The Role and Modulation of Pathogenic CCR6 Positive T Helper Cells in Rheumatoid Arthritis

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The work presented in this thesis was performed at the department of Rheumatology and the department of Immunology, Erasmus MC, Rotterdam, The Netherlands. Financial support was provided by Roche. Publication of this thesis was financially supported by Pfizer bv, AbbVie bv and the Erasmus University Rotterdam..

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Layout: Nikki Vermeulen - Ridderprint BV
Printing: Ridderprint BV - www.ridderprint.nl
Cover: Umberto Shtanzman - www.shutterstock.com

ISBN: 978-94-6299-210-8

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De rol en modulatie van pathogene CCR6 positieve
T helper cellen in reumatoïde artritis

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

woensdag 9 december 2015
om 15.30 uur

door

Sandra Maria Johanna Paulissen
geboren te Geleen

Promotiecommissie:

Promotor:	Prof.dr. J.M.W. Hazes
Overige leden:	Prof.dr. R.W. Hendriks Prof.dr. P.D. Katsikis Prof.dr. L.S. Taams
Copromotor:	Dr. E. Lubberts

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Chapter 1

Introduction

1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic chronic autoimmune disease which chiefly afflicts synovial joints. In industrialized countries, RA affects 0.5-1% of all adults, and it is more common in women than men. In early disease, a chronic inflammation is induced by pro-inflammatory cells migrating into the synovium. Synovial hyperplasia, angiogenesis and pannus tissue formation develop when the disease advances. If RA is not treated properly, these processes will eventually result in irreversible joint destruction and disability. Therefore, early detection of disease is of pivotal importance. RA disease etiology is not fully elucidated, but genetic susceptibility, autoantibody formation and environmental factors are identified as major RA risk factors [1-4]. An overview of these risk factors is provided in this chapter. Furthermore, the current knowledge regarding cells of the immune system, cells of the joint synovium and interactions between these cells, and signal molecules (mainly cytokines and chemokines) in RA pathogenesis is summarized and depicted in figure 1.

1.1 Genetics and environment in RA pathogenesis

From twin studies, the heritability of RA is estimated to be approximately 60%, and there are more than 100 genetic loci identified that are associated with susceptibility to RA. Among the best studied and largest genetic risk factors for RA are the human leukocyte antigen (HLA)-DRB1 alleles. HLA-DRB1 encodes for a protein that is part of a cell surface receptor, which is involved in antigen presentation by antigen-presenting cells (APCs). Antigens are presented to T cells that can be activated if their receptor recognizes the antigen. Several alleles of the HLA-DRB1 gene encode for a common amino acid sequence in the protein, called the shared epitope (SE). The hypothesis is that the presence of the SE enhances the efficiency of antigen presentation, leading to greater T cell activation [5].

Another strong and well-studied risk gene for RA is the missense variant of the protein tyrosine phosphatase non-receptor type 22 (PTPN22) gene. The tyrosine phosphatase that is encoded by the normal variant of PTPN22 regulates signaling through antigen and pattern-recognition receptors. The normal variant acts as a brake on T cell receptor (TCR) signaling, thereby inhibiting T cell activation, but stimulates signaling through pattern recognition receptors, leading to production of type I interferons by myeloid cells [6]. The variant of the gene associated with RA leads to a stronger inhibition of T and B cell receptor signaling [7]. It seems logical that this would lead to a lower risk of autoimmunity, but instead the risk variant of the PTPN22 gene is associated with higher risk of developing RA. It is postulated that the decreased signaling might cause a failure to delete autoreactive T cells and/or B cells, or might lead to insufficient activity of regulatory T cells (Tregs) [6, 8].

Next to genetic background, environmental factors also play a role in the development of RA. Tobacco smoking is a well-established environmental risk factor for developing RA. Smokers have a two-times higher risk of developing anti-citrullinated protein antibody (ACPA) positive RA than non-smokers [9].

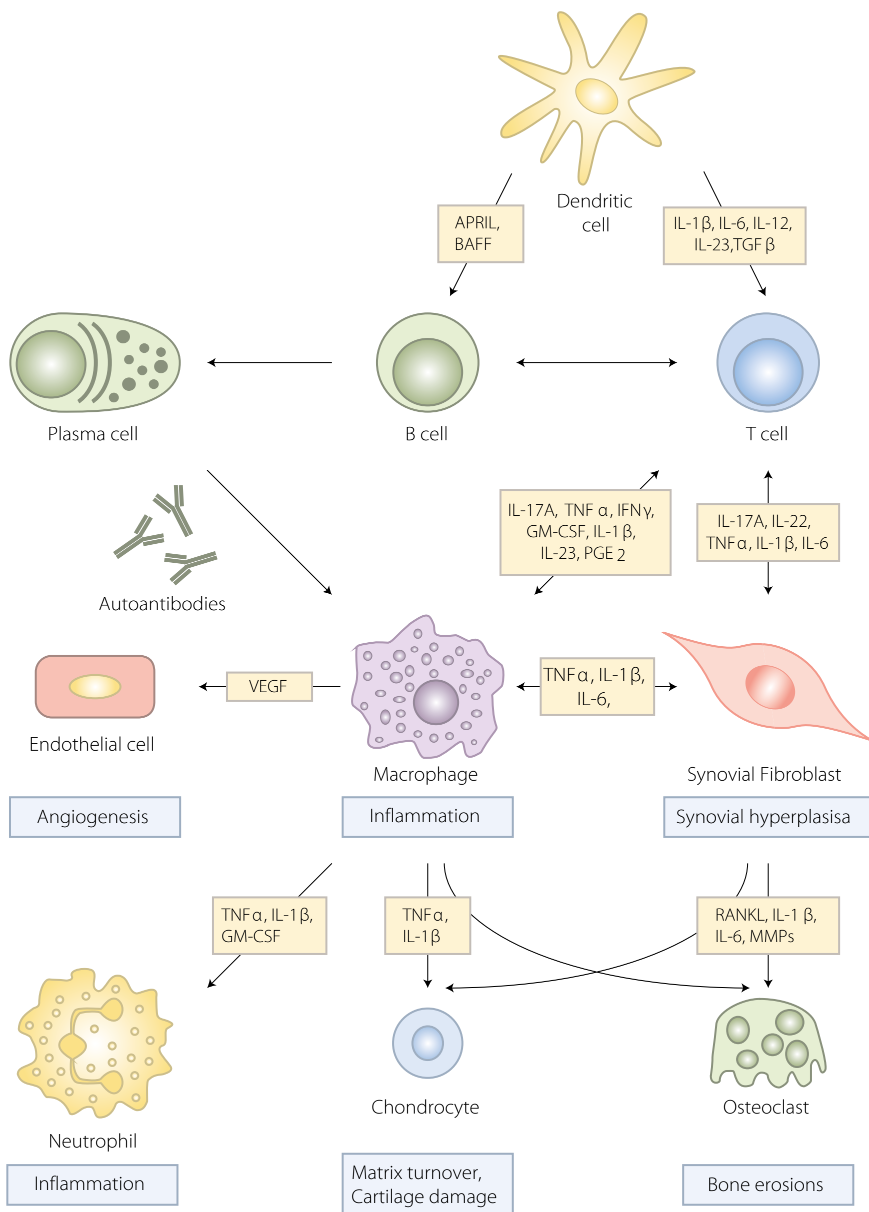

Figure 1. Pathogenesis of RA. Several cells that play a role in RA pathogenesis, and the cytokines, chemokines and other signaling molecules they produce are depicted.

Other environmental factors that might play roles in RA are the microbiome of the intestinal tract and the microbiome of the oral cavity. The role of the intestinal microbiome in RA has been studied mostly in animal models. Germ-free mice and rats were shown to be either protected, or more prone to develop arthritis, depending on the genetic background of the model. In RA patients, total bacterial counts are lower than in fibromyalgia patients, while several bacterial species (mainly *lactobacillus*) were increased, or even uniquely present, in RA patients [10]. Interestingly, presence of *Prevotella copri* correlated with early onset RA. Increased levels of *P. copri* correlated with a loss of bacterial species that are reported to be beneficial [11]. Although animal models and fecal research in RA patients indicate that the gut microbiome is altered in RA, the question remains whether the alterations are causative of RA, or a secondary effect of inflammatory processes in RA [10].

Evidence that the microbiome of the oral cavity might be important in RA comes mainly from the observation that RA patients have an increased incidence of periodontal disease (a bacterial infection of the gum tissue). Interestingly, a weak association between prior periodontal disease and RA was found, indicating that in a subgroup of patients periodontal disease might be causative in RA. However, periodontal disease and RA share common mechanisms in their pathogenesis, including the production of pro-inflammatory cytokines and destructive mediators, leading to inflammation and bone/tissue destruction [10]. Therefore, the activation of the immune system (regardless of the underlying cause), rather than changes in the microbiome of the oral cavity, might explain the observed association and co-occurrence between periodontal disease and RA.

1.2. Autoantibodies in RA

Antibodies are proteins produced by plasma cells (terminally differentiated B cells) that bind with high specificity to their antigen, which is usually a (unique) part of foreign objects like bacteria or viruses. In autoimmune diseases like RA, antibodies can be directed against antigens belonging to the own body (autoantibodies). Binding of any (auto)antibody to its antigen, leads to the formation of an immune complex, which in turn can lead to activation of immune cells (via Fc gamma receptors on these cells that bind the immune complex), and activation of the complement system (via binding and subsequent activation of complement proteins to the immune complex), thereby propagating inflammation [12].

Several autoantibodies are present in the serum of RA patients, and might play roles in disease initiation and propagation. The best studied of these are rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs), both of which are included in the 2010 RA diagnosis criteria of the joint American College of Rheumatology (ACR) and European League against Rheumatism (EULAR). RF is directed against a constant part of antibodies (it is an antibody against an antibody), and ACPAs are directed against citrullinated proteins. Citrullination is an enzymatic process, in which the amino acid arginine in a protein is converted to the amino acid citrulline, executed by peptidylarginine deiminase (PAD). For unknown reasons these citrullinated proteins can elicit antibody formation in RA. Both RF and ACPAs can be present years before onset of clinical RA, and their concentrations in serum rise at times close to onset of RA. The presence of ACPAs is more specific for RA than the presence of RF,

which is for example also present in the serum of patients with Sjögren's syndrome or systemic lupus erythematosus. ACPA positivity is more strongly associated with erosive disease. ACPAs might be a causal factor in pathophysiology in RA, because any immune complex can activate immune cells and activate the complement system, but in the case of ACPAs, the antigens are deposited (among others) in the joint and can contribute to local immune activation and inflammation [12, 13].

Especially in the case of ACPAs, the genetic background of the patient is also important. HLA-DRB1 SE alleles, smoking, and ACPA positivity are all linked to each other [5, 9, 14]. Smoking leads to increased citrullination of proteins in the lung. It is tempting to speculate that these citrullinated proteins are presented to T cells by APCs expressing the HLA-DRB1 SE, inducing activation of the T cells, which then promote ACPA production by B cells. In this way, a process starting in the lung could theoretically contribute to inflammatory processes in the joint [9].

Antibodies can be of several isotypes. Naïve (antigen-inexperienced) B cells produce only antibodies of the surface bound immunoglobulin (Ig)M type (the B cell receptor). When a B cell receptor binds its antigen, and is subsequently activated by a T cell via CD40/CD40L interaction, class switching is often induced. The B cell produces (soluble) antibodies with the same specificity (recognizing the same antigen), but with a different constant part of the antibody, which influences its properties. ACPAs are mainly of the IgG subtype, meaning that class switching has occurred, and implying that T cells activated the B cells [9, 15].

1.3. Involvement of the innate and adaptive immune system in RA

The immune system plays a very large role in RA induction and propagation. The immune system consists of two components: the innate and the adaptive immune system. The innate immune system is quickly activated and limits spreading of the infection; however, cells from the innate immunity are not specific to one antigen, but recognize conserved epitopes that are common among different pathogens. In contrast, the adaptive immune response takes several days to develop, but the cells from adaptive immunity recognize one specific antigen. This leads to a much stronger immune response, and memory cells will develop that will respond very quickly if a similar pathogen enters the body again.

The innate immune system forms the first line of defense, and includes mechanical barriers like the skin, and several cell types that express receptors that enable them to quickly recognize pathogens. These cells include monocytes, macrophages, neutrophils, natural killer (NK) cells and others. These cells destroy pathogens and infected cells, and they produce signal molecules (cytokines/chemokines) that activate and attract other cells, including those of the adaptive immunity, and promote inflammation. Furthermore, they process and present antigen to T cells, which belong to the adaptive immunity. In this context it is important to mention the dendritic cells (DCs), which are APCs that are very potent in activating naïve and memory T cells. DCs are vital in maintaining the balance between immunity and tolerance. Several cells of the innate immune system have well described roles in RA.

Monocytes and macrophages are present in inflamed RA joint synovium, and are an important source of pro-inflammatory cytokines [1, 16]. Neutrophil frequencies are increased in synovial fluid and

synovial tissue of RA patients, particularly in early disease stages, and play a role in joint destruction [17, 18]. NK cells are present and over-activated in RA synovium. They contribute to pathogenesis by producing pro-inflammatory cytokines [19]. In addition, antigen presenting DCs infiltrate the RA synovium [20], and both DCs and NK cells can provide co-stimulation to T cells [19-21].

The adaptive immune system consists of B and T cells. They have highly specific antigen receptors, which ensures that they respond only to one target. The main function of B cells is the production of very specific antibodies that bind to pathogens and mark them for clearance by other cells. In healthy persons, the majority of early B cells is autoreactive, but several checkpoints in B cell development insure that most, but not all, autoreactive B cells are removed [22]. In RA, there is an increase in autoreactive B cells, due to defects in these tolerance checkpoints. B cells in RA are pathogenic through several mechanisms, including autoantibody production, T cell activation and cytokine synthesis [12].

T cells are subdivided in two groups: most (~95%) have a TCR consisting of α and β chains, but some (~5%) express a TCR consisting of γδ chains. γδ T cells are mostly found in in mucosal and epithelial surfaces, and operate on the interface between innate and adaptive immunity. Several functions of γδ T cells might play a role in pathogenesis of RA: 1) they can act as APCs and provide co-stimulation, and DCs and γδ T cells stimulate each other's maturation and functions through cytokines; 2) they can produce pro-inflammatory cytokines like interferon (IFN)γ, tumor necrosis factor (TNF)α and interleukin (IL)-17; 3) they can give co-stimulation to B cells [23].

αβT cells can be subdivided into two main groups: the CD8+ cytotoxic T cells and the CD4+ T helper (Th) cells. CD8+ T cells primary function is killing of pathogen-infected cells. For the CD8+ T cell to 'know' which cell is infected and needs to be killed, it has to be instructed and activated first. This is done by APCs, including DCs, which display (pathogen) peptides on the major histocompatibility complex (MHC) class I (a molecule involved in antigen presentation that is expressed on almost all nucleated cells). Recognition of the (pathogen) peptide presented by the APC leads to activation and proliferation of the CD8+ cells. Activated CD8+ T cells kill infected cells by direct lysis (through production of perforin) or induction of apoptosis (through production of granzymes and activation of the FAS receptor). About 40% of the T cell population in RA synovial fluid consists of CD8+ T cells. CD8+ T cells in synovial fluid express higher levels of activation markers compared to CD8+ T cells in blood, and produce IFNγ, TNFα, IL-17 and IL-6. CD8+ T cells are likely to play a role in RA pathology, but are insufficiently researched, perhaps because their role in animal models is very conflicting (depleting CD8+ T cells leads to either disease exacerbation or improvement) [24].

CD4+ Th cells help B cells and CD8+ T cells with their function. For instance, Th cells help B cells in their differentiation process to plasma cells after T-B cell interaction through 2 interactions: T cell receptor binding to antigen-specific MHC class II (a molecule involved in antigen presentation, expressed on APCs and B cells) and binding of CD40-ligand (CD40L) on Th cells to CD40 on B cells. The Th cell population consist of several subpopulations: Th1 cells differentiate from naïve T cells under the influence of IL-2 and IL-12, have as major transcription factor T-box transcription factor (TBX)21 and produce high amounts of IFNγ; Th2 cells differentiate under the influence of IL-4, have as major transcription factor GATA domain

binding protein 3 (GATA3) and produce high amounts of IL-4 and IL-5; the regulatory T cell (Treg) differentiates under the influence of TGFβ, has as major transcription factor forkhead box P3 (Foxp3) and produces high amounts of IL-10; Th17 cells differentiate under influence of IL-1β and IL-6, have as major transcription factor retinoic acid receptor-related orphan receptor (ROR)γt and produce many cytokines, including IL-17A, IL-17F, IFNγ and IL-22. In addition, Th17 cells express the chemokine receptor C-C chemokine receptor type 6 (CCR6). There is another Th subset, the follicular Th (Tfh) subset, that differentiates when naïve Th cells recognize antigen presented by APCs in lymphoid tissue. The major transcription factor driving the Tfh program is B cell lymphoma 6 (Bcl-6), and they are characterized by the expression of C-X-C chemokine receptor type 5 (CXCR5) and the production of IL-21. Tfh have an important role in triggering the formation and maintenance of germinal centers (GCs). GCs are sites in secondary lymphoid tissue (spleen and the lymph nodes) that form during immune responses. Within GCs, Tfh mediate the selection and survival of B cells that differentiate into plasma cells or memory B cells. Therefore, Tfh have an important role in humoral immunity [25]. In RA patients, increased frequencies of Tfh and levels IL-21 in peripheral blood were found compared to healthy controls, and the levels of Tfh and IL-21 correlated with the level of ACPAs [26, 27]. Tfh were also found in rheumatoid synovium [28]. Tfh are thought play a role in the development of ectopic lymphoid structures (ELS), that form at sites of chronic inflammation (including the RA synovium). They range from T-B cell clusters to highly organized structures which contain GCs. In RA, ELS might play a role in selecting auto-reactive B cells, differentiation of plasma cells and auto-antibody production [29, 30], and are hypothesized to play a role in disease pathogenesis. Currently, of the Th subsets mainly Th1, Th17 and Tregs are thought to play roles in disease pathogenesis.

Cells from both the innate and adaptive immune system can activate each other, but also other non-immune cells that are present in the synovium: the synoviocytes.

1.4. Role of joint cells in inflammation and destruction

In a normal joint the synovial membrane is 1 to 2 cell layers thick and consists of type A fibroblast-like and type B macrophage-like synoviocytes that synthesize matrix constituents and synovial fluid. In RA these cells are inappropriately activated, leading to proliferation, cytokine production, pannus formation and production of catabolic enzymes such as metalloproteinases (MMPs) and cathepsins that can lead to degradation of the cartilage and bone.

Chondrocytes are the main cells present in cartilage, responsible for the production of the cartilaginous matrix, consisting mainly of proteoglycans and collagen. Under physiological conditions, the chondrocytes maintain homeostasis between matrix synthesis and degradation, and hardly any cells will be present in the matrix. In RA, due to the inflammation, there is pannus formation of synoviocytes, which can release proteinases that can digest the cartilage matrix components. Additionally, the chondrocyte itself, possibly activated by IL-1 and TNFα, may degrade matrix components via production of MMPs. Additionally, IL-1 and TNFα stimulated chondrocytes produce pro-inflammatory cytokines and chemokines, including IL-6 and IL-8. Therefore, chondrocytes may not only play a role in cartilage

destruction in RA, but also in the propagation of inflammation and the recruitment of immune cells to the synovium [31].

Osteoclasts are multinucleated cells derived from the monocyte lineage, and they are the only cells in the body capable of resorbing bone. Monocyte lineage cells migrate from the bone marrow into secondary lymphatic organs to the joint. The conditions necessary for migration are not completely known, but TNFα is thought to play a role. In the joint, the monocyte lineage cells are exposed to macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL), which differentiates them towards osteoclasts, a process that is finalized when they adhere to the bone. They adhere tightly to the bone, create an acidic milieu and break down bone by their production of matrix enzymes like MMPs, tartrate-resistant acid phosphatase type 5 (TRAP) and cathepsin K. Factors that directly or indirectly influence osteoclast differentiation are cytokines, like TNFα, IL-17 and IL-15, but also autoantibodies like ACPAs [32].

Synovial fibroblasts normally produce matrix constituents. In RA, synovial fibroblasts become activated, leading to inappropriate proliferation. Activation of synovial fibroblasts contributes to RA pathology [33-39].

2. Treatment and drug resistance

There is no cure for RA, but proper treatment can slow down the disease progression and improve symptoms. Usually a combination of disease-modifying anti-rheumatic drugs and anti-inflammatory medication is given. Treatment has the best result when it is started early and aggressively [40]. The different classes of medication used in RA are described below.

2.1. Glucocorticoids

Glucocorticoids (GC) are a class of steroid hormone drugs used in various diseases, including cancer and autoimmune diseases like RA. GCs used in treatment of RA include prednisone, prednisolone, cortisol and dexamethasone. GCs easily pass the cell membrane, and bind the glucocorticoid receptor (GR) in the cytosol. GC binding to the GR leads to translocation to the nucleus, where the complex binds to glucocorticoid response elements (GRE), located in the promotor regions of target genes. Binding to the GREs induces gene transcription, including transcription of anti-inflammatory agents. In addition, it has been postulated that GC-GR complexes can also directly bind the DNA, at the locations where the transcription factors NF-kB (major positive regulator of pro-inflammatory responses) and AP-1 (major positive regulator of cell differentiation and proliferation) would normally bind, resulting in down-regulation of pro-inflammatory processes and proliferation. GCs also have effects that are not mediated through manipulation of transcription (named non-genomic or non-nuclear effects). The best described of these is that GC binding to GR, via several steps, leads to reduced production of arachidonic acid, a key pro-inflammatory compound [41, 42].

2.2. Disease-modifying anti-rheumatic drugs

Disease-modifying anti-rheumatic drugs (DMARDs) are drugs that slow down disease progression, specifically by reducing the rate of bone- and cartilage damage. They are often classified as non-biological and biological DMARDs.

2.2.1. Non-biological DMARDS

DMARDs are first line treatment in RA. DMARDs used in the treatment of RA include methotrexate (MTX), sulfasalazine (SSZ), leflunomide and hydroxychloroquine (HCQ). These DMARDs can be given alone, or in combination with each other, which is often more effective than single treatment [43, 44].

MTX was originally developed for use in cancer treatment, because as a folate antagonist it inhibits DNA synthesis and consequently cell division. In RA, the dose used is much lower, and the mechanism by which it works remains elusive. At low dose MTX could still work as a folate antagonist, and perhaps only inhibit the proliferation of the fastest dividing lymphocytes, like T cells, thereby dampening the synovial inflammation. In addition, by inhibiting the enzyme dihydrofolate reductase, MTX might inhibit the accumulation of polyamides that contribute to tissue injury in RA. Another proposed mechanism of action is that MTX might reduce intracellular glutathione levels, leading to inhibition of macrophage and lymphocyte function. Lastly, MTX may be polyglutamated in the cell, and polyglutamated MTX can indirectly lead to release of adenosine, a potent anti-inflammatory compound. Many of these proposed mechanisms come from *in vitro* experiments and are highly hypothetical [45]. Despite being a first choice drug in RA management, the mechanism by which MTX works remains largely unknown.

SSZ is another DMARD that has been used in RA for decades, but also for SSZ the mechanism of action remains elusive. Proposed mechanisms are mainly anti-inflammatory: inhibition of the arachidonic acid pathway (which forms pro-inflammatory and pain-mediating compounds), folate antagonism and inhibition of T cell proliferation [46].

Leflunomide is an inhibitor of pyrimidine synthesis, which is vital for RNA and DNA synthesis. Especially fast dividing lymphocytes are hampered in their proliferation by leflunomide [47].

HCQ inhibits Toll-like receptor (TLR)-9, which plays a role in inducing inflammation by activating innate immunity. Furthermore, it is thought that HCQ disrupts antigen processing and presentation in macrophages, thus preventing activation of Th cells [48].

2.2.2. Biological DMARDS

While the other DMARDs often have a wide range of action, and the targets are often not well defined, DMARDs from biological origin are agents that have a very specific target. Several biological agents are in use in RA, which can broadly be subdivided in 2 categories: agents that target cytokines, and agent that target cell types.

Of the agents inhibiting cytokines, TNFα inhibitors are the most important and have become a central strategy in RA treatment, because of their broad anti-inflammatory effects and improvement in disease activity scores. Blockade of the IL-6R is also considered a major success in RA treatment; IL-6R

blockade improves acute phase response, synovitis and systemic features. Antagonism of the IL-1R was shown to be an effective anti-inflammatory agent in other autoimmune diseases, but use in RA seems limited.

There are several agents used in RA that target specific cell types. Anti-CD20 therapy (rituximab) has been shown to be effective in RA, and is directed against B cells. CD20 is expressed on almost all B cell stages, but is absent in plasma cells. CD20 is important for regulation of B cell differentiation, activation and optimal response to antigen. The CD20 antibody binds to CD20, depleting B cells via various mechanisms, including: 1) binding of the antibody-CD20 B cell complex to Fc-γ receptors on monocytes, leading to phagocytosis of the B cell; 2) crosslinking of CD20 by the antibody leads to altered calcium transport (CD20 is a calcium channel), leading to cell death [49].

Another cell-targeting antibody used in RA is abatacept, which depletes T cells. Next to recognition of antigen presented on MHC, T cells (CD4 and CD8, both memory and naïve) need co-stimulation for optimal activation and expansion. This co-stimulation is given by the interaction of CD28 on a T cell with CD80 (expressed on activated B cells and monocytes) or CD86 (expressed on APCs). Cytotoxic T lymphocyte-associated protein 4 (CTLA-4), which is normally expressed on T cells to terminate T cell activation, has a higher affinity for CD80 and CD86 than CD28. Abatacept is a fusion protein of CTLA-4 with part of an antibody, which thus prevents the delivery of the co-stimulation to the T cells. This leads to a state of anergy (failure to proliferate), and therefore a reduction in T cell numbers. Abatacept treatment has also shown effectivity in RA treatment [1, 50, 51].

More recently, janus kinases (JAK) inhibitors developed for use in RA. The JAKs are a family of intracellular signal transducers, important in cytokine signaling. When a cytokine binds its receptor, the receptor undergoes a conformational change that brings two JAKs in each other's proximity, and they phosphorylate each other. This complex than phosphorylates and activates signal transducer and activator of transcription (STATs). The STATs translocate to the cell nucleus, were they regulate transcription of selected genes that mediate cell proliferation and differentiation. Therefore, JAK inhibitors have pleiotropic effects, but also an increased risk of toxicity. JAK inhibition has shown efficacy in RA via various mechanisms, which are not fully elucidated, but include inhibition of CD80/CD86 expression in DCs and suppression of type I IFN signaling [52].

2.3. Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used as first-line agents for symptomatic relief, including RA. They inhibit cyclooxygenases (COX), enzymes that convert arachidonic acid to prostaglandins. Prostaglandins promote inflammation and have a role in pain sensation. NSAIDs use in RA reduces pain and stiffness, and potentially helps to suppress inflammation [53]. Many NSAIDs used in RA are COX-2 inhibitors. They reduce joint pain and improve motility, but do not inhibit disease progression [54]. PGE_2, the product of COX-2, induces Th17 polarization [55, 56].

2.4. Multidrug-resistance: transport pumps

Despite all the medication available mentioned above, not all patients respond equally well to therapy, especially to non-biological DMARDs. It is estimated that 30% of patients does not respond to, or becomes resistant to, DMARD therapy. One of the proposed mechanisms for this is multidrug resistance conferred by transporter pumps in the membrane of target cells [57]. These transporters belong to the family of ATP-binding cassette (ABC) transmembrane proteins. Under physiological circumstances, they clear the cells of foreign substances or toxins, but they are also able to remove certain drugs from the cell, thereby contributing to multidrug resistance (MDR) [58]. In the context of RA, several MDR-ABC transporters are known to be important. ABCC1 can transport MTX [59, 60] and chloroquine [61]; ABCG2 transports most small molecular DMARDS, such as MTX [62, 63], leflunomide [64, 65] and SSZ [66, 67]; and ABCB1 (also called MDR1 or P-gp) transports prednisolone [68], dexamethasone [69] and chloroquine [70]. Several correlations between the presence of MDR-ABC transporters and intracellular drug levels have been made. For example, increased expression of ABCB1 on PBMCs of RA patients correlated with lower intracellular dexamethasone levels [71-74]. Furthermore, activity of the ABCB1 was higher in a subgroup of therapy-resistant RA patients than in a subgroup of RA patients that responded well to therapy [71, 75]. Importance of the MDR-ABC transporters is also indicated by the correlation of gene polymorphisms with therapy resistance [58].

MDR-ABC transporters are expressed on various tissues, interestingly including lymphocytes. Especially ABCB1 expression on lymphocytes has been target of investigation. It is hypothesized that ABCB1 expression on lymphocytes has a role in the clearance of endogenously produced glucocorticoids, thereby controlling inflammation. However, since multiple RA drugs are also substrates for this receptor, the expression of ABCB1 on lymphocytes contributes to therapy resistance.

3. Aims of the thesis

In the last decades, great progress has been made in understanding the pathology of rheumatoid arthritis (RA), and in the development of therapeutics, which together lead to a significant reduction of disability and improved quality of life of patients with RA. Despite this progress, not all patients with RA respond to current treatment strategies. To improve and predict response to current RA treatment strategies, it will be essential to unravel the mechanisms underlying RA pathology and therapy resistance.

T cells and their cytokines are central players in RA pathogenesis. In particular, Th17 cells were extensively researched in the context of RA, and were shown to be able to activate and attract inflammatory cells to the joint, thus promoting RA inflammation and destruction. Next to the production of IL-17A, Th17 cells are characterized by the expression of CCR6. However, the pathological mechanisms underlying the action of CCR6+ Th cells are not completely understood. In addition, the CCR6+ Th cell population is comprised of various subpopulations with distinct characteristics, such as the expression of transcription factors, chemokine receptors and the production of cytokines. It is currently unclear whether all these subpopulations can be identified in patients with RA and which contribute to RA

pathology. Moreover, it is unclear how these cells respond to current treatment strategies in RA, and if their presence differs between different subsets of rheumatoid arthritis, such as auto-antibody positive and negative RA.

Therefore in **chapter 2,** a review is provided about the current understanding of specific characteristics and markers associated with CCR6+ Th cells, their role in RA pathogenesis and treatment approaches to target CCR6+ Th cells in RA.

One of the pathogenic activities of CCR6+ Th cells is the capacity to induce a pro-inflammatory loop when cultured together with RA-derived synovial fibroblasts (RASF). This loop is dependent on autocrine IL-17A production, and reflects inflammatory processes in the RA joint. In **chapter 3**, the mechanisms responsible for the autocrine IL-17A production were investigated.

CCR6+ Th cells can be divided into several subpopulations. In **chapter 4 and 5**, these subpopulations were investigated in peripheral blood and synovial fluid of patients with RA. The pathogenicity of these subpopulations was characterized, and expression of multidrug transporters by CCR6+ Th subpopulations was related to treatment response in patients with RA.

RA patients positive for the presence of serum ACPAs have a worse disease course with more development of erosions. It is postulated that the disease etiology in ACPA+ patients differs extensively from the etiology in ACPA- patients. In **chapter 6**, it was investigated whether the CCR6+ Th cell subpopulations were associated with the more severe disease course observed in ACPA+ patients with RA.

In addition to CCR6+ Th cells, other lymphocytes can express multidrug transporters. In **chapter 7**, the presence and activity of multidrug transporters on other lymphocyte populations were investigated in relation to DMARD and glucocorticoid therapy response in patients with RA.

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Chapter 2

The role and modulation of CCR6+ Th17 cell populations in rheumatoid arthritis

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Cytokine, 2015 Jul;74(1):43-53.

Abstract

The IL-17A producing T-helper-17 (Th17) cell population plays a major role in rheumatoid arthritis (RA) pathogenesis and has gained wide interest as treatment target. IL-17A expressing Th cells are characterized by the expression of the chemokine receptor CCR6 and the transcription factor RORC. In RA, CCR6+ Th cells were identified in peripheral blood, synovial fluid and inflamed synovial tissue. CCR6+ Th cells might drive the progression of an early inflammation towards a persistent arthritis.

The CCR6+ Th cell population is heterogeneous and several subpopulations can be distinguished, including Th17, Th22, Th17.1 (also called non-classic Th1 cells), and unclassified or intermediate populations. Interestingly, some of these populations produce low levels of IL-17A but are still very pathogenic. Furthermore, the CCR6+ Th cells phenotype is unstable and plasticity exists between CCR6+ Th cells and T-regulatory (Treg) cells and within the CCR6+ Th cell subpopulations. In this review, characteristics of the different CCR6+ Th cell populations, their plasticity, and their potential impact on rheumatoid arthritis are discussed.

Moreover, current approaches to target CCR6+ Th cells and future directions of research to find specific CCR6+ Th cell targets in the treatment of patients with RA and other CCR6+ Th cell mediated autoimmune-diseases are highlighted.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease mainly affecting synovial joints. In early disease, pro-inflammatory cells migrate to the synovium and induce a chronic inflammation. As the disease progresses, synovial hyperplasia, angiogenesis and pannus tissue formation will be induced. Without proper treatment, these processes will ultimately lead to irreversible joint destruction. Although RA disease etiology is still largely unknown, genetic susceptibility, auto-antibody formation and environmental factors, such as smoking, are major RA risk factors [1-3].

Inflammatory T cells and their cytokines play a central role in the pathogenesis of RA. Already early in the disease process inflammatory T cells and their cytokines are present in the joint synovium and contribute to the progression of an early synovial inflammation towards persistent and chronic arthritis [2, 4-6].

Cytokines produced by these synovial T cells include tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ) and interleukin-17A (IL-17A) [6-10]. Initially, the production of these pro-inflammatory cytokines was attributed to T-helper-1 (Th1) cells. Later it became clear that within Th cells, IL-17A production was restricted to a specific Th cell subpopulation, which was therefore named Th17. During the last decade of research great progress has been made in understanding the complexity of Th17 biology. In this regard, processes underlying Th17 differentiation and activation, as well as Th17-specific cytokines, transcription factors, chemokines, and other surface receptors were identified [11]. From these studies it also became clear that within human T cells, IL-17A expression is confined to memory T cell expressing the transcription factor RAR-related orphan receptor-C (RORC) and the chemokine receptor CCR6 [12, 13].

In patients with RA, CCR6+ Th cells have been found in the inflamed synovium and increased proportions of peripheral blood CCR6+ Th cells have been found in patients with early RA [14-16]. The characterization of CCR6+ Th cells revealed a pathogenic signature, including pro-inflammatory cytokine production, and *in vitro* studies showed potent pathogenic activity of CCR6+ Th cells obtained from patients with RA [14, 17].

However, the CCR6+ T cell population is heterogeneous and several subpopulations can be distinguished by differential expression of additional chemokine receptors [12, 18]. These CCR6+ Th subpopulations are all positive for RORC expression but differ in the level of cytokine production and expression of other transcription factors [19, 20]. Recent findings now show that the pathogenic signature is not confined to cells producing high levels of IL-17A, but that even CCR6+ Th cell populations producing low amounts of IL-17A display a pathogenic signature and activity [19]. In addition, it also becomes clear that the phenotype of CCR6+ T cells is unstable and that plasticity exist between CCR6+ T cell subpopulations and Th1 and Treg cells and between CCR6+ Th cell populations [21].

Here we summarize the current knowledge on the role of CCR6+ Th cells in the pathogenesis of RA. Moreover, we provide an overview of specific characteristics and markers associated with the effector function of CCR6+ Th cells. CCR6+ Th cell subpopulations and their plasticity are reviewed and placed in the context of RA. We also review the current approaches to target CCR6+ Th cells in RA. Finally, we

suggest future directions of research to find CCR6+ Th cell-specific targets, with therapeutic potential for the treatment of RA patients and potentially other CCR6+ T cell mediated autoimmune disorders.

2. Human CCR6+ T cell features under physiological circumstances

With the discovery of human IL-17A expressing CD4+ (Th) cells (currently referred to as Th17 cells) [11, 22], began the search for features that distinguish these cells from other Th cell subsets. Within Th cells, IL-17A expression is restricted to memory cells expressing the chemokine receptor CCR6 on their surface [12]. The only known ligand for CCR6 is the chemokine CCL20 [23, 24], which is highly expressed at sites of inflammation, including the inflamed RA joint synovium [25]. Upon binding of CCL20, CCR6+ Th cells become activated and migrate along the CCL20 gradient toward the RA joint synovium [25].

Under normal conditions, CCR6+ Th cells and their cytokines directly or indirectly induce and activate neutrophils and orchestrate the elimination of extracellular bacteria and fungi [11, 26, 27]. However, CCR6+ Th cells, and overproduction of their signature cytokines, are associated with persistent inflammation and autoimmune diseases, like RA [14, 28]. Currently, a broad array of effector molecules, surface receptors and transcription factors are identified which are involved in the effector function of CCR6+ T cells. These are summarized in the following paragraphs and depicted in figure 1.

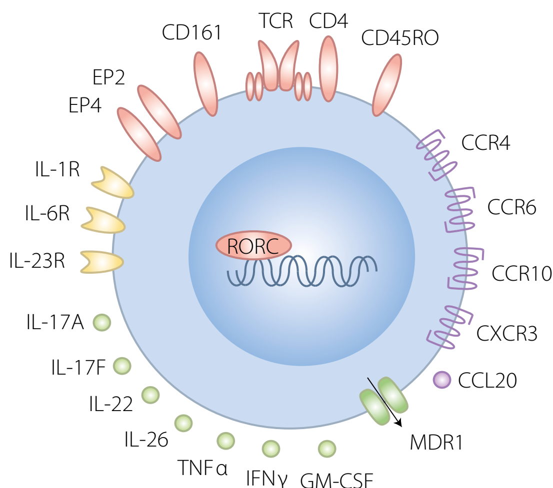

Figure 1. Phenotypical features of human CCR6+ Th cells. Within the CD4+ T cell population CCR6 is expressed by memory (CD4+CD45RO+) TCR expressing cells. All CCR6+ Th cells express the transcription factor RORC, the cytokine receptors IL-1R, IL-6R and IL-23R and the PGE_2 receptor (EP4). Subpopulations of CCR6+ Th cells express the PGE_2 receptor EP2, the c-type lectin CD161, the multi-drug transporter MDR1 and the chemokine receptors CCR4, CCR10 and CXCR3. Within Th cells the expression of the cytokines IL-17A, IL-17F, IL-22 and IL-26 and the chemokine CCL20 is confined to CCR6+ Th cells. In addition CCR6+ Th cells are capable of expressing the cytokines TNFα, IFNγ and GM-CSF.

2.1. CCR6+ Th cell associated cytokines IL-17A, IL-17F, IL-22 and IL-26

Expression of IL-17A, the signature cytokine of Th17 cells, is mainly confined to CD45RO+ (memory) CCR6+TCRαβ+ Th cells [12, 29, 30]. IL-17A binds to IL-17RA and IL-17RC and signals through the IL-17RA/IL-17RC complex. IL-17RA is ubiquitously expressed on many different cell types but the expression of IL-17RC is more restricted, and is mainly expressed on epithelial, endothelial and stromal cells [31, 32]. IL-17A binding to its receptor results in the production of wide array of effector molecules, such as pro-inflammatory cytokines (IL-1β and IL-6), chemokines (CCL20 and IL-8), and anti-microbial proteins [31].

Like IL-17A, IL-17F production by Th cells is confined to memory CCR6+ Th cells [12, 30]. IL-17F shares 50% homology with IL-17A [33]. Similar to IL-17A, IL-17F signals via IL-17RA and IL-17RC [31, 34]. IL-17F signaling induces similar effector molecules as IL-17A, but is less potent than IL-17A signaling. For example, IL-17A alone induces more genes in RA synoviocytes than IL-17F alone, and there were no genes that were induced by IL-17F but not by IL-17A [35, 36]. Interestingly, activated T cells are able to produce IL-17A/F heterodimers [37], of which the contribution to human disease remains unclear [36].
Within memory Th cells, IL-22 expression is confined to CCR6+ Th cells and Th1 cells [38]. IL-22 signals via the heterodimeric receptor consisting of IL-22R1 and the broadly expressed IL-10R2 [39]. IL-22R1 is mainly expressed on epithelial cells, keratinocytes and hepatocytes. In these cells, IL-22 signaling induces proliferation, and production of: 1) innate defensive mechanisms (β-defensins, S100 proteins, REG proteins and mucins); 2) pro-inflammatory mediators (IL-1β, IL-6 and G-CSF); 3) and chemokines (CXCL1, CXCL5 and CXCL9) [38, 40].

IL-26 is expressed by memory CCR6+ Th cells and signals via the heterodimeric receptor consisting of IL-20R1 and IL-10R2 [13, 41]. IL-20R1 is mainly expressed on epithelial cells, in which IL-26 induces IL-8 and TNFα expression [41].

2.2. TNFα, GM-CSF and IFNγ production by CCR6+ Th cells

TNFα is produced by many cell types, including stromal cells, macrophages and T cells including CCR6+ Th cells [14, 42] and signals via TNF-R1 and TNF-R2, of which the latter is specifically expressed by hematopoietic cells [42]. TNFα is a strong inducer of pro-inflammatory cytokine production by stromal cells, like IL-1β, IL-6, IL-8 and GM-CSF, and the pro-inflammatory mediator prostaglandin E_2 (PGE_2) [6, 8].

Granulocyte-macrophage colony stimulating factor (GM-CSF) is produced by, among others, CCR6+ Th cells and Th1 cells [43, 44]. It signals via the common β-chain and GM-CSF receptor-α heterodimer and stimulates macrophage and granulocyte activation, including pro-inflammatory cytokine production [45].

Within Th cells, IFNγ is produced by Th1 cells and CCR6+ Th cells [12, 14]. IFNγ signaling via the IFNγR activates macrophages and neutrophils by processes including the up-regulation of MHCII expression and antigen processing components [46].

2.3. Cytokine- and chemokine receptors

IL-1R1, IL-23R and the heterodimer IL-6Rα/gp130 are expressed by CCR6+ Th cells, enabling IL-1β, IL-23 and IL-6 signaling [11]. These cytokines are involved in the differentiation of IL-17 producing Th cells from naïve T cells and in the polarization of CCR6+ Th cells [17, 30, 47].

CCR6+ Th cells are able to express additional chemokine receptors on their cell surface, which are associated with different cytokine profiles (discussed in section 4). CCR6+ Th cells expressing CCR4 produce high levels of IL-17A, whereas CXCR3 expressing cells express high levels of IFNγ and low levels of IL-17A [12]. Moreover, CCR10 expressing CCR6+ Th cells produce IL-22 in the absence of IL-17A and IFNγ [18, 48]. In addition, IL-17A producing CCR6+ Th cells express the chemokine receptors CCR5, CXCR4, and CXCR6 [49].

