthritis
RF	rheumatoid factor
RT-PCR	reverse transcriptase-polymerase chain reaction
SF	synovial fluid
SHIP	src-homology 2-containing inositol 5' phosphatase
SIGIRR	single Ig IL-1-related receptor
SOCS	suppressor of cytokine signaling
SPF	specific-pathogen-free
STAT-1	signal transducer and activator of transcription-1
TNFα	tumor necrosis factor alpha
TNFAIP3	tumor necrosis factor alpha-induced protein 3 (also A20)
TGF	transforming growth factor
Th	T helper
TOLLIP	toll-interacting protein
TLR	toll-like receptor
TRAF	TNF receptor-associated factor
TRIF	TIR-domain containing adaptor protein inducing interferon-β
Treg	regulatory T cell

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Curriculum vitae

Kim Santegoets was born on the 5th of April 1985 in Boxmeer, The Netherlands. In 2003 she graduated from the Elzendaal college (VWO) in Boxmeer and started her bachelor education in Biomedical Sciences at the Radboud University in Nijmegen. As part of her bachelor she performed her first internship at the Laboratory of Pediatrics and Neurology at the Radboud UMC under the supervision of prof.dr. Bert van den Heuvel. During this internship she screened the factor H gene of patients with atypical hemolytic uremic syndrome (atypical HUS) for mutations. In 2006 she started the master program human pathobiology with a minor in virology. As part of the virology minor Kim went to Australia to perform research at Melbourne University in the department of Microbiology and Immunology. For 5 months she worked in the lab of dr. Barbara Coulson, where she studied the role of integrins and sialic acids in rotavirus binding to and infection of T cells. After traveling through Australia for the consecutive two months she came back to the Netherlands to perform her final internship at the Department of Rheumatology in the Radboud UMC under the supervision of dr. Timothy Radstake and Mark Wenink. Here she studied the effect of rheumatoid arthritis synovial fluid on Toll-like receptor signaling in dendritic cells, which formed the basis for Chapter 6 of this thesis. In 2008 she obtained her Master of Science degree cum laude.

In October 2008 Kim started her PhD at the department of Rheumatology at the Radboud UMC under the supervision of Prof.dr. Wim van den Berg and dr. Timothy Radstake. In 2012 Timothy Radstake became a professor at the UMC Utrecht and Kim joined him to finish her PhD at the department of Rheumatology & Clinical Immunology and the Laboratory of Translational Immunology at the UMC Utrecht. The results of her PhD research are described in this thesis. During her PhD Kim also followed some advanced immunology courses as part of the SMBWO Immunology training program. In addition, she presented her work at national and international Immunology and Rheumatology conferences.

Now Kim is working as a postdoc in the same laboratory studying possible prognostic biomarkers for and immunological mechanisms underlying successful cessation of anti-TNF treatment in RA patients.

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