Innate Immunity in Rheumatoid and Psoriatic Arthritis

Toll-like Receptors as Mediators of Disease

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The Dreamcatcher

Native Americans believe that the night air is filled with dreams both good and bad. The dream catcher when hung over or near your bed swinging freely in the air, catches the dreams as they flow by. The good dreams know how to pass through the dream catcher, slipping through the outer holes and slide down the soft feathers so gently that many times the sleeper does not know that he/she is dreaming. The bad dreams not knowing the way get tangled in the dream catcher and perish with the first light of the new day.

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General introduction

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(Auto)inflammation and autoimmunity

To protect the integrity of an organism against environmental and endogenous dangers nature has developed a complex system of defence mechanisms. This defensive system is known as inflammation. Inflammation is induced when (immune) cells recognize danger signals and start producing inflammatory mediators such as chemokines, cytokines and antimicrobial peptides. This attracts specialised immune cells to the compromised site in order to mount an inflammatory cascade aimed at containing the threat and consequently restoring immunological balance. Since pathogens, such as viruses and bacteria, are capable of causing major damage to the organism and can evolve rapidly, the immune system has to be capable of responding quickly and with great force. This poses the organism with an immense challenge since an exaggerated and prolonged inflammatory response can be deleterious and cause extensive damage by itself. Long-lasting or excessive inflammation might even lead to the induction of a self-perpetuating cycle known as autoimmunity, a sustained process in which constituents of the organisms' own tissues are recognized by the immune system as if they were foreign[1]. Another proposed model that might lead to chronic inflammation and consequent tissue damage is known as autoinflammation, herein tissue specific factors, including microtrauma and stress responses, lead to regional innate immune activation and persistent inflammation. It is essential to notice that an impaired immune reaction toward pathogens might also culminate in chronic inflammation due to the aberrant removal of these microbes or other inciting factors.

Autoimmune diseases are characterized by the presence of antibodies aimed at (disease-specific) endogenous ligands. Although the aetiology of autoimmune diseases is still largely unknown, it is widely recognized that a loss of immune tolerance to self components due to aberrant B and T cell reactivity is the shared basis of all autoimmune diseases. It is hypothesized that in genetically predisposed individuals an aberrant activation of the immune system might lead to a breach in tolerance, culminating in autoimmune disease. Other proposed models for the induction of autoimmunity are based on de-novo formation of autoantigens through mutations or protein modification, for example by citrullination, or on the observation that bacterial antigens can resemble self antigens. A large body of evidence points towards a seminal role for antigen-presenting cells (APCs) in the development of autoimmune diseases by forming a bridge between innate and adaptive immunity. In autoimmunity APCs appear to direct immune responses towards self by inducing the differentiation and proliferation of auto-reactive T cells, a crucial event in the pathogenesis of autoimmunity[2].

In contrast, (auto)inflammatory diseases are characterized by a lack of antibodies aimed against self antigens. The term autoinflammatory disease describes a group of disorders characterized by seemingly unprovoked inflammation without significant levels of autoantibodies and autoreactive T cells. While the disorders mostly associated with the term autoinflammation are rare inherited diseases such as familial Mediterranean fever, familial cold autoinflammatory syndrome and Muckle-Wells syndrome, other diseases such as Crohn's disease, Psoriasis, Psoriatic Arthritis (PsA) and Ankylosing Spondylitis also comply to this definition[3]. These diseases seem to be dictated by the innate immune system although it is clear that T cells also play an important role in their induction and maintenance. Until now the tissue site-specific factors that activate the immune system are unknown. These might be endogenous danger signals which induce aberrant immune responses or bacteria(l components) deposited at selective sites.

Two highly prevalent autoimmune and (auto)inflammatory diseases are, respectively, Rheumatoid Arthritis (RA) and Psoriatic Arthritis (PsA). RA and PsA share clinical features like the preferential inflammation of the small diarthrodial joints of the hands. However, the aetiology of both diseases seems to be clearly distinct. In the following chapters both diseases are discussed concerning their pathogenesis and the role APCs, such as dendritic cells and macrophages, and their Toll-like and Fc gamma receptors (TLRs, FcγRs) play in this. In the final chapter the aim and setup of this thesis is discussed.

Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a systemic autoimmune disease characterized by chronic symmetrical synovial inflammation of primarily the metacarpophalangeal, proximal interphalangeal and metatarsophalangeal joints of the hands and the feet. The aggresive front of the tissue, called pannus, invades and destroys local cartilage and bone resulting in characteristic focal bone loss and ultimately the complete destruction of the integrity of the joint. RA is a frequent disorder that affects 1% of the population worldwide thereby representing the most common inflammatory rheumatic condition. While RA existed in early native American populations several thousand years ago it seems to have made an appearance in Europe only after the 16th century, indicating that an infectious agent might play a role in the etiology of RA[4]. Despite the marked increase in the last decade in the armamentarium available to the modern rheumatologist only in a minor proportion of RA patients disease remission is achieved and a substantial number of RA patients are still therapy resistant, leading to severe disability and a decreased life expectancy.

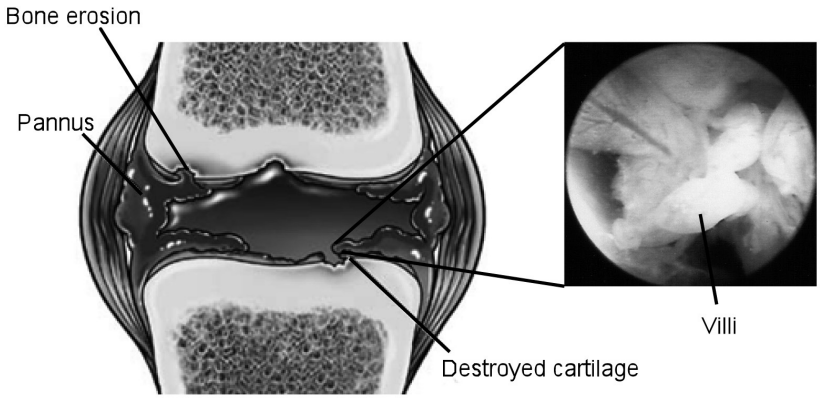

Figure 1

Representation of an afflicted joint in Rheumatoid Arthritis and a photograph of RA synovial villi growing into a joint cavity, as seen by knee arthroscopy.

During RA there is a massive influx of immune cells into the synovium and synovial cavity, including monocytes, macrophages, dendritic cells (DCs), neutrophils and T and B cells. Tissue damage and cell stress during synovial inflammation can lead to the production of heat shock proteins (HSPs), altered fibronectin or low molecular weight hyaluronan fragments and RNA release from necrotic cells. These are endogenous danger signals that in turn can activate synovial fibroblasts and APCs stimulating chronic inflammation[5,6]. The etiology of RA is still unclear although it is apparent that antigen-presenting cells and autoreactive T and B cells play a crucial role. This is exemplified by the therapeutic effect of CTLA4-Ig (Abatacept) which prevents the activation of T cells by APCs[7]. RA is typified by polyclonal B cell stimulation and production of autoantibodies targeting the synovial membrane, cartilage and underlying bone. 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.

Genetic evidence is supportive of an important role for APCs and their antigen-uptake receptors in the pathogenesis of RA. The strongest association with RA susceptibility and disease severity has been found in the HLA-DRB1 gene. Multiple RA risk alleles within the HLA-DRB1 gene share a conserved amino acid sequence and are therefore known as 'shared epitope' alleles. These polymorphisms are present in the epitope binding region of the major histocompatibility complex II (MHCII) and thereby probably influence antigen presentation by APCs and autoantibody production. More recently also non-HLA genes, such as PTPN22, FcγRs, STAT4 and IRF5, have been linked to RA susceptibility and

disease severity[8]. Since STAT4 and IRF5 are intricately involved in type I interferon signaling pathways it appears that the type I IFN system might play a role in the breaching of tolerance in RA. Supportive of this is the selective up regulation of type I IFN-response genes in peripheral blood cells from a subgroup of RA patients. In addition, it was recently reported that SLE features are common in RA patients given sufficient observation time and that these were associated with increased mortality.

Numerous autoantibodies have been discovered in sera and synovial fluid of RA patients. These antibodies may be directed to immunoglobulin G (rheumatoid factor (RF)), cartilage components, stress proteins, enzymes, nuclear proteins and citrullinated proteins such as fibrin or vimentin. In contrast to other antibodies including RF, anti-citrullinated protein antibodies (ACPAs) are almost exclusively found in RA patients, being the most specific serological markers of RA[9]. About 75% of RA patients have auto-antibodies against the Fc portion of IgG molecules (Reumatoid Factor, RF) and citrullinated proteins. The presence of RF and ACPAs is associated with a more aggressive disease course suggesting a pathogenic role in RA or common pathways leading to inflammation and tissue destruction and the production of these autoantibodies. Recent data shows that shared epitope alleles of the MHC-II are specifically associated with RF and ACPA positive RA. The most frequent proteins against which anti-citrullinated protein/peptide antibodies are produced are fibrinogen, vimentin and α-enolase, which are abundantly present in the RA synovium[10,11]. Necrotic cells in the RA synovium may release citrullinated proteins and activated citrullinating peptidylarginine deaminases (PADs). These enzymes can citrullinate proteins present in the inflamed joint. When these proteins are not degraded properly, they can be taken up by APCs and presented to T cells which in turn can trigger autoreactive B cells to produce ACPAs. Another major antigen against which immune responses are invoked in RA is heterogeneous nuclear ribonucleoprotein (hnRNP) A2. The hnRNPs are RNA binding proteins and they complex with heterogeneous nuclear RNA. These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport. HnRNP-A2, like citrullinated proteins and IgG, was found to be highly overexpressed in arthritic joints[12]. Locally produced ICs can fix complement and release chemotactic factors such as C5a, next to the induction of proinflammatory cytokine (e.g. TNFα) production by monocytes, DCs and macrophages, thereby aggravating local inflammation and promoting chronicity.

Psoriatic Arthritis

Psoriatic arthritis (PsA) is an inflammatory disease of the skin, joints and entheses leading to discomfort, pain and in some cases the aggressive destruction of joints (arthritis mutilans). Approximately 15-25% of psoriasis patients develop arthritis, the incidence of which rises with disease severity[13-15]. However, as revealed by ultrasound examination of tendons, many people with psoriasis also have (asymptomatic) abnormalities usually seen in psoriatic arthritis. In about 15% of people the arthritis starts before the psoriasis and may for that period of time be mislabelled. PsA in general involves fewer joints than RA. It is not uncommon to see just one or two joints involved. Inflammation of the distal interphalangeal joints is characteristic to PsA, especially in the presence of nail disease. In addition, spondylitis resembling Ankylosing Spondylitis is common in PsA and may be present in up to 30% of patients with PsA. The major enthesis in the human body is the Achilles tendon and this is commonly involved in PsA. However every singly tendon in the body may be affected explaining why some patients present with widespread pain.

Although the events leading to the development of psoriasis and PsA are unclear the underlying chronic inflammatory immune response is thought to be triggered by unknown environmental factors on a polygenic background with increased susceptibility. Predisposition to psoriasis and PsA is most strongly conferred by the HLA class I region. Other genetic risk factors implicate the IL-12p40/IL-23 pathway in the pathogenesis of both diseases. Resident keratinocytes and DCs, consisting of epidermal Langerhans cells and dermal DCs, make up the first line of defense in the skin against microbial pathogens and trauma[16]. Upon the encounter of danger signals these cells readily release antimicrobial peptides, chemokines and cytokines crucial for the induction of protective immune responses[17]. Recent studies have implicated an impaired barrier function of the skin as a major determinant in the induction of psoriasis. It was shown that mutations and polymorphisms in keratinocyte-expressed genes involved in physical barrier function or innate immunity are risk factors for developing psoriasis[18,19]. In addition GWAS studies demonstrated associations between inhibitors of Toll-like receptor (TLR) signaling (TNFAIP3 (A20), TNIP1) and psoriasis[20]. Current available data do not support the notion that autoantibodies or autoreactive T and B cells play an important role in causing PsA[16,21]. However, 15% of PsA patients have low titers of ACPAs while they demonstrate all the characteristic features of PsA[22,23]. T lymphocytes are shown to be important in the instigation and persistence of the chronic inflammatory response. Th1 and Th17 effector cells are thought to infiltrate the superficial perivascular skin early in

the development of psoriatic disease. There the T cells are activated by monocyte-derived APCs and subsequently produce copious amounts of inflammatory mediators[24]. The pathogenic role of activated $CD4^+$ T cells has been demonstrated by the clinical efficacy of drugs that inhibit T cell activation or deplete activated T cells[25]. Psoriatic lesions are further characterized by the presence of $CD8^+$ cytotoxic T cells in the epidemis, the segregation of neutrophils in epidermal microabscesses and, in early lesions, the accumulation of plasmacytoid DCs. These findings appear to imply that the aetiology of PsA is based on aberrances in the innate immune system followed by a dysregulated adaptive immune response. The interplay of innate and adaptive immunity in PsA is seen in the Koebner phenomenon. In this phenomenon physical trauma is thought to activate the innate immune system provoking the recruitment of immune cells, including T cells, and the subsequent development of a psoriatic lesion.

Recently, in Crohn's disease a primary innate immunodeficiency of macrophages leading to an impaired innate immune response and bacterial clearance has been demonstrated[26]. Like PsA, Crohn's disease is thought of as a dysfunctional barrier disease and both diseases share a remarkable overlap. PsA and Crohn's disease are, for example, both associated with HLA-B27 positivity, (sub)clinical eye and gut inflammation and the formation of tortuous vessels at sites of inflammation[13]. The compartments mostly affected during PsA, namely the skin, joints and entheses, are sites where bacterial components are likely preferentially deposited[27-29]. Microbial products are profoundly present in psoriatic skin lesions, but they also appear as thin deposits along the skin basement membrane of unaffected skin from psoriasis patients. No microbial products are found in skin from non-psoriatic individuals[29]. In synovial fluid from PsA patients a higher variety and concentration of bacterial DNAs are found compared to serum[27]. Furthermore PsA patients have higher antibody levels against various bacteria and bacterial constituents than psoriasis patients without articular involvement, Rheumatoid Arthritis (RA) patients or healthy controls[30-32]. Normal enthesis organs are avascular but tissue microdamage to entheses is common and appears to be associated with tissue repair responses and vessel ingrowth. This makes the enthesis organ a site where (molecules derived from) bacteria may preferentially localize. In the context of susceptibility-increasing genetic factors this might lead to the characteristic inflammatory responses of PsA. Altogether, the trapping of microbial products at specific compromised sites and the high levels of antibodies appear indicative of at least a perpetuating role for pathogens in PsA.

Dendritic cells and macrophages

Under the influence of distinct cytokines monocytes differentiate into specific effector cells, such as macrophages and DCs with distinct phenotypes and apparent roles in inflammation and immunity[33]. Through the production of a vast array of mediators and the instruction of the adaptive immune system the resulting subsets of monocyte-derived antigen-presenting cells control the progression or resolution of inflammatory processes[34]. GM-CSF and M-CSF are two cytokines important in the control of the numbers and function of macrophage lineage populations in steady state as well as in inflammatory conditions[35-37]. Both GM-CSF and M-CSF are survival factors for myeloid cells and induce their differentiation, resulting in discrete macrophage lineage populations. Human monocyte-derived macrophages have been divided in pro-inflammatory type-1, GM-CSF cultured macrophages (mϕ-1) and anti-inflammatory type 2, M-CSF cultured macrophages (mϕ-2). Mϕ-1 produce high levels of the pro-inflammatory cytokines TNFα, IL-6, IL-1β and IL-23 whereas mϕ-2 mainly produce IL-10 and trophic factors such as TGFβ and VEGF[34,38]. To study the biology of human DCs in vitro, monocytes are usually cultured in the presence of GM-CSF and IL-4. The resulting monocyte-derived DCs have the ability to take up, process and present foreign antigens and are, in contrast to mϕ-1 and mϕ-2, capable of readily inducing marked T cell responses[39].

Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system and are considered key regulators of T and B cell immunity owing to their superior ability to present antigens. DCs continuously probe their environment and are crucial for upholding tolerance or inducing adaptive immune responses aimed against invading pathogens[40]. A failure in one of these critical processes would have detrimental effects for the host, either by destruction of organs due to auto-reactivity or by invasion of harmful microorganisms. Upon encounter of a threat DCs take up the pathogen, process its antigens and then migrate through afferent lymphatic vessels into draining lymph nodes where they present the encountered antigen to T cells. The adaptive immune response evoked at that time is specifically aimed at the pathogen sensed by the DCs and should fit the level of intensity needed. To accurately restrain the threat but not culminate in excessive inflammation, which might lead to tissue damage and possibly even tolerance breakthrough, a balanced and well-controlled appearance of pro-inflammatory T cells is of paramount importance[41].

The function of DCs depends upon their state of maturation. As immature DCs they are distributed in the peripheral tissues were they sample the environment for so-called ‘danger signals’ that can be of exogenous and

endogenous nature. For this crucial task, DCs possess a specialized endocytic system consisting of a plethora of uptake receptors designed to deliver antigen to their compartments of processing. Upon the encounter of danger signals DCs commence a complex process generally termed as “maturation” which involves numerous pathways and greatly enhances their capacity for antigen processing, antigen presentation to naive T cells and the priming of specific T cell responses. In contrast to immature DC, mature DC home to the lymph nodes or secondary lymphoid organs guided by specific chemokine receptors such as CCR7 the expression of which is initiated by the maturation process. The general features encompassing DC maturation include the upregulation of co-stimulatory molecules, such as CD80 and CD86, the translocation of MHC molecules to the cell surface and the production of pro-inflammatory mediators, which latter is particularly dependent on the nature of the stimulus encountered. DCs not only play a role in the initiation of immunity but are also indispensable for the maintenance of tolerance. For this aim, DC capture antigens, even in the absence of overt infection or inflammation and present them in their steady-state immature phenotype to T cells rendering them anergic or inducing a regulatory T cell phenotype. Auto-immune diseases, such as RA, are characterized by a loss of tolerance to the body’s own constituents which results in a destructive process directed to a specific organ site. Since DC are believed to be key regulators in directing the fine balance between tolerance and immunity, a major goal in the treatment of autoimmune diseases would be to restrain autoimmunity-inducing antigen-presentation of self by DC and to generate tolerogenic DC capable of restoring homeostasis[42].

Activated macrophages and their inflammatory products play an important role in innate immunity and in the pathogenesis of autoimmune and inflammatory diseases. M-CSF is ubiquitously expressed during steady state conditions and is found in high levels in serum. M-CSF is thought to regulate the development and maintenance of tissue macrophage subpopulations that have important trophic and scavenger roles in tissue morphogenesis and function[43]. Macrophages resident at mucosal surfaces and the peritoneum have been demonstrated to display clear functional characteristics of mϕ-2. Mϕ-2 express the scavenger receptors CD163 and stabilin 1 and are highly phagocytic[44]. In addition to this it has been suggested that mϕ-2 might have a role in the suppression of tissue inflammation due to their preferential secretion of IL-10 and their ability to induce regulatory T cells[38,45]. GM-CSF has been depicted as a crucial mediator in both Th1 and Th2 mediated diseases[46,47]. Whereas under steady-state conditions GM-CSF is barely present, locally at sites of inflammation, such as the arthritic joint, GM-CSF is clearly detectable[48]. GM-CSF is produced by a wide array of

cells (Th1/Th17 cells, monocytes, fibroblasts, endothelial cells) upon activation with various inflammatory stimuli such as the cytokines IL-1β and TNFα (49-51). In its turn GM-CSF primes cells from the myeloid lineage for the release of pro-inflammatory cytokines thereby creating a self-perpetuating pro-inflammatory loop (52). Neutralization of GM-CSF inhibited lipopolysaccharide (LPS) induced lung-inflammation, with drastically lowered numbers of eosinophils and monocytes at the site of inflammation, and repressed the occurrence of inflammation and destruction in arthritis models (53-55). Underscoring the pro-inflammatory role of GM-CSF, Rheumatoid Arthritis disease severity flares during GM-CSF treatment of neutropenia (53,54,56,57).

Dendritic Cell induced T cell differentiation

With all their available tools, DCs are designed to control the T cell pool consisting of billions of different lymphocytes, which if left uncontrolled, will lead to severe disease conditions (reviewed in[58,59]). Therefore, DCs are often referred to as the 'generals' of the immune system controlling several immunological check- points both centrally as well as peripherally. Under steady-state conditions or after the resolution of infections T lymphocytes subjected to so-called 'tolerogenic DC' are deleted or made anergic. In situations which require an immune response helper T cells acquire the capacity to produce specific cytokines under direct supervision of DC. The adaptive immune response evoked is specifically aimed at the pathogen sensed by the DCs. To battle intracellular pathogens Th1 responses, characterized by IFNγ production, are mainly invoked while resistance to helminths is dependent on Th2 responses (IL-4, IL-5, IL-13) while Th17 (IL-17, IL-22) are important to fight extra-cellular pathogens. To accurately restrain a threat a balanced appearance of Th1, Th2 and Th17 cells at the compromised site is thus of paramount importance. However, next to their profitable role in the immunity against pathogens, Th1 as well as Th17 cells have been described as mediators of autoimmune pathology. It was recently suggested that the conditions during the initial exposure to antigens primes antigen-presenting cells thereby determining whether the resulting effector phase is either mainly a Th1 or a Th17 response, placing DCs in the centre of (auto)immunity[60,61].

How DCs are capable of inducing and controlling such a wide range of distinct T cells is still subject of research. The cytokines produced by DCs upon the activation with specific ligands has been shown to influence the $CD4^+$ T cell differentiation. For example, a high production of IL-12p70 strongly favors the development of Th1 while the absence of IL-12p70 production appears to favor

Th2 responses. In addition to Th1 and Th2 cells DCs have also been implicated in the promotion of Th17 cells, although the bacterial components and the type of pattern recognition receptors involved are not evidently recognized[62-64]. Recently the activation of the intracellular bacterial sensor nucleotide oligomerization domain 2 (NOD2) was found to be implicated in the differentiation of Th17 cells by the enhanced production of TLR induced IL-1 and IL-23[65]. In addition, whereas type 1 interferon signalling was found to be pivotal for the induction of Th1 responses it constrains Th17 development[66,67]. The cell surface expression of costimulatory molecules also has specific effects on the differentiation of $CD4^+$ T cells. The absence of CD28 activation by CD80/CD86 is thought to induce regulatory T cells while the expression of Jagged-1 on DCs favors the development of Th2[68]. The variety in immune deviation perfectly illustrates the necessity of the DC driven immune response for survival of the host. However, a failure to control or counter-act these pathways would lead to the opposite. Uncontrolled Th1 or Th17 responses lead to exaggerated tissue damage and possibly breakdown of tolerance, initiating autoimmunity or the creation of an inflammatory vicious circle leading to chronic (auto)inflammation. An exaggerated Th2 response might lead to hypersensitivity, asthma and fibrosis. Altogether, DC are crucial in maintaining immune homeostasis, upholding tolerance at the one hand and eliminating infections at the other.

Dendritic cells and macrophages in experimental arthritis

The first direct evidence for the involvement of DC in experimental arthritis originated probably from the experiments performed by Leung et al[69]. Here, the transfer of collagen-pulsed DC induced arthritis that was both DC as well antigen specific since the transfer of T cells and/or non-pulsed DC had no effect. Only very recently, an adoptive transfer model in adjuvant induced arthritis showed that the influx of $CD45^+MHCII^+$ (DC like cells) was already apparent at day 3 far before the influx of PMNs that usually occurs at day 12-14[70]. Interestingly, by day 14, the $CD45^+$MHC-II^+ cells constituted approximately half of all $CD45^+$ cells in the synovial compartment suggesting the implication of these cells as early as the initiation of the inflammatory circle.

Another well-defined mouse model for RA is collagen-induced arthritis (CIA). CIA is a multifaceted, immunologically mediated disease involving T cells, B cells and populations of inflammatory cells that infiltrate the joint tissue and induce pathology[71]. CIA is induced through immunization with bovine type II collagen (CII) emulsified in CFA. DC will present the CII to T cells leading to T helper cell

(Th) activation. Type II collagen (CII)-specific Th cells are necessary to help the B cells to produce anti-CII specific antibodies. The presence of CII-specific antibodies is crucial for the disease induction, as B cell-deficient mice are not able to develop arthritis[72] and as transfer of CII-specific antibodies is already sufficient to induce arthritis[73,74]. The main CII-specific antibodies that are produced are of the IgG2a isotype, which are produced with the help of Th cells. Recently it was unequivocally demonstrated that myeloid DCs are the APCs crucial for the breaching of tolerance in another experimental mouse model. The formation of autoantibodies was prevented when DCs were removed indicating their pivotal role in the induction of autoreactive T and B cells. In addition to this treating mice with modulated "tolerogenic" DCs reduces arthritis severity in the CIA model via various pathways. TNFα and IL-10 modulated DCs were demonstrated to skew the immune response towards a Th2-like phenotype thereby probably inhibiting the deleterious Th1 and Th17 responses. Dexamethason-treated DCs inhibit the pathogenic Th1 responses by the induction of IL-10-producing T cells. Furthermore DCs genetically engineered to express IL-4 are able to inhibit and suppress established CIA[75-78], presumably by promoting the Th2-axis. Also unmodulated immature DC exhibit tolerogenic properties and repetitive injections of immature DC suppresses CIA[79-81]. This suppression was mediated by expansion of regulatory T cells with high immunosuppressive potential[82,83]. Altogether, these data clearly indicate that DC are involved in the onset and perpetuation of the inflammatory circle of synovitis and suggest that DC targeting is a challenging approach to treat arthritis.

Dendritic cells and macrophages in Rheumatoid Arthritis

The first evidence that highlighted the potential role of DCs in RA came from the observation that DCs are able to infiltrate the synovial tissue and fluid, potentially contributing to disease initiation and/or perpetuation by mediating T cell activation[84-86]. Next to this, histopathological evidence from combined analysis of RA synovial sections and RA experimental models indicated that macrophages and DCs are abundantly present in RA synovial tissue and that DCs drive the formation of ectopic lymphoid organs often observed in RA synovium[87]. In the synovium, DCs are located in the perivascular regions and both immature and mature DCs are present in the synovial tissue. Whether myeloid DCs mature locally or are attracted both as immature and mature cell types remains a matter of debate although local maturation is likely, based upon the markers found in the synovial tissue compared with that seen in draining lymph nodes[88,89]. The number of circulating

myeloid DCs in peripheral blood is lower in RA patients suggesting that they are selectively homing to the inflamed joint[90]. However, the major source of tissue DCs are monocytes which infiltrate the site of inflammation while differentiating into distinct DC and macrophage phenotypes. Monocytes are found increased in the peripheral blood of RA patients, possibly in an attempt to keep up the numbers in the blood while a large proportion readily infiltrates the joints. Infiltrating CD68 positive macrophages are found in large numbers in inflamed synovium of RA patients and the change in CD68 positive macrophages upon treatment correlates strongly with the change in disease activity score (DAS)[91].

Highlighting the importance of APCs in the disease process it is clear that APCs and their cytokines are effective therapeutic targets in the treatment of RA. Therapies often used now are biologicals directed against effector molecules like TNFα (adalimumab, etanercept, and infliximab), IL-6 (tocilizumab) or IL-1 (anakinra), cytokines readily produced by APCs upon activation. Another effective therapy directed against APCs, highlighting their crucial role in RA, is CTLA4-Ig (Abatacept), which binds to CD80/CD86 on the APC and prevents T cell activation. As Steinman and Banchereau recently stated, it appears that "DCs are an early player in disease development and an unavoidable target in the design of treatments"[92].

Dendritic cells and macrophages in Psoriatic Arthritis

In psoriatic disease the role played by DCs and macrophages is apparent from studies ranging from mouse models to human in vitro studies and drug trials. While in normal human skin only DCs are present in both the epidermis (Langerhans cells) and the dermis (dermal DCs) in psoriatic skin macrophages are also found in considerable numbers[93]. In two different mouse psoriasis models the crucial role played by monocyte-derived cells was demonstrated by selective depletion. One study demonstrated that in mice with an epidermis-specific deletion of inhibitor of NF-kappaB (IkappaB) kinase 2 (IKK2), epidermal keratinocytes can initiate a hyperproliferative, inflammatory, psoriasis-like skin disease whose development requires skin DCs/macrophages but not granulocytes or T cells[94]. The CD18 hypomorphic psoriasis mouse model is characterized by the reduced expression of the common chain of beta2 integrins to only 2-16% of wild type levels. Activated APCs are significantly increased in lesional skin as well as in inflamed skin draining lymph nodes of affected CD18 hypomorphic mice and were identified as being an important source of TNFα. Both depletion of APCs and neutralization of TNF-α resulted in a significant alleviation of psoriasiform skin inflammation. Demonstrating that maintenance of psoriasiform skin inflammation

critically depends on the efficient recruitment and activation of DCs/macrophages[95].

The inflamed synovium from spondyloarthropathy patients, including PsA patients, is characterized by a higher vascularity and infiltration with "resident" $CD163^+$ macrophages and polymorphonuclear leukocytes and by lower levels of lining-layer hyperplasia, lymphoid aggregates, infiltrating "proinflammatory" macrophages, $CD1a^+$ cells and the number of $CD83^+$ dendritic cells than RA synovitis. Global disease activity in spondyloarthropathy patients correlated significantly with lining-layer hyperplasia as well as with inflammatory infiltration with macrophages, especially the $CD163^+$ subset, and with polymorphonuclear leukocytes[96,97].

In lesional psoriatic skin immunoreactivity for IL-12 and IL-23 is significantly enhanced as compared to non-lesional and normal skin. Since recent trials using an antibody against IL-12p40, the common component of IL-12 and IL-23, have shown great efficacy in treating psoriatic disease and both IL-12 and IL-23 immunoreactivity were readily detected in $CD68^+$ and some $CD163^+$ cells and $CD1a^+$ dendritic cells[98] the crucial role played by myeloid APCs in human psoriatic disease is apparent.

Fc gamma receptors

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. Both activating as well inhibitory FcγRs are expressed on immature DC and full maturation of DC results in a down regulation of FcγR expression, highlighting the deprived capacity of antigen uptake of mature DC. 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]. In fact, the balance between activating and inhibitory FcγR has been shown to be crucial in the susceptibility to and phenotype of various inflammatory conditions. The activating FcγR mediates its effect through an immunoreceptor tyrosine-based activation motif (ITAM). In contrast, the inhibitory FcγRIIb signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM). It is the phosphorylation of the 5'-phosphoinositol phosphatase SHIP by ITIM which inhibits the ITAM regulated signaling pathways so that the balance between these FcγR systems determines cell fate.

Fc gamma receptors in experimental arthritis

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, whereas deletion of activating FcγR subtypes has the opposite effect. Several studies have shown that the presence of activating FcγRs was associated with increased chondrocyte death and cartilage erosion and that FcγRIII knockout mice are protected from IC-induced arthritis. Inhibition of spleen tyrosine kinase (Syk), a key mediator of activating FcγRs and B cell receptor signaling, also shows suppression of inflammation and bone erosion, highlighting a possible role of activating FcγRs in arthritis models. On the other hand, deletion of the inhibitory FcγRII (mice lack FcγRIIa) induced arthritis even in non-susceptible mice. FcγRIIb not only inhibits activating FcγRs, but is also important for effective clearance of ICs. An important role for the inhibitory FcγRIIb in arthritis was further substantiated by the finding that arthritis can be induced with a single injection of IgG anti-collagen type II antibody in mice lacking this receptor[99,100]. 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.These data clearly show the importance of activating FcγRs on APCs in the initiation and progression of arthritis, which is in line with the *in vitro* data showing involvement of activating FcγRs in pro-inflammatory cytokine production and inhibition of this by FcγRIIb.

Fc gamma receptors in Rheumatoid Arthritis

A role for FcγRs in RA synovial inflammation is less clear, but accumulating evidence suggests that these receptors are of considerable importance. Until recently a major problem in the study of FcγR on human myeloid cells was the inability to distinguish FcγRIIa from FcγRIIb on the cellular surface. In the synovial tissue macrophages and DC express significantly higher levels of FcγRII and III when compared with those present in synovium from healthy controls. Similarly, it was found that monocyte-derived macrophages and DCs obtained from patients with RA express FcγRII at significantly higher levels than those from their healthy counterparts[101,102]. Coherently, APCs from RA patients have been shown to produce more TNFα upon IC triggering than healthy control APCs. In addition, ICs derived from RA synovial fluid have been shown to trigger the production of TNFα by monocytes via FcγRIIa.

Variants of the activating Fc gamma receptors FcγRIIIa and FcγRIIIb have been associated with RA susceptibility in Europeans, but not in Asians. The FcγRIIIa variant is less effective in IC binding, implicating impaired clearance of ICs might play a role in the pathogenesis of RA. The functional polymorphism of the FcγRIIb (I232T) has been identified as the strongest prognostic factor for radiological joint damage in RA and was found to modulate DC function, further substantiating the role of FcγRs in RA[103].

Since Syk is involved in FcγR and B cell receptor signaling inhibition of this tyrosine kinase could reduce IC mediated inflammation and B cell responses, suggesting potential in RA treatment. In fact a randomized clinical trial has already demonstrated superior clinical efficacy of an oral Syk kinase inhibitor, R788 compared to placebo[104].

Toll-like receptors

Toll-like receptors (TLR) are pattern-recognition receptors capable of potently initiating DC activation and maturation. TLRs recognize both endogenous molecules, released upon cell activation/damage, and a wide range of conserved constituents from pathogens. At present, 10 TLR subtypes have been identified in humans, each having its specific ligands, cellular localization and expression profiles. TLR2 (as heterodimer in combination 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 lipo-polysaccharides (LPS)[5]. For TLR5, also expressed extracellular, the identified ligand is flagellin, a component of flagellated bacteria. TLR3, TLR7, TLR8 and TLR9 are generally addressed as intracellular receptors located in endosomal compartments and are involved in the recognition of nucleic acids derived from viruses, bacteria and the host[105]. For TLR10, which is believed to originate from the TLR1/TLR6 precursor no ligand has been described thus far. Downstream TLR signaling involves a family of five adaptor (MyD88, Mal, TRIF, TRAM and SARM) proteins that couple protein kinases ultimately leading to activation of transcription factors among which nuclear factor κB (NF-κB), the mitogen activated kinase-like proteins (e.g. p38, ERK1/2, JNK) and members of the interferon regulatory factor (IRF) family are the most thoroughly investigated nowadays (reviewed in[5,105]) In contrast with that what was expected in the first years after its discovery, the TLR system is highly specific in that distinct cellular responses are observed depending upon the activated TLR. Much of this specificity is likely to come forth from the use of various co-molecules and down-stream adaptor pathways by the various TLR.

The ligation of TLRs results in a massive release of cytokines, creating an environment crucial for the appropriate adaptive immune response. However, TLRs are also highly suspected as conductors of autoimmunity. Most pathogens express several TLR ligands and numerous host-derived TLR ligands are generated in inflamed or degenerated tissue[6,106,107]. The activation of multiple TLRs leads to the abundant release of pro-inflammatory cytokines creating a volatile situation. In genetically predisposed individuals this might eventually lead to a breach in tolerance culminating in autoimmune disease[108]. In this context, the study of the control of TLR responses is of high relevance in diseases such as rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus. However, it is essential to notice that an impaired release of pro-inflammatory mediators by innate immune cells might culminate in chronic inflammation due to the aberrant removal of pathogens or other inciting factors which might underlie diseases such as Crohn's disease[26,109].

Activation of TLRs such as TLR4 and TLR7/8, and especially their simultaneous stimulation, results in DCs releasing copious amounts of IL-12p70, which favours the proliferation of Th1 cells while activation of TLR2 does not result in the release of IL-12p70 and is generally thought to induce a Th2 response[110]. While the function of TLR4 and TLR7/8 thus seems unambiguously aimed at the induction of a Th1 response, the precise function of TLR2 in immunity is less clear. To date, accumulating evidence points towards an, additional, regulatory role of TLR2 in inflammation[111,112]. Recently it was pointed out that there is interaction between TLR2 and other pattern recognition receptors demonstrating that TLR2 co-activation inhibits the release of IL-12p70 induced by R848 in synergistic combination with LPS, β-glucan or zymosan[113]. However, Re and Strominger demonstrated already in 2004 that TLR2 activation induces the rapid production of IL-10 inhibiting IL-12p35 and IP-10 production induced by TLR4[114]. While a panoply of negative regulators of TLR responses are known, such as IL-10 and many endogenous signaling inhibitors (eg Tumor Necrosis Factor Alpha-Induced Protein 3 (TNFAIP3 (A20)), Single Immunoglobulin and Toll-interleukin 1 Receptor (TIR) Domain (SIGIRR), Toll Interacting Protein (TOLLIP), Suppressor Of Cytokine Signaling 1 and 3 (SOCS1, SOCS3)), the mechanisms that regulate these factors in macrophages and DCs infiltrating an inflammatory lesion are largely unknown[115]. Most studies have focused on their induction by TLRs themselves and have concluded that their generation mostly functions to restrict the production of pro-inflammatory cytokines at later time points.

Toll-Like Receptors in experimental arthritis

Experimental arthritis models have contributed extensively to our understanding of the role of TLRs in arthritis. Classical animal models such as streptococcal cell wall (SCW) arthritis and autoimmune arthritis in IL-1 receptor antagonist-knockout (IL-1ra$^{-/-}$) mice are dependent on activation of the innate immune system via TLRs. The acute phase of SCW induced arthritis is dependent on TLR2, while the TLR dependency shifts towards TLR4 in the chronic phase. This occurs simultaneously with the transition from a macrophage driven arthritis to a T cell dependent process[116]. Arthritis development in IL-1ra$^{-/-}$ mice is also regulated by TLRs, TLR2 knockout mice develop a more severe arthritis while TLR4 knockouts are protected from severe arthritis. Selective blocking of TLR4 was also able to diminish the incidence and severity of experimental arthritis[117,118]. A role for TLRs is further supported by studies that show a self-limited form of arthritis in mice after synovial injection of TLR ligands, such as CpG DNA and dsRNA[119,120].

Toll-Like Receptors in Rheumatoid Arthritis

The identification of TLRs as receptors for conserved pathogen associated molecular patterns as well as endogenous ligands started a whole new field of research. Data derived from those studies forms the basis of our current way of thinking regarding the role TLRs play in RA. 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. Ligands for TLR4 (heat shock protein B8) and TLR3 (host dsRNA) are, for example, highly expressed and potentially released upon an initiating event that might be as trivial as a small trauma or viral infection[6,121]. Second, the expression of various TLR subtypes is clearly increased in the synovial compartment of RA patients compared to their healthy counterparts and TLR ligands induce an augmented inflammatory response by macrophages and DCs from RA patients. DC from RA patients demonstrated a clearly potentiated response to purified ligands for TLR2 and TLR4 whereas TLR3 and TLR7 responses were comparable between patients and controls[122,123]. In addition, RA synovial fibroblasts produce increased amounts of chemokines upon stimulation with TLR2 ligands. TLR involvement is further suggested by a functional variant of the TLR4 gene that reduces the response to LPS and thereby decreases the susceptibility to RA, although not significant in another study[124,125].

The intracellular localization of the nucleic acid sensing TLRs normally prevents aberrant activation of APCs by endogenous nucleic acids. In many autoimmune diseases however antibodies have been found aimed at nucleic acids or proteins bound to these, enabling endocytosis of these ICs resulting in TLR activation by normally harmless components of self. Recently, TLR8 has also been shown to be important for TNFα production in RA synovial tissue. Inhibition of TLR8 was able to inhibit spontaneous TNFα production by RA synovial membrane cultures[126]. Stimulation of TLR8 by an unknown ssRNA containing component present in the RA synovium appears to lead to pro-inflammatory cytokine production, including type I IFNs, and might support APC maturation and subsequent presentation of (self) peptides to (autoreactive) T cells, a crucial event in the pathogenesis of autoimmunity. It might be possible that the endocytosis pathway by which the ssRNA is delivered to the intracellular TLR8 is mediated via antibodies and FcγRs. A major candidate present in large amounts in the RA synovial cavity to which such antibodies might be aimed is hnRNP-A2[12]. HnRNP-A2 forms a complex with heterogeneous nuclear RNA and in that form it might be a stimulus for TLR8 when delivered to the intracellular endosomes upon endocytosis via FcγRs. Immune complexes in joints might thus provide a direct link to cytokine dependent inflammation in RA, via APCs.

Prove that TLRs play an important role in the pathogenesis of RA has to come from clinical trials aimed at dampening or modifying TLR responses. However, unknowingly, drugs directed against TLRs are already in use for decades. Hydroxychloroquine, which prevents intracellular TLR activation, in combination with methotrexate shows increased effectiveness compared to methotrexate alone. The effectiveness of direct targeting of TLRs in RA is already somewhat supported by a phase II clinical trial with chaperonin 10, which inhibits TLR2 and TLR4 signaling. No placebo was used in this trial, so confirmation of its potential is necessary, but it suggests quick and sustained improvement of symptoms during the 12 weeks follow up[127]. Long term controlled studies have to show if chaperonin 10 could be used in the treatment of RA or not.

Toll-Like Receptors in Psoriatic Arthritis

The role played by TLRs in psoriatic disease is still less extensively studied than in RA. However, recent findings implicate that innate immunity might play a pivotal role in psoriatic disease. From genetic studies associations between inhibitors of Toll-like receptor (TLR) and nucleotide-binding oligomerization domain containing 2 (NOD2) signaling (TNFAIP3 (A20), TNIP1) and psoriasis are apparent[20]. In addition, it was

shown that mutations and polymorphisms in keratinocyte-expressed genes involved in physical barrier function or innate immunity, are risk factors for developing psoriasis[18,19]. Immunohistochemistry demonstrated the (intracellular) expression of TLR2, 3 and 4 in keratinocytes and psoriatic skin showed a strong over expression of TLR2 in the epidermis compared with normal skin. Furthermore dermal DCs were demonstrated to express TLR2 and TLR4 but no TLR9 while epidermal DC (Langerhans cells) expressed TLR4 but no TLR2 or TLR9. Heat shock proteins 27, 60 and 70, endogenous ligands for TLRs, were present in psoriatic skin but not in healthy skin[93]. Activation via TLRs of infiltrating plasmacytoid DCs has been proposed to be an important pathogenic event in psoriasis/psoriatic disease. An aggravation and spreading of a psoriatic plaque was described upon the topical treatment with the TLR7 agonist imiquimod. This exacerbation was accompanied by a massive type I interferon production by infiltrating plasmacytoid DCs (pDCs)[128]. Especially pre-psoriatic skin was demonstrated to contain high numbers of pDCs and its chemotactic factor chemerin. Skin from chronic plaques showed low chemerin expression and few pDC in the dermis[129]. The role played by the activation of intracellular TLRs is also apparent from the use of chloroquine, hydroxychloroquine and quinacrine in the treatment of psoriatic disease for over 50 years[130]. These drugs, which were originally used as antimalarials, have an inhibitory effect on the signaling of the endocytic TLRs 3, 7, 8 and 9. A question that remained was how the shielded endocytic TLRs might be activated in psoriatic disease. Recently it was demonstrated that the antimicrobial peptide LL37 (cathelicidin), which is highly expressed in psoriatic skin, converts inert self-DNA and self-RNA released by dying cells into a potent trigger of type 1 interferon production by pDCs by binding the DNA/RNA to form complexes that are delivered to endocytic compartments in pDCs and myeloid DCs. In pDC, self-DNA-LL37 and self-RNA-LL37 complexes activate TLR9 and TLR7, respectively, and trigger the secretion of IFNα without inducing maturation or the production of IL-6 and TNFα. In addition, in contrast to self-DNA-LL37 complexes, self-RNA-LL37 complexes activate myeloid DCs via TLR8 which leads to the maturation of the DCs and the production of TNFα and IL-6. Self-RNA-LL37 complexes were demonstrated in psoriatic skin lesions and were associated with mature myeloid DCs in vivo[131,132].

Outline of this thesis

TLRs play an important role in the aetiology of many human diseases. They have been implicated in the resolution or induction and aggravation of infections, cancer and autoimmune and (auto)inflammatory diseases. Monocyte-derived

cells such as DCs and macrophages play a central role in the immune system. A better understanding of how TLR responses are regulated in these cells and what the consequences might be for the following adaptive immune response might open new opportunities for the treatment of some of the most debilitating and life-threatening diseases around. This thesis in general concerns the regulation of pro-inflammatory TLR responses in DCs and macrophages and specifically the role DCs and their TLRs might play in Psoriatic and Rheumatoid Arthritis.

Chapter 2 is a good example of the latter. In this chapter we aimed to delineate whether RA patients who were, in time, able to successfully discontinue their anti-rheumatic treatment had phenotypical and functionally different DCs compared to healthy controls or other RA patients. This chapter has a specific emphasis on the regulation of TLR4 responses by FcγRs. Chapter three further elucidates the role played by inhibitory and activating FcγRs on proinflammatory type 1 and anti-inflammatory type 2 macrophages and their regulation of Toll-like receptor responses. Much research has been focused on how immune reactions are ignited. How an immune reaction is terminated at a site of inflammation with a plethora of pro-inflammatory cytokines and endogenous danger signals present is much less extensively studied. In chapter 4 we describe a novel mechanism how the phenotype of proinflammatory type 1 macrophages and DCs might be forced into a more anti-inflammatory phenotype during the development of an inflammatory lesion, with grave impact on TLR responses. Proinflammatory responses induced by activation of TLR4 have been shown to be crucial in mouse models of arthritis while TLR2 appeared protective. During inflammation the presence of various endogenous ligands able to activate different TLRs appears inevitable. In chapter 5 and 6 we gained more insight in how the (simultaneous) activation of different TLRs influences the behaviour of DCs and other immune cells and we shed some light on the intracellular pathways involved. In addition, chapter 6 shines a new light on the decreased arthritis severity in $TLR2^{-/-}$ mice. Cytokine activated T cells and TLR ligands appear to play important roles in the imbalanced cytokine network in the arthritic joint. Chapter 7 describes the synergy of these two pathways in inducing cytokine production from type 1 macrophages and the effect Abatacept (CTLA4-Ig) has on these. Infectious agents have been implicated in the etiology of PsA and RA for decades. Chapter 8 focuses on the role the (dysfunctional) activation of PRRs/TLRs on monocyte-derived DCs by (myco)bacteria might play in PsA. Following chapter 8, chapter 8 focuses on why the immune response in PsA might derail with special emphasis on the “anti-inflammatory” type 2 macrophages. Chapter 10 elaborates on the implications the novel findings described in this thesis might have in the field of rheumatic diseases.

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The inhibitory FcγIIb receptor dampens Toll-like receptor 4 mediated immune responses and is selectively up regulated on dendritic cells from rheumatoid arthritis patients with quiescent disease

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Abstract

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[25,26,27]. 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 FcγRI (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 μM), 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 FcγRIIb. 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 FcγRs. 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).

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 1)**. 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

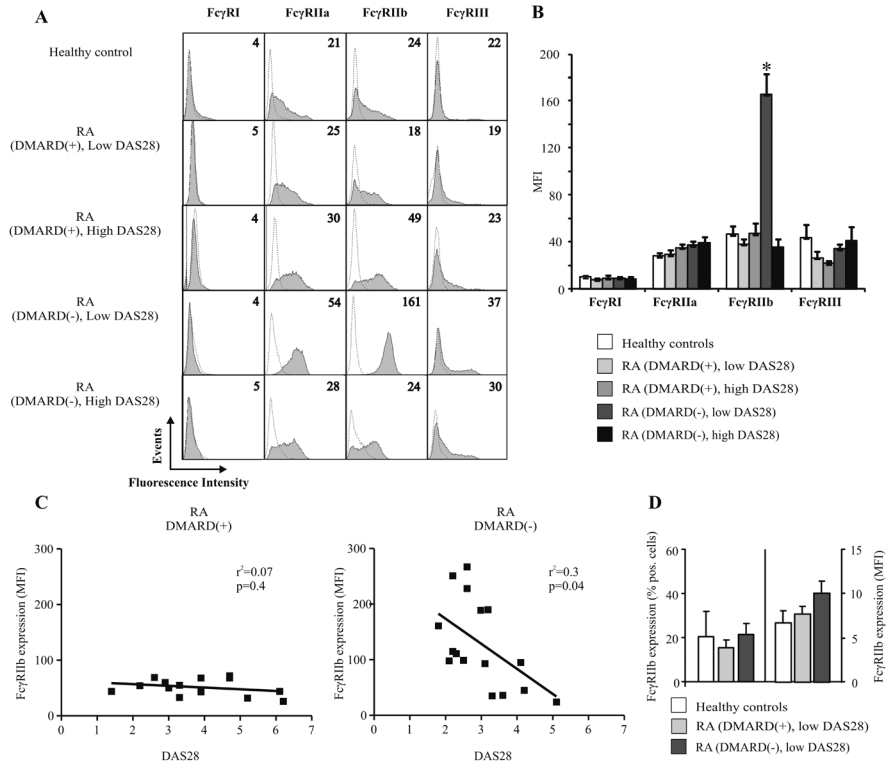

Figure 1

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), the expression of the inhibitory FcγRIIb is 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.