2.4. Surface receptors CD161, EP2 and EP4

A subgroup of CCR6+ Th cells express the c-type lectin CD161 [50], which is also expressed by natural killer cells (NK) and NK-T cells. CD161 function on CCR6+ Th cells is relatively unexplored [51]. CD161 has various ligands, and CD161-ligand binding has roles in: 1) cytokine production by T cells; 2) co-stimulation in the absence of CD28; and 3) chemotactic-independent trans-endothelial migration [51-53].

Th cells express the PGE_2 receptors, EP2 and EP4, and PGE_2 signaling induces IL-17A, IL-23R and IL-1R expression by CCR6+ Th cells [17, 54]. It was recently found that IL-17A producing T cells do not express EP2, because RORC directly represses EP2 expression [55]. However, in autoimmune disease conditions, EP2 expression on IL-17A producing T cells is induced and PGE_2 signaling results in a pathogenic phenotype including increased IFNγ, GM-CSF and IL-22 expression [55].

2.5. Transcription factors

An essential factor for the differentiation and function of IL-17A producing T cells is the transcription factor RAR-related orphan receptor C (RORC) [56, 57]. Interestingly, RORC expression is required for the expression of CCR6 by Th cells [13, 58]. In cooperation with IRF4, BATF and STAT3, RORC acts as a direct transcriptional regulator of IL-17A, IL-17F, IL-23R and GM-CSF [11]. The regulation of RORC induction and activity is complex and multiple positive and negative modulators are identified (reviewed by [11, 59, 60]).

3. CCR6+ Th cells in the pathogenesis of rheumatoid arthritis

Increased proportions of CCR6+ Th cells are found in peripheral blood of early and established RA patients [14]. In addition, CCR6+ Th cells are present in synovial fluid and in the inflamed synovial tissue of RA patients and juvenile idiopathic arthritis (JIA) patients. In these patients, CCR6+ Th cells proportions are higher in inflamed synovium than in peripheral blood [15, 16]. CCR6+ Th cells play a pivotal role in the development of RA, because they produce pro-inflammatory cytokines, attract and activate monocytes and neutrophils, activate synovial fibroblasts and induce osteoclast differentiation (figure 2) [6, 8].

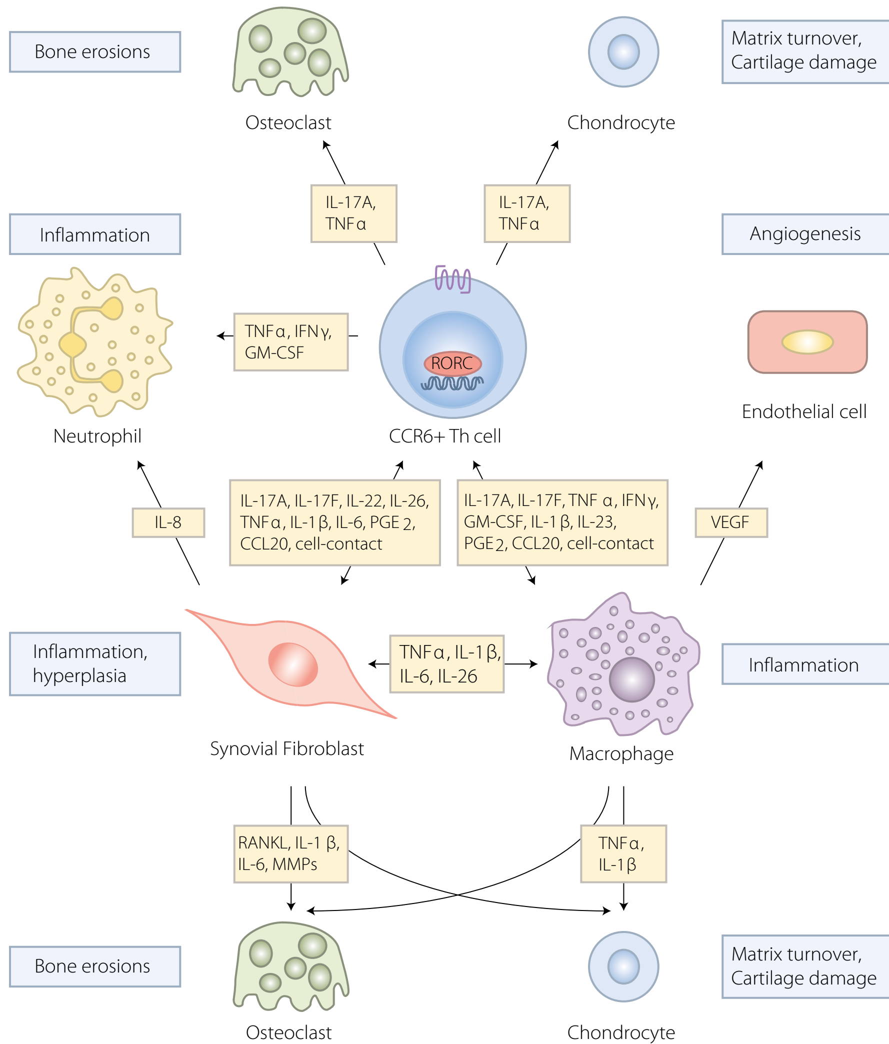

Figure 2. CCR6+ Th cells and their effector cytokines and chemokines play a central role in the pathogenesis of RA. Arrows originating from the CCR6+ Th cell indicate effector pathways directly mediated by CCR6+ Th cells resulting in the activation of synovial fibroblasts, macrophages, neutrophils, osteoclasts and chondrocytes. Bidirectional arrows indicate activating interactions between cell types. Arrows originating from other cell types than the CCR6+ Th cell indicate effector pathways indirectly induced by CCR6+ Th cells. The effector molecules and interactions involved in the indicated pathways are listed in yellow boxes. Clinical manifestations, associated with the activation of the indicated cell-types are listed in blue boxes.

3.1. CCR6+ Th cell effector cytokines in RA

The CCR6+ Th effector molecules IL-17A, IL-17F, IL-22, IL-26, TNFα, IFNγ, GM-CSF and the chemokine CCL20 are present in RA synovial fluid and are involved in RA pathogenesis [6, 25, 61-63]. Additionally, synovial CCR6+ Th cells expressing these cytokines and chemokines are found in established and active RA [15, 25, 64, 65].

Peripheral and synovial RA-derived CCR6+ Th cells are able to co-express cytokines, for example IL17A+TNFα+, IL17A+IL-22+ or IL17A+IFNγ+, double expressing cells [61, 65-67]. RA disease activity correlated positively with peripheral blood proportions of IL-17A+TNFα+ co-expressing (CCR6+) T cells and proportions of IL-17A expressing CCR6+ T cells [15, 67], indicating the important role of cytokines in RA pathogenesis.

3.2. Activation of synovial fibroblasts

Activated synovial fibroblasts play an important role in RA propagation [68, 69]. Synovial fibroblasts express receptors for the CCR6+ Th cell cytokines IL-17A, IL-17F and TNFα. Signaling by these cytokines, which have a synergistic effect on each other [22, 35, 70], and/or direct interaction with CCR6+ Th cells causes activation of synovial fibroblasts and induces pro-inflammatory cytokine (IL-1β, IL-6, IL-22, IL-26, GM-CSF) production [7, 22, 36, 62, 71-73]. Although synovial fibroblasts do express IL-22R1, IL-22 signaling does not contribute to CCR6+ Th cell induced pro-inflammatory cytokine production [65, 74]. Interestingly, synovial fibroblasts produce IL-1β and IL-6, and also express the IL-1β and IL-6 receptors, enabling autocrine IL-1β and IL-6 to enhance further activation [75].

CCR6+ Th cells are superior to CCR6- Th cells in activating synovial fibroblasts. While IL-17A and TNFα activate synovial fibroblasts to some extent, direct cell-cell contact between CCR6+ Th cells and synovial fibroblasts is required for strong activation. This suggests the presence of an additional co-stimulatory molecule, but which molecule or molecules are responsible for the interaction is not yet elucidated [14, 17, 64, 65].

Activation of synovial fibroblasts has multiple effects, which contribute to RA pathology: 1) The pro-inflammatory cytokines produced by synovial fibroblasts activate and/or recruit other synovial cells, including macrophages, chondrocytes and neutrophils [6, 36, 69]; 2) Upon CCR6+ T cell interaction, synovial fibroblasts produce matrix metalloproteinases (MMPs), which are involved in cartilage destruction and matrix turnover [14, 76]; 3) Synovial fibroblasts further respond to activation by producing chemokines, including IL-8, CCL7, CCL20 and CXCL12, which are involved in the recruitment and/or activation of cells like granulocytes, monocytes and CCR6+ Th cells [25, 36, 68].

Next to direct cell-cell activation, cytokine signaling can activate fibroblasts, with specific effects depending on the cytokine. TNFα and IFNγ signaling in synovial fibroblasts results in production of B cell activating factor (BAFF), which is involved in B cell survival and activation [77]. IL-17A signaling on synovial fibroblasts induces vascular endothelial growth factor (VEGF), which is involved in local angiogenesis [78]. In addition, IL-17A signaling is involved in inducing receptor activator of nuclear factor kappa-B ligand (RANKL) expression on fibroblasts [79]. RANKL is a key factor for the differentiation and

activation of bone resorbing osteoclasts [68]. RANKL expressing synovial fibroblasts might be involved in osteoclastogenesis [69, 80], because they induce PGE_2 and RANKL production by osteoblasts, which leads to osteoclast differentiation [81]. In this context, IL-17 producing Th cells were found in close proximity to osteoclasts in biopsies from inflamed joints [82].

3.3. Activation and recruitment of monocytes, macrophages and neutrophils

Monocytes and macrophages are present in the inflamed RA joint synovium, and are an important source of pro-inflammatory cytokines [2, 83]. Direct CCR6+ Th cell interaction or receptor binding of the cytokines IL-17A, IL-17F, IL-26, GM-CSF or IFNγ leads to macrophage activation and/or induces production of the pro-inflammatory cytokines TNFα, IL-1β, IL-6, IL-23 and GM-CSF. These cytokines in turn act on osteoblasts, chondrocytes, synovial fibroblasts and neutrophils [6, 62, 77], thus propagating RA pathogenesis. In addition, activated macrophages can produce BAFF and VEGF and chemokines including IL-8, CCL5, CCL20 [2, 84-86].

Neutrophil frequencies are increased in synovial fluid and synovial tissue of RA patients, particularly in early disease stages, and play a role in joint destruction [87, 88]. Cytokines produced by activated CCR6+ Th cells enhance neutrophil migration *in vitro*. GM-CSF, TNFα and IFNγ, but not IL-17A, produced by CCR6+ Th cells directly activate neutrophils, resulting in the expression of IL-8, TNFα, IL-1β and CCL20 [43, 87-90].

3.4. Polarization of CCR6+ Th cells by synovial fibroblasts and monocytes

CCR6+ cells activate fibroblasts and monocytes, as discussed above, but activated synovial fibroblasts and monocytes in turn can also polarize CCR6+ Th cells. When synovial fibroblasts interact with CCR6+ Th cells, a pro-inflammatory feed-back loop is induced, resulting in a specific up-regulation of IL-17A expression, but not IL-22, IFNγ and TNFα, by the CCR6+ Th cells. Direct cell-cell contact is indispensable for the induction of the pro-inflammatory loop [14, 64, 65, 91]. Synovial fibroblast production of PGE_2, and to a lesser extent IL-1β and IL-6, is responsible for the specific induction of IL-17A [17]. Other mechanisms that induce IL-17A production by CCR6+ T cells by synovial fibroblasts include hypoxia and TLR signaling [92-94]. In agreement with this, hypoxia inducible factor (HIF)-2α is expressed in RASF and up-regulates IL-6 expression, leading to Th17 polarization [95].

Next to synovial fibroblasts, activated monocytes, derived from peripheral blood or inflamed joints from RA patients, specifically induced Th17, but not Th1 or Th2 responses. This Th17 cell polarization was critically dependent on direct cell-cell contact [96-98] and on PGE_2 in combination with IL-1β and IL-23 [54, 99].

4. CCR6+ Th subpopulations

In many reports, in particular in the RA field, IL-17A producing Th cells are referred to as Th17 cells. Although the expression of IL-17A is a major characteristic of Th17 cells, these cells have additional features, as described above. By ignoring these features, such as CCR6 and RORC expression, pathogenic Th cell populations can be missed. Moreover, CCR6+ Th cells that do not express IL-17A *ex vivo*, can rapidly transform into IL-17A producers under inflammatory conditions *in vitro*. This suggests that *ex vivo* analysis of IL-17A expression may greatly underestimate the frequency and possibly pathogenicity of human Th17 cells [100].

It is proposed that not all CCR6+ subpopulations are equally pathogenic, regardless of cytokine profile [101, 102]. This is relevant for RA, since highly pathogenic CCR6+ Th cells displaying Th1 characteristics were identified in autoimmune disease settings [19]. This elegant paper shows that not all CCR6+ IL-17A producing subpopulations are equally pathogenic, and poses a strong appeal for better classification of Th17 subpopulations. Here we summarize the different properties and pathogenic features of distinctive CCR6+ and RORC+ subpopulations (figure 3).

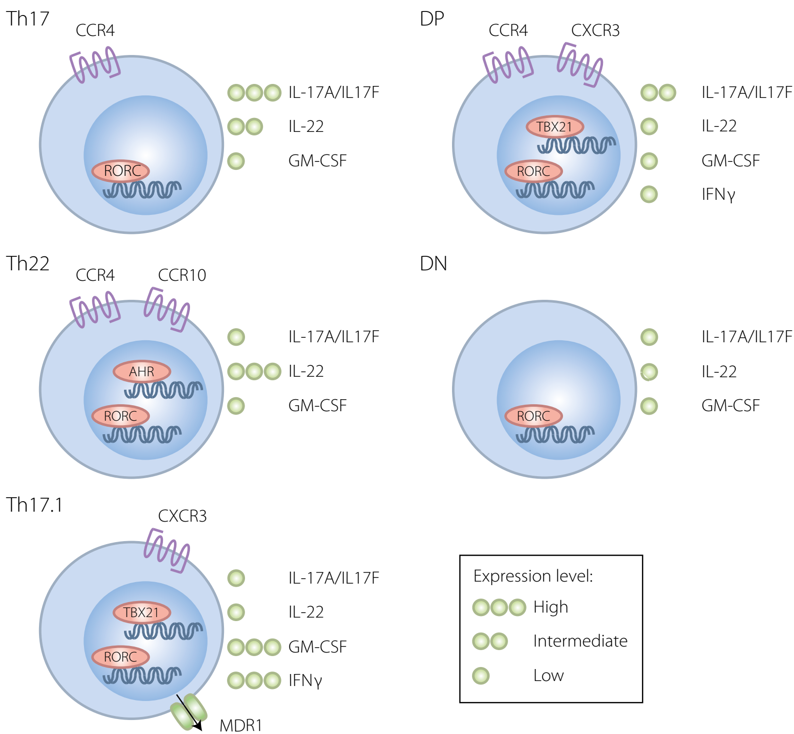

Figure 3. Human CCR6+ Th subpopulations. Although all CCR6+ subpopulations express RORC, they differ strongly in the expression of indicated transcription factors, chemokine receptors, MDR1 and in the capacity to produce effector cytokines upon TCR signaling.

4.1. Th17 cells

Several markers identify classic Th17 cells, defined as CCR6+ Th cells producing high amounts of IL-17A. Besides CCR6, Th17 cells selectively express CCR4, but not CXCR3 [12, 20, 29]. In a functional assay, classic Th17 cells were more resistant to autologous Treg suppression *in vitro* than Th1 and Th2 cells [29]. Classic Th17 cells were found in peripheral blood and synovial fluid of RA patients [15, 65].

4.2. Th22 cells

There is an ongoing debate whether Th22 cells are a separate population from Th17 cells [103]. Both express RORC and produce IL-17A, although the production of IL-17A is higher in Th17 cells, but in Th22 cells there seems to be an enrichment for the production of IL-22, and the transcription factor AHR is expressed next to RORC [18, 48]. In mice, the Th1-associated transcription factor TBX21 has a clear role in sustaining IL-22 expression in CD4+ T cells [104]. However, in human Th22 cells relatively low TBX21 expression levels were found [18, 48] and these murine findings have not yet been confirmed for human Th22 cells. Like Th17 cells, Th22 cells express high levels of CCR6 and CCR4, but in contrast to Th17 cells they also express CCR10 [18, 48]. Although Th22 cells play a pathological role in psoriasis, their role in RA is less clear [105]. IL-22 levels in serum of RA patients are higher than in healthy controls, and concentrations in synovial fluid are higher than serum. Serum levels of IL-22 are positively correlated with the development of erosions [106]. However, we have found that CCR6+ Th mediated synovial inflammation is not dependent on Th22 cells [65].

4.3. Th17.1 cells

Th17.1 cells, or non-classic Th1 cells, are CCR6+ Th cells with both Th17 and Th1 cell characteristics. Like Th17 cells, Th17.1 cells are positive for CCR6, RORC and IL-23R, but negative for CCR4 expression, and they produce IL-17A [12, 20, 29, 107]. In contrast to Th17 cells, but similar to Th1 cells, Th17.1 cells express TBX21 and CXCR3, and they produce high amounts of IFNγ [20].

Th17.1 cells are identified in many autoimmune diseases [102], including RA, where high proportions of Th17.1 cells are found in peripheral blood and at sites of inflammation, including the joint synovium [20, 61, 64, 107-109].

Th17.1 are hypothesized to be the most pathogenic of the CCR6+ subpopulations [102]. In comparison to Th17 cells, Th17.1 cells show an increased ability to proliferate in response to TCR signaling [20]. Furthermore, Th17.1 cells express high GM-CSF levels, and in disease settings a pathogenic signature was found including increased TBX21, STAT4 and RUNX expression and decreased IL-10 and AHR expression [44, 55]. Th17.1 cells specifically up-regulate multidrug resistance-1 (MDR1), a transmembrane pump that can transport substances out of the cell. The MDR1+ Th17 were considerably enriched for pro-inflammatory compounds and IL-23R but depleted in anti-inflammatory factors like IL-10. These pro-inflammatory and highly IL-23 responsive MDR1+Th17.1 cells were enriched in the affected gut tissue of patients with Crohn's disease. Most importantly, MDR1+Th17.1 cells were also shown *in vitro* to be resistant to glucocorticoid-mediated suppression, which might explain their enrichment in Crohn's disease [19].

4.4. Unclassified CCR6+ Th cells

The above described CCR6+ populations are, among others, distinguished by their selective expression of CXCR3 or CCR4. Besides these CXCR3 or CCR4 single positive cells, there are also CCR6+ Th cells double-positive (DP) or double-negative (DN) for CCR4 and CXCR3 expression [12]. DP CCR6+ Th cells have both Th17 and Th1 characteristics, suggesting that they are an intermediate Th17-Th17.1 population. The origin of DN Th cells is unclear. They possess Th17 cell characteristics, like RORC and CD161 expression, but IL-17A and IL-17F for example are expressed at a low level compared to the other CCR6+ subpopulations. Both DN and DP CCR6+ Th are present in both healthy and RA peripheral blood, and in RA synovium, although percentages vary extensively between patients. Furthermore, DN and DP CCR6+ Th populations of patients with RA more strongly activate synovial fibroblasts than Th1 and naïve cells (Paulissen *et al.*, unpublished results).

5. Plasticity of CCR6+ Th cells

Plasticity is a process in which a cell switches from one lineage to another lineage, or to an 'in-between' stage, which is usually determined by analyzing cytokine production and transcription factor expression [110]. Plasticity is at least partly regulated by epigenetic modifications of transcription factors and signature cytokines [111]. It is striking that CCR6+ Th cells stay very plastic throughout their entire differentiation stage [110, 112]. Plasticity between CCR6+ Th populations cells and conversion of Treg to Th17 cells have been reported, and will be summarized below.

5.1. Conversion of Th17 to Th17.1 cells

There are various findings supporting the conversion of Th17 to Th17.1 cells. For example, stimulation of human Th17 clones with IL-12 in the presence or absence of IL-23 leads to a down-regulation of RORC and IL-17 expression and an up-regulation of TBX21 and IFNγ expression [29, 113]. In the context of RA, clonal TCR repertoire analysis provided evidence for the conversion of IL-17A+ cells to IL-17A+IFNγ+ and IFNγ+ Th cells [107]. Of interest, the Th17-derived Th17.1 cells are linked to parameters of inflammation and are CD161+, indicating that this subpopulation might be more pathogenic at sites of inflammation than classic Th1 cells [108].

Not all subpopulations are equally stable. Under non-polarizing culture conditions IL-17A+ or IFNγ+ single-positive Th cells are relatively stable, because they mostly stay IL-17A+ or IFNγ+ single-positive. In contrast, IL-17A+IFNγ+ double-positive and IL-17A-IFNγ- double-negative cells convert mostly to IFNγ+ Th cells. This suggests that in particular IL-17A-IFNγ- and IL-17A+IFNγ+ CCR6+ Th cells are plastic and that they may resemble intermediate CCR6+ Th cell populations [113].

While some studies show Th17 cells converting to Th17.1 cells, little evidence is present for the reverse conversion of Th17.1 to Th17.

5.2. Plasticity from Treg cells to CCR6+ Th cells

Treg cells are involved in the suppression of T cell responses and are mainly characterized by expressing high levels of IL-2 receptor (CD25) and the transcription factor FOXP3 [114].

In peripheral blood and lymphoid tissue CCR6+CCR4+CXCR3- Th cells are identified that have characteristics of both Th17 (IL-17, CD161 and RORC expression) and Treg (IL-10, CTLA4 and FOXP3 expression) [115-118]. These Th17-like Tregs were found in patients with autoimmune diseases, including RA, where they are present in peripheral blood and synovial fluid [119-123]. Th17-like Tregs isolated from peripheral blood of RA patients showed suppressive activity *in vitro*, and their numbers in serum negatively correlated with inflammatory markers like CRP. Interestingly, Th17-like Tregs isolated from synovial fluid did not have suppressive activity, indicating that these Th17-like Tregs are protective in serum but pathogenic in synovial fluid [123].

In vitro, conversion of Treg cell to Th17-cells was accomplished upon IL-1β, IL-6 and IL-23 exposure or TLR2 signaling [115, 116, 124, 125]. It is very likely that plasticity is dependent on epigenetic modifications, since the presence of a histone deacetylase (HDAC) inhibitor almost completely blocked Th17 development [115, 116, 118].

6. Specific CCR6+ Th cell modulation

Th17 cells can be modulated by inhibiting their development, inhibiting cytokine production or disrupting signaling. Several compounds capable of inhibiting Th17 cells are or were being developed for use in RA, including antibodies against IL-17A, IL-17RA, and IL-23R, inhibitors for RORC and vitamin D. Here, pre-clinical or (where available) clinical data on the use of these compounds in RA is summarized.

6.1. IL-17A antibodies

Several clinical trials with antibodies against IL-17A have been performed, with varying results. Secukinumab (AIN457) is a human monoclonal IL-17A antibody. In an initial trial RA patients with active disease despite methotrexate (MTX) therapy were included. Compared to placebo, secukinumab therapy significantly increased the proportion of ACR20 responders and more strongly reduced C-reactive protein (CRP) and disease activity score in 28 joints (DAS28) [126]. In a follow-up phase II study, RA patients with active disease despite MTX treatment were included. ACR20 response was higher in the secukinumab treated group than in the placebo group, and DAS28-CRP was significantly lower. The overall safety profile was comparable between placebo and treatment groups [127]. In the same study, subjects were divided in a non-responder group (no ACR20 response reached at week 16) and a responder group (ACR20 response reached at week 16). In the responder group, 55% of patients on the second highest dose secukinumab reached ACR50 at week 52 [128]. Currently, Novartis is running phase III trials to investigate the long term efficacy, safety and tolerability in patients with RA and in anti-TNFα non-responders (clinicaltrails.gov identifiers: NCT01640938, NCT01377012, NCT01350804, NCT01770379, NCT01901900).

Ixekizumab (LY2439821) is a humanized IL-17A monoclonal antibody. Ixekizumab treatment significantly decreased DAS and CRP adjusted to baseline and significantly improved the proportion of ACR20 achievers compared to placebo (90% versus 56%). Adverse events were mostly mild and occurred in both placebo as treatment groups, and no dose-response relationships were noted [129]. In a subsequent phase II study, ixekizumab was given to RA patients who were biologicals-naïve or to patients with an inadequate response to anti-TNFα treatment. In both groups, ACR20 and ACR50 rates were significantly higher in treatment group compared to placebo group, and CRP adjusted for baseline was significantly improved. Adverse events occurred with similar frequencies in treatment and placebo groups [128]. Eli Lilly is currently developing ixekizumab for use in psoriasis and psoriatic arthritis.

6.2. IL-17RA antibody

Brodalumab (AMG 827) is a human monoclonal antibody directed against IL-17RA. A phase I study showed that brodalumab lead to an inhibition of IL-17R signaling, and adverse events were reported in comparable frequencies between treatment and placebo groups. However, results concerning efficacy were discouraging: rates of ACR20 and ACR50 achievers were comparable in treatment and placebo groups [130]. In a subsequent phase II study no significant changes were found in ACR20, ACR50 or ACR70, DAS28 change from baseline, or CRP levels changed from baseline. The study concludes that these results do not support further development of brodalumab for RA [131].

6.3. RORC inhibitors

Several inhibitors of RORC have been described in literature. These RORC inhibitors specifically inhibited Th17 differentiation, reduced expression of IL-17A and other Th17 signature genes, like IL-23R. Some of these inhibitors have been tested in animal RA models and have shown delayed incidence and/or reduced severity of experimental arthritis and experimental autoimmune encephalomyelitis [132, 133]. To our knowledge, no clinical studies investigating RORC inhibitors are currently running (http://clinicaltrials.gov/).

6.4. IL-23 antibodies

Ustekinumab is a human monoclonal antibody directed against the p40 subunit shared by IL-23 and IL-12. It is approved in the US and Europe for the treatment of moderate to severe plaque psoriasis and moderate to severe psoriatic arthritis. In these diseases, it is safe and efficient [134]. Concerning RA, Janssen Research and Development completed a phase II study in RA patients in which they investigated ustekinumab and guselkumab, a human monoclonal antibody directed against the p19 subunit of IL-23. No data of this trial have been published yet (clinicaltrials.gov identifier NCT01645280).

6.5. Vitamin D and COX-2 inhibitors

A meta-analysis of the literature concerning vitamin D suggests that low vitamin D intake is associated with a higher risk of RA development. Furthermore, vitamin D level is associated with RA activity [135]. One randomized controlled trial compared a triple DMARD therapy with or without supplementation of the active vitamin D metabolite, 1,25-(OH)2D3. The combination of triple DMARD therapy with 1,25-(OH)2D3 led to higher pain relief, but no significant differences in DAS28 were found [136].

The targets of vitamin D are pleiotropic, but one possible beneficial immunomodulatory mechanism is through direct inhibition of CCR6+ Th cell cytokine expression and activity [64, 137]. TNFα inhibition does not disrupt the Th17 pathway, and IL-17 inhibition does not reduce the production of TNFα [14]. There is some evidence that anti-TNFα treatment in RA patients leads to increased IL-17A+CD4+ cells and increased IL-17A serum levels [138, 139]. *In vitro* studies showed that in comparison to anti-TNFα mono-treatment, the combination of anti-TNFα and vitamin D has additional suppressive effects on CCR6+ Th cytokine expression and activity [64].

Recently, a clinical trial has been completed in which RA patients with low vitamin D levels were given vitamin D or placebo for a year. Results have not yet been published (clinicaltrails.gov identifier NCT00423358). More randomized controlled trials are needed to establish efficacy in RA patients.

COX-2 inhibitors are used in RA for symptomatic relief. It reduces joint pain and improves motility, but does not inhibit disease progression [140]. PGE_2, the product of COX-2, induces Th17 polarization [54, 99]. Recently, we described that the COX-2 inhibitor celecoxib reduces IL-17A production by CCR6+ Th cells in a co-culture system with synovial fibroblasts. Moreover, in this system celecoxib reduced pro-inflammatory cytokine and MMPs production. Interestingly, this was accompanied by an increase in TNFα production by CCR6+ Th cells, which could explain why disease progression is not inhibited by celecoxib treatment [17]. Therefore, the combination of COX-2 inhibitors with biologicals (such as anti-TNFα) could be valuable in RA treatment.

6.6. CCR6-CCL20 inhibitors

CCR6 and its ligand CCL20 are of pivotal importance in Th17 trafficking to the RA synovium, and is therefore an interesting treatment target in RA [25, 61]. To our knowledge, no inhibitors of CCR6 and CCL20 are currently under clinical investigation for RA, but a CCL20 antibody (GSK3050002) is under investigation for use in ulcerative colitis (clinicaltrials.gov identifier: NCT01984047).

7. Conclusion and future directions for research

Th17 cells play an important role in RA, by virtue of the (pro-inflammatory) cytokines they produce and because they attract and/or activate cells important in the inflamed joint (e.g. neutrophils, macrophages, osteoclasts and synovial fibroblasts). Cells belonging to the Th17 lineage can be divided into several subpopulations. All of these subpopulations share Th17 properties like RORC, CCR6 and IL-23R expression, and all of them can produce IL-17A, albeit in different quantities. The question remains whether the subpopulations are truly separate, or whether they represent plasticity and heterogeneity

within the Th17 lineage. Several reports indicate that Th17 cells can convert to Th17.1 cells (section 5.1). To confirm that this process is truly plasticity, fate mapping of single cells should be performed. Although the published literature is indicative of plasticity, the possibility that the observed variation in CCR6+ subsets reflects the large heterogeneity and instability of the CCR6+ subsets remains open. Additionally, Th17-like Tregs have been identified in multiple diseases, including RA, and might also play a role in disease propagation. The CCR6+ subpopulations are proposed to differ in their relative pathogenicity, for example, Th17.1 cells are more enriched in pathogenic factors than classic Th17 cells. Since all of these subpopulations would be (at least partly) identified by analyzing IL-17A production or CCR6 expression, the two most used strategies in literature, it is very desirable to better classify the heterogeneous CCR6+Th17 cell populations present in the RA joint or peripheral blood. Better characterization of the presence and specific functions of these different CCR6+ subpopulations would potentially elucidate more of the role of Th17 cells in RA, and even give some insight in therapy resistance (the presence of transport pumps on specific subpopulations for example). The current standard ways of analyzing Th17 cells in RA could very well underestimate their importance. Importantly, while CCR6 expression is stable on proliferating cells, even after multiple divisions, it is transiently down-regulated upon TCR activation [141]. This implicates that frequencies of activated CCR6+ cells might be underestimated, especially at sites of inflammation, where activated T cells will be present. Additionally, characteristics of CCR6+ cells might wrongly be attributed to CCR6- cells.

Efficacy in treating RA with the above described antibodies in clinical trials varies extensively. Especially interesting are the large differences found in the efficacy of the two anti-IL-17A antibodies and the anti-IL-17RA antibody. These differences could be due to lack of power or other weaknesses in the experimental design. However, it is intriguing that secukinumab treatment identified a clear responder and non-responder group, and increasing the doses did not significantly improve responses in the non-responder group [142]. Stratifying the results of clinical trials might show that, for a proportion of patients, treatment may be very effective, but the relevance might not show when all patients (responders and non-responders) are treated as one group. In this context, one study found that IL-17 mRNA was expressed in 28% of RA patients, in contrast to TNFα, which was expressed in all patients [9]. IL-17 concentration in synovial fluid varies extensively between patients, and is indeed undetectable in a large fraction of patients [10]. Personalized medicine uses diagnostic testing for selecting the appropriate therapy. In RA, considerable proportions of patients respond insufficiently to medication, including anti-TNFα treatment and DMARDs [38]. Personalized medicine seems an ideal approach for the treatment of the very heterogeneous disease RA, but better predictors for response to treatment need to be identified first.

Another issue that remains unresolved is the T cell receptor specificity of CCR6+ Th cells. It is of vital importance to determine which proteins activate the CCR6+ Th cells, and at which location this happens, in order to understand the chronic joint inflammation in RA.

MicroRNAs (miRNAs) are small non-coding RNAs, complementary in sequence to coding RNAs. They bind to the coding RNA, thereby inactivating it, thus controlling gene translation. miRNAs with changed

expression levels in RA have been found, some of which are pro-inflammatory and some are anti-inflammatory [143]. Currently, little is known about the specific cell types that express these miRNAs, but it is likely that this is very cell specific [144]. Recently, long non-coding RNA (lnc-RNA) molecules have been identified to be specifically expressed in Th17 cells [145]. Unraveling specific production in CCR6+ Th cells will help to unravel the role of non-coding RNA molecules in RA and might lead to potential new treatment targets.

Th cells are not the only cells that express CCR6. Natural killer (NK) cells and innate lymphoid cells (ILCs) also express CCR6. ILC group-3 (ILC3) cells express RANK, RANKL, RORC, IL-23R and have the ability to produce IL-17, GM-CSF and IL-22. Their role in RA is still unexplored, but based on these features, they might play an important role in RA [146, 147]. NK cells also express CCR6 and IL-23R, and are found in RA peripheral blood and in synovium, where they are present in an early stage, and are enriched compared to blood. NK cells produce IFNγ, TNFα and IL-22, and a subset can produce IL-17A. NK cells in the synovium are over-activated and contribute to disease propagation [148, 149].

CCR6+ cells play various pro-inflammatory roles in RA, and the production of IL-17A explains only part of their pathogenicity. Future research should focus on the pathologic potential of specific CCR6+ subpopulations and the importance of plasticity. Therapy focussed on inhibiting both CCR6+ cells and the TNFα pathway is potentially clinical more valuable than blocking either pathway alone.

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Chapter 3

Synovial fibroblasts directly induce Th17 pathogenicity via the cyclooxygenase/prostaglandin-E_2 pathway, independent of IL-23

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The Journal of Immunology, 2013 Aug 1;191(3):1364-72.

Abstract

Th17 cells are critically involved in autoimmune disease induction and severity. Recently, we showed that Th17 cells from patients with rheumatoid arthritis (RA) directly induced a pro-inflammatory loop upon interaction with RA-synovial-fibroblasts (RASF), including increased autocrine IL-17A production. To unravel the mechanism driving this IL-17A production we obtained primary CD4+CD45RO+CCR6+ (Th17) cells and CD4+CD45RO+CCR6- (CCR6-) T cells from RA patients or healthy individuals and co-cultured these with RASF. IL-1β, IL-6, IL-23p19 and cyclooxygenase-2 (COX-2) expressions and prostaglandin-E_2 (PGE_2) production in Th17-RASF cultures were higher than in CCR6- T cell-RASF cultures. Cytokine neutralization showed that IL-1β and IL-6, but not IL-23, contributed to autocrine IL-17A induction. Importantly, treatment with celecoxib, a COX-2 inhibitor, resulted in significantly lower PGE_2 and IL-17A, but not IFN-γ production. Combined celecoxib and TNFα blockade more effectively suppressed the pro-inflammatory loop than did single treatment, as shown by lower IL-6, IL-8, matrix metalloproteinase-1 (MMP-1) and MMP-3 production. These findings show a critical role for the COX-2/PGE_2 pathway in driving Th17-mediated synovial inflammation in an IL-23 and monocyte independent manner. Therefore, it would be important to control PGE_2 in chronic inflammation in RA and potentially other Th17-mediated autoimmune disorders.

Introduction

T-helper 17 (Th17) cells help protect a host against infection – especially infection with extracellular bacteria and fungi [1-3] – but on the other hand have a pathogenic role in autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), psoriasis and inflammatory bowel disease (IBD) [4-9]. The Th17 subset belongs to the class of CD4+ T-helper cells. Th17 cells produce cytokines like IL-17A, IL-17F and IL-22 [10-14]. Furthermore, they can induce expression of their master transcription factor retinoid-related orphan receptor-gt (RORgt), as well as CCR6 cell-surface expression [15-17].

Recently we have shown that in the early stage of RA, Th17, but not Th1 cells, are potent activators of synovial fibroblasts derived from patients with RA (RASF). This activation results in autocrine IL-17A production, which in turn creates a pro-inflammatory loop characterized by up-regulation of the pro-inflammatory cytokines IL-6 and IL-8, and the cartilage degrading enzymes MMP-1 and MMP-3 [18]. This loop may be an important pathway in the progression of arthritis, and possibly in other Th17 diseases as well. The autocrine IL-17A production by Th17 cells is critical to sustain the pro-inflammatory loop [18, 19], but the mechanism underlying the autocrine IL-17A induction is still unknown.

IL-1β, IL-6 and IL-23 promote Th17 cell differentiation. Adding IL-1β to naive T cells induces expression of RORgt and production of IL-17A and IFN-γ, whereas IL-6 addition sustains RORgt expression and induces production of IL-17A, but not IFN-γ. IL-23 promotes the pathological behavior of Th17 cells [10, 20-25].

Interestingly, also prostaglandin E_2 (PGE_2), a lipid mediator that induces inflammation and fever, participates in Th17 expansion and differentiation [26, 27]. PGE_2 is produced when arachidonic acid (AA) is converted by cyclooxygenase (COX), which has two isoforms. COX-1 is expressed constitutively; COX-2 is inducible and is up-regulated at inflammation and infection sites [28-30]. Up-regulation of COX-2 induction and PGE_2 production occurs in response to pro-inflammatory stimuli, including IL-1β, IL-17A and/or TNFα [31-37]. PGE_2, via receptor binding (EP1, EP2, EP3 and EP4), induces production of pro-inflammatory cytokines [28, 29]. PGE_2 has a pathogenic role in murine inflammatory disease models, possibly via the IL-23/IL-17 axis [38-40].

PGE_2 may be involved in pathogenesis in autoimmunity via Th17 cells, which express EP2 and EP4 receptors. PGE_2 binding to these receptors promotes Th17 differentiation [26, 28, 29] and IL-1β/IL-23 mediated Th17 expansion [27-29, 41, 42]. Simultaneously, RORgt expression increases [26, 41], and there is evidence for a more pathogenic Th17 phenotype from the increased expression of cytokines, chemokines and chemokine receptors including IL-17A, IL-17F, CCL20, CCR6 and CCR4 [26, 42]. Furthermore, PGE_2 indirectly promotes Th17 expansion, because it stimulates IL-23 production by dendritic cells (DCs) [27]; and it alters the Th1/Th17 balance in favor of Th17 by decreasing Th1 differentiation and IFN-γ production [26, 28, 41].

Here we show that the autocrine IL-17A production induced by the interaction of Th17 cells and RASF is critically dependent on the cyclooxygenase/prostaglandin-E_2 pathway.

Materials and Methods

Subjects

Blood samples from six treatment-naive early RA patients (5 women and 1 man, mean age±SD; 57.5±9.5, mean DAS28±SD; 4.39±0.82) were studied. All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA. None of the patients had been taking disease modifying anti-rheumatic drugs. Buffy coats from healthy individual blood donors were obtained from Sanquin Bloodbank (Rotterdam, the Netherlands). This study was embedded in the Rotterdam Early Arthritis Cohort Study (REACH) and approved by the Medical Ethics Review Board of Erasmus MC Rotterdam.

Flow cytometry, antibodies and cell sorting

Monoclonal antibody preparations, intracellular cytokine detection and flow cytometry were performed as described previously [43]. For intracellular cytokine detection by flow cytometry, cells were stimulated for 4 hours with 50 ng/ml PMA, 500 ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO) and Golgistop (BD Biosciences, San Diego, CA). The following monoclonal antibodies (MoAb) were purchased from BD Biosciences: CD45RO, CCR6 and CD4. IL-17A MoAb was purchased from eBioscience (San Diego, CA). Samples were acquired on a FACSCantoII flow cytometer and analyzed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR). Cells were gated on the lymphocyte fraction. T cell populations were sorted from PBMC isolated from buffy coats from healthy individual blood donors and from peripheral blood from the RA subjects using a FACSAria cell sorter (BD Biosciences). Purity of the obtained T cell populations was ≥ 98%.

Cell cultures

RASF isolation and subsequent culture was performed as described previously [18]. In brief, $1.0x10^4$ or $2.0x10^4$ RASF were seeded in a flat bottom plate. After 24 hours, RASF were cultured with or without $2.5x10^4$, $1.0x10^5$ or $2.0x10^5$ allogeneic Th17 cells obtained from healthy individuals or the six subjects. Cells were stimulated with soluble αCD3 and αCD28 (0.3 µg/ml and 0.4 µg/ml respectively, (Sanquin, Amsterdam, The Netherlands) and cultured for 2 or 3 days in a final volume of 200 µl IMDM medium (Lonza, Basel, Switzerland), supplemented with 10% FCS, 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamine and 50 µM β-mercapto-ethanol (Merck, Darmstadt, Germany). Cultures were grown in the absence or presence of 5-25 µM celecoxib (Sigma-Aldrich, St. Louis, MO), 10 µg/ml etanercept (Wyeth Pharmaceuticals Inc., Collegeville, PA,) 25 µg/ml tocilizumab (Roche Pharmaceuticals, Basel, Switzerland), 2.5 µg/ml αIL-1β, 25 µg/ml αIL-6R, 10 µg/ml αIL-10, 2.5 µg/ml sIL-17R, 10 µg/ml αIL-15, and 2.5 µg/ml αIL-23p19 (all from R&D systems, Minneapolis, MN). In addition, RASF monocultures were grown in the absence or presence of 10 ng/ml IL-17A (R&D systems) and 10 ng/ml TNFα (Invitrogen, Carlsbad, CA). To measure DNA synthesis, cells were pulsed with thymidine [^{3}H] for 16–20 h, harvested, and counted using standard methods. For analysis of cell cycle status of Th17 cells, DNA content was determined after fixing in ice-cold ethanol and subsequent staining in PBS containing 0.02 mg/ml propidium iodide,

0.1% v/v Triton X-100, and 0.2 mg/ml RNase. CFSE labeling of cells and annexin V and 7-AAD staining to determine proliferation and apoptosis of Th17 cells was performed as described previously [44].

PGE_2, MMPs and cytokine measurements

PGE_2 expression was determined using Parameter PGE_2 ELISA (R&D systems). IL-1β, IL-6, IL-8, IL-10 and IFN-γ expressions were determined using ELISA (Invitrogen). IL-17A, IL-22, TNFα, MMP-1 and MMP-3 expressions were measured using Duoset ELISA (R&D systems). ELISA was performed according to the manufacturer's instructions. IL-23 was expression was detected by ELISA using the combination of biotin-conjugated anti-human IL-12/23p40 (eBioscience) and anti-human IL-23p19 (R&D systems).