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 FcγRIIb 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)**.

Immune complexes 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 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

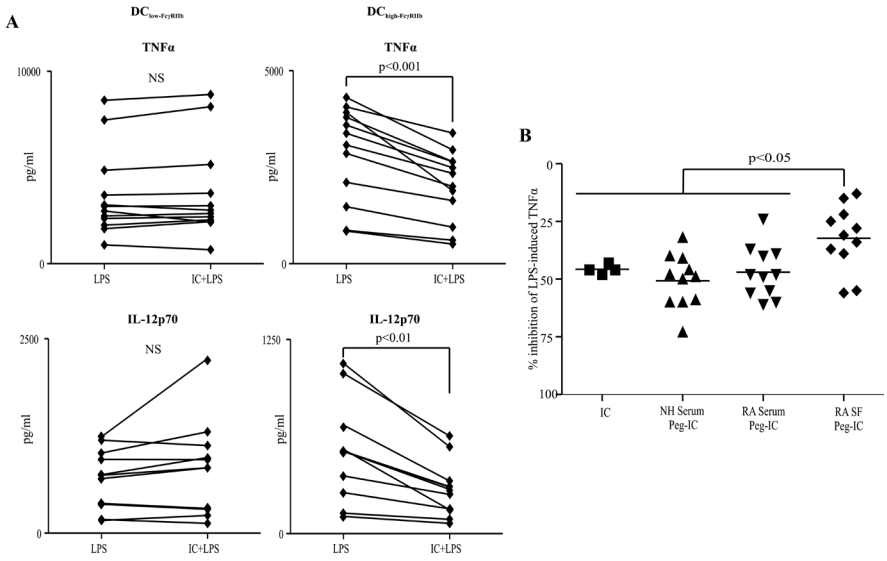

Figure 2

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.

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 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 $DC_{high\text{-}Fc\gamma RIIb}$ 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, $DC_{high\text{-}Fc\gamma RIIb}$ 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 $DC_{low\text{-}Fc\gamma RIIb}$. 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 $DC_{high\text{-}Fc\gamma RIIb}$ 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 $DC_{low\text{-}Fc\gamma RIIb}$ 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 $DC_{high\text{-}Fc\gamma RIIb}$ co-incubated with the combination of LPS and IC strongly increased the potential of T cells to secrete IL-13 compared to $DC_{high\text{-}Fc\gamma RIIb}$ incubated with LPS alone, whereas this effect was absent on $DC_{low\text{-}Fc\gamma RIIb}$ **(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)**.

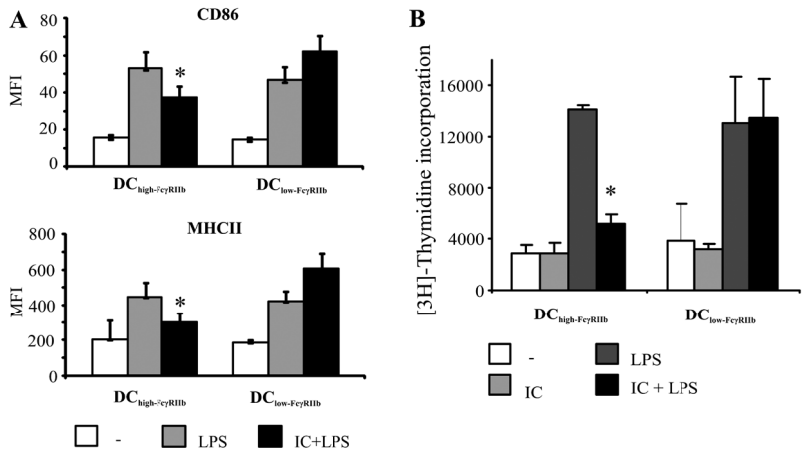

Figure 3

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

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 $DC_{high\text{-}Fc\gamma RIIb}$ 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 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 $DC_{low\text{-}Fc\gamma RIIb}$ to induce regulatory T cells (Treg). To this end, $CD3^+CD25^-CD127^+$ T cells were stimulated with $DC_{high\text{-}Fc\gamma RIIb}$ or $DC_{low\text{-}Fc\gamma RIIb}$ 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].

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 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 FcγRIIb was solely responsible for the inhibitory effect of IC on TLR4 immune responses.

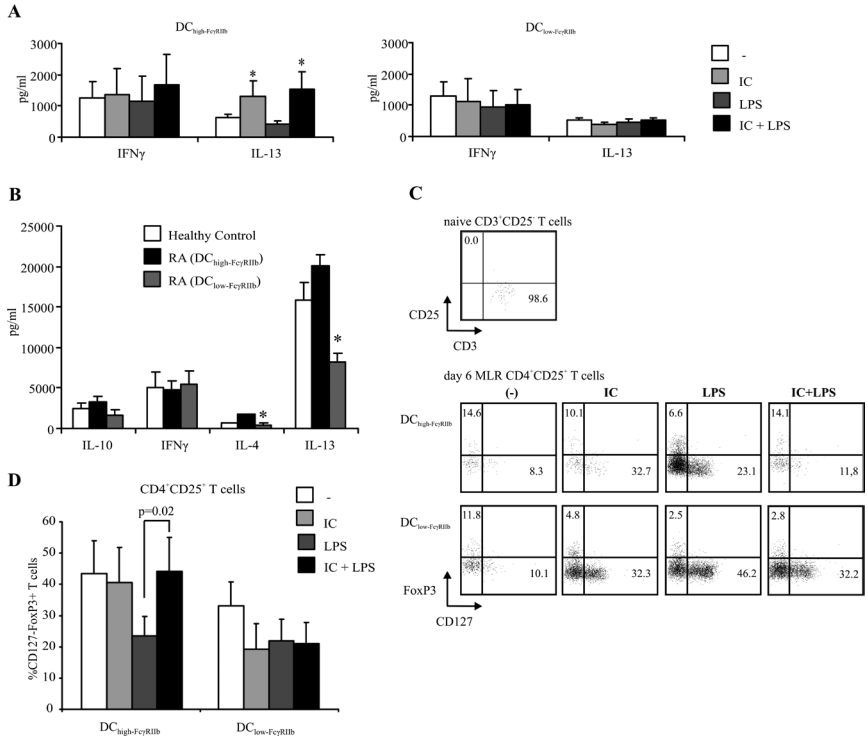

Figure 4

$DC_{high\text{-}Fc\gamma RIIb}$ 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 and 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 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 Figure 6e)**.

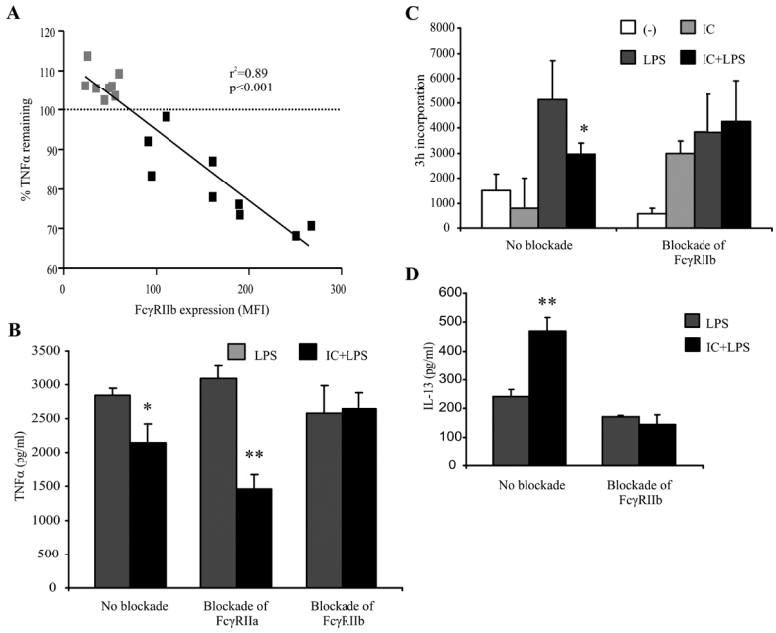

Figure 5

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.

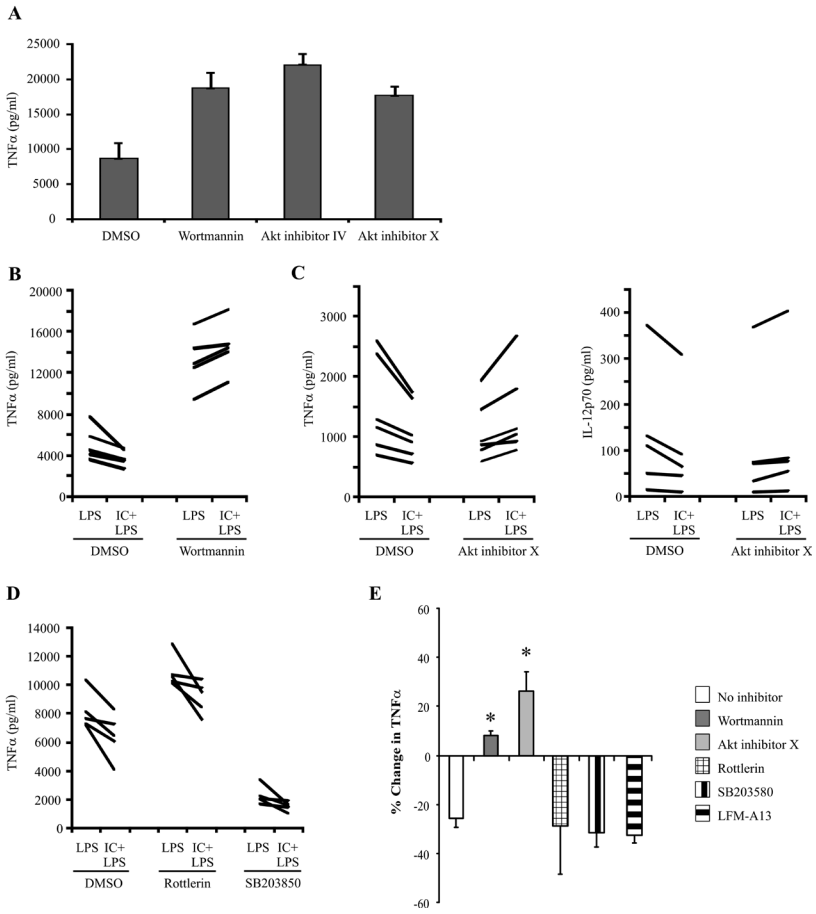

Figure 6

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, c and d**) LPS-induced TNFα secretion with and without co-stimulation of $DC_{high\text{-}Fc\gamma RIIb}$ 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 $DC_{high\text{-}Fc\gamma RIIb}$ from 5-6 DMARD(-) RA patients in each figure. **(e)** The percentage change in LPS-induced TNFα secretion with and without co-stimulation of $DC_{high\text{-}Fc\gamma RIIb}$ 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 $DC_{high\text{-}Fc\gamma RIIb}$ of 3-6 DMARD(-) RA patients. * indicates a p-value < 0.05 compared to $DC_{high\text{-}Fc\gamma RIIb}$ stimulated in the absence of an inhibitor.

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 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 FcγRIIb 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 $DC_{high\text{-}Fc\gamma RIIb}$ 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[16,62]. 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 $DC_{high\text{-}Fc\gamma RIIb}$ 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.

Taken together, here we show for the first time, that 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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Functional consequences of differences in Fc gamma receptor expression on pro- and anti-inflammatory macrophages

3

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Abstract

Macrophages are a very heterogeneous subset of myeloid cells with both anti- and pro-inflammatory functions. In autoimmune diseases like rheumatoid arthritis they play a major role in amplifying local inflammation and their activation occurs in the presence of abundant amounts of immune complexes. Depending on the balance between activating Fc gamma receptors (FcγR) and the inhibitory FcγRIIb, immune complexes can further activate or inhibit cell activation. The purpose of this study was to determine FcγR expression on pro- and anti-inflammatory macrophages and identify the functional consequences this has on immune complex uptake and macrophage activation via toll-like receptors (TLRs) and activated T cells. Human monocytes were isolated and differentiated into pro-inflammatory type-1 (mϕ-1) and anti-inflammatory type-2 (mϕ-2) macrophages in the presence of GM-CSF and M-CSF, respectively. FcγR expression on mϕ-1 and mϕ-2 was measured by flow cytometry and functional consequences were evaluated by stimulating the macrophages with either TLR ligands or activated T cells in the presence or absence of immune complexes. TNFα and IL-10 production were determined. Anti-inflammatory mϕ-2 express high levels of the activating FcγRIIa and FcγRIII and low levels of the inhibitory FcγRIIb. Contrastingly, pro-inflammatory mϕ-1 have much higher FcγRIIb levels and lower levels of the activating FcγRs. Immune complexes can inhibit TNFα production by mϕ-1 after TLR stimulation, while mϕ-2 are unaffected. This inhibition is dependent on FcγRIIb and subsequent PI3K activation. Mϕ-1, but not mϕ-2, produce high levels of TNFα upon co-culture with cytokine activated T cells, which can be inhibited by the presence of immune complexes. Immune complex uptake by mϕ-2 is increased compared to mϕ-1, corresponding with higher expression of activating FcγRs. In conclusion, immune complexes can inhibit TLR responses and T cell mediated activation of mϕ-1 and thereby control its pro-inflammatory phenotype, while no inhibition is seen in mϕ-2 which express very low levels of the inhibitory FcγRIIb.

Introduction

Macrophages are a very heterogeneous subset of immune cells with functions ranging from maintaining tissue homeostasis to fighting infections. Depending on micro environmental signals present in a tissue, monocytes that exit the bloodstream will differentiate into different types of macrophages. Two important survival and differentiation factors for macrophages are macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). M-CSF is ubiquitously expressed during steady-state conditions and induces anti-inflammatory macrophages that promote tissue repair and remodeling[1]. GM-CSF, on the other hand, is barely present in steady state conditions but is clearly detectable at sites of inflammation, such as an arthritic joint[2]. GM-CSF is produced by both immune cells and non immune cells such as fibroblasts and endothelial cells upon activation by various stimuli including the pro-inflammatory cytokines TNFα and IL-1β[3,4]. Corresponding with this in vivo distribution, human monocyte derived macrophages cultured in the presence of GM-CSF (type 1 macrophages, mϕ-1) have a pro-inflammatory phenotype, while macrophages cultured in the presence of M-CSF (type 2 macrophages, mϕ-2) have a more anti-inflammatory phenotype, marked by high levels of IL-10 production upon stimulation[5]. Recently, these macrophage subtypes have been extensively phenotyped; however specific knowledge of Fc gamma receptors on these macrophage subsets is still missing[5,6].

Macrophages play an important role in autoimmune diseases like rheumatoid arthritis (RA). In an inflamed joint macrophages are one of the main effector cells present and their levels correlate with disease activity and joint destruction (7,8). Macrophages are the main producers of pro-inflammatory cytokines, including TNFα and IL-6. The importance of these cytokines is exemplified by the efficacy of current therapeutics neutralizing these cytokines in the treatment of RA. In an arthritic joint, macrophages can be activated by TLR ligands (both endogenous and exogenous) and activated T cells, but also by the presence of immune complexes that are present in most RA patients. Immune complexes are recognized by Fc gamma receptors present on the membrane of antigen presenting cells. The Fc gamma receptor (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). FcγRIIa, FcγRIIb and FcγRIII are involved in the recognition of immune complexes and IgG coated particles/bacteria, while FcγRI mainly recognizes monomeric IgG. The balance between the activating and the inhibitory FcγRs on the cell membrane

determines the response of macrophages to immune complexes. Therefore, it is important to know the exact FcγR expression on different macrophage subtypes to understand their role in (auto) immunity and their behavior in the presence of immune complexes. Major factors hindering the extensive examination of different FcγR expression on myeloid cells, was the lack of specific antibodies to discriminate between the activating FcγRIIa and the inhibitory FcγRIIb. The development of the FcγRIIb specific antibody 2B6 gives us the opportunity to look specifically at FcγRIIa and FcγRIIb, which have very different functions[9]. Signaling via FcγRIIb can inhibit phagocytosis and cell activation mediated by the activating FcγRs, but FcγRIIb recently has been shown to have a more broad function in regulating immune function. Immune complex binding to FcγRIIb can modulate DC maturation and cytokine production upon TLR4 triggering[10,11]. This direct link between the Toll like receptor signaling and FcγR receptor signaling puts FcγRIIb at a central location in the pathogenesis of autoimmune diseases like RA and systemic lupus erythematosus (SLE), since TLR signaling and immune complexes have been shown to play important roles in these diseases. The broad immune regulatory function of FcγRIIb made us wonder if it could also influence the T cell mediated activation of macrophages.

Therefore, we determined the FcγR expression on type 1 and type 2 macrophages, two extremes in the whole range of anti- and pro-inflammatory macrophage subtypes. We further looked at the functional consequences of differential FcγR expression by evaluating the effect on immune complex uptake, but also on the modulation of macrophage activation via both TLRs and activated T cells in the presence of immune complexes.

Materials and Methods

Culture of monocyte-derived type 1 and type 2 macrophages and Tck cells

Peripheral blood mononuclear cells were isolated from heparinized venous blood of healthy volunteers using density-gradient centrifugation over Ficoll-Paque (Amersham Bioscience). The local Medical Ethics Committee approved the study protocol. Monocytes and CD4+ T cells were obtained using CD14 and CD4 microbeads respectively, and MS columns (Miltenyi Biotec). Monocyte-derived type 1 macrophages (mϕ-1) and type 2 macrophages (mϕ-2) 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, respectively. Macrophages were cultured in 6 well plates (Corning) with $0{,}5x10^6$ cells per well in 2 ml of culture medium (RPMI-1640 Dutch modification (Invitrogen) supplemented with 10% FCS, antibiotic-

antimycotic and L-glutamine (Invitrogen)). New culture medium with the same supplements (1 ml) was added at day 3 after which the cells 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) at a density of $5x10^6$ cells/ml for 6 days.
For the western blot experiments monocyte derived DCs have been cultured from CD14+ monocytes by culturing them for 6 days in the presence of GM-CSF (800 U/ml; R&D) and IL-4 (500 U/ml; R&D).

Phenotypical analysis

Using standardized flow cytometry protocols as described previously[12], phenotypical analysis of mϕ-1 and mϕ-2 was performed. Mϕ-1 and mϕ-2 were characterized for their FcγR expression using antibodies against FcγRI (CD64, clone 10.1; Dako) and FcγRIII (CD16, clone DJ130c; Dako), clone IV.3 which preferentially binds to FcγRIIa (Medarex) and the FcγRIIb specific antibody 2B6 (Macrogenics). Furthermore the expression of CD14, CD163 (both BD Biosciences) and MHC-II DR/DP (clone Q1514) was determined. Cells were analyzed using Cell Quest software for the proportion of positive cells and the mean fluorescence intensity relative to cells stained with the appropriate IgG isotypes.

Stimulation of monocyte-derived type 1 and type 2 macrophages

At day 6 mϕ-1 and mϕ-2 were harvested, washed, counted and replated in a concentration of $0.5x10^6$ cells/ml in 96 well (100 μl) culture plates. Cells were stimulated with TLR agonists for 20-24 hours in the presence or absence of heat-aggregated human immunoglobulins (IC, used in a concentration of 50μg/ml), prepared as previously described[13]. The concentration in which the TLR agonists were used was as follows: LPS (100 ng/ml, E. coli 0111:B4, Sigma-Aldrich), R848 (2 μg/ml, InvivoGen) and Pam3CSK4 (P3C; 5 μg/ml, EMC Microcollections). The used E. coli Lipopolysaccharide was double-purified at our laboratory according to the two-step phenol-water extraction method to remove any contaminating proteins resulting in purified LPS (14). Mϕ-1 and mϕ-2 were also co-cultured with cytokine-activated T cells for 24 hrs in a 1:5 ratio. This was performed in the presence or absence of IC prestimulation (50μg/ml) of macrophages for 1 h.
To determine the role of the inhibitory FcγRIIb in response to IC stimulation, FcγRIIb was specifically blocked before IC addition. FcγRIIb blocking was performed by 30 min pre-incubation of macrophages with 30 μg/ml 2B6 antibody at 4°C before stimulation with IC and TLR agonists. To determine the involvement of PI3K in the FcγRIIb mediated interference with TLR signaling, macrophages were also stimulated with IC and LPS in the presence of PI3K inhibitors. Macrophages were

pre-treated with the PI3K specific inhibitors Wortmannin (0.1 μM; Calbiochem) or LY294002 (10 μM; Calbiochem) for 1 h at 37°C before stimulation with IC and LPS.

Measurement of cytokines in culture supernatants

Levels of IL-6, IL-10 and TNFα 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.

Phagocytosis assays

Phagocytosis assays were performed with fluorescently labeled ICs, prepared as previously described (15). Mϕ-1 and mϕ-2 were incubated with FITC-labeled ICs (50 μg/ml) for 30 min at 37°C. The amount of IC uptake was determined by flow cytometry.

Western blot

moDCs were harvested 30 min after stimulation, washed in PBS and lysed in lysisbuffer. Protein concentrations were measured and equal amounts (50 μg) of protein were loaded on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were then blocked with 5% nonfat dried milk in TBST (15 mM Tris-HCL (pH 7.4), 150 mM NaCl, 0.1% Tween 20). Blots were probed overnight with a mouse antibody specific for phosphorylated SHIP and AKT (Cell signaling technologies) or with a mouse antibody specific for IκBα, according to the manufacturers protocol. The membranes were subsequently treated with the appropriate secondary antibodies and visualized by the ECL Western blotting detection kit (Pierce).

Statistical analysis

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

Results

Mϕ-1 express high levels of the inhibitory FcγRIIb, while mϕ-2 express higher levels of the activating FcγRIIa and FcγRIII

To date, mounting evidence suggests pivotal differences in macrophage subsets and supports a central role of Fc gamma receptor expression in part explaining the specific function of these subsets. Therefore, we evaluated the expression of

activating and inhibitory FcγRs on pro- and anti-inflammatory mϕ-1 en mϕ-2. To confirm the phenotype of our mϕ-1 en mϕ-2 we first analyzed their expression of CD14, CD163 and MHC-II. According to literature the expression of CD14 and CD163 was higher on mϕ-2, while the expression of MHC-II was slightly increased on mϕ-1 (Figure 1A)[5,6]. The monomeric IgG receptor FcγRI was similarly expressed in mϕ-1 and mϕ-2, while FcγRIII mean fluorescence intensity (MFI, reflects expression per cell) was highly increased on mϕ-2 compared to mϕ-1 (Figure 1B). Investigating the activating and inhibiting subtype of FcγRII separately, we observed a marked difference between the mϕ-1 en mϕ-2. Whereas the activating FcγRIIa is expressed higher on mϕ-2, the inhibitory FcγRIIb expression was markedly higher on mϕ-1. More specifically, the FcγRIIb/FcγRIIa ratio was 1.17 for mϕ-1 and 0.36 for mϕ-2. Thus, mϕ-2 have a skewed FcγR balance toward the activating FcγRs whereas FcγR expression on mϕ-1 is skewed towards the inhibitory FcγRIIb. In contrast with MFI, the percentage of positive cells was similarly distributed between mϕ-1 and mϕ-2 for all FcγR subtypes (Figure 1B).
The capacity to take up immune complexes is an important function of macrophages and dendritic cells. To evaluate the functionality of this altered activating/inhibitory FcγR balance, we investigated whether the mϕ-1 and mϕ-2 display a different uptake of ICs. By adding fluorescently labeled ICs to both macrophage subtypes, we determined that mϕ-2 show a significantly increased potential for IC uptake compared to mϕ-1 (Figure 1C), likely caused by their enhanced expression of FcγRIIa and FcγRIII.

Immune complexes inhibit TLR induced cytokine production by mϕ-1 but not by mϕ-2.

It has been shown previously that DCs from RA patients having quiescent disease express high levels of FcγRIIb and are able to inhibit TLR4 responses when co-stimulated with immune complexes[10]. To further evaluate the functional consequences of the differential expression of inhibitory and activating FcγRs on mϕ-1 and mϕ-2 we studied the role of immune complexes on TLR induced cytokine production. We found that mϕ-1 produce high levels of TNFα and low levels of IL-10 upon TLR stimulation, while mϕ-2 are marked by their relatively high IL-10 production and low production of pro-inflammatory cytokines which corroborates the literature (Figure 2A)[5]. After co stimulation with LPS and ICs, mϕ-1 were able to significantly attenuate TNF production compared to those stimulated with LPS alone, while IL-10 production is relatively unaffected (Figure 2A). 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 on mϕ-2. In fact, mϕ-2 show a trend towards increased production of TNFα. Following

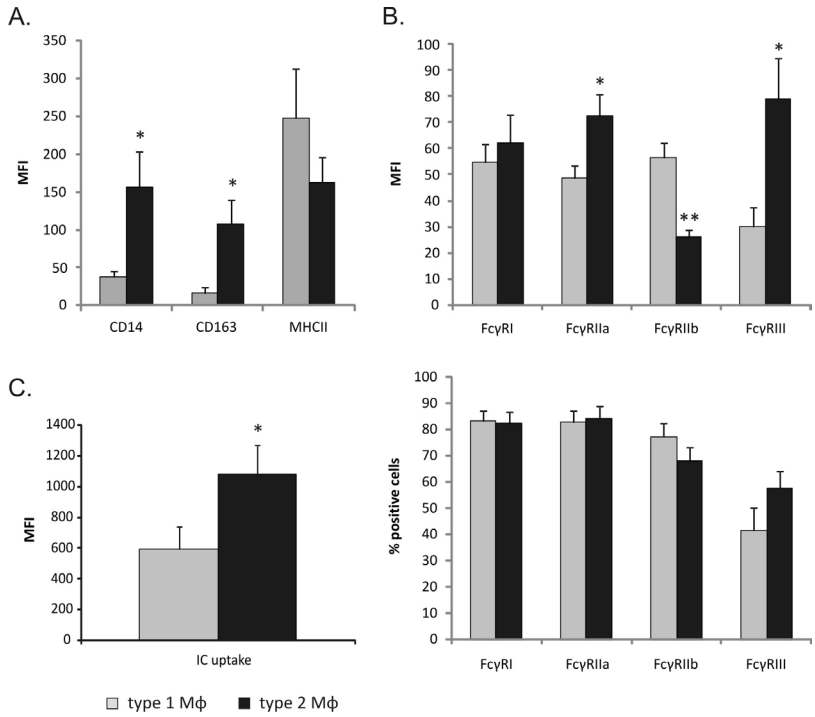

Figure 1

Mϕ-1 express high levels of the inhibitory FcγRIIb, while mϕ-2 express higher levels of the activating FcγRIIa and FcγRIII. Monocytes were cultured for 6 days with GM-CSF or M-CSF into mϕ-1 and mϕ-2. (**A**) CD14, CD163 and MHC-II expression (mean fluorescence intensity (MFI)) were determined by flow cytometry. Bars are mean and SEM from 6 donors. **(B**) The FcγR expression of the mϕ-1 and mϕ-2 was measured by flow cytometry. The upper panel shows the MFI and the lower panel the percentage positive cells from the same experiments. Bars are mean and SEM from 11 donors from 5 independent experiments. **(C)** Mϕ-1 and mϕ-2 were incubated with fluorescent labelled ICs (50 μg/ml) for 30 min at 37°C. IC uptake was determined by flow cytometry. Figure shows mean and SEM of 4 independent experiments. * $p < 0.05$ compared to mϕ-1, ** $p < 0.001$ compared to mϕ-1.

experiments learned that the inhibitory effect of IC on TLR signaling by mϕ-1 is not limited to TLR4, but also extends to TLR2 and TLR7/8 (Figure 2B). Again, IL-10 production was not clearly affected by the presence of ICs and this effect on TNFα production was not present in mϕ-2 (Figure 2B and data not shown).

Immune complexes can inhibit mϕ-1 activation by activated T cells

It has been advocated that macrophages that enter an arthritic joint do not only get activated by the presence of (endogenous) TLR ligands, but also by the presence

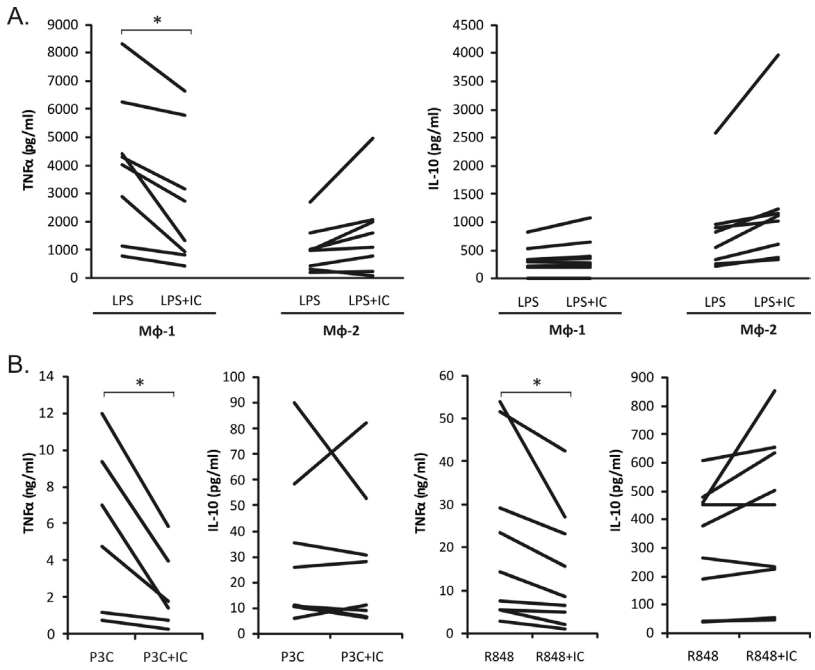

Figure 2

Immune complexes can inhibit TLR2, 4 and 7/8 induced cytokine production in mϕ-1. Monocytes were cultured for 6 days into mϕ-1 and mϕ-2, after which they were harvested, washed and replated in 96 well plates. **(A)** $5x10^4$ macrophages per well were stimulated with LPS (100 ng/ml) in the presence or absence of ICs (50 μg/ml). After 24 hours, supernatants were collected and analyzed for TNFα and IL-10 levels. **(B)** Mϕ-1 were stimulated with P3C (5 μg/ml) or R848 (2 μg/ml) in the presence or absence of ICs. After 24 hours, supernatants were collected and analyzed for TNFα and IL-10 levels. * $p < 0.05$ difference with and without IC.

of already activated T cells. T cells from RA synovial tissue or synovial fluid have been shown to mainly induce TNFα expression by monocytes/macrophages in a cell-cell contact dependent manner[16,17]. The phenotype of RA synovial fluid or synovial tissue T cells is comparable to T cells cultured in the presence of TNFα, IL-2 and IL-6[16,18]. To be able to mimic the in vivo RA situation more closely, we studied whether the difference in FcγR expression between mϕ-1 and mϕ-2 would affect the interaction of mϕ with these so-called cytokine activated T cells (Tck). Tck induced significant levels of TNFα production by the mϕ-1, while they hardly induce any cytokine production by the more anti-inflammatory mϕ-2 (data not shown). More importantly, pre-stimulation of mϕ-1 with ICs dramatically reduced the TNFα release by these cells with more than 50% upon TCK stimulation (Figure 3).

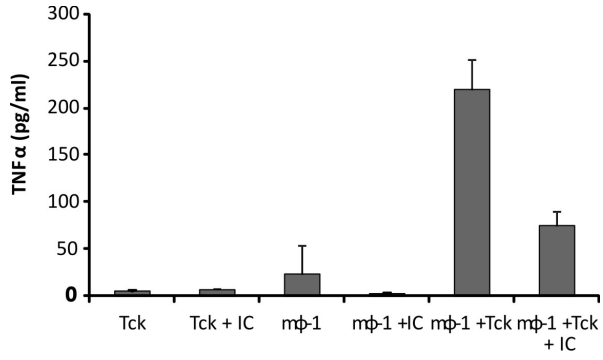

Figure 3

IC can inhibit T cell mediated macrophage activation in mϕ-1. Monocytes were cultured for 6 days into mϕ-1. From the same donor, CD4+ T cells were simultaneously cultured with IL-2 (25 ng/ml), IL-6 (100 ng/ml) and TNFα (25 ng/ml). At day 6 the macrophages and cytokine activated T cells (Tck) were harvested, washed and cultured together in a ratio of 1:5 in the presence or absence of ICs for 24 hours before collecting the supernatant. Bars are mean and SEM from three independent experiments.

The inhibitory effect of immune complexes is mediated via FcγRIIb and the PI3K/AKT pathway, preventing IκBα degradation

To determine whether the high FcγRIIb expression on mϕ-1 was responsible for the inhibitory effect of ICs on TLR signaling in these cells, we used a blocking antibody against FcγRIIb. Blocking of FcγRIIb fully abrogated the inhibitory effect of ICs on TLR induced TNFα production (Figure 4A). In dendritic cells our group has previously shown that the PI3K/Akt pathway is involved in the crosstalk between FcγRIIb and TLR4[10]. To test if this pathway is also involved in macrophages, we blocked PI3K with two different inhibitors before stimulation of mϕ-1 with ICs and LPS. The IC mediated inhibition of LPS induced TNFα production was clearly abrogated in the presence of either Wortmannin or LY294002, confirming the role of the PI3K pathway in FcγRIIb signaling in mϕ-1 (Figure 4B). The different effects of Wortmannin and LY294002 on TNF production upon TLR stimulation alone have been previously described and are caused by inhibitory effects of LY294002 on mRNA synthesis unrelated to PI3K function[19].

To further understand the mechanism involved in FcγRIIb-TLR4 crosstalk, we used DCs from RA patients with high and low FcγRIIb expression as described in our previous report[10]. Using this system we were able to investigate potential differences in FcγRIIb expression without the interference of other FcγRs, since these were similar in the FcγRIIb high and the FcγRIIb low DCs. Western Blot analysis demonstrated that the presence of ICs during the stimulation by LPS

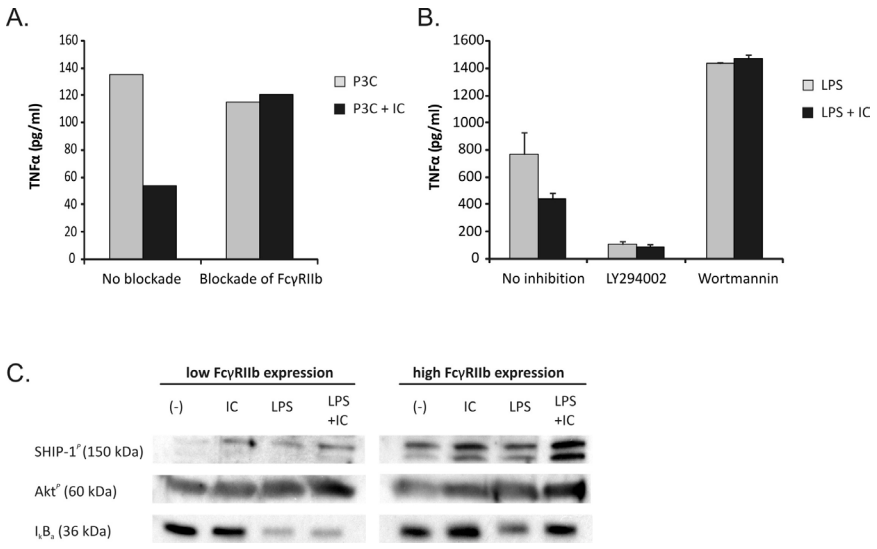

Figure 4

TLR inhibition by IC binding to FcγRIIb is mediated via PI3K. Monocytes were cultured for 6 days into mϕ-1 after which they were harvested, washed and replated in 96 well plates. **(A)** Mϕ-1 were stimulated with P3C and IC with and without blockade of FcγRIIb by the specific 2B6 antibody (30 μg/ml). After 24 hours, supernatants were collected and TNFα levels were analyzed. Bars show a representative experiment which was replicated three times. **(B)** Mϕ-1 were treated for 1 hour with PI3K inhibitor wortmannin (100 nM) or LY294002 (10 μM), prior to stimulation with LPS and ICs. After 24 hours, supernatants were collected and analyzed for TNFα levels. Bars are mean and SD of duplicate wells representative of 3 experiments. **(C)** $DC_{high\text{-}Fc\gamma RIIb}$ derived from RA patients who had successfully discontinued anti-rheumatics and $DC_{low\text{-}Fc\gamma RIIb}$ obtained from RA patients on DMARDs were stimulated for 30 minutes with medium, ICs, LPS or with the combination of LPS and ICs before protein isolation for Western Blot. The protein content of all cell lysates was measured to ascertain equal loading. Analysis was done on the protein content of the DCs derived from the same RA patient on phosphorylated SHIP, phosphorylated AKT and IκBα. Blots are representative for the results from 3 RA patients in each group.

resulted in markedly increased levels of phosphorylated SHIP in $DC_{high\text{-}Fc\gamma RIIb}$ whereas the addition of ICs had no effect on phosphorylated SHIP in $DC_{low\text{-}Fc\gamma RIIb}$ (Figure 4C). In addition, $DC_{high\text{-}Fc\gamma RIIb}$ displayed slightly increased levels of phosphorylated Akt upon the addition of ICs and LPS demonstrating that the increased phosphorylation of SHIP was not inhibiting the activation of Akt. Furthermore, the degradation of IκBα induced by LPS was abrogated by the addition of ICs to $DC_{high\text{-}Fc\gamma RIIb}$, while IκBα degradation was unaffected in $DC_{low\text{-}Fc\gamma RIIb}$ (Figure 4C). These results suggest that the activation of FcγRIIb inhibits the release of the p65 subunit of NFκ-B upon the stimulation of $DC_{high\text{-}Fc\gamma RIIb}$ with LPS culminating in a decreased release of pro-inflammatory cytokines.

Discussion

Mϕ-1 and mϕ-2 have been extensively characterized in the past few years[5,20]. Here we focused on FcγR expression on these macrophage subsets with special emphasis on the balance between activating and inhibitory FcγR subtypes. The increased FcγRIII expression on mϕ-2 and similar levels of FcγRI between mϕ-1 and mϕ-2 are confirming previous reports (5,21). Specifically determining the expression of FcγRIIa and FcγRIIb provided novel information regarding the inhibitory versus activating balance of FcγRs on these macrophages. Mϕ-1 have an increased expression of the inhibitory FcγRIIb, whereas mϕ-2 have a low expression of FcγRIIb and a high expression level of FcγRIIa (and FcγRIII), which most importantly, had clear consequences for multiple cell functions. TNFα production upon TLR and T cell mediated activation of mϕ-1 was inhibited by the presence of immune complexes, which was not the case for mϕ-2. Mϕ-2 were more efficient in immune complex uptake compared to mϕ-1, seemingly in line with the increased expression of the activating FcγRs.

So far, two studies have shown the capacity of FcγRIIb to inhibit TLR4 signaling[10,11]. Here, we demonstrate that the inhibitory effect of immune complex binding to FcγRIIb on TLR signaling is not specific for TLR4, but also applies to TLR2 and TLR7/8 on mϕ-1 further substantiating the pivotal role of the FcγR balance in regulation of the immune response. Our group previously described the involvement of the PI3K/Akt pathway in FcγRIIb-TLR4 crosstalk in human DCs[10]. Here we show that the PI3K pathway is also critically involved in immune complex mediated suppression of mϕ-1 TLR responses. In addition, we demonstrated that FcγRIIb activation leads to a clear and immediate inhibition of LPS induced IκBα degradation, implicating direct inhibition of the TLR4 signaling pathway. This indicates that de novo production of inhibitory mediators such as prostaglandin E2, as was suggested by Zhang et al, might not play a crucial role in the inhibition of TLR responses by FcγRIIb (Zhang et al, 2009). It has been described that signaling via ITAM containing FcγRs can interfere with TLR signaling[22]; however in our study we clearly show a selective effect in cells that express high levels of the ITIM containing FcγRIIb while cells with low FcγRIIb expression do not show inhibition. Even more, the inhibitory effect was completely prevented by blocking immune complex binding to FcγRIIb using a specific blocking antibody. More research is needed to determine the exact mechanisms of IC interference with TLR signaling.

In arthritis the pivotal role for FcγR has been often addressed. Two recent studies regarding macrophage responses to ICs in RA have been done on mϕ-2. They show that ICs formed by citrullinated fibrinogen and ACPA IgG can trigger activation of mϕ-2 mainly via FcγRIIa in combination with TLR4[23,24]. However, it would be interesting to further investigate the response not only by mϕ-2 but also by mϕ-1 under these circumstances. Since the ratio of FcγRIIb /FcγRIIa is clearly different on mϕ-1, these cells might show a quite different response to these TLR ligand containing ICs. This might help to explain better why some patients have high and some have low disease activity despite the presence of these antibody complexes in both groups. Moreover, not many CD163 positive macrophages (mϕ-2) are found in RA synovial tissue and joint inflammation in RA is better correlated with mϕ-1 derived cytokines[25]. Mϕ-1 would thus be an interesting macrophage subtype to look at in the context of RA.

T cell mediated macrophage activation seen in RA synovial tissue is dependent on cell-cell contact between memory $CD4^+CD45RO^+$ T cells and macrophages involving CD69, CD18 and CD49d[18]. Cytokine activated T cells resemble the functional properties of synovial T cells[16] and we show that ICs can inhibit Tck induced TNFα production by mϕ-1. Thus far, no direct interactions are known between FcγR signaling and CD69, CD18 or CD49d signaling. Hence, our work justifies more research focused at deciphering potential mechanisms involved in FcγRIIb inhibition on T cell mediated macrophage activation.

Next to regulating cell activation, FcγRs are also involved in endocytosis of small immune complexes and phagocytosis of IgG coated particles. The role for FcγRIIb in these two processes has been extensively investigated and appeared to be different in endocytosis compared to phagocytosis. While FcγRIIb has been demonstrated to inhibit phagocytosis induced via activating FcγRs, it does not inhibit endocytosis and it even actively participates in this process[26]. The increase of immune complex endocytosis in mϕ-2 is therefore most likely not a direct effect of lower FcγRIIb expression but merely an increased expression of the other FcγRs. Leidi et al. looked at phagocytosis of rituximab-coated B cells and found a two to three times increased rituximab-coated B cell uptake by mϕ-2 compared to mϕ-1[21]. Although FcγRIIb is more likely to actively inhibit phagocytosis in these settings, the difference is quite similar to what we see with fluorescent immune complexes. These findings support an important role for FcγR bearing macrophages not only in disease pathogenesis, but also in the treatment of RA.

FcγR expression is also closely linked to the pathogenesis of SLE and although macrophages were not originally considered a major contributor to SLE, recent studies have demonstrated a prominent role for macrophages in the pathogenesis

of SLE. In biopsies of kidney tissue from SLE patients activated macrophages are abundantly present, while they are not found in healthy kidneys[27]. Animal models demonstrate that mice with elevated numbers of circulating bone marrow-derived macrophage progenitor cells spontaneously develop an SLE phenotype, while the removal of macrophages prevents this[28,29]. Bergtold et al show that FcγRs on circulating hematopoietic cells are necessary for development of nephritis in (NZBxNZW)F1 mice. Even more, in the absence of activating FcγRs on hematopoietic cells, restoration of FcγR expression on monocytes/macrophages only is enough to induce development of glomerulonephritis in these mice[30]. In SLE there is also a strong interplay between the FcγR system and TLRs. The immune complexes present in SLE patients mostly consist of autoantibodies directed against DNA or RNA binding proteins, which can function as triggers for TLR7, 8 or 9. The findings that in both RA and SLE immune complexes are present which contain TLR ligands even further substantiates the importance of the interplay between these receptors. Further research would be necessary to determine the role of FcγR expression on multiple macrophage and dendritic cell subtypes regarding responses to these disease related immune complexes. Targeting FcγRIIb, especially on mϕ-1 since these are capable of reacting vigorously to TLR stimulation, might thus be a valuable new target in SLE.

In conclusion, we demonstrate that mϕ-1 and mϕ-2 display a different FcγR balance with high FcγRIIb expression on mϕ-1 and high FcγRIIa and FcγRIII expression on mϕ-2. This results in quite different responses to immune complexes, with an inhibitory function for immune complexes in combination with TLRs or cytokine activated T cells in mϕ-1 but not in mϕ-2. These differences in FcγR balance might be used for therapeutic targeting of specific macrophage subsets covering a wide spectrum of clinical conditions depending on what type immune reaction is desired.

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Mac-1 controls the pro-inflammatory phenotype of type 1 macrophages and Dendritic Cells

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Abstract

Macrophages perform dichotomous tasks; they are mediators of inflammation yet are capable of suppressing immune responses and mediate tissue homeostasis. The exact mechanisms by which macrophages shift their phenotype is unknown. We hypothesized that accumulation of macrophages during inflammation induces a shift from a pro-inflammatory to an anti-inflammatory phenotype, aimed at dampening the immune response. Pro-inflammatory type-1 (mϕ-1) but not anti-inflammatory type-2 macrophages were highly sensitive to homotypic cell-cell contact. Cell-cell contact led to mϕ-1 producing dramatically lower levels of TNFα, IL-1β and IL-6, while the IL-10 production was increased upon stimulation with TLR ligands or *M. tuberculosis*. This phenotypical shift depended on Mac-1, signaling via p38, the activation of COX2 and the subsequent up regulation of SOCS-3. Correspondingly, the pro-inflammatory cytokine production by dendritic cells as well as their ability to induce T cell differentiation into pro-inflammatory Th1 cells was restricted by cell-cell contact. Concluding, Mac-1 activation during macrophage and dendritic cell differentiation serves as a natural inhibitory mechanism designed to dampen chronic inflammation. This places signaling via Mac-1 at a centre stage in the regulation of macrophage and dendritic cell behavior in a wide range of diseases including autoimmune diseases as well as cancer, elucidating novel therapeutic avenues.

Introduction

Upon entering tissues or sites of inflammation 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 the resulting subsets of immune cells are able to control the progression or resolution of inflammatory processes [1]. Activated macrophages and their inflammatory products play a key role in innate immunity and in the pathogenesis of autoimmune and inflammatory diseases. GM-CSF and M-CSF are two cytokines important in the control of the differentiation and function of macrophage lineage populations in steady state as well as in inflammatory conditions [2-4]. Human monocyte-derived macrophages have been divided in pro-inflammatory type-1, GM-CSF cultured macrophages (mϕ-1) and anti-inflammatory type 2, M-CSF cultured macrophages (mϕ-2). Mϕ-1 produce high levels of the pro-inflammatory cytokines TNFα, IL-6 and IL-1β whereas mϕ-2 mainly produce IL-10 and trophic factors such as TGFβ and VEGF [1;5]. With regard to inflammation M-CSF and GM-CSF seem to play different and even competing roles.

GM-CSF has been depicted as a crucial mediator in various diseases [6;7]. Whereas under steady-state conditions GM-CSF is barely present, locally at sites of inflammation GM-CSF is clearly detectable [8]. GM-CSF is produced by a wide array of cells (e.g. monocytes, fibroblasts, endothelial cells) upon activation with various stimuli such as the pro-inflammatory cytokines IL-1β and TNFα [9-11]. In its turn GM-CSF primes cells from the myeloid lineage for the release of pro-inflammatory cytokines and the differentiation of monocytes into proinflammatory macrophages [12]. Neutralization of GM-CSF inhibited lipopolysaccharide (LPS) induced lung-inflammation and repressed the occurrence of inflammation and destruction in arthritis models while Rheumatoid Arthritis disease severity flares during GM-CSF treatment of neutropenia [13;13;14;14-17]. Regarding its role in infection GM-CSF was found invaluable for the formation of granulomas and the containment of *M. tuberculosis* [18]. In contrast to GM-CSF, M-CSF is constitutively expressed during steady state conditions and is found in high levels in serum. M-CSF is thought to regulate the development and maintenance of tissue macrophage subpopulations that have important trophic and scavenger roles in tissue morphogenesis and function [19]. In addition to this, it has been suggested that mϕ-2 might have a role in the suppression of tissue 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 [5;20].

Macrophage activation needs to be tightly controlled to enable them to rapidly mount responses to inciting pathogens but avoid excessive tissue damage by an over activate immune reaction. The activation of macrophages occurs for a large part via the activation of Toll-like receptors (TLRs). TLRs are essential pattern recognition receptors of the immune system widely expressed on a large variety of cells [21;22]. Most pathogens express several microbial TLR ligands and numerous host-derived TLR ligands are generated in inflamed or degenerated tissue [23-25]. In this context, the study of the control of TLR responses is of high relevance in inflammatory/autoimmune diseases including inflammatory bowel disease, atherosclerosis and rheumatoid arthritis as well as infectious diseases such as tuberculosis. While a panoply of negative regulators of TLR responses are known, such as IL-10 and many endogenous signaling inhibitors (e.g. Tumor Necrosis Factor Alpha-Induced Protein 3 (TNFAIP3 (A20)) and Suppressor Of Cytokine Signaling 1 and 3 (SOCS1, SOCS3)), the mechanisms that regulate these factors in macrophages and DCs infiltrating an inflammatory lesion are largely unknown [26]. Different receptors have been shown to be able to inhibit TLR responses in macrophages and DCs. FcγRIIb activation by immune complexes inhibits TLR4 responses by monocyte-derived DCs while the activation of β2-integrins by fibrinogen inhibits TLR and interferon signalling in macrophages. Interestingly, β2-integrins are known to play a role in cell-cell contact and homotypic aggregation of macrophages. This suggests that they might play a role in modulating the pro-inflammatory phenotype of macrophages without the presence of other external stimuli [27].

Since monocytes that enter a site of inflammation become highly pro-inflammatory macrophages under the influence of GM-CSF we hypothesized that there should be a negative feedback mechanism restricting the pro-inflammatory phenotype of mϕ-1 when at a site of inflammation sufficient amounts of macrophages have infiltrated. This might be an explanation for the ability of macrophages to perform such dichotomous tasks in an inflammatory microenvironment such as the clearance of pathogens and in time the removal of cellular debris and the restoration of tissue homeostasis. The current article supports this hypothesis by demonstrating that homotypic cell-cell contact via Mac-1 is a potent modulator of their pro-inflammatory phenotype. This phenotypical change, occurring when large and sufficient amounts of macrophages have infiltrated, appears to be aimed at dampening inflammation and restoring tissue integrity by the production of IL-10 and VEGF. This feedback mechanism was found to depend on signalling via the MAPK p38, the production of prostaglandins and the subsequent up regulation of SOCS-3. It thus appears that this pathway might play

an important regulatory role in shaping the extra-(and intra)cellular environment during inflammation. In diseases like Rheumatoid Arthritis, in which there is a skewed balance towards proinflammatory cytokines, this pathway might be exploited to reduce the production of these, while in cancer, the inhibition of this pathway might reduce the presence of suppressive tumor-associated macrophages leading to better anti-tumor responses.

Materials and Methods

Culture of monocyte-derived mϕ-1, mϕ-2 and DCs

PBMCs were isolated from heparinized venous blood of healthy volunteers by using density-gradient centrifugation over Ficoll-Paque (Amersham Bioscience). The local Medical Ethics Committee approved the study protocol. Monocytes were obtained using CD14 microbeads and MS columns (Miltenyi Biotec). Monocyte-derived mϕ-1, mϕ-2 or DCs were generated by culturing isolated monocytes in the presence of GM-CSF (800 U/ml; R&D), M-CSF (50 ng/ml, R&D) or IL-4 (500 U/ml; R&D) in combination with GM-CSF (800 U/ml), respectively, for 6 days. Mϕ/DCs were cultured in 6 or 24 wells plates (Corning) with the number of cells per well as indicated in 2 ml of culture medium (RPMI-1640 Dutch modification (Invitrogen Life Technologies) supplemented with 10% FCS, antibiotic-antimycotic and L-glutamine (Invitrogen Life Technologies). Fresh culture medium (1 ml) with the same supplements was added at day 3 after which the mϕ/DCs were harvested at day 6. Experiments performed in 12 well transwell plates (Corning) were carried out as follows: none or $0.5x10^6$ mϕ-1 were cultured for 6 days in the insert with or without none, $0.5x10^6$ or $2x10^6$ cells in the bottom well and after 6 days of differentiation the mϕ-1 were harvested. In some experiments we collected the media at day 6 from mϕ-1 cultured in a low ($0.5x10^6$ / well) and a high density ($2x10^6$ / well) and cultured newly isolated monocytes in this medium in a low concentration ($0.5x10^6$ / well) for 6 days with 1:1 fresh medium containing GM-CSF (800 U/ml).

In blocking experiments antibodies against LFA-1 (clone L15, 5 μg/ml), Mac-1 (clone Bear-1, 5 μg/ml) or CD18 (clone or L19, 5 μg/ml) or against IL-6 receptor (10 μg/ml, R&D), IL-10 receptor (10 μg/ml, R&D) and TGFβ (10 μg/ml, kindly gifted by Dr. R. Lafyatis, Boston University, Boston, USA) were added to the wells at the beginning of the culture. As a control antibody a mouse IgG_1 isotype control was used (10 μg/ml, eBioscience).

In experiments with intracellular signalling molecule/enzyme inhibition the following inhibitors were added at the beginning of culture and at day three: p38 (SB203850, 10 μM), Akt inhibitor X (0.5 μM), PI3K (Wortmannin, 10 nM), Erk

(PD98059, 2 μM), JNK inhibitor III (0.1 μM, with appropriate negative control in same concentration), Syk inhibitor II (0.1 μM), PP2 (0.5 μM), COX-2 inhibitor I (10 μM) (all purchased from Calbiochem).
In experiments determining the role of IFNβ and TGFβ in the differentiation of mϕ-1, monocytes were cultured into mϕ-1 in 6 well plates in a density of $0.5x10^6$ / well or $2x10^6$ / well for 6 days with or without IFNβ (5 ng/ml, Invitrogen) or TGFβ (5 ng/ml, Invitrogen) and subsequently harvested and stimulated.

Phenotypical analysis

Using standardized flow cytometry protocols as described previously [28], phenotypical analysis of mϕ-1, mϕ-2 and monocyte-derived DCs was performed. Mϕ-1 and mϕ-2 were characterized for their expression of CD163, TLR2, TLR4, CD14, CD86 (all BD Biosciences) and MHC-II DR/DP (clone Q1514). DCs were characterized by staining with mAbs against human CD80 (BD Biosciences), CD86 and MHC-II DR/DP. Cells were analyzed for the proportion of positive cells and the mean fluorescence intensity relative to cells stained with the appropriate IgG isotypes.

Phagocytosis assays

Phagocytosis assays were performed with labeled beads (2 μM carboxylate modified FluoSpheres, nile red 535/575nm, Invitrogen) or with labeled *Escherichia coli* (K-12 strain, Alexa Fluor 488 BioParticles, Invitrogen). Macrophages were harvested at days six, washed and replated in 96 wells plates in a concentration of $5x10^4$/100 μl/well. The labeled beads or *E. coli* were added to reach a final concentration of 10 beads per cell or 50 μg/ml. The macrophages were left on ice or were incubated at 37°C for 15, 30 or 60 minutes. Hereafter the cells were harvested and analyzed by flow cytometry for the amount of *E. coli* or beads bound and taken up.

Stimulation of monocyte-derived mϕ-1, mϕ-2 and DCs

Harvested day 6 monocyte-derived mϕ-1, mϕ-2 and DCs were washed, counted and plated in a concentration of $0.5x10^6$ DCs/ml and transferred to 24 well (1 ml) or 96 well (100 μl) culture plates. Cells were then stimulated with TLR agonists for 16-24 hours. For experiments in which mRNA levels were determined, TRIzol was added to monocyte-derived mϕ-1 immediately upon harvesting at day six. The concentration in which the TLR agonists were used was as follows: LPS (100 ng/ml, *E. coli* 0111:B4, Sigma-Aldrich), R848 (2 μg/ml, InvivoGen), Pam3CSK4 (5 μg/ml, EMC Microcollections). The used *E. coli* LPS was double-purified at our laboratory according to the two-step phenol-water extraction method to remove

any contaminating proteins resulting in purified LPS [29]. The dessicated *Mycobacterium tuberculosis* (H37Ra) was obtained from Difco and used in a concentration of 100 μg/ml. As a dectin-1 ligand Curdlan (50 μg/ml, Wako) was used.

Autologous mixed leukocyte reaction (MLR)

At day 6 DC were harvested, washed and resuspended in a concentration of $100x10^3$ DC/ml in culture medium. $5x10^3$ DC were replated in 96 round bottom well plates and were left unstimulated or were stimulated with R848 (2 μg/ml). $CD4^+$ T cells from PBMCs from the same healthy controls were obtained by negative selection using microbeads and MS columns (Miltenyi Biotec). $CD4^+CD45RA^+$ naïve T cells were separated from the $CD4^+$ population by the use of microbeads aimed at CD45RO. $50x10^3$ $CD4^+CD45RA^+$ T cells were added to the DCs in 96 round bottom well plates. At day six of the MLR cells were incubated with PMA (50 ng/ml, Sigma) and Ionomycin (1 μg/ml, Sigma) 12 hours before the collection of supernatants.

Measurement of cytokines in culture supernatants

Levels of IL-10, TNFα, IL-12p70, IL-6, IL-1β, VEGF, IFNγ, IL-13 and IL-17A 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.

RNA isolation and real-time PCR

Total RNA was extracted in 1 ml of TRIzol reagent. Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems). All PCRs were performed with SYBR Green Master mix (Applied Biosystems), 10 ng cDNA, and a primer concentration of 300 nmol/L in a total volume of 20 μl. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the gene of interest of each sample with the Ct values of the reference genes GAPDH (ΔCt) and were deployed as relative expression ($2^{-\Delta Ct}$). Software package Primer Express Version 2.0 (Applied Biosystems) was used to identify appropriate primer sets for *TLR2*, *TLR4*, *TLR7*, *TLR8*, *IFNβ*, *IRF7, TGFβ*, *SOCS1*, *SOCS3, TOLLIP, SIGIRR* and *A20*.

Statistical analysis

Differences between groups were analyzed using the Mann-Whitney U test, when appropriate a paired students T test was used. P values less than 0.05 were considered significant.

Results

Culture density affects the pro-inflammatory phenotype and function of mϕ-1 which resembles that of alternatively activated macrophages

In order to test our hypothesis that the phenotype of macrophages is influenced by the accumulation of monocytes during inflammation, resulting in a shift towards macrophages aimed at suppressing inflammation and restoring tissue homeostasis, we started by culturing monocytes for 6 days in the presence of GM-CSF or M-CSF to yield type 1 and type 2 mϕ, respectively, and analysed their phenotype. According to the literature the expression of CD163, CD14 and TLR2 was higher on mϕ-2 while the expression of CD86 and MHCII was higher on mϕ-1 [30]. The expression of TLR4 and Dectin-1 did not differ between the two mϕ subtypes **(Figure 1A)**. Next we cultured the monocytes in increasing concentrations with either GM-CSF or M-CSF. On day 6 the macrophages were harvested, counted and replated in 96 wells plates at a fixed concentration of $5x10^4$/100 μl per well before stimulation with LPS. In line with literature type 1 mϕ were capable of producing much higher levels of TNFα, IL-1β and IL-6 compared to type 2 mϕ. Strikingly, culturing monocytes into type 1 mϕ in high concentrations, mimicking an inflammatory setting with a high influx of monocytes/macrophages, resulted in a markedly 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 greatly dependent on cell concentration and not significantly present in type 2 mϕ. Although there was a trend towards even less TNFα and IL-6 production by type 2 mϕ cultured at higher concentrations. As with TLR4 activation similar effects were noted upon stimulation with a TLR2 or TLR7/8 ligand as well as with *M. tuberculosis* **(Figure 1C)**. These observations indicate that high cell density orchestrates a significant chance in cell behaviour that is unique for type I mϕ.

Based upon our early observations, we evaluated whether mϕ-1 cultured in higher densities would also gain an increased capacity to phagocytose particles mirroring so-called alternatively activated mϕ. Indeed, increasing cell densities in mϕ-1 boosted their ability to phagocytose labelled beads as well as labelled *Escherichia coli* considerably, whilst the expression of pivotal antigen uptake receptor was untouched **(Figure 2A, B, C)**. As described for "alternatively activated" macrophages [31] mϕ-1 cultured at high density, in contrast to those cultured at a low density, responded vigorously to Dectin-1 ligation, a pattern recognition receptor important in the recognition of *Candida spp.* [32], by releasing massive amounts of TNFα and IL-6 as well as IL-10 **(Figure 2D and data not shown)**.

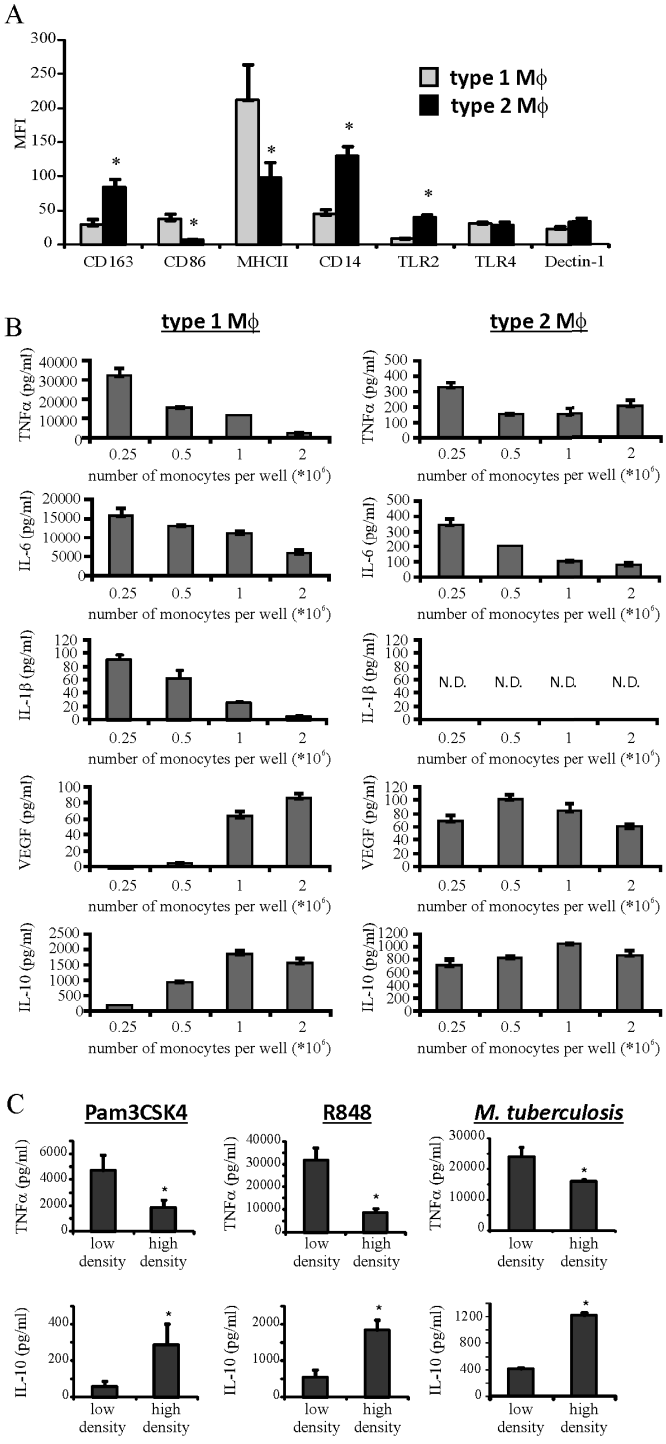
A
MFI
type 1 Mϕ
type 2 Mϕ
CD163
CD86
MHCII
CD14
TLR2
TLR4
Dectin-1
B
type 1 Mϕ
type 2 Mϕ
TNFα (pg/ml)
IL-6 (pg/ml)
IL-1β (pg/ml)
VEGF (pg/ml)
IL-10 (pg/ml)
N.D.
number of monocytes per well (*10^6)
C
Pam3CSK4
R848
M. tuberculosis
low density
high density

Figure 1

Differentiation of monocytes into type 1 mϕ in increasing concentrations shifts their behaviour from a highly pro-inflammatory to an anti-inflammatory phenotype. (A) Monocytes were cultured with GM-CSF or M-CSF after which they were analysed for their expression of various markers. Bars represent means and SEM of 5 independent experiments. * $p<0.05$ compared to type 1 mϕ. (B) Mϕ-1 and mϕ-2 were cultured in increasing concentrations, were harvested at day 6 and stimulated with LPS (100 ng/ml) for 24 hours before cytokine measurement of the supernatants. Results are presented as mean and SD of three replicates representative of three independent experiments. N.D. is not detectable. (C) Monocytes were cultured at a density of $0.5x10^6$ (low density) or $2.0x10^6$ (high density) per well in 6 wells plates for 6 days with GM-CSF in equal amounts of culture medium. At day 6 the mϕ-1 were harvested, washed, replated in 96 wells plates with $5x10^4$ macrophages per well and stimulated with Pam3CSK4, R848 or desiccated M. Tuberculosis (C). Supernatants were collected after 24 hours and analysed for their TNFα and IL-10 content. Data are presented as means and SEM of four independent experiments. * $p<0.05$ compared to mϕ-1 cultured in low density.

The shift towards a more anti-inflammatory mϕ-1 is dependent on cell-cell contact controlled by the β2-integrin Mac-1

As with a higher amount of monocytes in a 6 wells plate, culturing mϕ-1 in 24 wells plates induced increased IL-10 secretion upon TLR stimulation, while the release of TNFα was decreased **(Figure 3A and data not shown)**. This latter effect was not explained by higher amounts of GM-CSF since the addition of increasing amounts of GM-CSF had no effect **(data not shown)**. Also, cell survival was unaffected underscoring the idea that this effect is solely explained by an increased cell density **(Figure 3B).** β2-integrins are known to play a role in cell-cell contact and homotypic aggregation of macrophages [27]. Blocking of the common β chain CD18, which associates with CD11a, CD11b, CD11c and CD11d to form distinct functional heterodimers (LFA-1, Mac-1, gp150,95 and CD11d/CD18, respectively), during mϕ-1 differentiation under high-density circumstances completely prevented the aggregation of the monocytes/macrophages while blocking of CD11a alone did not **(Figure 3C)**. Blocking of CD18 on high density cultured mϕ-1 led to macrophages producing significantly less IL-10 and more TNFα upon LPS stimulation. In contrast, blocking of CD11a did not affect the release of these cytokines **(Figure 3D)**. Specific blockade of Mac-1 (CD11b/CD18) resulted in the same effect as blockade of CD18 and was similar upon the stimulation with other TLR ligands and *M. tuberculosis* **(Figure 3D and data not shown)**. When GM-CSF macrophages were cultured in low concentrations the addition of CD18 blocking antibodies did not have an effect on the release of IL-10 or TNFα upon TLR4 activation **(Figure 3E and data not shown)**. Blockade of CD18 or CD11b of high density mϕ-1 at the time of stimulation with LPS did not

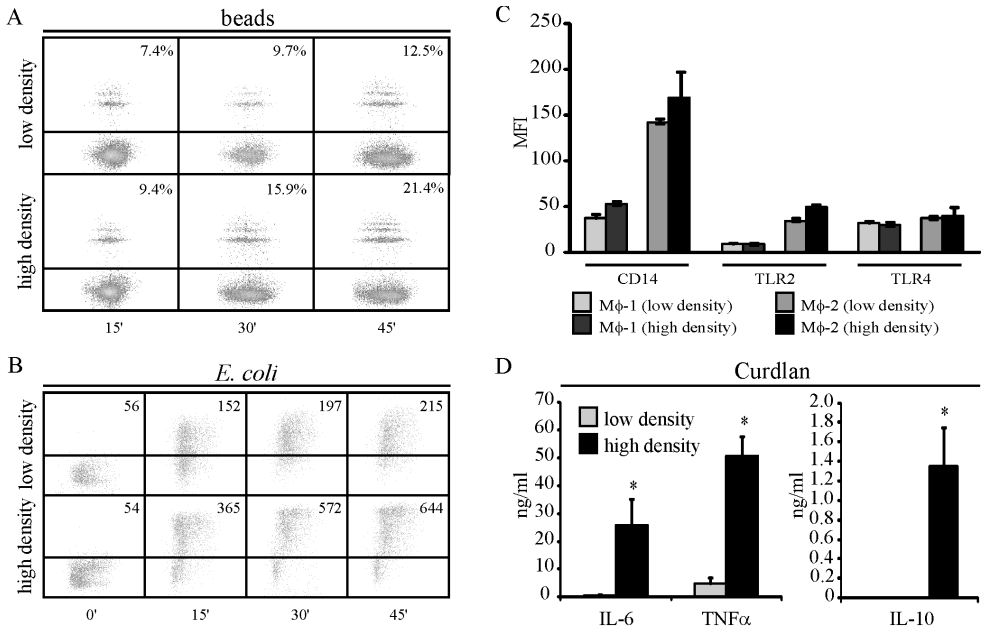

Figure 2

Type 1 macrophages cultured at high density display characteristics of alternatively activated macrophages. Monocytes were cultured in a low ($0.5x10^6$ per well in 6 wells plates) or a high density ($2x10^6$ per well in 6 wells plates) into Mϕ-1 after which they were analysed for their ability to phagocytose labelled beads (A) or labelled *E. coli* (B) by flowcytometry. Numbers are percentage positive cells (A) and mean fluorescence intensity (B). Data are representative for three independent experiments. (C) Mϕ-1 and mϕ-2 were cultured at low and high density and the expression of CD14, TLR2 and TLR4 was determined at day 6. Bars are mean and SEM of four independent experiments (D) Mϕ-1 were cultured at low and high density for 6 days and subsequently stimulated with curdlan for 24 hours. Levels of TNFα and IL-6 were measured in the supernatants. Bars are mean and SEM of four independent experiments, * $p<0.05$ compared to low density mϕ-1.

influence their release of IL-10, TNFα and IL-6 **(data not shown)**. Taken together, our observations show the pivotal role of Mac-1 in the control of cell function in inflammatory conditions where high cell densities are likely.

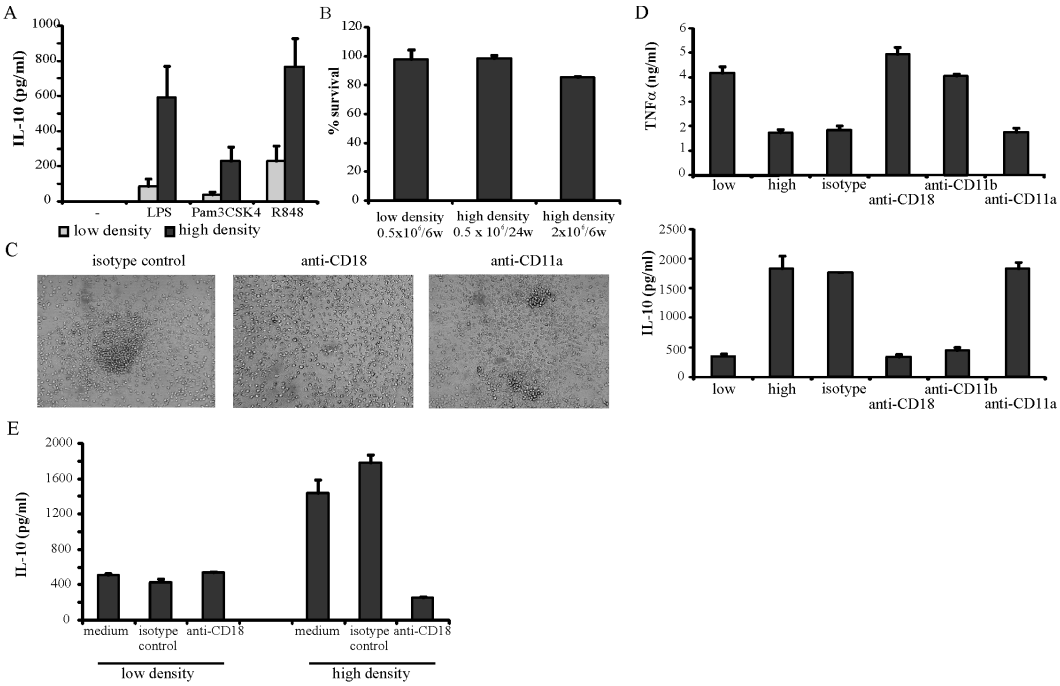

Figure 3

Blocking CD18 inhibits the differentiation of mϕ-1 towards more anti-inflammatory macrophages. (A) $0.5x10^6$ monocytes were cultured into mϕ-1 in either a 6 wells plate well (low density) or a 24 wells plate well (high density) and subsequently stimulated with LPS, Pam3CSK4 or R848. After 24 hours the supernatants were analysed for their IL-10 content. Bars represent mean and SEM of 5 independent experiments. * $p<0.05$ (B) Monocytes were cultured into mϕ-1 with $0.5x10^6$ in a 6 or a 24 wells plate or with $2x10^6$ in a 6 wells plate. At day 6 the mϕ-1 were harvested and counted. The amount of counted cells was related to the amount of monocytes that were added to the wells at the start of the culture (% survival). Data are presented as means and SEM of three independent experiments. (C) Monocytes were cultured into mϕ-1 at high density in the presence of blocking antibodies against CD18 or CD11a or mouse IgG1 control antibodies (all 5 μg/ml). At day 3 photographs were taken at an original magnification of 100x. (D) Low density mϕ-1 (low), high density mϕ-1 (high) and high density mϕ-1 cultured in the presence of an isotype control antibody or blocking antibodies against CD18, CD11b or CD11a were stimulated with LPS for 24 hours. The IL-10 and TNFα levels were measured in the supernatants. Bars are mean and SD of triplicate wells representative of three experiments. (E) $0.5x10^6$ monocytes were cultured into mϕ-1 in a 6 wells plate (low density) or a 24 wells plate (high density) in the presence of blocking antibodies against CD18, a control antibody or medium. At day 6 the macrophages were stimulated with LPS after which the IL-10 levels in the supernatants were determined. Bars are mean and SD of triplicate wells representative of three independent experiments.

The shift towards a more anti-inflammatory mϕ-1 is mediated by a soluble factor

Fuelled by our observations we sought evidence for the possibility that these effects were caused by soluble factors. In this light it was interesting to take note that increasing the concentration of macrophages in the bottom well of a trans-well system induced a more anti-inflammatory phenotype of the macrophages in the upper well **(Figure 4A)**. In addition, culturing mϕ-1 at a low concentration in day 6 medium from mϕ-1 cultured at a high concentration produced markedly elevated levels of IL-10 and decreased levels of TNFα compared to macrophages cultured in medium from mϕ-1 cultured at low density **(data not shown)**. We set to determine whether cytokines known to be able to dampen the pro-inflammatory phenotype of myeloid cells would play a role in the β2 integrin mediated phenotypical shift. We determined the basal mRNA expression of TGFβ and IFNβ as well as the basal secretion of IL-6 and IL-10, since these have been implicated in the induction of anti-inflammatory macrophage cell types and found that the level of TGFβ was up regulated while IFNβ and IRF7 expression was unaffected **(Figure 4B)**. Mϕ-1 were found to spontaneously produce slightly higher levels of IL-6 when cultured in high concentrations while IL-10 was not produced (**data not shown).** We determined whether the inhibition of IL-6 or TGFβ would mimic the effect of blocking CD18. Blocking IL-6 receptor or neutralising TGFβ during the differentiation of mϕ-1 had no significant effect on the production of IL-10 so neither appeared to be involved in the effect caused by β2 integrin activation via cell-cell contact **(Figure 4C)**. In contrast, specific and dose-dependent inhibition of COX2 led to the complete reversal of the Mac-1 activation induced anti-inflammatory phenotype of mϕ-1 cultured at high density **(Figure 4D, E)**. These observations thus identified prostaglandins as key mediators orchestrating the anti-inflammatory mϕ-1 phenotype upon increasing cell densities.

Signalling through MAPK p38 underlies the shift towards a more anti-inflammatory mϕ-1 phenotype and increases the expression of SOCS-3

To further delineate the signalling pathways involved in the Mac-1 mediated induction of an anti-inflammatory phenotype in mϕ-1 we cultured mϕ-1 at a high density in the presence of various inhibitors of intracellular signalling molecules. In addition to COX2 the inhibition of p38 MAPkinase resulted in a reduction of the release of IL-10 to levels produced by mϕ-1 cultured at low density **(Figure 5A)**. To determine whether this effect was not due to a direct inhibitory effect on TLR signalling we measured the release of TNFα. The release of TNFα was increased upon TLR stimulation when signalling via p38 was blocked during culture **(Figure 5B)**, stressing the role played by p38 in Mac-1 signalling as was previously proposed [33].

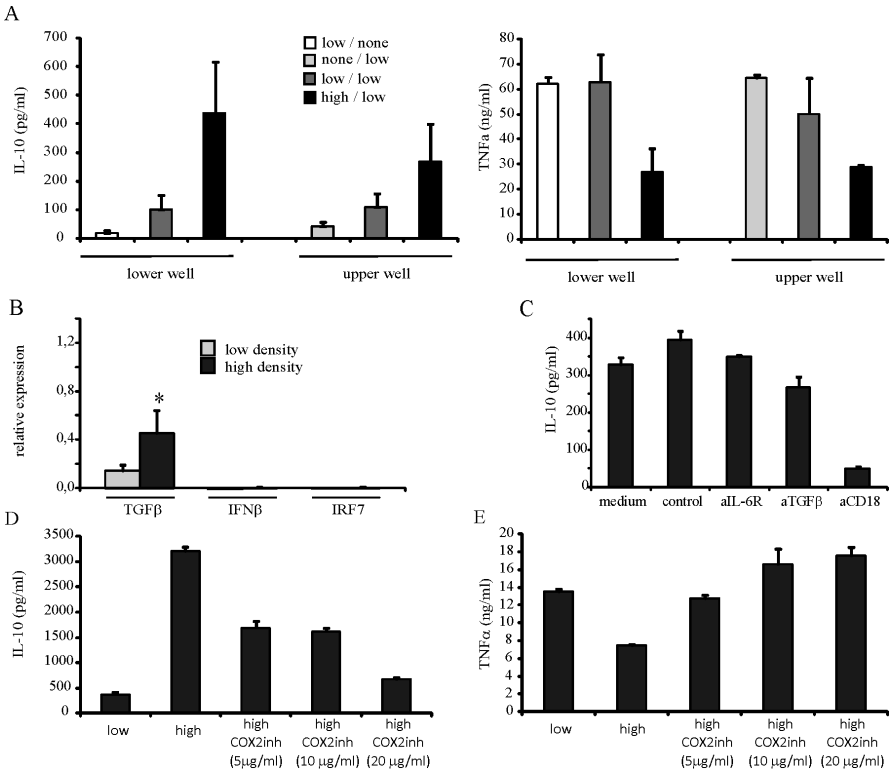

Figure 4

The anti-inflammatory mϕ-1 phenotype induced by cell-cell contact is carried over by a soluble factor and can be halted by inhibiting COX2. (A) Monocytes were cultured 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 mϕ-1 were stimulated with LPS and after 24 hours the supernatants were analysed for their IL-10 (left panel) or TNFα (right panel) content. (B) Mϕ-1 cultured in a low or high density were analysed for their mRNA expression of *TGF*β, *IFN*β, *IRF7* by real-time PCR. Expression is relative to GAPDH expression. Data are presented as the mean ± SEM of three independent experiments. * $p<0.05$ compared to mϕ-1 cultured in low density. (C) Mϕ-1 were cultured at high density in the presence of medium, control antibody, a TGFβ neutralising antibody or blocking antibodies against IL-6 receptor or CD18. After their differentiation they were stimulated with LPS and the released IL-10 levels were measured. Bars are mean and SD of triplicate wells representative of three experiments. Low density mϕ-1 and high density mϕ-1 were cultured either in the absence or presence of increasing amounts of COX2 inhibitor after which they were harvested and stimulated with LPS for 24 hours before collection of the supernatants. The levels of IL-10 (D) and TNFα (E) were measured in the supernatants. Bars are mean and SD of duplicate wells representative of three experiments.

Next we evaluated the expression of intracellular inhibitors of TLR signalling pathways SOCS-1, SOCS-3, TOLLIP, SIGIRR and A20 and observed that SOCS3 was up regulated significantly when mϕ-1 were cultured in high concentrations, an effect which was modulated by Mac-1 signalling **(Figure 5C, D)**. Moreover, functional p38 and COX2 were necessary for the induction of SOCS3 **(Figure 5E)**. These results clarify a new potent anti-inflammatory feedback mechanism in the innate immune response. Accumulation of macrophages in inflammation appears to invoke an anti-inflammatory phenotype in macrophages through cell-cell contact by Mac-1, leading to signalling via p38, thereby inducing the production of COX2 dependent prostaglandins which modulate macrophage phenotype by increasing the expression of SOCS3 and enabling the marked production of IL-10.

The inflammatory phenotype of monocyte-derived dendritic cells is strictly controlled by cell-cell contact

We next sought evidence for a similar role for integrin biology in the regulation of monocyte-derived DC behaviour. As with mϕ-1, DCs cultured in high concentrations produced increased level of IL-10 and decreased levels of IL-12p70 and TNFα **(Figure 6A, B and C)**. No significant differences were found in the expression levels of MHCII, CD80 or CD86 on DCs cultured at a low or high density on a basal level or upon stimulation with R848 **(data not shown)**. Since DCs are the professional antigen-presenting cells of the immune system we wondered whether the induction of T cell proliferation and differentiation would also be affected when DCs were cultured in higher concentrations. DCs cultured at low density induced a polarization of autologous naive T cells into T cells capable of releasing strikingly higher levels of IFNγ and they induced an increased proliferation of autologous naive T cells **(Figure 6D and 6E).** This demonstrates that the phenotype of DCs shifts towards a phenotype less capable of inducing strong Th1 responses when differentiated in the close presence of homotypic neighbouring cells.

TGFβ plays a restricting role in the development of a more anti-inflammatory mϕ-1 phenotype

Since TGFβ is found in high levels during chronic inflammatory processes we wondered what the effect of TGFβ would be on the differentiation of high and low density mϕ-1. We found that TGFβ had no effect on the differentiation of low density mϕ-1. However, when mϕ-1 were cultured at high concentrations in the presence of TGFβ, increased TNFα production but suppressed IL-10 secretion upon TLR stimulation was observed, as well as decreased basal SOCS-3 expression **(Figure 7A and B)**. In line with the increased TNFα (and IL-10) production upon Dectin-1 stimulation when mϕ-1 were cultured at high cellular numbers, addition

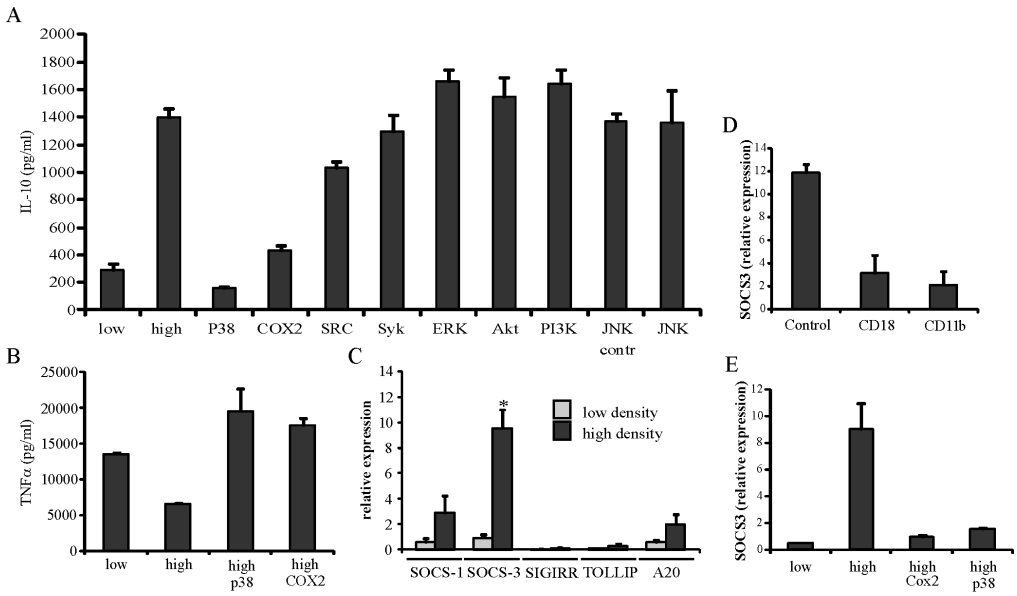

Figure 5

The anti-inflammatory mϕ-1 phenotype induced by Mac-1 activation is dependent on the MAPkinase p38. (A) Mϕ-1 were cultured in a low concentration (low) or a high concentration (high) or at a high concentration in the presence of inhibitors for p38, COX2, SRC family kinases, Syk, Erk, Akt, PI3K or JNK. At day 6 the resulting mϕ-1 were harvested and stimulated with LPS. After 24 hours the supernatants were harvested and analyzed for their IL-10 content. Data are mean and SD of duplicate wells. Data are representative of 3 independent experiments. (B) Similar experiments as described for panel A were performed with low and high density mϕ-1 and high density mϕ-1 in the presence of inhibitors for p38 or COX2. The production of TNFα after 24 hours of stimulation with LPS was determined. Data are mean of duplicate wells and SD. Data are representative of 3 independent experiments. (C) Mϕ-1 cultured in a low or high density were analysed for their mRNA expression of *SOCS-1*, *SOCS-3*, *SIGIRR, TOLLIP* and *A20*. Expression is relative to GAPDH expression (x100). Data are presented as the mean ± SEM of four independent experiments. * $p<0.05$ compared to mϕ-1 cultured in low density. (D) Mϕ-1 cultured at high density in the presence of an isotype control antibody (control) or blocking antibodies against CD18 or CD11b were analysed for their mRNA expression of *SOCS-3*. Expression is relative to GAPDH expression (x100). Data are presented as mean ± SD of triplicate wells. Data are representative of 3 independent experiments. (E) Mϕ-1 cultured at low density or high density in the absence or presence of an inhibitor for COX2 or p38 were analysed for their mRNA expression of *SOCS-3*. Expression is relative to GAPDH expression (x100). Data are presented as mean ± SD of duplicate wells. Data are representative of 3 independent experiments.

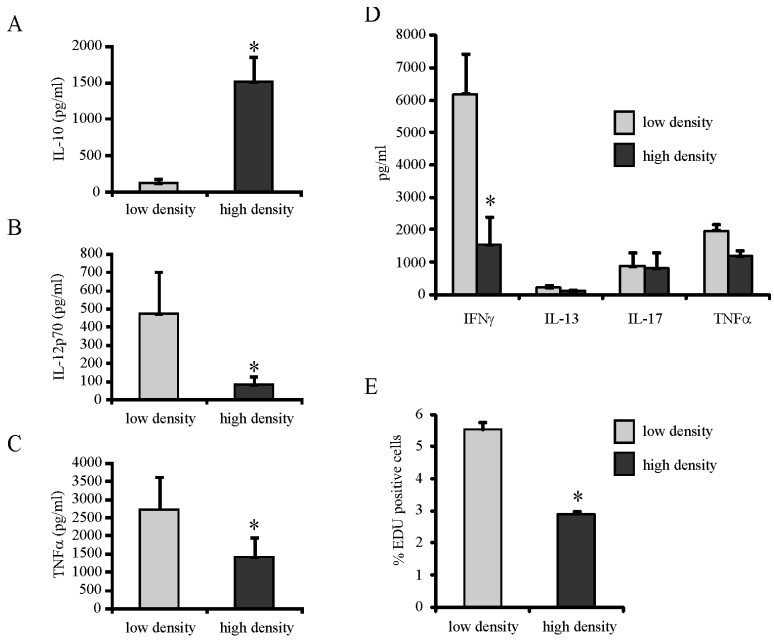

Figure 6

DCs are subject to down modulation of their pro-inflammatory phenotype when cultured at a higher density. DCs cultured in 6 wells plates with either $0.5x10^6$ (low density) or $2x10^6$ (high density) cells per well were harvested at days 6 and stimulated with R848 for 24 hours. The levels of IL-10 (A), IL-12p70 (B) and TNFα (C) in the supernatants were measured by Bio-plex technology. Data are presented as means and SEM of 5 independent experiments. DCs cultured in 6 wells plates with either $0.5x10^6$ (low density) or $2x10^6$ cells (high density) per well were harvested at days 6, washed and added 1:10 to autologous $CD4^+CD45RA^+$ T cells. R848 was added to the wells in a concentration of 2 μg/ml and the DCs and T cells were subsequently cultured for 6 days before stimulation with PMA and Ionomycin (D) or for 3 days before the addition of EDU to determine proliferation rates (E). After 24 hours the supernatants were harvested and the levels of IFNγ, IL-13, IL-17 and TNFα were measured or the T cells were harvested and analysed by flowcytometry for their incorporation of EDU. Data are presented as means and SEM of 4 independent experiments. * $p<0.05$ compared to DCs cultured in low density.

of TGFβ prevented this increased response and reduced it to levels found with mϕ-1 cultured at low densities **(Figure 7C and data not shown)**.

This shows that TGFβ can have clear proinflammatory effects on mϕ-1 increasing their responsiveness towards TLR ligands. This indicates that the presence of TGFβ during chronic inflammation might support the continuation of inflammatory processes.

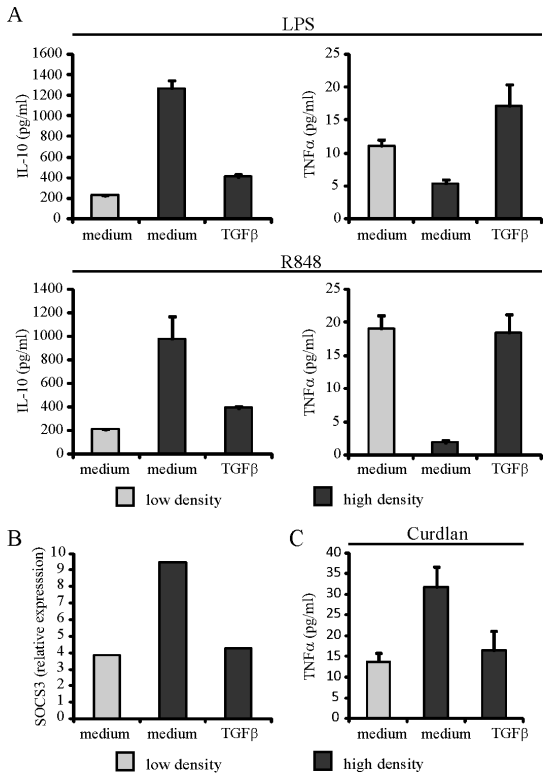

Figure 7

The Mac-1 mediated anti-inflammatory mϕ-1 phenotype is inhibited by TGFβ**.** 0.5x10^6 (low density) or 2x10^6 (high density) monocytes were cultured in a 6 well plate into mϕ-1, in addition monocytes were cultured in a concentration of 2x10^6/well with GM-CSF in the presence of TGFβ (5 ng/ml) for 6 days. At day 6 the mϕ-1 were harvested, washed and stimulated with LPS, R848 (A) or curdlan (C) before the measurement of IL-10 and TNFα Bars are mean of duplicate wells and SD. Data are representative of four independent experiments. (B) In similar experiments the expression of *SOCS3* was determined by RT-PCR. Expression is relative to GAPDH expression (x100). Data are representative of three independent experiments.

Discussion

In an inflammatory lesion monocytes are one of the first cells to appear. The infiltrating monocytes differentiate into macrophages, a phenomenon that is under strict control of factors produced locally upon activation by pathogen or damage associated molecular patterns or inflammatory mediators. The resulting macrophages are highly capable of producing proinflammatory cytokines when

they encounter pro-inflammatory stimuli. The invoked immune response will lead to tissue damage culminating in the release of endogenous ligands recognized by TLRs, which in turn will activate macrophages and DCs to produce high levels of proinflammatory cytokines starting a vicious cycle of damage and inflammatory mediator release. So far, no clear mechanism explains the restoration of tolerance once a vicious cycle has been incited. This manuscript provides compelling evidence for a model in which cell-cell contact caused by the influx of inflammatory cells itself is able to dampen this self-perpetuating loop. Cell-cell contact potently reduces the release of pro-inflammatory cytokines by type 1 macrophages while increasing the release of the anti-inflammatory cytokine IL-10. This change in phenotype was dependent on signalling through the β2 integrin Mac-1 resulting in the up regulation of SOCS3. The increased release of IL-10 and up regulated expression of SOCS3 was dependent on the MAPkinase p38 and COX2. Our results extend on findings described in a recent study in which β2 integrin stimulation with fibrinogen interfered with TLR signalling resulting in increased IL-10 and decreased TNF and IL-6 mRNA levels [33]. We excluded IL-6, IL-10 and IFNβ as playing prominent roles in the described phenomenon. In contrast, TGFβ which is highly present in many chronic inflammatory conditions can inhibit the anti-inflammatory phenotype induced by β2 integrin mediated cell-cell contact, thereby possibly contributing to chronic inflammation. These findings highlight the importance of Mac-1 in the control of the inflammatory response by, among others, enabling mϕ-1 to produce high levels of IL-10, thereby preventing exaggerated tissue damage and restoring immune homeostasis and possibly even prevent tolerance breakthrough.

Upon infection, deficiencies in IL-10 production were shown to lead to exacerbated tissue damage and increased mortality resulting from excessive immune activation [34]. Nevertheless aberrant IL-10 production can inhibit the proinflammatory response to pathogens to the extent that they escape immune control [35]. It was recently demonstrated that overexpression of IL-10 in $CD68^+$ mϕ highly increases the susceptibility of mice to *M. tuberculosis* [36]. The production of IL-10 by macrophages thus needs to be tightly controlled explaining why its production is only initiated in mϕ-1 later in the inflammatory response when substantial numbers have infiltrated. Earlier production of ample amounts of IL-10 would most likely undermine the capability of the immune system to contain the inciting pathogen. An insufficient production of IL-10, on the other hand, allows the initial proinflammatory mechanisms to culminate, leading to massive immunopathology. Next to infections the capability of macrophages to produce IL-10 appears important in the suppression of atherosclerosis and arthritis [37;38]. In contrast the

presence of macrophages in tumors which produce IL-10 is correlated with progression of tumor growth and metastasis [39]. The regulation of the production of IL-10 by macrophages and DCs is thus a crucial point in immunity in various diseases and appears to be regulated by Mac-1 signalling.