Quantitative real-time PCR analysis

RNA extraction and cDNA synthesis were performed as described previously [43]. Primers were designed with ProbeFinder software and probes were chosen from the universal probe library (Roche Applied Science, Indianapolis, IN). Quantitative real-time PCR (RT-PCR) was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster. City, CA) and analyzed using SDS v2.3 software (Applied Biosystems). The transcription values were relative against hypoxanthine–guanine phosphoribosyltransferase (Hprt) transcription. Primers used in this study (gene; forward primer 5'-3', reverse primer 5'-3', probe no.): Cox1; agggtttggcatgaaacccta, ctctgctgccatctccttct, 12 and Cox2; cttcagcatcagtttttcaag, tcaccgtaaatatgatttaagtccac, 23, Hprt; tgaccttgatttattttgcatacc, cgagcaagacgttcagtcct, 73, IL-1β; agctgatggccctaaacaga, tcggagattcgtagctggat, 85, IL-6; gatgagtacaaaagtcctgatcca, ctgcagccactggttctgt; 40, IL-12/23p40; ccctgacattctgcgttca, aggtcttgtccgtgaagactcta, 37, IL-23p19; tgttccccatatccagtgtg, tcctttgcaagcagaactga, 76, RORC; cagcgctccaacatcttct, ccacatctcccacatggact, 69, and IL-15; caaacaacagtttgtcttctaatgg, gacaatatgtacaaaactctgcaaaaa, 65.

Statistical analysis

Differences between experimental groups were tested with a two-sided paired t-test using Prism software v5.04 (GraphPad Software Inc. La Jolla, CA). *P*-values <0.05 were considered significant.

Results

Limited contribution of IL-6 and IL-1β to the induction of IL-6, IL-8, MMPs and autocrine IL-17A production upon Th17-RASF interaction

To investigate the mechanism driving autocrine IL-17A production by Th17 cells upon interaction with RASF, we first focused on the pro-inflammatory cytokines IL-1β, IL-6 and IL-23, which are required for Th17 cell differentiation [10, 20-25]. Therefore, primary CD4+CD45RO+CCR6+ (Th17) and CD4+CD45RO+CCR6- (CCR6- T cells) cells were sorted from peripheral blood of healthy individuals and co-cultured with RASF. Gene transcription and protein analyses showed that the Th17-RASF cultures were highly enriched for the Th17-specific transcription factor RORC and IL-17A production (figure 1A). Moreover, IL-1β and IL-6

gene transcription and IL-6 protein expression in Th17-RASF cultures were significantly higher than those in Th1-RASF cultures (figure 1A). IL-1β protein production was not detectable in the Th17-RASF cultures (detection limit 10 pg/ml, data not shown). To confirm the expression of IL-1β in our system, we used higher numbers of Th17 cells and RASF. When Th17 cell numbers were increased at least 4 times and RASF 2 times, IL-1β expression was detected (supplementary figure 1).

To investigate the contributions of IL-1β and IL-6 in autocrine IL-17A production, neutralizing antibodies against these cytokines were added to Th17-RASF cultures. Blockade of IL-1β and IL-6 activity significantly suppressed IL-17A production (~21% and ~26% compared to no blockade, respectively). This blockade had no measurable effects on IFN-γ and TNFα production. In addition, blockade of IL-1β, but not IL-6, had limited suppressive effects on IL-8, MMP-1 and MMP-3 expressions in Th17-RASF cultures. Combined blockade of IL-1β and IL-6 had an additional effect on the suppression of IL-6 expression (figure 1B).

IL-23p19 transcription was higher in Th17-RASF cultures than in Th1-RASF cultures. However, IL-12/23p40 transcription was undetectable (figure 1C). Subsequently, no IL-23 protein was detected in supernatant of Th17-RASF cultures. Moreover, blocking of IL-23 in Th17-RASF cultures had no effect on the expression of pro-inflammatory cytokines and MMPs induced by the pro-inflammatory loop upon Th17-RASF interaction (supplementary figure 2).

These data show that the pro-inflammatory cytokines IL-1β and IL-6, which are involved in Th17 differentiation, are indeed induced in Th17-RASF cultures. However, these findings do not fully explain how IL-17A production and the pro-inflammatory loop are driven in the Th17-RASF cultures, suggesting that additional mechanisms are involved.

Celecoxib treatment reduces PGE_2 and autocrine IL-17A production in Th17-RASF cultures

Gene expression profiles from RASF cultured with primary Th17 cells or CCR6- T cells, obtained from peripheral blood of treatment-naive patients with early RA [18], revealed that COX-2 transcription was highly up-regulated in Th17-RASF cultures. This is particularly interesting since COX-2 is involved in PGE_2 synthesis, which has been shown to induce Th17 expansion [26, 27, 42]. To validate these findings, primary peripheral blood Th17 and CCR6- T cells obtained from healthy volunteers were cultured with RASF. The gene encoding for COX-2, but not the gene encoding for COX-1, was highly up-regulated (~10 fold) in Th17-RASF cultures compared to CCR6- T cell-RASF cultures. In addition, PGE_2 production was significantly increased in Th17-RASF cultures (figure 2A).

To investigate the roles of COX-2 and PGE_2 in the production of IL-17A, Th17-RASF cultures were treated with celecoxib, a specific inhibiter of COX-2 activity. This resulted in a dose dependent decrease of IL-17A and PGE_2 production in supernatant. Compared to the control situation, the decrease in IL-17A production as a result of celecoxib treatment (25μM) was ~73% (figure 2B). Moreover, celecoxib treatment resulted in a significant reduction of the fraction of IL-17A, but not IFN-γ producing cells (figure 2C). Celecoxib treatment did not affect IFN-γ and IL-22 production, while TNFα and IL-10 production were induced (figure 2D). The reduction of IL-17A expression by celecoxib was not caused by altered cell proliferation, apoptosis and cell cycle status (figure 2E-H).

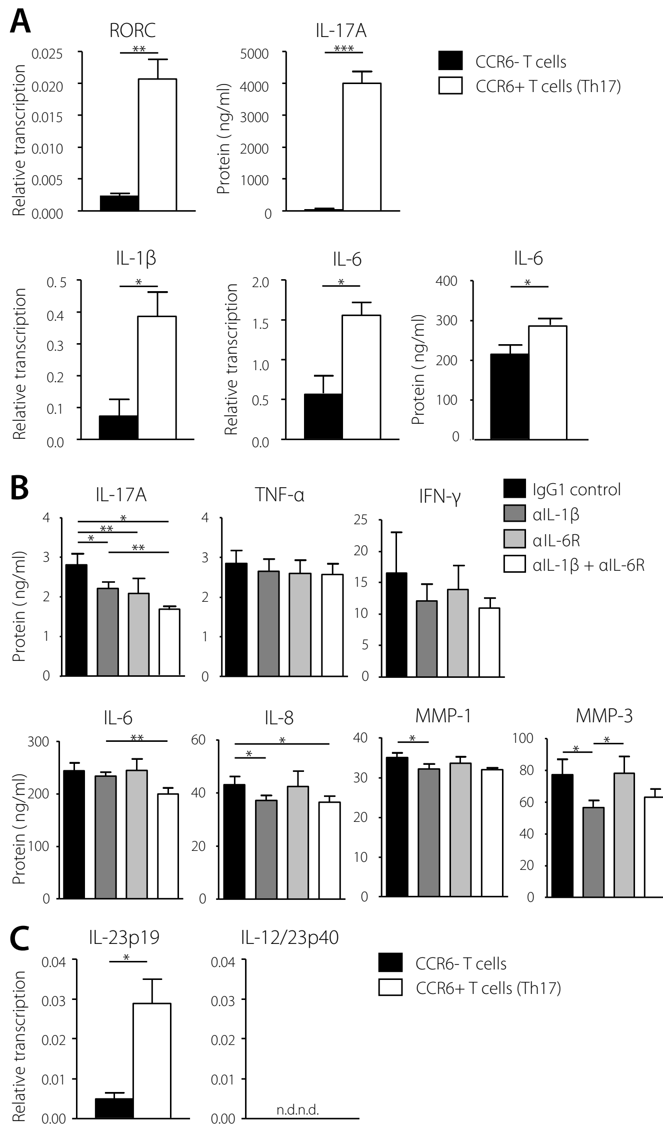


Figure 1. Limited contribution of IL-1β and IL-6 to the perseverance of the pro-inflammatory loop induced upon Th17-RASF interaction. Sorted primary memory CCR6- T cells and/or CCR6+ T cells (Th17) from healthy individuals were stimulated with αCD3/αCD28 and cultured with RASF for 2 days. (**A**) IL-1β, IL-6 and RORC gene transcription levels were detected by quantitative RT-PCR and IL-6 and IL-17A protein levels were detected by ELISA in CCR6- T cell-RASF cultures (black bars) or in Th17-RASF cultures (white bars). Gene transcription levels were normalized against HPRT values. (**B**) Th17-RASF cultures were stimulated with αCD3/αCD28 and cultured for 2 days in the presence or absence of αIL-1β (2.5 µg/ml) and/or αIL-6R (25 µg/ml). Expression of indicated cytokines and MMPs determined by ELISA in supernatant of Th17-RASF cultures in absence (black bars) or presence of αIL-1β (dark grey bars), αIL-6R (light grey bars) or both (white bars). (**C**) IL-23p19 and IL-12/23p40 gene transcription levels were detected by quantitative RT-PCR and normalized against HPRT gene expression values. Mean and SEM are given for 6 healthy donors. Results are representative for at least 3 independent experiments (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

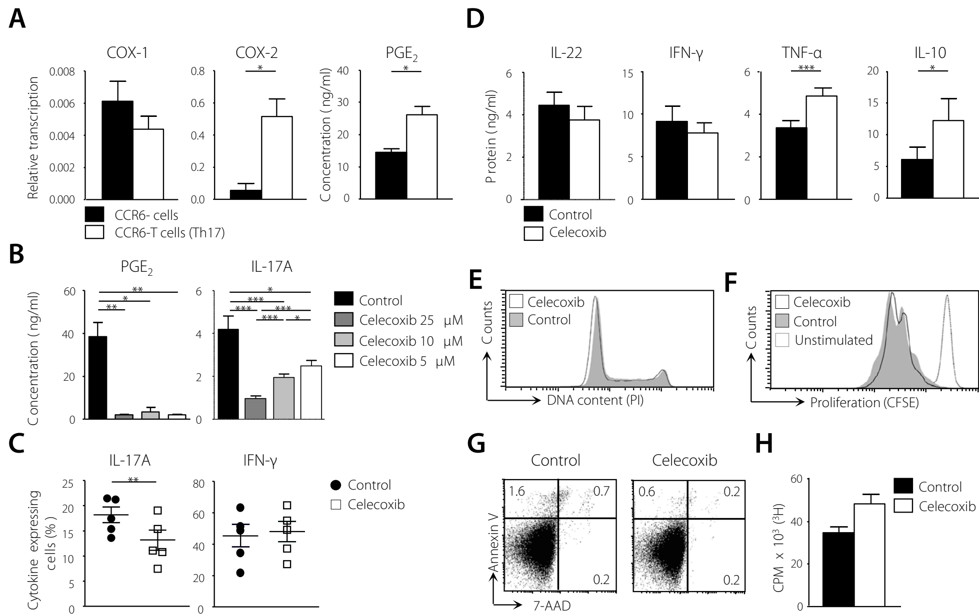


Figure 2. The COX-2/PGE_2 pathway is critically involved in autocrine IL-17A production by Th17-RASF co-cultures. (**A**) Th17-RASF and CCR6- T cell-RASF cultures were stimulated with αCD3/αCD28 and cultured for 2 days. PGE_2 production was detected by ELISA and COX-1 and COX-2 gene expression were analyzed by quantitative RT-PCR in Th17-RASF (white bars) or CCR6- T cell-RASF cultures (black bars). Mean and SEM are given (*n*=4-6). (**B-H**) Th17-RASF cultures were stimulated with αCD3/αCD28 and cultured for 2 days with celecoxib (25 µM) or otherwise stated. (**B**) Detection of PGE_2 and IL-17A production by ELISA in Th17-RASF cultures with celecoxib (25, 10 or 5 µM) or control condition (black bars). Mean and SEM are given (*n*=4-6). (**C**) Flow cytometric analysis of intracellular IL-17A and IFN-γ expression by Th17 cells cultured for 2 days with RASF with celecoxib (white squares) or control condition (black circles). Mean and SEM are given (*n*=4-5). (**D**) Production of indicated cytokines detected by ELISA in Th17-RASF cultures with celecoxib (white bars) or control condition (black bars). Mean and SEM are given (*n*=5-10). (**E**) Representative histogram showing cell cycle distribution by propidium iodide (PI) staining. (**F-G**) Flow cytometric analysis for CFSE expression (**F**) and 7-AAD and Annexin V (**G**) by Th17-RASF cultures with or without celecoxib treatment. Representative histograms or dot plots are given. (**H**) Proliferative response to celecoxib treatment, as determined by [^{3}H] thymidine incorporation. Mean and SEM are given (*n*=3). Results are representative for at least 2 independent experiments (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

In addition to PGE_2, IL-15 has also been shown to induce IL-17A expression in PBMC and T cells obtained from patients with RA [45, 46]. We detected IL-15 expression in both CCR6- T cell-RASF co-cultures and Th17-RASF co-cultures, although the expression was highest in CCR6- T cell-RASF co-cultures. To analyze the contribution of IL-15 to the induction of IL-17A, we added αIL-15 to the co-cultures. Although IL-15 blockade in Th17-RASF co-cultures reduced the production of IL-17A, the effects were limited compared to celecoxib treatment. Combining IL-15 blockade and celecoxib treatment had no additional effect compared to celecoxib treatment only (supplementary figure 3).

These findings clearly show that COX-2 expression and PGE_2 production are induced in Th17-RASF cultures. Moreover, PGE_2 suppression by the COX-2 inhibiter celecoxib resulted in a significant reduction of IL-17A production in Th17-RASF cultures.

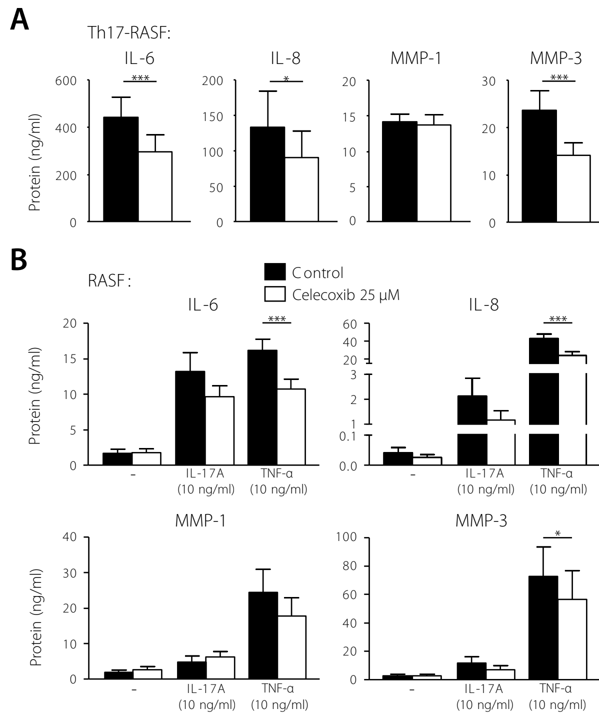


Figure 3. Celecoxib inhibits IL-6, IL-8 and MMP-3 expression in Th17-RASF co-cultures. Th17-RASF co-cultures were stimulated with αCD3/αCD28 and cultured for 2 days in the presence or absence of celecoxib (25 μM). RASF monocultures were cultured for 3 days in the presence or absence of celecoxib (25 μM), IL-17A (10 ng/ml) and TNFα (10 ng/ml). (**A**) Detection of indicated cytokines and MMPs by ELISA in supernatant of Th17-RASF co-cultures in control condition (black bars) or with celecoxib (white bars). Mean and SEM are given (n=10). (**B**) Detection of indicated cytokines and MMPs by ELISA in supernatant of RASF cultures in control condition (black bars) or with celecoxib (white bars). Mean and SEM are given (n=5). Results are representative for at least 2 independent experiments (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Inhibition of COX-2 activity suppressed expression of pro-inflammatory cytokines and MMPs in Th17-RASF cultures

Upon interaction with Th17 cells, RASF are activated and produce the pro-inflammatory cytokines IL-6, IL-8, MMP-1 and MMP-3 [18]. Celecoxib treatment significantly inhibited these processes with regard to IL-6, IL-8 and MMP-3 production (figure 3A). Celecoxib had limited effects on RASF directly, because treatment of RASF monocultures with celecoxib did not change the production levels of IL-6, IL-8, MMP-1 and MMP-3. In addition, celecoxib treatment did not reduce the production of these cytokines in RASF monocultures when IL-17A was added to the cultures, but when TNFα was added celecoxib effectively reduced the production of IL-6, IL-8 and MMP-3 (figure 3B).

These findings show that celecoxib treatment of Th17-RASF cultures not only reduces IL-17A expression, but also the pro-inflammatory mediators IL-6, IL-8 and MMP-3.

The suppressive effects of celecoxib in Th17-RASF cultures involves IL-17A inhibition

The inhibitory effect of celecoxib treatment on PGE_2 production was accompanied by reduced IL-17A production and a reduced fraction of IL-17A producing Th17 cells (figure 2). In earlier studies, we showed that the pro-inflammatory loop induced upon interaction of Th17 cells and RASF is dependent on IL-17A [18, 19]. To investigate whether the inhibitory effect of celecoxib was dependent on the inhibition of IL-17A production, Th17-RASF cultures were treated with celecoxib and/or sIL-17R to neutralize IL-17A activity. Compared to sIL-17R only treatment, combining celecoxib and sIL-17R treatment results in increased TNFα, but not of IFN-γ production. Moreover, the combination of celecoxib and sIL-17R treatment had an additional effect on IL-6 and MMP-3 suppression, compared to sIL-17R only treatment (figure 4).

When taken together, these findings show that celecoxib treatment of Th17-RASF cultures involves IL-17A suppression. However, in comparison to sIL-17R treatment, celecoxib has additional stimulatory effects on TNFα expression and inhibitory effects on IL-6 and MMP-3 in Th17-RASF cultures.

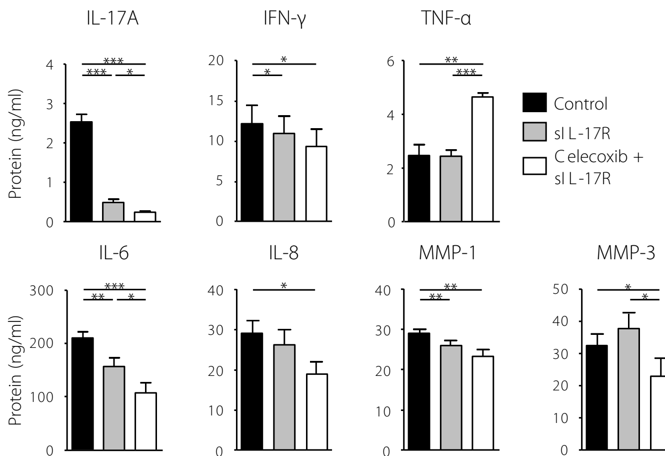


Figure 4. The suppressive action of celecoxib in Th17-RASF cultures involves inhibition of IL-17A production. Th17-RASF cultures were stimulated with αCD3/αCD28 and cultured for 2 days with sIL-17R (2.5 µg/ml, light grey bars), celecoxib and sIL-17R (25 µM and 2.5 µg/ml respectively, white bars) or control condition (black bars). ELISA analysis for the production of IL-17A, IFN-γ, TNFα, IL-6, IL-8, MMP-1 and MMP-3 by Th17-RASF cultures under indicated conditions. Mean and SEM are given (n=5). Results are representative for at least 2 independent experiments (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Combining celecoxib and TNFα blockade in Th17-RASF cultures has additional value on the suppression of cytokines and MMPs

Recently, we showed that besides IL-17A, TNFα was involved in the induction of the pro-inflammatory loop in Th17-RASF cultures [18, 19]. Furthermore, the combination of TNFα and IL-17A blockade more strongly suppressed IL-6, IL-8, MMP-1 and MMP-3 production in these Th17-RASF cultures than blocking only IL-17A or only TNFα [18]. In this study celecoxib treatment suppressed IL-17A production, therefore we investigated a possible additional value of celecoxib compared to TNFα blockade. Th17-RASF cultures were treated with etanercept, a soluble TNFα receptor, in the presence or absence of celecoxib.

In contrast to celecoxib treatment, TNFα blockade had no effects on PGE_2 and IL-17A production (figure 5A-B). In line with our previous findings [18], TNFα blockade inhibited IL-6, IL-8, MMP-1 and MMP-3 production (figure 5C). Importantly, combining celecoxib and TNFα blockade had an additional value compared to TNFα blockade only in suppressing IL-6 and IL-8 expression (figure 5C).

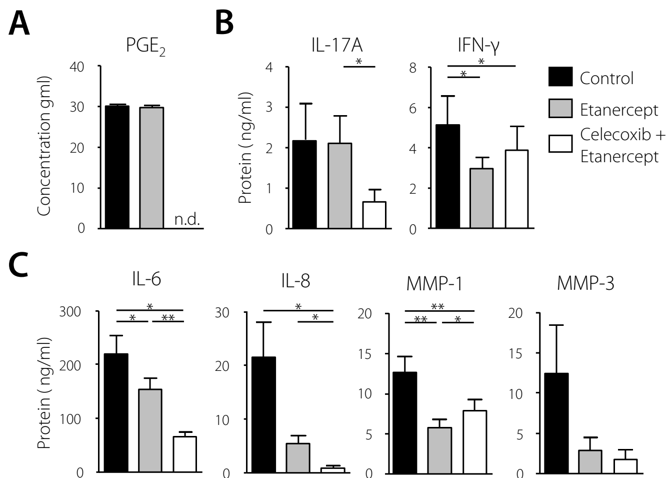


Figure 5. Combining celecoxib and TNFα blockade has additional value to TNFα blockade alone in suppressing IL-6 and IL-8 expression in Th17-RASF cultures. (**A-C**) Th17-RASF cultures were stimulated with αCD3/αCD28 and cultured for 2 days in the absence (black bars) or presence of etanercept only (10 μg/ml) (grey bars) or the combination of etanercept and celecoxib (25 μM) (white bars). ELISA analysis for the production of PGE_2 (**A**) IL-17A and IFN-γ (**B**) and IL-6, IL-8, MMP-1 and MMP-3 (**C**) by Th17-RASF cultures under indicated conditions. Mean and SEM are given (n=4). Results are representative for at least 2 independent experiments (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

The decrease in pro-inflammatory cytokine and MMPs expression after celecoxib treatment was accompanied by an increase in IL-10 concentration (figure 2D), However, IL-10 blockade did not reverse the effects of celecoxib treatment on IL-17A, IFN-γ, IL-6, IL-8, MMP-1 or MMP-3 production in Th17-RASF cultures (supplementary figure 4). This indicates that the effects of celecoxib treatment in these cultures were independent of IL-10 induction.

These data show that in contrast to celecoxib, TNFα blockade has no suppressive effects on PGE_2 and IL-17A production in Th17-RASF cultures. Moreover, combining celecoxib treatment and TNFα blockade has an additional value in suppression of IL-6 and IL-8 production in these cultures.

Celecoxib, but not IL-1β and IL-6 blockade, inhibits autocrine IL-17A production in co-cultures of RASF and Th17 cells from treatment-naive patients with early RA

To investigate the contribution of IL-1β and IL-6 to Th17 polarization under pathological conditions, RASF were cultured with primary Th17 cells, isolated from peripheral blood of treatment-naive patients with early RA. These Th17-RASF cultures were treated with αIL-1β and/or tocilizumab, a humanized monoclonal against the IL-6R. Single or combined treatment of Th17-RASF cultures with αIL-1β and tocilizumab had no effects on the production of IL-17A. However, both αIL-1β and tocilizumab inhibited the expression of IL-6 and IL-8 production. In addition, treatment of Th17-RASF cultures with αIL-1β suppressed the production of both MMP-1 and MMP-3 (figure 6A).

In contrast to αIL-1β and tocilizumab, treatment of these Th17-RASF cultures with celecoxib resulted in a significantly lower (~ 4 fold, $p < 0.05$) IL-17A production compared to the control conditions. Celecoxib treatment induced the production of TNFα production, but had no effects on IFN-γ production. Furthermore, celecoxib treatment resulted in a significantly reduced IL-6, IL-8, MMP-1 and MMP-3 production in the Th17-RASF cultures (figure 6B).

Collectively, these data show that PGE_2 production via COX-2 activation is the main contributing factor in driving IL-17A production when Th17 cells interact with RASF. Consequently, treatment with the COX-2 inhibiter celecoxib results in inhibition of the pro-inflammatory loop induced upon Th17-RASF interaction.

Discussion

Recently we showed that when Th17 cells interact with RASF, a pro-inflammatory loop is induced, resulting in increased autocrine IL-17A production [18]. This IL-17A/TNFα dependent loop may be an important mechanism in the role of Th17 cells in the progression of chronic persistent inflammation in arthritis, and possibly in other Th17-mediated diseases. In the present study we showed that PGE_2 production via COX-2 activation is the main contributing factor in driving IL-17A production when Th17 cells interact with RASF (figure 7). Consequently, treatment with the COX-2 inhibiter celecoxib resulted in a specific inhibition of IL-17A production, which led to reduced synovial inflammation, as was shown by reduced IL-6, IL-8, MMP-1 and MMP-3 expression (figure 7).

The pro-inflammatory cytokines IL-1β, IL-6 and IL-23 are required for Th17 cell differentiation [25]. Therefore their effect on the autocrine IL-17A induction upon Th17-RASF interaction was investigated. Although IL-1β and IL-6, but not IL-23, were increased in the Th17-RASF cultures compared to Th1-RASF cultures, neutralization experiments showed a limited role of these cytokines in the total production of IL-17A in Th17-RASF cultures. In concordance with this, neutralization of IL-6 and IL-1β in a co-culture of mesenchymal cells and PBMC only marginally reduced IL-17A production [47].

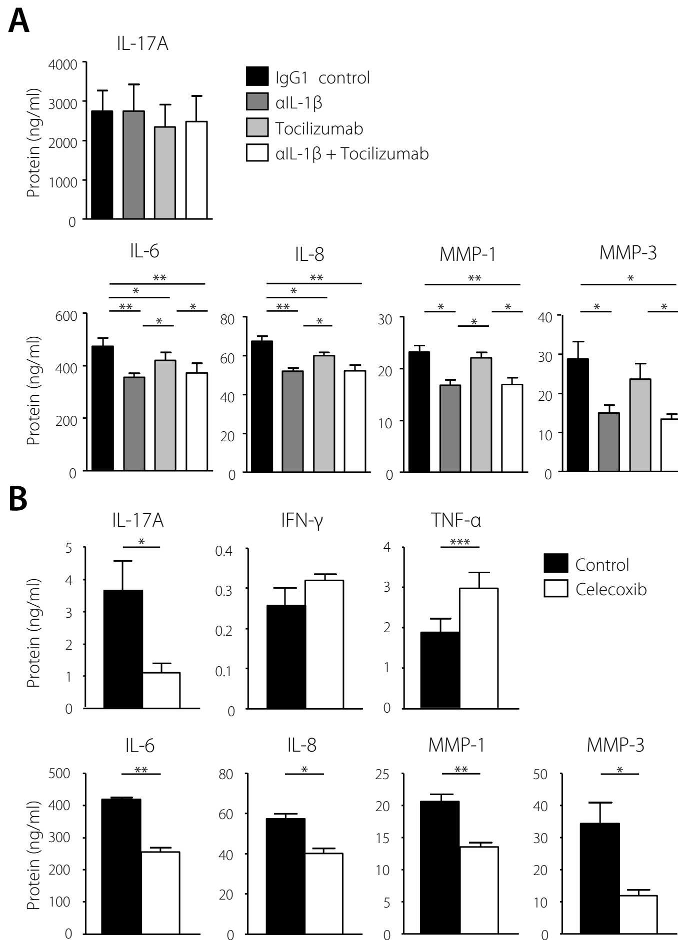


Figure 6. Effects of IL-1β and IL-6 blockade and celecoxib treatment in RASF cultured with primary Th17 cells obtained from patients with RA. (**A-B**) Primary Th17 cells were sorted from peripheral blood of treatment-naive patients with early RA. These Th17 cells were cultured with RASF in the presence of αCD3/αCD28; (**A**) without blocking antibodies (black bars) or with 2.5 μg/ml αIL-1β (dark grey bars) or 25 μg/ml tocilizumab (light grey bars) or αIL-1β and tocilizumab (white bars) or (**B**) celecoxib (25 μM) (white bars) or in control condition (black bars). Detection of IL-17A, IFN-γ, TNFα, IL-6, IL-8, MMP-1 and MMP-3 production by ELISA in supernatant of Th17-RASF cultures under indicated conditions. Mean and SEM are given (n=4-6). Results are representative for at least 2 independent experiments (* = $p < 0.05$, ** = $p < 0.01$).

Blockade of IL-1β and/or IL-6 signaling was able to suppress IL-17A production by peripheral blood Th17 cells obtained from healthy volunteers, but not from patients with early RA (Figure 1B and 6A). In an earlier study we found that RA Th17 cells are more polarized to the IL-17A producing phenotype than healthy Th17 cells [18]. It may be possible that because of this polarization, RA Th17 cells are less sensitive for IL-1β and IL-6 signaling, which are especially important during differentiation and polarization, compared to healthy Th17 cells. This polarization status may reflect in a higher activity of RA Th17 cells,

explaining the higher levels of IL-6 induced by RA Th17 cells (~500 ng/ml, Figure 6A), compared to healthy Th17 cells (~250 ng/ml, Figure 1B).

Next to IL-1β, IL-6 and IL-23, PGE_2 has been reported to induce IL-17A expression by Th17 cells [26, 27, 41, 42]. Treatment of Th17-RASF cultures with celecoxib reduced the production of PGE_2 and IL-17A. The effects of celecoxib on IL-6, IL-8 and MMPs production were explained for a large part by IL-17A blockade, as celecoxib inhibited IL-8 and MMP-1 production in a similar extent as sIL-17R. However, in comparison to IL-17A blockade, celecoxib had additional inhibitory effects on IL-6 and MMP-3 expression. This indicates that besides IL-17A, celecoxib may affect additional factors as well. These may include mechanisms involved in pro-inflammatory cytokine and MMP production by activated RASF (figure 3B).

The observed decrease in pro-inflammatory cytokine and MMP expression after celecoxib treatment was accompanied by increased IL-10 production. Boniface *et al*. found that PGE_2 through EP4 receptor signaling inhibits IL-10 expression [26]. Blocking PGE_2 by celecoxib in our co-cultures may lead to an inverse process leading to increased IL-10 production. The induction in IL-10 production may explain the inhibitory effects of celecoxib on the pro-inflammatory loop. However, IL-10 blockade did not reverse the effects of celecoxib treatment in Th17-RASF cultures. This shows that the inhibitory action of celecoxib on the pro-inflammatory loop is independent of IL-10.

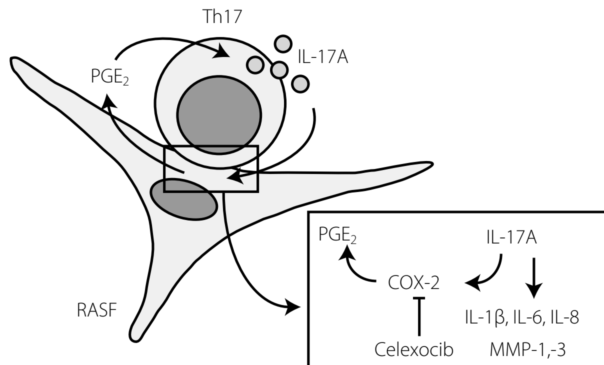


Figure 7. Conceptual model of Th17 cell mediated synovial inflammation. Activated Th17 cells secrete the pro-inflammatory cytokine IL-17A. Upon interaction of activated Th17 cells with RASF, IL-17A induces production of the pro-inflammatory cytokines, IL-1β, IL-6 and IL-8 and the tissue degrading enzymes MMP-1 and MMP-3 by RASF. Simultaneously, IL-17A induces COX-2 expression, which results in PGE_2 synthesis and secretion. Next, PGE_2 induces autocrine IL-17A expression by Th17 cells enabling the development of a pro-inflammatory loop. This loop may explain the progression of an early synovial inflammation towards chronic destructive arthritis. The induction of autocrine IL-17A expression and hereby the pro-inflammatory loop is blocked by treatment of the COX-2 inhibiter celecoxib.

In addition, we observed increased TNFα levels after celecoxib treatment. It was shown that celecoxib increases TNFα production in LPS-stimulated human monocytes, in human RA synovial membranes cultures, and in whole blood of human subjects following celecoxib treatment *in vivo* [48], which is in concordance with our results. In the present study, TNFα blockade has no suppressive effects on PGE_2 and IL-17A production in Th17-RASF cultures. However, combining celecoxib treatment and TNFα blockade has an additional value in suppression of IL-6 and IL-8 production in these cultures. These data show that celecoxib and TNFα blockade both down-regulate inflammation, but they exert their effects via different routes.

Many reports on the effects of PGE_2 on Th17 differentiation and expansion show that these effects are IL-23 dependent [26, 27, 37, 42]. A proposed model is that IL-23 and PGE_2 act synergistically to achieve these Th17 effects, possibly via PGE_2 mediated up-regulation of IL-23R [26], but our results show an IL-23 independent effect of PGE_2. Even though IL-23p19 transcription was induced, IL-23 protein expression was not detectable in Th17-RASF cultures and subsequently IL-23 blockade did not affect IL-17A production. These data are in line with the findings observed by Brentano et al. [49], who showed that the IL-23-subunit p19 is abundantly expressed in RA synovial tissue, but that no functional IL-23, consisting of both the p19 and p40 subunit can be detected in RA synovial fluid. In this context our findings show that, upon Th17-RASF interaction, PGE_2 induces autocrine IL-17A production in an IL-23 independent manner.

In addition to PGE_2, IL-15 has also been shown to induce IL-17A expression in PBMC and T cells obtained from patients with RA [45, 46, 50]. In line with this, IL-15 blockade resulted in a suppression of IL-17A production in Th17-RASF cultures. However, the contribution of IL-15 to the total IL-17A production is limited, as celecoxib treatment in the Th17-RASF cultures resulted in a significant stronger suppression of IL-17A production in comparison to IL-15 blockade (Supplementary figure 3). IL-15 has been found to up-regulate COX-2 expression in synoviocytes [51]. This may explain our finding that combined celecoxib treatment and IL-15 blockade has no additional inhibiting effects on IL-17A production compared to celecoxib only treatment. Furthermore, IL-15 may also function as an activator of T cell activation and proliferation [46]. In line with this, IL-15 blockade suppressed IL-17A, but also TNFα and IFN-γ production in PBMC and T cells from patients with RA [45, 46]. In contrast, PGE_2 is a specific inducer of IL-17A, since celecoxib treatment leads specifically to IL-17A suppression and not to TNFα, IFN-γ or IL-22 suppression.

Our results and aforementioned studies suggest that controlling PGE_2 might be important in the context of Th17-mediated (auto-immune) diseases. However, there are also studies that suggest that PGE_2 does not stimulate Th17 responses but instead inhibits them, thereby controlling inflammation [29, 52-54]. For example, when PGE_2 was added to naive T cells which are cultured under Th17 polarizing conditions, IL-17A production was inhibited [53]. In addition, in co-cultures of Th17 and mesenchymal stem cells (MSCs), a cell type which inhibits T cell activation and proliferation, COX inhibition reversed the inhibition of Th17 polarization by MSCs [54]. These reports illustrate that the immunomodulatory role of PGE_2 on T cells and Th17 cells in particular is context and cell differentiation status dependent. Along this line, the effects of PGE_2 and thereby celecoxib treatment may depend on inflammatory conditions.

This may explain a stronger celecoxib effect on MMP-1 production by RASF cultures with RA obtained Th17 cells (figure 6) compared to RASF cultures with Th17 cells obtained from healthy volunteers (figure 3). In addition, inflammatory conditions may explain the more pronounced effects of IL-1β and IL-6 blockade in RASF cultures with Th17 cells from patients with RA on IL-6, IL-8 and MMP-1 production when compared with Th17 cells from healthy volunteers (figure 1B and 6A).

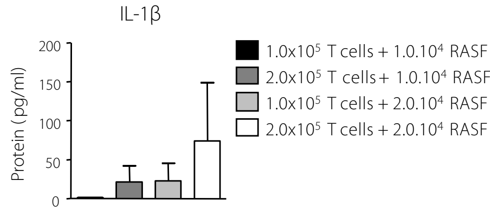
This study showed an important role for PGE_2 in IL-17A production in RA synovial inflammation. Because PGE_2 is produced in many inflammatory diseases, the mechanism reported here may be important in other Th17-mediated autoimmune diseases in which Th17 cells are implicated including psoriasis, MS and IBD [8, 9]. The induction of TNFα in the Th17-RASF cultures by celecoxib may explain why the inhibition of COX-2 activity reduces joint pain and improve motility in humans, but does not inhibit disease progression [48]. Future research should carefully address whether the inducing effect of celecoxib on TNFα can be compensated with anti-TNFα treatment in vivo.

Collectively, here we show a critical role for the COX-2/PGE_2 pathway in driving Th17-mediated synovial inflammation in an IL-23 and monocyte independent manner. Moreover, inhibiting PGE_2 production in addition to TNFα blockade may be an important mechanism to control chronic inflammation in RA and potentially other Th17-mediated autoimmune disorders.

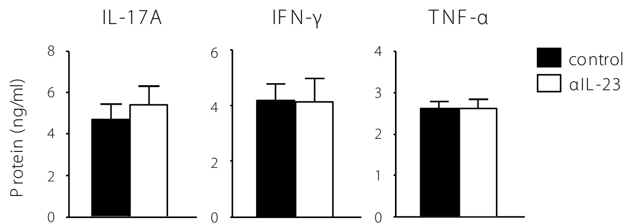
Acknowledgements

We would like to thank B. Bartol, H. Bouallouch-Charif, and H. Vroman for technical assistance at various stages of the project.

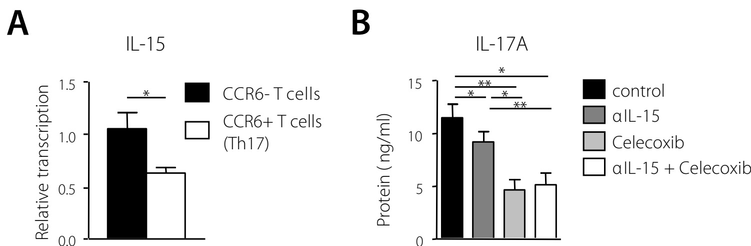
Supplementary figures



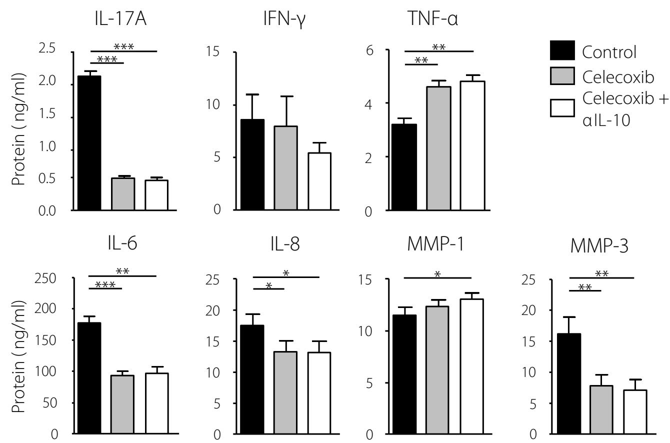
Supplementary figure 1. Detection of IL-1β in Th17-RASF co-cultures. $1.0x10^5$ or $2.0x10^5$ Th17 cells were co-cultured with $1.0x10^4$ or $2.0x10^4$ RASF, stimulated with αCD3/αCD28 and cultured for 3 days. Expression of IL-1β was determined by ELISA in supernatant. Mean and SEM are given (*n*=3).



Supplementary figure 2. Suppression of the pro-inflammatory loop in Th17-RASF cultures is IL-23 independent. Th17-RASF cultures were stimulated with αCD3/αCD28 and cultured for 3 days with αIL-23p19 (2.5 μg/ml, white bars) or in control condition (black bars). IL-17A, IFN-γ, TNFα, IL-6, IL-8, MMP-1 and MMP-3 production by Th17-RASF cultures under indicated conditions was determined by ELISA analysis. Mean and SEM are given (*n*=5). Results are representative for at least 2 independent experiments.



Supplementary figure 3. IL-15 blockade suppresses IL-17 in Th17-RASF cultures, but IL-15 has a minor contribution to the total production of IL-17 in these cultures. (**A**) CCR6- T cell-RASF cultures (black bars) and Th17-RASF cultures (white bars) were stimulated with αCD3/αCD28 and cultured for 2 days. IL-15 gene transcription levels were detected by quantitative RT-PCR and normalized against HPRT values. (**B**) Th17-RASF cultures were stimulated with αCD3/αCD28 and cultured for 3 days without (black bars) or with αIL-15 (10 μg/ml, dark grey bars), celecoxib (25 μM, light grey bars) or both celecoxib and αIL-15 (white bars). Production of IL-17A under indicated conditions was determined by ELISA. Mean and SEM are given (*n*=5).



Supplementary figure 4. Celecoxib mediated suppression of the pro-inflammatory loop in Th17-RASF cultures is IL-10 independent. Th17-RASF cultures were stimulated with αCD3/αCD28 and cultured for 3 days in the absence (black bars) or presence of celecoxib (25 μM) (grey bars) or the combination of celecoxib and αIL-10 (10 μg/ml) (white bars). Detection of IL-17A, IFN-γ, TNFα, IL-6, IL-8, MMP-1 and MMP-3 production by ELISA in supernatant of Th17-RASF cultures under indicated conditions. Mean and SEM are given (*n*=4). Results are representative for at least 2 independent experiments (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

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Chapter 4

IL-17/Th17 mediated synovial inflammation is IL-22 independent

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Annals of the Rheumatic Diseases, 2013 Oct;72(10):1700-7.

Abstract

Background IL-17A and Th17 cells are critically involved in T cell-mediated synovial inflammation. Besides IL-17A, Th17 cells produce IL-22. Recently, Th22 cells were discovered, which produce IL-22 in the absence of IL-17. However, it remains unclear whether IL-22 and Th22 cells contribute to T cell-mediated synovial inflammation. Therefore, we examined the potential of IL-22 and Th22 cells to induce synovial inflammation and whether IL-22 is required for T cell-mediated experimental arthritis.

Methods Peripheral and synovial Th17 and Th22 cells were identified and sorted from patients with rheumatoid arthritis (RA). Co-culture experiments of these primary T cell populations with RA synovial fibroblasts (RASF) were performed. The *in vivo* IL-22 contribution to synovial inflammation was investigated by inducing T cell-mediated arthritis in IL-22 deficient mice and wild-type mice.