β2 integrins are heterodimers consisting of the common β chain CD18 and either CD11a, CD11b, CD11c or CD11d forming LFA-1, Mac-1/CR3, p150,95/CR4 or CD11d/CD18, respectively. β2 integrins mediate adhesion of leucocytes to the endothelium facilitating the extravasation of immune cells into tissues [40]. Next to this β2 integrins have been shown to be important in monocyte/macrophage homotypic aggregation and their formation into multinucleated cells such as osteoclasts and foreign body giant cells [27]. Recently, a new role for β2 integrins in the resolution of inflammation has been described. It was found that CD18 hypomorph mice infected with *Borrelia burgdorferi* developed an aggravated carditis and demonstrated a tendency towards an increased arthritis severity. This significant increase in carditis disease severity was not due to an increased *B. burgdorferi* burden but was marked by a massively increased infiltration of macrophages into the heart and an increased production of MCP-1 [41]. It thus appears that in mice with low levels of β2 integrins the resolution phase of the immune response, by shifting the phenotype of the infiltrating macrophages into a more anti-inflammatory one, is most likely not induced to a sufficient extent resulting in the observed aggravated innate immune response. The intricate involvement of β2 integrins in the immune system was also demonstrated by the increased severity of a psoriasis model in CD18 hypomorph mice previously demonstrated to crucially depend on activated macrophages [42]. The diminished expression of CD18 impaired the contact between regulatory T cells and DCs and the regulatory T cells additionally displayed decreased levels of membrane bound TGFβ resulting in dysfunctional regulatory T cells [43]. The increased disease severity could only partly be restored by adoptive transfer of wild type regulatory T cells indicating that other mechanisms might play additional roles in the increased severity, such as the mechanism described in the current manuscript. In cancer CD18 positive macrophages have been shown to promote metastasis and outgrowth of breast cancer and tumors in CD18 hypomorphic mice are much more sensitive to radiotherapy [44;45]. Together with the data demonstrating that COX2 inhibition increases the effect of immunotherapy and reduces the presence of tumor-associated suppressive macrophages [46] these findings underscore the importance of the described inhibitory pathway and the therapeutic possibilities of abrogating this pathway in cancer.

Furthermore, a recent report demonstrated that the phenotype of wound macrophages changes over time. In the beginning the macrophages have a pro-inflammatory phenotype which develops into a more anti-inflammatory macrophage phenotype after 7 days when the amount of monocytes in the wound has increased 40 fold. This was independent of both IL-4 or IL-13, but might be fully dependent on β2 mediated cell-cell contact [47]. We thus established a new mechanism independent of the known polarizing agents GM-CSF and M-CSF by which the phenotype of macrophages is regulated likely playing a major role in immune regulation.

TGFβ appears to have dual roles in inflammation. While its anti-inflammatory functions are apparent from literature [48], its pro-inflammatory effects are less clear although it was demonstrated that the inhibition of ALK5 led to a decreased production of TNFα by type 1 macrophages [49]. This is in line with our results showing that TGFβ can prevent the phenotype shift towards more anti-inflammatory type 1 macrophages when cultured at high density, resulting in high levels of TNFα and IL-6 after stimulation with TLR ligands. Thereby the presence of TGFβ could prevent the dampening of inflammation when large amounts of monocytes have entered a site of inflammation, promoting chronic inflammation. 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 [50]. 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.

Altogether, we demonstrate that the pro-inflammatory phenotype of mϕ-1 is tightly controlled by cell-cell contact via the β2 integrin Mac-1. This phenotypic shift was dependent on p38 and COX2 and was transferable by a soluble factor, implicating the release of prostaglandins as major players. This feedback mechanism ultimately results in the resolution of inflammation and the restoration of tissue homeostasis. Our observations suggest an important role for the inhibition of TGFβ at the site of inflammation in the treatment in chronic inflammatory conditions. Local efforts to restore the naturally existing capacity to limit inflammatory responses would be an important step towards patient tailored medicine.

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Type I interferons might form the link between Toll-like receptor (TLR) 3/7 and TLR4-mediated synovial inflammation in rheumatoid arthritis (RA)

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Abstract

Rheumatoid arthritis (RA) has been associated with an increased risk of infections, but the underlying pathways have not yet been identified. Toll-like receptors (TLR) probably play a role in synovial inflammation and may also contribute to the understanding of the role of infections in RA. The objective of this study was to investigate if the synovial expression of TLR3 and TLR7 in RA correlates with that of inflammatory cytokines, and to assess whether this has functional consequences for local cytokine production and to study potential links between the TLR3/7 axis and TLR4 in RA synovium. Immunohistochemistry was used to study the expression of TLR3, TLR7, interferon α (IFNα), tumour necrosis factor α (TNFα) and interleukins IL1β, IL12, IL17 and IL18 in RA synovium obtained by arthroscopy from 34 patients with RA. Monocytes, monocyte-derived dendritic cells (MoDCs) and RA synovial fibroblasts were stimulated via TLR3 (poly-IC) and TLR7 (loxorubin), after which IL1β, IL6 and TNFα were measured by Luminex bead array technology. Following preincubation with IFNα, IL1β and IL18, TLR3 and TLR7 mRNA expression was assessed using real-time PCR. Cytokine production after preincubation with IFNα and subsequent TLR stimulation was measured. Synovial TLR3/7 expression was co-expressed with IFNα, IL1b and IL18, but not with TNFα, IL12 and IL17. Stimulation of TLR3/TLR7 on monocytes, MoDCs or synovial fibroblasts led to secretion of type I IFN but no biologically active IL1β or IL18 could be detected. Type I IFNa increased TLR3/7 mRNA expression whereas IL1β and IL18 did not. In spite of the fact that the mRNA level of TLR4 remained unchanged, IFNa enhanced the response to TLR4 agonists, a phenomenon that was clearly more marked in patients with RA.

Type I interferons are highly co-expressed with TLR3/TLR7 in RA synovium. They enhance TLR3/TLR7-mediated cytokine production and also TLR4-mediated responses.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease which is characterised by chronic inflammation of the synovial joints. Although the cause of RA is still unidentified, a role for both genetic and environmental factors has repeatedly been advocated. As environmental factors, it has been suggested that bacteria and/or viruses may trigger autoimmunity in the host, and evidence for the presence of at least some viruses – including cytomegalovirus,[1,2] Epstein-Barr virus[3] and parvovirus B19[4] – has been demonstrated. In line with these observations, it has been shown that dsRNA, which is a common feature of viruses, exerts clear arthrogenic properties,[5] further substantiating the potential role of viruses in RA. Although recent studies have indicated a type I interferon (IFN) signature in the RA synovium, the underlying pathway for the role of type I IFN remains unknown.[6]

Accumulating evidence points to a role for the Toll-like receptor (TLR) family in the type I IFN-mediated response. TLR belong to the family of pattern recognition receptors, which were first identified to recognise microbial components known as pathogen-associated patterns. TLR are constitutively expressed by numerous immune cells and designed to detect and eliminate invading pathogens by activation of both innate as well as adaptive immune responses. TLR3/7 and 9 serve as receptors for viral nucleic acids, by which means they have a key role in antiviral immunity by inducing type I IFN production.[7,8,9] In contrast, TLR2 and TLR4 elicit immune responses on binding of antigens from bacteria and host-derived molecules (so-called endogenous ligands or alarmins), leading to the production of inflammatory mediators including tumour necrosis factor α (TNFα) and interleukin 1β (IL1β).[10,11,12,13] The increased expression of various TLR subtypes in the synovial tissue of patients with RA further substantiates the potential role of TLR in RA.

Recently, the role of TLR in arthritis was highlighted in experimental models.[14,15] Although TLR and its ligands are abundant in the synovial compartment of patients with RA, it is hard to conceive that any trigger of a single TLR subtype would be sufficient to convert tolerance to immunity. If this is the case, then autoimmunity should regularly follow from infections. It is therefore more likely that more then one TLR ligand is needed for the initiation of a chronic and persisting inflammatory response as is seen in RA. Accordingly, recent research suggested that simultaneous or sequential triggering of different pathways is perhaps needed to set off autoimmunity.[16] It is thus tantalising to speculate that simultaneous triggering of different TLR pathways might initiate a series of events

that form the basis of the breakthrough of tolerance. Previous evidence from our group demonstrated a high expression of TLR2, 3, 4 and 7 in the synovial tissue from patients with RA and a synergistic effect with regard to cytokine production after stimulation of dendritic cells (DC) with two (or more) TLR subtypes. Here we sought evidence for a potential link between TLR2/4 and TLR3/7 pathways in RA inflammation.

Our study showed that IL1β, IL18 and IFNα (the classic type I interferon) are co-expressed with TLR3/7 in RA synovium. In addition, we provide evidence that type I IFN increases the expression of TLR3 and TLR7 which, on stimulation, lead to an increased production of cytokines. Interestingly, type I IFN also induced a clear augmentation of TLR4-mediated production of pro-inflammatory mediators including TNFα, IL1β and IL18, which was clearly more potent in RA. These data show that TLR3/7-mediated stimulation indirectly lowers the threshold for TLR4-mediated immune activation, setting the stage for the vicious circle of inflammation observed in RA. Together, these observations underscore the potential role of viruses in RA and provide a rationale for interference with TLR signalling in this condition.

Methods

Study population

For immunohistochemistry, synovial biopsy specimens were obtained from the medial and lateral suprapatellar pouch of 24 patients with RA by small needle arthroscopy. A mean of 30 samples were obtained at each biopsy. For in vitro experiments, heparinised venous blood was collected from 10 patients with RA, 11 healthy volunteers and 3 patients with systemic sclerosis (SSc) of a diffuse cutaneous subtype. All the patients with RA were attending the Department of Rheumatology of the Radboud University Nijmegen Medical Centre; they fulfilled the American College of Rheumatology criteria for RA, gave informed consent and had a disease activity score (DAS) of >3.2, reflecting moderate to high disease activity.[17] All the patients with SSc fulfilled the preliminary criteria of the American College of Rheumatology for SSc.[18] SSc was classified as either a limited subtype or a diffuse subtype according to the extent of skin involved, as proposed by Leroy *et al*.[19] Patients using high-dose prednisolone (>10 mg/day) and those receiving intra-articular steroids or anti-cytokine therapies (anti-TNFα and/or IL1Ra) were excluded from the study. For the arthroscopy, patients who had a history of bleeding, infectious disorders or were currently pregnant or lactating were excluded.

Immunohistochemical staining of synovial biopsies

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. Staining of IL1β, IL12, IL17, IL18 and TNFα was performed as described previously.[20,21,22] For TLR3, TLR7 and IFNα staining, sections were incubated for 60 min with monoclonal antibodies against human TLR3 (T-17), human TLR7 (V-20) or IFNα (FL-198), which were all obtained from Santa Cruz, California, USA. After this, endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 15 min and subsequently the appropriate biotinylated secondary antibody (mouse anti-goat (Jackson ImmunoResearch, West Grove, Pennsylvania, USA)/swine anti-rabbit (DakoCytomation, Glostrup, Denmark)) was incubated for 30 min. For TLR3 and TLR7 staining, Vectastain ABC (Vector Laboratories, Burlingame, California, USA) reagent was incubated for 30 min, developed with diaminobenzidine (Sigma, St Louis, Missouri, USA) and counterstained with haematoxylin for 30 s. For IFNα staining, sections were incubated with streptavidin peroxidase (DakoCytomation), developed with diaminobenzidine and counterstained with haematoxylin for 30 s. Staining was semi-quantitatively scored on a 5-point scale (scores 0–4) at 200× magnification where a score of 0 represents no or minimal staining, 1 represents 10–20% positive cells, 2 represents 30–40%, 3 represents 50–60% and 4 represents staining of >60% of the cells.

Isolation and culturing of monocytes and monocyte-derived dendritic cells (MoDCs)

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood using density gradient centrifugation over Ficoll-Paque (Amersham Biosciences, Roosendaal, The Netherlands). Low-density cells were collected and washed with citrated phosphate buffered saline/5% fetal calf serum (FCS). For monocytes, the CD14+ cell fraction was isolated using MACS cell separation according to the manufacturer's instructions. Briefly, PBMC were incubated with anti-human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min, cells washed and the CD14+ fraction was separated from the CD14− fraction over a MACS MS separation column (Miltenyi Biotec). The CD14+ cell fraction was eluted from the column, washed again and resuspended in a concentration of 0.5×10^6 cells/ml in RPMI 1640 Dutch modification (Invitrogen Corporation, Carlsbad, California, USA) supplemented with 10% FCS, plated in 6-well plates and cultured overnight at 37°C and 5% carbon dioxide.

For MoDCs, PBMC were allowed to adhere for 1 h at 37°C in RPMI 1640 Dutch modification supplemented with 2% human serum (PAA Laboratories, Pasching, Austria) in 25 cm^2 cell culture flasks (Corning, New York, USA). Adherent

monocytes were cultured in RPMI-1640 Dutch modification supplemented with 10% FCS and antibiotic-antimycotic (Life Technologies) in the presence of IL4 (500 U/ml, Schering-Plough, Amstelveen, The Netherlands) and granulocyte-macrophage colony stimulating factor (800 U/ml, Schering-Plough) for 6 days. Fresh culture medium with the same supplements was added on day 3. Immature DCs were harvested at day 6, resuspended in fresh cytokine-containing culture medium, transferred to 6-well culture plates (Corning) in a concentration of 0.5×10^6 cells/ml and cultured for 16 h at 37°C and 5% carbon dioxide.

Isolation and culturing of RA synovial fibroblasts

Immediately after surgery the synovial tissue was minced and digested with dispase at 37°C for 60 min. After washing, the cells were grown in Dulbecco's minimum essential medium (Gibco Invitrogen, Basel, Switzerland) supplemented with 10% FCS, 50 IU/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES and 0.2% Fungizone (all from Gibco Invitrogen). Cell cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% carbon dioxide. For the experiments, cultured synovial fibroblasts between passages 4 and 8 were grown in 12-well culture plates (6×10^4 synovial fibroblasts/well) and cultured for 16 h at 37°C and 5% carbon dioxide.

Stimulation of monocytes, MoDCs and RA synovial fibroblasts

To study TLR mRNA expression on cytokine stimulation, after a resting period of 16 h monocytes (which express TLR7), MoDCs and synovial fibroblasts (which both express TLR3) were stimulated for 8 h with TNFα, IL1β, IFNα, IL12, IL17, IL18 (all R&D Systems, Minneapolis, Minnesota, USA). Culture supernatants were removed and 1 ml TRIzol reagent (Sigma) was added to the cells and stored at −20°C until RNA isolation was performed.

To study functional upregulation of TLR, after a resting period of 16 h monocytes, MoDCs and RA synovial fibroblasts were stimulated with IFNα (R&D Systems) for 24 h and subsequently stimulated with the TLR3 and TLR7 agonists poly(I:C) and loxoribin, respectively (both Invivogen, San Diego, USA) or medium. All non-TLR4 ligands were tested for lipopolysaccharide (LPS) contamination using the Lumilus assay and all were negative. After a further 24 h the culture supernatants were collected and stored at −20°C until cytokine measurement was performed. In addition, to investigate the role of type I IFN, various cell types were preincubated with 100 U/ml IFNα for 16 h.

RNA isolation and real-time PCR

Total RNA was extracted in 1 ml TRIzol reagent, an improved single-step RNA isolation method based on the method described by Chomczynski *et al.*[23] Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems, Foster City, California, USA). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, with data collection in the last 30 s. All PCR were performed with SYBR Green Master mix (Applied Biosystems), 10 ng cDNA and a primer concentration of 300 nmol/l in a total volume of 25 μl. Quantification of the PCR signals was performed by comparing the cycle threshold value (C_t) of the gene of interest of each sample with the C_t values of the reference gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Primer sequences for gene expression analysis for hGAPDH, hTLR3, hTLR7 and IL1β are shown in table 1.

Table 1 Oligonucleotide primers for quantitative PCR analysis

cDNA	Forward primer	Reverse primer
hGAPDH	ATC TTC TTT TGC GTC GCC AG	TTC CCC ATG GTG TCT GAG C
hTLR3	AGA GTT GTC ATC GAA TCA AAT TAA AGA G	CAT TGT TCA GAA AGA GGC CAA AT
hTLR7	TGC CAT CAA GAA AGT TGA TGC T	GGA ATG TAG AGG TCT GGT TGA AGA G
hIL1β	AAT CTG TAC CTG TCC TGC GTG TT	TGG GTA ATT TTT GGG ATC TAC ACT CT

Software package Primer Express Version 2.0 (Applied Biosystems) was used to identify appropriate primer sets. All sequences are presented in the 5′→3′ direction.
GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; TLR, Toll-like receptor.

Measurement of cytokines in culture supernatant

TNFα, IL1β, IL6, IL10 and IL12p70 levels were measured in the supernatant of the cell cultures using commercially available kits (Bio-Rad Laboratories, Hercules, California, USA) according to the manufacturer's instructions.[24] Cytokine levels were measured and analysed using the Bio-Plex system (Bio-Rad Laboratories) and data analysis was performed with Bio-Plex Manager software (Bio-Rad Laboratories). The secretion of IL18 was measured using an ELISA (Invitrogen, Biosource, USA) carried out using the manufacturer's instructions. For the measurement of IFNα and IFNβ, ELISA kits were used and the ELISAs were performed according to the manufacturers' protocols (Cell Science, Canton, Massachusetts, USA).

Statistical analysis

Correlations of the expression of TLR and cytokines in human synovial biopsies were calculated using the Pearson correlation test. Differences in mRNA expression and cytokine production on cell stimulation with cytokines and TLR agonists were calculated using the Mann-Whitney U test. p Values were two-sided and the level of significance was set at $p<0.05$.

Results

Clinical features

The clinical and demographic features of the patients included in the immunohistochemistry studies are presented in table 2. All patients had active disease as defined by the inclusion criteria (DAS >3.2). Not surprisingly, patients with severe knee joint arthritis tended also to have more active disease. The patients with RA included in the in vitro studies on DCs (n = 10) also had active disease with a mean (SD) DAS of 3.8 (0.4) and were comparable with the patients included in the studies on synovial tissues.

Table 2 Clinical and demographic data of patients with rheumatoid arthritis according to clinical status of biopsied joint

	Moderate arthritis (n = 12)	Severe arthritis (n = 12)
Median (range) age (years)	61 (41–75)	59 (45–83)
Gender (F/M)	5/7	8/4
Rheumatoid factor positivity (%)	92	100
Mean (range) disease duration (years)	10 (3–29)	11 (3–26)
Mean (SD) DAS	5.0 (1.0)	5.5 (1.5)
Median (range) number of previous DMARDs	4 (2–8)	5 (2–7)
Knee joint examination		
Pain at rest (n)	2	9
Pain at inspection (n)	6	12
Median (range) score*	1 (0–2)	2 (1–3)
Swelling (n)	9	12
Median (range) score†	1 (0–1)	2.5 (2–3)
Effusion (n)	5	12

*Pain score: 0 = none, 1 = pain reported on asking, 2 = patient winces, 3 = patient withdraws.
†Swelling score: 0 = absent, 1 = mild, 2 = moderate, loss of bony loss or bony contour, 3 = severe bulging.
DAS, disease activity score; DMARDs, disease-modifying antirheumatic drugs.

Correlation of TLR3/7 expression with IL1β, IL18 and IFNα in RA synovium

Synovial biopsy specimens from 24 patients with active RA (DAS >3.2) stained for TLR subtypes (TLR2, 3, 4 and 7) and inflammatory cytokines IL12, IL17, IL18 and IL1β were used to study the association between TLRs and inflammatory mediators.[20,21,22] Sequential slides from those used for the aforementioned markers were then stained for IFNα (fig 1A). TLR3/7 expression was correlated with the levels of expression of IFNα, IL1β and IL18 but not with IL12, IL17 and TNFα. Not unexpectedly, IFNα was clearly correlated with TLR3 and TLR7 expression in the lining and sublining of synovial biopsies (fig 1B, table 3). Moreover, both IL1β and IL18 were correlated with the expression of TLR in the lining, although these correlations were weaker than that observed between TLR and type I IFN. In contrast, neither IL1β nor IL18 was associated with either TLR3 or TLR7 expression in the sublining. Neither TNFα, IL12 nor IL-17 were correlated with the TLR expression levels in RA synovium.

Table 3 Correlation between TRL3/7 expression and type I IFN, IL1 and IL18 in RA synovium

	Correlation (r)	p Value
Correlation TLR–type I IFN expression		
TLR3–type I IFN		
Lining	0.76	0.02
Sublining	0.77	0.01
TLR7–type I IFN		
Lining	0.70	0.04
Sublining	0.84	0.01
Correlation TLR–IL1 expression		
TLR3–IL1		
Lining	0.54	0.02
Sublining	0.22	NS
TLR7–IL1		
Lining	0.32	NS
Sublining	0.17	NS
Correlation TLR–IL18 expression		
TLR3–IL18		
Lining	0.48	0.04
Sublining	0.11	NS
TLR7–IL18		
Lining	0.48	0.04
Sublining	0.16	NS

IFN, interferon; IL, interleukin; NS, not significant; RA, rheumatoid arthritis; TLR, Toll-like receptor.

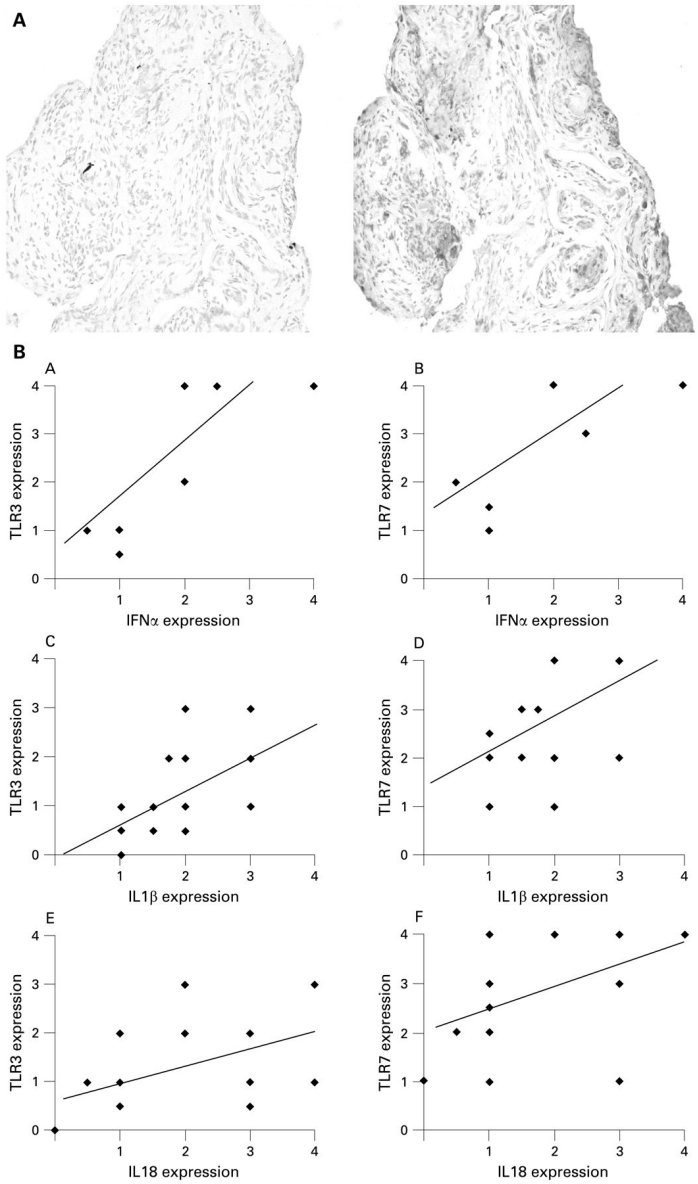

Figure 1

Increased expression of type I interferon (IFN) in rheumatoid arthritis (RA) synovium and association of Toll-like receptor (TLR) 3/7 with IFNα, interleukin (IL)1β and IL18. (A) Expression of IFNα in RA synovium (right panel) and isotype control staining (left panel). Magnification 200×. (B) Staining of RA synovial tissue with a specific antibody against IFNα showing the association between TLR3/7 and IFNα, IL1β and IL18 in RA synovial tissue. Synovial tissue sections were counterstained with haematoxylin.

TLR3/7-mediated stimulation of monocytes, MoDCs or synovial fibroblasts could not explain its correlation with IL1β/IL18

Since we found a correlation between TLR3/7 expression and the presence of IFNα, IL1β and IL18, we further investigated the functional relation between these mediators and TLR3/7. It is generally accepted that stimulation of TLR3/7 leads to the production of type I IFN DCs. In line with this, we found that TLR3/7-mediated stimulation of MoDCs resulted in clearly enhanced protein levels of both IFNα and IFNβ that reached similar levels in healthy donors and patients with RA (fig 2A). To investigate the potential relation between TLR3/7 and IL1β, TLR3-expressing MoDCs and synovial fibroblasts were stimulated with poly(IC) and TLR7-expressing monocytes were stimulated with loxoribine. All cell types investigated showed markedly increased expression of IL1β mRNA upon stimulation of either TLR3 or TLR7; however, IL1β protein could not be detected. The stimulation of monocytes, MoDCs or synovial fibroblasts with TLR3/7 did not lead to the secretion of IL18 proteins.

Regulation of TLR3/7 expression by IL1β, IL18 and type I IFN

Since TLR3/7 stimulation leads to the production of IFNα but not to IL1β or IL18, we investigated whether the correlation between TLR3/7 and IL1β/IL18 could be explained by IL1β/IL18-induced upregulation of these receptors. Monocytes (TLR7-expressing cells), MoDCs (TLR3-expressing cells) and RA synovial fibroblasts (TLR3-expressing cells) were cultured in the presence of IFNα, IL1β and IL18. IFNα significantly enhanced TLR3 mRNA expression on RA synovial fibroblasts and MoDCs and TLR7 mRNA expression on monocytes (fig 2B). This increase in TLR expression was similar in patients with RA (n = 5) and in healthy volunteers (n = 5), and was specific for TLR3 and TLR7 since the expression of TLR2 and TLR4 was not altered. In contrast, IL1β and IL18 had no effect on the expression of TLR3/7, excluding a direct role in their co-expression with TLR3/7.

Functionality of IFNα-mediated upregulation of TLR3/7

As we found that the expression of TLR3 and TLR7 was strongly upregulated by IFNα, we examined whether this enhanced TLR expression was functional in terms of increased TLR-mediated cytokine production. TLR7-expressing monocytes and TLR3-expressing MoDCs and RA synovial fibroblasts (n = 8) were incubated with IFNα or medium and TLR3 or TLR7 were stimulated with poly(IC) and loxoribine as appropriate. As anticipated, TLR7 stimulation on monocytes and TLR3 stimulation on MoDCs and RA synovial fibroblasts led to production of IL6 and TNFα, which was significantly enhanced when the cells were preincubated with IFNα compared with cells stimulated with TLR3/7 ligands alone (fig 3). These data show that the upregulation of TLR3/7 by type I IFN is functional.

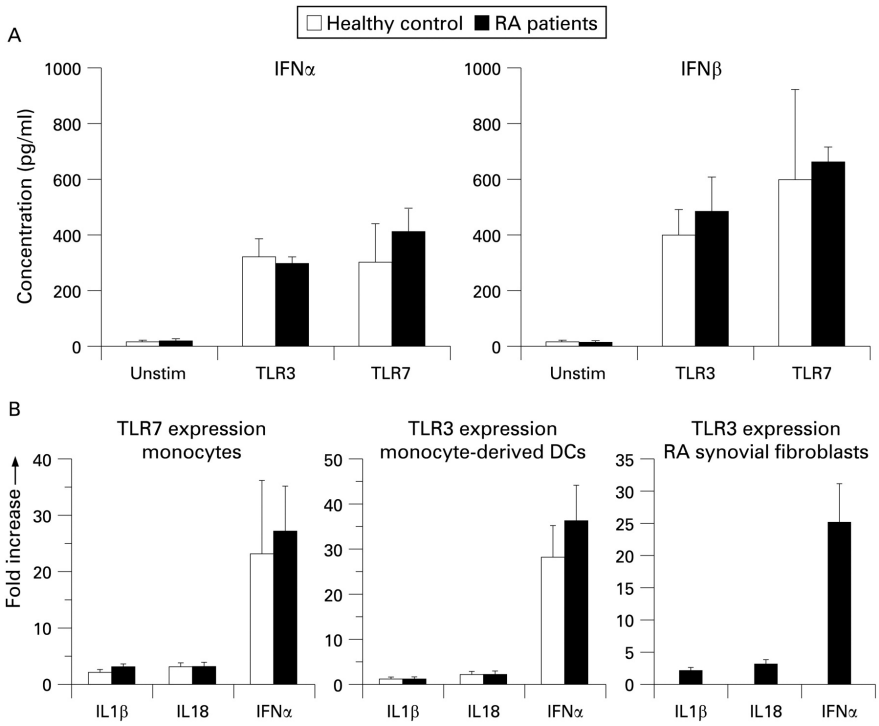

Figure 2

Effects of type I interferon (IFN), interleukin (IL)1β and IL18 on Toll-like receptor (TLR) 3/7 expression. (A) IFNα and IFNβ protein levels were measured using ELISA techniques. Both TLR3 and TLR7 agonists increased the production of IFNα and IFNβ by monocyte-derived dendritic cells (DCs). No differences in IFN type I secretion were observed between DCs from healthy controls (open bars) and patients with rheumatoid arthritis (RA) (solid bars). (B) IL1β, IL18 and IFNα induced TLR3/7 mRNA expression by monocytes, monocyte-derived DC and RA synovial fibroblasts from patients with RA (n=5) and healthy controls (n=5). Cells were incubated with 20 ng IL1β, IL18 and 100 IU/ml IFNα for 8 h and TLR mRNA expression was determined by real-time PCR. Data shown as mean (SD).

Augmentation of TLR4-mediated cytokine production by type I IFN provides the missing link explaining the correlation between TLR3/7 and IL1/IL18 expression

Since TLR4 triggering is recognised as a potent inducer of inflammatory mediators including IL1β and IL18, we postulated that stimulation of TLR3/7 leading to the production of type I IFN perhaps augments the TLR4 response ending up in the production of IL1β and IL18. To test this we prestimulated MoDCs with IFNα and then stimulated them with the TLR4 agonist LPS. Surprisingly, prestimulation with

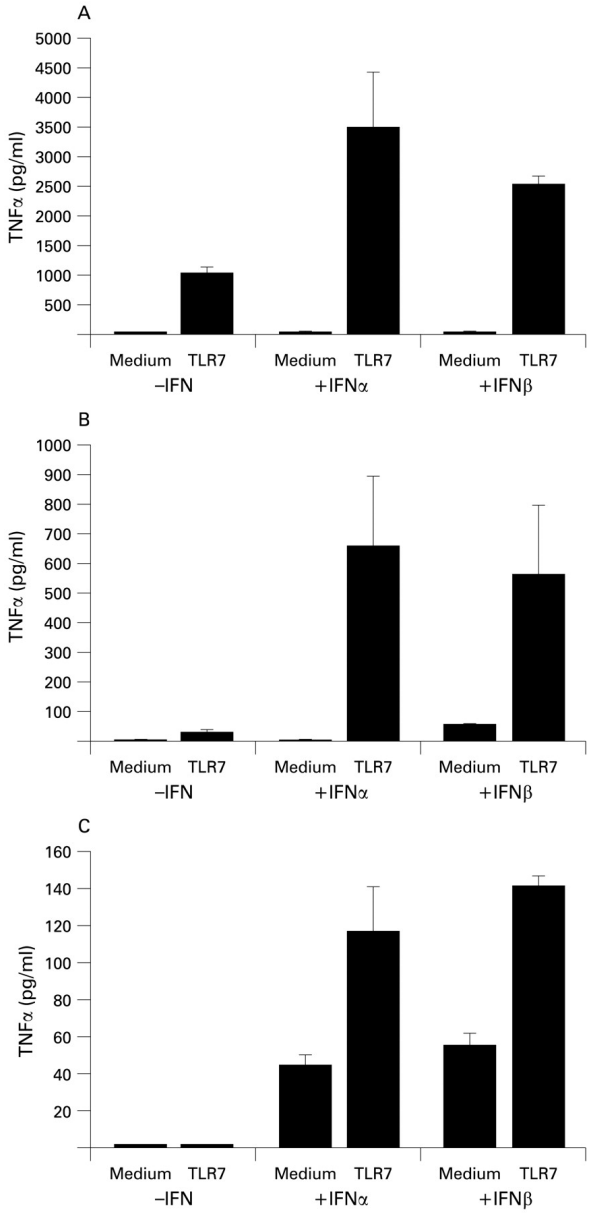

Figure 3

Functionality of increased Toll-like receptor (TLR) 3/7 expression by type I interferon (IFN). (A) Monocytes, (B) monocyte-derived dendritic cells (DCs) and (C) rheumatoid arthritis (RA) synovial fibroblasts from patients with RA (n = 5) were stimulated with TLR3/7-specific ligands after incubation with medium only or medium containing IFNα 100 U/ml for 10 h. Data shown as mean (SD). TNFα, tumour necrosis factor α.

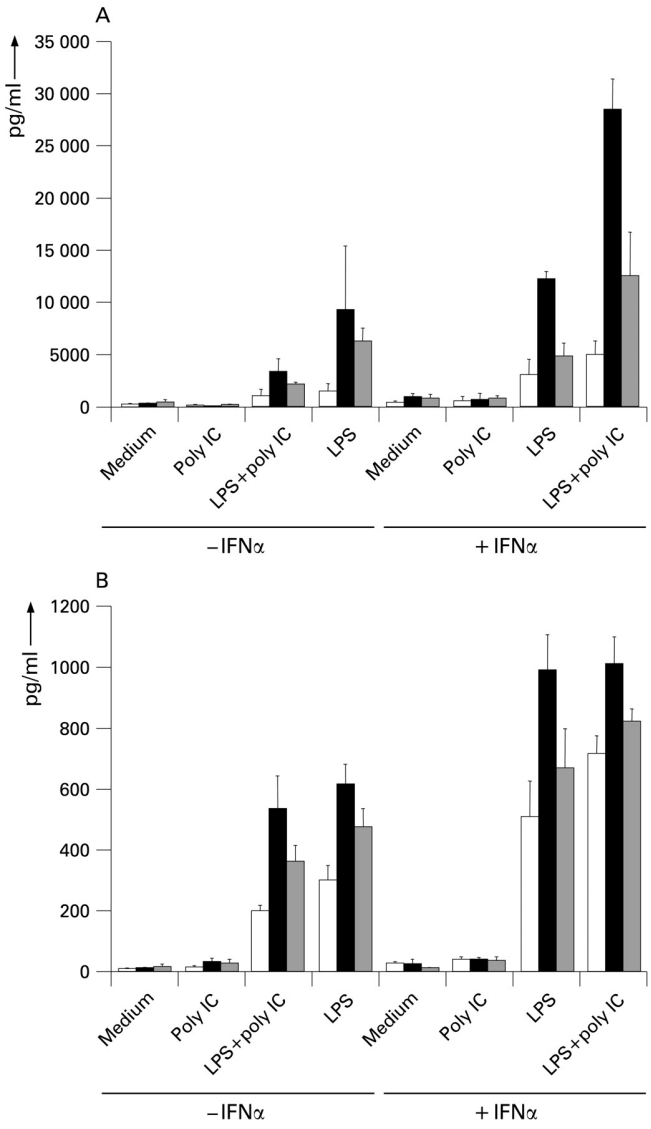

Figure 4

Augmentation of Toll-like receptor (TLR) 4 responses by preincubation with type I interferon (IFN). Secretion of tumour necrosis factor α (TNFα, upper panel) and interleukin 18 (IL18, lower panel) by monocyte-derived dendritic cells (DCs) co-incubated for 24 h with agonists for TLR3 (TLR3), TLR4 (purified LPS) or the combination of both. TNFα and IL18 secretion was compared between DCs prestimulated with 100 U/ml IFNα for 10 h and those not prestimulated. DCs from healthy individuals (n=5), patients with rheumatoid arthritis (RA, n=4) and patients with systemic sclerosis (SSc, n=3) were compared. Data shown as mean (SD).

IFNα resulted in a marked potentiation (threefold) of TLR4-mediated secretion of TNFα by DC from healthy controls (3044 pg/ml vs 1000 pg/ml, p=0.01, n=5; fig 4). In addition, preincubation with type I IFN augmented TNF production by the combination of TLR3/4 ligands by almost threefold (1880 pg/ml vs 5010 pg/ml, p<0.01). Compared with DC from healthy controls, stimulation of DC from patients with RA with TLR4 led to significantly greater production of TNF, as previously described.[22] Notably, DC from patients with RA (n=4) produced a fourfold higher level of TNFα on stimulation with IFNα/LPS (12344 pg/ml vs 3044 pg/ml, p=0.001) and IFNα/LPS/Poly-IC (28477 pg/ml vs 8484 pg/ml), p=0.01) than DC from healthy individuals. To investigate whether the augmented TLR4-mediated stimulation by type I IFN could underlie the association between TLR3/7 and IL18, we measured the production of this crucial mediator. In corroboration with TNFα, the secretion of IL18 was markedly increased following prestimulation with type I IFN and stimulation with LPS (2155 pg/ml vs 4855 pg/ml, p=0.02). The addition of a TLR3 agonist, however, did not amplify IL18 production as is seen with TNF secretion, which is in line with our observation that TLR3/7 stimulation does not result in IL18/IL1β production. The secretion of IL1β showed the same trend, but the absolute levels by MoDCs was low (data not shown). Together these data imply that the TLR4 response is potentiated by prestimulation with type I IFN, and this is even more pronounced in patients with RA. To investigate whether the IFN-mediated augmentation of the TLR4 response is RA-specific, we included DC from patients with SSc (n=3) in the analysis. Although the absolute production of pro-inflammatory mediators tends to be higher than that seen in healthy controls, the magnitude of potentiation of the TLR4 response is similar to that observed in healthy controls and thus smaller than that in RA.

Discussion

In this study we have shown that TLR3 and TLR7 in synovial tissue from patients with RA is associated with the presence of IL1β, IL18 and type I IFN. Since TLR3/7-mediated cell activation did not result in IL1β/IL18 secretion and TLR3/7 expression was not regulated by these mediators, we sought evidence for the underlying mechanism to explain the relationship between these mediators in the synovium. Preincubation of DCs with IFNα led to clear augmentation of TLR4-mediated responses. It is therefore tempting to speculate that TLR3/TLR7 triggering leads to the production of type I IFN which, in turn, augments TLR4-mediated triggering leading to the production of pro-inflammatory mediators, explaining the observed correlation between TLR3/7 and IL1β/IL18 expression. Since ligands for the

TLR3/7 axis and TLR4 axis are abundantly present in the synovial compartment of patients with RA, this chain of events is likely to contribute to the inflammatory cascade in this condition.

IFN type I is the key cytokine that regulates the innate immune response against viruses.[25] Type I IFN is released upon transcription of interferon regulating factor (IRF)-3 or IRF-7, which can be induced following TLR3- or TLR7-mediated cell activation. TLR3 and TLR7 are highly expressed in synovial tissue from patients with RA,[22] and TLR3/7 ligands such as cytomegalovirus, Epstein-Barr virus and parvovirus B19 have also been demonstrated in the joints of patients with RA.[1,3,26] The observation that a type I IFN signature is present in the synovium in a substantial proportion of patients with RA supports the notion that TLR3 and/or TLR7 triggering is likely to occur in RA synovium. In view of this, it is tempting to speculate that exposure to TLR3/TLR7 agonists might sensitise the synovial milieu for TLR4 ligands via IFNα production which, in turn, acts as a key regulator in the sustained inflammation in RA. For several reasons, IFNβ therapy has been expected to have a beneficial effect in RA comparable to that seen in multiple sclerosis.[27] However, IFNβ therapy was shown to be effective in animal models of collagen-induced arthritis but ineffective in several clinical trials in RA.[28,29,30] The fact that IFN type I strongly upregulates TLR3/TLR7 expression and tunes TLR4 responses which, in turn, are continuously stimulated by ligands present in the synovial joints might, in fact, maintain the inflammatory processes and therefore lead to failure of IFN therapy. Identification of endogenous TLR agonists is of great interest in terms of autoimmune disorders. In particular for TLR4, several endogenous agonists have been described to date—for example, hyaluronan fragments, heparan sulfate, fibronectin and (small) heat shock proteins—which can all be released in RA joints as a result of inflammation-induced tissue injury and cell stress.[11,12,13,31] For instance, Brentano *et al* described an endogenous ligand for TLR3, as endogenous RNA released from necrotic synovial fluid cells was able to activate RA synovial fibroblasts in a TLR3-dependent fashion.10 Highly conserved RNA sequences within small nuclear ribonucleoprotein particles are able to activate immune cells via TLR7 and could act as an endogenous autoantigen in systemic lupus erythematosus.[32,33] Thus, triggering of the TLR3/7 pathways does not necessarily result from viruses but might well originate from the host itself. TLR-mediated immune responses in the synovial joints might lead to the release of endogenous TLR ligands originating from cells under stress or tissue damage. It is therefore not unlikely that a self-sustaining loop of TLR activation and generation of new endogenous TLR ligands might lead to a chronic inflammatory process as occurs during RA.

It has recently been demonstrated that stimulation of several different TLRs at the same time leads to synergistically induced levels of pro-inflammatory mediators.[22,34] Our data indicate that cytokine levels induced in a synergistic fashion by co-stimulation of TLR3 and TLR4 were even more enhanced when cells were preincubated with type I IFN. The exact mechanisms underpinning the augmented TLR4 response after preincubation with type I IFN is not yet understood. From our data, we can conclude that this phenomenon is not explained by an increased expression of TLR4 itself since preincubation with type I IFN does not affect its mRNA expression. Perhaps the explanation for this lies in the upregulation of adaptor molecules or downregulation of intracellular inhibitors that are part of the TLR4 pathways.[35] Since the augmentation of TLR4 responses by type I IFN was significantly more potent in patients with RA, further investigation into the causative pathways is highly desirable because it might open novel insights into treating this condition selectively.

In conclusion, this study has shown that the expression of TLR3 and TLR7 is associated with IFNα, IL1β and IL18 in synovial tissue from patients with RA. Furthermore, we have demonstrated the involvement of IFNα in the regulation of TLR3/7 expression and function and also in an augmented TLR4-mediated response. These observations might, at least partly, explain the role of type I IFN in the inflammatory cascade of RA.

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TLR2 promotes Th2/Th17 responses via TLR4 and TLR7/8 by abrogating the type I IFN amplification loop

6

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Abstract

Toll Like Receptor (TLR) 2 plays an important role in the removal of gram-positive bacteria, contrastingly it also appears to have important protective effects against unrestrained inflammation and subsequent organ injury during infection and autoimmunity. We hypothesized that TLR2 tunes the phenotype of dendritic cells (DCs) activated through other TLRs thereby fulfilling a crucial role in the modulation of the immune response. TLR2 potently inhibited TLR4 and TLR8 induced cytokine production by human DCs. The inhibitory effect of TLR2 on the release of TNFα but not of IL-12p70 was mediated by PI3K. TLR2 inhibits the production of IL-12p70 by dampening the type 1 interferon (IFN) amplification loop. When DCs were triggered with the potent synergistic combination of LPS (TLR4) and R848 (TLR8) in conjunction with a TLR2 ligand a clear shift from Th1 to more Th2 and Th17 prone responses in the naïve and memory T cell subpopulations was observed. This shift in T cell responses was inherent to the inability of TLR2 stimulated DCs to produce IL-12p70 and was dependent on the production of IL-1 and IL-6.

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells which continuously probe their environment for invading microbes and are crucial for upholding tolerance or inducing immune responses[1]. Upon activation DCs elicit T cell responses crucial for the protection against invading microbes. The adaptive immune response evoked is specifically aimed at the pathogen sensed by the DCs. To accurately restrain this threat a balanced appearance of Th1, Th2 and Th17 cells at the compromised site is of paramount importance. However, next to their profitable role in the immunity against pathogens, Th1 as well as Th17 cells have been described as mediators of autoimmune pathology. It was recently suggested that the conditions during the initial exposure to antigens primes antigen-presenting cells thereby determining whether the resulting effector phase is either a Th1 or a Th17 response, placing DCs in the centre of (auto)immunity[2-4].

Toll like receptors (TLRs) are essential pattern recognition receptors of the immune system widely expressed on a large variety of cells[5]. An important role for TLRs involves their expression on DCs which enables them to sense invading pathogens and mount an appropriate immune response. As most pathogens express several microbial TLR ligands and numerous host-derived TLR ligands are generated in inflamed or degenerated tissue, simultaneous activation of multiple TLRs *in vivo* during chronic inflammatory diseases is conceivable. In this context, the study of the interaction and crosstalk between TLRs is of high relevance in chronic inflammatory diseases including inflammatory bowel disease, atherosclerosis and rheumatoid arthritis (RA). Recently, the presence of TLR ligands was addressed in RA patients[6-8] and enhanced expression of various TLRs in RA synovium further substantiates their role in RA[9,10].

The ligation of TLRs results in a massive release of cytokines, creating an environment crucial for the appropriate adaptive immune response. Activation of TLRs such as TLR4 and TLR7/8 results in DCs releasing IL-12p70, consisting of IL-12p35 and IL-12p40 subunits, which favours the proliferation of Th1 cells while activation of TLR2 does not result in the release of IL-12p70 and is generally thought to induce a Th2 response[11]. The production of IL-12p70 depends on the type 1 interferon (IFN) amplification loop in which an initial small production of IFN leads to the additional transcription of IFN genes and IL-12p35. Interferon Regulatory Factors (IRF) 1, 7 and 8 have been demonstrated to be intricately involved in this process leading to IL-12p70 production and Th1 responses[12-14]. In addition to Th1 and Th2 cells DCs have also been implicated in the promotion of

Th17 cells, although the bacterial components and the type of pattern recognition receptors involved are not evidently recognized[15,16]. Recently the activation of the intracellular bacterial sensor nucleotide oligomerization domain 2 (NOD2) was found to be implicated in the differentiation of Th17 cells by the enhanced production of TLR induced IL-1 and IL-23[17]. In addition, whereas type 1 interferon signalling was found to be pivotal for the induction of Th1 responses it also constrains Th17 development[18,19]. Interestingly, it was recently demonstrated that adoptive transfer of both IL-12p70 (Th1) as well as IL-23-polarized (Th17) T cells resulted in a clinically indistinguishable experimental autoimmune encephalitis. The histopathological features, however, were very distinct with myeloid cell rich infiltrates in IL-12p70-driven disease whereas IL-23-driven lesions were prominently populated by neutrophils[20].

While the function of TLR4 and TLR7/8 thus seems unambiguously aimed at the induction of a Th1 response, the precise function of TLR2 in immunity is less clear. In experimental autoimmune arthritis, TLR2 deficiency results in more severe arthritis, whereas TLR4 deficiency leads to protection against severe destructive disease[21]. To date, accumulating evidence points towards an inhibitory role of TLR2 in inflammation. For instance, TLR2 mediates the intracellular survival of certain bacteria, such as *Porphyromonas gingivalis*, in macrophages[22] and the absence of TLR2 during experimental *Mycobacterium tuberculosis* infection led to increased mortality due to uncontrolled inflammation and injury of the lungs[23]. These studies demonstrate that TLR2 signals might have important protective effects against unrestrained inflammation and subsequent organ injury. Similarly, *Gerosa et al.* pointed out that there is interaction between TLR2 and other pattern recognition receptors demonstrating that TLR2 co-activation inhibits the release of IL-12p70 induced by R848 in synergistic combination with LPS, β-glucan or zymosan[24]. Fuelled by these observations, we aimed to further explore the role of TLR2 co-stimulation in controlling TLR mediated responses and to delineate the underlying pathways. Here, we report that TLR2 was able to specifically restrain the TLR4 and/or TLR7/8 induced production of a wide array of cytokines while leaving the cytokine production induced by TLR3 or TLR5 untouched. Importantly we disclose the mechanisms by which TLR2 exerts its suppressive function and demonstrate that co-activation of TLR2 leads to a clear shift in the DC mediated T cell differentiation from a Th1 response to a Th2 and Th17 prone response. This shift was inherent to the inability of TLR2 stimulated DCs to produce IL-12p70 via the abrogation of the type 1 IFN amplification loop and was dependent on IL-1 and IL-6. Collectively, this points towards a prominent role for TLR2 mediated signalling in (auto)immunity by coordinating the effector phase of an immune response towards a predominant Th1 or Th17 mediated process.

Materials and Methods

Isolation and culture of monocyte-derived Dendritic Cells and BDCA-1 $(CD1c)^+$ myeloid DCs

PBMCs were isolated from heparinized venous blood of healthy volunteers by using density-gradient centrifugation over Ficoll-Paque (Amersham Bioscience). Monocytes were obtained using CD14 microbeads and MS columns (Miltenyi Biotec). The positive selection kit for BDCA-1 $(CD1c)^+$ from Miltenyi Biotec was used to isolate myeloid DCs. Monocyte-derived DCs were generated by culturing isolated monocytes in the presence of IL-4 (500 U/ml; Schering-Plough) and GM-CSF (800 U/ml; Schering-Plough) for 6 days. Fresh culture medium was added at day 3. The local Medical Ethics Committee approved the study protocol.

Phenotypical analysis of monocyte-derived DCs

Using standardized flow cytometry protocols as described previously[25] phenotypical analysis of monocyte-derived DCs was performed. DCs were characterized by staining with mAbs against human CD80 (BD Biosciences), CD83 (Beckman Coulter), CD86 (BD Pharmingen) and MHC-II DR/DP (clone Q1514). Cells were analyzed for the proportion of positive cells and the mean fluorescence intensity relative to cells stained with the appropriate IgG isotypes.

Stimulation of monocyte-derived DCs and myeloid DCs

Freshly isolated myeloid DCs and day 6 monocyte-derived DCs were plated in a concentration of $0.5x10^6$ DCs/ml and transferred to 24 well (1 ml) or 96 well (100 μl) culture plates. Cells were then stimulated with TLR agonists for 16-24 hours. For experiments in which mRNA levels were determined monocyte-derived DCs were stimulated for 4 hours before they were put in TRIzol. The concentration in which the TLR agonists were used is as follows unless otherwise described: LPS (LPS, 100 ng/ml, E. coli 0111:B4, Sigma-Aldrich), R848 (2 μg/ml, InvivoGen), Pam3CSK4 (5 μg/ml, EMC microcollections), Poly(I:C) (25 μg/ml, InvivoGen), S. Typhimurium Flagellin (1 μg/ml, InvivoGen), FSL-1 (Pam2Cys, 1 μg/ml, EMC microcollections). The used E. coli Lipopolysaccharide was double-purified at our laboratory according to the two-step phenol-water extraction method to remove any contaminating proteins resulting in purified LPS (pLPS)[26]. Blocking of TLR2 was accomplished by using antibodies specific for TLR2 (clone TL2.1, isotype IgG2a, 30μg/ml, eBioscience). As a control antibody clone eBM2a was used (isotype IgG2a, 30 μg/ml, eBioscience). In certain experiments DCs were pre-treated with the following for 1h at 37°C before adding TLR ligands: Wortmannin (PI3K inhibitor, 100 nM, Calbiochem), PD98059 (Erk inhibitor, 20 μM,

Calbiochem), antibody against IL-10 (3 μg/ml, R&D systems), IFNα and IFNβ1 (both 100 IU/ml, R&D systems), blocking antibodies specific for IFNαR2 (Interferonsource.com, 30 μg/ml). Supernatants were collected after 24 hours for cytokine measurements.

Mixed leukocyte reaction (MLR)

At day 7 DC were harvested from their 24 well plates, washed in PBS and resuspended in a concentration of $100x10^3$ DC/ml in culture medium. $5x10^3$ DC were replated in 96 round bottom well plates. $CD4^+$ T cells from PBMCs from healthy controls were obtained by negative selection using microbeads and MS columns (Miltenyi Biotec). Next $CD4^+CD45RA^+$ naïve T cells were separated from the $CD4^+CD45RO^+$ T cells by the use of microbeads aimed at CD45RO. $50x10^3$ $CD4^+CD45RA^+$ or $CD4^+CD45RO^+$ T cells were added to the DCs in 96 round bottom well plates. T cell proliferation was monitored at day three during the MLR by tritiated thymidine incorporation (0.5 μCi). The tritiated thymidine incorporation is expressed as mean count per 5 min and SD of at least quadruplicate measurements. At day six of the MLR cells were incubated with PMA (50 ng/ml, Sigma) and Ionomycin (1 μg/ml, Sigma) 12 hours before the collection of supernatants. In some MLR experiments antibodies or recombinant human IL-12p70 (5 ng/ml) were added to the wells at day 1 of the MLR. Antibodies used were aimed against IL-12p35 (10 μg/ml), IL-12p40 (10 μg/ml), IL-6R (10 μg/ml), OX40L (10 μg/ml) and TGFβ (100 ng/ml, kindly gifted by Dr. R. Lafyatis, Boston University, Boston, USA), additional experiments were performed with IL-1ra (1 μg/ml).

Measurement of cytokines in culture supernatants

Levels of IL-10, TNFα, IL-12p70, IL-6, MCP-1, IFNγ, IL-4, IL-13 and IL-17A 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. The levels of IL-23 (R&D systems, Minneapolis, USA), IL-1α and IL-1β (Biosource, Breda, The Netherlands) were measured by specific ELISA. The minimum detection limits for IL-23 was 32 pg/ml, and for IL-1alpha and IL-1beta both 3.2 pg/ml.

Intracellular cytokine staining

At day 6 of MLR with $CD4^+CD45RA^+$ or $CD4^+CD45RO^+$ T cells and DCs stimulated with pLPS and R848 with or without Pam3CSK4 the cells were stimulated for 4 hours with PMA (50 ng/ml; Sigma) and Ionomycin (1 μg/ml, Sigma) in the presence of Golgiplug (BS biosciences) according to manufacturers protocol. Subsequently the cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD biosciences) and then intracellularly stained with anti-IFNγ-PE (eBiosciences), anti-IL-17-FITC (eBiosciences) and anti-IL-13-APC (eBiosciences) or with the corresponding isotype controls. Samples were measured on a FACS Calibur and data were analyzed using the CellQuest-Pro software (BD biosciences) and WinMDI 2.8.

RNA isolation and real-time PCR

Total RNA was extracted in 1 ml of TRIzol reagent. Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems). All PCRs were performed with SYBR Green Master mix (Applied Biosystems), 10 ng cDNA, and a primer concentration of 300 nmol/L in a total volume of 20 μl. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the gene of interest of each sample with the Ct values of the reference genes GAPDH or GUS (ΔCt) and were deployed as relative expression ($2^{-\Delta Ct}$). Software package Primer Express Version 2.0 (Applied Biosystems) was used to identify appropriate primer sets for *IRF1*, *IRF7*, *IRF8*, *IFNα*, *IFNβ*, *IFNλ1*, *SOCS1*, *SOCS3* and *A20*.

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

Human embryonal kidney 293 (HEK293) cells stably expressing TLR4, MD2 and CD14 were obtained from Invivogen and cultured according to the manufacturers guidelines. After the cells reached confluency in flat bottom 96 well plates stimulations were performed. HEK293-TLR4/MD2-CD14 were stimulated with medium or pLPS (100 ng/ml or 1 μg/ml) together with medium or Pam3CSK4 (5 or 10 μg/ml). After 24 hours supernatants were collected and the levels of IL-8 were measured using commercially available kits (Bio-Rad) according to the manufacturer's instructions with the Bio-Plex system.

Western Blot analysis

DCs were harvested at fixed time-points after stimulation, washed in PBS and lysed in buffer. To ascertain equal loading the protein content of all cell lysates was measured. 50 μg of protein was resolved on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were then blocked with 5%

nonfat dried milk in TBST (15 mM Tris-HCL (pH 7.4), 150 mM NaCl, 0,1% Tween 20). Blots were probed overnight with mAb specific for STAT-1 and phosphorylated STAT-1 (Cell signaling technologies) according to the manufacturer's protocol. The membranes were subsequently treated with the appropriate secondary antibodies. Immunoreactive bands were visualized by the ECL Western blotting detection kit (Pierce).

Statistical analysis

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

Results

TLR2 inhibits TLR4 and TLR7/8 but not TLR3 and TLR5 mediated production of pro-inflammatory mediators by Dendritic Cells

Previous observations indicated cross-talk between TLR2 and TLR4 on DCs[27]. To evaluate the concept that TLR2 inhibits the production of pro-inflammatory mediators upon TLR4 mediated activation of DCs we co-stimulated DCs with increasing concentrations of Pam3CSK4 (TLR2/1 ligand) and purified LPS (pLPS, TLR4 ligand). The release of both TNFα and IL-12p70 after the stimulation of DCs with pLPS was dose-dependently inhibited by the presence of Pam3CSK4 **(Figure 1A)**. Additional experiments using monoclonal anti-TLR2 antibodies, HEK293 cells expressing TLR4, MD2 and CD14, a ligand for TLR2/6 (FSL-1 or Pam2Cys), alternating order of ligand addition and increasing LPS dosages clearly demonstrated that the inhibitory effect was TLR2 specific **(Figure 1B/E)**, not due to physical interaction prior to engagement of TLR4 by pLPS **(Figure 1C)**, independent of the order of addition of pLPS/Pam3CSK4, as long as Pam3CSK4 was added within two hours of pLPS **(Figure 1D)**, and was present over all LPS dosages (1 ,10, 100 ng/ml and 1 and 2 μg/ml) **(data not shown)**.

In line with these observations, the addition of Pam3CSK4 also markedly inhibited the secretion of IL-10, IL-6 and IL-23 whereas MCP-1 showed the opposite effect **(Figure 2A)**. Based upon apparent dissimilarities in the signalling cascades of the different TLR subtypes, we subsequently evaluated the effect of TLR2 on TLR3, TLR5 and TLR7/8. Stimulation of DCs with R848 (TLR7/8 ligand) resulted in the production of IL-10, TNFα, IL-12p70, IL-6, IL-1γ, IL-1γ, IL-23 and MCP-1, which was markedly inhibited by the addition of Pam3CSK4 for all mediators except IL-6 and MCP-1, underscoring the potency of the inhibitory effect of TLR2 **(Figure 2A)**. Strikingly, concomitant stimulation of DCs with Pam3CSK4 and poly(I:C) (TLR3

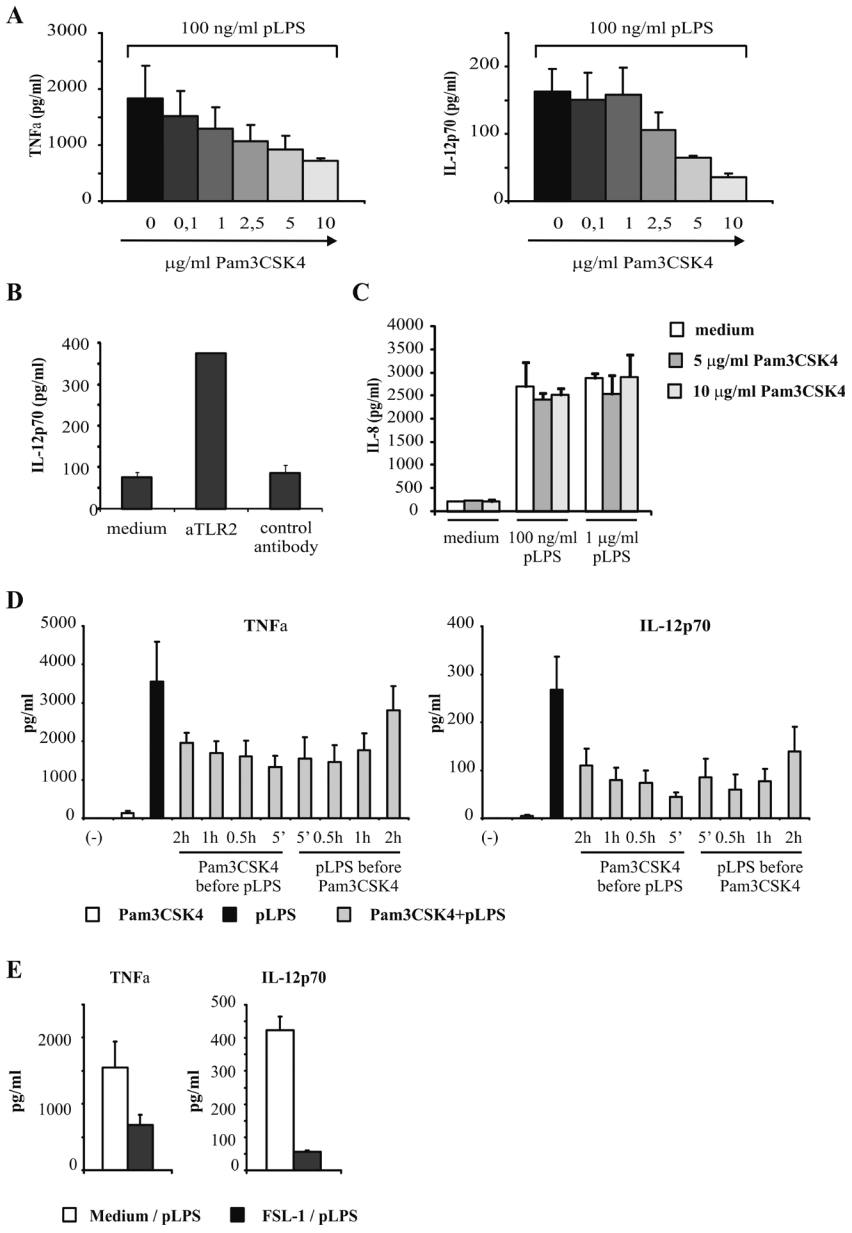

Figure 1

The TLR4 mediated production of TNFα and IL-12p70 is dose-dependently inhibited by the co-stimulation of TLR2 on human monocyte-derived DCs. (**A**) Monocyte-derived DCs were stimulated with 100 ng/ml pLPS and increasing amounts of Pam3CSK4. After 24 hours supernatants were collected and measured for their TNFα and IL-12p70 content by Luminex.

Bars represent mean and SEM of three independent experiments. (**B**) DCs were activated with the combination of Pam3CSK4 (5 μg/ml) and pLPS (100 ng/ml) in the presence of blocking antibodies for TLR2 or of isotype control antibodies. Bars represent mean and SD of triplicate wells. Data are representative of three independent experiments. (**C**) HEK293-TLR4/MD-2/CD14 cells were stimulated with pLPS (100 ng/ml and 1 μg/ml) and/or Pam3CSK4 (5 and 10 μg/ml) for 24 hours after which the levels of released IL-8 were measured. Bars represent mean and SD of triplicate wells. (**D**) Surrounding the pLPS activation of DCs Pam3CSK4 was added at various time-points ranging from 2 hours before to 2 hours after the stimulation with pLPS. Supernatants were collected after 24 hours and the levels of TNFα and IL-12p70 were determined. Bars represent mean and SEM of three independent experiments. (**E**) DCs from three healthy volunteers were stimulated with pLPS (100 ng/ml) with or without FSL-1 (Pam2Cys, 1 μg/ml). In line with the results obtained by the activation of TLR2/1 by Pam3CSK4, the triggering of TLR2/6 by FSL-1 inhibited TLR4 mediated TNFα and IL-12p70 secretion. Bars represent mean and SEM of five independent experiments.

ligand) and Flagellin (TLR5 ligand) did not result in the inhibition of cytokine production **(Figure 2B)**. In fact, the addition of Pam3CSK4 to either poly(I:C) or flagellin augmented the cytokine response compared to that of the single ligands. To assess the in-vivo relevance of our observations and extend the findings by *Gerosa et al* [24], we assessed whether the inhibitory effect of TLR2 was present in freshly isolated myeloid DCs ($CD11c^+$/$BDCA\text{-}1^+$). Although the level of IL-12p70 secretion by myeloid DCs induced by the single ligands pLPS or R848 was very low compared to monocyte-derived DCs, the inhibitory effect of Pam3CSK4 was clearly present on the pLPS and R848 induced IL-12p70 and TNFα production **(Data not shown and Figure 2C)**.

The inhibitory effect of TLR2 on the release of TNFα but not of IL-12p70 is mediated by PI3K

IL-10 has been implicated in many of the regulatory functions mediated by TLR2 signalling[27]. However, in support of the findings by *Gerosa et al.* we demonstrate that the addition of IL-10 neutralising antibodies had no effect on the TLR2 mediated suppression of pLPS and R848 induced IL-12p70 production nor on the inhibition of the release of TNFα **(Figure 3A)**. The addition of IL-10 neutralising antibodies did however lead to the increased release of TNFα and IL-12p70 demonstrating it's regulatory role independent of TLR2.

The stimulation of DCs with TLR2 ligands was previously shown to lead to a potent phosphorylation of Erk1/2 leading to a reduced ability of the DCs to produce IL-12p70 upon the activation of TLR2[28,29]. To evaluate whether the phosphorylation of Erk1/2 was implicated in the dampening of TLR4 and TLR7/8 responses by TLR2, DCs were exposed to an inhibitor of Erk1/2 before the

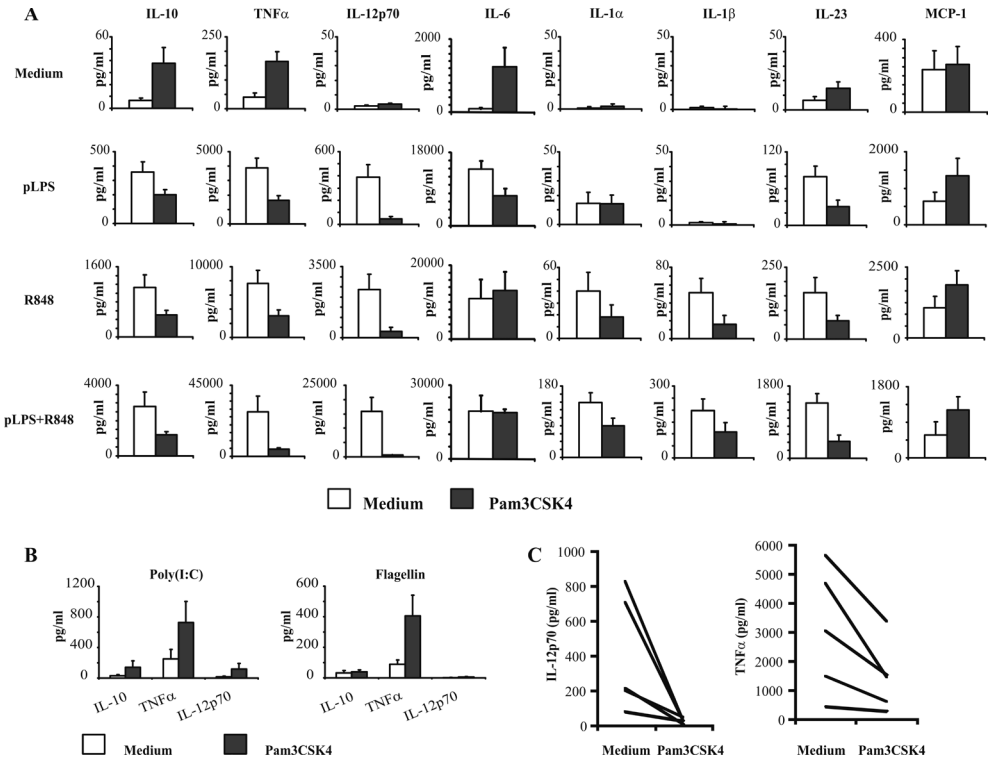

Figure 2

TLR2 activation on monocyte-derived and blood DCs inhibits the production of a wide array of cytokines induced by TLR4 and/or TLR8 activation, not by TLR3 or TLR5 activation. (**A**) DCs from 10 healthy volunteers were stimulated with pLPS (100 ng/ml), R848 (2 μg/ml) or the synergistic combination of pLPS and R848 with or without Pam3CSK4 (5 μg/ml). TLR2/1 activation markedly inhibited most of the TLR4 and/or TLR8 mediated cytokine secretion, only the release of IL-6 was unchanged in the presence of R848 while the release of MCP-1 was increased with all stimulations. Bars represent mean and SEM of ten independent experiments. (**B**) The release of IL-10, TNFα and IL-12p70 in the supernatants of DCs stimulated with TLR3 ligand (Poly(I:C), 25 μg/ml) or TLR5 ligand (S. Typhimurium Flagellin, 1 μg/ml) for 16-24 hours were measured by Luminex (n=10 independent experiments, mean±SEM). (**C**) BDCA-1$^+$ myeloid DCs were isolated from venous blood and stimulated with pLPS (100 ng/ml) in combination with R848 (2 μg/ml) in the presence or absence of Pam3CSK4 (5 μg/ml). Lines represent the results from five experiments with myeloid DCs from five healthy individuals.

stimulation with TLR ligands. No effect on the inhibition by TLR2 was observed, demonstrating that the phosphorylation of Erk1/2 by TLR2 does not play a role in the inhibition of TLR4 and TLR7/8 responses **(Figure 3A)**. TLR2 has been shown to signal through PI3K, which is a known inhibitor of the TLR4 pathway. Taking this into account, we used the specific inhibitor Wortmannin for the inhibition of the PI3K pathway. A clear dependence on the presence of functional PI3K was observed for TLR2 to exert its full inhibitory effect on the release of TNFα and IL-10 **(Figure 3B and data not shown)**. However, suppression of IL-12p70 secretion by TLR2 was not influenced by the blocking of the function of PI3K **(Figure 3B)** implicating that TLR2 inhibits via multiple pathways.

The TLR2 mediated inhibition of IL-12p70 secretion is IRF1/8, STAT1 and SOCS1 dependent

Having demonstrated the capacity of TLR2 to potently suppress IL-12p70 production in a PI3K-independent manner we set to determine how TLR2 induces this inhibitory effect. An endogenous type 1 IFN amplification loop has been shown to be essential in the production of IL-12p70 by DCs 14. Upon TLR ligation an initial production of IFNα4 and IFNβ genes leads, via activation of the IFN receptor, to the next stage in which additional IFN genes and IL-12p35 are induced[30,31]. In order to study whether the type 1 IFN amplification loop was abrogated by the activation of TLR2 we measured the mRNA levels of IFNα, IFNγ and IFNλ1 in DCs after 4 hours of stimulation with pLPS or pLPS and R848 with or without Pam3CSK4. IFNλ1 expression was recently shown to depend on signaling pathways shared with IFNγ and to play a role in dampening DC induced development of Th2[32,33]. The presence of TLR2 ligands potently decreased the mRNA levels of IFNγand IFNλ1 induced by pLPS alone and by the combination of pLPS and R848 underscoring that TLR2 might inhibit TLR4 and TLR7/8 responses by interfering in the type 1 IFN amplification loop **(data not shown and Figure 4A)**. IFNα mRNA was hardly detectable. Next, exogenous IFNα and IFNβ were added to DCs in conjunction with pLPS which resulted in a synergistically increased secretion of IL-12p70. This however had no effect on TLR2 mediated inhibition, suggesting that abrogation of the initial type I IFN release is not essential **(Figure 4B)**. The common IFNα receptor 2 (IFNαR2) was blocked to determine whether the type 1 IFN amplification loop does play a role in our system. The blockade of IFNαR2 led to a clearly decreased LPS and R848 induced production of IL-12p70 but again did not affect the inhibitory capacity of TLR2 ligation **(Figure 4C)**.

To deepen our understanding of the TLR2 mediated inhibition of the type 1 IFN amplification loop, we studied the downstream mediators of the type 1 IFN

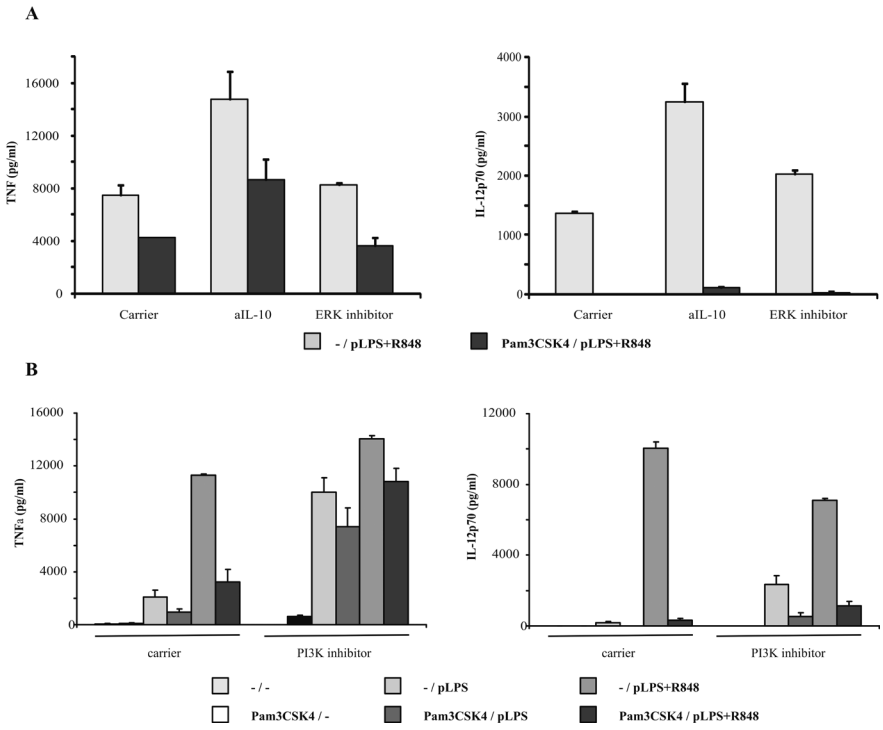

Figure 3

The PI3K pathway is crucial in the inhibition of TLR4 and TLR8 induced TNFα by TLR2. (**A**) Monocyte-derived DCs were cultured for 6 days before the stimulation with pLPS and R848 with or without Pam3CSK4 and either in the presence of the appropriate carrier solution (DMSO), neutralising antibodies against IL-10 (3 μg/ml) or the specific ERK inhibitor PD98059 (20 μM). After 24 hours supernatants were collected and measured for their content of TNFα and IL-12p70. (**B**) The LPS- or LPS+R848-induced TNFα and IL-12p70 secretion with and without co-stimulation of DCs by Pam3CSK4 was measured by luminex technology in the absence or presence of the specific inhibitor for PI3K (100 nM Wortmannin). Inhibition of the PI3K pathway largely inhibited TLR2 mediated TLR4 inhibition of TNFα release while no effects were observed on the release of IL-12p70. Results are the mean and SEM of three independent experiments.

pathway IRF1, 7 and 8. Upon TLR activation IRF7 is essential for the initial type 1 IFN production while IRF8 is indispensable for the induction of IL-12p70 production during the second phase of the type 1 IFN amplification loop. IRF1 has an important role in the amplification of IRF8 mediated IL-12p70 release. In our experimental setup, IRF1, IRF7 and IRF8 mRNA was readily present in the DCs after the activation of TLR4 alone and by the activation of both TLR4 and TLR7/8. Interestingly, the addition of TLR2 ligands had no effect on the mRNA level of IRF7

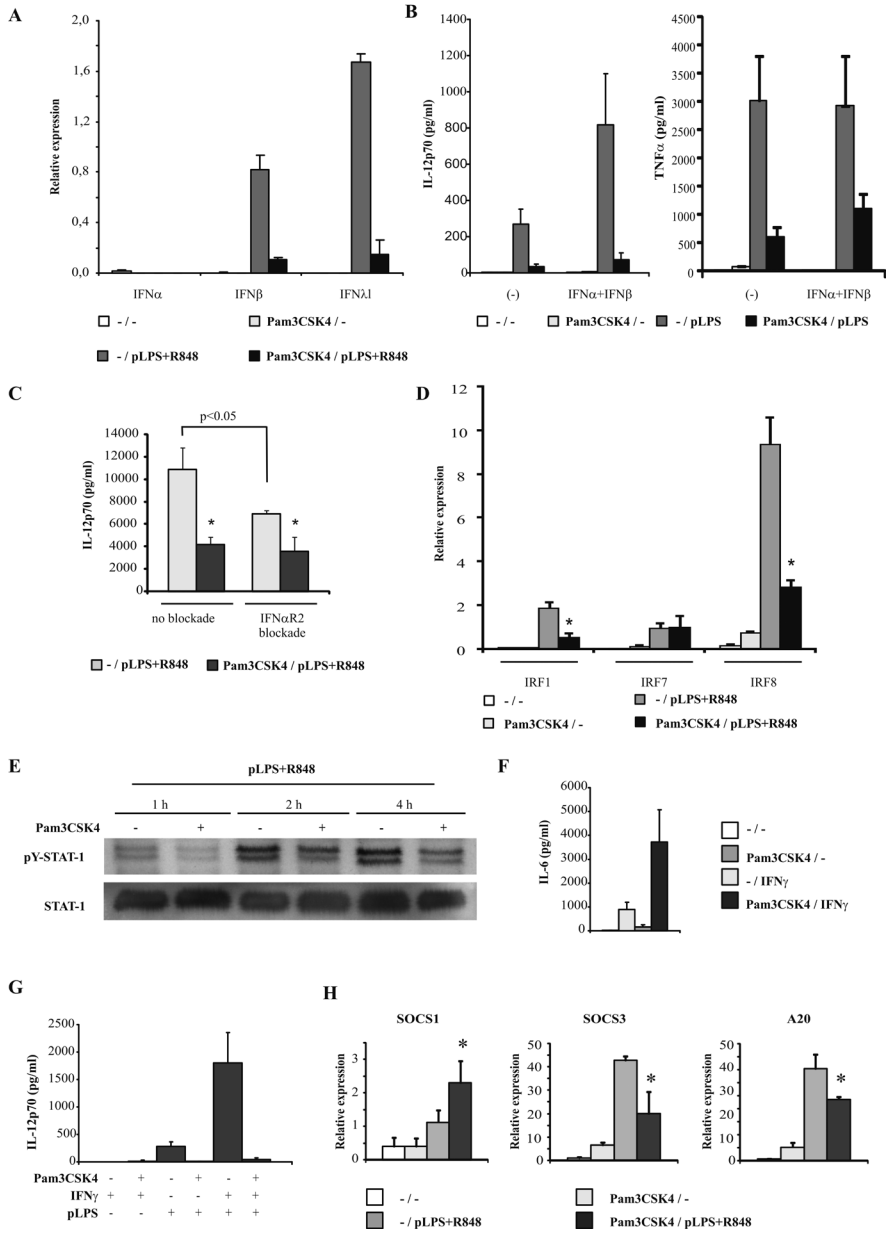

Figure 4

The type 1 IFN amplification loop is inhibited by TLR2 activation. (**A**) Real-time PCR of the expression of *IFNα*, *IFNβ* and *IFNλ1* mRNA in DCs stimulated with medium or pLPS and R848 with or without Pam3CSK4 for 4 hours. Expression is relative to *GAPDH* expression.

(**B**) The production of IL-12p70 by DCs stimulated with medium or pLPS with or without Pam3CSK4 in the presence or absence of IFNα (100 IU/ml) and IFNβ (100 IU/ml) was measured after 24 hours. (**C**) The pLPS and R848 induced IL-12p70 secretion with and without co-stimulation of DCs by Pam3CSK4 was measured by luminex technology in the absence or presence of neutralising antibodies against IFNα Receptor 2. Inhibition of the type 1 IFN receptor inhibited TLR4 and TLR8 induced IL-12p70 release with no additive effect on the inhibition evoked by TLR2 co-ligation. (**D**) Real-time PCR of the expression of *IRF1, IRF7 and IRF8* mRNA in DCs stimulated with medium or pLPS and R848 with or without Pam3CSK4 for 4 hours. Expression is relative to *GAPDH* expression. (**E**) DCs derived from healthy controls were stimulated for 1, 2 and 4 hours with pLPS and R848 or with the combination of Pam3CSK4, LPS and R848 before protein isolation for Western Blot. The protein content of all cell lysates was measured to ascertain equal loading. Analysis was done on the protein content of the DCs regarding STAT-1 and phosphorylated STAT-1. Blots are representative for the results from 3 independent experiments with DCs from 3 individuals. (**F**) DCs primed with IFNγ or not were stimulated with pLPS with or without Pam3CSK4 for 24 hours after which the IL-12p70 levels were measured in the supernatants. (**G**) DCs from 3 healthy volunteers were stimulated with medium, Pam3CSK4, IFNγ or the combination of Pam3CSK4 and IFNγ. The release of IL-6, IL-10, TNFα and IL-12p70 in the supernatants was measured after 24 hours by Luminex. Data are mean and SEM of three independent experiments. (**H**) Real-time PCR of the expression of *SOCS1, SOCS3 and A20* mRNA in DCs stimulated with medium or pLPS and R848 with or without Pam3CSK4 for 4 hours. Expression is relative to *GAPDH* expression. Data are presented as the mean ± SEM of at least five independent experiments.

but significantly reduced IRF1 and IRF8 mRNA levels induced by pLPS alone and by the combination of pLPS and R848 **(data not shown and Figure 4D)**. Next to IRFs, STAT1 signaling is essential for the induction of IL-12p70 production by DCs via the IFN amplification loop. To study the role for STAT-1 inhibition by Pam3CSK4 in more detail we evaluated the effect of Pam3CSK4 on the level of TLR4 and TLR7/8 -induced phosphorylation of STAT-1. At 60, 120 and 240 minutes the tyrosine phosphorylation status of STAT-1 was determined upon the activation of DCs with pLPS and R848 in the presence and absence of Pam3CSK4. There was a clear inhibition of the pLPS and R848 induced phosphorylation of STAT-1 by Pam3CSK4 **(Figure 4E)**.

In order to determine whether TLR2 activation would also inhibit IFNγ induced STAT-1 dependent cytokine production by DCs we co-stimulated DCs with IFNγ and Pam3CSK4 and monitored the secretion of inflammatory mediators such as IL-6, TNFα, IL-10 and IL-12p70. IFNγ dependent activation of DCs resulted in a low production of IL-6 while IL-10, TNF and IL-12p70 were undetectable. Stimulation with both Pam3CSK4 and IFNγ however resulted in a synergistic release of IL-6 suggesting that TLR2 stimulation does not act via direct inhibition of STAT-1 phosphorylation **(Figure 4F)**. The production of IL-10, TNFα and IL-12p70 was comparable to that induced by TLR2 activation alone **(data not shown)**. Since

the cross-talk between TLR4 and IFNγ requires IRF1 and IRF8 we aimed to investigate whether this synergy would be dampened by the presence of TLR2 ligands. A clear synergy in the release of IL-12p70 was observed when DCs were activated with both IFNγ and pLPS, a response fully abrogated by the addition of Pam3CSK4 **(Figure 4G)**.

The SOCS1 protein has been implicated in the control of various cytokine and TLR responses by the inhibition of JAK/STAT pathways[34]. Recently a role for this protein was described in the dampening of TLR responses by the TAM receptors dependent on the type 1 IFN amplification loop[35]. Indeed, a potent facilitating effect of TLR2 on the expression of SOCS1 induced by pLPS and R848 was observed whereas the expression of SOCS3 and A20 mRNA was decreased **(Figure 4H)**.

Co-stimulation with TLR2 ligands does not influence maturation of DC or T cell proliferation but skews T cell priming to a Th2/Th17 response

In order to investigate the inhibitory capacity of Pam3CSK4 on phenotypical DC maturation, DCs were stimulated with pLPS or R848 and the expression of multiple surface markers was studied. Both stimulation with pLPS and R848 led to a rapid up regulation of MHC II, CD80, CD83 and CD86 that was not influenced by the addition of Pam3CSK4 **(Figure 5A)**. In line with this observation, the rate of T cell proliferation induced by DC stimulated with TLR4 and TLR7/8 was unaltered by the addition of TLR2 ligands **(Figure 5B)**. Based on the strong inhibition of IL-12p70 release we postulated that co-stimulation of DCs with Pam3CSK4 alters DC mediated T cell priming leading to a more Th2 prone immune response even in combination with potent inducers of Th1 responses such as pLPS and R848. To test this, DCs were stimulated with pLPS and R848 in the presence or absence of Pam3CSK4 and subsequently co-incubated with naïve $CD4^+CD45RA^+$ T cells or memory $CD4^+CD45RO^+$ T cells. The stimulation of DCs with Pam3CSK4 in combination with pLPS and R848 suppressed the IFNγ release from the naïve T cell population, and resulted in an increased release of IL-4, IL-13 and IL-17 compared to DCs stimulated with pLPS and R848 alone **(Figure 6A).** Additionally, a significant increase in IL-4, IL-13 and IL-17 release was found in cultures of $CD4^+CD45RO^+$ memory T cells with DCs co-activated with Pam3CSK4 **(Figure 6B)**. Next we performed intracellular staining for IFNγ, IL-13 and IL-17 of naïve $CD4^+CD45RA^+$ T cells and memory $CD4^+CD45RO^+$ T cells. In line with the levels of released cytokines we observed that the percentage of IL-13 as well as IL-17 positive cells in the naïve T cell population doubled upon co-activation of the DCs with Pam3CSK4 **(Figure 6C)**. In addition the level of IFNγ positive cells decreased with approximately 10% **(data not shown)**. These findings were comparable to

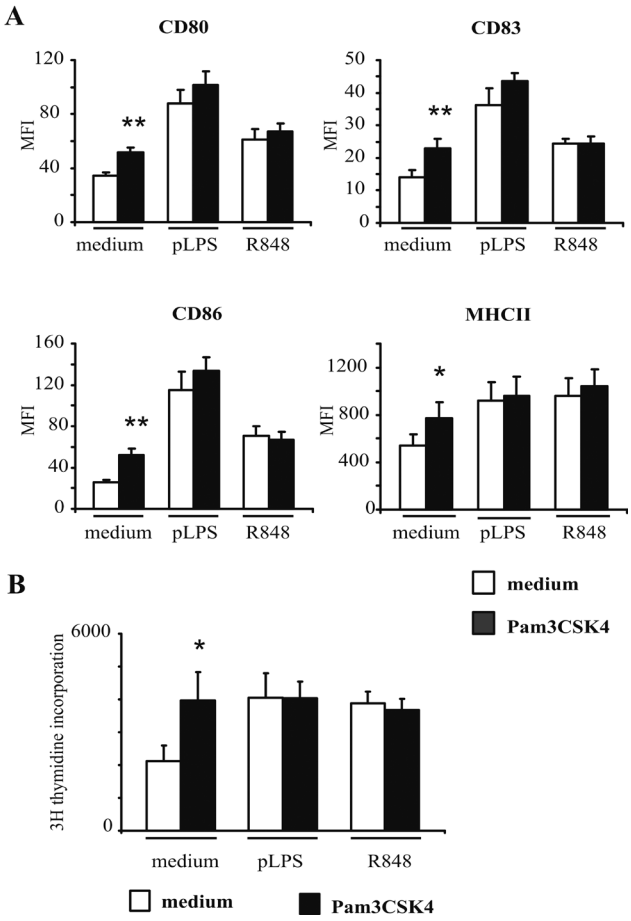

Figure 5

Co-stimulation of TLR2 with TLR4 or TLR8 does not affect DC phenotypic maturation or T cell proliferation. (**A**) The up regulation of CD80, CD83, CD86 and MHC-II expression initiated by stimulation of TLR4 (pLPS (100 ng/ml)) or TLR8 (R848 (2 μg/ml)) is not modified by co-incubation of DCs with a TLR2 ligand (Pam3CSK4 (5 μg/ml)). DCs were cultured for 24 hrs with medium, LPS (100 ng/ml) or R848 (2 μg/ml) alone or in combination with Pam3CSK4 (5 μg/ml). Expression of cell surface markers was performed using flow cytometry. (**B**) Co-incubation of DCs with the combination of LPS (100 ng/ml) or R848 (2 μg/ml) and Pam3CSK4 (5 μg/ml) had no marked effect on T cell proliferation measured by 3H-Thymidine incorporation at day 3 of the MLR compared to those stimulated with LPS or R848 alone. Data are mean and SEM of five independent experiments. * = $p<0.05$ and ** = $p<0.01$ compared to co-incubation with medium alone.

those in the memory T cell cultures in which co-activation of DCs with Pam3CSK4 led to a 50% increase in IL-13 producing T cells while the level of cells only producing IL-17 increased with 100% **(data not shown and Figure 6D)**. As in the cultures with naïve T cells the level of IFNγ positive cells decreased with approximately 10% **(Figure 6D)**.

The inhibition of IL-12p70 production by co-stimulation with Pam3CSK4 underlies the shift towards Th2 and Th17 responses

Since the activation of TLR2 led to an almost complete abrogation of the production of IL-12p70 and it shifted T cell responses towards Th2 and Th17 responses we surmised that these phenomena were related. The presence of neutralising antibodies for IL-12p70 and the co-stimulation of the DCs with Pam3CSK4 led to equal increases in IL-13 and IL-17 production. The neutralisation of IL-12p70 did not have an additive effect on the co-stimulation of DCs with Pam3CSK4 in up regulating the production of IL-13 or IL-17 **(Figure 7A and 7B)**. In addition we performed MLR experiments with naive T cells in which we added recombinant human IL-12p70. The addition of IL-12p70 restored the production of IFNγby T cells differentiated under the influence of DCs matured in the presence of Pam3CSK4 and pLPS and R848, up to the levels found when T cells were cultured with DCs exposed to pLPS and R848 alone while the production of IL-13 was also significantly reduced **(Figure 7C)**. These data underscore the fundamental principle that the activation of TLR2 on DCs abrogates their ability to produce IL-12p70 leading to a shift towards Th2 and Th17 cells in the naïve and memory T cell populations.

Nowadays, multiple factors have been identified as driving forces in the induction of Th17. Here we tested whether these factors were responsible for Th17 priming by Pam3CSK4 co-stimulated DCs. To this aim, DCs matured in the presence of Pam3CSK4, pLPS and R848 and allogeneic memory $CD4^+$ T cells were cultured for 6 days in the presence of control IgGs, IL-1 receptor antagonist (IL-1ra) and antibodies against IL-6R, TGFβ, OX40L, IL-12p40 or IL-12p35. After six days the differentiated T cells were stimulated with PMA and ionomycin after which the IL-17 content of the supernatants was measured. In line with literature[36], the neutralisation of IL-6 and IL-1α/β and especially the neutralisation of both resulted in a clear reduction of IL-17 production by the differentiated $CD4^+CD45RO^+$ T cells whereas the addition of anti-TGFβ, anti-OX40L, anti-IL-12p40 and anti-IL12p35 had no effect **(Figure 7C)**.

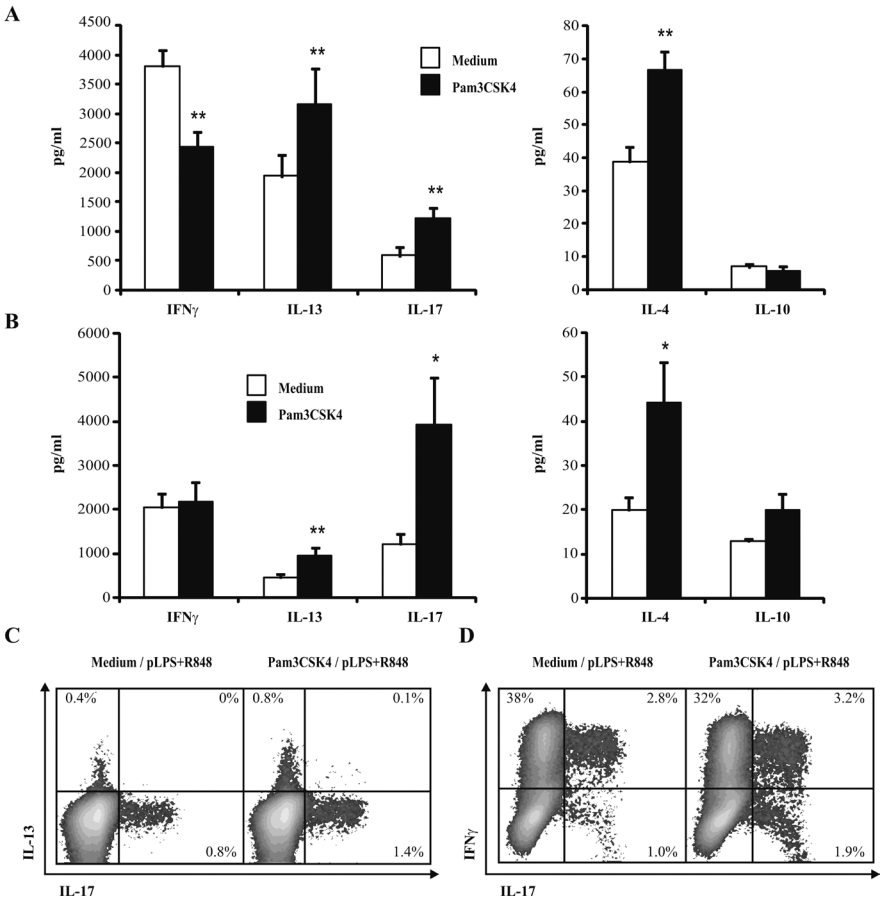

Figure 6

Co-stimulation of TLR2 with TLR4 or TLR8 skews the immune response towards Th2/Th17. Naive $CD4^+CD45RA^+CD45RO^-$ T cells (**A**) and memory $CD4^+CD45RA^-CD45RO^+$ T cells (**B**) isolated from the venous blood of healthy controls were added to DCs activated with pLPS and R848 and with or without Pam3CSK4 in a ratio of 10:1 and were cultured for 6 days before the addition of PMA (50 ng/ml) and ionomycin (1 μg/ml). After 12h supernatants were collected for the measurement of the released levels of IFNγ, IL-4, IL-13, IL-10 and IL-17. Data are mean and SEM of four independent experiments including eight individuals. * = $p<0.05$ and ** = $p<0.01$ compared to co-incubation with medium.

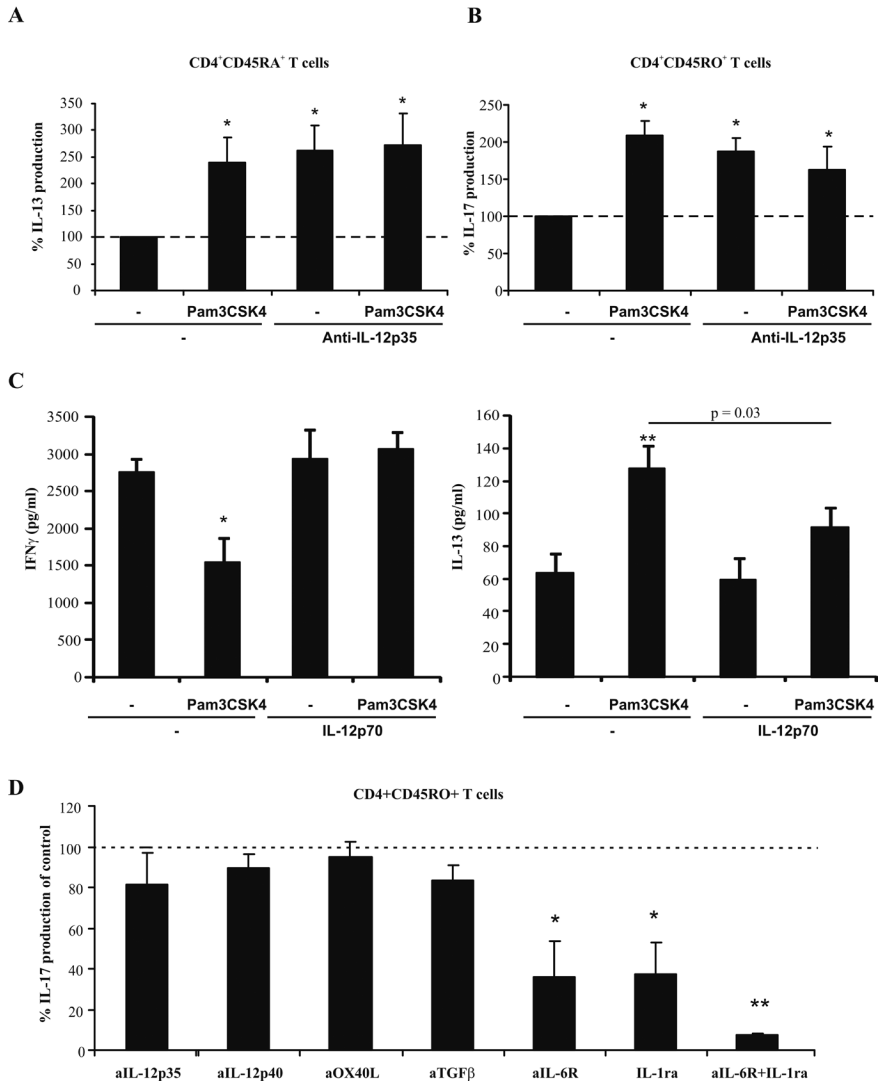

Figure 7

TLR2 stimulation mediates its TH17 inducing effect via the inhibition of IL-12p70 secretion. Naive $CD4^+CD45RA^+CD45RO^-$ T cells (**A**) and memory $CD4^+CD45RA^-CD45RO^+$ T cells (**B**) isolated from the venous blood of healthy controls were added to pLPS and R848 stimulated DCs with or without co-stimulation with Pam3CSK4 in a ratio of 10:1 and were cultured for 6 days in the absence or presence of antibodies against IL-12p35 before the addition of PMA (50 ng/ml) and ionomycin (1 μg/ml). After 12h supernatants were collected for the measurement of the released levels of IL-13 and IL-17 for the naïve and memory T cell population, respectively. Levels of cytokine release were compared to those produced by T cells cultured with pLPS and R848 stimulated DCs. (**C**) Memory $CD4^+CD45RA^-CD45RO^+$

T cells were added to DCs activated with the combination of Pam3CSK4, pLPS and R848 in a ratio of 10:1 and were cultured for 6 days in the presence of antibodies against IL-12p35, IL-12p40, OX40 Ligand, TGFβ, IL-6 Receptor or IL-1ra or the combination of an antibody against IL-6R and IL-1ra before the addition of PMA (50 ng/ml) and ionomycin (1 μg/ml). After 12h supernatants were collected for the measurement of the released levels of IL-17. Levels of IL-17 were compared to those produced by T cells cultured with DCs activated with the combination of Pam3CSK4, pLPS and R848 in the absence of neutralizing antibodies. Data are mean and SEM of three independent experiments. * = $p<0.05$ and ** = $p<0.01$ compared to co-incubation with medium alone.