Results Peripheral Th17 and Th22 cell populations were increased in patients with RA and present in RA synovial fluid. In T cell-RASF co-cultures, IL-22 in the presence of IL-17A had limited effects on IL-6, IL-8, MMP-1 and MMP-3 production. Furthermore, primary peripheral blood and synovial Th17 cells were more potent in the induction of these factors by RASF compared to Th22 cells. In line with this, similar synovial inflammation and disease severity was found between IL-22 deficient and wild-type mice in T cell-mediated experimental arthritis.

Conclusion These findings show that IL-17A/Th17 cell-mediated synovial inflammation is independent of IL-22 and Th22 cells. This implies that targeting IL-17A/Th17 cells, rather than IL-22/Th22 cells, should be the focus for treatment of T cell-mediated synovial inflammation.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by synovial inflammation and destruction of cartilage and bone [1, 2]. Pro-inflammatory cytokines, such as TNF-α and IL-17A, play a central role in RA [3, 4]. IL-17A producing T-helper-17 (Th17) cells are implicated in the pathogenesis of RA and other T cell-mediated diseases [5-10]. In patients with RA elevated proportions of peripheral and synovial Th17 cells were found [11-14] and the pathogenic role for IL-17A in arthritis has been shown in experimental mouse models [15, 16]. Furthermore, we have shown that Th17 cells from patients with RA, induce a pro-inflammatory loop upon interaction with RA synovial fibroblasts (RASF). This loop includes autocrine IL-17A production, which may explain the progression of an early inflammatory arthritis to a chronic destructive arthritis [14, 17].

Besides IL-17A, Th17 cells express the cytokine IL-22 [18, 19]. Among target cells of IL-22 are mainly tissue epithelial cells. IL-22 is involved in antimicrobial defense and tissue regeneration [20-22]. The IL-22 receptor is not expressed by immune cells and no effects of IL-22 have been identified in these cells. In addition to T cells, TCRγδ, lymphoid tissue inducer (LTi) and NK cells also produce IL-22 [23, 24]. Recently, the novel human Th22 cell subset was identified producing high levels of IL-22 in the absence of IL-17A production. Th22 cells are implicated in skin homeostasis and pathology [25] and IL-22 production in T cells is dependent on the aryl hydrocarbon receptor (AHR) and the transcription factor ROR-C [26, 27]. Besides cytokine production, Th17 and Th22 cells can be characterized by a specific chemokine receptor expression pattern. Whereas both Th17 and Th22 cells are negative for CXCR3 expression and positive for CCR6 and CCR4 expression, only Th22 cells are positive for the expression of CCR10 [25, 27, 28].

In patients with RA increased IL-22 expression levels or elevated proportions of IL-22 producing T cells were found in peripheral blood and in the inflamed synovium [11, 29-31]. The pathological role of IL-22 in RA has been suggested by an association of IL-22 serum levels with bone erosions. Also elevated Th22 and Th17 cell proportions were found in patients with RA compared to healthy controls [32-34]. In addition, RASF have been shown to produce IL-22 and respond to IL-22 with increased proliferation and chemokine production [35]. Moreover, IL-22 deficient (IL-22$^{-/-}$) mice develop less severe collagen induced arthritis (CIA) compared to wild-type mice, indicating an involvement of IL-22 in the pathogenesis of CIA [36].

However, the involvement of IL-22 in autoimmune disease development appears to be context dependent. In skin inflammatory disorders, such as psoriasis, IL-22 acts as a key mediator of pathogenic psoriatic skin features [37]. Moreover, IL-22 produced by innate and adaptive immune cells is required for imiquimod induced skin inflammation in mice [38]. In contrast, IL-22 is not required for the development of experimental autoimmune encephalomyelitis [39] and IL-22 is even protective for experimental inflammatory bowel disease [40]. These discrepancies in the role of IL-22 in autoimmune disease development and the various IL-22 target cells and sources of IL-22 production prompted us to investigate the direct effect of IL-22 and Th22 cells in T cell-mediated synovial inflammation. Using human *in vitro* T cell-RASF co-cultures and murine T cell-mediated experimental arthritis, we show that IL-22 and Th22 cells do not contribute to IL-17/Th17 mediated synovial inflammation.

Methods

Subjects

For this report 10 healthy volunteers (8 women and 2 men, mean age ± SD; 47.4 ± 24.5), 10 treatment naive patients with early RA (6 women and 2 men, mean age ± SD; 49.7 ± 13.7) and 8 patients with established RA and active disease were studied. All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA. Blood was obtained at the second visit after obtaining informed consent. Clinical and laboratory data of treatment naive patients with early RA are shown in supplementary table 1. This study was embedded in the Rotterdam Early Arthritis Cohort Study (REACH) and was approved by the Medical Ethics Committee of the Erasmus MC Rotterdam.

Animals

IL-22$^{-/-}$ mice on the C57BL/6 background [21] were kindly provided by Dr. Wenjun Ouyang, Genentech Inc., USA. Wild-type C57BL/6 mice were purchased from Harlan Laboratories B.V. (Horst, the Netherlands). Mice were kept under specific pathogen free conditions and provided with food and water *ad libitum*. Mice between 8-12 weeks of age were used for experiments. All experiments were approved by the Erasmus MC Animal Ethics Committee (DEC).

Flow cytometry and cell sorting

Monoclonal antibody preparations, intracellular cytokine detection, flow cytometry and cell sorting were described previously [14, 41]. The following human monoclonal antibodies (MoAb) were obtained from BD Biosciences (San Diego, CA): CD45RO, CCR6, CD4 and IFN-γ. IL-22 and IL-17A MoAb were obtained from eBioscience (San Diego, CA). Murine CD4, CD8, IL-17A and IFN-γ MoAb were obtained from BD Biosciences and TCRγδ MoAb from Biolegend Inc. (San Diego, CA). Samples were acquired on a FACScantoII flow cytometer (BD Biosciences) and analyzed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR). T cell populations were sorted from peripheral blood mononuclear cells (PBMC) or synovial fluid mononuclear cells (SFMC) using a FACSAria cell sorter (BD Biosciences).

Cell cultures

RASF isolation and subsequent culture has been described [14]. 10.0 x 10^3 RASF were co-cultured with sorted allogeneic 25.0 x 10^3 peripheral blood total CCR6+ T, Th17 or Th22 cells or 3.0-10.0x10^3 synovial fluid Th17 or Th22 cells. Cells were cultured for 72 hours in Iscove's Modified Dulbecco's Media (IMDM, Lonza, Verviers, Belgium), supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamin (Lonza) and 50 µM β-mercapto-ethanol (Merck, Darmstadt, Germany) and stimulated with soluble αCD3 and αCD28 (0.3 µg/ml and 0.4 µg/ml respectively, Sanquin, Amsterdam, The Netherlands). Cells were cultured in the absence or presence of 10 µg/ml neutralizing IL-22 Moab (R&D systems, Minneapolis, MN) or 2 µM FICZ (Enzo Life Sciences Inc., Farmingdale, NY)

Cytokine measurements

Human IL-6, IL-8 and IFN-γ production was determined using ELISA (Invitrogen). Human, IL-17A, IL-22, TNF-α, MMP-,1 MMP-3 and murine IL-17 expression was measured using Duoset ELISA (R&D systems, Minneapolis, MN). ELISA was performed according to the manufacturer's instructions.

T cell-mediated arthritis

8 µg/ml methylated bovine serum albumin (mBSA, Sigma-Aldrich, St. Louis, MO) was emulsified in an equal volume of complete freund's adjuvant (CFA), containing 1 µg/ml heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories Inc., Detroit, Mi). At day –7, mice were immunized by intra-dermal injection of 100 µl mBSA/CFA emulsion into the tail base. On day 0, arthritis was induced by injecting mice intra-articularly (i.a.) with 60 µg mBSA in 6 µl 0.9% NaCl into both knee joints. The arthritis severity was scored macroscopically, 7 days after immunization, on a scale of 0 to 2. Rear limbs were removed and prepared for histology. Sections were hematoxylin and eosin stained as previously described [41]. The analysis of murine IL-17A cytokine expression in synovial washouts was described earlier [41]. To determine mBSA specific effector cell responses, draining lymph node cells were cultured in the presence or absence of 50 ug/ml mBSA for 3 days. IL-17A production was measured by ELISA. To measure DNA synthesis, cells were pulsed at day 2 with thymidine [^{3}H] for 16–20 h, harvested, and counted using standard methods.

Statistical analysis

Differences between experimental groups were tested with a two-sided paired t-test or stated otherwise, using Prism software v5.04 (GraphPad Software Inc. La Jollla, CA). *P*-values <0.05 were considered significant.

Results

Both Th17 and Th22 cells are increased in peripheral blood of RA patients and present in the inflamed synovium

Recently, we have shown that the proportion of IL-17A and IL-22 producing CD4+CD45RO+ (memory) CCR6+ T cells was increased in patients with early RA [14, 29]. Through a flow cytometric approach combining specific chemokine and cell surface receptor expression it is now possible to distinguish in more detail Th17 and Th22 cells, without the need to perform intracellular cytokine stainings [25, 27, 28]. First, CD25-CCR6+ T cells were gated to exclude regulatory T cells. Subsequently, Th17 and Th22 cells were defined within this CCR6+ gate as CXCR3-CCR4+ whereby Th22 cells, but not Th17 cells were CCR10+ (Figure 1A).

By following this gating strategy, significantly increased peripheral blood memory total CCR6+ T cell, Th17 cell and Th22 cell proportions were identified within the total memory T cell population of patients with early RA compared to age and sex matched healthy controls. The increase of Th17 and Th22 cell proportions (~2.5 and ~3.3 fold respectively) was even relatively larger than the total CCR6+ T cell population (~1.6 fold) (Figure 1B).

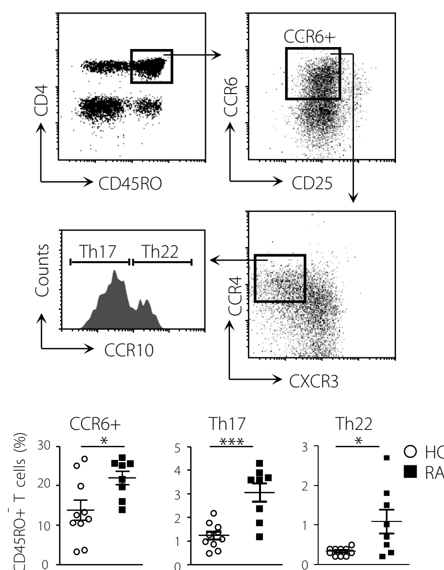

Figure 1 Th17 and Th22 cell populations are increased in peripheral blood of patients with early RA compared to age and sex matched healthy individuals. (**A**) Flow cytometric gating strategy for the identification of peripheral blood Th17 and Th22 populations. CCR6+ T cells are defined as CD4+CD45RO+CCR6+CD25- and within this CCR6+ T cell population, Th17 cells are defined as CXCR3-CCR4+CCR10- and Th22 cells as CXCR3-CCR4+CCR10+ (**B**) Flow cytometric analysis for total CCR6+ T, Th17 and Th22 cells in PBMC of patients with early RA (black squares) and age and sex matched healthy controls (HC, open circles). Mean and SEM are given in a scatter plot for 10 healthy controls (HC) and 8 RA patients. For statistical analysis a two-sided unpaired t-test was performed (* = $p < 0.05$, *** = $p < 0.001$).

In addition, total memory CCR6+ T cell, Th17 cell and Th22 cell populations were present in matched peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) from patients with established RA and active disease. Moreover, total memory CCR6+ T cell, Th17 cell and Th22 cell proportions were similar in SFMC compared to PBMC (Figure 2A). The presence of Th17 (IL-17A+IL-22+/-) and Th22 (IL-17A-IL-22+) cells in SFMC was also indicated by performing intracellular IL-17A, IL-22 and IFNγ stainings (Figure 2B).

These data show that in addition to the total memory CCR6+ T cell population, Th17 and Th22 cells are increased in peripheral blood of patients with early RA and that both Th17 and Th22 cells are present in synovial fluid.

IL-17A, but not IL-22 is up-regulated in CCR6+ T cell-RASF cultures

The interaction of CCR6+ T cells and RASF induces a pro-inflammatory loop leading to increased autocrine IL-17A production [14]. To verify whether this interaction leads to increased IL-22 production as well, CCR6+ T cell cultures with or without RASF were analyzed for IL-17A, IL-22 and TNF-α production. Increased IL-17A producing CCR6+ T cell proportions and increased IL-17A protein levels were detected in CCR6+ T cell RASF co-cultures compared to CCR6+ T cell mono-cultures as shown before [14]. In

contrast, no difference was found in the fraction of IL-22 producing CCR6+ T cells as well as IL-22 and TNF-α protein levels (Figure 3A and 3B). This clearly shows a specific induction of IL-17A, but not of IL-22 and TNF-α production by CCR6+ T cells upon RASF interaction.

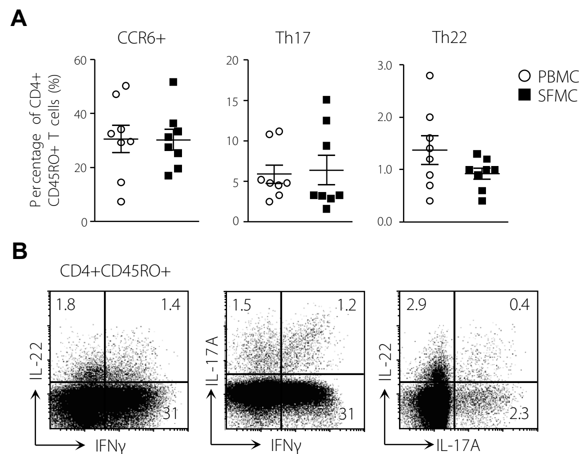

Figure 2 Identification of Th17 and Th22 cells in peripheral blood and synovial fluid of patients with established RA. (**A**) Flow cytometric analysis for total CCR6+ T cells, Th17 and Th22 cells in PBMC (open circles) and SFMC (black squares). Mean and SEM are given in a scatter plot for 8 patients with established RA and active disease per group. (**B**) Flow cytometric analysis for intracellular IL-17A, IL-22 and IFN-γ cytokine expression in CD4+CD45RO+ synovial T cells in patients with established RA. Numbers in representative dot plots indicate the proportion of cytokine expressing cells per quadrant.

IL-22 has limited effects on IL-6, IL-8, MMP-1 and MMP-3 production in CCR6+ T cell-RASF cultures

Upon interaction with CCR6+ T cells, RASF produce pro-inflammatory mediators such as IL-6, IL-8 and tissue destructive enzymes, such as matrix metalloproteinase-1 (MMP-1) and MMP-3. This production is largely dependent on IL-17A and TNF-α [14]. However, the contribution of IL-22 to IL-6, IL-8, MMP-1 and MMP-3 induction is unclear. Therefore, CCR6+ T cell-RASF co-cultures were performed wherein IL-22 signalling was neutralized by anti-IL-22 antibodies, or wherein IL-22 production was induce by an AHR agonist, 6-formylindolo[3,2-b]carbazole (FICZ) [27]. IL-22 neutralization had no effects on IL-17A, IL-6, IL-8, MMP-1 and MMP-3 production in CCR6+ T cell-RASF co-cultures (Figure 4). Treatment of CCR6+ T cell cultures with FICZ resulted in a ~4.7 fold induction of IL-22 expression. This had a slight, but not significant inhibitory effect on IL-17A production in CCR6+ T cell-RASF co-cultures. This effect on IL-17A was accompanied by a significant reduction of IL-6, but not of IL-8, MMP-1 and MMP-3 production. These findings show that IL-22 has limited effects on pro-inflammatory cytokine and MMP production in CCR6+ T cell-RASF co-cultures in the presence of IL-17A.

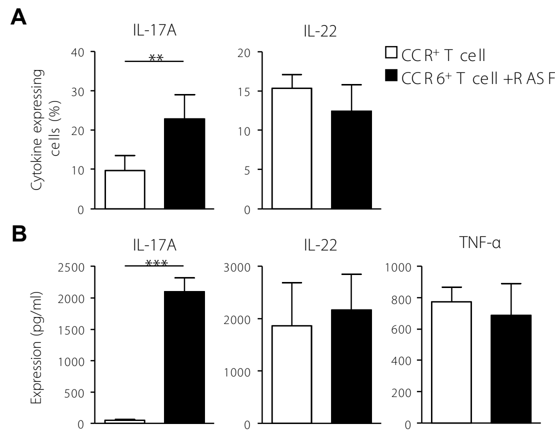

Figure 3 IL-22 production by CCR6+ T cells is not induced upon interaction with RASF. (**A-B**) Cultures of 25.0 x 10^3 sorted CCR6+ T cells from patients with early RA in the presence or absence of 10.0 x 10^3 RASF. Cells were cultured for 3 days in the presence of αCD3 and αCD28. (**A**) Flow cytometric analysis for intracellular IL-17A and IL-22 expression in CCR6+ T cell cultures (white bars) and CCR6+ T cell RASF co-cultures (black bars). (**B**) IL-17A, IL-22 and TNF-α expression levels were determined by ELISA in supernatant of CCR6+ T cell cultures (white bars) and CCR6+ T cell RASF co-cultures (black bars). Mean and SEM are given for 5-7 treatment-naive early RA patients per group (** = $p < 0.01$, *** = $p < 0.001$).

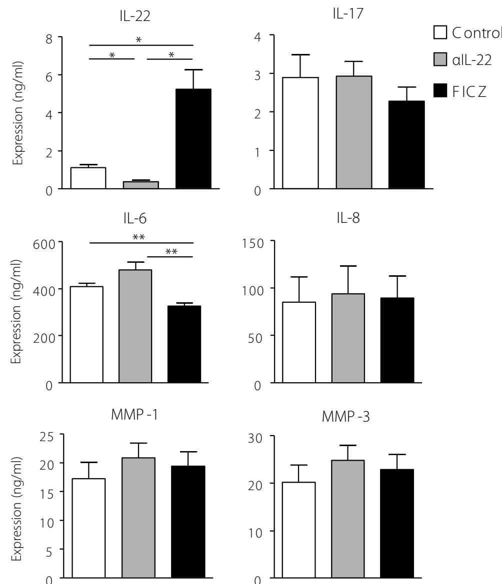

Figure 4 IL-22 has limited effects on pro-inflammatory cytokines and MMPs produced by RASF upon interaction with CCR6+ T cells. 10.0 x 10^3 RASF were co-cultured with 25.0 x 10^3 sorted CCR6+ T cells from patients with early RA. Cells were cultured for 3 days in the presence of αCD3 and αCD28 and in the absence or presence of αIL-22 (10 μg/ml) or FICZ (2 μM). Indicated cytokines and MMPs were determined in supernatant by ELISA. Mean and SEM are given for 5 treatment-naive early RA patients per group. Results are representative for at least 3 independent experiments (* = $p < 0.05$, ** = $p < 0.01$).

Th22 cells are less potent inducers of IL-6, IL-8 and MMP-1 production by RASF compared to Th17 cells

In addition, the effects of primary Th17 or Th22 cells from patients with early RA on RASF were investigated. Therefore, primary Th17 and Th22 cells were sorted according to the gating strategy as shown in figure 1A and cultured in the presence of RASF. To verify the phenotype of the sorted Th17 and Th22 cells, intracellular flow cytometric staining for IL-17A and IL-22 was performed. Th17 and Th22 cells both produced IL-17A and IL-22, but IL-17A production was higher in Th17 cells (4.4% vs. 1.3% in Th22) and IL-22 production was higher in Th22 cells (9.3% vs. 2.0% in Th17) (Figure 5A). These differences were reflected by protein expression levels in the culture supernatant. Th17 cells expressed higher levels of IL-17A compared to Th22 cells and IL-22 was expressed by both Th17 and Th22 cells. Furthermore, slightly higher levels of IFN-γ and TNF-α were expressed by Th17 cells compared to Th22 cells (Figure 5B).

Importantly, compared to peripheral blood Th17 cells, Th22 cells were less efficient in the induction of IL-6 and MMP-1 production by RASF (Figure 5B). To verify whether this phenomenon is also true for synovial Th22 cells, synovial Th17 and Th22 cells were sorted and co-cultured with RASF. After co-culture, IL-17A and IFN-γ expression was restricted to Th17-RASF co-cultures, whereas both Th17-RASF and Th22-RASF co-cultures expressed IL-22 and TNF-α. Compared to synovial Th17 cells, synovial Th22 cells are less potent in inducing IL-6 and MMP-3 production by RASF (Figure 5C).

When taken together, compared to peripheral and synovial Th17 cells, Th22 cells are less efficient in inducing pro-inflammatory cytokine and MMP production by RASF.

IL-22 does not contribute to IL-17A/Th17 mediated synovial inflammation in antigen induced arthritis

To investigate whether IL-22 directly contributes to T cell-mediated synovial inflammation *in vivo*, the murine mBSA antigen induced arthritis (AIA) model was used. This model is largely T cell-mediated, dependent on IL-17A and positive for IL-22 expression IL-22 [41, 42]. AIA was induced in IL-22 deficient (IL-22$^{-/-}$) mice and wild-type mice. Knee swelling in time was measured and arthritis severity was macroscopically scored. No difference in either knee swelling, macroscopical inflammation score, histology and IL-17A production between arthritic wild-type and IL-22$^{-/-}$ mice was detected, indicating that in the presence of IL-17A, IL-22 is not directly involved in T cell-mediated synovial inflammation in AIA (Figure 6A-D). This was further supported by similar CD4, CD8 and TCRγδ T cell proportions, and similar proportions of IL-17A and IFN-γ producing CD4 T cells in arthritic IL-22$^{-/-}$ and wild-type draining lymph nodes (Figure 6E). In addition, similar mBSA specific proliferation or cytokine production responses were observed between arthritic IL-22$^{-/-}$ and wild-type draining lymph node cells (Figure 6E and 6F).

Taken together, these findings show that lack of IL-22 has no *in vivo* contribution to IL-17A/Th17 mediated synovial inflammation in AIA.

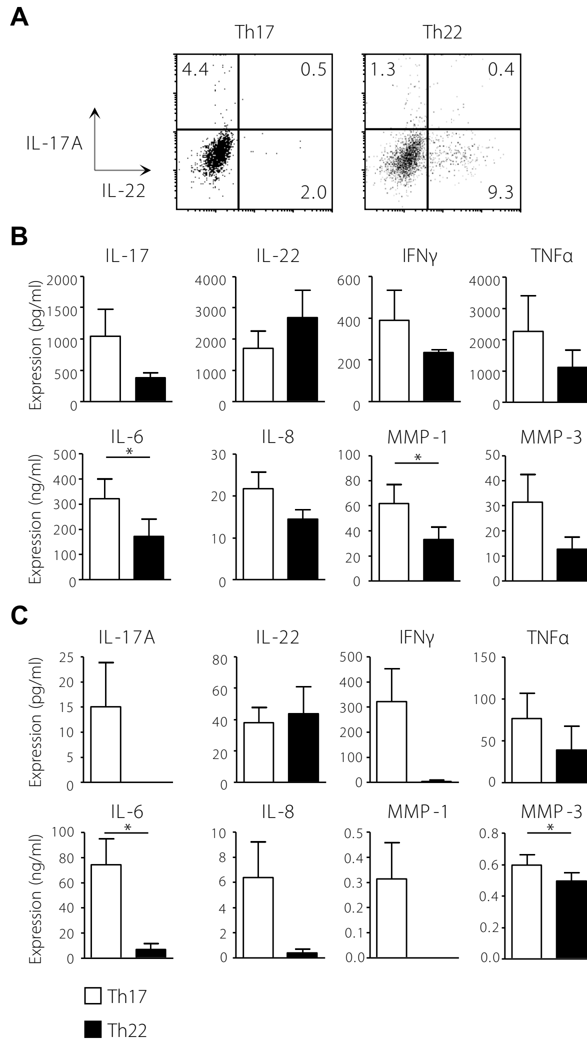

Figure 5 Effects of primary Th17 and Th22 of patients with early RA in RASF co-cultures. (**A-B**) 10.0 x 10^3 RASF were co-cultured with 25.0 x 10^3 sorted primary Th17 or Th22 cells from patients with early RA. Cells were cultured for 3 days in the presence of αCD3 and αCD28. (**A**) Flow cytometric analysis for intracellular IL-17A and IL-22 expression by primary Th17 or Th22 cells, co-cultured for 3 days with RASF. Numbers in representative dot plot represents proportion of cytokine expressing cells per quadrant. (**B**) Indicated cytokines and MMPs were determined in supernatant by ELISA. Mean and SEM are given for 5 treatment-naive early RA patients per group. (**C**) 10.0 x 10^3 RASF were co-cultured with 3.0 – 10.0 x 10^3 sorted primary synovial fluid Th17 or Th22 cells from patients with early RA. Indicated cytokines and MMPs were determined in supernatant by ELISA. Mean and SEM are given for 4 patients with established RA per group (* = $p < 0.05$, ** = $p < 0.01$).

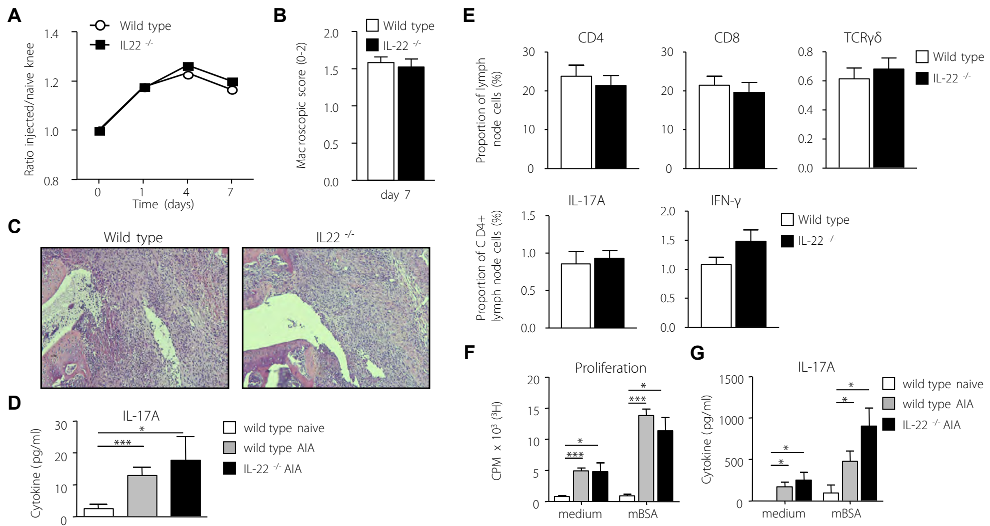

Figure 6 Effects of IL-22 deficiency in antigen induced arthritis (AIA). Arthritis was induced in both IL-22$^{-/-}$ and wild type mBSA immunized mice by an intra-articular injection with 60 µg mBSA. (**A**) Synovial inflammation of wild-type (black squares) and IL-22$^{-/-}$ mice scored as the ratio between knee thickness before onset and 1, 4 and 7 days after onset of arthritis. Mean is given for 24 knee joints of 12 mice per group per time point. (**B**) Macroscopical scores of synovial inflammation of wild-type (white bars) and IL-22$^{-/-}$ (black bars) mice, 7 days after onset of arthritis. Mean and SEM are given for 24 knee joints of 12 mice per group. (**C**) Representative histological (H&E) stainings of knee joint sections obtained 7 days after immunization from wild-type and IL-22$^{-/-}$ mice. (**D**) Expression of IL-17A synovial washouts of naive wild type (white bars), arthritic wild type (grey bars) and arthritic IL-22$^{-/-}$ mice (black bars). Mean and SEM are given for 8 synovial washouts per group (**E**) Flow cytometric analysis for the population of CD4, CD8 and TCRγδ T cells and intracellular IL-17A and IFN-γ expression obtained from wild-type (white bars) and IL-22$^{-/-}$ mice (black bars), 7 days after the induction of arthritis. Data are obtained from at least 2 separate experiments. Mean and SEM are given for 6 mice per group. (**F-G**) MBSA specific responses of draining lymph node cells from naive wild type (white bars), arthritic wild type (grey bars) and arthritic IL-22$^{-/-}$ mice (black bars), cultured in the presence or absence of mBSA. (**F**) Proliferative response to mBSA stimulation, as determined by [^{3}H] thymidine incorporation. (**G**) IL-17A response to mBSA stimulation. Mean and SEM are given for 4-9 mice per group. For statistical analysis a two-sided unpaired t-test was performed. (* = $p < 0.05$, *** = $p < 0.001$).

Discussion

By using cultures with primary human T cells of treatment naive patients with early RA and T cell-mediated arthritis in IL-22$^{-/-}$ mice, we showed that IL-17A/Th17 mediated synovial inflammation is independent of IL-22. IL-22 produced by CCR6+ T cells had limited effects on RASF produced pro-inflammatory cytokines IL-6, IL-8 and tissue destructive enzymes MMP-1 and MMP-3. In addition, primary human peripheral or synovial Th22 cells were markedly less potent than Th17 cells in inducing IL-6, MMP-1 and MMP-3 production by RASF. Moreover, deficiency of IL-22 *in vivo* has no significant effect on IL-17A/Th17 mediated synovial inflammation in AIA.

Despite, the limited observed effects of IL-22 and Th22 cells on IL-17A/Th17 mediated synovial inflammation, increased IL-22 levels or Th22 cell proportions are found in patients with early RA and

are present in synovial fluid. Interestingly, Th22 cells correlate with elevated proportions of Th17 cells in patients with RA [32]. This and the shared developmental program and dependence on similar transcriptional regulators and cytokines, such as ROR-C and IL-23 [19], may imply that IL-22 expression is commonly accompanied with IL-17A mediated function. This may be highly relevant in local mucosal immune responses against microorganisms [19, 23, 24], whereas this is less relevant in non-mucosal environments, such as the joint synovium. We did find increased IL-17A, but not IL-22 production by CCR6+ T cells in the presence of RASF. The finding of increased IL-22 levels or Th22 in patients with RA, suggest additional signaling events or cellular interactions to induce IL-22 expression. In this line, addition of IL-23 or LPS activated monocytes were able to induce the induction of IL-22 in our system (data not shown).

On the other hand, IL-22R is expressed by RASF and within synovial tissue IL-22 is produced by RASF [35], indicating a function for IL-22 in the inflamed synovium. Treatment of RASF with IL-22 has been shown to induce proliferation and expression of chemokines, such as CCL2 [35]. However, in our T cell-RASF co-cultures we were not able to identify an effect of IL-22 on the expression of CCL2 (data not shown). This lack of effect together with no inducing effects on the pro-inflammatory mediators IL-6 and IL-8 produced by RASF indicates that in comparison to IL-17A, which is a potent inducer of IL-6 and IL-8 [14], IL-22 has limited effects in T cell-mediated synovial inflammation.

Moreover, the effect of IL-22 is likely dependent on the stage of synovial inflammation. In IL-1Ra$^{-/-}$ mice, IL-17A is already expressed in the early stages of inflammation, whereas IL-22 is mainly expressed in highly inflamed synovia [43]. This may explain the mild effect on arthritis severity in IL-1Ra$^{-/-}$ mice after anti-IL-22 treatment compared to anti-IL-17A treatment [43]. The expression of IL-22 in later stages of arthritis and our finding that IL-22 deficiency has no effect in T cell-mediated arthritis, shows that IL-22 has a secondary effect on synovial inflammation rather than a direct role in the induction of synovial inflammation.

It might be that IL-22 has a role in the induction of bone erosions in later stages of RA. This would be in line with the finding that IL-22 expression levels correlate with bone erosions and the induction of osteoclastogenesis by RASF induced RANKL production [12, 34, 44].

The observation that the increase of IL-22 levels and Th22 cells in patients with RA correlates with elevated Th17 cells and progression of bone erosions [12], would argue for the use of IL-22 or Th22 cells as a biomarker in RA. For this purpose a combination of chemokine receptors and cytokines will be preferable to distinguish pure Th17 and Th22 cell populations (Figure 1)

From the findings that synovial inflammation was not affected in IL-22$^{-/-}$ mice it can be concluded that local IL-22 produced by adaptive or innate immune cells have no direct contribution to the induction of T cell-mediated synovial inflammation. However, this is in disagreement with findings obtained by collagen induced arthritis (CIA) in IL-22$^{-/-}$ mice, in which arthritis incidence and severity was lower compared to wild-type mice [36]. On the other hand, our data are in line with other experimental auto-immune disease models, such as EAE, in which IL-22 is not required for the induction of encephalomyelitis [39]. A possible explanation for these differences is the underlying pathological

mechanism in these experimental autoimmune models. Whereas AIA and EAE are largely dependent on cellular immune responses, CIA is both dependent on humoral and cellular responses, suggesting a more important role of IL-22 in the humoral response and less in the cellular response [36, 41, 45]. After CIA induction high total IgG and collagen specific IgG levels were found in IL-22$^{-/-}$ mice [36], suggesting a role of IL-22 in IgG production by B cells. In this context, a current study performed in our laboratory (Corneth et al., unpublished), confirmed the data as published by Geboes et al. [36], that IL-22 deficiency in the CIA model results in decreased arthritis incidence and severity. However, we observed impaired germinal center formation terminal B cell differentiation in IL-22$^{-/-}$ mice. As IL-22 is expressed in lymphoid organs the suppressive effects of IL-22 deficiency on arthritis severity may be caused by intrinsic defects in germinal center formation and altered kinetics of collagen specific antibody production in IL-22$^{-/-}$ mice.

IL-22 serum levels in patients with RA correlated with serum titers of antibodies against citrinulated peptides [44]. It would be of interest to investigate whether these antibodies are of high affinity and whether the kinetics of antibody production is altered in IL-22$^{-/-}$ mice.

Studying the role of IL-22 is complicated, because (1) IL-22 is expressed by multiple cells of the immune system, such as T cells, NK cells and lymphoid tissue inducer cells, (2) IL-22 is expressed in different tissues, including the skin, gut and lymph nodes and (3) IL-22 has multiple context dependent functions, such as wound healing and microbial defence [1-2]. The fact that there are contrasting results of studies regarding IL-22 and its pro-inflammatory, protective or no effects in different diseases or disease models may be inherent to these context dependent effects of IL-22. In psoriasis for example IL-22 can synergize with other proinflammatory cytokines to induce many of the pathogenic phenotypes from keratinocytes and exacerbate disease progression in psoriasis. In contrast, IL-22 plays a beneficial role in IBD by enhancing barrier integrity and epithelial innate immunity of the intestinal tract [21, 40, 46, 47]. Moreover, IL-22 has protective roles in airway inflammation, protects against liver pathology during malaria infection [48-50]. When taken together, IL-22 does not contribute to the induction of IL-17A/Th17 mediated synovial inflammation. This implies that treatment of patients with early RA should focus on targeting IL-17A and Th17 cells, rather than on targeting IL-22.

Acknowledgements

We would like to thank H. Vroman, M. Brem, B. Bartol, H. Bouallouch-Charif and N. Kops and the people from the EDC Animal Facility (Erasmus MC Rotterdam) for technical assistance at various stages of the project.

Supplementary Table

Supplementary Table 1. Clinical and laboratory data

ACR 1987 criteria	(No patients (%))*
Morning stiffness	5/8 (63)
Arthritis of 3 or more joint areas	6/8 (75)
Arthritis of hand joints	7/8 (88)
Symmetric arthritis	7/8 (88)
Rheumatoid nodules	0/8 (0)
Serum rheumatoid factor positif	5/8 (63)
Serum ACPA positif	7/8 (88)
Bone erosions	0/8 (0)
Clinical parameters	**(mean ± S.E.M.; range)**
DAS28	4.3 ± 0.4 (2.7-5.8)
Swollen joint count	3.6 ± 0.8 (0-7)
Tender joint count	5.3 ± 1.5 (0 -13)
Disease duration (months)	4.3 ± 1.2 (1-11)

*All patients were classified as RA ≥ 4 criteria

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Chapter 5

IL-17A-low CCR6+ Th cell populations of patients with rheumatoid arthritis are pathogenic, multidrug resistant and associated with DMARD and glucocorticoid treatment response

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Abstract

Background CCR6+ T-helper (Th) cells are implicated in rheumatoid arthritis (RA). However, within the CCR6+ Th population various subpopulations are present and the clinical relevance of each subpopulation is unclear. Therefore, we characterized CCR6+ Th subpopulations with regard to pathogenic potential and treatment outcome in RA.

Methods Within CCR6+ Th cells from RA patients, CCR4+CXCR3- (Th17), CCR4-CXCR3+ (Th17.1), CCR4/CXCR3 double-positive (DP) and double-negative (DN) cells were distinguished and/or sorted by flow cytometry. These subpopulations were: analyzed for Th17/Th1-associated factors; co-cultured with RA-derived synovial fibroblasts (RASF); related to disease-modifying antirheumatic drugs (DMARDs) and glucocorticoid (GC) therapy response; analyzed regarding the expression of multidrug transporters MDR1 and MRP1 and drug efflux potential.

Results All CCR6+ Th subpopulations were RORC+ and present in RA peripheral blood and synovial fluid. Despite differential IL-17A, IL-17F, IFNγ and TBX21 expression, all subpopulations, including IL-17A low-producing Th17.1, DP and DN cells, showed pathogenic activity in the induction of IL-1β, IL-6, IL-8, COX-2 and MMP-3 expression by RASF. Despite strongly varying MDR1 and MRP1 expression, all subpopulations, and particularly Th17.1 and DN cells, displayed higher drug efflux potential compared to Th1. DMARD/GC therapy induced MRP1 expression by CCR6+ Th subpopulations, and DMARD/GC non-response was associated with increased drug efflux potential by these populations.

Conclusion Despite distinct differences in Th17/Th1 characteristics, including IL-17A expression, all CCR6+ Th subpopulations display pathogenic activity. Future treatment strategies towards CCR6+ Th cells in RA should focus on targets shared by all subpopulations. Personalized treatment strategies may be improved by using drug efflux potential of CCR6+ Th cells to monitor DMARD/GC response.

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease mainly characterized by joint inflammation. Without appropriate treatment an early inflammation may develop to a chronic destructive arthritis, leading to irreversible cartilage- and bone destruction and physical disability [1, 2]. Initial RA treatment commonly includes disease-modifying antirheumatic drugs (DMARDs), such as methotrexate (MTX), and/or glucocorticoids (GCs). However, a large fraction (~25-45%) of patients with RA does not respond to, or becomes resistant to this initial therapy [3-5]. Therefore it is necessary to identify cells critically involved in the pathogenesis of RA and in the lack of DMARD/GC therapy response. T-helper (Th) cells, and in particular Th cells expressing the C-C chemokine receptor type 6 (CCR6) are implicated in RA pathogenesis [2, 6]. CCR6+ Th cells are characterized by expression of the transcription factor retinoic acid receptor-related orphan receptor C (RORC) and excretion of the cytokines interleukin (IL)-17A, IL-17F, interferon (IFN)γ and IL-22 [7-9]. Evidence is now emerging that CCR6+ Th cells are involved in the development of an early inflammation towards a chronic destructive arthritis [10]. For example, in peripheral blood of treatment naïve patients with early RA, proportions of CCR6+ Th cells are higher than in healthy persons, and these cells are present in RA synovium [11-14]. Moreover, we recently found that memory CCR6+ Th cells can induce a pro-inflammatory feedback loop upon interaction with RA-derived synovial fibroblasts (RASF), resulting in the production of pro-inflammatory mediators, including IL-1β, IL-6, IL-8 and PGE_2, and tissue destructive mediators like matrix metalloproteinase-1 (MMP-1) and MMP-3. This loop induced by CCR6+ Th cells is critically dependent on IL-17A, TNFα and PGE_2 [14-16].

Differential chemokine receptor expression is instrumental to distinguish human Th cell populations. For example CCR6- Th cells positive for either CCR4 or CXCR3 are enriched for respectively Th2 and Th1 characteristics: CCR6-CCR4+ Th cells express high levels of IL-4 and GATA3, whereas CCR6-CXCR3+ Th cells express high levels of T-box transcription factor 21 (TBX21) and IFNγ [17]. Interestingly, CCR6+ Th are heterogeneous in their ability to co-express chemokine receptors. Based on differential CXCR3, CCR4 and CCR10 expression various CCR6+ Th subpopulations can be identified with specific cytokine production and transcription factor expression profiles [18, 19]. IL-17A and IL-17F producing cells are mainly found within CCR4+CXCR3- cells, representing the classic Th17 cell profile. Th22 cells, which mainly produce IL-22, are restricted to CCR10+ cells [13, 20, 21]. Recently we showed that Th22 cells were less potent than Th17 cells in inducing inflammatory mediator production by RASF [13].

CCR6+CXCR3+CCR4- cells have characteristics of both Th17 and Th1 cells, such as RORC and TBX21 expression and IL-17A and IFNγ production and are therefore named non-classic Th1 or Th17.1 cells [19, 22, 23]. Interestingly, Th17.1 cells were described to be enriched in pathogenic factors and expressed multidrug resistance protein 1 (MDR1), an ATP-binding cassette (ABC) transporter that is expressed on the cell membrane and is able to export GCs out of the cell. Accordingly, MDR1+ Th17.1 cells were resistant to GC suppression, and these cells were found in the gut of Crohn's disease patients [18]. Besides Th17, Th22 and Th17.1 additional CCR6+ Th populations can be identified that are double-positive (DP) or double-negative (DN) for CXCR3 and CCR4 expression.

Although CCR6+ Th cells as a whole population are implicated in RA, it is unclear to what extent the various CCR6+ Th subpopulations, characterized by distinct cytokine, transcription factor and multidrug transporter profiles, contribute to the pathogenic activity of CCR6+ Th cells and the response on DMARD/GC therapy. Therefore, our main goals in the present study were: (i) to identify CCR6+ Th cell subpopulations in peripheral blood and synovial fluid of patients with RA, (ii) to characterize the cytokine and transcription factor profile of these subpopulations (iii) to characterize the pathogenic potential and the drug efflux potential of the subpopulations and (iiii) to relate these findings to DMARD/GC therapy response.

Methods

Patients

Peripheral blood mononuclear cells (PBMCs) were sampled from treatment naïve, early RA patients at baseline and after 6 months of DMARD/GC therapy. In addition paired PBMC and synovial fluid mononuclear cells (SFMCs) were obtained from patients with established and inflammatory RA. All patients met the American College of Rheumatology 2010 revised criteria for RA, and none had been taking DMARDs before starting the therapy. Samples from 16 therapy responders and 18 non-responder patients were studied. Response to therapy was defined as a disease activity score in 44 joints (DAS44) ≤ 1.6 after 3, 6, 9 and 12 months of treatment. Non-response to therapy was defined as a DAS44 ≥ 2.4 after 3 months of treatment and a DAS44 ≥1.6 after 6, 9 and 12 months of treatment. At baseline, patients were randomly assigned to one out of three treatment strategies: A) combination therapy, consisting of MTX, sulfasalazine (SSZ) and hydroxychloroquine (HCQ), with GCs intramuscularly; B) combination therapy with an oral GC tapering scheme; or C) MTX with an oral GC tapering scheme (study described in [4]). Furthermore, non-responders were given etanercept after the initial 3 months treatment. Characteristics of these patients at baseline and at 6 months after the start of DMARD/GC therapy are shown in table 1.