Discussion

In the present study, we identify TLR2 as a potent modulator of TLR4 and TLR7/8 mediated immune responses confirming the recent observations by *Gerosa et al* [24]. TLR2 on the one hand suppresses the release of TLR4 and TLR7/8 induced pro-inflammatory cytokine release but on the other hand orchestrates the skewing of the immune response either toward Th1 or Th2 and Th17. In addition, we unravel two novel pathways by which TLR2 mediates its suppressive function baring potential for therapeutic intervention in immune diseases. By increasing the transcription of SOCS1 and decreasing the phosphorylation of STAT-1, transcription of type 1 IFNs and the transcription factors IRF1 and IRF8, TLR2 potently inhibits the type 1 IFN amplification loop, thereby abrogating the production of IL-12p70. Moreover, the production of TNFα is suppressed by the activation of the intracellular inhibitor PI3K by TLR2. We further demonstrate that the inhibition of IL-12p70 release by TLR2 underlies the shift from a Th1 to a more Th2/Th17 prone response.

The combination of archetypical constituents of a certain pathogen recognized by different pattern recognition receptors is thought to give specificity to our innate immune response. In this light, it can be envisaged that the myriad of host-derived TLR ligands generated in inflamed or degenerated tissue are able to modify these responses or in itself create specific immune responses. The role played by the different TLRs in immune activation was scrutinized extensively over the last years. The combined activation of certain TLR pathways has demonstrated some striking synergistic responses in the release of pro-inflammatory cytokines. The activation of TLR4 and TLR7/8 on DCs for example leads to the release of IL-12p70 and IL-23 that far surpasses the release by the activation of either TLR alone resulting in a prominent Th1 response[10,11]. Since the ubiquitous nature of endogenous proteins able to trigger TLRs is such that if unchecked the host would be overwhelmed by immune activation, several immune checkpoints

must be in place. A potential inhibitory or immune tuning role of TLRs has not been tested to a large extent in the literature.

An inhibitory function for TLR2 has been proposed in various settings. In contrast to TLR4 knockout mice, $TLR2^{-/-}$ mice are less susceptible to lethal infections with *Yersinia enterocolitica* or *Candida albicans*[37,38]. Next to the interference in the removal of pathogens the inhibitory effects mediated by TLR2 seem to have a bright side. In the absence of TLR2, uncontrolled inflammation and excessive injury of tissues in response to infections or during autoimmune processes have been observed[21,23]. Thereby, these studies demonstrate that TLR2 signals might have important protective effects against destructive chronic immune responses although the underlying pathways explaining these phenomena remain unidentified thus far. The present study establishes a role for TLR2 in providing an inhibitory signaling unit able to vigorously dampen TLR4 and TLR7/8 mediated activation of DCs and provides rational for the downstream pathways involved. For instance, PI3K, which functions at the early phase of TLR signaling and modulates the magnitude of the primary activation, was found to mediate the TLR2 induced inhibition of the release of TNFα while it did not affect IL-12p70 secretion. IL-12p70 secretion in turn was inhibited by the dampening of the type 1 IFN amplification loop seemingly by the increased transcription of SOCS1. PI3K as well as SOCS1 have been implicated as crucial negative regulators of Th1 responses by suppressing the production of IL-12p70[34,39]. The absence of SOCS1 in DCs enables them to invoke a potentiated Th1 response resulting in an enhanced antigen-specific anti-tumour immunity[40,41]. Likewise in an experimental arthritis model the severity of synovial inflammation and joint destruction was increased in the absence of SOCS1[42]. In *Chlamydia pneumoniae* infection it was shown that SOCS1 inhibited the expression of type 1 IFNs and IFNγ and suppressed *C. pneumoniae* induced lethal inflammation but impaired effective bacterial clearance[43]. These observations corroborate with our results and demonstrate that TLR2 is able to up regulate the expression of SOCS1 in DCs, thereby reducing Th1 inflammatory responses.

The ability of the immune system to mount an apt response to the wide array of different microbes is seminal to the protection of the host against these invading pathogens as well as the limitation of damage evoked by this response. In this light, the paradigm that activation of multiple TLRs by pathogens acts as a combinatorial code to induce a desired immune response is tempting. In addition this conceptual framework can be translated to the situation of autoimmunity in that the environment is thought to orchestrate the type of immune response.

Although the inhibitory role of TLR2 seems clear, in the removal of gram-positive bacteria, such as *Streptococcus Pneumoniae* and *Staphylococcus aureus*, TLR2 signalling is of vital importance[44-46]. We demonstrated that the activation of TLR2 in the presence of ligands for TLR4 and TLR7/8 leads to a shift from Th1 to Th17 responses. This implies that pathogens harboring archetypical constituents that trigger multiple TLR systems are recognized and processed resulting in an apt T cell response, notably a predominant Th17 response when TLR2 is added to the equation. In accordance with this notion Th17 cells seem to be essential in the clearance of both *S. Pneumoniae* and *S. aureus*[47,48]. Many experimental autoimmune models are induced in the presence of complete Freund's adjuvant (CFA) and are dependent on the presence of Th17 cells while the severity of the disease processes is aggravated by the removal of Th1 cells or the neutralization of IFNγ. The active component of CFA consists of sonicated *Mycobacterium tuberculosis*. *M. tuberculosis* is recognized by multiple pattern recognition receptors among which TLR2, TLR4, TLR9 and the NOD proteins seem to be the most dominant. In addition, it was recently demonstrated that IL-17 producing $CD4^+$ T cells are intricately involved in the first phase of the immune response against *M. tuberculosis*[49]. Accordingly, in our system *M. tuberculosis* activated DCs did not produce IL-12p70 and were able to induce elevated levels of IL-17 producing T cells **(unpublished results)**. In line with these findings in experimental autoimmune encephalitis, evoked in the presence of CFA, type 1 IFN receptor mediated signalling pathways, important for the establishment of Th1 responses, constrained Th17 mediated inflammation[19]. These recent findings put previous observations by our laboratory, in which it was demonstrated that STAT-1-/- mice show an exacerbation of zymosan induced arthritis[50], in a new light. Zymosan was demonstrated to induce strong Th17 responses[24,51], by removing the STAT-1 mediated IL-12p70/Th1 brake a fulminate inflammatory response mediated by Th17 cells can be expected. This underscores the delicate balance the immune system has to cope with keeping the different Th cell subsets in check thereby regulating immunity and tolerance.

In summary, we demonstrate that TLR2 is a potent modulator of the immune system by abrogating the type 1 IFN amplification loop thereby inducing a shift from Th1 to Th2 and Th17 responses. This puts TLR2 in the middle of the immune network deciding whether the effector response against microorganisms or in autoimmunity is mainly Th1 or Th17 mediated. Modulation of TLR2 mediated signalling might thus provide novel avenues for the intervention within a broad spectrum of immune diseases.

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

7

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Abstract

One of the major pathways underlying RA is the aberrant production of pro-inflammatory cytokines by infiltrating $CD68^{+}CD163^{-}$ macrophages. Macrophage activation in turn reflects the actions of a variety of pathways including those mediated by activated T cells and critically TLR ligands. We investigated whether Abatacept might reduce proinflammatory cytokine production by macrophages arising upon contact with cytokine activated T cells and/or stimulation with TLR ligands. Peripheral blood derived proinflammatory macrophages and cytokine stimulated T cells (Tck) were generated in vitro and added together in the presence of Abatacept or a control Ig, with or without TLR ligands. The production of cytokines was determined by multiplex methodologies. Abatacept potently reduced Tck-induced production of TNFα by macrophages. Tck and TLR ligands synergistically induced the production of proinflammatory cytokines by macrophages. This was especially the case for IL-12p70 which was solely produced when the macrophages were activated by both TLR ligands and Tck. Abatacept significantly reduced IL-12p70 production.

Infiltrating macrophages in the RA synovium are likely targets for activated T cells to induce the production of TNFα. Moreover in the presence of TLR ligands Tck stimulate macrophages to produce highly increased levels of TNFα, IL-6 and even IL-12p70. 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 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 GM-CSF (mϕ-1) exhibit the same characteristics as these infiltrating macrophages [2].

One crucial pathway mediating synovial macrophage activation is that driven by 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 demonstrated that human peripheral blood lymphocytes when cultured in the presence of IL-2, IL-6 and TNFα (cytokine stimulated T cells, Tck) display the same characteristics as activated synovial T cells [4].

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 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 IFNγ have been demonstrated to be potent in the induction of proinflammatory cytokine production by macrophages. Since cytokine stimulated T cells 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 (APC) such as DCs and macrophages by binding to CD80 and CD86 on the APC thereby preventing binding to CD28 on the T cell, 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 CTLA-4, which, upon stimulation, are transported readily

to the cell surface. Activation of mouse dendritic cells via CD80/86 by CTLA-4 expressing T cells resulted in the production of IFNγ by the dendritic cells [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-1 macrophages and Tck. PBMCs were isolated from buffycoats from healthy blood donors by using density-gradient centrifugation over Histopaque (Sigma). 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 6 wells plates in 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, NY). 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) 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 Ag, combined with a human IgG1 C region; it was used as a control fusion protein (control Ig) in these studies. 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 per well in 96 well culture plates. For the cell-contact assay after extensive washing $2x10^5$ cytokine-activated T cells were added to the mϕ-1 in the absence or 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 hours, the supernatants were collected for analysis of cytokine levels. For the TLR stimulation TLR agonists were added

to the mϕ-1 either in the presence or absence of Tck and/or Abatacept/control protein and after 24 hours the supernatants were harvested. LPS was used in a concentration of 1 ng/ml (E. coli 0111:B4, Sigma-Aldrich) and R848 was used in a concentration of 1 μg/ml (InvivoGen).

Measurement of cytokines in culture supernatants. Levels of TNFα, IL-12p70, IL-6 and IL-10 were measured in the supernatants using commercially available kits (BioSource International, Camarillo, CA) according to the manufacturer's instructions. Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad).

Statistical analysis. Differences between groups were analyzed using the Mann-Whitney U test or the paired T-test when applicable. 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 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 co-cultures (**Figure 1B**). 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 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 and especially IL-6 was observed when mϕ-1 were exposed to a TLR4 or TLR7/8 ligand together with Tck (**Figure 2A and B).** Abatacept did not significantly reduce the production of TNFα or IL-6 under these circumstances (**data not shown**).

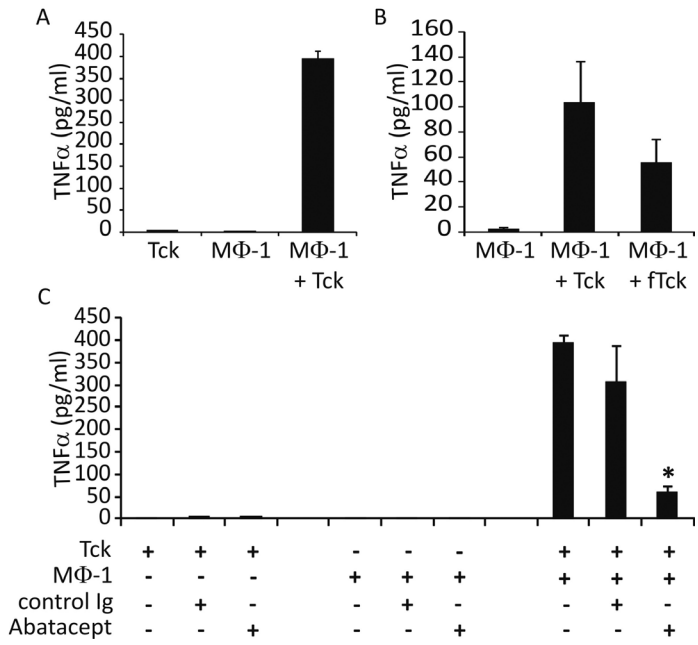

Figure 1

Mϕ-1 readily produce TNFα upon exposure to Tck which is inhibited by Abatacept. **A**, Mϕ-1 were stimulated with Tck for 24 hours 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 PFA for 24 hours after which the supernatants were measured for the presence of TNFα. Data are from triplicate wells and are representative of two 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 hours 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. * = $p<0.05$ compared to control Ig.

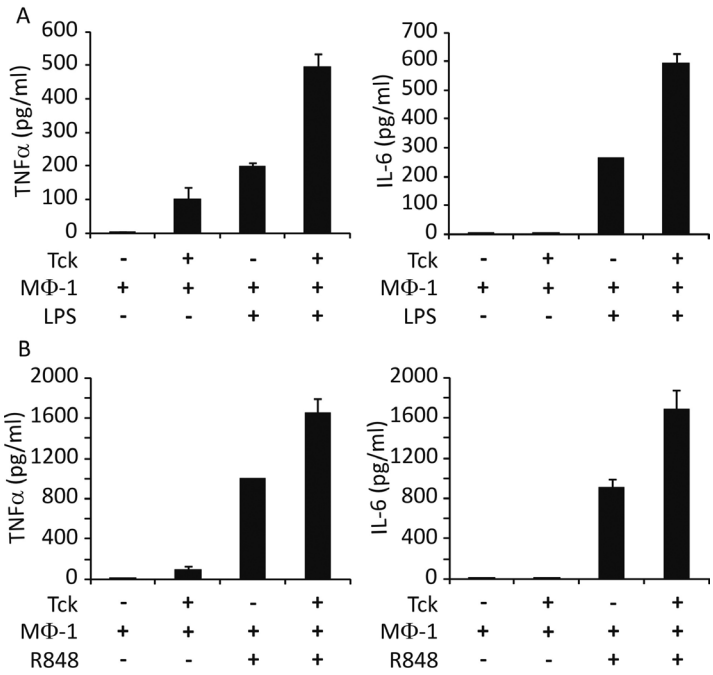

Figure 2

The combination of Tck and TLR ligands synergistically induce the production of TNFα and IL-6 by mϕ-1. Mϕ-1 were unstimulated or stimulated with Tck or LPS(**A**)/R848(**B**) or a combination of both. After 24 hours the supernatants were collected and measured for their TNFα (left panels) and IL-6 (right panels) content. Data are from duplicate wells and are representative of three individual experiments with similar results. Bars are mean and SD.

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 did not induce IL-12p70 production. However, stimulation with either TLR4 or TLR7/8 ligands in combination with Tck resulted in clear release of IL-12p70 (**Figure 3A**). Blockade of CD80/86 by Abatacept resulted in a significant reduction in the production of IL-12p70 (**Figure 3B**). Abatacept had no effect on cytokine production induced by TLR ligands alone providing evidence that the effect of Abatacept is not due to inhibitory signaling through CD80/86 but was due to the blockade of Tck-mϕ interactions (**data not shown**).

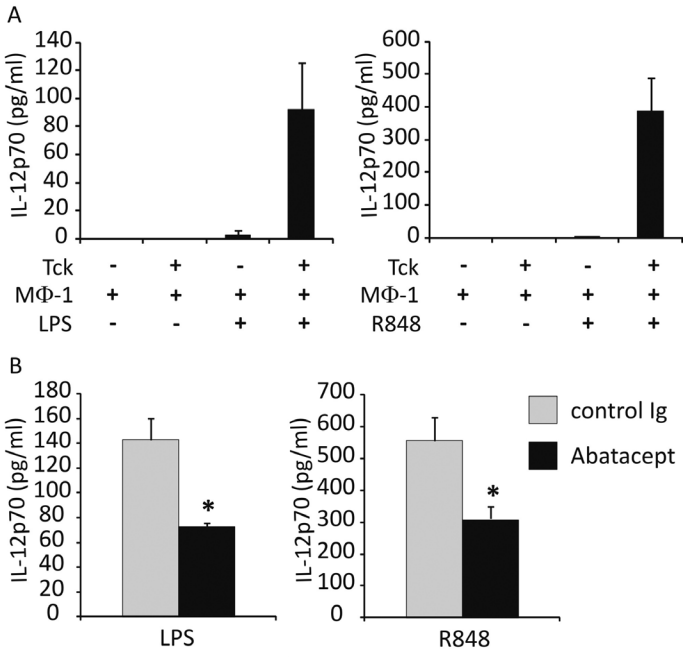

Figure 3

The combination of Tck and TLR ligands licenses mϕ-1 to produce IL-12p70 which is dampened by the presence of Abatacept. **A**, Mϕ-1 were unstimulated or stimulated with Tck or LPS(left panel)/R848(right panel) or a combination of both. After 24 hours the supernatants were collected and measured for their IL-12p70 content. **B**, Mϕ-1 were stimulated with Tck and LPS (left panel) or R848(right panel) in the presence of the control Ig (grey bars) or Abatacept (black bars). After 24 hours the supernatants were collected and measured for their IL-12p70 content. Data are from duplicate wells and are representative of three individual experiments with similar results. Bars are mean and SD. * = $p<0.05$ compared to control Ig.

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 by mϕ-1, activated by Tck and a TLR ligand, by approximately 50%, whereas the production of TNFα was unaffected. Th1 cells and NKT 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 NKT cells [15-17]. The IL-12p70/IFNγ axis was recently demonstrated, using the K/BxN serum transfer model and $IL\text{-}12p35^{-/-}$ mice, to promote antibody-induced joint inflammation underscoring the role played by IL-12p70 in arthritis [13].

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. This 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 dendritic cells [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. The production of IFNγ by macrophages upon Tck activation might explain our results regarding the highly increased release of IL-12p70. IFNγ is known to potently increase the release of IL-12p70 by macrophages

upon TLR stimulation. 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 specifically binding a receptor known to play a role in this process, Brennan et al did use a blocking antibody against the β2 integrin LFA-1 in experiments with Tck and macrophages [4]. This had no effect on the level of TNFα produced, substantiating that a role for steric hindrance might be minimal.

In conclusion, we demonstrated that infiltrating proinflammatory macrophages in the RA synovium are likely targets for activated T cells to induce the production of TNFα. Moreover in the presence of TLR ligands, which are present plentiful in the arthritic joint, activated T cells stimulate mϕ-1 to produce highly increased levels of TNFα, IL-6 and even IL-12p70. Abatacept was shown to potently interfere in these pathways.

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Impaired Dendritic Cell Proinflammatory Cytokine Production in Psoriatic Arthritis

8

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Abstract

The pathogenesis of Psoriatic Arthritis (PsA) remains poorly understood. The underlying chronic inflammatory immune response is thought to be triggered by unknown environmental factors potentially arising from a defective immune function. An impaired acute inflammatory response by Dendritic Cells (DCs) might compromise the clearance of bacteria and predispose to chronic inflammation. The cytokine production by DCs from healthy controls, Rheumatoid Arthritis, PsA and psoriasis patients towards *Mycobacterium tuberculosis, Mycobacterium avium paratuberculosis* and a range of other bacteria and TLR ligands was determined. Phenotypical differences involved in cellular responses against (myco)bacteria were determined by qPCR and flow cytometry. The secretion of proinflammatory cytokines by PsA DCs was impaired upon in vitro challenge with mycobacteria and TLR2 ligands. This impairment was associated with elevated serum CRP levels. The expression of TLR2 and other receptors known to mediate mycobacterial recognition was unaltered. In contrast, the intracellular TLR inhibitors SOCS3 and A20 (TNFAIP3) were more highly expressed in DCs from PsA patients. PsA DCs further demonstrated up regulated levels of ATG16L1, NOX2 and LL37; molecules implicated in the immune response against intracellular bacteria.

Our findings indicate that DCs from PsA patients have a disordered immune response towards some species of (myco)bacteria. This might predicate to impaired immune responses to, and in turn impaired clearance of, these bacteria setting the stage for the chronic inflammation of joints, entheses, skin and the gut.

Introduction

Psoriatic arthritis (PsA) is a common heterogeneous inflammatory disease of the skin, joints and entheses leading to discomfort, pain and in some cases the aggressive destruction of joints[1-3]. Like other spondyloarthropathies, PsA is associated with HLA-B27 positivity and (sub)clinical eye and gut inflammation[1]. Although the pathogenesis of PsA is unclear the underlying chronic inflammatory immune response, like in psoriasis, might be triggered by unknown environmental factors that function on the background of a polygenic susceptibility. Current available data do not support the notion that (autoreactive) T cells play an important role in the etiology of psoriasis nor PsA[4;5]. Analysis of intra-articular T-cells demonstrated that almost all expanded T cell clones were non-autoantigen driven, inflammation-related expansions underscoring the notion that autoreactive T cells might not be crucial in PsA[5]. However, T lymphocytes are important in the instigation and persistence of the chronic inflammatory process. Recent studies have implicated an impaired barrier function of the skin as playing a role in the induction of psoriasis. It was shown that mutations and polymorphisms in keratinocyte-expressed genes involved in physical barrier function or innate immunity are risk factors for developing psoriasis[6;7]. In addition GWAS studies demonstrated associations between inhibitors of Toll-like receptor (TLR) signaling (TNFAIP3 (A20) and TNIP1) and psoriasis[8]. These findings appear to imply that psoriasis is, at least in part, a disease of the innate immune system. We wondered whether the pathogenesis of PsA might also, at least in part, be due to a defect in innate immunity.

The first line of immune defense in tissues against pathogens is made up by resident Dendritic Cells (DCs)[4]. Upon the encounter of danger signals, recognized by pattern recognition receptors (PRR), DCs readily release antimicrobial peptides, chemokines and cytokines crucial for the induction of protective immune responses[9]. An important subset of the family of PRRs consists of the TLRs[10]. DC activation through TLRs results in the production of cytokines, enhanced expression of co-stimulatory and MHC molecules and enhanced bacterial killing. The initial release of cytokines and chemokines by DCs upon the encounter of a threat is pivotal in the creation of a microenvironment aimed at the immediate containment of the inciting pathogen or environmental factor. In order to prevent excessive inflammation TLR signaling is under tight control of an array of intracellular inhibitory molecules such as the suppressor of cytokine signaling (SOCS) proteins and TNFAIP3 (A20)[11;12]. However, it is essential to note that an impaired release of pro-inflammatory mediators might culminate in chronic

inflammation due to the aberrant removal of pathogens or other inciting factors.

The inciting environmental factors in the pathogenesis of PsA are unknown. However evidence points towards a pathogenic role for bacteria. The compartments mostly affected during PsA, namely the skin, joints and entheses, are likely sites where bacterial components are preferentially deposited[13-15]. In synovial fluid from PsA patients a higher variety and concentration of bacterial DNAs were found compared to serum (13). The trapping of microbial products at specific compromised sites appears indicative of at least a perpetuating role for pathogens in PsA. Furthermore PsA patients were demonstrated to have higher antibody levels against various bacteria and bacterial constituents than psoriasis patients without articular involvement, Rheumatoid Arthritis (RA) patients or healthy controls[16-18]. One bacterial type of particular interest in the pathogenesis of PsA are myco-bacteria. High levels of antibodies against 65-kDa and 48/45 doublet antigens from myco-bacteria have been found in psoriasis and these correlated with disease severity[19]. In addition, in placebo-controlled studies immunotherapy with *Mycobacterium vaccae* for psoriatic arthritis resulted in significant reductions of important disease parameters[20].

These findings prompted us to hypothesize that decreased immune reactivity towards (myco)bacteria might underlie PsA. PsA might thus be thought of as a dysfunctional barrier disease in which insufficient removal of invading pathogens by the immune system and subsequent deposition of bacteria at distal preferential sites results in local chronic inflammation. To determine the validity of our hypothesis we aimed to define whether DCs from PsA patients have an impaired immune response towards (myco)bacteria. We observed a striking inability of DCs from PsA patients to produce pro-inflammatory cytokines upon the exposure to mycobacteria. The inability to react adequately to these pathogens was associated with the presence of elevated CRP levels and was related to a reduced function of TLR2.

Materials and Methods

Study population

Eleven RA patients, fifteen PsA patients and eight plaque-type psoriasis patients attending the Department of Rheumatology or the Department of Dermatology at the Radboud University Nijmegen Medical Centre and thirteen Dutch healthy controls were included. In addition six PsA patients attending the Centre for Rheumatic diseases at the Glasgow Royal Infirmary and six healthy controls

resident in Glasgow were included. All patients fulfilled the American College of Rheumatology criteria for RA or the CASPAR criteria for psoriatic arthritis at the time of disease diagnosis and gave their informed consent. Patients using biological agents and/or high dose prednisolone were excluded from the study. The local Medical Ethics Committees approved the study protocol.

Isolation and culture of monocyte-derived Dendritic Cells

PBMCs were isolated from heparinized venous blood of patients and healthy volunteers by using density-gradient centrifugation over Ficoll-Paque (Amersham Bioscience). Monocytes were obtained using CD14 microbeads and MS columns or an AutoMACS (Miltenyi Biotec). Monocyte-derived DCs were generated by culturing isolated monocytes in the presence of IL-4 (500 U/ml; Schering-Plough) and GM-CSF (800 U/ml; Schering-Plough) for 6 days. Fresh culture medium with growth factors was added at day 3.

Flow cytometry of monocyte-derived DCs

Using standardized flow cytometry protocols phenotypical analysis of monocyte-derived DCs and macrophages was performed. Cells were characterized by staining with mAbs against human CD80 (BD Biosciences), CD86 (BD Pharmingen), MHC-II DR/DP (clone Q1514) and CD14, TLR2, DC-SIGN, Mannose Receptor (all BD Biosciences) and Dectin-1 (R&D). Cells were analyzed for the proportion of positive cells and the mean fluorescence intensity. As a control cells were stained with the appropriate IgG isotypes. For the determination of the binding and up take of the TLR2/1 ligand Pam3CSK4, rhodamine-labelled Pam3CSK4 (5 μg/ml, Invivogen) was added for 15 or 30 minutes at 37°C to DCs in 96 wells plates ($5x10^4$, 100 μl total volume).

Stimulation of monocyte-derived DCs

Day 6 monocyte-derived DCs were plated in a concentration of $0.5x10^6$ DCs/ml and transferred to 24 well (1 ml) or 96 well (100 μl) culture plates. Cells were then stimulated with TLR agonists for 16-24 hours. For experiments in which mRNA levels were determined monocyte-derived DCs were stimulated for 4 hours before they were put in TRIzol. The concentration in which the agonists were used was as follows unless stated otherwise: LPS (LPS, 100 ng/ml, E. coli 0111:B4, Sigma-Aldrich), Pam3CSK4 (5 μg/ml, EMC microcollections), FSL-1 (Pam2Cys, 1 μg/ml, EMC microcollections), MDP (2 μg/ml, Invivogen), heat-killed *M. tuberculosis* (10 or 100 μg/ml, Gibco), *S. pyogenes* cell wall fragments (1 or 10 μg/ml), heat-killed *M. avium paratuberculosis* ($5x10^6$/ml), heat-killed *K. pneumoniae* ($1x10^6$/ml) (all three were kindly gifted by L.A.B. Joosten, Department of Internal Medicine,

Radboud University Nijmegen Medical Centre), heat-killed *S. mutans* ($1x10^6$/ml) and heat-killed *E. coli* ($1x10^6$/ml). Supernatants were collected after 24 hours for cytokine measurements.

Measurement of cytokines in culture supernatants

Levels of IL-10, TNFα, IL-12p70, IL-6, IL-8 and IL-1β were measured in the supernatants using commercially available kits (Bio-Rad or BioScource) 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.

RNA isolation and real-time PCR

Total RNA was extracted in 1 ml of TRIzol reagent. Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems). All PCRs were performed with SYBR Green Master mix (Applied Biosystems), 10 ng cDNA, and a primer concentration of 300 nmol/L in a total volume of 20 μl. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the gene of interest of each sample with the Ct values of the reference genes *GAPDH* (ΔCt) and were deployed as relative expression ($2^{-\Delta Ct}$ x100). Software package Primer Express Version 2.0 (Applied Biosystems) was used to identify appropriate primer sets. Primers were as followed: GAPDH forward ATCTTCTTTTGCGTCGCCAG, reverse TTCCCCATGGTGTCTGAGC; SOCS1 forward CCCTGGTTGTTGTAGCAGCTT, reverse TTGTGCAAAGATACTGGGTATATGT; SOCS3 forward TCGGACCAGCGCCACTT, reverse CACTGGATGCGCAGGTTCT; SIGIRR forward TGAAAGACGGGCTTCCATTG, reverse TTCAGTGCTGGTCACGTTGAC; TOLLIP forward GCAGTACGGAGGCGCAGTGG, reverse CAGGCGCAGTCGGCAGTAGG; A20 forward AAGAAACTCAACTGGTGTCGAGAA, reverse TGCCGTCACCGTTCGTT; ATG16L1 forward AAAGAGCTTGCAGAAGCAGC, reverse GTCATGTCCACAGATGTGCC; NOX2 forward CCCTCCTATGACTTGGAAATGGA, reverse TGGTTTTGAAAGGGTGAGTGAC; LL37 forward CAGCAGTCACCAGAGGATTGT, reverse CAGCAGGGCAAATCTCTTGTTA; IRGM1 forward CATCACTATGGCAGGGGACT, reverse AGTTCTCCAGGGTTGTGGTG.

Statistical analysis

Differences between groups were analyzed using the Mann-Whitney U test. Paired student's T-test was used when differences were evaluated between different stimulations within donors. P values less than 0.05 were considered significant.

Results

Decreased proinflammatory cytokine production by PsA DCs upon exposure to *M. tuberculosis*

Monocytes were isolated from the venous blood of PsA (n=15) and RA (n=11) patients attending the outpatient clinic of the Department of Rheumatic Diseases at the Radboud University Nijmegen Medical Centre and from healthy controls (n=13). All PsA patients had peripheral arthritis. Patient characteristics are shown in **table 1**. After culturing the monocytes for 6 days in the presence of GM-CSF and IL-4 the resulting DCs were harvested. In order to evaluate the innate immune reaction towards *M. tuberculosis* in the RA and PsA patient populations and in healthy controls, DCs were exposed to *M. tuberculosis* or were left unstimulated. PsA patients produced significantly lower levels of TNFα and IL-6 when stimulated with *M. tuberculosis* compared to healthy controls and RA patients **(Figure 1)**. The production of IL-10 upon *M. tuberculosis* stimulation demonstrated the same trend although statistical significance was not reached **(Figure 1)**. The levels of IL-8 released by healthy control, RA and PsA DCs upon the stimulation with *M. tuberculosis* were similar while the levels of IL-12p70 and IL-1β were undetectable in all groups **(Figure 1 and data not shown)**. To determine whether the reduced

Table 1 Patient characteristics

	PsA	RA
Patients, n	15	11
Age in yr, mean (range)	55 (31-78)	62 (51-81)
Disease duration (yr); mean (range)	8 (1-41)	13 (1-28)
MTX use, n	6	6
SASP use, n	3	4
Other DMARD, n	2	0
Prednisolon, n	1	3
No DMARD/prednisolon, n	4	0
NSAID use, n	5	7
BSE, mean (range)	9.5 (2-30)	12.4 (3-30)
CRP ≥ 5, n	5	4
Rheumatoid factor positivity, n	1	11
TJC, mean (range)	1.4 (0-6)	1.2 (0-10)
SJC, mean (range)	1.9 (0-6)	1.7 (0-5)
DAS28, mean (range)	n.a.	3.1 (1.8-7.1)

n.a. not applicable, TJC Tender Joint Count, SJC Swollen Joint Count, DAS28 Disease Activity Score (28 joints)

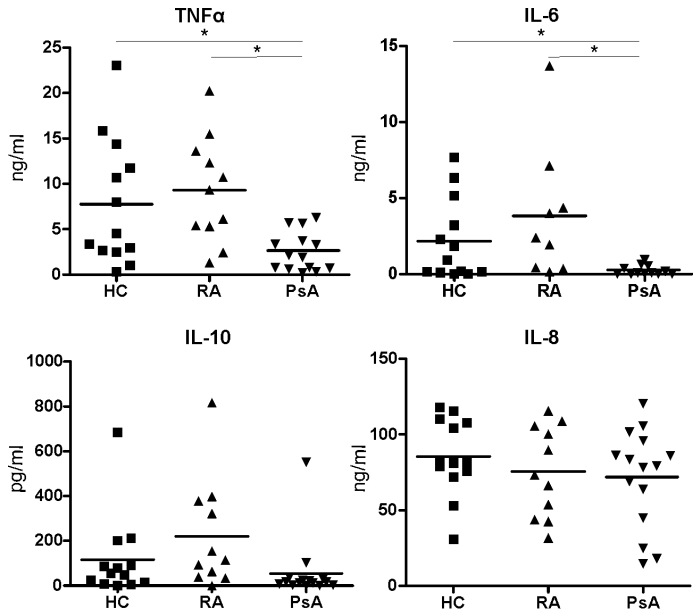

Figure 1

Cytokine production by DCs from healthy controls, RA and PsA patients upon *M. tuberculosis* exposure. Secretion of TNFα, IL-6 , IL-10 and IL-8 by DCs from healthy controls (n=13), RA (n=11) and PsA (n=15) patients stimulated with 100 μg/ml heat-killed *M. tuberculosis*. Lines represent means. Groups were compared using the Mann-Whitney U test. * = $p<0.05$ compared between two groups.

cytokine production was reflected in a decreased phenotypical maturation we measured the expression of CD80, CD86 and MHCII of healthy control and PsA DCs before and after the activation with *M. tuberculosis*. No differences were found in the induction of these DC maturation markers **(data not shown)**. In contrast to DCs from PsA patients, DCs from psoriasis patients did not demonstrate a hampered cytokine production when exposed to *M. tuberculosis* **(Figure 2A)**. To determine whether this abnormality reflected a peculiar geographic phenomenon, monocyte-derived DCs were cultured from six PsA patients attending the Centre for Rheumatic diseases and six healthy controls resident in Glasgow. Similar to the previous results with Dutch PsA DCs, PsA DCs from Scottish patients produced significantly less TNFα and IL-6 when exposed to heat-killed *M. tuberculosis* compared to healthy control DCs (**Figure 2B and C**).

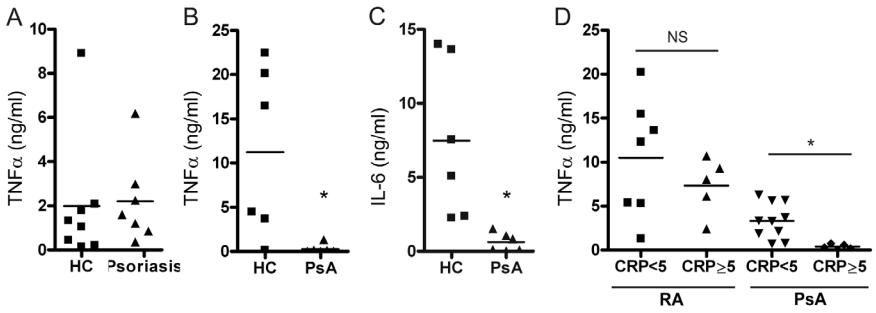

Figure 2

Cytokine production by DCs from psoriasis patients and DCs from healthy controls and PsA patients from an independent centre upon *M. tuberculosis* exposure. Secretion of TNFα by DCs from healthy controls (n=8) and psoriasis patients (n=7) stimulated with 100 μg/ml heat-killed *M. tuberculosis* **(A)**. Secretion of TNFα (**B**) and IL-6 (**C**) by DCs from healthy controls (n=6) and PsA patients attending the Centre for Rheumatic diseases at the Glasgow Royal Infirmary (n=6) stimulated with 100 μg/ml heat-killed *M. tuberculosis*. Release of TNFα by DCs from Dutch RA and PsA (**D**) patients with a CRP below 5 mg/L or equal or above 5 mg/L (RA<5 mg/L, n=7 and ≥5 mg/L, n=5; PsA<5 mg/L, n=10 and ≥5 mg/L, n=5) upon *M. tuberculosis* (100 μg/ml) stimulation. Groups were compared by means of the Mann-Whitney U test.* = $p<0.05$.

The dysfunctional reaction of PsA DCs towards *M. tuberculosis* is related to the presence of CRP

To determine whether disease activity was related to the cytokine production by DCs upon stimulation with *M. tuberculosis* we looked at the venous CRP levels at the time when blood was obtained for our experiments. In our experimental cohort 4 of the 11 RA patients and 5 of the 15 PsA patients had a CRP above detection limit (5 mg/L). DCs from RA patients with a noticeable CRP did not produce significantly different amounts of TNFα compared to RA patients without an elevated CRP upon *M. tuberculosis* stimulation. In contrast, the DCs from PsA patients with a detectable CRP did produce significantly lower amounts of TNFα compared to PsA patients with a CRP below 5 mg/L **(Figure 2D)**. The production of TNFα by RA or PsA DCs upon stimulation with *M. tuberculosis* in this population was independent of age, medication, disease duration, erythrocyte sedimentation rate and gender **(data not shown)**.

The dysfunctional proinflammatory cytokine release by PsA DCs is selective for certain bacterial species

To determine whether the effects found were selective for *M. tuberculosis* or could be extrapolated to other bacteria we performed experiments in which we stimulated

DCs from PsA patients and healthy controls with heat-killed *M. avium paratuberculosis*, *S. pyogenes* cell wall fragments, heat-killed *S. mutans*, *E. coli* or *K. pneumonia*. Comparable to the stimulation with *M. tuberculosis*, activation of PsA DCs with *M. avium paratuberculosis* led to the release of markedly lower levels of TNFα **(Figure 3A)**. Stimulation of healthy control and PsA DCs with *S. pyogenes*, *S. mutans*, *E. coli* or *K. pneumonia* led to the release of similar amounts of TNFα **(Figure 3B, C, D and data not shown)**. These data support the notion that the impaired innate immune reaction of PsA DCs is restricted to certain pathogens and is not due to a general incompetence of the DCs.

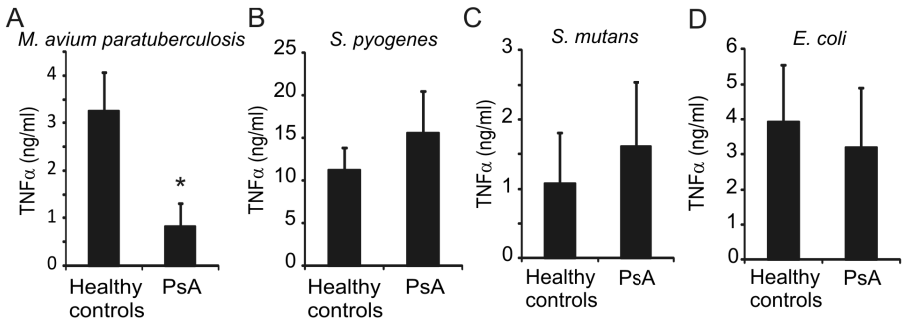

Figure 3

Cytokine production by DCs from healthy controls, RA and PsA patients upon *M. avium paratuberculosis, S. pyogenes* cell wall fragments*, S. mutans* or *E. coli* exposure. (**A**) TNFα release by DCs from healthy controls (n=5) and PsA (n=5) patients stimulated with $1x10^7$/ml *M. avium paratuberculosis*. (**B**) Secretion of TNFα by DCs from healthy controls (n=6) and PsA (n=7) patients stimulated with 10 μg/ml *S. pyogenes* cell wall fragments. TNFα production by DCs from healthy controls (n=5) and PsA (n=5) patients stimulated with $1x10^6$/ml *S. mutans* (**C**) or *E. coli* (**D**). Bars are mean and SEM. Groups were compared by means of the Mann-Whitney U test.* = $p<0.05$.

PsA DCs produce less proinflammatory cytokines when exposed to specific PRR ligands and this is correlated with a diminished reaction towards *M. tuberculosis*

To determine whether the aberrant immune reaction towards mycobacteria by PsA DCs might be due to a dysfunction of specific PRRs, we stimulated DCs from healthy controls and PsA patients with different ligands for PRRs suspected or known to play a role in the innate recognition of mycobacteria by DCs. DCs from PsA patients produced less TNFα upon the activation of TLR2/1 (Pam3CSK4) and upon the activation of both NOD2 (MDP) and TLR2/1 compared to healthy control DCs **(Figure 4A)**. Stimulation of TLR2/6 (FSL-1) and the activation of both NOD2 and TLR2/6 demonstrated the same trend but did not yet reach significance. When TLR4 (LPS) was added to the combinations the difference in TNFα production by HC and PsA DCs disappeared displaying the selectivity of the dysregulated cytokine production by PsA DCs and the delicate interactions between different PRRs **(data not shown)**. In addition, TLR4 alone or dectin-1 activation (curdlan) of healthy control and PsA DCs did not lead to differences in TNFα release either **(data not shown)**. The release of TNFα upon the stimulation of TLR2/1 or TLR2/1 and NOD2 was highly correlated with the production of TNFα upon stimulation of DCs with *M. tuberculosis* **(Figure 4B and C)**. TNFα production upon the TLR4 or dectin-1 ligation was not correlated with the production of TNFα upon *M. tuberculosis* stimulation of DCs **(data not shown)**. This strengthens the notion that signalling through TLR2 is an important determinant of the magnitude of the cytokine response upon the stimulation of DCs with heat-killed *M. tuberculosis*.

PsA DCs express unaltered levels of PRRs but significantly increased levels of SOCS3 and A20

We next determined whether the observed deregulated innate immune response by PsA DCs was due to an altered expression level of specific PRRs. The expression of CD14 and TLR2 was not altered on PsA DCs compared to healthy control DCs, although the levels were, as expected, extremely low **(Figure 5A)**. To determine whether there is a difference in the regulation of the expression of TLRs which might be overlooked due to the very low expression, we determined the expression of TLR2, 4, 7 and 8 mRNA in healthy control and PsA DCs. No significant differences were found between healthy control and PsA DCs **(data not shown)**. We additionally measured the expression of Mannose Receptor, DC-SIGN and Dectin-1, the expression of which was also identical on DCs from PsA patients compared to healthy control DCs **(Figure 5A and data not shown)**. To determine whether the uptake and binding of TLR2 ligands by PsA DCs was

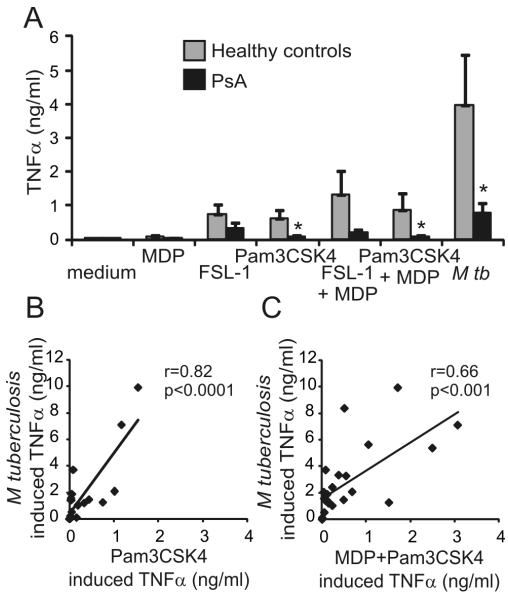

Figure 4

Pattern recognition receptor stimulation of DCs from healthy controls and PsA patients. (**A**) Secretion of TNFα by DCs from healthy controls (n=6) and PsA (n=5) patients stimulated with ligands for NOD2 (MDP, 1 μg/ml), TLR2/6 (FSL-1, 1 μg/ml), TLR2/1 (Pam3CSK4, 5 μg/ml), combinations of these or with 100 μg/ml *M. tuberculosis*. Bars are mean and SEM. *=p<0.05. Correlation between the secretion of TNFα by DCs stimulated with ligands for TLR2/1 (Pam3CSK4, 5 μg/ml) (**B**) or TLR2/1 and NOD2 (MDP, 1 μg/ml) (**C**) with 100 μg/ml *M. tuberculosis*.

altered compared to healthy control DCs rhodamine-labelled Pam3CSK4 was added to the DCs. The uptake/binding of the TLR2 ligand by PsA DCs was not different from that by healthy control DCs **(Figure 5B).** The difference in cytokine production between healthy control and PsA DCs is thus not due to a difference in expression of PRRs nor is it due to a decreased capacity to bind TLR2 ligands. Since TLR are under the strict control of intracellular inhibitory proteins we wondered whether an elevated expression of these would underlie the restricted cytokine production by PsA DCs. Increased mRNA levels were observed for SOCS3 and A20 in unstimulated DCs from PsA patients with an active disease, while SOCS1, TOLLIP and SIGIRR were not significantly different **(Figure 5C)**. RA patient DCs demonstrated similar levels of SOCS3 and A20 compared to healthy control DCs (**data not shown**).

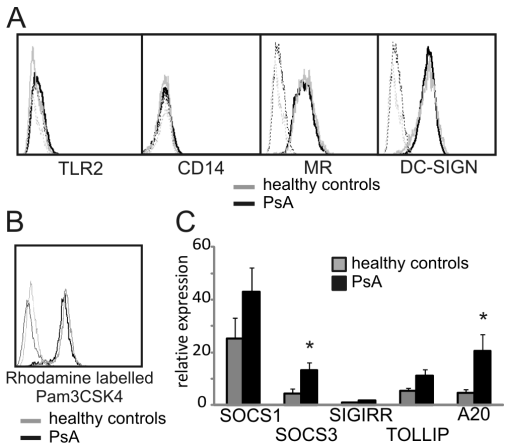

Figure 5

Expression of PRRs and intracellular signalling inhibitors. (**A**) Expression of TLR2, CD14, mannose receptor (MR) and DC-SIGN on unstimulated DCs from a healthy control (grey lines) and a PsA patient (black lines) cultured simultaneously. Data are representative of three independent experiments. (**B**) Binding and up take of Rhodamine-labelled Pam3CSK4 by DCs from a healthy control and a PsA patient cultured simultaneously. Data are representative of two independent experiments. (**C**) mRNA expression of *SOCS1*, *SOCS3*, *SIGIRR*, *TOLLIP* and *A20* relative to *GAPDH* expression (x100) in unstimulated DCs from healthy controls (n=7) and PsA patients (n=8) cultured simultaneously. Data are mean and SEM. Groups were compared by means of the Mann-Whitney U test.* = $p<0.05$.

DCs from PsA patients express enhanced levels of genes involved in the immune response against intracellular (myco)bacteria

M. tuberculosis infected alveolar and monocyte-derived macrophages exhibit increased expression of LL37 (cathelicidin)[21]. We tested whether DCs from PsA patients with active disease also had increased expression of LL37 and found clearly up regulated levels of LL37 in PsA DCs compared to healthy control and psoriasis DCs **(Figure 6A)**. The difference between PsA DCs and RA DCs demonstrated a clear trend but did not reach significance. Next we evaluated the mRNA levels of the autophagy genes ATG16L1 and IRGM1 and of the signalling molecule NOX2. Both ATG16L1 and IRGM1 have been linked with Crohn's disease susceptibility and the immune response against mycobacteria, whereas NOX2 is known to induce LL37. In addition to LL37, increased expression levels of ATG16L1 and NOX2 have been reported in macrophages from mice infected with mycobacteria[22;23]. Both ATG16L1 and NOX2 were found up regulated in DCs from PsA patients compared to healthy control, RA and psoriasis DCs **(Figure 6B and C)**. No IRGM1 mRNA was detectable in monocyte-derived DCs **(data not shown)**.

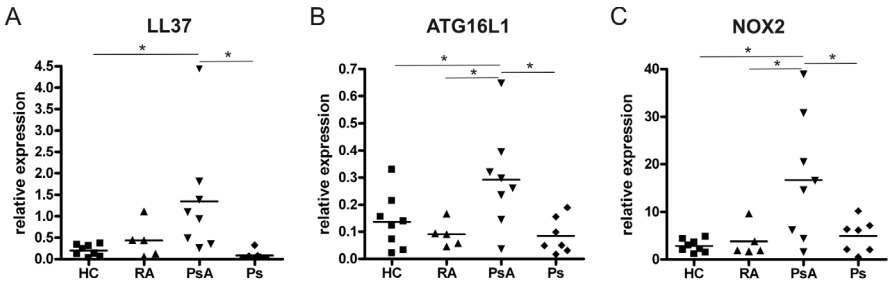

Figure 6

mRNA expression of mediators involved in the immune response against intracellular (myco)bacteria. The expression of Cathelicidin/LL37 (**A**), ATG16L1 (**B**) and NOX2 (**C**) mRNA was determined in DCs from healthy controls (n=8) and RA (n=5), PsA (n=8) and psoriasis (n=7) patients. Values are relative to *GAPDH* expression (x100). Groups were compared by means of the Mann-Whitney U test.* = $p<0.05$.

Discussion

Although considerable advances have been made in understanding the pathogenesis of PsA, the principal nature of the disease as an epithelial or immunological disorder are as yet enigmatic. Our observations reveal a fundamental deficit in innate immune recognition, particularly of mycobacterial species, that might underlie the initiation and/or perpetuation of PsA. This impaired function coincides with highly up regulated expression of molecules intimately involved in the regulation of the immune response against (intracellular) pathogens. These findings were selective for PsA patients and not even found for psoriasis patients. This appears to underscore that psoriasis and PsA might have a different underlying aetiology despite their clear clinical overlap. Genetic research is in line with this, demonstrating that first-relatives from PsA patients have a forty-fold increased risk of developing the disease which is much more than found for psoriasis[24]. This, together with a substantial body of evidence originating from previous studies prompted us to propose a mechanism that ultimately might lead to the disclosure of the environmental factor(s) fundamental to the pathology of PsA.

The role played by bacteria in the pathogenesis of spondyloarthropathies, such as Crohn's disease, psoriatic disease and ankylosing spondylitis, has been subject of investigation for decades. Diseases like PsA and ankylosing spondylitis often coincide with (subclinical) gut inflammation reminiscent of Crohn's disease.

Common genetic and immunological mechanisms seem to underlie skin, gut and joint inflammation in these diseases[25]. Since patients with Crohn's disease were recently shown to have an impaired acute inflammatory response in the skin and consequently an impaired removal of bacteria, accumulating evidence suggests that diseases like PsA and Crohn's disease might be two sides of the same coin[26]. An aberrant innate immune reaction by resident immune cells towards certain bacteria appears to underlie both diseases. Whereas Smith et al [27] demonstrated dysfunctional cytokine secretion upon the exposure of macrophages from Crohn's disease patients to heat-killed *E. coli*, we show here that DCs from PsA patients have a grossly impaired function when stimulated with mycobacteria, possibly facilitating the insufficient removal of pathogens culminating in chronic inflammation. In Crohn's disease primary innate immunodeficiency of macrophages leading to an impaired innate immune response and bacterial clearance has been demonstrated[27]. Recently a strong genetic and functional link between susceptibility to leprosy, a disease caused by *Myco-bacterium leprae*, and predisposition to Crohn's disease has been established[28;29]. Mycobacteria are ubiquitous in the environment and possess considerable stealth and capacity to adapt to environmental changes[30;31]. Mycobacteria have evolved strategies to survive within myeloid cells by preventing phagosome-lysosome fusion[32]. In Crohn's disease a pathogenic role for *M. avium paratuberculosis* has been suggested for years[33]. A possible role for mycobacteria in Crohn's disease appears to be underlined by the strong genetic link between the susceptibility to leprosy and the predisposition to Crohn's disease[28;29]. Some evidence also suggests a role for mycobacteria in PsA, vaccination with mycobacteria, for example, reduces disease severity[20].

The impaired immune response against *M. tuberculosis* by PsA DCs was related to the presence of CRP. This presents us with the question whether the aberrant DC phenotype is a true contributing factor to the disease or if it's merely a consequence of the disease process. If it is just a consequence of the chronic inflammatory process in PsA the increased expression of LL37 and ATG16L1 is remarkable. The antimicrobial peptide LL37 is up regulated during infection with *M. tuberculosis* in human macrophages and keratinocytes[34;35]. Next to its direct antimicrobial activity LL37 was shown to be a mediator of autophagy, an important anti-mycobacterial defense. In addition LL37 was shown to be highly expressed in psoriatic skin and able to bind self-DNA and RNA. DCs efficiently transport these complexes to endosomes where they activated nucleic acid-sensing TLRs leading to proinflammatory cytokine production[36;37]. Our data demonstrating that LL37 is highly up regulated in DCs from PsA patients thus might suggest a

protective mechanism of the immune system against mycobacteria whereas it might also perpetuate the ongoing disease process in PsA. The increased expression of ATG16L1 in PsA DCs also underscores the possible involvement of intracellular (myco)bacteria in PsA. ATG16L1 is an autophagy-related molecule associated with Crohn's disease, autophagy is important in the cellular immune response against intracellular (myco)bacteria by increasing killing and peptide presentation[38;39]. The increased expression of ATG16L1 found in PsA DCs might be a forced act in response to a (myco)bacterial infection. This is in line with the increased expression of NADPH oxidase 2 (Nox2) in PsA DCs. Nox2 activity is a key signal for the production of cathelicidin/LL37 and the induction of autophagal degradation of phagosomes[22;40]. The seemingly extreme readiness of PsA DCs to counteract an infection with an intracellular pathogen appears contradictory to an impaired clearance as we have suggested. However, it is clear from literature that the production of proinflammatory cytokines is crucial for the prevention of infections[41]. The activation of these intracellular defenses thus appears to act as a rescue mechanism preventing the full-blown infection of the host.

The reduced immune reaction against mycobacteria by PsA DCs appears to be, at least in part, mediated by the increased expression of the inhibitory proteins A20 and SOCS3. Interestingly, genome-wide association studies linked A20 with psoriasis susceptibility[8]. A20 is an ubiquitin-modifying enzyme that restricts TNF receptor and TLR induced signals. A20-deficient cells in addition demonstrated dramatically amplified responses to NOD2 activation, a PRR linked to Crohn's disease and psoriasis susceptibility[12]. We found that, next to TLR2 stimulation, NOD2 stimulation of PsA DCs was hampered, underscoring the involvement of A20 in the observed dysfunctional immune response by PsA DCs. SOCS proteins regulate JAK/STAT signaling pathways and interfere in TLR signaling[42]. SOCS3 is up regulated in human macrophages infected with mycobacteria rendering them unresponsive to IFNγ, and hindering the clearance of bacteria[43].

The correlation between the decreased proinflammatory cytokine production by PsA DCs upon the exposure to mycobacteria, NOD2 and TLR2 ligands could be expected since NOD2 and TLR2 activation have been shown to be crucial in the recognition of mycobacteria although other immunoreceptors have also been implicated[44;45]. This is in sharp contrast to *S. pyogenes* for which a MyD88 dependency in its recognition was shown which was not crucially dependent on either TLR1, TLR2, TLR4, TLR9 or TLR2/6[46]. This indicates that for the recognition of *S. pyogenes* a combination of multiple TLRs act in concert, which is not affected in PsA DCs. The recognition of *E. coli* and *K. pneumoniae* appears to be mainly

mediated through TLR4 which might explain the unhampered recognition of this gram-negative pathogen by PsA DCs[47]. Together these findings indicate that the hampered innate immune response by PsA DCs is selective for pathogens triggering specific combinations of PRRs, possibly mainly those triggering TLR2.

In conclusion, we have uncovered an aberrant immune response by DCs from PsA patients towards specific intracellular (myco)bacteria which might underlie their impaired removal in the tissues affected in this condition. Intriguingly, this altered response was absent in psoriasis patients assuming a different pathogenic mechanism compared with psoriatic arthritis. Further studies are warranted to further define the underlying circuitry leading to this impaired immune function.

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Macrophage Dysfunction in Psoriatic Arthritis

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Abstract

The pathogenesis of Psoriatic Arthritis (PsA) remains poorly understood. The underlying chronic inflammatory immune response is thought to be triggered by unknown environmental factors potentially arising from a defective barrier function of the skin as well as an impaired immune function of Dendritic Cells (DCs). "Anti-inflammatory" $CD163^+$ macrophages (mϕ-2) are highly present in psoriatic synovium. We determined whether these mϕ-2 have an aberrant phenotype compared to those from healthy controls thereby contributing to chronic inflammation. Mϕ-2 from healthy controls and PsA patients were analysed for their expression of various cell surface receptors as well as their cytokine production when exposed to a range of bacteria and single Toll-Like receptor (TLR) ligands. Differences between healthy control and PsA mϕ-2 involved in suppressing DC function were determined by stimulation assays and mixed leucocyte reactions. CCR5 was significantly increased on mϕ-2 from PsA patients compared to healthy controls while the expression of CCR1, TLR2 and CD14 was unaltered. Further research revealed that PsA mϕ-2 secreted more IL-6 upon incubation with heat-killed *Escherichia coli* and *E. coli* lipopolysaccharide, were less efficient in taking up *E. coli* and were incapable of suppressing pro-inflammatory cytokine production by autologous DCs compared with mϕ-2 from their healthy counterparts . Further reflecting their disordered function was the finding that in allogenic T cell assays PsA mϕ-2 are incapable of suppressing DC induced IL-17 and TNFα production by T cells. In addition naive $CD4^+$ T cells from PsA patients already have an intrinsic increased tendency to polarize into IL-17 producing T cells.
In this manuscript we demonstrate the dysfunction of "anti-inflammatory" $CD163^+CCR5^+$ mϕ-2 in PsA. This implicates mϕ-2 as an important defective cell type in PsA facilitating chronic inflammation instead of exerting their normal function of restoring immune homeostasis.

Introduction

Psoriatic arthritis (PsA) is an inflammatory disease of the skin, joints and entheses leading to discomfort, pain and in some cases the aggressive destruction of joints (arthritis mutilans). Approximately 15-25% of psoriasis patients develop arthritis, the incidence of which rises with disease severity (1-3). Although the events leading to the development of psoriasis and PsA are unclear the underlying chronic inflammatory immune response is thought to be triggered by unknown environmental factors on a polygenic background with increased susceptibility. Recent studies have implicated an impaired barrier function of the skin as a major determinant in the induction of psoriasis. It was shown that mutations and polymorphisms in keratinocyte-expressed genes involved in physical barrier function or innate immunity, are risk factors for developing psoriasis (4,5). The pathogenic role of activated $CD4^+$ T cells has been demonstrated by the clinical efficacy of drugs that inhibit T cell activation or deplete activated T cells (6). These findings appear to imply that the aetiology of PsA is based on aberrances in the innate immune system followed by a dysregulated adaptive immune response.

In psoriatic disease the role played by DCs and macrophages is apparent from studies ranging from mouse models to human in vitro studies and drug trials. While in normal human skin only DCs are present in both the epidermis (Langerhans cells) and the dermis (dermal DCs) in psoriatic skin macrophages are also found in considerable numbers (7). The inflamed synovium and skin from PsA patients is characterized by a marked infiltration with "resident" $CD163^+$ macrophages. CD163 is a marker for type 2 macrophages (mϕ-2) which are highly phagocytic, are resident at mucosal surfaces and the peritoneum, and have been been suggested to play a role in the suppression of tissue inflammation due to their secretion of IL-10 and their suppressive effect on the development of IFNγ producing T cells (8,9). By culturing monocytes with M-CSF Mϕ-2 can be differentiated *in vitro* (9). The function of $CD163^+$ macrophages in psoriatic disease is not fully understood. Global disease activity in patients with spondylarthropathy, including PsA patients, correlated significantly with the infiltration of $CD163^+$ macrophages (10)-(11). $CD163^+$ macrophages were highly increased in psoriatic lesional skin and returned to normal levels after anti-TNFα treatment (12). It was shown that in psoriasis $CD163^+$ macrophages were not immunostimulatory and were, like in vitro M-CSF-cultured $CD163^+$ macrophages, (virtually) unable to polarize T cells to produce IL-17 or IFNγ (13).

Bacteria have long been thought to play a role in many autoinflammatory and autoimmune diseases. Macrophages of Crohn's disease patients have been shown to have an aberrant immune reaction towards heat-killed *E. coli* leading to the delayed removal of these pathogens when injected intradermally, possibly contributing to the chronic inflammatory process (14). PsA and Crohn's disease are both thought of as dysfunctional barrier diseases and both diseases share a remarkable clinical and genetic overlap. The compartments mostly affected during PsA, namely the joints and entheses, are sites where bacterial components are preferentially deposited (15-17). PsA patients have higher antibody levels against various bacteria and bacterial constituents than Rheumatoid Arthritis (RA) patients or healthy controls (18-20). Mycobacteria have been suspected of playing a role in Crohn's disease as well as psoriatic disease (21). The presence of *M avium subspecies paratuberculosis* reactive T cells with a Th1 or Th1/Th17 phenotype strengthens a possible role of mycobacteria in Crohn's disease (22). In addition, a strong genetic and functional link between susceptibility to leprosy, a disease caused by *Mycobacterium leprae*, and predisposition to Crohn's disease has been established (23,24). In psoriatic disease high levels of antibodies against 65-kDa and 48/45 doublet antigens from mycobacteria have been found and these correlated with disease severity (25). The hypothesis that certain bacteria might play a role in PsA was strengthened by our finding that DCs from PsA patients with an active disease have a severely impaired innate immune reaction towards mycobacteria (Wenink et al. submitted). Taken together, a hypothetical framework can be formed for the pathogenesis of PsA in which an initial decreased response by DCs towards certain (myco)bacteria would lead to an insufficient removal of these bacteria and the initiation of inflammatory responses. However, how this inflammatory response derails and becomes chronic is left unanswered. A potentially altered function of the abundantly present $CD163^+$ Mϕ-2 is an interesting hypothesis to work with in an attempt to answer this issue.

Here we demonstrate that $CD163^+$ macrophages from PsA patients display a remarkably different phenotype and function compared with those from healthy controls. Our data thus demonstrate that mϕ-2 in PsA fail to inhibit inflammatory responses which potentially underlies the ongoing inflammatory response in these patients. Therefore, restoring the function of mϕ-2 in PsA is an attractive novel therapeutic target in this condition.

Materials and Methods

Study population

Nine PsA patients attending the Department of Rheumatology at the Radboud University Nijmegen Medical Centre and nine Dutch healthy controls were included. In addition eight PsA patients attending the Centre for Rheumatic diseases at the Glasgow Royal Infirmary and eight healthy controls resident in Glasgow were included. All patients fulfilled CASPAR criteria for psoriatic arthritis at the time of disease diagnosis and gave their informed consent. Patients using biological agents and/or high dose prednisolone were excluded from the study or added separately to analyze the effect of anti-TNFα treatment. The local Medical Ethics Committees approved the study protocol.

Isolation and culture of monocyte-derived type-2 macrophages and Dendritic Cells

PBMCs were isolated from heparinized venous blood of healthy volunteers and PsA patients by using density-gradient centrifugation over Ficoll-Paque (Amersham Bioscience). Monocytes were obtained using CD14 microbeads and MS columns or an AutoMACS (Miltenyi Biotec). Monocyte-derived macrophages were generated by culturing isolated monocytes in the presence of M-CSF (50 ng/ml, Invitrogen). Monocyte-derived DCs were generated by culturing isolated monocytes in the presence of IL-4 (500 U/ml; Schering-Plough) and GM-CSF (800 U/ml; Schering-Plough) for 6 days. Fresh culture medium with the appropriate growth factors was added at day 3.

Flow cytometry of monocyte-derived type-2 macrophages

Using standardized flow cytometry protocols phenotypical analysis of monocyte-derived macrophages was performed. Cells were characterized by staining with mAbs against human CCR1, CCR5 (both R&D) and CD14 and TLR2 (both BD Biosciences). Cells were analyzed for the proportion of positive cells and the mean fluorescence intensity. As a control cells were stained with the appropriate IgG isotypes. For the determination of the binding and up take of *E. coli*, *E. coli* (k-12) fluorescein-labelled Bioparticles (25 μg/ml, Invitrogen) were added for 15, 30 or 45 minutes at 37°C to macrophages or DCs in 96 wells plates ($5x10^4$, 100 μl total volume).

Stimulation of monocyte-derived macrophages

Day 6 monocyte-derived macrophages were replated in a concentration of $0.5x10^6$ /ml in 96 well (100 μl/well) culture plates. Cells were then stimulated with heat-killed bacteria or TLR agonists. The concentration in which the stimuli were

used was as follows: LPS (LPS, 100 ng/ml, *E. coli* 0111:B4, Sigma-Aldrich), Pam3CSK4 (5 μg/ml, EMC microcollections), R848 (2 μg/ml, Enzo life sciences) heat-killed *M. tuberculosis* (100 μg/ml, Gibco), heat-killed *P. gingivalis* ($1x10^6$/ml, Invitrogen) and heat-killed *E. coli* ($1x10^6$/ml). Supernatants were collected after 24 hours for cytokine measurements.

DC and macrophage co-stimulations

After harvesting at day 6 of culture $5x10^4$ monocyte-derived macrophages and $5x10^4$ DCs were replated separately in 96 well culture plates. To determine the effect of the presence of macrophages on DC (and vice versa) $5x10^4$ macrophages together with $5x10^4$ DCs were put in 96 wells plates, as a control $1x10^5$ macrophages and $1x10^5$ DCs were also plated separately in 96 wells plates. Cells were then stimulated with heat-killed *M. tuberculosis* (100 μg/ml, Gibco) in a total volume of 200 μl. Supernatants were collected after 24 hours for cytokine measurements.

T-cell assays

At day 6 monocyte-derived macrophages and DCs were harvested and resuspended in a concentration of $1x10^5$ cells/ml in culture medium. $5x10^3$ macrophages and/or $5x10^3$ DCs were replated in 96 round bottom well plates. $CD4^+$ T cells from PBMCs from healthy controls were obtained by negative selection using microbeads and MS columns or AutoMacs (Miltenyi Biotec). Next $CD4^+CD45RA^+$ naïve T cells were separated from the $CD4^+CD45RO^+$ T cells by the use of microbeads aimed at CD45RO. $50x10^3$ $CD4^+CD45RA^+$ T cells were added to the round bottom wells with the DCs, macrophages or macrophages+DCs. Next lipoarrabinomannan (10 μg/ml, Invitrogen) or heat-killed M. tuberculosis (100 μg/ml) was added to the round bottom wells. At day five of the MLR cells were incubated with PMA (50 ng/ml, Sigma) and Ionomycin (1 μg/ml, Sigma) 12 hours before the collection of supernatants. In addition T-cell experiments were performed in which unstimulated or *M. tuberculosis* (24 hours, 100μg/ml) stimulated $5x10^3$ DCs from healthy controls or CD3/CD28 expander beads in medium from the *M. tuberculosis* stimulated healthy control DCs (50 μl/well) were co-cultured with allogeneic $CD4^+CD45RA^+$ T cells from healthy controls or PsA patients for five days before the stimulation with PMA (50 ng/ml, Sigma) and Ionomycin (1 μg/ml, Sigma) 12 hours before the collection of supernatants.

Measurement of cytokines in culture supernatants

Levels of IL-10, TNFα, IL-6, IFNγ, IL-17 and IL-13 were measured in the supernatants using commercially available kits (Bio-Rad or BioScource) 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 the Mann-Whitney U test. The paired students T-test was used where appropriate. P values less than 0.05 were considered significant.

Results

Selective increased expression of CCR5 on mϕ-2 from PsA patients

The inflamed synovium in Psoriatic Arthritis is characterized by the infiltration of high numbers of $CD163^+$ mϕ-2. The role played by mϕ-2 in PsA not fully understood. Mϕ-2 have been suggested to suppress tissue inflammation by their preferential secretion of IL-10 and their suppressive effect on the development of IFNγ producing T cells (8,9). To determine their possible role in the perpetuation of tissue inflammation in PsA we first characterized their phenotype. While the expression of CD14, TLR2, mannose receptor (MR) and CCR1 was unaffected on PsA mϕ-2 compared to healthy control mϕ-2, monocyte-derived mϕ-2 from PsA patients express elevated levels of CCR5 (**Figure 1a**). This increased expression of CCR5 was not present on monocytes (**Figure 1b**). Mϕ-2 from PsA patients on anti-TNFα treatment demonstrated CCR5 levels comparable to healthy controls (**Figure 1c**). In contrast, type-1 GM-CSF cultured macrophages and monocyte-derived DCs do not express considerable levels of CCR5 and Mϕ-2 from rheumatoid arthritis patients did not demonstrate elevated levels of CCR5 (**data not shown**). These findings might explain the marked and selective prevalence of mϕ-2 in the inflamed synovium of PsA patients.

Mϕ-2 from PsA patients produce increased levels of IL-6 when exposed to *E. coli* and phagocytose *E. coli* less efficiently

To further characterize PsA mϕ-2 we stimulated them with heat-killed *Mycobacterium tuberculosis*, *Escherichia coli* or *Porphyromonas gingivalis*. It was demonstrated that exposure of PsA mϕ-2 to *E. coli* leads to an exaggerated IL-6 production while the release of TNFα or IL-10 was not significantly altered. The cytokine production of PsA mϕ-2 was comparable to healthy control mϕ-2 upon exposure to *M. tuberculosis* or *P. gingivalis* (**Figure 2A**). To determine whether this increased released of IL-6 by PsA mϕ-2 was due to an altered response towards TLR triggering we stimulated PsA mϕ-2 with TLR ligands specific for TLR2/1

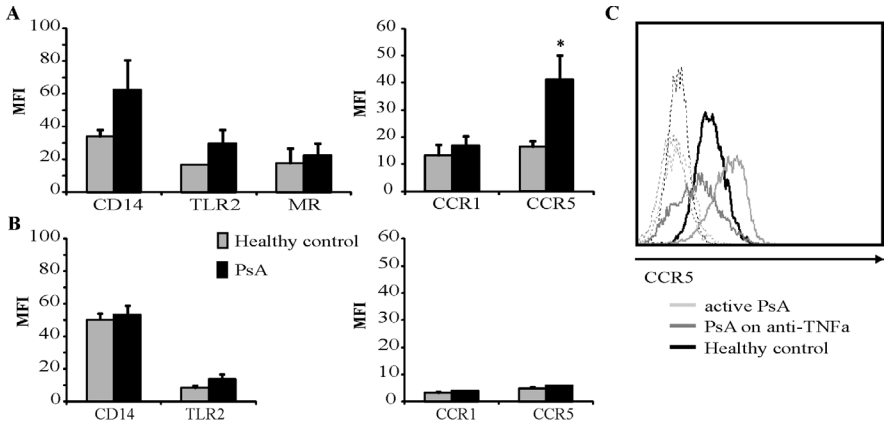

Figure 1

Increased expression of CCR5 on PsA mϕ-2. Staining of surface markers on mϕ-2 from healthy controls (n=8) and PsA patients (n=8) revealing no significant differences in CD14, TLR2, mannose receptor (MR) and CCR1 expression while the expression of CCR5 is highly increased on PsA mϕ-2 (**A**). Staining of CD14, TLR2, CCR1 and CCR5 on monocytes from healthy controls (n=4) and PsA patients (n=4) (**B**). Bars are mean and SEM. * = p=0.03. Expression of CCR5 on mϕ-2 from a healthy control, a PsA patient not on anti-TNFα therapy and a PsA patient on anti-TNFα therapy (**C**). Dotted lines are isotype controls, straight lines are CCR5 staining. Data for PsA patients on anti-TNFα therapy are representative for 2 patients in 2 independent experiments.

(Pam3CSK4), TLR4 (*E. coli* lipopolysaccharide) or TLR7/8 (R848). In line with their reaction towards heat-killed *E. coli*, PsA mϕ-2 produced increased levels of IL-6 when exposed to purified *E. coli* lipopolysaccharide (**Figure 2B)**.

In Crohn's disease deficient clearing of intradermally injected heat-killed *E.coli* has been demonstrated. Since PsA patients often have subclinical gut inflammation we wondered whether PsA mϕ-2 (and DCs) would demonstrate a reduced ability to phagocytose heat-killed *E. coli*. In comparison to healthy control mϕ-2, PsA mϕ-2 were less efficient in the up take of labelled *E. coli* (**Figure 2C**). DCs from PsA patients demonstrated the same defect. Even after 45 minutes there was still a significant deficiency in the uptake of E coli by the PsA DCs and macrophages (**Figure 2D and data not shown**). The uptake of E coli was due to active phagocytosis since the same assay on ice did not show any increase in the level of staining beyond the background (**data not shown**).

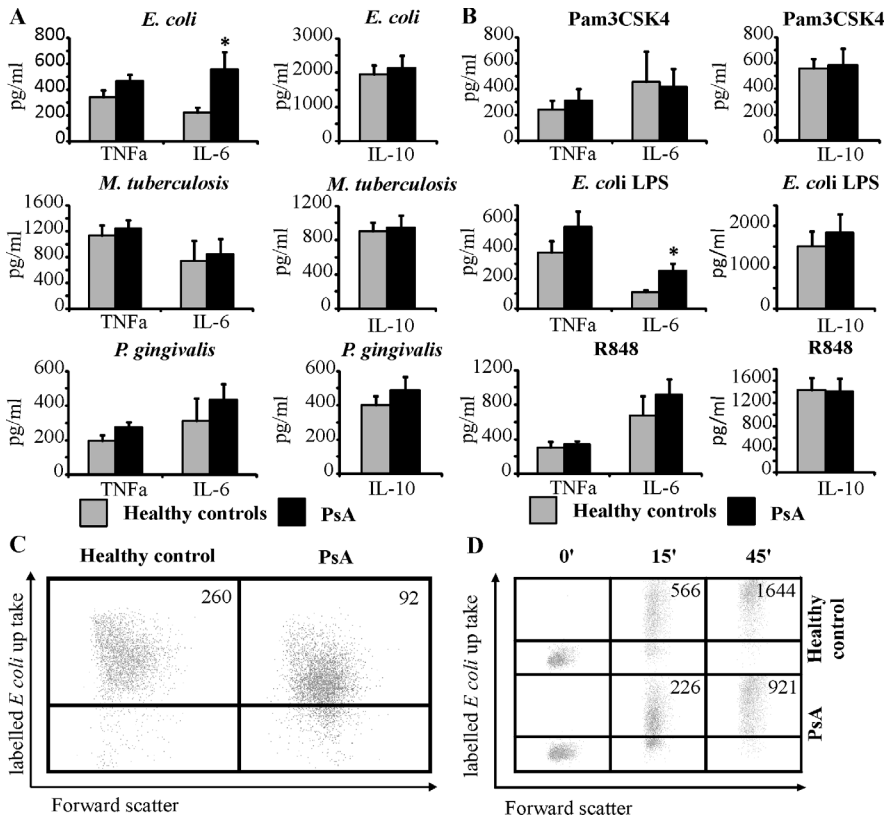

Figure 2

Increased production of IL-6 by PsA mϕ-2 upon stimulation with *E. coli.* Mϕ-2 from healthy controls (n=9) and PsA patients (n=9) stimulated with heat-killed bacteria (**A**) or single TLR ligands (**B**) demonstrating an increased release of IL-6 by PsA macrophages upon stimulation with heat-killed *E. coli* or *E. coli* lipopolysaccharide (LPS). (**C**) Mϕ-2 from a healthy control and a PsA patient were incubated with *E. coli* fluorescein-labelled Bioparticles for 15 minutes at 37°C. PsA mϕ-2 less efficiently phagocytised labelled *E.coli* compared to healthy control mϕ-2. Data are representative for 2 independent experiments with mϕ-2 from in total 2 healthy controls and 2 PsA patients. (**D**) DCs from a healthy control and a PsA patient were incubated with *E. coli* fluorescein-labelled Bioparticles for 0, 15 and 45 minutes at 37°C. PsA DCs less efficiently phagocytised labelled *E.coli* compared to healthy control DCs after both 15 and 45 minutes. Data are representative for 3 independent experiments with mϕ-2 from in total 3 healthy controls and 3 PsA patients.

PsA mϕ-2 are less capable of reducing pro-inflammatory cytokine production by DCs and $CD4^+$ T cells

Since mϕ-2 have been described as important mediators of tissue homeostasis and appear to be "anti-inflammatory" we wondered whether their suppressive character might be dysfunctional in PsA. Previously we demonstrated that reduced cytokine production by PsA DCs upon exposure to mycobacteria might underlie the disease process in PsA. We wondered whether the presence of mϕ-2 would alter the cytokine production of DCs upon exposure to *M. tuberculosis*. In co-cultures with autologous DCs PsA mϕ-2 were less capable of suppressing the *M. tuberculosis* induced production of TNFα or increasing the production of IL-10 in comparison to healthy control mϕ-2 (**Figure 3A).** In control experiments doubling the number of DCs resulted in the release of approximately twice the level of TNFα, IL-6 and IL-10 produced while doubling the number of mϕ-2 resulted in an unaltered release of TNFα and IL-10 while the release of IL-6 was increased with fifty percent (**data not shown**). In line with these results, in MLR experiments PsA mϕ-2 were incapable of suppressing the polarization of TNFα and IL-17 while healthy control mϕ-2 potently reduced the capability of T cells to produce TNFα and IL-17 upon polarization after the stimulation with lipoarrabinomannan (a cell wall constituent of all mycobacteria) (**Figure 3B**). This effect was not due to an increase in the number of antigen-presenting cells nor was it due to a decreased survival of PsA mϕ-2 and above all, successful treatment with anti-TNFα appeared to reinstate their capacity to suppress T cell polarization into TNFα and IL-17 producing cells (**data not shown**).

$CD4^+CD45RA^+$ T cells from PsA patients demonstrate an increased tendency to polarize into IL-17 producing T cells

To determine whether naive $CD4^+$ T cells from PsA patients possess a phenotype skewed towards the increased polarization of IL-17 producing T cells we stimulated $CD4^+CD45RA^+$ T cells from healthy controls and PsA patients with allogenic healthy control DCs. Naive T cells from PsA patients were more prone to polarize into IL-17 producing T cells compared to those from healthy controls when exposed to allogenic healthy control DCs stimulated with *M. tuberculosis* but not when exposed to immature unstimulated DCs (**Figure 4A and B**). To determine whether this was due to a different reaction towards (healthy control) DCs naive $CD4^+$ T cells from healthy controls and PsA patients were cultured in the presence of $CD3^+CD28^+$ beads and supernatant from healthy control DCs stimulated with M. tuberculosis (to mimick an inflammatory environment). In line with the results obtained with healthy control DCs, PsA T cells activated with $CD3^+CD28^+$ beads were more prone to polarize into T cells capable of producing IL-17 compared to healthy control naive $CD4^+$ T cells (**Figure 4C**).

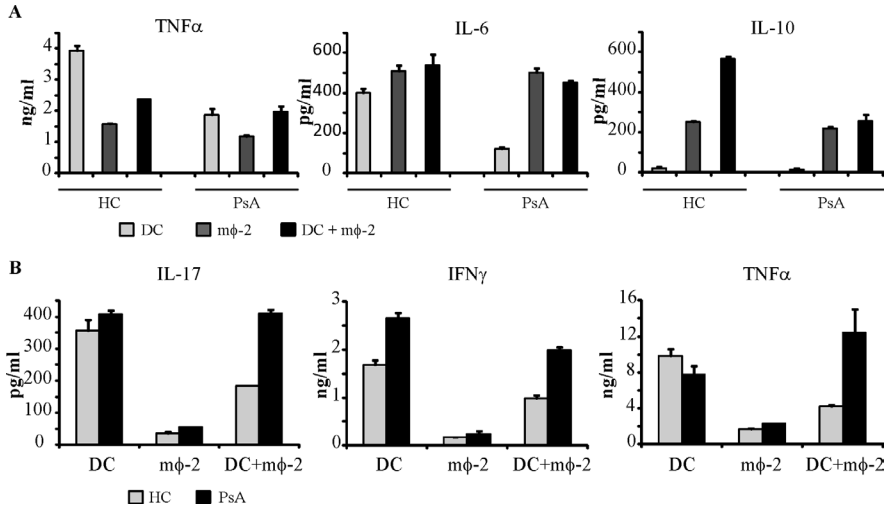

Figure 3

PsA mϕ-2 are less efficient suppressors of DC mediated inflammatory responses. DCs and mϕ-2 from a healthy control (HC) and a PsA patient were stimulated separately ($5x10^4$ mϕ-2 or DCs/well in a total volume of 200 μl) or together ($5x10^4$ mϕ-2 and $5x10^4$ DCs/well in a total volume of 200 μl) with heat-killed *M. tuberculosis*. Released levels of TNFα, IL-6 and IL-10 in the supernatants were measured after 24 hours (**A**). Bars are mean from duplicate wells and SD. Data are representative for 3 independent experiments with in total 3 healthy controls and 3 PsA patients. (**B**) Allogenic $CD4^+$ T cells from a healthy control were cultured during five days in the presence of DCs, mϕ-2 or DCs together with mϕ-2 from a healthy controls or a PsA patient, as a stimulus lipoarrabinomannan (a cell wall constituent of all mycobacteria) was added to the wells. After 5 days of culture the cells were activated with PMA and Ionomycin to induce cytokine production. After 12 hours the supernatants were collected and measured for their content of IL-17, IFNγ and TNFα. Bars are mean from duplicate wells and SD. Data are representative for 3 independent experiments with in total 3 healthy controls and 3 PsA patients.

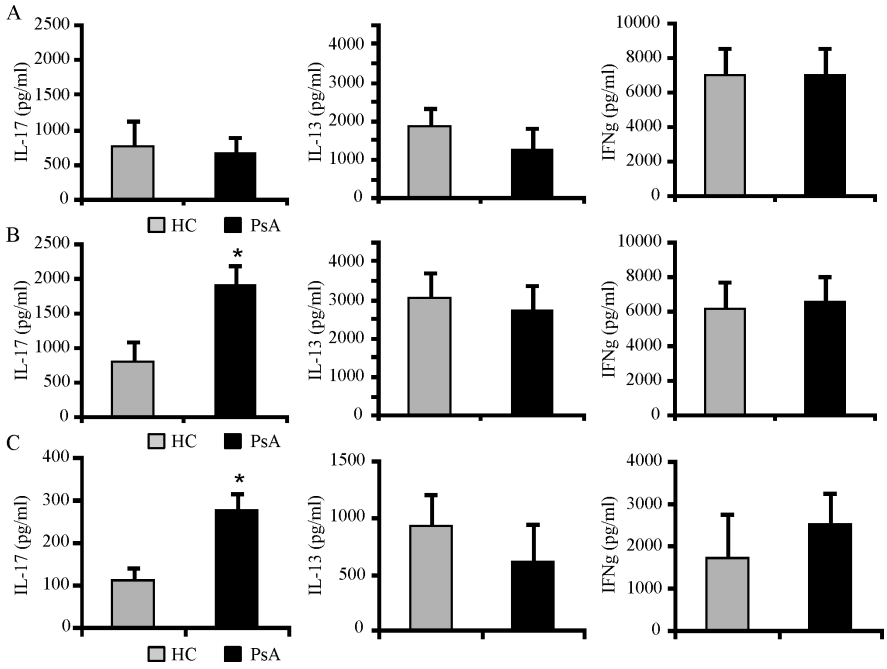

Figure 4

Naive $CD4^+$ T cells from PsA patients have an increased tendency to polarize into IL-17 producing T cells. T-cell experiments were performed in which unstimulated (A) or *M. tuberculosis* stimulated DCs (B) from healthy controls or CD3/CD28 expander beads in medium from *M. tuberculosis* stimulated healthy control DCs (C) were co-cultured with allogeneic $CD4^+CD45RA^+$ T cells from healthy controls (HC, n=6) or PsA patients (n=7) for five days. Before the collection of supernatants at day six of culture stimulation took place with PMA and Ionomycin for 12 hours. Levels of IL-17, IL-13 and IFNγ were measured in the supernatants. Bars are mean and SEM. * = $p<0.05$.

Conclusion

The inflamed synovium and skin from PsA patients is characterized by a marked infiltration with $CD163^+$ macrophages. CD163 is a marker for type 2 macrophages (mϕ-2) which are highly phagocytic and have been implicated in the suppression of tissue inflammation due to preferential IL-10 secretion and suppressive effects on the polarization of Th1 cells (8,9). The function of $CD163^+$ macrophages in psoriatic arthritis is not fully understood. However, several observations pinpointed $CD163^+$ macrophages as potential key players in PsA. For instance, PsA disease

severity correlated significantly with the infiltration of $CD163^+$ macrophages (10) and $CD163^+$ macrophages were highly increased in psoriatic lesional skin but returned to normal levels after anti-TNFα treatment (12).

Here we demonstrate that $CD163^+$ type 2 macrophages from PsA patients have an markedly aberrant phenotype and function which suggests their direct implication in PsA for several reasons which will be discussed below. First, a higher CCR5 expression was clearly present on PsA $CD163^+$ type 2 macrophages. CCR5 expression has been linked to various pathological processes. $CCR5^{-/-}$ mice, for example, demonstrated an increased activation of alternatively activated macrophages, a stronger Th2 response, decreased inflammation and longer allograft survival in a transplant model (26). Moreover, in bleomycin induced pulmonary fibrosis CCR5 deficiency led to a decrease in fibrotic changes induced by bone-marrow derived macrophages and fibrocytes (27). In this light it is interesting to note that psoriasis is associated with concomitant fibrotic diseases of the liver and the lungs (28). Fibrotic changes along the sacroiliac joints and spine are also common in PsA. The increased expression of CCR5 on monocytes and macrophages in PsA might thus be involved in these pathological changes. The increased expression of CCR5 on $CD163^+$ macrophages in PsA is in line with the increased presence of $CD163^+$ macrophages in the synovium of PsA patients since these cells were the only monocyte-derived cell type expressing CCR5 at substantial levels and the ligands for CCR5 are clearly present in the synovial cavity in PsA {Fiocco, 2010 407 /id}. A clue to why PsA patients express elevated levels of CCR5 might be the finding that CCR5 is elevated on monocyte-derived and alveolar macrophages during *M. tuberculosis* infection (29) and that antigens from *M. avium complex* up regulate the expression of CCR5 on monocytes. This is in line with our previous report demonstrating a dysfunctional response by PsA DCs towards mycobacteria possibly facilitating chronic inflammation due to a decreased removal of these pathogens (Wenink et al, submitted).

Second, mϕ-2 from PsA patients have an altered response to specific TLR ligands. Here we show that the production of IL-6 is higher in PsA mϕ-2 stimulated with *E. coli*. In PsA Th17 have been implicated as an important mediator of disease (30). In this context the increased production of IL-6 might be of importance by facilitating the development of Th17 since IL-6 has been shown to be crucial in the development of this pathogenic T cell subset (31). Besides, we demonstrated that DCs and mϕ-2 from PsA patients were less efficient in the uptake of *E. coli* compared to those from healthy individuals. This is in line with that observed in patients with Crohn's disease were heat-killed *E. coli* is less efficiently cleared

when injected intradermally possibly leading to increased inflammatory responses (14). Deficient clearing of *E. coli* might thus also occur in PsA due to hampered phagocytosis by macrophages and DCs. Subsequently, this series of events might lead to a self-perpetuating loop were persistence of microorganisms lead to chronic activation of immune cells and might relate to the large proportion of PsA patients who have (subclinical) gut inflammation. Finally, PsA macrophages are shown to have an impaired anti-inflammatory response. This might explain why despite the prominent presence of "anti-inflammatory" mϕ-2 in inflamed tissues the inflammation ensues in PsA. PsA mϕ-2 lack the capability to negatively influence pro-inflammatory DC functions while their cytokine production towards most ligands and mycobacteria is intact. This, in combination with the proneness of PsA naive T cells to polarize into IL-17 producing T cells, explains the high occurrence of Th1/Th17 in PsA and the chronic inflammatory responses evoked.
Altogether, these results support a hypothetical framework in which a dysfunctional innate immune response towards certain (myco)bacteria by resident myeloid DCs leads to the recruitment of type 2 macrophages which, under normal circumstances, would dampen the immune response. However due to a dysfunctional anti-inflammatory capacity and possibly the persistence of the (intracellular) bacterium the immune response persists, resulting in chronic inflammation enhanced by the influx of inflammatory Th subsets. Our observations provide important clues in the understanding of the pathogenesis of PsA. Further research focussed on the precise mechanisms underlying the aberrant response of antigen presenting cells in this condition is highly justified since it is likely to open novel avenues towards a patient tailored therapy approach.

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10 Final considerations and future perspectives

Final considerations

Rheumatoid and Psoriatic Arthritis are two diseases frequently seen in the clinic of a Rheumatology department. They are both diseases of the immune system and share common features like the inflammation of the small diarthrodial joints of the hands. While the occurrence of autoantibodies and bone destruction are high in RA, in PsA they are rarely seen. This underscores the distinct pathological mechanism underlying both diseases. While RA is thought of as a classic auto-immune disease characterized by autoreactive T cells, PsA appears to have more in common with autoinflammatory or dysfunctional barrier diseases such as Crohn's disease. Recent reports, however, have demonstrated that around 15% of PsA patients have antibodies against citrullinated proteins, the hallmark autoantibodies of RA. Both diseases have in common that their pathological processes are characterized by a massive influx of immune cells such as T cells, macrophages, DCs, B cells and neutrophils. These immune cells form a complex network which appears to underlie the disease process. Over the last decades the pathways leading to the instigation and perpetuation of both diseases have been subject of investigation.

In recent years the role played by TLRs in the initiation and/or perpetuation of both diseases has become apparent from both animal and human studies. TLRs are expressed by a wide range of immune and resident tissue cells. Upon TLR activation these cells start producing high amounts of cytokines, chemokines and other inflammatory mediators. In addition TLR activation of DCs leads to their differentiation from cells focused on sampling their environment and maintaining tolerance to cells capable of inducing adaptive immune responses. These important roles played by TLRs puts them in the spotlight as drivers of immune responses and possibly as agents causing autoimmunity and auto(inflammation). Proinflammatory cytokines such as IL-6 and TNFα have been shown to be crucial in the pathogenesis of synovitis. The neutralization of both cytokines has been proven to be efficacious in the treatment of RA and PsA. The inhibition of TLR4 and TLR8 has been shown to decrease the TNFα production in RA synovium *ex vivo* implicating their role in the maintenance of the inflammatory milieu. Another pathway that leads to the production of proinflammatory cytokines in RA synovium is the contact between activated synovial T cells and macrophages, demonstrating the intricate network of immune cells perpetuating the disease process.

Although the neutralization of TNFα or IL-6 is an effective treatment for RA it lacks both specificity and efficiency. The lack of specificity is due to the neutralization

of the cytokine targeted throughout the body. This leads to an increased risk of serious infections such as the well-known increased risk of the resurgence of a latent *M tuberculosis* infection. The lack in efficiency is apparent from the still small amount of patients that reaches true remission. The cause for this lacking efficiency might lie in the redundancy of many cytokines produced during immune activation or more, the role played by TNFα is likely taken over by other cytokines such as IL-1 and IL-6. For this reason studies have been done in which combinations of different antibodies were used. This led however, to an unacceptable increase in the occurrence of infections. Due to these restrictions it appears worthwhile to investigate the regulation of the production of these harmful inflammatory mediators. By understanding how the immune system gets activated and which pathways might be used to selectively undermine the ongoing immune response we aimed to get leads on novel pathways which might be used to treat diseases, such as RA, more targeted, leading to less side effects and possibly greater therapeutic efficacy.

Fc gamma Receptor IIb and Toll-Like Receptors

Our quest began with the question why some RA patients in time are able to discontinue their medication use, while the large majority of patients has to use potent immunosuppressive drugs for life. We observed that monocyte-derived DCs from patients capable of dampening their disease themselves, resulting in a very low disease activity without the help of medication, had a very high expression of the inhibitory FcγRIIb on their cell surface (**Figure 1**).

These patients had a clear diagnosis at their time of entry in our outpatient clinic, some with a very high disease activity and already the presence of bony erosions. In time however their disease activity waned and they were taken of their medication. Since we had found that there were elevated levels of endogenous TLR4 ligands in serum and synovial fluid of RA patients and DCs from RA patients reacted more potently towards certain TLR4 ligands we wondered whether FcγRIIb might be able to interfere in the TLR4-mediated activation of DCs. Activation of DCs from RA patients who had successfully discontinued their medication use and thus had a high expression of the inhibitory FcγRIIb, with immune complexes significantly reduced the release of TNFα or IL-12p70 upon TLR4 activation. This inhibitory function of immune complexes was lost when FcγRIIb was blocked with specific antibodies demonstrating that FcγRIIb was responsible for the dampening effect of the immune complexes. This inhibition of cytokine production was shown to depend on the activation of the PI3K-Akt pathway by FcγRIIb. The inhibition of these intracellular molecules would increase the cytokine production when TLR4 was activated and it blocked the inhibitory

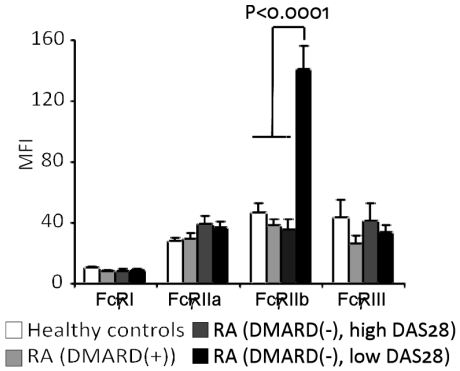

Figure 1

Highly increased expression of FcγRIIb on DCs from RA patients who were able to succesfully discontinue the use of immunosuppressives (RA (DMARD(-), low DAS28)). MFI = mean immune fluorescence as measured by flowcytometry.

effect of FcγRIIb activation. We proposed a model by which FcγRIIb is able to dampen TLR4 responses. In this model the activation of a large number of FcγRIIb molecules draws a large part of the intracellular SHIP molecules to the cell surface away from the intracellular parts where it counteracts the effects of PI3K-Akt on TLR4. This leads to an increased inhibitory effect of phosphorylated Akt on the TLR4 pathways leading to a decreased proinflammatory cytokine production (**Figure 2**). This model was supported by Western Blot data demonstrating that activation of high numbers of FcγRIIb and TLR4 led to a highly increased phosphorylation of SHIP and a decreased degradation of IκBα while the phosphorylation of Akt was also increased.

In addition we demonstrated that the activation of FcγRIIb on DCs has profound effects on the instruction of T cell responses by the DCs. FcγRIIb activation led to DCs capable of inducing strong Th2 responses and it would even restore the ability of TLR4 activated DCs to induce regulatory T cells to the same level as immature DCs. Both Th2 and regulatory T cells have been shown to be important in the restoration of immune homeostasis during Th1 and/or Th17 mediated inflammation. Interestingly, recently it was described that immune complexes containing citrullinated fibrinogen, present in seventy percent of ACPA-positive RA patients, would induce the vast production of TNFα by macrophages via the dual engagement of TLR4 and FcγRs [1]. Overexpression of FcγRIIb thus seems a likely candidate to counteract the proinflammatory effect of these immune complexes on macrophages and DCs.