Table 1 Characteristics of DMARD/GC non-responding and responding patients with early RA

Characteristic	Non-responders (n=18)	Responders (n=16)	p-value
Treatment (A/B/C)	2/3/13	7/4/5	0.041*
Sex (female/male)	15/3	7/9	0.015
Age (mean ± SD, years)	55.20 ± 11.08	46.68 ± 16.76	0.087
Duration of complaints (mean ± SD, days)	140.39 ± 86.99	137.13 ± 83.30	0.91
Erosions at baseline n (%)	2 (11.1)	4 (25.0)	0.30
RF positive at baseline n (%)	16 (88.9)	12 (75.0)	0.30
ACPA positive at baseline n (%)	12 (66.7)	13 (81.3)	0.35
DAS44 T0 (mean ± SD)	3.51 ± 0.54	3.21 ± 0.73	0.18
DAS44 T3 (mean ± SD)	3.24 ± 0.45	0.83 ± 0.39	<0.001
DAS44 T6 (mean ± SD)	3.05 ± 0.74	0.78 ± 0.46	<0.001

*calculated using chi-square test

Flow cytometry, cell sorting and antibodies

Monoclonal antibody stainings, intracellular cytokine and transcription factor detection and flow cytometry were performed as described previously [13]. MDR1-PE (clone UIC2) and isotype control IgG2a κ-PE (clone MOPC-173) were bought from BioLegend (San Diego, CA). MRP1-FITC (clone QCRL) and isotype control IgG1-FITC (clone 11711) were purchased from R&D systems (Minneapolis, MN). ABCG2-PE (clone 5D3) and isotype control IgG2b κ-PE (clone MPC-11) were bought from BioLegend. Other fluorochrome-labeled antibodies were purchased from eBioscience (San Diego, CA), BD Biosciences (San Diego, CA), BioLegend and R&D systems. Samples were acquired on a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR). T cells were sorted with a FACSAria cell sorter (BD Biosciences). Purity of the obtained T cell populations was ≥ 98%.

Cell culture and dye efflux analysis

RASF isolation and subsequent culture was performed as described previously [14]. In brief, $1.0x10^4$ RASF were seeded in a 96-well flat bottom plate. After 24 hours, RASF were co-cultured with $1.0x10^4$ allogeneic T cells. Cells were stimulated with 0.3 µg/ml soluble αCD3 and 0.4 µg/ml αCD28 (Sanquin, Amsterdam, The Netherlands) and cultured for 4 days in Iscove's Modified Dulbecco's Media (IMDM, Lonza, Basel, Switzerland), supplemented with 10% fetal calf serum (FCS, Invitrogen, Carlsbad, CA), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamine (Lonza) and 50 µM β-mercapto-ethanol (Merck, Darmstadt, Germany).

Calcein-AM and Rhodamine-123 (Rh123) (Sigma-Aldrich, St. Louis, MO) were dissolved in DMSO and ethanol respectively. Rh123 or calcein-AM were added to $\sim 1.5x10^{\wedge 6}$ PBMCs in a final concentration of 0.5 µg/ml (Rh123) and 0.025 µg/ml (Calcein-AM) and incubated for 30 minutes in the dark at 4° C. After a PBS wash the cells were incubated at 37° C for 2 hours in the presence or absence of MK571 (100 µg/ml, Sigma-Aldrich) or valspodar (5 µg/ml, Sigma-Aldrich).

Enzyme-linked immunosorbent assay (ELISA)

IFNγ, IL-6 and IL-8 expression was determined using ELISA (Invitrogen). IL-17A, TNFα, MMP-1 and MMP-3 expression was measured by Duoset ELISA (R&D systems). ELISA was performed according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (PCR) analysis

RNA extraction and cDNA syntheses were performed as described previously [24]. Primers were designed with ProbeFinder software and probes were used from the universal probe library (Roche Applied Science, Indianapolis, IN). Quantitative real-time PCR (RT-PCR) was performed and analyzed using the ViiA7 sequence detection system and software (Life Technologies, Carlsbad, CA). Hypoxanthine–guanine phosphoribosyltransferase (HPRT) was used to normalize gene transcription. Primer and probe sequences are available upon request.

Statistical analysis

Differences between experimental groups were tested with unpaired student's T test or paired student's T test using Prism software v5.04 (GraphPad Software Inc. La Jolla, CA), unless otherwise indicated. *P*-values <0.05 were considered significant.

Results

Within the CCR6+ Th cell population from patients with RA, subpopulations are present with distinct Th1 and Th17 cell characteristics.

We recently found increased proportions of peripheral blood CCR6+ Th cells in treatment naïve patients with early RA. These CCR6+ Th cells were potent stimulators of synovial fibroblast activation [14, 16]. However, the CCR6+ Th cell population is heterogeneous, and by using CCR4 and CXCR3 expression various CCR6+ Th cell subpopulations can be identified in peripheral blood of early, treatment naïve RA patients: CCR6+CCR4+CXCR3- Th cells consist of both Th17 and Th22 cells [13, 20, 21]; and CCR6+CCR4-CXCR3+ cells are Th17.1 cells [18, 19, 25]. Two additional CCR6+ Th cell populations were identified that are either double-positive (DP) or double-negative (DN) for CCR4 and CXCR3 expression (Figure 1A). In treatment naïve patients with early RA, CCR6+ Th cells on average consists mostly of Th17/Th22 cells (~45±11 %), followed by Th17.1 (~22±9 %), DP (~17±12 %) and DN cells (~16±8 %). A large variation exists between patients, in particular in the Th17.1, DN and DP CCR6+ Th populations (Figure 1B). Similar to treatment naïve patients with RA, all CCR6+ Th populations were present in peripheral blood and synovial fluid of patients with established RA (Figure 1C). Th17.1, in contrast to Th17, of healthy individuals possesses characteristics of both Th17 and Th1 cells, such as the expression of RORC and TBX21 and the production of IL-17A and IFNγ [18, 19]. To investigate these characteristics in treatment naïve patients with early RA, Th17/Th22 and Th17.1 cells, but also DN and DP CCR6+ Th cells were sorted (Figure 1D) and analyzed for the transcription of Th17 and Th1 associated genes. Th1 and naïve T cells were used as reference populations. Compared to naïve and Th1 cells all CCR6+ Th populations were positive for RORC expression. In contrast, TBX21 was differentially expressed among the CCR6 Th subpopulations, with the highest expression in Th17.1 cells. In addition, IL-17A gene expression was found to be relatively high in Th17 cells and low in Th17.1, DN and DP cells. In contrast, relatively high IFNγ gene expression levels were observed for Th17.1 cells in comparison to the other populations (Figure 1E).

When taken together, these data show that CCR6+ Th cell subpopulations, which display distinct Th17 and Th1 characteristics, are present in both treatment naïve patients with early RA and in established RA patients.

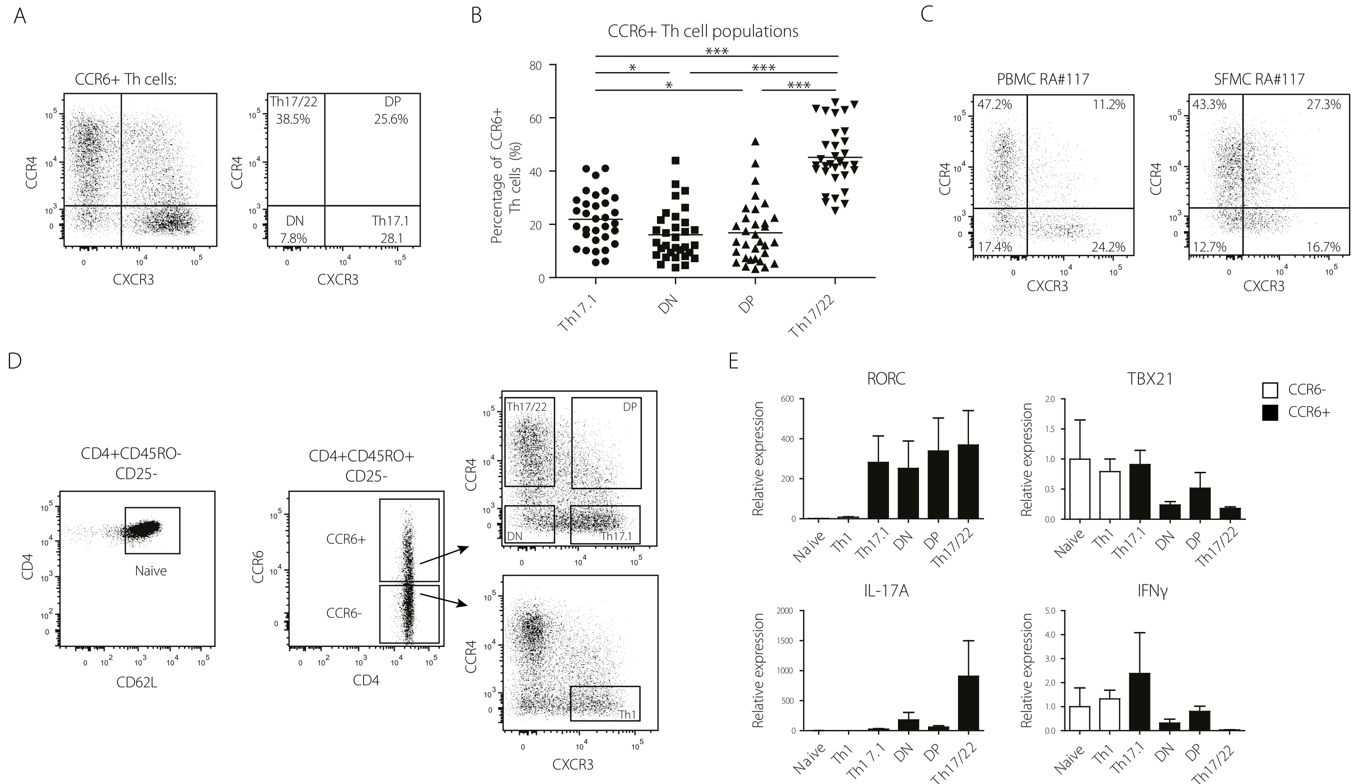


Figure 1 CCR6+ Th cell subpopulations in peripheral blood and synovial fluid are distinguished by a differential chemokine receptor and transcription factor expression profile. (**A**) Left: representative dot plot showing CCR4 and CXCR3 expression of CCR6+ Th cells from a treatment-naïve patient with early RA. Right: numbers indicate the proportion of cells per quadrant. (**B**) quantification of indicated CCR6+ Th subpopulations as proportions of peripheral blood CCR6+ Th cells from 32 treatment-naïve early RA patients. (**C**) Representative dot plots of CCR4 and CXCR3 expression by CCR6+ Th cells from paired peripheral blood and synovial fluid of a patient with established RA. (**D**) Gating strategy used to sort the indicated peripheral blood CD4+ T cell populations from patients with early RA. (**E**) Quantitative RT-PCR analysis of RORC, TBX21, IL-17A and IFNγ transcription by the indicated CCR6- T cell (white bars) and CCR6+ T cell (black bars) populations. Mean and SEM are given for 5 patients per group. For statistical analysis a two-sided paired t-test was performed (* = $p < 0.05$, *** = $p < 0.001$).

Chapter 5

Despite differences in Th17 and Th1 characteristics all CCR6+ Th cell subpopulations display high pathogenic potential.

To further assess the characteristics and pathogenicity the CCR6+ Th cell populations, Th17, Th17.1, DP and DN cells were sorted from treatment naïve patients with early RA and co-cultured with RASF. We recently found that Th22 cells are present in peripheral blood and synovial fluid of RA patients, but they are not involved in synovial inflammation [13]. By sorting for CCR10- cells, Th22 cells were excluded from the analysis. Primary Th1 and naive Th cells served as reference populations. Upon co-culture with RASF the CCR6+ Th cell populations retained their Th17 associated transcriptional profile as indicated by the expression of RORC. TBX21 remained differentially expressed within the CCR6+ Th cell populations, with relatively high expression in Th17.1 cells. The Th17 cell characteristics of all CCR6+ populations were further confirmed by CD161 expression, a surface receptor associated with human Th17 cells [26] (Figure 2A).

Next, the cytokine production of the CCR6+ Th cell populations was assessed. All populations produced IL-17A, with highest production by Th17 cells. While IFNγ was mainly produced by Th17.1 cells, TNFα was produced by all CCR6+ Th cell populations. Th17 and DP Th cells produced the highest levels of IL-22 and IL-17F (Figure 2B).

The pathogenic potential of the CCR6+ Th cell subpopulations was assessed from the induction of pro-inflammatory mediators produced upon co-culture with RASF. Despite the differential IL-17A and IFNγ production, all CCR6+ Th cell populations were strikingly more potent than Th1 and naïve Th cells to induce IL-1β, COX-2, IL-6, IL-8 and MMP-3 expression (Figure 2C).

These findings show that all CCR6+ Th cell populations have Th17 characteristics, such as RORC, CD161, and IL-17A expression, although to a varying extent. These populations differ in Th1 characteristics. Despite these differences, all CCR6+ T cell populations were strikingly potent in inducing pro-inflammatory mediators upon co-culture with RASF.

IL-17A attributes to the pathogenicity of all CCR6+ Th cell populations.

All CCR6+ Th cell populations expressed IL-17A upon co-culture with RASF, albeit to a varying extent. To identify the contribution of IL-17A to the induction of IL-6 and IL-8, IL-17A effects were neutralized by adding soluble IL-17 receptor (sIL-17R/Fc) to the co-cultures. This had no effect on RORC expression, indicating that the Th17 characteristics of the CCR6+ Th cell populations had been maintained (Figure 3). IL-17 neutralization had no observable effects on IFNγ and TNFα expression by these co-cultures. In contrast, it inhibited IL-6 and IL-8 production. Regardless of IL-17 neutralization, all CCR6+ Th cell populations remained more potent than Th1 and naive Th cells in inducing IL-6 and IL-8 production by RASF.

When taken together, CCR6+ Th cells show high pathogenic potential in the induction of IL-6 and IL-8 by RASF, which is partly dependent on IL-17A.

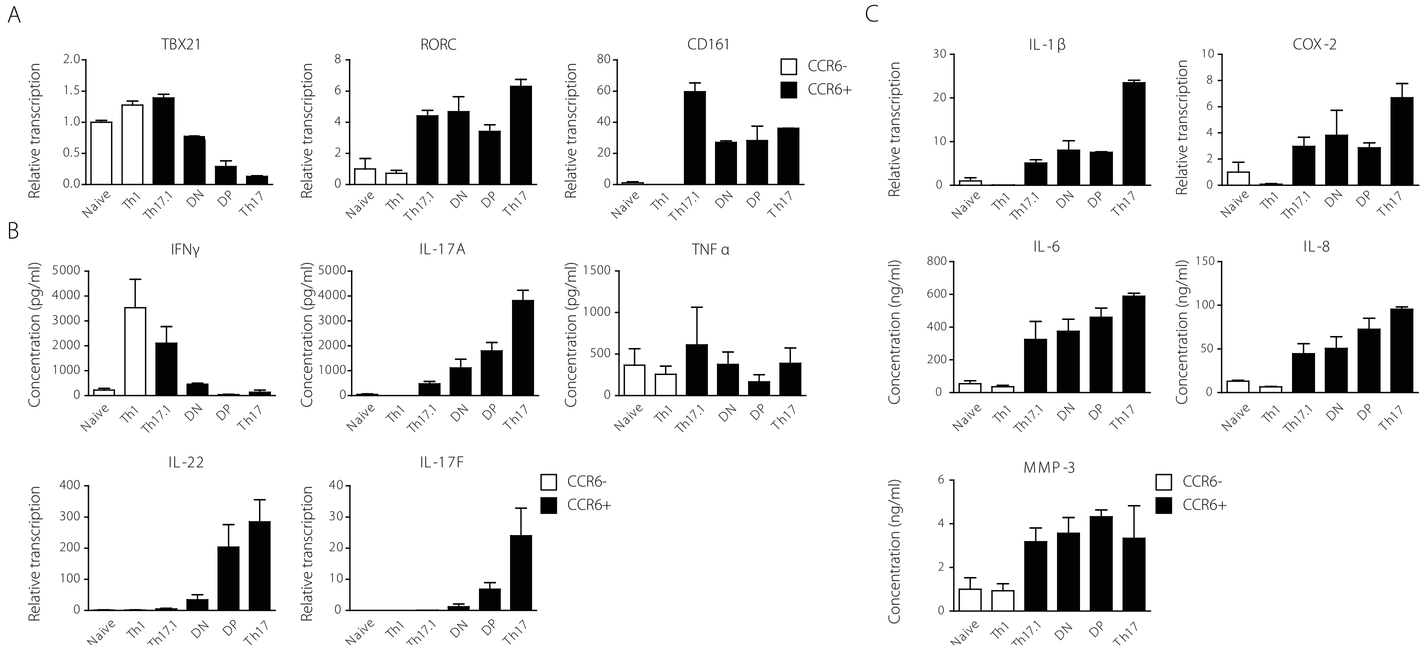


Figure 2 Despite differential Th1 and Th17 characteristics all CCR6+ Th cell populations display higher pathogenic activity in comparison to Th1 and naive CD4+ T cells. (**A-C**) 1.0 x 10^4 RASF were co-cultured for 3 days with 1.0 x 10^4 sorted primary Th1, naive CD4+ T cells and indicated CCR6+ Th cell populations from treatment-naive patients with early RA. (**A**) Quantitative RT-PCR analysis of TBX1, RORC and CD161 transcription by the indicated CCR6- T cell (white bars) and CCR6+ T cell (black bars) populations upon co-culture with RASF. (**B**) Analysis of IFNγ, IL-17A and TNFα production by ELISA and IL-22 and IL-17F transcription by quantitative RT-PCR by the indicated CCR6- T cell (white bars) and CCR6+ T cell (black bars) populations upon co-culture with RASF. (**C**) Analysis of IL-1β and COX-2 transcription by quantitative RT-PCR and IL-6, IL-8 and MMP-3 expression by ELISA for the indicated CCR6- T cell (white bars) and CCR6+ T cell (black bars) populations upon co-culture with RASF. Mean and SEM are given for 2-5 patients per group. Results are representative for at least 2 independent experiments.

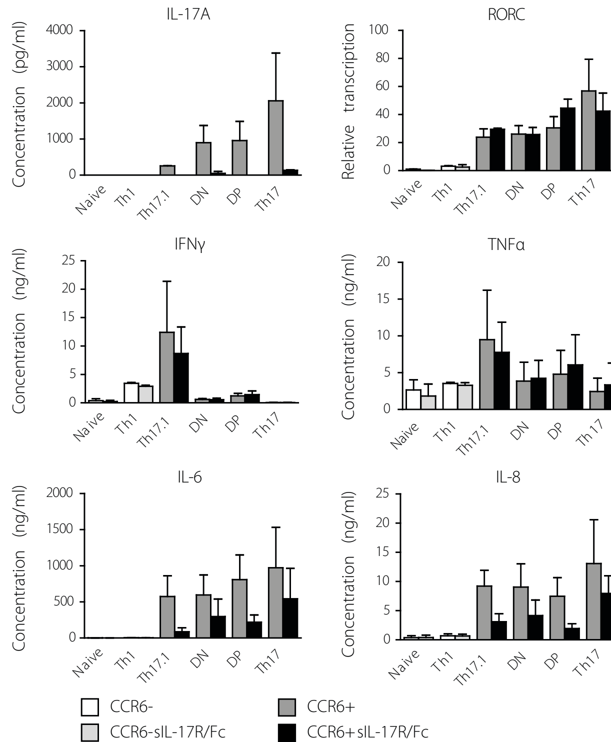


Figure 3 The pathogenic activity of CCR6+ T cell populations is partly dependent on IL-17A. 1.0 x 10^4 RASF were co-cultured with 1.0 x 10^4 sorted primary Th1, naive CD4+ T cells and indicated CCR6+ T cell populations from treatment-naive patients with early RA. Cells were cultured for 3 days in the presence or absence of sIL-17R/Fc. Analysis of IL-17A, IFNγ, TNFα, IL-6 and IL-8 expression by ELISA and RORC transcription by quantitative RT-PCR in CCR6- T cell (white bars) and CCR6+ T cell (black bars) populations upon co-culture with RASF. Mean and SEM are given for 3-5 patients per group. Results are representative for at least 2 independent experiments.

All CCR6+ Th subpopulations express MDR1 and MRP1, but Th17.1 and DN CCR6+ Th cells have the highest efflux activity.

Recently, it was shown that mainly Th17.1 cells express high levels of MDR1 and are resistant to suppression by glucocorticoids [18]. To assess drug resistance in the CCR6+ Th subpopulations from treatment naïve patients with early RA, we analyzed MDR1 expression by flow cytometry. CCR6+ Th cells expressed higher levels of MDR1 than CCR6- Th cells, as assessed by the mean fluorescent intensity (MFI) (Figure 4A). In patients with RA, Th17.1 and surprisingly DN CCR6+ Th cells expressed significantly ($p<0.0001$) higher MDR1 levels in comparison to Th1, Th17 and DP cells (Figure 4B). To investigate multidrug resistance potential, efflux of rhodamine-123 (Rh123) can be used. Rh123 is a fluorescent dye which can be taken up by all Th cells, and is exported by MDR1+ Th cells [18, 27]. In line with the higher MDR1 expression levels by CCR6+ Th cells, a larger fraction of Rh123- cells was observed in CCR6+ Th cells (39.5% of CCR6+ Th vs 11.3% of CCR6- Th cells) (Figure 4C). In comparison to Th1 cells, all CCR6+ Th cells except Th17 cells showed significantly larger ($p<0.0001$) fractions of Rh123- cells (Figure 4D).

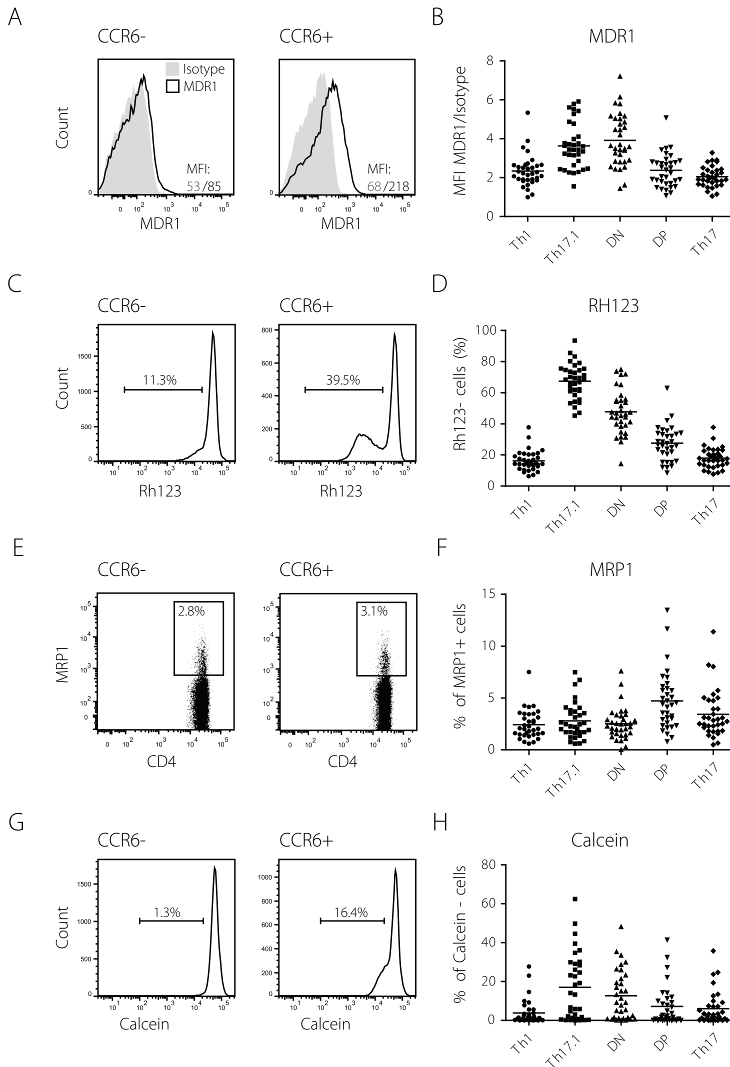


Figure 4 Th17.1 and DN CCR6+ Th express high MDR1 levels and display high dye efflux activity. (**A**) Histogram showing the mean fluorescent intensity (MFI) of MDR1 in the total memory Th CCR6- fraction (left) or memory Th CCR6+ fractions (right) of a treatment-naïve early RA patient. The isotype control antibody is shown in the filled gray plot. (**B**) Normalized MDR1 expression levels for the indicated cell populations. (**C**) Histogram showing Rh123 efflux by memory CCR6- Th (left) or CCR6+ Th cells (right) of a treatment-naïve early RA patient. Numbers indicate the proportions of Rh123 negative cells. (**D**) Proportions of Rh123 negative cells in the indicated populations. (**E**) Representative dot plots displaying MRP1 expression by CCR6- Th (left) and CCR6+ Th cells (right) of a patient with early RA. (**F**) Proportions of MRP1 expressing cells in the indicated populations. (**G**) Histogram showing calcein negative cells in CCR6- Th (left) or CCR6+ Th cells (right) of a patient with early RA. The horizontal line indicates the cut-off point for calcein negative cells. (**H**) Numbers indicate the proportion of calcein negative cells in the indicated populations. Mean is given in scatter plots for 34 early treatment-naïve RA patients. For statistical analysis a two-sided paired t-test was performed.

Substantial differences in the proportions of Rh123- Th17.1 (67±11%), DN (48±14%) and DP (28±11%) cells can be observed which do not appear to correlate with the MDR1 expression levels (Figure 4B and D). Therefore, we analyzed to what extent MDR1 expression levels within CCR6+ subpopulations and Th1 correlated with Rh123 export in those populations. Interestingly, this correlation varied extensively, with the highest level of correlation found in Th1 and Th17 (both 27%, p=0.002) and low levels of correlation (3.6-5.4%, none significant) found in Th17.1, DN and DP (Supplementary Figure 1A). Therefore, it appears additional transporters were involved. A candidate transporter was ABCG2, which is involved in MTX efflux and expressed in the RA synovium [28, 29]. However, we were not able to detect ABCG2 protein expression by CCR6- and CCR6+ Th cells of patients with RA (data not shown). Another potential candidate may be multidrug resistance-associated protein (MRP)1 (also called ABCC1), which is expressed by lymphocytes of patients with RA and is down-regulated upon methotrexate treatment [30]. Similar fractions of MRP1 expressing CCR6+ and CCR6- Th cells were observed (Figure 4E). However, within the CCR6+ Th cells, Th17 and DP had significantly larger fractions (p≤0.0005) of MRP1+ cells in comparison to Th1, Th17.1 and DN cells (Figure 4F). T cells are able to take up non-fluorescent calcein-AM, which will be converted to fluorescent calcein. MRP1+ cells are capable of transporting calcein, but not calcein-AM, enabling calcein efflux to function as a read out of multidrug transporter activity [27]. This analysis showed lower calcein efflux in CCR6- Th cells compared to CCR6+ Th cells, which was reflected by higher calcein- fractions of all CCR6+ Th cell populations in comparison to Th1 cells (Figure 4G and H). A large variation in the proportion of calcein- cells between patients with RA was observed.

Strikingly, the fraction of MRP1 expressing cells is highest in Th17 and DP cells, whereas Th17.1 and DN cells are the best exporters of calcein (p<0.0001 versus Th1, DP and Th17). Therefore, we investigated to what extent MRP1 expression correlated with calcein efflux. In all CCR6+ subpopulations and Th1, the proportion of MRP1+ cells correlated significantly with the proportion of calcein- cells, and the level of correlation varied from 12-19% (Supplementary Figure 1B). This indicates the involvement of MRP1 independent mechanisms in calcein efflux.

These findings show that all CCR6+ Th cell populations express MDR1 and MRP1, although to a varying extent. In particular Th17.1 and DN cells express high MDR1 levels and show high efflux capacity. These data also indicate that additional drug transporters are present, and that presence/activity of these transporters differs between subpopulations.

MDR1 and MRP1 expression and efflux capacity by CCR6+ Th subpopulations do not distinguish non-responders and responders to DMARD/GC therapy at baseline.

Patients with early RA are often treated with DMARDs, such as MTX, and GCs. However, a large fraction (~25-45%) of patients with RA does not respond to, or becomes resistant to this initial therapy [3-5]. The observed pathogenic potential of CCR6+ Th cell populations and the differential MDR1 and MRP1 expression and efflux capacity led us to investigate whether CCR6+ Th populations predict the lack of response in DMARD/GC therapy. At baseline similar proportions of memory Th cells and CCR6+ Th cells were observed between DMARD/GC therapy responders and non-responders (Figure 5A).

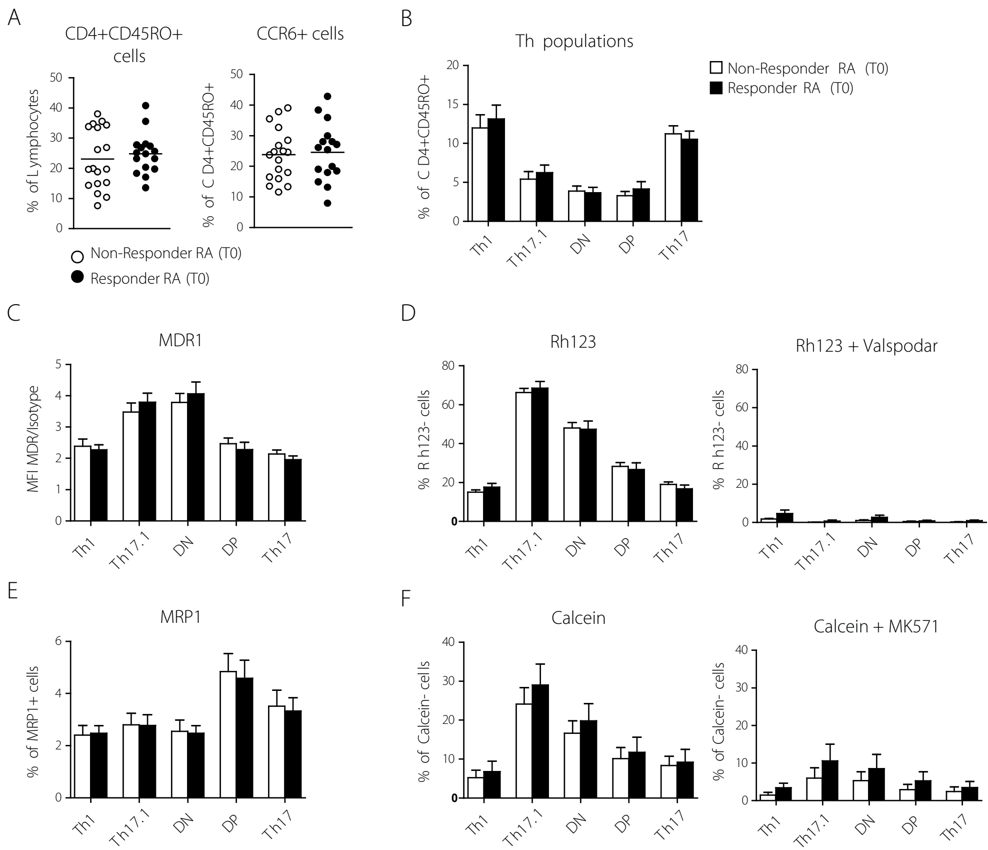


Figure 5 Patients with RA not-responding and responding to DMARD/GC therapy have similar MDR1 and MPR1 expression and dye efflux capacity at baseline. (**A**) Proportions of memory Th cells (left) or memory CCR6+ Th cells (right) of early treatment-naïve RA patients not responding (open circles) or responding (filled circles) to therapy at baseline. (**B**) Proportions of indicated cell populations of non-responders (open bars) and responders (filled bars) to DMARD/GC therapy at baseline. (**C-F**) Comparison of MDR1 and MRP1 expression and dye efflux of DMARD/GC therapy responder (filled bars) or non-responder (open bars) patients at baseline. (**C**) Normalized MDR1 expression levels for the indicated cell populations. (**D**) Proportions of Rh123 negative cells in the indicated cells populations without (left) or with (right) addition of the MDR1 inhibitor valspodar. (**E**) Proportions of MRP1 expressing cells in the indicated cell populations. (**F**) Proportions of calcein exporting cells in the indicated populations without (left) or with (right) addition of the MRP1 inhibitor MK571. Mean and SEM are shown for early treatment-naïve RA patients not responding (n=18) or responding (n=16) to therapy. For statistical analysis a two-sided unpaired t-test was performed.

Furthermore, we could not detect significant differences in proportions of CCR6+ Th subpopulations at baseline between responders and non-responders (Figure 5B). MDR1 expression levels and the fraction of Rh123- cells were not different between the patient groups (Figure 5C and D). In contrast, the level of correlation between MDR1 expression levels and Rh123 export varied extensively between the patient groups. MDR1 expression on Th1 and Th17 correlated significantly with Rh123 efflux in all patients together (Supplementary Figure 1A). Upon closer examination, these correlations are only significant

in responders: Th1 R^2=0.70, p<0,0001 and Th17 R^2=0.45, p=0,004; non-responders Th1 R^2=0.13, p=0.13 and Th17 R^2=0.13, p=0.14 (Supplementary table 1). This strongly indicates that additional multidrug transporters play a role in both patients groups, but mostly in non-responders. Valspodar is an inhibitor of MDR1 activity. To verify that Rh123 efflux was a result of drug transporter activity in CCR6+ Th cells, valspodar was added to the Rh123 stained cells. Valspodar inhibited at least 94% of Rh123 efflux in the CCR6+ Th cells of both patient groups (Figure 5D).

In addition to MDR1, levels of MRP1+ and fractions of calcein- CCR6+ Th populations were similar in non-responders and responders (Figure 5E and F). Again, the level of correlation between fractions of MRP1 and calcein export varied to a great extent between responders and non-responders. In both patient groups together, these correlations were significant for Th1 and all CCR6+ subpopulations. After reexamination of the data, these correlations were only significant for non-responders (R^2 between 0.33-0.52; all p-values≤0.01) but not for responders (Supplementary Table 1). This indicates that mainly in responders, other drug transporters play a role in effluxing calcein. To establish that calcein efflux was a consequence of multidrug transporter activity, MK571, an inhibitor of MRP1 activity, was added to calcein stained cells. MK571 inhibited 54-74% of the calcein efflux of the CCR6+ Th cell populations, and no differences were observed between responder and non-responders (Figure 5F).

These findings show that at baseline, MDR1 and MRP1 expression and Rh123 and calcein efflux capacity by CCR6+ Th cell populations are not predictive of DMARD/GC response. In addition, these data suggest that other drug transporters are involved, and that presence/activity of these transporters is different between responders and non-responders.

DMARD/GC treatment induces MRP1 expression and increases efflux capacity by CCR6+ Th cells of non-responder patients.

RA patients that respond to DMARD/GC therapy could not be distinguished from those not responding to therapy by analyzing MDR1 and MRP1 expression and efflux capacity at baseline. However, exposure to DMARDs and steroids can induce multidrug transporter expression [31, 32]. Therefore, we compared the non-responders and responders at baseline and after 6 months of DMARD/GC treatment.

Fractions of total memory CD4+ cells did not change in both groups (Figure 6A), but reduced CCR6+ Th proportions were observed in the therapy non-responder group (Figure 6B). In particular, the fractions of DP and Th17 subpopulations of non-responders decreased after treatment (Figure 6C). whereas in responders the fraction of Th17.1 cells even increased after treatment (Figure 6D).

MDR1 expression levels did not significantly differ after treatment in CCR6+ and CCR6- Th cells from responders and non-responders (Figure 6E). However, MDR1 expression levels on Th17.1 and Th17 cells were significantly decreased after treatment in non-responders (Supplementary Table 2). Conversely, Rh123 efflux increased in CCR6+ Th cells (Figure 6F), and reached statistical significance for DN and DP subpopulations (p<0.05, Supplementary Table 2).

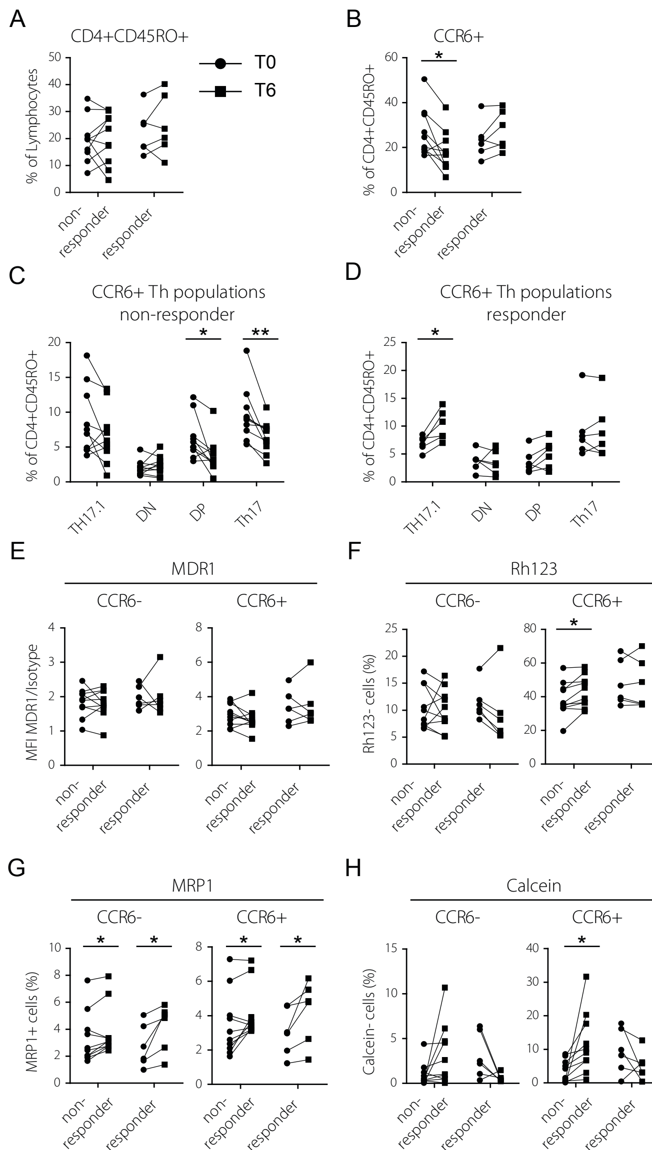


Figure 6 Effects of 6 months treatment on CCR6+ Th subpopulation distribution, MDR1 and MRP1 expression and dye efflux function. (**A-B**) Proportions of memory Th cells (**A**) and CCR6+ memory Th cells (**B**) at baseline (T0) and after 6 months (T6) of treatment in RA patients not responding or responding to DMARD/GC therapy. (**C-D**) Distribution of the indicated CCR6+ Th subpopulations in RA patients not responding (**C**) or responding (**D**) to therapy at T0 and T6. (**E-F**) MDR1 expression levels (**E**) and proportions of Rh123 negative cells (**F**) in CCR6- Th (left) and CCR6+ Th cells (right) of indicated patient groups and time-points. The MFI of MDR1 is normalized for the isotype control antibody. (**G-H**) Proportions of MRP1 expressing cells (**G**) and calcein negative cells (**H**) in CCR6- Th (left) and CCR6+ Th cells (right) of indicated patient groups and time-points. Data is shown for RA patients not responding (n=10) or responding (n=6) to therapy at T0 and T6. For statistical analysis a two-sided paired t-test was performed (* = $p < 0.05$).

The fractions CCR6+ and CCR6- Th cells expressing MRP1 significantly increased after treatment in both responders and non-responders (Figure 6G). In non-responders, statistical significance ($p<0.05$) was reached for Th17.1 and Th17 cells, and in responders for Th17.1 (Supplementary Table 2). However, similar calcein efflux by CCR6- Th cells of both responders and non-responders was observed. Of interest, in non-responders, calcein efflux by CCR6+ Th cells is significantly increased after treatment (figure 6H), and was also statically significant ($p<0.05$) for Th17.1 cells (Supplementary Table 2). In contrast, 5 out of 6 responders showed decreased calcein efflux by CCR6+ Th cells after treatment.

When taken together, DMARD/GC therapy induces MRP1 expression in both CCR6- and CCR6+ Th cells. However, only in DMARD/GC therapy non-responders increased efflux capacity of CCR6+ Th cells developed over time.

Discussion

CCR6+ Th cells and their cytokines are implicated in RA. However, the CCR6+ Th cell population is heterogeneous and distinct CCR6+ Th cell subpopulations can be identified. In the current study we characterized these subpopulations in the context of RA.

The CCR6+ Th populations Th17, Th17.1 and CXCR3/CCR4 DN and DP cells were found to be present in peripheral blood and synovial fluid of patients with RA. Despite the marked differences in Th17 and Th1 cell characteristics, all CCR6+ Th populations possess more pathogenic potential compared to Th1 and naive Th cells. All CCR6+ Th populations express multidrug transporters, but in particular Th17.1 and DN CCR6+ Th cells possess high MDR1 expression levels and drug efflux potential. DMARD/GC therapy increased MRP1 expression disregard of therapy response. However, drug efflux potential of CCR6+ Th cells increased only in patients with RA not responding to therapy, implicating that these cells are involved in therapy response.

Whereas Th17 cells are well studied in the context of disease [2, 10, 33-35], only recently evidence emerged concerning the pathogenic properties of Th17.1 cells, which were linked to inflammatory bowel disease and multidrug resistance [18]. Concerning the DP and DN CCR6+ Th cells even less information is available and they are, to the best of our knowledge, not studied so far in RA. However, these subpopulations might be very relevant, since our study shows that Th17.1, DN and DP CCR6+ Th populations together form the majority of CCR6+ Th cells in peripheral blood and synovial fluid of patients with RA. In addition, these subpopulations show pathogenic potential in the context of RA.

The ontogeny of the CCR6+ Th cell subpopulations is not well elucidated. Culturing Th17 cells in the presence of IL-12 up-regulates TBX21 expression and IFNγ expression, while blocking IL-12 prevents this process. This indicates that Th17.1 cells might be derived from Th17 cells [7, 36, 37]. However, culturing *Candida albicans* primed monocytes with naïve T cells led to development of cells with Th17.1 characteristics, pointing to a direct origin of Th17.1 cells from naïve T cells [38]. The ontogeny of the DN and DP CCR6+ Th subpopulations is unclear. The CCR6+ Th subset is very plastic, making it possible that these subpopulations are intermediate populations [36, 39, 40]. This may be a likely scenario for DP cells, as these cells in comparison to Th17 and Th17.1 express intermediate levels of IL-17A, IFNγ and TBX21.