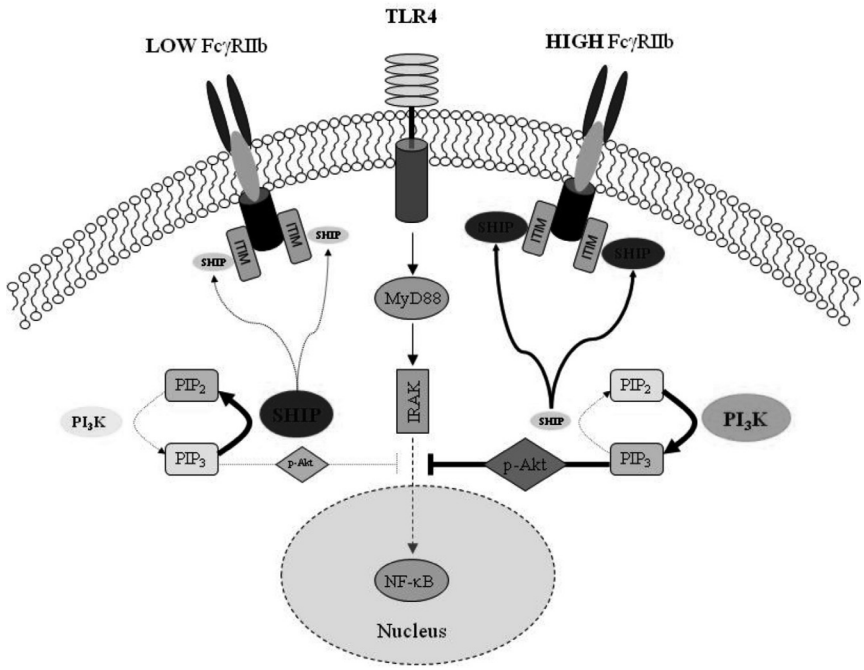

Figure 2

Hypothetical model explaining the inhibition of TLR4 signalling upon the activation of FcγRIIb. The activation of FcγRIIb translocates SHIP molecules away from the cytoplasm towards the cell membrane. This drains the intracellular compartment of SHIP where it no longer counteracts the effects of PI3K on the processing of PIP2 into PIP3. This leads to an increase in phosphorylated Akt subsequently inhibiting the TLR4 signaling pathway resulting in a decrease in IκBα degradation and a decrease in proinflammatory cytokine production.

Next we aimed to determine whether the dampening effect of FcγRIIb was specific for TLR4. However, since RA patients capable of successfully discontinuing their medication use are rare we sought a cell type which would express FcγRIIb readily at high levels. Macrophages have been shown to express many FcγRs. As previously described under the influence of various factors such as M-CSF and GM-CSF monocytes can differentiate into different subsets of macrophages ranging from anti-inflammatory type 2 macrophages to proinflammatory type 1 macrophages. GM-CSF is produced heavily at sites of inflammation, such as the arthritic joint, upon the activation of stromal and immune cells with endogenous damage associated or exogenous pathogen associated molecular patterns. In addition, GM-CSF induces a proinflammatory state in many cells, especially

monocytes/macrophages leading to the differentiation of type 1 macrophages capable of producing copious amounts of proinflammatory cytokines. Considering these characteristics we hypothesized that there should be mechanisms at work aimed at dampening the proinflammatory effect of GM-CSF to prevent chronic inflammation. In contrast to type 2 macrophages (and DCs) from healthy controls, type 1 macrophages indeed expressed very high levels of FcγRIIb. Activation of FcγRIIb on type 1 macrophages did not only inhibit TLR4-mediated cytokine release but also TLR2- and TLR7/8-mediated cytokine production but not the cytokine production induced by Dectin-1. In addition, FcγRIIb was found to inhibit the release of TNFα by type 1 macrophages induced upon contact with cytokine stimulated T cells further emphasizing the role played by FcγRIIb in suppressing inflammation. This shows that FcγRIIb has a broad but specific inhibitory effect on various extracellular and intracellular receptors expressed by (proinflammatory) monocyte-derived macrophages.

Mac-1 and type 1 macrophages

In addition to our portrayal of the inhibitory function of FcγRIIb, many pathways/factors have been described capable of dampening inflammation, such as IL-10 and the intracellular expression of TLR pathway inhibitors (eg SOCS proteins, A20, SIGIRR etc). However, how these factors are regulated in monocytes/macrophages/DCs prior to their initial exposure to inflammatory mediators and their release of (proinflammatory) cytokines is largely unknown. We demonstrated that the accumulation of monocyte-derived cells already has a grave impact on their differentiation. Type 1 macrophages differentiated at high concentrations produced significantly higher levels of IL-10 while they produced far lower levels of the proinflammatory cytokine TNFα. This anti-inflammatory phenotype seems in part due to the up regulation of intracellular TLR pathway inhibitors, especially SOCS3. These data appear to point to an in vivo mechanism in which the accumulation of sufficient amounts of macrophages at a site of inflammation induces an anti-inflammatory phenotype in the infiltrating macrophages aimed at dampening the immune response. Not only type 1 macrophages were subject to this regulatory mechanism but also DCs, leading to a potently decreased production of IL-12p70 and a decreased ability to support T cell responses. This negative feedback system depended on cell-cell contact via the β2-integrin Mac-1, signaling via p38 and the activation of COX2 resulting in the production of prostaglandins. In line with these results it was recently demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit COX2 increased the spontaneous release of TNFα while decreasing the release of IL-10 [2]. This strengthens the notion that the mechanism we described is functioning *in vivo*

and regulates macrophage behavior under inflammatory conditions. TGFβ, present in large amounts in many chronic inflammatory diseases , was capable of invalidating this shift towards an anti-inflammatory macrophage phenotype. The identification of this new inhibitory feedback mechanism and the inhibitory effect TGFβ has on this might lead to new therapeutics or the reconsideration of old ones (COX2 inhibition with NSAIDs) for chronic inflammatory diseases.

TLR2 and type 1 Interferons

Type 1 interferons (IFN), and especially IFNα, have been linked to many human diseases including PsA and RA. In PsA the accumulation of plasmacytoid DCs, the main producers of IFNα, has been shown to precede the occurrence of Psoriatic plaques and the application of a TLR7/8 agonist to psoriatic skin potently exacerbated the disease. In RA it was demonstrated that IFNα is expressed in synovium and its expression correlated with the expression of certain TLRs and proinflammatory cytokines. Another clue to the importance of type 1 IFN in the pathogenesis of RA is the finding that a significant proportion of RA patients displays a type 1 IFN signature in their mononuclear cells from peripheral blood and that serum of RA patients activates plasmacytoid DCs to produce IFNα [3]. IFNα is able to prime other cells of the immune system. Importantly exposure of myeloid DCs to IFNα increases their reactivity to TLR ligands. Activation of myeloid DCs with type 1 IFN and LPS, for example, led to a synergistic release of IL-12p70. Recent advances in the literature have demonstrated that IL-12p70, next to the induction of Th1 cells, is important in the induction of follicular T helper cells (Tfh cells). Tfh cells help the development of antibody responses by B cells by the production of IL-21 and inducible costimulatory (ICOS). Tfh cells appear to play an important role in the development of many autoimmune diseases by facilitating the production of pathogenic autoantibodies and the formation of germinal centers [4;5]. Type 1 IFN might thus underlie autoimmunity in RA by supporting the production of IL-12p70. In this light our finding that TLR2 is capable of potently inhibiting the production of IL-12p70 by abrogating the type 1 IFN loop sheds a new light on the role played by TLR2 in autoimmunity.

TLR2 was demonstrated to be a potent inhibitor of TLR4 and TLR7/8 induced cytokine production by monocyte-derived DCs. TLR4 and TLR8 have both been demonstrated to play a role in the “spontaneous” cytokine production by RA synovial tissue. Alike for FcγRIIb the inhibition by TLR2 of TLR4 mediated TNFα release was prevented by inhibiting PI3K. However, whereas the inhibitory effect of FcγRIIb on the production of IL-12p70 was also mediated by PI3K, this was not the case for TLR2. TLR2 appeared to inhibit the production of IL-12p70 by up

regulating the transcription of SOCS1 (**Figure 3**). The production of IL-12p70 depends on the type 1 interferon (IFN) amplification loop in which an initial small production of interferon leads to the additional transcription of interferon genes and IL-12p35. Interferon Regulatory Factors (IRF) 1, 7 and 8 have been demonstrated to be intricately involved in this process leading to IL-12p70 production and Th1 responses. Co-activation of TLR2 with TLR4 and TLR7/8 led to a DC phenotype capable of inducing increased Th2 and Th17 responses while slightly decreasing the presence of Th1 cells compared to DCs stimulated with TLR4 and TLR7/8 alone. It would be interesting to determine whether the presence of follicular helper T cells decreases when DCs are co-incubated with TLR2 ligands.

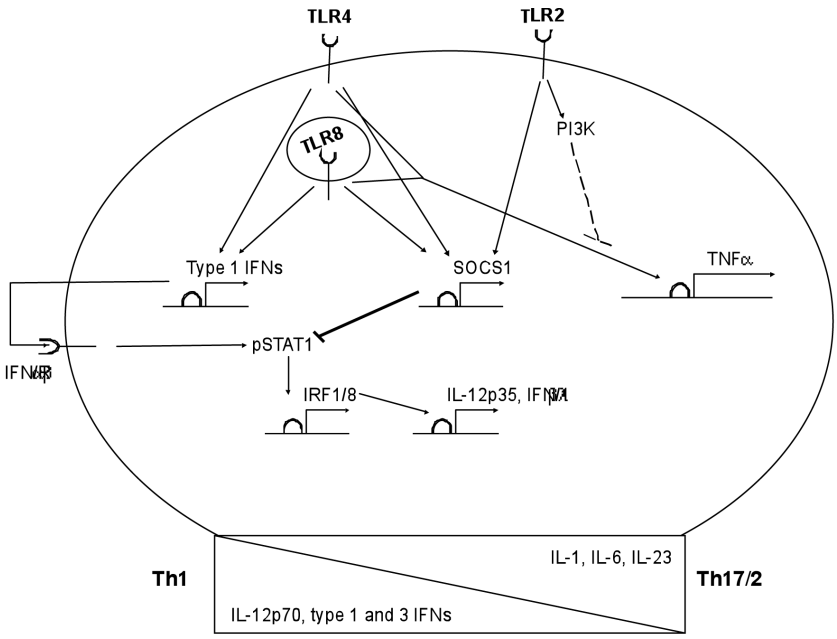

Figure 3

Diagram representing the pathways TLR2 exploits to inhibit TLR4 and TLR7/8 mediated cytokine production. Activation of TLR2 inhibits the release of cytokines induced by TLR4 or 7/8 stimulation via two pathways. The production of TNFα is reduced via the activation of the PI3K pathway while the release of IL-12p70 is hampered by interference in the interferon-feedback loop probably at least in part due to the up regulation of SOCS1. A molecule known to inhibit STAT-1 phosphorylation. TLR2 co-activation with TLR4 and 7/8 leads to the inability of the DCs to produce copious amounts of IL-12p70. This has important positive effects on the ability of the DCs to skew T cell diffferentiation away from Th1 and towards Th2 and Th17 responses.

These data demonstrate that TLR2 activation on DCs has pronounced effects on their cytokine production when other TLRs are simultaneously triggered, something that might occur in inflammatory conditions such as RA, and on their preferential Th cell induction. TLR2 might be a useful target in many autoimmune diseases. However due to its dual role in the immune response, activation of TLR2 leads to the production of large amounts of proinflammatory cytokines by certain cell types such as type 1 macrophages, direct stimulation of TLR2 seems ill-advised.

Abatacept and activated T cells

The intricate network of cytokines present in arthritis joints is essential to the disease processes of RA and PsA. Different pathways have been proposed by which the production of cytokines by DCs and macrophages might be induced. The induction via TLRs has been discussed extensively in this thesis. Another stimulus important for the activation of macrophages in RA appears to be the direct activation via activated T cells. Monocytes/macrophages were demonstrated to readily produce TNFα when exposed to activated synovial T cells. This was shown to depend on cell-cell contact of memory $CD4^{+}CD45RO^{+}$ T cells and monocytes/macrophages via CD69, CD18 and CD49d and was independent of the production of soluble mediators by the T cells. $CD4^{+}CD45RO^{+}$ T cells are the main infiltrating cells in the pannus underlining their pivotal role in the pathogenesis of RA, possibly via the activation of other immune cells such as macrophages. Cytokine stimulated T cells (Tck) display the same characteristics as activated synovial T cells. Just like Tck, synovial T cells induced the release of TNFα but not Il-10 by monocytes, thereby possibly creating an unbalanced proinflammatory cytokine environment likely setting the stage for chronic inflammatory responses. Abatacept (Orencia) is a fusion protein composed of an immunoglobulin Fc part and the extracellular domain of CTLA-4. Abatacept is a selective costimulation inhibitor as it blocks costimulation via CD80/86 of T cells by DCs. The mode of action of Abatacept is thought to depend on the inhibition of the full activation of T cells. We have demonstrated a new mechanism by which Abatacept might be able to dampen inflammation. *In vitro* Abatacept prevented the activation of type 1 macrophages by Tck implying that *in vivo* Abatacept might block the activation of synovial macrophages by activated synovial T cells resulting in an inhibition of the local production of TNFα. Thereby Abatacept might, in addition to its effect on the T cell compartment, undermine the proinflammatory cytokine network dampening the chronic inflammatory response. In addition we demonstrated that TLR activation and Tck exposure synergistically induce the production of especially IL-12p70 by type 1 macrophages which was dampened by the presence of Abatacept.

Psoriatic Arthritis and (myco)bacteria

Bacteria have long been thought to play a role in many (auto)inflammatory and autoimmune diseases. Proposed roles for bacteria range from molecular mimicry, in which a component from bacteria is thought to mimick a self peptide leading to an autoimmune response, to the deposition of bacterial constituents in synovium, thought to lead to pattern recognition receptor activation and the initiation/ perpetuation of joint inflammation. From animal models it is clear that the gut flora has a profound impact on the immune system and especially on T cell differentiation. IL-1Ra$^{-/-}$ mice housed under germ-free conditions do not develop arthritis and upon stimulation splenocytes from these mice produce hardly any IL-17. Recently it was demonstrated that a restricted number of bacteria, prototypically the nonculturable *segmented filamentous bacterium*, were crucial in the induction and maintenance of intestinal immune responses, such as the differentiation of Th1 and Th17 [6]. The fact that a selective type of bacteria has the prerogative to shape the immune system implies that a dysfunctional handling of certain bacteria might lead to a thwarted immune system possibly leading to immune diseases. Crohn's disease patients, for example, have been shown to have an aberrant immune reaction towards intradermally injected heat-killed *Escherichia coli* leading to the delayed removal of these pathogens. Eventually this impaired removal of pathogens might lead to chronic inflammation.

Mycobacteria have been suspected of playing a role in Crohn's disease as well as psoriatic disease [7]. Recently, the presence of *M avium subspecies paratuberculosis* reactive T cells with a Th1 or Th1/Th17 phenotype strengthened a possible role of mycobacteria in the inflammation seen in Chron's disease [8]. In addition, it was demonstrated that *M avium paratuberculosis* actively invades the human gut epithelial goblet cells of the small intestine, inducing severe tissue damage and inflammation [9]. In psoriatic disease high levels of antibodies against 65-kDa and 48/45 doublet antigens from mycobacteria have been found and these correlated with disease severity. Significantly more psoriasis patients showed "false" positive tuberculin skin test results than other dermatology patients and the tuberculin skin test correlated with the psoriasis area and severity index score (PASI). In addition, in placebo-controlled studies, immunotherapy with *Mycobacterium vaccae* for psoriasis and psoriatic arthritis resulted in significant reductions of important disease parameters [10].

The hypothesis that certain (myco)bacteria might play a role in PsA was strengthened by our finding that DCs from PsA patients with an active disease have a severely impaired innate immune reaction towards mycobacteria **(Figure 4)**.

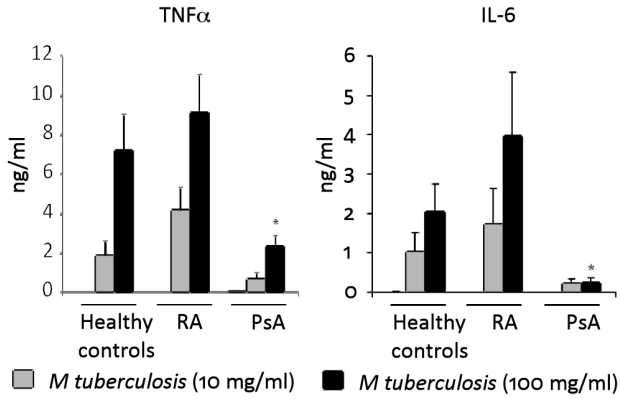

Figure 4

Clearly reduced cytokine production by PsA DCs upon stimulation with M tuberculosis in comparison to healthy controls or RA patients. * $p<0.01$ compared to healthy control or RA patients.

The cytokine production by DCs upon exposure to other bacteria such as *E coli*, *Klebsiella pneumonia* and *Streptococcus pyogenes* was not impaired, highlighting the selective nature of the weakened immune response. This striking inability of PsA DCs to respond to mycobacteria was found in two independent cohorts of PsA patients, at the Radboud University Medical Centre Nijmegen and at the Western Infirmary Glasgow.

The impaired cytokine production upon exposure to mycobacteria was positively correlated with the cytokine production upon exposure to TLR2, TLR7/8 and NOD2 ligands but was not dependent on their expression. The selective up regulation of SOCS3 and A20 mRNA found in DCs from active PsA patients might be an explanation for the dampened cytokine production. Further support for the role of certain intracellular bacteria in PsA was found in the fact that molecules heavily involved in the defence against intracellular pathogens (NOX2 and LL37) were highly up regulated in DCs from PsA patients. In mice infected with *M tuberculosis* these molecules were also found up regulated in monocytes and monocyte-derived macrophages. These data prompted us to hypothesize that an initial decreased response by DCs towards certain (myco)bacteria would lead to an insufficient removal of these bacteria and the initiation of an inflammatory response. However, why this inflammatory response derails and becomes chronic is another question. We demonstrated that $CD163^{+}$ type 2 macrophages from PsA patients have an elevated expression of CCR5. $CCR5^{-/-}$ mice demonstrate

an increased activation of alternatively activated macrophages, decreased inflammation, longer allograft survival and less pulmonary fibrosis in allograft survival and fibrosis models [11;12]. Psoriatic disease is characterized by an increase in the presence of fibrotic diseases of the liver and the lungs. Fibrotic changes along the sacroiliac joints and spine are also common in PsA. The increased expression of CCR5 on type 2 macrophages in PsA might be involved in these pathological changes. This is in line with the increased presence of $CD163^+$ macrophages in the synovium of PsA patients since these cells were the only monocyte-derived cell type expressing CCR5 at considerable levels. A clue to why PsA patients express elevated levels of CCR5 might be the finding that CCR5 is elevated on monocyte-derived and alveolar macrophages during *M tuberculosis* infection and that antigens from *M avium complex* up regulate the expression of CCR5 on monocytes. In addition to the preferential recruitment of $CCR5^+$ $CD163^+$ macrophages in PsA we have demonstrated that these macrophages have an impaired anti-inflammatory function while their cytokine production is unhampered. While type 2 macrophages from healthy controls were capable of suppressing proinflammatory cytokine production and T cell polarization by DCs, type 2 macrophages from active PsA patients did not have these suppressive capacities. These results support a hypothetical framework in which a dysfunctional innate immune response towards certain (myco)bacteria by resident myeloid DCs leads to the hampered removal of these pathogens and increased inflammatory responses. The subsequent preferential recruitment of type 2 macrophages would, under normal circumstances, dampen the immune response. However, due to a dysfunctional anti-inflammatory capacity and possibly the persistence of the (intracellular) bacterium the immune response persists resulting in chronic inflammation.

Conclusion

This thesis encompasses new insights into a range of immunological processes and in the pathogenesis and possible resolution of RA and PsA. The main hypothetical framework these studies were based on, was the idea that Toll-like receptors play an intricate role in many inflammatory diseases. FcγRIIb and TLR2 activation was shown to dampen TLR4 and 7/8 responses in DCs and type 1 macrophages, significantly down regulating their production of proinflammatory cytokines. Both FcγRIIb and TLR2 would shift DC induced T cell responses towards Th2 responses even in the presence of strong Th1 inducers like TLR4 and TLR7/8 ligands. Th2 responses have been shown to decrease the inflammatory responses in both RA and PsA. Another regulatory mechanism utilized by the

immune system in order to prevent chronic inflammation is the inhibitory effect of the accumulation of high levels of macrophages and DCs at a site of inflammation. Accumulation of cells led to a potently decreased ability of DCs and macrophages to produce proinflammatory cytokines while the production of IL-10 and VEGF were increased. This mechanism was dependent on the β2 integrin Mac-1 and the production of prostaglandins. In addition the ability of DCs to support autologous T cell responses was strongly reduced. Along with TLR ligands inflammatory T cells have been demonstrated to be potent inducers of TNFα production by macrophages in RA. Abatacept was capable of blocking this proinflammatory cross-talk leading to a marked reduction in TNFα production elucidating another mode of action for this effective drug. While RA is thought to be dependent on an overactive immune system we demonstrated that PsA might, at least in part, depend on an insufficient immune response towards (myco)bacteria by DCs. The aberrant immune reaction leading to PsA seemed to be supported by type 2 macrophages lacking their characteristic anti-inflammatory behaviour. However, much more research is needed to reach the full potential of the mechanisms and novel findings described in this thesis.

Future perspectives

DCs regulate both central and peripheral tolerance and have successfully been used in treating animal models of autoimmune diseases. The central role played by DCs in maintaining tolerance has been supported by the finding that constitutive ablation of DCs results in spontaneous fatal autoimmunity [13]. In contrast, during the onset of autoimmunity DCs are thought to be critical for priming of self-reactive T cells, underscoring their central role in immunity and tolerance. The role played by myeloid DCs in RA has become increasingly clear over the last decade. With animal models demonstrating that DCs are crucial in the breach of self tolerance in an arthritis model [14] to the demonstration in this thesis that DCs from RA patients capable of successfully discontinuing treatment have a phenotype aimed at restoring immune balance [15], a central and regulating role for DCs in RA seems apparent. Dysfunctional DCs in addition seem to play a role in the pathogenesis of PsA as we have demonstrated that DCs from PsA patients are incapable of reacting appropriately to mycobacterial insults. This might lead to a lagged immune response and consequently an increased persistence of pathogens leading to chronic inflammation. It appears that for both RA and PsA the statement of Steinman and Banchereau "*DCs are an early player in disease development and an unavoidable target in the design of treatments*" [16] is appropriate.

A novel perspective of looking at disease and trying to find new treatment modalities and understanding is to investigate the immune system of those unique RA patients who reach remission and are no longer in need of medication. The elevated expression of FcγRIIb on DCs from these patients already appears to be a valid target. Increasing the expression of FcγRIIb on DCs from RA patients might lead to the decreased release of proinflammatory cytokines and a shift in T cell responses towards Th2 and regulatory T cell responses thereby restoring immune homeostasis. To be able to do this we first need to investigate how FcγRIIb expression is regulated in general and in these "disease-free" RA patients. Therefore we need to further elucidate on the DC phenotype of these patients, among others by gene expression profiling and by investigating the role played by epigenetics in these patients. Further research is ongoing in our lab aimed at delineating the factors important in the up regulation of FcγRIIb on DCs. This might result in our ability to exploit this novel but internal regulatory mechanism used by the immune system itself. Thereby we might be able to dampen inflammation in a more natural manner via various routes (dampening proinflammatory cytokine production, shifting T cell responses to Th2, increasing regulatory T cell numbers) and possibly more specifically aimed at the deranged immune response and thus with less side effects. Preliminary data demonstrate that, like the induction of an anti-inflammatory type 1 macrophage phenotype, the expression of FcγRIIb is increased upon homotypic cell-cell contact. How this up regulation is mediated is currently under investigation. A possible mechanism might be the transfer of microparticles loaded with FcγRIIb from one cell to the other, a mechanism with possible therapeutic implications.

However we should not forget to investigate other immune-inhibitory pathways in these specific RA patients. Potential other targets are regulatory T cells, plasmacytoid DCs and macrophages. These cell types have been described to have potent immune modulating potential. Investigation could be aimed at the analysis of the phenotype and anti-inflammatory potential of these cell types in these patients. Since monocyte-derived DCs from these patient express elevated levels of FcγRIIb it would be interesting to see whether macrophages from these patient also have an increased expression of FcγRIIB with functional consequences. Another interesting question is whether RA patients who have a low disease activity and who might be taken of their medication successfully already have a tendency to express elevated levels of FcγRIIb. Answering this will unravel whether this might be a method to screen patients who will be able to handle their disease themselves and might thus be taken of medication with confidence. However, it might demonstrate that the use of medication suppresses the organisms own

tendency to up regulate FcγRIIb in order to dampen inflammation, which would underscore the potential therapeutic effect of up regulating FcγRIIb artificially.

Another immune inhibitory pathway laid bare in this thesis which might be exploited for the treatment of RA is the Mac-1 mediated anti-inflammatory DC and macrophage phenotype. Homotypic cell-cell contact was demonstrated to induce an anti-inflammatory cell phenotype in otherwise pro-inflammatory macrophages and DCs. This was mediated via a signalling cascade involving MAPK p38, COX2 and SOCS proteins leading to a suppressed TNFα production and a highly increased release of IL-10 upon TLR stimulation. Since TLR activation of DCs and macrophages appears to play an important role in the pathogenesis of RA exploitation of this pathway might potentially be beneficial. This appears in contrast to the wide-spread use of COX2 inhibiting NSAIDs in RA. However, the ability of NSAIDs to ameliorate pain and tenderness does not prevent disease progression in rheumatoid arthritis. Even more, in line with our results, it was established that the addition of COX2 inhibitor to RA synovial tissue increased the release of TNFα while the production of IL-10 was suppressed [2]. This in combination with the notion that TLR8 inhibition potently reduced the spontaneous release of TNF from synovial tissue warrants the further investigation of our proposed feedback mechanism [17]. Thus instead of inhibiting the COX2 pathway it might be of interest to activate Mac-1 e.g. via agonistic antibodies, stimulate the production of COX2 dependent prostaglandins or to induce the expression of SOCS proteins via, for example, small molecule agonists. These small molecule agonists might be used enveloped in liposomes thereby mainly targeting monocytes and monocyte-derived cells. However, since PGE2 has been implicated in the induction of Th17 responses [18] and Th17 have been described to mediate osteoclast activation-formation [19] caution is warranted.

Another major inhibitory pathway we have described is the inhibitory effect of activating TLR2. However due to its dual role in the immune response, activation of TLR2 leads to the production of large amounts of proinflammatory cytokines by certain cell types such as type 1 macrophages, direct stimulation of TLR2 seems ill-advised. A possible way to go about is by learning which pathways TLR2 uses to exert its beneficial effects and utilize these. We have made a start by demonstrating that SOCS1 is specifically up regulated by TLR2 co-activation. The artificial up regulation of SOCS1 might tune the immune response towards a Th2 type response and away from the deleterious Th1 response characteristic of RA. In addition it might prevent the production of autoantibodies by decreasing the presence of IL-12p70 dependent follicular T cells thereby abrogating B cell

maturation and memory. Modulation of TLR responses instead of completely blocking them might even be more effective due to a reversal of the immune reaction away from Th1 towards Th2. RA appears to be a mainly Th1 driven disease and atopic patients with RA have a marked less active disease. In addition, DCs from RA patients capable of halting their use of immunosuppressive drugs had an highly increased capability of inducing Th2 cells and IL-4 has shown potent effects in suppressing inflammation in mouse models. Further elucidation of the inhibitory effect of TLR2 on DCs might result in targeted therapies aimed at inducing DCs capable of subverting Th1 responses in RA into Th2 or regulatory T cell responses thereby dampening inflammation.

For PsA, other treatment modalities appear to be warranted. The apparent dysfunction of DCs in producing the necessary amounts of proinflammatory cytokines as well as the presence of large amounts of bacterial products in the blood and joints of patients with psoriatic disease underscores the defective function of the immune system as a major component of disease pathogenesis in PsA [20;21]. Although the neutralization of TNFα has proven to be highly effective in PsA, additional therapy aimed at boosting or restoring the immune barrier function of the body might have beneficial effects on the recurrence/exacerbation of PsA. NSAIDs have an important part in the treatment of spondylarthropathies possibly in part due to the increased ability of DCs/macrophages to respond to (myco) bacteria. Much more research is necessary to understand the complex interplay of the immune system and microbiota in PsA. Interesting is to investigate whether psoriasis patients and PsA patients differ in their response towards (myco) bacteria to determine disease/stage specificity and using a broader panel of bacteria to further determine specificity. Since 16S rRNA can be used to determine the presence of mycobacterial ribosomal RNA it could be a tool to determine its presence in blood (e.g. in monocytes) and in synovial tissue and fluid. Thereby more circumstantial evidence for a role for mycobacteria in PsA might be uncovered. Direct evidence might be found by using drugs aimed against mycobacteria (antibiotics, vitamin D) and correlating change in disease activity with the presence of (myco)bacterial components. Rifampicin, an antibiotic used for the treatment of mycobacterial infections, already has demonstrated some effectiveness in the treatment of psoriasis [22;23]. Autophagy is an essential cellular process by which cells degrade their own components. Recent developments have revealed that the autophagy pathway and its proteins also play an important role in inflammation and immunity. In DCs autophagy processes degrade bacteria, influence cytokine production upon contact with bacteria and regulate the correct presentation of bacterial components to T cells [24]. It thus appears apparent that

autophagy might play an important role in PsA. We found that the transcription of NOX2 and LL37, proteins important in the induction of autophagy following TLR2 and NOD2 activation, are highly increased in PsA DCs. Together with the recent description that the induction of autophagy limits the production of proinflammatory cytokines upon TLR activation [25] this underscores the possible importance in the aberrant immune reaction towards mycobacteria by PsA DCs. This warrants further in depth research into the regulation of autophagy in PsA. A dysfunction in autophagy might underlie PsA characterized by an aberrant innate immune and autophagy reaction of DCs followed by the dysfunctional presentation of bacterial components to T cells leading to inappropriate immune activation and eventually chronic inflammation. A first step might be to further delineate the autophagy pathway induced by TLR2 activation and investigate whether this signaling route is enhanced in PsA DCs (e.g. by measuring the NOX2-dependent production of reactive oxygen species upon the engagement of TLRs which mediate the recruitment of the autophagy protein LC3 to the phagosome). Understanding of the involvement of the autophagy pathway in DC behavior in PsA might lead to new therapeutic modalities specifically aimed at the causative immune dysfunction.

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11

Nederlandstalige samenvatting

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Het immuunapparaat is cruciaal in de afweer tegen bedreigingen van buitenaf, zoals bacteriën, virusen en schimmels. Hierbij zijn veel verschillende soorten (immuun)cellen, betrokken. Deze cellen kunnen bedreigingen herkennen en hierop actie ondernemen door een ontsteking te veroorzaken gericht tegen deze bedreiging. Naast gevaren van buitenaf kan het immuunapparaat ook reageren op schade van het lichaam zelf zodat dit opgeruimd wordt. Naast alle goede eigenschappen van het immuunapparaat kan het zich echter ook tegen ons keren. Dit gebeurt bijvoorbeeld in Reumatoïde Artritis waarbij bestanddelen van het gewricht zelf door het immuunapparaat worden gezien als bedreigingen waarop er een immuunreactie ontstaat die leid tot ontsteking van het gewricht. Afweercellen kunnen bedreigingen en schade herkennen via Toll-like Receptoren waarna de immuunreactie begint. In dit proefschrift hebben we onderzoek gedaan naar de rol van Toll-like Receptoren en Fc gamma Receptoren op dendritische cellen (DCs) en macrofagen in Reumatoïde Artritis en Artritis Psoriatica. Daarnaast hebben we gekeken naar de regulatie van Toll-like Receptor reacties in deze cellen. DCs zijn cellen die belangrijk zijn in het herkennen van bacteriën, virussen en schimmels waarna ze een immuunreactie opstarten door de activatie van andere immuuncellen zoals T- en B-cellen zodat de bedreiging wordt beperkt. Macrofagen zijn cellen met zeer uiteenlopende functies. Er is een grote verscheidenheid aan macrofagen, van macrofagen die in staat zijn om veel ontstekingsmoleculen te produceren en zo belangrijk zijn in het ontstaan van een ontstekingsreactie, tot macrofagen die met name gericht zijn op juist het onderdrukken van immuunreacties. Toll-like Receptoren zijn receptoren die bestanddelen van bacteriën en virussen kunnen herkennen en vervolgens de betrokken cel kunnen activeren tot het produceren van stoffen die belangrijk zijn in de afweerreactie van het lichaam. Daarnaast kunnen Toll-like Receptoren stoffen herkennen die vrijkomen bij schade in het lichaam, dit om immuunreacties op te roepen welke belangrijk zijn voor het opruimen van de ontstane schade. Nu is het echter zo dat wanneer er te veel van dat soort ontstekingsstoffen vrijkomen of er volgt een te sterke immuunreactie er een vicieuze cirkel kan optreden waardoor er een blijvende (chronische) ontsteking ontstaat. In RA zijn er veel stoffen aanwezig in het gewricht die Toll-like Receptoren kunnen binden en daardoor een immuunreactie op kunnen wekken. Deze stoffen lijken belangrijk in het ontstaan en aanwezig blijven van de ontsteking in de gewrichten. Als tegen-reactie tegen de activatie van Toll-like Receptoren heeft het lichaam verschillende mechanismen ontwikkeld om hun reactie te onderdrukken. Wij hebben gevonden dat de Fc gamma Receptor IIb in staat is om Toll-like Receptor

reacties te onderdrukken (**hoofdstuk 2 en 3**). Fc gamma Receptoren zijn receptoren die antistoffen binden die zelf weer kunnen binden aan allerlei bestanddelen van bacteriën, virussen of onderdelen van het lichaam zelf. De meeste Fc gamma Receptoren hebben een activerend effect op de cel, maar Fc gamma Receptor IIb is uniek door zijn remmende functie. Daarnaast hebben we aangetoond dat patiënten met Reumatoïde Artritis die, na in het begin van de ziekte behandeld te zijn geweest met immuun-onderdrukkende middelen, uiteindelijk zonder medicijnen kunnen, een hele hoge aanwezigheid hebben van Fc gamma Receptor IIb op hun DCs. Daarmee kunnen deze patiënten heel goed Toll-like Receptor reacties onderdrukken in DCs. Hierdoor maken deze DCs vervolgens veel minder ontstekingseiwitten en zorgen ze ervoor dat er T cellen ontstaan (T helper 2 en regulatoire T cellen) die belangrijk zijn in de onderdrukking van immuunreacties. Dit is mogelijk een verklaring waarom deze Reumatoïde Artritis patiënten geen medicijnen meer nodig hebben, aangezien hun lichaam/ immuunsysteem een eigen methode heeft ontwikkeld om de ontsteking te onderdrukken. In andere Reumatoïde Artritis patiënten, die wel krachtige medicatie nodig hebben, zou het juist kunnen zijn dat de Fc gamma Receptor IIb onvoldoende hoog wordt waardoor ze juist de ziekte krijgen of blijven houden. Een mogelijke nieuwe therapie zou dus het ophogen van de aanwezigheid van Fc gamma Receptor IIb kunnen zijn op DCs. Hiervoor moet duidelijk zijn hoe dit in cellen gereguleerd is. Op dit moment wordt er aldus in ons lab onderzoek gedaan naar de onderliggende regel mechanismen.

Aangezien macrofagen zulke uiteenlopende functies vertonen en ze belangrijk zijn in zowel het starten van een immuunreactie en tevens juist het onderdrukken hiervan wilden wij bekijken of macrofagen die binnen komen in een ontsteking van aard veranderen gedurende de immuunreactie. Onze hypothese was dat gedurende de ontsteking de aard van de macrofagen van ontstekings-veroorzakend naar ontstekingsremmend zou veranderen en dat dit zou samenhangen met de hoeveelheid macrofagen die aanwezig is (hoe meer macrofagen hoe minder pro-ontsteking ze hoeven te zijn omdat er dan voldoende aanwezig zijn om de bedreiging aan te pakken). Wij vonden dat deze hypothese, in ieder geval *in vitro* (in het lab), klopt. Macrofagen die normaalgesproken een ontstekingsveroorzakende aard hebben, kregen een ontstekingsremmende aard wanneer ze met veel cellen bij elkaar werden gekweekt. Ze werden in staat tot het produceren van veel moleculen die ontsteking onderdrukken en de productie van ontstekingsmediatoren werd onderdrukt na Toll-like Receptor stimulatie (**hoofdstuk 4**). Dit bleek te berusten op de productie van een oplosbare factor, meest waarschijnlijk prostaglandine-E2. Dit molecuul is belangrijk in de progressie van tumoren en remming

van dit molecuul leidt tot een toegenomen weerstand tegen bacteriën. Veelgebruikte pijnstillers in Reumatoïde Artritis zoals diclofenac en naproxen, die horen tot de groep van de NSAIDs (non-steroidal anti-inflammatory drugs), werken door de onderdrukking van een molecuul (COX2) wat voor de productie van prostaglandine-E2 zorgt. Recent heeft men laten zien dat deze middelen zorgen voor een verhoogde productie van pro-ontstekings moleculen in RA gewrichtsweefsel. Onze resultaten laten zien dat dit wel eens zou kunnen komen doordat de ontwikkeling van een anti-ontstekings macrofaag wordt voorkomen.

Er bestaan tien verschillende Toll-like Receptoren (Toll-like Receptor 1-10) met allemaal hun eigen mogelijkheid om verschillende moleculen te herkennen en vervolgens hun cel verschillende reacties te laten vertonen. Dit is belangrijk in het afweersysteem omdat er zo onderscheid kan worden gemaakt tussen verschillende bacteriën, virussen en schimmels die een bedreiging kunnen vormen. Uit dierproefonderzoek is gebleken dat Toll-like Receptor 2 een onderdrukkende werking heeft op het ontstaan van gewrichtsontstekingen (artritis) terwijl Toll-like Receptor 4 juist belangrijk is in het ontstaan van deze ontsteking. Toll-like Receptor 4 bindende moleculen (liganden) zijn aanwezig in het gewricht van RA patiënten. Wij hebben laten zien dat Toll-like Receptor 2 in staat is om de sterke Toll-like Receptor 4 (en Toll-like Receptor 7/8) reacties te onderdrukken in DCs (**hoofdstuk 6**). Deze onderdrukking vindt plaats via verschillende routes in de cel. Een belangrijke remming vindt plaats via de activatie van SOCS1, een molecuul dat activatie via interferon type 1 kan remmen (**hoofdstuk 5**). Interferon type 1 is belangrijk in de afweer tegen virussen maar lijkt ook een belangrijke rol te hebben in het ontstaan van autoimmuunziekten zoals Reumatoïde Artritis. Deze remmende route via TLR2 zou dus een aanknopingspunt kunnen zijn voor nieuwe therapieën.

In Reumatoïde Artritis wordt sinds een aantal jaren gebruik gemaakt van moleculen die speciaal ontwikkeld zijn om specifiek in te grijpen in het immuun systeem. Bekende moleculen die ontwikkeld zijn,zijn de anti-TNFα middelen die dit eiwit, zeer belangrijk in ontsteking, neutraliseren. Daarnaast zijn er moleculen ontwikkeld die de communicatie tussen DCs en T-cellen beperken. Abatacept is zo'n molecuul. Wij hebben gevonden dat dit medicijn nog andere effecten heeft waardoor het ontstekingsreacties kan onderdrukken. Het blijkt dat het ervoor zorgt dat geactiveerde T-cellen, die aanwezig zijn in het gewricht bij Reumatoïde Artritis patiënten, veel minder in staat zijn om macrofagen aan te zetten tot de productie van TNFα (**hoofdstuk 7**). Dit is een direct mechanisme waardoor het effect van Abatacept nog beter verklaard zou kunnen worden.

Artritis Psoriatica is een aandoening waarbij er naast gewrichtontstekingen sprake is van ontstekingen in de huid zoals bij psoriasis. Waar er bij Reumatoïde Artritis sprake is van een duidelijke auto-immuun component is dit in Artritis Psoriatica minder duidelijk. Artritis Psoriatica behoort tot de groep van ziekten genaamd spondylarthropathieën, andere ziekten in deze groep zijn onder andere de ziekte van Crohn en de ziekte van Bechterew. Zoals bij Crohn wordt er bij psoriasis gesproken over een mogelijke oorzaak door een verminderde afweerfunctie van weefsels tegen aanvallen van buiten af door bacteriën. In Crohn is er sprake van een chronische ontsteking van de darmwand. Men heeft laten zien dat mensen die deze ziekte hebben minder goed in staat zijn om een immuunreactie op te roepen tegen bepaalde bacteriën waardoor deze waarschijnlijk langer aanwezig blijven in de darm en daardoor voor een chronische ontsteking zorgen. In dit proefschrift laten wij zien dat DCs van patiënten met PsA minder goed in staat zijn om te reageren op mycobacteriën(**hoofdstuk 8**). Mycobacteriën zijn overal om ons heen, op onze huid en in onze darmen. Het zou kunnen zijn dat door de verminderde verwijdering van mycobacteriën uit huid en darmen, door de slechtere herkenning door DCs, bestanddelen van deze bacteriën of hele bacteriën zich via het bloed verplaatsen naar de gewrichten en pezen waarna er daar ontstekingen ontstaan. Dit zou waarschijnlijk nog niet zo'n groot probleem zijn als de onderdrukkende mechanismen in het immuunsysteem goed zouden werken in deze patiënten. Het lijkt er echter op dat anti-ontsteking macrofagen die specifiek worden aangetrokken naar de ontstekingsplekken en normaal gesproken de immuunreactie onderdrukken, niet goed werken. Hierdoor wordt de immuunreactie niet geremd maar gaat juist door (**hoofdstuk 9**). Door een verminderde herkenning van mycobacteriën door DCs kunnen deze bacteriën zich dus mogelijk meer dan normaal vestigen in het lichaam waarna er een chronische ontstekingsreactie ontstaat van huid, gewrichten en pezen zoals gezien in Artritis Psoriatica. Vervolgens blijven deze ontstekingen chronisch aanwezig omdat de aangetrokken ontstekingsonderdrukkende macrofagen hun werk onvoldoende doen.

In dit proefschrift hebben we meerdere onderdelen van het immuunapparaat bekeken die betrokken zijn bij het ontstaan van Reumatoïde Artritis en Artritis Psoriatica. We hebben ons met name gericht op de rol die gespeeld wordt door DCs en hun Toll-like Receptoren. Het blijkt dat DCs mogelijk een grote rol spelen in Reumatoïde Artritis, patienten die namelijk uiteindelijk zonder medicijnen kunnen blijken een hele hoge aanwezigheid te hebben van de remmende Fc gamma Receptor IIb op hun DCs waarmee Toll-like Receptor reacties kunnen worden geremd. Mogelijk dat hierdoor hun ziekte wordt onderdrukt wat zou

betekenen dat DCs een centrale rol spelen in het (remmen van het) ziekteproces. Daarnaast hebben we een aanwijzing gevonden dat DCs niet goed werken in Artritis Psoriatica. Ze zijn niet goed in staat om mycobacteriën te herkennen via Toll-like Receptoren waardoor deze mogelijk minder makkelijk verwijderd worden en vervolgens zorgen voor een (te) sterke immuunreactie. Dit kan vervolgens leiden tot ontstekingen van onder andere huid, gewrichten en pezen zoals gezien in Artritis Psoriatica. Door het beter begrijpen van de ziekteprocessen in Reumatoïde Artritis en Artritis Psoriatica en de rol die DCs en Toll-like Receptoren hierin spelen kunnen we in de toekomst mogelijk nog betere en meer gerichte medicijnen ontwikkelen tegen deze veel voorkomende ziekten.

List of publications

List of abbreviations

Over de auteur

Dankwoord

12

List of publications

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List of abbreviations

ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatism
APC	antigen-presenting cell
ATG16L1	autophage-related protein 16-1
CCR	credence clearwater revival (or in this thesis: C-C motif receptor)
CD	cluster of differentiation
CRP	C-reactive protein
DAMP	damage associated molecular pattern
DAS28	disease activity score for 28 joints
DC	dendritic cell
$DC_{low\ Fc\gamma RIIb}$	DC expressing a low Fc gamma receptor IIb level
$DC_{high\ Fc\gamma RIIb}$	DC expressing a high Fc gamma receptor IIb level
DMARD	disease modifying anti-rheumatic drug
DMARD(-) RA	RA patients having a DAS28<3.2 not on DMARD therapy
DMARD(+) RA	RA patients having a DAS28<3.2 on DMARD therapy
dsRNA	double-stranded ribonucleic 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
HAGGs	heat-aggregated gamma globulins
HE	heamatoxylin/eosin
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
Mϕ-1	type 1 macrophage
Mϕ-2	type 2 macrophage
MACS	magnetic bead activated cell sorter
(m)Ab	(monoclonal) antibody
MAPK	mitogen-activated protein kinase
M-CSF	macrophage-colony stimulating factor
MDP	muramyl dipeptide
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHC II	MHC class II
MTX	methotrexate
MyD88	myeloid differentiation factor 88
NFκB	nuclear factor kappa -light-chain-enhancer of activated B cells

NOD	nucleotide-binding oligomerization domain
NOX2	nicotinamide adenine dinucleotide phosphate-oxidase 2
NSAID	non-steroidal anti-inflammatory drug
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
ST	synovial tissue
STAT-1	signal transducer and activator of transcription-1
TNFα	tumor necrosis factor alpha
TNFAIP3	tumor necrosis factor alpha-induced protein 3
Th	T helper
TOLLIP	toll-interacting protein
TLR	toll-like receptor
TRAF	TNF receptor-associated factor
TRIF	TIR-domain containing adaptor protein inducing IFNbeta
Treg	regulatory T cell

Over de auteur

Mark Herald Wenink werd geboren op 11 augustus 1977 te Almelo. Zijn lagere school tijd bracht hij de door op De Kei te Aadorp. In 1995 voltooide hij het VWO op het OSG Erasmus te Almelo. Na het behalen van het VWO diploma begon hij aan de studie Technische Bedrijfskunde aan de Technische Universiteit Twente. Na een jaar verruilde hij deze studie voor de studie Biomedische Wetenschappen aan de Katholieke Universiteit Nijmegen (thans Radboud Universiteit Nijmegen). Na deze afgerond te hebben stapte hij in 2000 over naar de studie Geneeskunde aan dezelfde Universiteit.

In 2004 behaalde hij zijn artsexamen waarna hij werd aangenomen voor de opleiding tot Reumatoloog aan het UMC St. Radboud en in 2005 startte hij zijn promotie onderzoek op de afdeling Reumatische Ziekten. Subsidie werd gevonden middels een AGIKO. Gedurende zijn promotie deed Mark onderzoek naar de functie van Toll-like en Fcγ receptoren in ontstekingen en specifiek in reumatoide artritis en artritis psoriatica. De resultaten staan beschreven in dit proefschrift. Dit onderzoek werd verricht onder leiding van Dr. Timothy Radstake en onder supervisie van Prof. Dr. Wim van den Berg en Prof. Dr. Piet van Riel. De bevindingen werden middels lezingen gepresenteerd op verscheidene internationale congressen. Travel Grants werden verkregen voor het congres van de Federation of Clinical Immunology Societies (FOCIS) te San Francisco in 2009 en de European workshops on Immune-mediated Inflammatory Disease (ewIMID) in 2009 en 2011. In 2010 deed Mark gedurende 5 maanden onderzoek bij het Institute of Infection, Immunity and Inflammation van de universiteit van Glasgow onder leiding van Prof. Dr. Iain McInnes.

De vooropleiding Algemeen Interne Geneeskunde voor de opleiding tot Reumatoloog werd gestart in februari 2010 in het Radboud University Nijmegen Medical Centre (hoofdopleider Prof. Dr. Jacqueline de Graaf).Van september 2011 tot april 2012 deed Mark naast zijn klinische opleiding een dag in de week onderzoek op de afdeling Reumatische Ziekten. Momenteel heeft hij zijn klinische werkzaamheden vier maanden opgeschort voor het verrichten van onderzoek als post-doc bij het Laboratory of Translational Immunology aan het UMC Utrecht onder leiding van Prof. Dr. Timothy Radstake waarbij hij zich onder andere richt op de rol van autofagie in de pathogenese van artritis psoriatica.

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