It may be possible that in comparison to healthy individuals, pro-inflammatory conditions, such as in patients with RA, result in different peripheral and/or synovial CCR6+ Th cell population distribution. This may also explain the large variation in proportions of DN and DP CCR6+ Th cells between patients with RA.

The CCR6+ Th cells populations can be further distinguished by IL-17A production levels (Th17.1 low, Th17 high and DN and DP intermediate). Despite this difference all subpopulations were able to induce expression of MMP-3 and pro-inflammatory mediators such as IL-1β, IL-6, IL-8 and COX-2 upon co-culture with RASF. Neutralization of IL-17A could only partly inhibit the pathogenic potential of these cells, indicating that other pro-inflammatory mediators or interactions play a role as well. Likely candidates are TNFα and the COX-2 product PGE_2, which are both known to be involved in RASF activation [15, 16] and are induced by all CCR6+ Th subpopulations in co-culture with RASF. IFNγ was highly produced by Th17.1 cells. It is unlikely, however, that IFNγ production itself is responsible for the increased pathogenic potential of Th17.1 cells, given the finding that IFNγ producing Th1 cells show markedly less pathogenic activity compared to Th17.1 cells, even when compared to CCR6+ populations wherein IL-17A activity is blocked. These data show that IL-17 neutralization alone is not sufficient to completely inhibit the pathogenic activity of CCR6+ Th subpopulations in RA. A potential therapeutic target could be RORC which is expressed by all CCR6+ Th subpopulations. Initial studies concerning RORC inhibition show encouraging results in the suppression of Th17 signature gene expression and inflammation in murine autoimmune disease models [41, 42]. Multidrug resistance induced by ABC drug efflux transporters is proposed as one of the underlying mechanisms in the lack of DMARD and/or GC therapy response patients with RA [3]. However, target cells and markers to predict and monitor drug resistance in RA are missing. Interestingly, pro-inflammatory Th17.1 cells were recently found in patients with inflammatory bowel disease that expressed MDR1 and were resistant to GC [18]. In this study we not only confirm these results in RA, but also further indicate that DN CCR6+ Th cells express high levels of MDR1 and possess drug efflux potential. Moreover, we find that within all CCR6+ Th subpopulations, cells are present that express MRP1. This is of particular interest since MRP1 is able to efflux MTX [43, 44].

In this study we found that DMARD/GC treatment including MTX resulted in increased MRP1 expression by both CCR6+ and CCR6- Th cells of patients with RA. This effect was observed in both patients that responded, and patients that did not respond to DMARD/GC therapy. Therefore, MRP1 expression itself cannot explain the differences in RA treatment outcome. This notion is further supported by our findings that efflux capacity is induced by CCR6+ Th populations from DMARD/GC therapy non-responder patients only.

It may be possible that other drug transporters are involved in the increased efflux capacity in non-responder patients. The family of ABC transporters consists currently of 48 members [45] and it is very likely that additional transporters are involved in the induction of efflux capacity in DMARD/GC therapy non responders patients. This is supported by our finding that correlations between MDR1 expression levels and Rh123 efflux, and between fractions of MRP1 expressing cells and calcein efflux, varied extensively between cell subsets and between non-responders and responders. Future research should

carefully address which transporters are responsible for drug resistance in non-responders, and which cell types are involved. Furthermore, pro-inflammatory mediators such as TNFα may be involved in the regulation of multidrug transporters. For example, TNFα decreases MDR1 expression, whereas it induces MRP1 expression in various systems [46, 47]. It may also possible that other mechanisms are involved in controlling intracellular concentrations of drugs such as DMARDs and GCs. In this context, carriers are identified involved in the uptake of MTX [48].

Our findings indicate that measuring efflux capacity, by fluorescent dyes such as calcein and Rh123, rather than measuring multidrug transporter expression, would be a reliable marker of DMARD/GC responses.

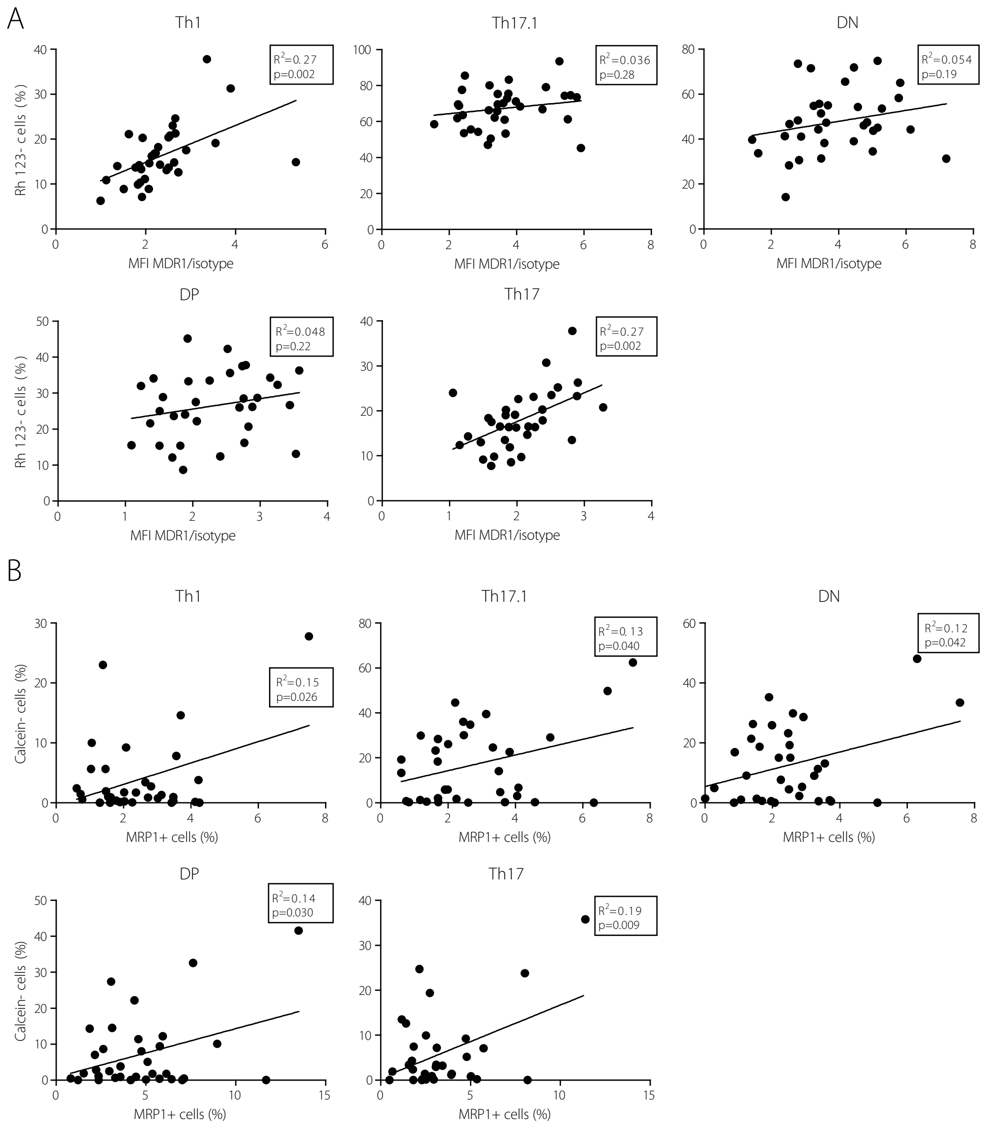
MDR1/MRP1 expression and MDR1/MRP1 function on CCR6+ Th subpopulations did not discriminate between responders and non-responders to therapy at baseline. Therefore, future research is required to unravel the underlying mechanism of multidrug transporter expression in early untreated RA patients, and to identify possible markers that can predict treatment response.

Our study identified CCR6+ Th cell subpopulations with marked differences in Th17 and Th1 characteristics in peripheral blood and synovial fluid of patients with RA. Despite these differences all populations possess RA related pathogenic potential. Moreover drug efflux activity of CCR6+ Th cell populations was associated with DMARD/GC treatment response. Further studies are required to find common modulation strategies for all CCR6+ Th cell populations. The results presented here implicate that efflux dyes can be used to monitor DMARD and/or GC therapy response in patients with RA.

Acknowledgments

We would like to thank Dr. P.H.P. de Jong for assistance in selecting the RA patient cohort.

Supplementary figures



Supplementary Figure 1 Correlation between efflux function and multidrug transporter expression in RA patients (n=34). (**A**) Correlation of the percentage of Rh123- cells with the MFI of MDR1 in indicated cell population in early treatment-naïve RA patients. (**B**) Correlation of the percentage of calcein- cells with the percentage of MRP1 expressing cells in indicated cell population in early treatment-naïve RA patients. Pearson correlation test was used to calculate the correlation coefficients (R^2) and p-values.

Supplementary Tables

Supplementary Table 1 Correlation between efflux function and multidrug transporter expression in early treatment-naïve RA patients not responding (n=18) or responding (n=16) to therapy.

	Non-Responder		Responder	
	R^2	p value	R^2	p value
MFI MDR1/%Rh123-				
Th1	0.13	0.13	0.70	<0.001
Th17.1	0.07	0.29	0.02	0.65
DN	0.03	0.48	0.08	0.30
DP	<0.01	0.86	0.12	0.20
Th17	0.13	0.14	0.45	<0.01
%MRP1+/%calcein-				
Th1	0.33	0.01	-0.07	0.31
Th17.1	0.49	<0.01	-0.03	0.51
DN	0.39	<0.01	-0.05	0.39
DP	0.42	<0.01	-0.04	0.45
Th17	0.52	<0.001	-0.07	0.32

Pearson correlation test was used to calculate the correlation coefficients (R^2) and p-values.

Supplementary Table 2 Effects of 6 months treatment on MDR1 and MRP1 expression and dye efflux function.

	Non-Responder			Responder		
	t=0	t=6	p value	t=0	t=6	p value
MFI MDR1/Isotype (sd)						
Th17.1	4.0 (0.9)	3.4 (0.9)	0.03	4.4 (0.9)	4.5 (1.2)	0.90
DN	4.1 (1.3)	4.0 (1.9)	0.74	5.1 (2.0)	4.4 (2.1)	0.36
DP	2.5 (0.5)	2.1 (0.4)	0.09	2.5 (0.6)	2.6 (0.8)	0.50
Th17	2.1 (0.4)	1.9 (0.3)	0.02	2.3 (0.9)	2.3 (0.9)	0.84
% Rh123- cells						
Th17.1	61.7 (12.0)	65.5 (11.5)	0.16	75.0 (12.2)	72.2 (12.4)	0.33
DN	42.9 (7.5)	48.5 (9.6)	0.04	58.2 (11.8)	52.1 (15.8)	0.14
DP	26.3 (9.0)	31.1 (9.1)	0.04	37.6 (15.0)	37.0 (18.7)	0.80
Th17	16.5 (6.1)	17.7 (5.3)	0.42	19.6 (9.4)	20.2 (10.5)	0.69
% MRP1+cells						
Th17.1	2.6 (1.4)	3.8 (1.3)	0.02	2.8 (1.4)	3,6 (1.5)	0.01
DN	2.3 (1.4)	3.1 (1.4)	0.10	2.7 (1.4)	3.4 (2.1)	0.23
DP	4.9 (2.6)	5.1 (2.4)	0.52	4.2 (1.9)	5.9 (2.7)	0.07
Th17	3.6 (1.9)	4.2 (1.7)	0.04	3.1 (1.4)	4.3 (2.2)	0.19
% Calcein- cells						
Th17.1	5.5 (5.3)	17.4 (12.9)	0.03	14.2 (10.4)	8.2 (6.7)	0.28
DN	6.7 (6.8)	12.4 (9.9)	0.17	14.4 (8.7)	7.1 (3.6)	0.11
DP	2.4 (2.0)	6.5 (9.2)	0.21	5.1 (3.8)	1.5 (1.4)	0.12
Th17	1.3 (1.6)	5.6 (6.3)	0.06	4.4 (3.8)	1.0 (0.7)	0.10

Data is shown for RA patients not responding (n=10) or responding (n=6) to therapy at T0 and T6. For statistical analysis a two-sided paired t-test was performed.

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Chapter 6

CCR6+ Th cell populations distinguish ACPA positive from ACPA negative rheumatoid arthritis

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Arthritis Research and Therapy, in press.

Abstract

Introduction Patients with rheumatoid arthritis (RA) can be separated into two major subpopulations based on the absence or presence of serum anti-citrullinated protein antibodies (ACPAs). The more severe disease course in ACPA+ RA and differences in treatment outcome between these subpopulations suggest that ACPA+ and ACPA- RA are different disease subsets. The identification of T-helper (Th) cells specifically recognizing citrullinated peptides, combined with the strong association between HLA-DRB1 and ACPA positivity, point toward a pathogenic role of Th cells in ACPA+ RA. In this context we recently identified a potential pathogenic role for CCR6+ Th cells in RA. Therefore, we examined whether Th cell populations distributions differ by ACPA status.

Methods We performed a nested matched case-control study including 27 ACPA+ and 27 ACPA- treatment-naive early RA patients matched for disease activity score in 44 joints, presence of rheumatoid factor, sex, age, duration of complaints and presence of erosions. CD4+CD45RO+ (memory) Th cell distribution profiles from these patients were generated based on differential chemokine receptor expression and related with disease duration.

Results ACPA status was not related to differences in total CD4+ T cell or memory Th cell proportions. However, ACPA+ patients had significantly higher proportions of Th cells expressing the chemokine receptors CCR6 and CXCR3. Similar proportions of CCR4+ and CCR10+ Th cells were found. Within the CCR6+ cell population, four Th subpopulations were distinguished based on differential chemokine receptor expression: Th17 (CCR4+CCR10-), Th17.1 (CXCR3+), Th22 (CCR4+CCR10+) and CCR4/CXCR3 double-positive (DP) cells. In particular, higher proportions of Th22 (p=0.02), Th17.1 (p=0.03) and CCR4/CXCR3 DP (p=0.01) cells were present in ACPA+ patients. In contrast, ACPA status was not associated with differences in Th1 (CCR6-CXCR3+; p=0.90), Th2 (CCR6-CCR4+; p=0.27) and T-regulatory ($CD25^{hi}$FOXP3+; p=0.06) cell proportions. Interestingly, CCR6+ Th cells were inversely correlated with disease duration in ACPA- patients (R^2=-0.35; p<0.01) but not in ACPA+ (R^2<0.01; p=0.94) patients.

Conclusions These findings demonstrate that increased peripheral blood CCR6+ Th cells proportions distinguish ACPA+ RA from ACPA- RA. This suggests that CCR6+ Th cells are involved in the differences in disease severity and treatment outcome between ACPA+ and ACPA- RA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic synovial joint inflammation and auto-antibody presence [1, 2]. The presence of serum anti-citrullinated protein antibodies (ACPAs) is highly specific for RA, and ~70% of patients with RA are ACPA+ [3-5]. Moreover, ACPAs are a useful marker for RA diagnosis as they can be present several years before clinical onset [6, 7].

The disease course in ACPA+ patients is worse than in ACPA- patients, as shown, for example, from more development of erosions; and treatment outcomes differ between these groups [8-17]. Moreover, associations between the HLA-DRB1 shared epitope (SE) alleles, PTPN22 gene polymorphisms and smoking have been found in ACPA+ patients [4, 18-21] .

The association between HLA-DRB1 SE and ACPA positivity implicates a role for MHC class II-dependent CD4+ T cell activation in ACPA+ RA [22]. In line with this, ACPAs are of the IgG subtype, which indicates that ACPA-producing B cells have undergone T cell-dependent class switching [23]. Moreover, citrullinated epitope specific T cells have been identified in ACPA+ patients [24-26].

CD4+ T helper (Th) cells and their cytokines play a central role in RA pathogenesis [27]. In early RA, pro-inflammatory T cells migrate to inflammatory sites and contribute to disease progression [27-29]. Cytokines produced by T cells, such as TNFα and IL-17A, are involved in activation of local cells and in inflammatory cell recruitment [29-31].

Th cell populations are characterized by differential chemokine receptor expression. For instance, IFNγ producing Th1 cells are CCR6-CXCR3+CCR4-, and IL-4 producing Th2 cells are CCR6-CXCR3-CCR4+ [32]. IL-17A and IL-22 producing cells are primarily found in the heterogeneous CCR6+ T cell population, with its subpopulations based on CXCR3, CCR4 and CCR10 expression. CCR6+ cells with Th17 characteristics are CXCR3-CCR4+CCR10- and CCR6+ cells with Th22 characteristics are CXCR3-CCR4+CCR10+ [33-35]. CCR6+CXCR3+CCR4- T cells exhibit both Th17 and Th1 features and are named non-classic Th1 or Th17.1 cells [36-38].

Recently we identified a potential role for CCR6+ Th cells in the pathogenesis of RA. In particular CCR6+ Th cells and not CCR6- Th cells were potent inducers of synovial fibroblast activation. This resulted in a pro-inflammatory feedback loop leading to the induction of pro-inflammatory mediators, such as IL-1β, IL-6 and PGE_2 and the tissue degrading enzymes MMP-1 and MMP-3. This loop was dependent on TNFα and IL-17A and may play an important role in the progression of an early inflammation towards a chronic persistent arthritis [30, 31].

The strong indications of T cell involvement in ACPA+ RA, and the clinical and molecular differences between ACPA+ and ACPA- disease, prompted us to investigate differences in Th cell populations between ACPA+ and ACPA- RA patients. In this report we describe that ACPA+ patients differ from ACPA- patients by significantly higher memory CCR6+ Th cell proportions. These findings suggest that pathogenic memory CCR6+ Th cells may be involved in the worse disease course observed in ACPA+ RA patients.

Methods

Patients

We performed a nested matched case-control study including 27 ACPA+ and 27 ACPA- treatment-naive early RA patients matched for disease activity score in 44 joints (DAS44), presence of rheumatoid factor (RF), sex, age, duration of complaints and presence of erosions. All patients met the American College of Rheumatology 2010 revised criteria for RA. None had been taking disease modifying anti-rheumatic drugs. Baseline characteristics on which was matched did not significantly differ between groups. Patients were not matched on tender joint count, swollen joint count, C-reactive protein levels (CRP), erythrocyte sedimentation rate (ESR) and titers of RF. Swollen joint count and titers of RF were significantly different between ACPA+ and ACPA- patients (Table 1).

This study was embedded in the Treatment in the Rotterdam Early Arthritis Cohort Study (tREACH) and approved by the Medical Ethics Review Board of Erasmus MC Rotterdam. Written informed consent from all patients participating in this study was obtained.

Table 1 Baseline characteristics of ACPA+ and ACPA- treatment-naive patients with early RA

Characteristic	ACPA+ patients (n=27)	ACPA- patients (n=27)	p value
DAS44 score (mean ± SD)	3.12 ± 0.82	3.14 ± 0.96	0.89
RF positive n (%)	21 (78)	16 (59)	0.07
Sex (female/male)	19/8	18/9	1.0
Age (mean ± SD, years)	48.2 ± 13.4	51.8 ± 13.8	0.31
Duration of complaints (mean ± SD, days)	159.9 ± 87.2	179.1 ± 103.5	0.29
Erosions n (%)	5 (19)	5 (19)	1.0
Tender joint count	9.93 ± 7.23	9.00 ± 7.90	0.30
Swollen joint count	6.63 ± 5.31	9.44 ± 6.67	0.02
ESR (mm/hr)	27.6 ± 22.5	26.3 ± 24.1	0.49
CRP (mg/l)	13.3 ± 30.3	11.1 ± 9.93	0.57
RF titer (IU/ml)	163 ± 327	64.3 ± 157	0.01

Flow cytometry and cell culture

Monoclonal antibody stainings, transcription factor detection and flow cytometry were performed as described previously [35]. Fluorochrome labeled antibodies were purchased from eBioscience (San Diego, CA), BD Biosciences, BioLegend (San Diego, CA) and R&D systems (Minneapolis, MN). Fixable Viability Dye and FOXP3 staining buffer sets were purchased from eBioscience. Samples were acquired on a LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR). Cells were gated on the lymphocyte fraction. Th cell populations were sorted with a FACSAria cell sorter (BD Biosciences). Purity of the obtained Th cell populations was ≥ 98%. Sorted Th cell populations were stimulated with 0.3 μg/ml soluble αCD3 and 0.4 μg/ml αCD28 (Sanquin, Amsterdam, The Netherlands) and cultured for 4 days as described previously [31].

Quantitative real-time PCR analysis

RNA extraction and cDNA syntheses were performed as described previously [39]. Primers were designed with ProbeFinder software and probes were used from the universal probe library (Roche Applied Science, Indianapolis, IN). Quantitative real-time PCR (RT-PCR) was performed and analyzed using the ViiA7 sequence detection system and software (Life Technologies, Carlsbad, CA). Hypoxanthine–guanine phosphoribosyltransferase (HPRT) was used to normalize gene transcription. The following primers and probes (forward, reverse, probe no.) were used: HPRT (5′- tgaccttgatttattttgcatacc-3′, 5′-cgagcaagacgttcagtcc-3′, 73), IL-17A (5′-tgggaagacctcattggtgt-3′, 5′-ggatttcgtgggattgtgat-3′, 8), IFNγ (5′-ggcattttgaagaattggaaag-3′, 5′-tttggatgctctggtcatctt-3′, 21), RORC (5′-cagcgctccaacatcttct-3′, 5′-ccacatctcccacatggact-3′, 69), and TBX21 (5′-tgtggtccaagtttaatcagca-3′, 5′-tgacaggaatgggaacatcc-3′, 9).

Statistical analysis

Differences between experimental groups were tested with Wilcoxon matched-pairs signed-ranks test using Prism software v5.04 (GraphPad Software Inc. La Jolla, CA), unless otherwise indicated. *P*-values <0.05 were considered significant.

Results

Elevated proportions of CCR6+ and CXCR3+ Th cell subpopulations in ACPA+ patients with early RA

In a previous study we found that the proportion of CD4+CD45RO+ (memory) T cells in PBMC of treatment-naive early RA patients was higher than that in healthy controls [31]. Therefore, we first checked for differences in the total CD4+ T cell or the memory CD4+ T cell populations between the 27 matched ACPA+ and ACPA- subjects. Flow cytometry showed similar proportions of both populations (Figure 1A and B).

Memory CD4+ T cell populations can be characterized by differential expression of the chemokine receptors CCR4, CCR6, CCR10 and CXCR3 and the surface receptor CD161 [32-34, 36]. Receptor expression analysis revealed significantly higher proportions of CCR6 and CXCR3 expressing memory CD4+ T cells in ACPA+ patients compared to ACPA- patients. No significant differences were found for Th cells expressing CCR4, CCR10 or CD161 (Figure 1C).

Elevated proportions of Th22, Th17.1 and unclassified CCR6+ Th cells in ACPA+ patients with early RA

T cells co-express different chemokine receptors on their surface. Specific combinations of CCR4, CCR6, CCR10 and CXCR3 are expressed by human Th cell populations. We applied a chemokine receptor gating strategy to identify memory CD4+ T cells with a Th1, Th2, Th17, Th22 or a Th17.1 profile. Within the CD4+CD45RO+CD25- T cell population, cells positive for CCR6 expression were gated. Within this CCR6+ population, Th17 cells were gated as CXCR3-CCR4+CCR10- and Th22 cells as CXCR3-CCR4+CCR10+ [33-35]. Th17.1 cells were gated as CXCR3+CCR4-. Using this gating strategy (Figure 2A), an unclassified

subpopulation was identified, that was double-positive (DP) for the expression of CCR4 and CXCR3. Recently we validated the gating strategy for Th17 and Th22 cells of patients with RA [35]. To validate the gating strategy for the other CCR6+ subpopulations we sorted Th1, Th17, Th17.1 and CCR4/CXCR3 DP CCR6+ Th cells from patients with RA and analyzed their Th17 and Th1 profile by the transcription levels of IL-17A, IFN-γ, RORC and TBX21. These analyses confirmed the expression profile of Th1, Th17 and Th17.1 as reported previously [36-38]. The CCR4/CXCR3 CCR6+ DP were IL-17A low and RORC+ with intermediate IFN-γ and TBX21 levels (Figure 2B). This gating strategy was applied to PBMCs of ACPA+ and ACPA- early RA patients. Proportions of the CCR6+ Th cell subpopulations Th22, Th17.1 and CCR4/CXCR3 DP Th cells were significantly higher in ACPA+ than in ACPA- patients. No statistical significant (p=0.10) difference was reached for the distribution of Th17 cells between ACPA+ and ACPA- patients (Figure 2C).

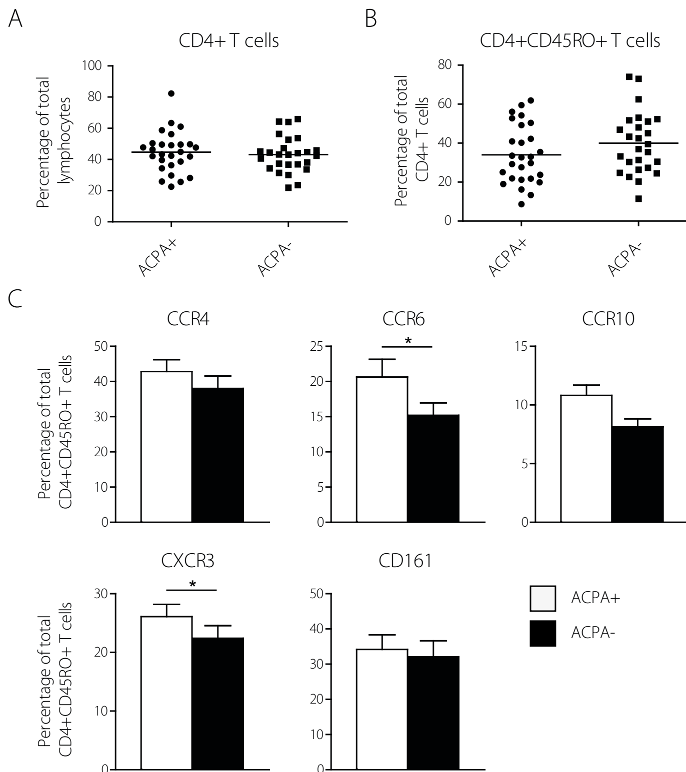

Figure 1 Proportions of chemokine receptor expressing memory Th cells differ between ACPA+ and ACPA- RA patients. (**A**) Fraction of CD4+ T cell population within the total lymphocyte population of 27 ACPA+ and 27 ACPA- patients with RA. (**B**) Fraction of memory CD4+ T cell population within the total CD4+ T cell population of 27 ACPA+ and 27 ACPA- patients with RA. (**C**) Chemokine receptor and CD161 expression on peripheral blood memory (CD45RO+) CD4+CD25- T cells from matched ACPA+ and ACPA- patients with RA, measured by flow cytometry. For statistical analysis Wilcoxon matched-pairs signed-ranks test was performed (* = $p < 0.05$).

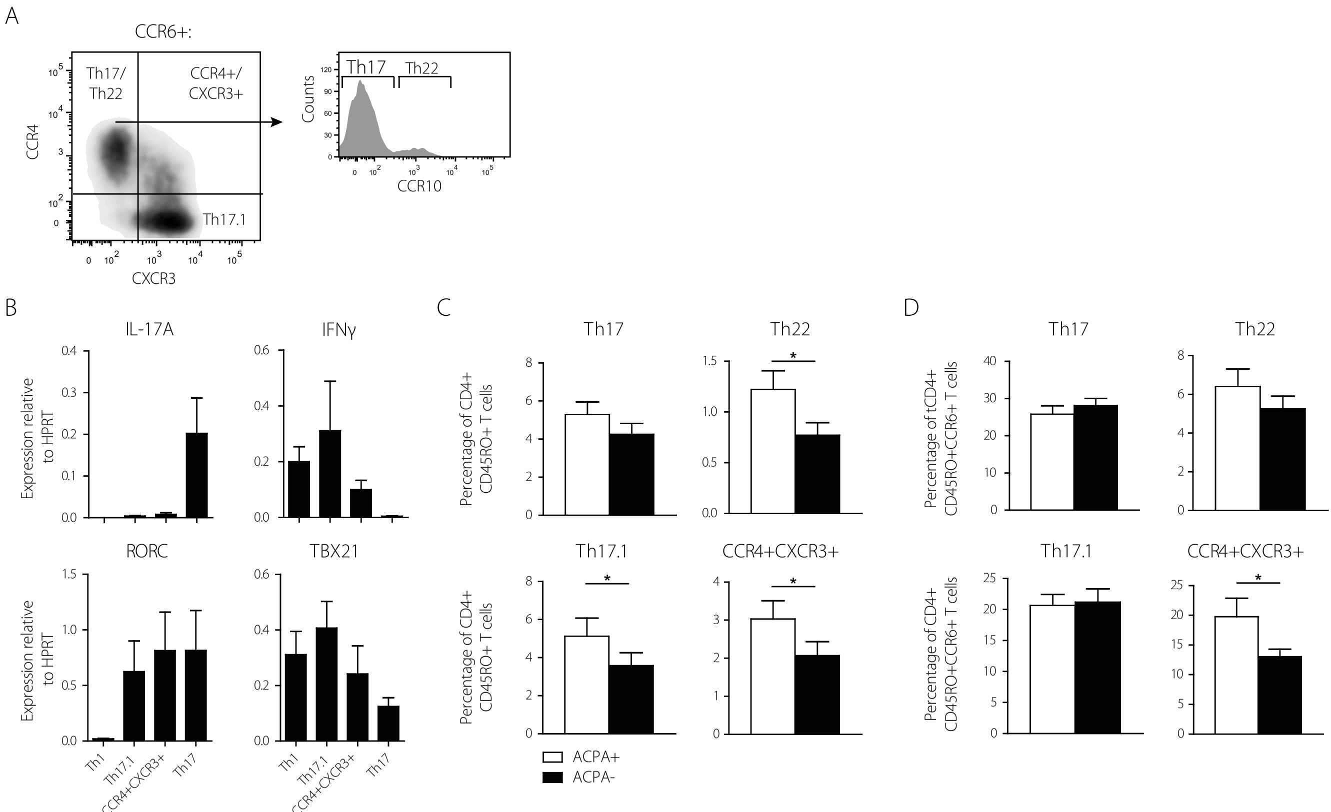

Figure 2 Memory CCR6+CD4+ T cell subpopulations are increased in ACPA+ patients compared to matched ACPA- patients. (**A**) Gating strategy for the identification of peripheral blood Th17, Th17.1, Th22 and CCR4/CXCR3 DP cell subpopulations. CCR6+ cells were gated on CD4+CD45RO+CD25- T cells. (**B**) Real-time PCR expression analysis for IL-17, IFNγ, RORC and TBX21 in sorted Th1, Th17, Th17.1 and CCR4/CXCR3 DP CCR6+ Th cells obtained from patients with RA (8-10 patients per population). Prior to RNA isolation cells were stimulated with antiCD3/CD28 and cultured for 3 days. (**C-D**) Proportions of the indicated CD4+ T cell subpopulations within the total memory CD4+ T cell population (**C**) and memory CCR6+CD4+ T cell population (**D**) of 27 ACPA+ and 27 ACPA- patients with RA. For statistical analysis Wilcoxon matched-pairs signed-ranks test was performed (* = $p < 0.05$).

To investigate whether the observed increases in Th22, Th17.1 and CCR4/CXCR3 DP memory Th cell populations in ACPA+ were due to the overall increase in CCR6+ Th cells in these patients (Figure 1C) or that specific increases were taking place, the CCR6+ Th cell populations were expressed as proportion of total CCR6+ Th cells. Interestingly, the proportions of all CCR6+ subpopulations were comparable between ACPA+ and ACPA- patients, except the proportion of CCR4/CXCR3 DP Th cells, that was significantly higher in ACPA+ patients than ACPA- patients (Figure 2D).

These findings show that, in ACPA+ patients, proportions of Th22, Th17.1 and CCR4/CXCR3 DP subpopulations were significantly larger than in ACPA- patients. These increases are mainly attributed to the observed increase in the proportion of total CCR6+ Th cells.

Th1 and Th2 proportions are similar in ACPA+ and ACPA- patients with RA, but the CCR4/CXCR3 DP CCR6- Th cell subpopulation is elevated in ACPA+ patients

Similar as described above, CCR6- Th cells were gated within the CD4+CD45RO+CD25- T cell population. Cells with a Th1 and Th2 profile were gated as CXCR3+CCR4- and CXCR3-CCR4+ respectively [32]. CCR4/CXCR3 DP cells were identified in the CCR6- Th cell fraction (Figure 3A). The combination of FOXP3 and high CD25 expression by memory CD4+ T cells was used to identify T regulatory (Treg) cells (Figure 3B).

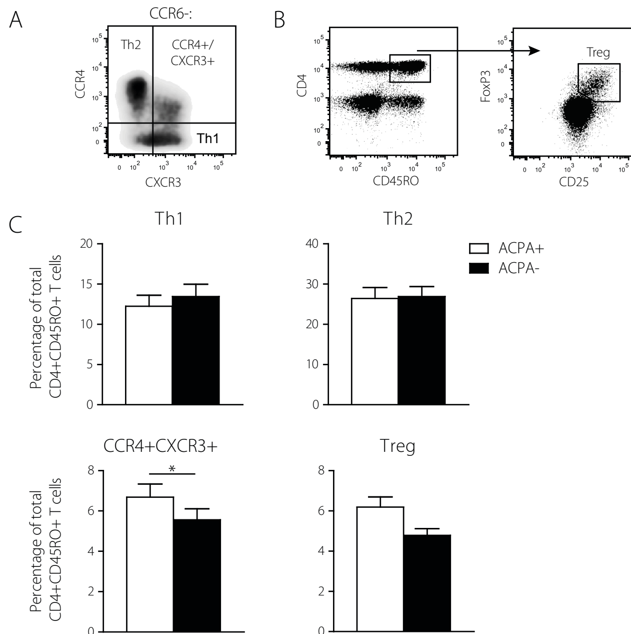

Figure 3 Differences in CCR6-CD4+ T cell subpopulations and Tregs between ACPA+ patients and matched ACPA- patients. (**A**) Gating strategy for the identification of peripheral blood Th1, Th2 and CCR4/CXCR3 DP cell subpopulations. CCR6- cells were gated on CD4+CD45RO+CD25- T cells. (**B**) Gating strategy to identify Treg ($CD25^{hi}$FOXP3+) cells within the memory CD4+ T cell population. Cells were gated on the total lymphocyte population. (**C**) Proportions of the indicated CD4+ T cell subpopulations within the total memory CD4+ T cell population of 27 ACPA+ and 27 ACPA- patients with RA. For statistical analysis Wilcoxon matched-pairs signed-ranks test was performed (* = $p < 0.05$).

Proportions of Th1 and Th2 cells did not differ between the groups. In contrast, the CCR4/CXCR3 DP CCR6- Th cell subpopulation was higher in ACPA+ patients than in ACPA- patients (Figure 3C). Moreover, we found a trend for larger Treg proportions in ACPA+ patients than in ACPA- patients (p=0.06).

Disease duration correlates with CCR6+ Th cell proportions in ACPA-, but not ACPA+ patients

ACPA+ and ACPA- patients with RA have a similar clinical presentation in the very early phase of disease [40], but ACPA+ RA is associated with a more severe disease course and erosions [8-13, 41]. Therefore, we investigated whether the differing CCR6+ Th cell, CCR6- Th cell and Treg proportions between ACPA+ and ACPA- patients were associated with patient-reported disease duration. Disease duration was not associated with CCR6+ Th cell proportions in ACPA+ patients, whereas it was significantly inversely correlated with CCR6+ Th cell proportions in ACPA- patients (Figure 4). Additionally, disease duration was significantly positively correlated with CCR6- Th cell proportions in ACPA-, but not in ACPA+ patients (Table 2).

In contrast, neither in ACPA+ nor in ACPA- patients disease duration was associated with Treg proportion. Further analysis of the CCR6+ Th cell compartment in ACPA- patients showed that Th17 cells and CCR4/CXCR3 DP CCR6+ Th cells had a significant inverse correlation with the disease duration. Within the CCR6- Th cell subpopulations, no significant correlations were found (Table 2). In addition, we found a small but significant inverse correlation between the disease duration with the DAS in all patients together (R^2=-0.07, p<0.05), but not in ACPA+ patients only (R^2=-0.13, p=0.06) and ACPA- patients only (R^2=-0.04, p=0.31).

These data show that CCR6+ Th cell subpopulations are inversely correlated with disease duration in ACPA- patients but not in ACPA+ patients.

Table 2 Correlation of Th cell populations (% memory Th cells) with disease duration

	ACPA+ patients		ACPA- patients	
Population	**R^2**	**P value**	**R^2**	**P value**
CCR6+ total	<0.01	0.94	-0.35	<0.01
Th17.1	0.02	0.50	-0.13	0.09
Th22	-0.04	0.37	-0.10	0.14
Th17	<0.01	0.91	-0.27	0.01
CCR4/CXCR3 DP CCR6+	<0.01	0.69	-0.36	<0.01
Treg	<0.01	0.76	0.04	0.31
CCR6- total	<-0.01	0.82	0.37	<0.01
Th1	0.09	0.14	<0.01	0.79
Th2	<-0.01	0.99	0.03	0.43
CCR4/CXCR3 DP CCR6-	0.02	0.51	-0.11	0.13

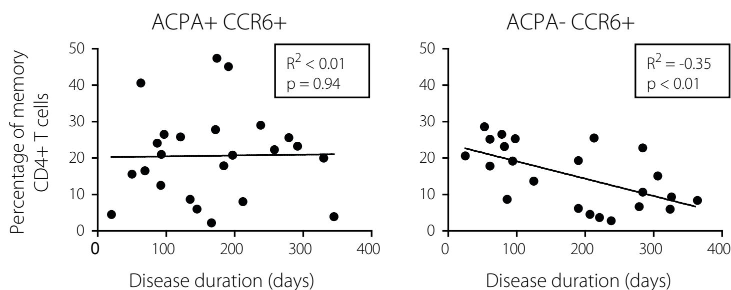

Figure 4 Inverse correlation between proportions of CCR6+CD4+ T cell subpopulations and disease duration in ACPA- patients. Correlation of the percentage of CCR6+CD4+ cells (as percentage of total memory CD4+ T cells) with self-reported disease duration in ACPA+ and ACPA- patients with RA. Pearson correlation test was used to calculate the correlation coefficients (R^2) and p-values.

Discussion

In the present study we found that ACPA+ patients have a higher proportion of peripheral CCR6+ Th cells than ACPA- patients. Chemokine receptor expression analysis revealed an increase in Th22, Th17.1 and CCR4/CXCR3 DP CCR6+ Th cells. These increases are mainly attributed to the observed increase in the proportion of total CCR6+ Th cells. In addition, ACPA+ patients had higher proportions of CCR4/CXCR3 DP CCR6- Th cells than ACPA- patients, but did not differ in Th1 and Th2 proportions. We also found increases in Th17 and Treg proportions in ACPA+ patients, but these differences did not reach statistical significance. Furthermore, in ACPA+ patients the proportion of CCR6+ Th cells was not correlated to disease duration, whereas in ACPA- patients proportions of CCR6+ Th cells were negatively correlated with increasing disease duration.

Our findings that the distribution of Th cell populations is dependent on ACPA status is in line with previous studies linking ACPA+ with the CD4+ T cell component: (i) There is a strong association between ACPA positivity and MHC class II-restricted HLA-DRB1 SE alleles; (ii) ACPAs are mainly of the IgG subtype, which are normally synthesized after T cell-mediated immunoglobulin loci class switching; (iii) citrullinated peptide specific CD4+ T cells are present in ACPA+ patients [23-25, 42]. Furthermore, PTPN22 gene polymorphisms may be involved in the formation of citrullinated peptide-specific CD4+ T cells and therefore be a risk factor for ACPA+ RA [43].

The identification of increased CCR6+ Th populations in ACPA+ RA suggest that these cells are implicated in the more severe disease course of patients with ACPA+ RA. In this context, we recently identified a potential pathogenic role for CCR6+ Th cells obtained from treatment naïve patients with early RA. This included the role of CCR6+ Th cells as potent inducers of a pro-inflammatory loop, driven by autocrine IL-17A production and resulting in the induction of IL-1β, IL-6, IL-8, PGE_2 and MMPs by synovial fibroblasts [31, 35]. This may suggest that CCR6+ Th cells are involved in the amplification of inflammatory reactions resulting in the more severe disease course observed in ACPA+ RA.

Given this pathogenic role of CCR6+ Th cells, it is of particular interest that we found no correlation between the proportion of CCR6+ Th cells and disease duration in ACPA+ patients, while in ACPA- patients

proportions of CCR6+ Th cells were negatively correlated with increasing disease duration. However, the time of onset of disease is self-reported, and therefore an estimation. Additionally, it is possible that the worse disease course in ACPA+ patients might lead to earlier recognition of disease onset by ACPA+ patients than by ACPA- patients, skewing the estimated time of onset [8-10]. Nevertheless, these data might suggest that CCR6+ Th cells are involved in the maintenance of inflammation in ACPA+ RA and may underlie the differences in treatment outcome between ACPA+ and ACPA- RA.

In a previous study it was found that IL-17A responses by CD4+ T cells of ACPA+ patients with RA were induced after culture with citrullinated peptides. This was not the case for PBMCs from healthy controls. In addition, the level of IL-17A production correlated strongly with the level of proliferation in response to citrullinated peptides [26]. This indicates that IL-17 producing T cells, and therefore CCR6+ Th cells as well, might be particularly important in responses to citrullinated protein. Interestingly, it has been suggested that a SNP in the CCR6 gene, which is associated with RA, is more strongly linked to ACPA+ RA than ACPA- RA [44-46]. Future research should clarify whether CCR6+ Th cells have T cell receptors specific for citrullinated peptides. Alternatively, it would be interesting to compare CCL20 levels in synovial fluid of ACPA+ and ACPA- patients, since CCL20 is the only known ligand for CCR6 [47, 48]. Higher CCL20 levels in synovial fluid of ACPA+ patients could also account for higher CCR6+ Th cell numbers.

The CCR6+ Th population is heterogeneous, and based on differential chemokine receptor expression various subpopulations can be identified. The roles and contribution of these CCR6+ Th populations in the severity of ACPA+ RA are unclear. Recently we found that Th22 cells were not required for Th17/IL-17 mediated synovial inflammation [35]. On the other hand, Th22 cells were shown to be associated with erosive disease and serum IL-22 levels correlate with serum ACPA titers [49, 50]. Moreover IL-22 was able to promote osteoclastogenesis by inducing RANKL in synovial fibroblasts [50].

The ontogeny of the Th17.1 CCR6+ Th cell subpopulation is unclear. Th17.1 cells might be derived from Th17 cells, as culturing of human Th17 cells in the presence of IL-12 up-regulates Th1 characteristics like TBX21 and IFNγ and down-regulates Th17 characteristics like RORC and IL-17, leading to a Th17.1-like phenotype [51-53]. On the other hand, Th17.1 cells may also originate directly from human naive T cells. Upon interaction with *Candida albicans* primed monocytes, naive T cells develop into cells with Th17.1 characteristics [54]. Recently, particular Th17.1 cells were found to have a pathogenic signature, specifically those that expressed the transporter protein multi-drug resistance type 1 (MDR1), and thereby became unresponsive to glucocorticoids [37]. The pathogenic signature and drug-resistance suggest the clinical importance of Th17.1 cells in RA. The origin and development of the CCR4/CXCR3 CCR6+ and CCR6- Th subpopulations are also ill-defined, and these populations might resemble intermediate or transitional Th cells. Research to factors that foster the development of these cells is lacking, but one possibility is that the micro-environment, such as concentrations of the cytokines IL-12, IL-23 and IL-6 that are important in Th1 and Th17 differentiation, plays a role. More research is needed to investigate the ontogeny, stability, characteristics and functions of these subpopulations.

Surprisingly, we found higher Treg proportions in ACPA+ patients, although the difference did not reach statistical significance (p=0.06). Tregs normally play an immune suppressive role. It might be that these increased Tregs are a induced as a feedback mechanism to control the increased proportions of CCR6+ Th cells. However, Tregs are able to convert to Th17 cells [55, 56]. Especially these converted cells are key to the development of autoimmune arthritis [57]. Future research should point out whether the Tregs in (ACPA+) RA patients are functional and could convert to Th17 cells.

In this study we have found that Th cell distributions are associated with ACPA status. In particular CCR6+ Th cell proportions were higher in ACPA+ RA in comparison to ACPA- RA. Moreover, CCR6+ Th cells are inversely correlated with disease duration in ACPA- patients but not in ACPA+ patients. These findings point toward a pathogenic role for CCR6+ Th cells in the more severe disease course of patients with ACPA+ RA and imply a role for CCR6+ Th cells in the differences observed in the treatment outcome of patients with ACPA+ and ACPA- RA.

Acknowledgements

We thank B. Bartol, H. Bouallouch-Charif and Patrick S. Asmawidjaja for technical assistance with flow cytometry based purification of T cell populations.

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Chapter 7

Multidrug resistant lymphocytes of patients with rheumatoid arthritis are predictive for DMARD and glucocorticoid treatment response

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Abstract

Background A large fraction of patients with RA does not respond to treatment with glucocorticoids (GCs) and disease-modifying anti-rheumatic drugs (DMARDs), or becomes resistant in time. Multidrug resistance may underlie the lack of response, but markers to predict or monitor drug resistance in RA are lacking. In this study, drug efflux capacity and multidrug transporter expression were investigated on various lymphocyte populations in relation to DMARD/GC response.

Methods Peripheral and/or synovial lymphocytes were obtained from: healthy individuals, established inflammatory arthritis patients and DMARD/GC therapy responders and non-responders at baseline and after 6 months of therapy. Drug efflux capacity, indicated by rhodamine-123 (Rh123) and calcein transport, and MDR1, MRP1 and ABCG2 expression were analysed for total lymphocytes, as well as for B, CD4+ T, CD8+ T and natural killer (NK) cells.

Results Compared to healthy individuals, lymphocytes from patients with inflammatory arthritis showed a higher drug efflux capacity and expressed higher MDR1 and MRP1 levels. DMARD/GC non-response was associated with lower MDR1 expression and Rh123 efflux by naïve CD8+ T cells at baseline and increased MRP1 expression and calcein efflux after 6 months of treatment. Blocking MRP1 activity partially inhibited calcein efflux by peripheral and synovial lymphocytes, indicating additional MRP1-independent drug resistance.

Conclusion Multidrug resistant lymphocytes are present in RA. In addition, MDR1 expression and activity in naïve CD8+ T cells was identified as a discriminating marker to distinguish therapy responders from non-responders. Implications of these findings might lead to improved personalized RA treatment strategies.

Introduction

RA is a systemic autoimmune disease, characterized by joint inflammation, synovial hyperplasia, and auto-antibody formation [1]. Without appropriate treatment, an early inflammation may develop into a chronic persistent arthritis associated with irreversible cartilage- and bone destruction. Currently, there is no cure for RA, and early treatment strategies mainly focus on controlling inflammation, slowing down disease progression and achieving disease remission. Initial RA treatment commonly includes DMARDs such as methotrexate (MTX), sulfasalazine (SSZ) and hydroxychloroquine (HCQ) and GCs such as dexamethasone and prednisolone. Unfortunately, a large fraction of ~25-45% of patients with RA does not respond to, or becomes resistant to, DMARD and/or GC treatment [2-4].

One of the proposed mechanisms for this lack of response on DMARD/GC therapy is multidrug resistance through ATP-binding cassette (ABC) transporters [2, 5]. ABC transporters are expressed within the cell membrane and are under physiological circumstances involved in the cellular efflux of xenobiotics. However, various drugs are also substrates for ABC transporters. Increased expression levels or increased activity of these transporters can lead to lower intracellular drug concentrations and insufficient therapy responses. Multidrug resistance has been researched extensively in cancer [6], but implications for ABC transporters in the context of RA treatment only start to emerge [2]. For example, MRP1 (also called ABCC1) can transport methotrexate (MTX) [7, 8], HCQ and chloroquine (CQ) [9, 10]; ABCG2 transports most small molecular DMARDS, such as MTX, polyglutamated MTX [11-13], leflunomide (LEF) [14, 15] and SSZ [16, 17]; and MDR1 (also called ABCB1 or P-glycoprotein) transports prednisolone [18], dexamethasone (DEX) [19] and (H)CQ [10, 20].

ABC transporters are expressed in many tissues and cells, including RA synovial tissue [21]. Lymphocytes from patients with established RA express higher MDR1 levels than lymphocytes from healthy controls [22-24]. Of interest, MDR1 efflux activity is higher in RA patients compared to healthy controls, and is highest in patient subgroups that are therapy resistant [22, 23, 25, 26]. Expression of the ABCG2 transporter was found in RA synovial tissue before and after DMARD treatment, but not in synovial tissue from non-inflammatory orthopedic controls. Non-responders to DMARD therapy had higher levels of ABCG2 positive cells, consisting of mainly macrophages, after treatment than responders [14]. Furthermore, SSZ induces ABCG2 expression on a tumor T cell line *in vitro*, leading to SSZ, MTX and LEF resistance [27]. MRP1 is expressed in synovial tissue [28] and peripheral lymphocytes [29] of RA patients. Synovial expression levels are higher in RA patients receiving MTX compared to patients not receiving MTX [28]. *In vitro*, chloroquine (CQ) can up-regulate MRP1 expression, leading to DEX and CQ resistance [9].

Lymphocytes express ABC transporters [2, 5, 30] and are involved in RA pathogenesis [1]. Furthermore, the mechanisms underlying the effect of DMARDs include the inhibition of lymphocyte proliferation [31-33]. These findings together imply a role for multidrug transporters expressed by lymphocytes in the lack of treatment response of patients with RA on DMARD/GC therapy. However, markers to predict or monitor DMARD/GC treatment response are lacking. Therefore, our main goals in this study were: (i) to identify which lymphocyte populations in RA express MDR1, MRP1 and ABCG2 and show drug efflux

activity, (ii) to quantify expression and efflux of these ABC transporters in lymphocyte populations of DMARD/GC therapy responders and non-responders at baseline and after 6 months of treatment and (iii) to relate these findings to local synovial lymphocyte populations.

Methods

Patients

Peripheral blood mononuclear cells (PBMCs) were obtained from treatment naïve patients with early RA at baseline and 6 months after the start of DMARD/GC therapy, and from healthy individuals (2 males, 6 females, mean age 49 years). PBMC and autologous synovial fluid mononuclear cells (SFMCs) were obtained from patients with inflammatory arthritis. All RA patients treated with DMARD/GC therapy met the American College of Rheumatology 2010 revised criteria for RA. None had been taking DMARDs before the start of therapy. Samples from 18 therapy responders and 18 non-responder patients were studied. Responding to therapy was defined as a disease activity score in 44 joints (DAS44) ≤1.6 after 3, 6, 9 and 12 months of treatment. Non-response to therapy was defined of a DAS44 ≥2.4 after 3 months of treatment and a DAS44 ≥1.6 after 6, 9 and 12 months of treatment. At baseline, patients were randomly allocated to one out of three treatment strategies: A) combination therapy consisting of MTX, SSZ and HCQ with GCs intramuscularly; B) combination therapy with an oral GC tapering scheme; or C) MTX with an oral GC tapering scheme (study described in [3]). In addition, non-responders were given etanercept after the initial 3 months treatment. Characteristics of these patients at baseline and at 6 months after the start of DMARD/GC therapy are shown in table 1. Peripheral blood and synovial fluid were studied from patients with inflammatory arthritis (2 psoriatic arthritis, 1 juvenile idiopathic arthritis, 1 oligoarthritis, 1 undifferentiated ankylosing spondylitis and 2 RA).

This study was embedded in the Treatment in the Rotterdam Early Arthritis Cohort Study (tREACH) and approved by the Medical Ethics Review Board of Erasmus MC Rotterdam.

Table 1 Characteristics of patients with early RA responding and not responding to DMARD/GC therapy

Characteristic	Non-responders (n=18)	Responders (n=18)	p-value
Treatment (A/B/C)	2/3/13	7/6/5	0.023*
Sex (female/male)	15/3	8/10	0.014
Age (mean ± SD, years)	55.20 ± 11.08	47.32 ± 16.01	0.095
Duration of complaints (mean ± SD, days)	140.39 ± 86.99	136.72 ± 82.76	0.90
Erosions at baseline n (%)	2 (11.1)	4 (22.2)	0.39
RF positive at baseline n (%)	16 (88.9)	14 (77.8)	0.39
ACPA positive at baseline n (%)	12 (66.7)	14 (77.8)	0.47
DAS44 T0 (mean ± SD)	3.51 ± 0.54	3.30 ± 0.75	0.34
DAS44 T3 (mean ± SD)	3.24 ± 0.45	0.83 ± 0.37	<0.001
DAS44 T6 (mean ± SD)	3.05 ± 0.74	0.82 ± 0.46	<0.001

*calculated using chi-square test

Cell culture and drug efflux capacity analysis

Rhodamine-123 (Rh123) and Calcein-AM (Sigma-Aldrich, St. Louis, MO) were dissolved in ethanol and DMSO respectively. Approximately 1.5x10^6 PBMCs or SFMCs were suspended in Iscove's Modified Dulbecco's Media (IMDM, Lonza, Basel, Switzerland), supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamin (Lonza) and 50 µM β-mercapto-ethanol (Merck, Darmstadt, Germany). To stain the cells, Rh123 or calcein-AM were added to a final concentration of 0.5 µg/ml (Rh123) and 0.025 µg/ml (Calcein-AM) and incubated on ice in the dark for 30 minutes. Cells were washed with PBS and incubated for 2 hours at 37° C in IMDM supplemented as above with or without the MDR1 inhibitor valspodar (5 µg/ml, PSC883 Sigma-Aldrich) or the MRP1 inhibiter, MK-571 (100 µg/ml, Sigma-Aldrich).

Flow cytometry and antibodies

Monoclonal antibody (MoAb) stainings and flow cytometry were performed as described previously [34]. Antibodies to analyze multidrug transporter expression included MDR1-PE (clone UIC2) and isotype control IgG2a κ-PE (clone MOPC-173, BioLegend, San Diego, CA), MRP1-FITC (clone QCRL) and isotype control IgG1-FITC (clone 11711, R&D systems, Minneapolis, MN) and ABCG2-PE (clone 5D3) and isotype control IgG2b κ-PE (clone MPC-11 BioLegend) Fluorochrome-labeled antibodies used to distinguish lymphocyte populations were purchased from eBioscience (San Diego, CA), BD Biosciences (San Diego, CA), BioLegend and R&D systems. Moab labeled and Rh123 or calcein-AM stained cells were acquired on a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR). Apoptotic cells were excluded using fixable viability dye (eBioscience) and cells were gated on the lymphocyte fraction.

Statistical analysis

Differences between experimental groups were tested with unpaired student's T test or paired student's T test using Prism software v5.04 (GraphPad Software Inc. La Jolla, CA), unless otherwise indicated. *P*-values <0.05 were considered significant.

Results

Elevated MDR1 and MRP1 expression on lymphocytes of patients with inflammatory arthritis

Previous reports have shown increased expression of the multidrug transporter MDR1 [22-24] in PBMCs of patients with inflammatory arthritis. However, it is not clear whether other transporters, such MRP1 and ABCG2, are increased as well in inflammatory arthritis and whether the expression of multidrug transporters is restricted to a particular lymphocyte population. Therefore, we analyzed the expression of the multidrug transporters MDR1, MRP1 and ABCG2 in different peripheral blood lymphocyte populations of patients with inflammatory arthritis and compared this to lymphocytes obtained from healthy individuals.

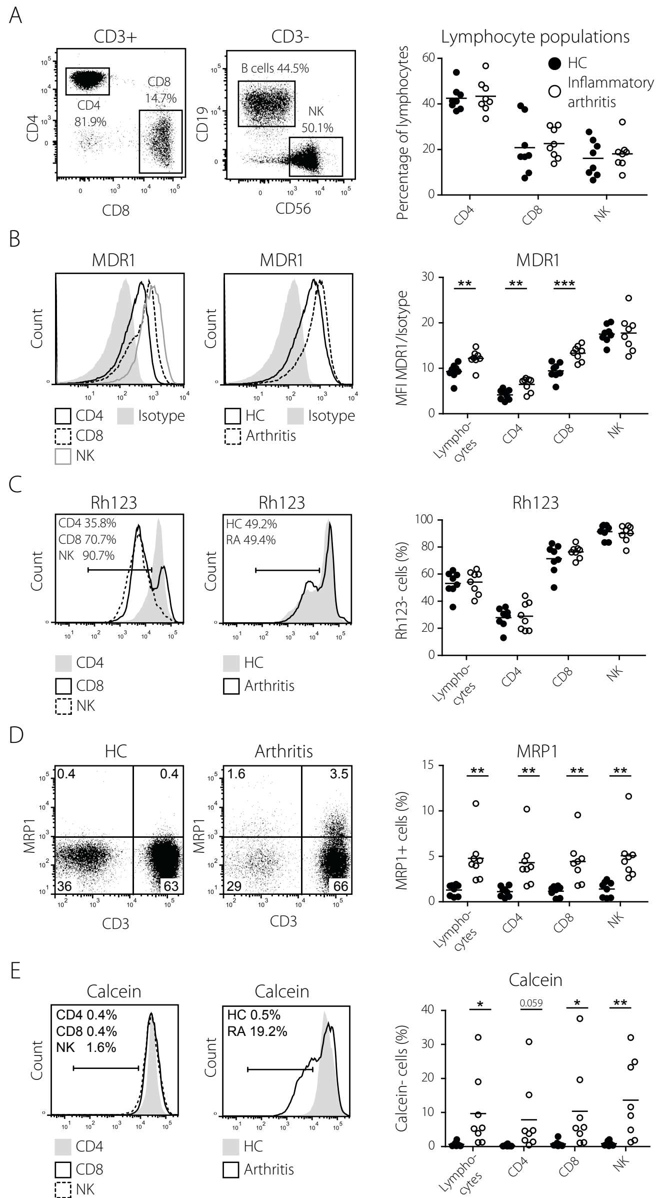


Figure 1 MDR1 and MRP1 expression levels are increased in peripheral blood lymphocyte populations of patients with inflammatory arthritis compared to healthy controls (HC). (**A**) Left and middle: representative dot plots indicating the flow cytometry gating strategy for peripheral blood lymphocyte populations. Right: percentages of CD4+ and CD8+ T cells and NK cells in total lymphocytes. (**B**) Left: representative histogram showing MDR1 expression in indicated cell populations of a HC. Middle: representative histogram showing MDR1 expression in lymphocytes of a HC and a patient with inflammatory arthritis. Right: quantification of MDR1 mean fluorescent intensity (MFI) divided by isotype control MFI in indicated cell populations. (**C**) Left: representative histogram showing Rh123 efflux by indicated populations of a HC. Percentages of Rh123- cells are given. Middle: representative histogram showing Rh123 efflux

by lymphocytes of a HC and a patient with inflammatory arthritis. Right: Quantification of the fraction of Rh123- cells in indicated cell populations. (**D**) Left and middle: representative dot plots showing the fraction of MRP1 expressing CD3+ and CD3- lymphocytes of HC (left) and a patient with inflammatory arthritis (middle). Right: proportions of MRP1 expressing cells in the indicated cell populations. (**E**) Left: representative histogram showing calcein efflux by indicated populations of a HC. Percentages of calcein- cells are given. Middle: representative histogram showing calcein efflux by lymphocytes of a HC and a patient with inflammatory arthritis. Right: Quantification of calcein- cells in the indicated cell populations. Mean is given in scatter plots for 8 HC (filled circles) and 8 patients with inflammatory arthritis (open circles). For statistical analysis a two-sided unpaired t-test was performed (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

To identify lymphocyte populations by flow cytometry a gating strategy was used, wherein T cells were distinguished by CD3 expression. Within this CD3+ fraction, T-helper (Th) cells were identified as CD4+ and cytotoxic T cells as CD8+. In the CD3- fraction, B cells were identified by CD19 expression, and natural killer (NK) cells by CD56 expression. Within the total lymphocyte population CD4+ and CD8+ T cell and NK cell proportions did not differ between healthy controls and patients with inflammatory arthritis (Figure 1A).

MDR1 expression was detectable in lymphocytes, and levels differed markedly between CD4+, CD8+ and NK cells from both healthy controls and patients. The expression of MDR1 was significantly higher on lymphocytes and specifically on CD4+ and CD8+ T cells from patients with inflammatory arthritis than from healthy controls (Figure 1B).

To establish MDR1 activity, efflux of the fluorescent dye Rhodamine-123 (Rh123) can be used. Rh123 is taken up by lymphocytes, and can be exported by MDR1 expressing lymphocytes, and is therefore often used as a marker of MDR1 activity [13, 35, 36]. In particular NK and CD8+ T cells had a high Rh123 efflux activity as observed by a large fraction of Rh123- cells (90.7% and 70.7% respectively) whereas in the CD4+ T cell population a smaller Rh123- fraction (35.8%) was present. However, no differences in Rh123 efflux activity in total lymphocytes, CD4+, CD8+ and NK were observed between healthy controls and patients (Figure 1C). In addition, healthy individuals and patients with inflammatory arthritis had similar peripheral B cell proportions, which did not differ in MDR1 expression and Rh123 efflux activity (Supplementary Figure 1A-C). In addition to increased MDR1 expression, significantly larger proportions of MRP1 expressing lymphocytes were present in patients with inflammatory arthritis compared to healthy individuals (~5.0% and ~1.0% respectively). Fractions of MRP1+ cells were comparable between CD4+, CD8+, NK and B cells (Figure 1D and Supplementary Figure 1D).

A common assay to measure MRP1 activity is the analysis of cellular calcein efflux. In this assay, non-fluorescent calcein-AM is taken up by living cells, which will be converted intracellularly to fluorescent calcein. Cells expressing MRP1 are capable of transporting calcein, enabling calcein efflux as a read out of drug transporter activity [13, 37, 38]. In agreement with a higher fraction of MRP1 expressing lymphocytes in patients, increased calcein efflux activity was present, indicated by larger fractions of calcein- lymphocytes in patients (~10.0%) compared to healthy individuals (~1.0%). The fractions of calcein- cells were comparable between CD4+, CD8+, NK and B cells (Figure 1E and Supplementary Figure 1E).

The expression of the multidrug transporter ABCG2 was below the detection limit in lymphocytes of both patients and healthy controls (data not shown) and was therefore not further investigated.

Taken together, these findings show that lymphocytes, including CD4+ and CD8+ T, NK and B cells from patients with inflammatory arthritis express higher MRP1 and in particular CD4+ and CD8+ T cells express higher MDR1 levels compared to healthy individuals.

Lower MDR1 expression and activity on CD8+ T cells of patients with RA not responding on DMARD and steroid therapy

As initial therapy, patients with RA are often treated with DMARDs and GCs. In our study, responding to DMARD/GC therapy was defined as a DAS44 ≤1.6 after 3, 6, 9 and 12 months of treatment. Non-response to therapy was defined as a DAS44 ≥2.4 after 3 months of treatment and a DAS44 ≥1.6 after 6, 9 and 12 months of treatment [39].

Since increased MDR1 and MRP1 expression levels were detected in patients with inflammatory arthritis, we investigated whether MDR1 and MRP1 expression levels and efflux activity of peripheral lymphocytes were different at baseline between DMARD/GC therapy responders and non-responders. This analysis showed that CD8+ T cells from non-responders expressed significantly lower MDR1 levels and displayed less efflux activity as assessed by Rh123 transport at baseline than CD8+ T cells from responders. Other lymphocyte populations did not differ between responders and non-responders (Figure 2A and Supplementary Figure 2B, C).

To verify that Rh123 efflux was a result of MDR1 activity in lymphocyte populations, the specific MDR1 inhibitor valspodar was added to the Rh123 stained cells [40]. Adding valspodar reduced the fraction of Rh123- lymphocytes from 45.0% to 9.6%. The remaining fraction of Rh123- cells in lymphocytes consists mainly of NK cells. After valspodar treatment, similar Rh123- CD8+ T cell fractions between therapy responders and non-responders were observed. This indicates that the difference in Rh123 efflux in CD8+ T cells was mainly caused by lower MDR1 activity in CD8+ T cells of therapy non-responders (Figure 2B).

Valspodar treatment was not sufficient to completely prevent Rh123 efflux in lymphocytes and in particular NK cells, which implies the presence of MDR1 independent Rh123 efflux mechanisms. Therefore we analyzed to what extent MDR1 expression levels within lymphocytes populations correlated with Rh123 export in those populations. Interestingly, correlation levels varied strongly between populations and patient groups. In CD8 cells from non-responders, MDR1 levels correlated significantly with fractions of Rh123- cells (R^2= 0.26 p=0.03), while no such correlation was found in responders (R^2= 0.02 p=0.61). CD4+ and NK cells showed no significant correlations, in both groups. The absence of correlations may indicate the presence of MDR1 independent Rh123 efflux mechanisms in lymphocytes of patients with RA (Supplementary Table 1).

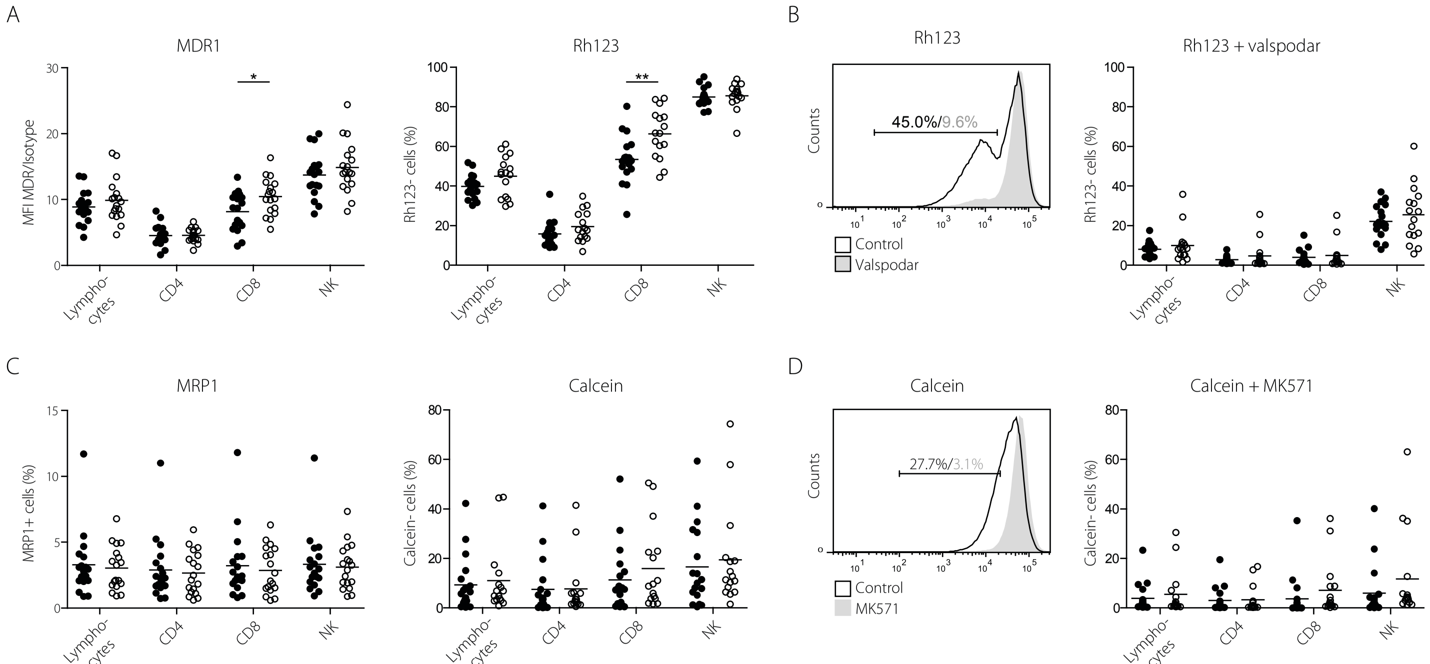


Figure 2 Lower MDR1 expression and lower efflux activity by CD8+ lymphocytes of patients with RA not responding on DMARD/GC therapy. (**A**) MFI of MDR1 divided by MFI of an isotype control antibody (left) or proportions of Rh123- cells (right) in the indicated lymphocyte populations of RA patients that responded or not responded on DMARD/GC at baseline (T0). (**B**) Left: Representative histogram showing Rh123 efflux in the presence (gray) or absence (black line) of valspodar by total lymphocytes from a treatment naïve patient with early RA. Right: proportions of Rh123- cells in the presence of valspodar in the indicated lymphocyte populations. (**C**) Proportions of MRP1 expressing cells (left) or calcein- cells (right) in the indicated lymphocyte populations of DMARD/GC therapy non-responders and responders at baseline. (**D**) Left: Representative histogram showing calcein efflux in the presence (gray) or absence (black line) of MK571 by total lymphocytes from a treatment naïve patient with early RA. Right: proportions of calcein- cells after MK571 treatment by the indicated lymphocyte populations. Mean is given in scatter plots for 18 non-responders (filled circles) and 16-18 responders (open circles) to therapy. For statistical analysis a two-sided unpaired t-test was performed (*= $p < 0.05$, ** = $p < 0.01$).

Fractions of MRP1 expressing lymphocytes and activity of calcein efflux activity were not significantly different between therapy responders and non-responders at baseline (Figure 2C and Supplementary Figure 2D, E). MRP1 expression levels and fractions of calcein- lymphocytes correlated significantly in the lymphocyte populations CD4+ and CD8+ cells from both responders and non-responders, and in NK cells from non-responders (Supplementary Table 1). Adding MK571, an inhibitor of MRP1 [40], to calcein-AM stained lymphocytes, reduced the fraction of calcein- cells (27.7% versus 3.1%). Fractions of calcein- lymphocytes were comparable between non-responders and responders after MRP1 inhibition by MK571 (Figure 2D).

In summary, these data show that patients with RA who do respond to DMARD and steroid therapy can be distinguished from non-responders by lower Rh123 efflux and MDR1 expression by CD8+ T cells.

Lower MDR1 expression and activity on naïve CD8+ T cells of therapy non-responder patients with RA

The observed differences between therapy responders and non-responders in MDR1 expression and activity on CD8+ T cells were not related to differences in CD8+ T cell proportions of total lymphocytes (Figure 3A). Within CD8+ T cells, naïve cells (Tn, CD45RO-), effector memory cells (Tef, CD45RO+CD62L-) and central memory cells (Tcm, CD45RO+CD62L+) can be identified. Similar proportions of these CD8+ T cell populations were observed between therapy responders and non-responders (Figure 3B). MDR1 expression levels varied between these populations, e.g. Tn have higher MDR1 levels compared to Tcm and Tef. In addition, MDR1 expression was significantly lower on Tn, but not on Tcm and Tef, from therapy non-responders than from responders (Figure 3C). In comparison to Tcm and Tef, Tn were more effective in the efflux of Rh123. Tn and Tef cells from therapy non-responders were less effective in the efflux of Rh123 (Figure 3D). Curiously, efflux of Rh123 correlated significantly to MDR1 levels on all CD8 populations in both patient groups, except for Tn from responders (Supplementary Table 1). Adding valspodar to Rh123 stained cells resulted in the inhibition of Rh123 efflux in all CD8+ T cell populations. Similar proportions of remaining Rh123- CD8+ T cell populations of both therapy responder and non-responders cells were observed after valspodar treatment. This indicates that the observed differences in Rh123 export by CD8+ T cells between responders and non-responders were associated with MDR1 activity (Figure 3E).

Together, these data identified MDR1 expression and activity in CD8+ Tn in particular as a discriminative marker to distinguish therapy responders from non-responders at baseline.

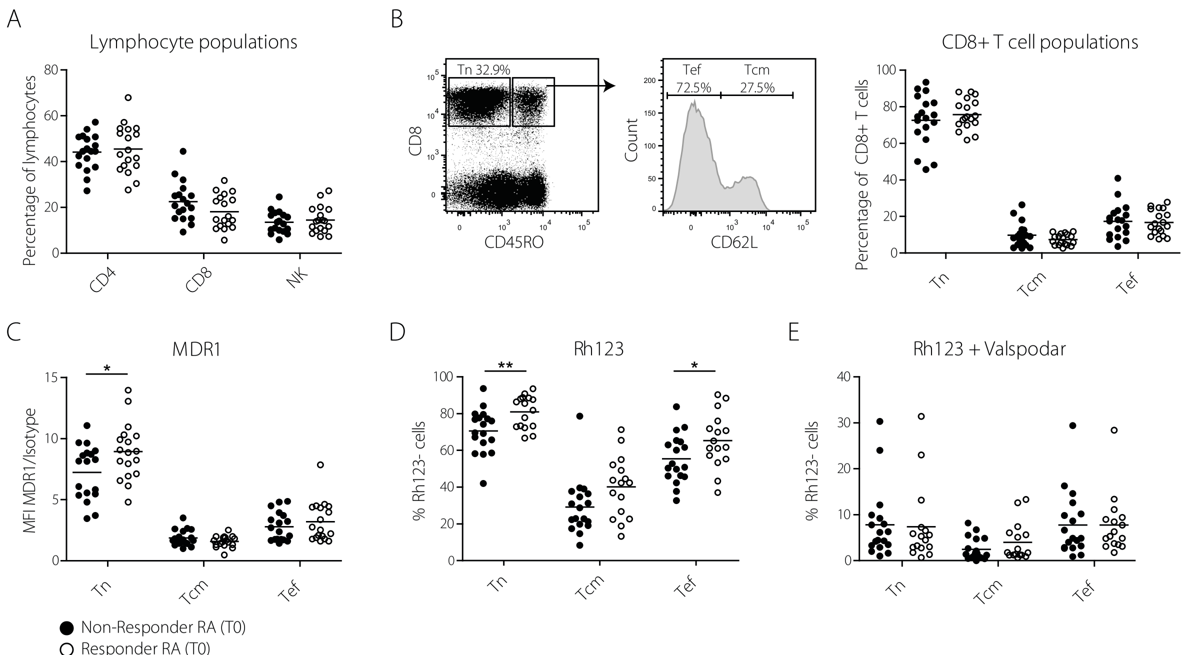


Figure 3 Lower MDR1 expression and Rh123 efflux by naïve CD8+ T cells of DMARD/GC non-responders than responders at baseline. (**A**) Proportions of CD4+ T, CD8+ T and NK cells in total lymphocytes of RA patients at baseline (T0), who did or did not respond to DMARD/GC therapy. (**B**) Left and middle: flow cytometry gating strategy for the identification of CD8+ T cell subpopulations; naïve (Tn), central memory (Tcm) and effector memory (Tef). Cells were gated within the lymphocyte fraction, and were CD3+CD4-. Right: proportions of the indicated populations in total CD8+ T cells. (**C**) MFI of MDR1 divided by the MFI of an isotype control by the indicated CD8+ T cell populations. (**D-E**) Proportions of Rh123- cells in the indicated populations without (**D**) or with (**E**) valspodar added. Mean is given in scatter plots for 18 non-responders (filled circles) and 16-18 responders (open circles) to DMARD/GC therapy. For statistical analysis a two-sided unpaired t-test was performed (*= $p < 0.05$, ** = $p < 0.01$).

Increased calcein efflux capacity in lymphocytes of RA patients not responding to DMARD and steroid therapy

To investigate whether MDR1 and MRP1 can be used to monitor multidrug resistance during treatment, PBMCs before (T0) and after 6 months (T6) of DMARD/GC treatment were compared in therapy responders and non-responders. Mean DAS44 (± SD) of responders at baseline and T6 were respectively 3.14 ± 0.68 and 0.89 ± 0.45, mean DAS44 (± SD) of non-responders at T0 and T6 were respectively 3.51 ± 0.56 and 3.06 ± 0.78. Treatment did not influence MDR1 expression and Rh123 efflux on total lymphocytes and CD4+ T, CD8+ T, NK and B cells from responders and non-responders. Adding valspodar to the cells showed that most of the Rh123 efflux was due to MDR1 activity (Figure 4A, B and Supplementary Table 2).

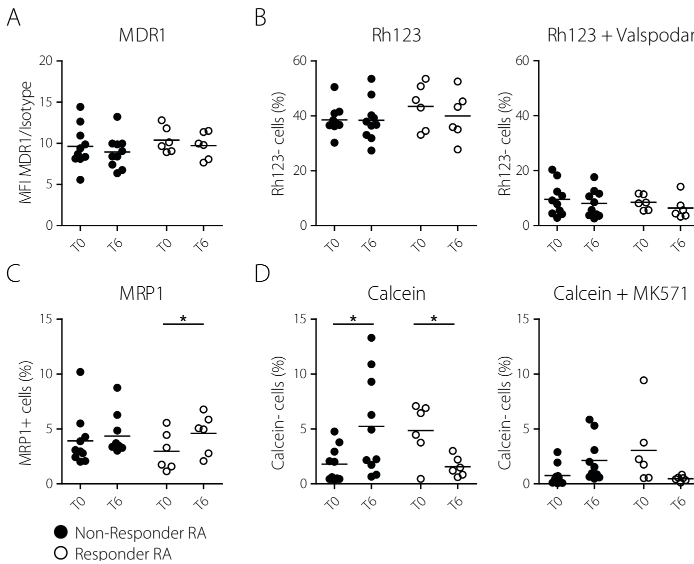


Figure 4 DMARD/GC treatment induces calcein efflux in lymphocytes from therapy non-responders, while inhibiting calcein efflux in lymphocytes from responders. (**A**) MFI of MDR1 divided by the MFI of an isotype control by total lymphocytes of RA patients at baseline (T0) and after 6 months (T6) of DMARD/GC treatment. (**B**) Proportions of Rh123- lymphocytes without (left) or with (right) valspodar added. (**C**) Proportions of MRP1 expressing lymphocytes. (**D**) Proportions of calcein- lymphocytes without (left) or with (right) MK571 added. Mean is given in scatter plots for 10 non-responders (filled circles), and 6 responders (open circles) to therapy, at T0 and T6. For statistical analysis between T0 and T6 a two-sided paired t-test was performed (*= $p < 0.05$).

In contrast, fractions of MRP1 expressing lymphocytes were significantly increased after treatment in therapy responders, but not in non-responders (Figure 4C). This was significant for all lymphocyte populations (Supplementary Table 2). However, calcein efflux was significantly increased in non-responders and significantly decreased in responders. These effects were present in all lymphocyte populations (Figure 4D and Supplementary Table 2). Blocking of MRP1 activity by MK571 resulted in a residual fraction (~2.1%) of calcein- lymphocytes of therapy non-responders at T6, whereas in responders

a residual fraction below 0.5% was detected at T6. This indicates that the increase in calcein efflux in upon treatment in therapy non-responders may reflect additional MRP1 independent mechanisms (Figure 4D).

Taken together, these results indicate that increased calcein efflux is a marker of DMARD and therapy non-response in patients with RA.

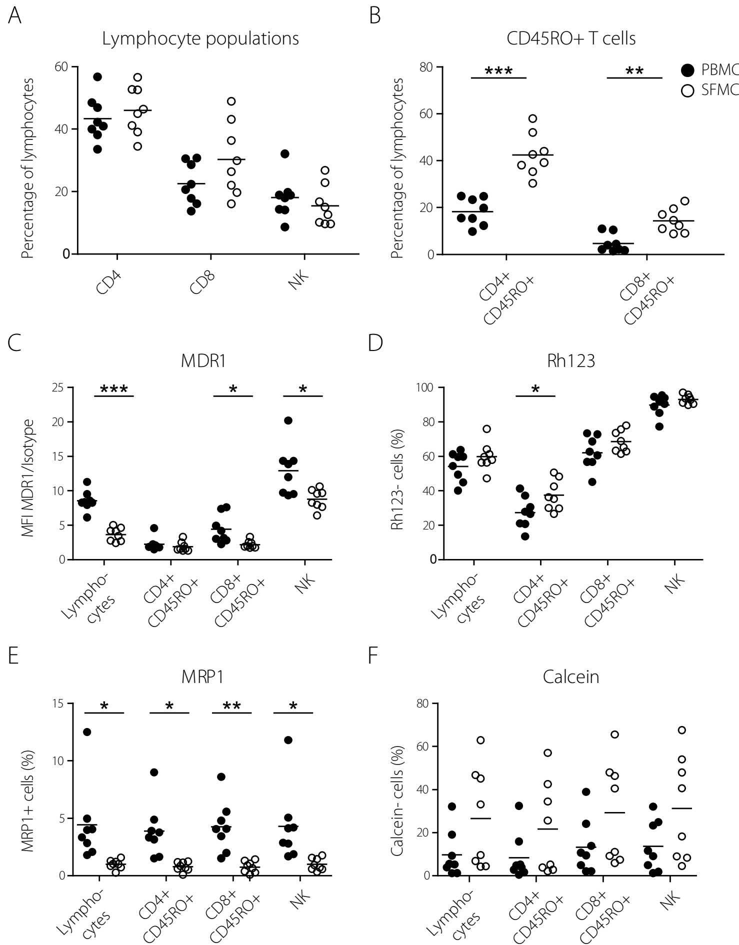


Figure 5 Synovial lymphocytes from patients with inflammatory arthritis express lower MRP1 and MDR1 levels, while their efflux activity is higher compared to peripheral lymphocytes. (**A-B**) Proportions of CD4+ T, CD8+ T and NK cells (**A**) or memory CD4+ and CD8+ T cells in (**B**) in either peripheral blood or synovial fluid lymphocytes of patients with inflammatory arthritis. (**C**) MFI of MDR1 divided by the MFI of an isotype control by indicated lymphocyte populations. (**D**) Proportions of Rh123- cells of indicated lymphocyte populations. (**E**) Proportions of MRP1 expressing lymphocyte populations. (**F**) Proportions of calcein- cells in indicated populations. Mean is given in scatter plots for matched PBMCs (filled circles) and SFMCs (open circles) from 8 established arthritis patients. For statistical analysis a two-sided paired t-test was performed (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Lower MRP1 and MDR1 expression levels, but increased efflux activity by synovial lymphocytes in comparison to paired peripheral blood lymphocytes

As a next step we investigated whether MDR1 and MRP1 expression and activity in peripheral blood lymphocytes are reflected in local synovial fluid lymphocytes. The expression levels and activity of MRP1 and MDR1 were investigated in lymphocytes obtained from synovial fluid and compared to autologous peripheral blood, sampled at the same time, from inflammatory arthritis patients. Fractions of Th cells, CD8+ T cells and NK cells did not differ significantly between SFMC and PBMC (Figure 5A), but fractions of CD45RO+ (memory) CD4+ and CD8+ T cells were significantly increased in synovial fluid compared to peripheral blood (Figure 5B). Therefore MDR1 and MRP1 expression was assessed on memory CD4+ and CD8+ T cells, rather than on the total CD4+ and CD8+ populations. MDR1 levels were significantly lower on synovial fluid memory CD8+ T and NK cells, but in not memory CD4+ T cells in comparison to their peripheral counterparts (Figure 5C). However, Rh123 efflux is similar in memory CD8+ T and NK cells and even significantly higher in memory CD4+ T cells in the synovial fluid than in peripheral blood (Figure 5D). In addition to MDR1, also MRP1 expression levels were significantly lower in all synovial lymphocyte populations (Figure 5E). In contrast, synovial lymphocyte calcein efflux is increased (although not statistically significant) compared to peripheral lymphocytes in the majority of patients (Figure 5F).

These data show that synovial lymphocytes have lower MDR1 and MRP1 expression levels compared to peripheral lymphocytes. The increase in calcein efflux capacity of synovial lymphocytes suggests the presence of additional MRP1 independent mechanisms. Therefore, calcein efflux rather than MDR1 and MRP1 expression levels reflect potential drug efflux capacity of lymphocytes.

Discussion

A large fraction of patients with RA does not respond to, or becomes resistant to DMARD/GC therapy and markers to predict or monitor this treatment response are lacking. The results presented here show that the multidrug transporters MDR1 and MRP1, but not ABCG2 are expressed at higher levels in patients with inflammatory arthritis compared to healthy individuals. In contrast to MRP1, MDR1 is differentially expressed between RA lymphocyte populations. Here we found that in particular low MDR1 expression and drug efflux activity of naïve CD8+ T cells were markers that predict the lack of DMARD/GC response at baseline. Moreover, we found that DMARD/GC treatment resulted in increased calcein efflux capacity of lymphocytes from therapy non-responders that is likely both MRP1-dependent and -independent, whereas in therapy responders, calcein drug efflux was decreased over time. This indicates that calcein efflux, rather that MRP1 expression, can be used to monitor DMARD/GC therapy response in RA.

Our finding that lymphocytes from patients with inflammatory arthritis express higher MDR1 levels than lymphocytes of healthy controls confirm previously published results [22-24]. MDR1 expression and activity varies extensively in lymphocyte populations from healthy individuals [41]. Correspondingly, MDR1 expression and efflux activity varied between lymphocyte populations in RA patients, with relatively high MDR1 expression and efflux capacity on NK and CD8+ T cells and low on CD4+ T cells. Only by performing lymphocyte specific analysis, we were able to detect lower MDR1 expression and activity on naïve CD8+ T cells at baseline from patients not responding to DMARD/GC therapy.

The underlying mechanism for lower MDR1 levels in CD8+ T cells from patients not responding to DMARD/GC therapy is unclear. It may be an intrinsic difference between non-responders and responders, since naïve CD8+ T cells are involved, which have not experienced antigen activation. It could also be possible that lower MDR1 levels reflect other processes, such as the up-regulation of other multidrug transporters. The family of ABC transporters consists of 48 members [6], and the regulation of ABC transporter expression is complex [42]. For example, in various systems TNFα decreases MDR1 expression, whereas it induces MRP1 and ABCG2 expression [43, 44].

MDR1 is known to be involved in transport of GCs, but not MTX [2]. A speculative hypothesis is that DMARD/GC non-responders have an up-regulation of other transporters involved in MTX transport. However MRP1, which is capable to efflux MTX [7, 8], is not differentially expressed in DMARD/GC therapy responders and non-responders at baseline. Furthermore, instead of efflux transporters other mechanisms might play a role in determining intracellular drug concentrations. For example, carriers have been described that are involved in MTX uptake in the cell [5, 10, 13].

We found no changes in MDR1 expression levels after DMARD/GC treatment, while previous research found either higher [23, 26, 45] or lower [24] MDR1 expression levels and activity on lymphocytes from non-responders after treatment. These different results may reflect the combined use of DMARDs and GCs in our study: MTX is able to inhibit MDR1 expression, whereas GCs induce MDR1 expression [23].

ABCG2 expression was previously found in the RA synovium, but the ABCG2 positive cells were mainly macrophages and cells from the endothelial lining. Only weak expression was detected on T cells [14]. In line with this, we could not detect ABCG2 expression in lymphocytes from healthy controls and established arthritis patients (data not shown). Therefore, lymphocytes might not be the primary cells of interest in ABCG2 mediated drug excretion.

In contrast to MDR1, limited research has been performed in RA concerning MRP1. We found that in patients with inflammatory arthritis MRP1 expression is present at similar levels in all lymphocyte populations, but it is expressed at significantly lower levels in healthy individuals. MRP1 expression can be detected already in treatment naïve patients with early RA, indicating that inflammatory mediators might be involved in the regulation of MRP1 expression. Indeed, TNFα for example is known to induce MRP1 expression [43]. In the case of DMARD therapy MRP1 expression and activity are highly relevant, since MTX and HCQ are MRP1 targets [7-10]. Exposure to MTX and HCQ may explain the expansion of MRP1 expressing lymphocytes that we observed in non-responders. However, MRP1 expression is not a suitable marker for DMARD/GC therapy response, since MRP1 is also up-regulated (although not significantly) in patients that do respond to DMARD/GC therapy.

Calcein efflux is often used as a marker for MRP1 activity, although it is plausible that other multidrug transporters can also export calcein [13]. In lymphocytes from patients with inflammatory arthritis both MRP1 and calcein efflux are up-regulated in comparison to healthy individuals. However, blocking MRP1 activity with MK571 only partially reduces the calcein efflux in patients not responding to DMARD/GC therapy. In addition, patients responding to DMARD/GC therapy up-regulate MRP1 expression but show a decreased calcein efflux. These findings suggest that calcein efflux is regulated via MRP1 dependent and -independent mechanisms, and that MRP1 expression and calcein efflux do not necessarily correlate.

This is further supported by the comparison of paired synovial and peripheral blood lymphocytes of patients with inflammatory arthritis. Although synovial lymphocytes expressed lower MRP1 and MDR1 levels than peripheral blood lymphocytes, their Rh123 efflux capacity was comparable, and calcein efflux capacity was even increased. As mentioned before, regulation of ABC transporter expression is very complex and it is possible that other ABC transporters are up-regulated in the synovium. In line with this, pro-inflammatory mediators, such as TNFα, and hypoxia may be involved in ABC transporter regulation, since the synovium is a pro-inflammatory, hypoxic environment [10, 42, 46].

Because of these discrepancies between MRP1 expression and activity, calcein efflux activity, rather than MRP1 expression, may serve as an ideal marker for DMARD/GC therapy response in RA. Calcein efflux capacity (i) is low in lymphocytes of healthy individuals (ii) is increased in both peripheral blood and synovial lymphocytes of patients with RA (iii) decreases in RA patients that do respond and increases in patients that do not respond to DMARD/GC therapy and (iv) is similar between lymphocyte populations. Future research should be performed identifying the underlying MRP1-independent mechanism in the efflux of calcein from lymphocytes in patients with RA.

In our study, only non-responders to DMARD/GC therapy received TNFα inhibitors after 3 months. Treatment with TNFα blockers can reduce MDR1 expression on peripheral lymphocytes [23, 47]. Although we see no significant changes in MDR1 expression after 6 months of treatment in the non-responders, we cannot exclude that TNFα inhibitors may influence ABC transporter expression on lymphocytes of these patients.

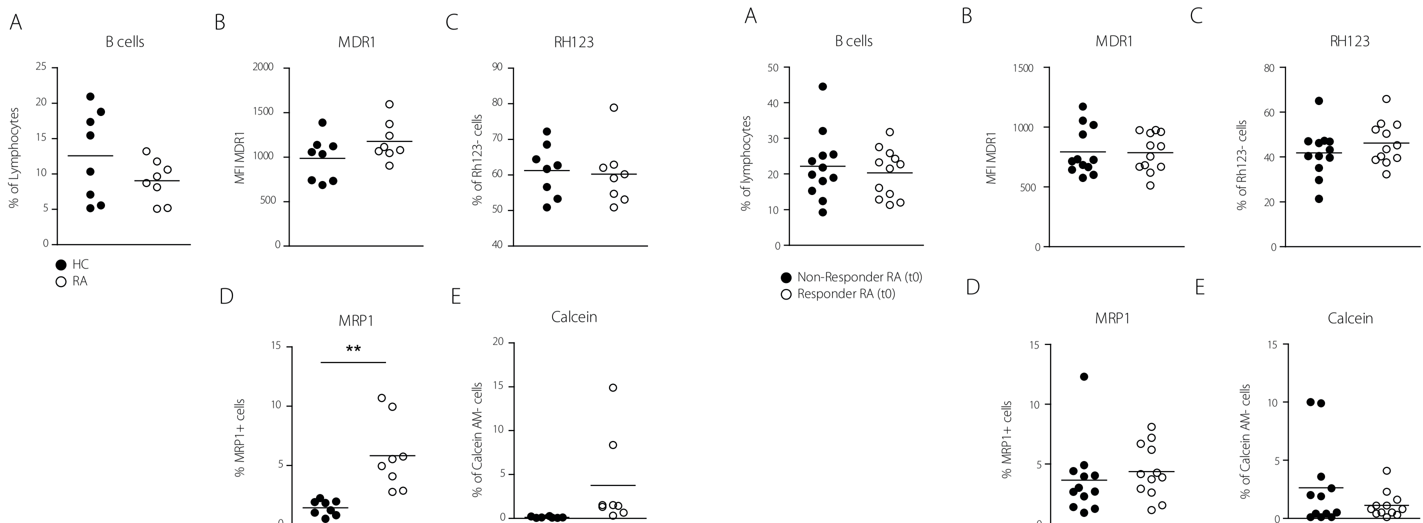
DMARD combination therapy is more effective than monotherapy [3, 48]. Interestingly, it appeared that especially combinations of DMARDs that are all ABCG2 substrates are more effective than DMARD monotherapy [5]. This indicates that combination DMARD therapy might be more effective, at least partly, by saturating the ABCG2 transporters. Also in the cohort that we used in this study, multiple DMARD therapy (MTX, SSZ and HCQ) with GC was more effective than MTX with GC [3]. It is conceivable that triple DMARD therapy in this cohort is more effective, at least in part, because the DMARDs are substrates of the same transporters [5, 10, 27]. The presence of many substrates might saturate the transporters, thereby leading to higher intracellular drug concentrations, especially in the case of HCQ, which is not only a MDR1 and MRP1 substrate, but blocks these transporters as well [10].

In conclusion, we report here that multidrug resistant lymphocytes are present in patients with RA and that these lymphocytes can be used to predict and monitor DMARD/GC treatment response. These findings might lead to improved personalized RA treatment and monitoring, and reduce the number of RA patients not responding to DMARD/GC therapy.

Acknowledgments

We would like to thank Dr. P.H.P. de Jong for assistance in selecting the RA patient cohort

Supplementary figures



Supplementary figure 1 ABC transporter expression on and efflux activity by B cells in peripheral blood of healthy controls and patients with inflammatory arthritis. (**A**) B cell proportions in peripheral lymphocytes. (**B**) MFI of MDR1 on B cells. (**C**) Proportions of Rh123- B cells. (**D**) Proportions of MRP1 expressing B cells. (**E**) Proportions of calcein- B cells. Mean is given in scatter plots for 8 healthy controls (filled circles) and 8 patients with inflammatory arthritis (open circles). For statistical analysis a two-sided unpaired t-test was performed (** = $p < 0.01$).

Supplementary figure 2 ABC transporter expression on and efflux activity of B cells in peripheral blood of treatment naïve, early RA patients that do or do not respond to DMARD/GC therapy at baseline (T0). (**A**) B cell proportions in peripheral blood lymphocytes. (**B**) MFI of MDR1 on B cells, corrected for MFI of isotype control. (**C**) Proportions of Rh123- B cells. (**D**) Proportions of MRP1 expressing B cells. (**E**) Proportions of calcein- B cells. Mean is given in scatter plots for 12 non-responders (filled circles) and 12 responders (open circles) to therapy. For statistical analysis a two-sided unpaired t-test was performed.

Supplementary Tables

Supplementary Table 1 Linear regression analysis of MDR1 and MRP1 expression with Rh123 and calcein efflux of lymphocytes of DMARD/GC therapy responder and non-responder patients with RA at baseline.

	Non-Responder		Responder	
	R^2	p value	R^2	p value
MFI MDR1/%Rh123-				
CD4	-0.03	0.49	0.13	0.16
NK	0.01	0.66	-0.14	0.16
CD8	0.26	0.03	0.02	0.61
CD8 Tn	0.40	<0. 01	<0.01	0.86
CD8 Tcm	0.34	0.01	0.34	0.02
CD8 Tef	0.60	<0.001	0.46	<0. 01
%MRP1+/%calcein-				
CD4	0.52	<0.001	0.32	0.02
NK	0.44	<0.01	0.21	0.07
CD8	0.54	<0.001	0.58	<0.001

Pearson correlation test was used to calculate the correlation coefficients (R^2) and p-values.

Supplementary Table 2 MDR1 and MRP1 expression and Rh123 and calcein efflux in lymphocyte populations from DMARD/GC non-responders and responders, at baseline (T0) and after 6 months of treatment (T6).

	Non-Responder			Responder		
	t=0	t=6	p value	t=0	t=6	p value
MFI MDR1/Isotype (sd)						
Lymphocytes	9.6 (2.5)	8.9 (2.0)	0.58	10.4 (1.6)	9.7 (1.6)	0.27
CD4	4.1 (1.0)	3.7 (1.0)	0.20	4.7 (0.9)	3.8 (0.9)	0.14
CD8	9.2 (2.3)	8.1 (1.9)	0.12	11.1 (2.2)	10.0 (1.9)	0.07
NK	16.1 (3.6)	14.1 (2.0)	0.13	16.8 (3.0)	15.0 (2.4)	0.19
B (MFI MDR1)	945 (270)	817 (189)	0.12	924 (143)	880 (163)	0.47
% Rh123- cells						
Lymphocytes	38.5 (5.2)	38.4 (7.6)	0.97	43.5 (8.3)	40.0 (8.8)	0.46
CD4	14.9 (4.4)	14.1 (3.9)	0.54	16.9 (5.0)	15.4 (4.8)	0.37
CD8	51.5 (12.8)	54.7 (14.6)	0.19	64.3 (8.8)	59.5 (10.6)	0.12
NK	86.4 (4.3)	76.8 (12.3)	0.02	84.9 (6.0)	85.3 (9.1)	0.27
B	37.9 (9.0)	38,1 (11.6)	0.93	48,5 (9.4)	42.3 (9.8)	0.13
% MRP1+ cells						
Lymphocytes	3.9 (2.5)	4.4 (1.8)	0.26	3.0 (1.8)	4.6 (1.8)	0.02
CD4	3.2 (2.1)	3.6 (1.9)	0.05	2.6 (1.7)	4.1 (1.7)	0.03
CD8	3.5 (2.2)	3.8 (1.9)	0.21	2.6 (1.7)	4,2 (1.7)	0.02
NK	3.7 (2.2)	4.2 (1.7)	0.11	2.8 (1. 4)	4.1 (1.5)	0.04
B	5.5 (2.5)	5.3 (2.2)	0.88	3.5 (1.5)	6.7 (3.6)	0.04
% Calcein- cells						
Lymphocytes	1.8 (1.6)	5.2 (4,5)	<0.05	4.9 (2.6)	1.6 (0.9)	0.04
CD4	1.2 (1.3)	4.2 (4.4)	0.07	3.0 (2.3)	0.9 (0.6)	0.09
CD8	2.6 (2.5)	7.4 (6.0)	0.02	8.2 (5.4)	2.6 (2.1)	0.04
NK	3.2 (2.8)	8.6 (6.2)	0.02	6.8 (3.4)	3.1 (2.0)	0.10
B	0.2 (0.2)	1.0 (1.2)	<0.05	1.1 (1.2)	0.1 (0.1)	0.08

Means and standard deviations (SD) are given for 10 non-responders at T0 and T6, and 6 responders at T0 and T6. For statistical analysis between T0 and T6 a two-sided paired t-test was performed.

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Chapter 8

General discussion

RA affects 0.5-1.0% of the adult population world-wide. During the last decades, great progress has been made in understanding the pathogenesis of RA, and new and targeted therapies have been developed, such as inhibitors of TNFα, IL-6R and B cells. Although these therapies have greatly improved symptoms of RA [1], some patients do not respond to, or become resistant to therapy, leading to disability, reduced quality of life, cardiovascular and other comorbidities [2-6]. Therefore, there is still a need for therapies that target different (cytokine) pathways than the ones currently applied.

In 2005 Th17 cells were discovered, and these cells are thought to play a role in the development of many autoimmune diseases, including RA [1]. Th17 cells can be distinguished from other subsets by the expression of CCR6 [7]. CCR6+ Th cells and their cytokines have been extensively researched in the context of RA, and were shown be involved in RA inflammation and destruction [1]. However, CCR6+ Th cells are a very heterogeneous subset, and the contributions of the CCR6+ Th subpopulations to RA pathology are not well understood.

In this thesis the pathogenic role and the modulation of CCR6+ Th cells in RA was investigated. For these studies, peripheral blood donated by healthy persons, and peripheral blood and synovial fluid donated by RA patients were used. The results described in this thesis shed light on the pathogenesis of CCR6+ Th cells in (especially early) rheumatoid arthritis and highlight their potential as treatment target.

Main findings of the thesis

- COX-2/PGE_2 is critically involved in autocrine IL-17A production by CCR6+ Th cells upon co-culture with RASF. COX-2 inhibition in CCR6+ Th/RASF cultures reduces the production of the pro-inflammatory mediators IL-17A, IL-6, IL-8 and IL-1β and of the tissue-destructive MMPs by RASF. Moreover, COX-2 inhibition combined with TNFα blockade had additional value to TNFα blockade alone in reducing CCR6+ Th cell activity. **Chapter 3**
- Both in human and murine arthritis IL-17/Th17-mediated synovial inflammation is IL-22/Th22 independent. Th17 cells were more potent than Th22 cells in inducing a pro-inflammatory loop upon co-culture with RASF. In AIA, IL-22 deficiency does not affect arthritis severity. **Chapter 4**
- Th17, Th17.1, DP and DN cells are identified in RA peripheral blood and synovial fluid. Despite differing in Th17/Th1-associated properties, all CCR6+ Th cell subpopulations showed high pathological activity in co-culture with RASF. All CCR6+ Th subpopulations expressed MDR1 and MRP1, but Th17.1 and DN CCR6+ Th cells are *in vitro* potentially most drug resistant. **Chapter 5**
- ACPA+ early RA patients have higher proportions of CCR6+ memory Th cells (specifically Th22, Th17.1 and DP cells) in peripheral blood than matched ACPA- patients. CCR6+ Th cells are inversely correlated with disease duration in ACPA- patients but not in ACPA+ patients. CCR6+ Th cells might be involved in the differences in disease severity and treatment outcome between ACPA+ and ACPA- RA. **Chapter 6**
- MDR1 expression and activity on peripheral lymphocytes, mainly on naïve CD8+ T cells, can be used to predict DMARD and glucocorticoid treatment response. Moreover, MRP1 expression and efflux potential of lymphocytes from patients with RA are induced by combined DMARD and glucocorticoid treatment, and can be used to monitor the response to this treatment strategy. **Chapter 7**

PGE_2 induces autocrine IL-17A production

Recently, our group has shown that in the early stage of RA, CCR6+ Th memory cells, are potent activators of RASF. This activation results in the creation of a pro-inflammatory loop, characterized by up-regulation of IL-6, IL-8, MMP-1 and MMP-3, and depends on autocrine IL-17A production [8]. The autocrine IL-17A production by CCR6+ Th cells is critical to sustain the pro-inflammatory loop [8, 9], but the mechanism underlying the autocrine IL-17A induction was still unknown. In chapter 3 we show that the COX-2 inhibitor celecoxib reduces IL-17A production by CCR6+ Th cells in a co-culture system with synovial fibroblasts. Moreover, in this system celecoxib reduced pro-inflammatory cytokine and MMPs production. Interestingly, this was accompanied by an increase in TNFα production by CCR6+ Th cells, which could explain why disease progression is not inhibited by celecoxib treatment [10]. Inhibiting PGE_2 production in addition to TNF-α blockade may be an important mechanism to control chronic inflammation in RA and potentially other Th17-mediated autoimmune disorders.

Th22 and classic Th17 cells in synovial inflammation

Besides IL-17A, CCR6+ Th cells express the cytokine IL-22 [11, 12]. In RA, elevated Th22 and Th17 cell proportions were found compared to healthy controls [13-15], and IL-22 producing T cells were found in peripheral blood and in the inflamed synovium of RA patients [16-19]. IL-22 serum levels correlate positively with bone erosions, suggesting a pathogenic role of IL-22 [20]. Moreover, IL-22 deficient (IL-22$^{-/-}$) mice develop less severe collagen induced arthritis (CIA) compared to wild-type mice [21]. In contrast, IL-22 is not required for the development of experimental autoimmune encephalomyelitis [22] and IL-22 is even protective for experimental inflammatory bowel disease [23]. Therefore, the role of Th22 cells in RA is controversial.

In chapter 4 we sought to clarify the role of Th22 cells in synovial inflammation. Using human *in vitro* T cell-RASF co-cultures, we show that classic Th17 cells, but not Th22 cells, activate pro-inflammatory cytokine and MMP production by RASF. In addition, in murine T cell-mediated experimental arthritis (antigen-induced arthritis, AIA), we show that IL-22 does not contribute to IL-17/Th17 mediated synovial inflammation [24]. The increase in Th22 levels and IL-22 in RA patients [16-19] might be a consequence, rather than a cause of inflammation. IL-22 is involved in antimicrobial defense and tissue regeneration [25-27], and might therefore be upregulated as a result of the inflammatory and destructive processes in RA.

In addition to T cells, TCRγδ, lymphoid tissue inducer (LTi) and NK cells also produce IL-22 [28, 29], and their potential roles in synovial inflammation are not addressed in the co-culture that we used. However, the onset and severity of AIA were comparable in IL-22$^{-/-}$ and wild-type mice, indicating that IL-22, independent of the cellular source, has no role in synovial inflammation. In contrary, another group has found that IL-22$^{-/-}$ mice develop less severe CIA than wild-type mice [21]. AIA is a mainly T cell driven model, whereas in CIA B cells and immune complex mediated processes also play a major role. This suggests that IL-22 is more important in B cell than T cell immunity, but more research is needed to clarify the possible connection between IL-22 and B cells.

The RASF/CCR6+ Th cell co-culture system as a model for RA joint inflammation

In chapter 3 and chapter 4 we investigated synovial inflammation using a co-culture system of CCR6+ Th cells (derived from healthy persons or early RA patients) and RASF. Interaction between the CCR6+ Th cells and RASF results in the induction of a pro-inflammatory feed-forward loop, depending on autocrine IL-17A production [8]. This loop may be an important pathway in the progression of arthritis, and possibly the interaction of Th17 cells with stromal cells may drive chronicity in other Th17 diseases as well. However, in this system we only study CCR6+ Th cells and synovial fibroblasts, while many more cell types are present in the inflammatory synovial joint. For example, monocytes, derived from peripheral blood or inflamed joints from RA patients, specifically induced Th17, but not Th1 or Th2 responses. This Th17 cell polarization was dependent on cell-cell contact [30-32] and on PGE_2 in combination with IL-1β and IL-23 [33, 34]. Furthermore, CCR6+ Th cells can attract and activate neutrophils and macrophages to sites of inflammation, and can also interact with other cells present in the synovium, like CD8+ T cells,

B cells, osteoclasts and chondrocytes (see chapter 2 for a more complete overview). These cells also interact with each other, via cytokines, chemokines and/or direct interactions, thereby activating each other and propagating the inflammatory processes.

Our RASF-Th cell co-cultures study only a small part of the many interactions in the synovium, but because of the limited cell types present in the co-culture system, it is also possible to study the RASF/T cell interaction in detail, adding to our understanding of these cells. Furthermore, many of the cytokines that are thought to play important roles in RA pathogenesis are also expressed in our co-culture system, like TNFα, IL-1β, IL-17A, IL-6 and IFNγ. The expression of these cytokines, and in addition chemokines, PGE_2 and metalloproteinases enables us to investigate the mechanisms of action of agents used in the treatment of RA (like TNF inhibitors and IL-6R inhibitors), but also compounds that are not yet approved like blockers of IL-17A and vitamin D [8, 10]. Furthermore, inhibitors of inflammation (anti-TNF and vitamin D) were comparable in efficacy in the RASF/CCR6+ Th cell co-culture and in explants of synovial biopts, which contain many cell types [9].

The CCR6+ Th cell/RASF co-culture has enhanced our understanding about how these cells interact and how these cells contribute to inflammation. In the future, adding more cell types to the co-culture, or add cytokines produced by these cell types, to better mimic the complex synovium is likely to lead to more insights.

Non-response to therapy and multidrug resistance

RA patients are usually treated with a combination of DMARDs and GCs. While this combination is usually effective, about 25-45% of RA patients does not respond to, or becomes resistant to, DMARD treatment [2, 3]. Furthermore, not all patients respond well to GC treatment [4]. One of the mechanisms involved in not responding to, or becoming resistant to drugs is multidrug resistance through cellular expression of ABC transporters. Multidrug resistance has been extensively researched in cancer [35], but has received little attention in RA. Interestingly, it is hypothesized that DMARD combination therapy is more effective than monotherapy [3, 36] because drugs that are substrates for the same ABC transporters saturate these transporters, leading to higher intracellular drug concentrations [37-39].

ABC transporters are expressed in many tissues and cells, including lymphocytes [2, 38, 40]. The expression of ABC transporters on lymphocytes, and the involvement of lymphocytes in RA pathogenesis [1], led us to investigate which ABC-expressing lymphocyte populations can be used to predict and monitor treatment in RA patients. In chapter 4 and 7, we found expression of MDR1 and MPR1 in peripheral and synovial lymphocytes from established arthritis patients, and lymphocytes from these patients are able to export fluorescent dyes. Within RA patients, responders can be distinguished from non-responders by higher levels and activity of MDR1 on naïve CD8+ T cells. DMARDs and glucocorticoid treatment induced calcein-AM efflux in all lymphocyte populations, including CCR6+ Th cells, in non-responders, but reduced efflux in responders. Rh123 efflux was unchanged in total lymphocytes after DMARD and glucocorticoid treatment, but efflux was induced in CCR6+ Th cells from non-responders.

Surprisingly, in our study responders had higher MDR1 expression levels on CD8+ T cells at baseline than non-responders, and synovial lymphocytes expressed lower levels of ABC transporters than matched peripheral lymphocytes. The regulation of ABC transporter expression is very complex, and many factors can influence ABC transporter expression, like cytokines and hypoxia [41, 42]. Especially in the highly inflammatory environment of the synovium, ABC transporter expression might be regulated different than in the peripheral blood. Strikingly, while synovial lymphocytes express lower levels of MDR1 and MPR1 than peripheral lymphocytes, the synovial lymphocytes efflux more fluorescent dyes. The ABC transporter family is quite large (at least 48 family members are known [35]), and it well possible that several transporters can transport the same dye (or drugs), explaining the difference in MDR1/MRP1 expression levels and the efflux of 'corresponding' dyes [43]. More research into the expression of ABC transporters in RA is needed. Because of the unavailability of antibodies against many ABC family members [38], analysis of RNA expression levels would be a suitable starting point to identify ABC members with roles in drug resistance in RA.

However, the biggest hurdle in research to treatment failure, is the fact that many factors define whether treatment will be successful. For RA, as well as for (almost) all other drugs, the ADME (absorption, distribution, metabolism and excretion) principles are applicable: the drug needs to be absorbed and distributed into the body, without metabolic inactivation (or with metabolic activation), and should not be excreted before it can reach its target. If the drug reaches its target cell/organ in physiological relevant concentrations, there are still many factors that can hamper its activity. For example: 1) Some drugs are taken up into the cell by passive diffusion through the membrane, but some need carriers to enter the cell [42]. For instance, carriers are known to play a role in MTX uptake in the cell, next to passive diffusion [38, 39, 43]. 2) Within the cell the drug could be metabolized to a less active or inactive form. 3) Some patients have better cellular repair mechanisms than others, thereby overcoming drug toxicity. 4) Some patients are able to export drugs out of the cell through ABC transporters [42]. All of these factors define whether drug treatment will be successful in the individual case, but it is currently impossible to account for all these factors in *in vivo* research, especially in the individual case. *In vitro* research however typically only focusses on one or several of these factors, and cannot take the others into account, rendering the results only partly applicable to the *in vivo* situation. Defining the contribution of each of the above factors will be a major challenge in research to treatment non-response. In this context, I support Márki-Zay *et al*. [38], who stated: "Due to clinical diagnostic limitations, therapy resistance can only be defined by lack of clinical response. In order to develop drugs… the resistance and response must be defined at a cellular level."

CCR6+ Th subpopulations

As stated in the introduction, IL-17A producing Th cells are often referred to as Th17 cells, but by only analyzing IL-17A producing Th cells, pathogenic Th cell populations with Th17 characteristics can be missed [44]. On the basis of CXCR3 and CCR4 expression, four CCR6+ subpopulations can be distinguished: Th17, Th17.1, DP and DN cells. In chapter 4 we showed that Th22 cells have less pathogenic potential

than Th17 cells in RA, therefore Th22 cells were not included in the research described in subsequent chapters. In chapter 5, the CCR6+ Th cell subpopulations were analyzed for their pathologic potential in activating fibroblasts, and their resistance to drugs was evaluated by MDR1 and MPR1 expression, and by fluorescent dye efflux. We found that Th17, Th17.1, DP and DN CCR6+ Th cells were all present in the peripheral blood of RA patients in both early and established RA and in synovial fluid of a patient with established RA and active disease, indicating their relevance at the main site of inflammation in RA. Although the CCR6+ Th cell populations differ markedly in Th17 and Th1 cell characteristics (the expression of IL-17A and IFNγ, respectively), they all possess more pathogenic potential than Th1 and naive Th cells, as shown by the inflammatory and destructive proteins produced by the fibroblast upon co-culture. This underscores the importance of better classifying CCR6+ Th subpopulations, since also the subpopulations that produce relatively low amounts of IL-17A were shown to be pathogenic. Furthermore, all CCR6+ Th subpopulations express MDR1 and MPR1, but based on fluorescent dye efflux, mainly Th17.1 and DN CCR6+ Th cells have the potential to be drug resistant, indicating the importance of these cells in RA.

The ontogeny of the four CCR6+ Th cell populations is not elucidated. IL-12 is able to up-regulate TBX21 expression and IFNγ expression in Th17 cells, indicating that Th17.1 cells could be derived from Th17 cells [45-47]. On the other hand, cells with Th17.1 characteristics develop when naïve T cells are cultured *Candida albicans* (a commensal fungus that is controlled by IL-17/Th17 [48-50]) primed monocytes, pointing to a direct origin of Th17.1 cells from naïve T cells [51]. No research to the ontogeny of the DN and DP CCR6+ Th subpopulations has been published to date. The CCR6+ Th subset is very plastic [45, 52, 53], making it possible that these subpopulations are intermediate populations. However, it seems irrational that Th17 cells switching to or from Th17.1 cells could either go through a DN or a DP stage. Furthermore, on average, almost 20% of all CCR6+ Th cells in RA patients is DP and almost 20% is DN, which seems a high number for cells that plastic and are moving through a DN or DP phase to become Th17 or Th17.1. This indicates that all CCR6+ Th cells might be separate subpopulations (although with a high level of plasticity) with distinct functions.

In line with this, studies have been published that describe separate roles for Th17.1 and Th17 cells. The same study that showed that *Candida albicans* primed monocytes gave rise to a Th17.1 like phenotype, also showed that *Staphylococcus aureus* (a commensal bacterium that is controlled by IL-17/Th17 [48-50]) primed monocytes gave rise to a Th17 phenotype with a higher percentage of IL-17A producing cells and lower percentages of IFNγ producing or IFNγ/IL-17A double-producing cells. This indicates that at least Th17 and Th17.1 have different functions *in vivo*. In line with this, CCR6+ Th cells producing both IL-17A and IFNγ (Th17.1 phenotype) are thought to be involved in anti-tumor immunity, while other CCR6+ Th cells are thought to be protective for the tumor (mainly cells co-expressing IL-10) [54]. Additionally, in Crohn's disease, the cells found in the activated gut were mainly Th17.1 cells [55].

For Th17 and Th17.1 cells different functions have been established, and therefore it seems likely that also the DN and DP CCR6+ Th cells have specific functions, as is also indicated by the distinct cytokine and transcription factor profile in these cells that we described. However, further research is needed

to clarify the ontogeny, stability and function of these populations. Furthermore, the question remains which factors (cytokines/chemokines/stimulatory receptors etc.) are responsible for the observed pathogenicity of these subpopulations. Neutralization of IL-17A could only partly inhibit the pathogenic potential of these cells, showing the involvement of other factors. IFNγ is not likely the responsible factor, since IFNγ producing Th1 cells show markedly less pathogenic activity compared to Th17.1 cells (which produce high levels of IFNγ), even when compared to Th17.1 cells wherein IL-17A activity is blocked. Further research should clarify the mechanisms through which CCR6+ Th subpopulations exert their pathogenic effects.

One possibility, as discussed in chapter 2, is that CCR6+ Th cells express specific microRNAs (miRNAs), which are involved in post-transcriptional regulation of gene expression, or long non-coding RNAs (lnc-RNAs), which are involved in regulation of gene transcription, post-transcription regulation and epigenetic regulation. Unraveling specific production of lncRNAs and miRNAs in CCR6+ Th subpopulations might help to unravel the role of non-coding RNA molecules in pathogenicity of RA and might lead to potential new treatment targets.

We found that mainly Th17.1 and DN cells are efficient in efflux of Rh123 and calcein. Little research has been performed to drug resistance in CCR6+ Th cells. Recently, particular Th17.1 cells, but not Th17 cells, were found to express MDR1 and to be resistant to glucocorticoid suppression. These cells were enriched in the affected gut tissue of patients with Crohn's disease (34). The pathogenic signature and drug-resistance of Th17.1 and DN CCR6+ Th cells suggest the clinical importance of these cells in RA. It would be very interesting to investigate whether these cells are enriched in the synovial fluid of RA patients, and if so, whether the presence of these cells in the synovium correlates to drug resistance.

Autoantibody positive RA

About 70% of patients with RA are ACPA+, and the presence of ACPAs is highly specific for RA [56-58]. ACPA+ patients have a worse disease course than ACPA- patients, as shown, for example, from more development of erosions; and treatment outcomes differ between these groups [59-68]. The striking differences in ACPA+ and ACPA- disease might even mean that they should be treated as different disease subsets [69]. Th cells are implicated in the development of ACPAs [70, 71], which prompted us to investigate if there are differences in Th cell populations in ACPA+ and ACPA- patients. In chapter 6 we found that ACPA+ patients had significantly higher proportions of Th22, Th17.1 and DP cells. Importantly, both Th17.1 and DP cells were found in chapter 5 to be highly pathogenic, as shown by their ability to induce pro-inflammatory mediator and tissue-destructive MMPs production by RASF. Additionally, Th17.1 cells showed an increased potential for multidrug resistance. Interestingly, CCR6+ Th cells are inverse correlated with disease duration in ACPA- patients but not in ACPA+ patients. This suggests that CCR6+ Th cells are involved in the maintenance of inflammation in ACPA+ RA. If (part of) these CCR6+ Th cells are very drug resistant, this could partly underlie the differences in treatment outcome observed between ACPA+ and ACPA- RA.

Presence of HLA-DRB1 SE is associated with ACPA positivity [70]. Furthermore, PTPN22 polymorphisms are associated with RA, and carriers of the PTPN22 risk allele had higher ACPA levels before onset of RA [72]. Interestingly, in a humanized mouse model, citrullination of proteins increased the affinity of the peptide bond to the SE, leading to T helper cell activation [73]. IL-17A+ Th cells, but not IFNγ+ Th cell numbers of patients with RA were increased after culture with citrullinated peptides. In addition, the level of IL-17A production correlated strongly with the level of proliferation in response to citrullinated peptides [74]. Putting these findings together, the following mechanistic model for citrullination and risk genes, based on the model by Klareskog can be proposed [69]: proteins are citrullinated, for example because of smoking or prior infection with *p.gingivalis* [69, 75]. The citrullinated proteins are presented by antigen presenting cells, which, because of the presence of the SE, bind the antigens with high affinity, leading to a higher density of HLA-peptide complexes on the membrane. The higher density of these complexes surpasses the threshold for Th cell activation, and mainly CCR6+ Th cells are activated [73, 74]. Activated CCR6+ Th cells will give help to B cells, causing production of ACPAs. In the case of persons carrying the risk variant for PTPN22, autoreactive B and T cells are present [76, 77], which are possible already citrullinated peptide-specific, and are therefore easily activated in the presence of citrullinated protein.

Future research should clarify the T cell receptor specificity of CCR6+ Th cells. It is of vital importance to determine which proteins activate the CCR6+ Th cells, and the location where this happens, in order to understand the chronic joint inflammation in RA.

Next to ACPAs and RF, new autoantibodies have recently been discovered in the serum of RA patients. Carbamylation is a post-translational protein modification that can be targeted by autoantibodies named anti-CarP antibodies [78]. Similar to ACPAs, these autoantibodies are present before disease onset [79] and predict a disease course with more development of erosions [78]. Furthermore, patients with RA have autoantibodies against proteins with malondialdehyde-acetaldehyde (MAA) adducts, which is another post-translational modification [80]. ACPAs, anti-CarP antibodies and anti-MAA antibodies all arise as a result of a post-translational modification of the protein. However, these modifications are generated by different and unrelated mechanisms, indicating that perhaps in RA there is an underlying, more general problem with post-translational modification [81]. Darrah and Andrade suggest in their editorial that the neutrophil, a cell that is abundantly present in the (early) RA joint, and is capable of all three modifications, might play a currently underappreciated role in the pathogenesis of this disease. In this context, neutrophils that form neutrophil extracellular traps (a chromatin meshwork containing immunostimulatory proteins including cytokines) contain citrullinated proteins, which might lead to formation of antibodies against these proteins [82]. The question remains how these antibodies/and or neutrophils can be present for a long time without immediately causing disease.

Concluding remarks

Despite the progress made in understanding RA pathology and the developments in RA treatment strategies, a large fraction of patients with RA does not respond to, or becomes resistant to current therapies. To improve RA therapy it is of critical importance to unravel the underlying mechanisms in RA pathology and therapy resistance. In this thesis, the pathogenic role and modulation of CCR6+ Th cells in RA was explored. These investigations revealed that all CCR6+ Th cells, except Th22 cells, are potent activators of RASF and therefore possess pathogenic potential in RA. However, CCR6+ Th subpopulations differ in their Th1/Th17-related characteristics and are able to produce various pro-inflammatory cytokines. Neutralizing a single cytokine was insufficient to completely inhibit the pathogenic action of CCR6+ Th cells. Therefore, treatment approaches directed against CCR6+ Th cells in RA should aim at combined inhibition of pro-inflammatory mediators. Alternatively, novel therapeutics can be developed that affect the development or function of all CCR6+ Th populations, such as inhibition of RORγt. Development of such therapeutics might be particularly relevant for RA patients with ACPA positivity, because these patients have higher proportions of CCR6+ Th cells than ACPA- patients. Furthermore, lymphocytes (including CCR6+ Th subpopulations) of RA patients express multidrug transporters and show potential drug efflux capacity. The expression of multidrug transporters and the efflux capacity of lymphocytes was associated with DMARD and glucocorticoid response in patients with RA. These findings underline the potential of using multidrug transporter expression and efflux capacity in the prediction and monitoring of treatment responses in RA. Future research should focus on implementing these findings in personalized RA treatment strategies.

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Chapter 9

Addendum

Summary

In the last decades, great progress was made in understanding rheumatoid arthritis (RA) pathology and in the development of therapeutics. This together led to a significant reduction of disability and improved quality of life of patients with RA. Despite this progress, some patients with RA do not respond to treatment and some become therapy resistant in time. To improve current RA treatment strategies, it will be essential to unravel the mechanisms underlying RA pathology and therapy resistance.

T cells, and in particular Th17 cells and their cytokines, are implicated in the pathogenesis of RA. Next to the production of IL-17A, Th17 cells are characterized by the expression of the chemokine receptor CCR6. However, the pathogenic mechanisms underlying the action of CCR6+ Th cells are not completely understood. In addition, the CCR6+ Th cell population is comprised of various subpopulations. It is currently unclear whether all these subpopulations can be identified in patients with RA and which contribute to RA pathology. Moreover, it is unclear how these cells respond to current treatment strategies in RA, and if their presence differs between different subsets of RA, such as auto-antibody positive and negative RA.

In **chapter 3**, we examined the mechanism underlying autocrine IL-17A production by CCR6+ Th cells upon their interaction with RA derived synovial fibroblasts (RASF). Cyclooxygenase (COX)-2 and its product prostaglandin-E_2 (PGE_2) were identified as critical regulators of IL-17A production in RASF/CCR6+ Th cell cultures. In addition, COX-2 inhibition reduced expression of pro-inflammatory mediators such as IL-6 and IL-8 and matrix-destructive metalloproteinases (MMPs). Interestingly, COX-2 inhibition combined with TNFα inhibition had additional suppressive effects on these factors in comparison to TNFα only inhibition. This indicates that in the treatment of patients with RA combining TNFα inhibition with COX-2 or IL-17A inhibition may have additional value to anti-TNFα alone.

Next to IL-17A, CCR6+ Th cells produce IL-22. CCR6+ Th cells which produce IL-22 but no or low levels of IL-17A are classified as Th22 cells. The role of Th22 cells and IL-22 in RA is controversial. In **chapter 4**, both Th17 and Th22 cells in synovial fluid of RA patients were identified. Peripheral and synovial Th22 cells were less efficient than Th17 cells in the induction of pro-inflammatory cytokines and MMPs by RASF. Moreover, blocking IL-22, in contrary to blocking IL-17A signaling, in RASF/CCR6+ Th cell co-cultures did not reduce IL-6, IL-8 and MMPs production. In antigen-induced arthritis (AIA), IL-22 deficient and wild-type mice had similar arthritis severity, including parameters of synovial inflammation. These findings show that Th17/IL-17A mediated synovial inflammation is IL-22/Th22 independent.

Next to Th17 and Th22 cells, several other subpopulations are present in CCR6+ Th cells, based on CXCR3 and CCR4 expression. In **chapter 5**, Th17, Th17.1, CXCR3/CCR4 double positive (DP), and CXCR3/CCR4 double negative (DN) CCR6+ populations in peripheral blood and synovial fluid of RA patients were identified. The production of IL-17A, IL-17F and interferon (IFN)γ varied extensively within the CCR6+ Th subpopulations. Despite this, all of the subpopulations were very effective in inducing the production of IL-6, IL-8 and MMPs by RASF compared to Th1 cells. Lymphocytes can express transport pumps on the cell membrane that actively transport drugs out of the cell and that are associated with drug resistance. All of the subpopulations expressed transporters, but the Th17.1 and DN cells were

most effective in efflux. These CCR6+ Th subpopulations are present in peripheral blood and inflamed synovium of RA patients, and might be particularly pathogenic and drug resistant.

RA patients positive for serum anti-citrullinated protein antibodies (ACPAs) have a worse disease course than ACPA- patients. Th cells are implicated in ACPA production by B cells. In **chapter 6**, we found that ACPA+ early RA patients had higher proportions of CCR6+ Th cells in peripheral blood in comparison with ACPA- patients. This increase was also observed for the CCR6+ Th subpopulations Th22, Th17.1 and DP cells. Furthermore, CCR6+ Th cells were inversely correlated with disease duration in ACPA- patients but not in ACPA+ patients. These findings implicate that CCR6+ Th cells might be involved in the worse disease outcome observed in ACPA+ RA.

In chapter 5 we found that response to therapy is associated with the expression of multidrug transporters on the cell membrane of CCR6+ Th cells. In **chapter 7**, we found that at baseline, therapy responders had higher proportions of multidrug resistance protein (MDR)1 expressing naïve CD8 T cells than non-responders. After 6 months of treatment, increased multidrug resistance-associated protein (MRP)1 expression was observed in both patient groups. However, as a result of therapy, MRP1 efflux activity in lymphocytes was reduced in therapy responders, but not in therapy non-responders. These results indicate that MDR1 and MRP1 expression and activity on peripheral lymphocytes can be used to predict and monitor treatment response.

The data presented in this thesis shed light on the pathogenic role and modulation of CCR6+ Th cells in RA. Future research should focus on the implementation of these findings in CCR6+ Th targeted therapies and in personalized RA treatment strategies.

Samenvatting

Gedurende de afgelopen jaren is er veel vooruitgang geboekt in het begrijpen van de pathologie van reumatoïde artritis (RA) en in het ontwikkelen van therapieën. Voor RA patiënten heeft dit geleid tot een aanzienlijke reductie van lichamelijke beperkingen en een verbeterde kwaliteit van leven. Desondanks reageren niet alle RA patiënten goed op therapie, en sommigen worden resistent na verloop van tijd. Om de huidige RA behandelingsstrategieën te verbeteren is het essentieel om de mechanismen die ten grondslag liggen aan RA pathologie en therapieresistentie te ontrafelen.

Van T cellen, en voornamelijk van Th17 cellen en hun cytokines, wordt gedacht dat ze een rol spelen in de pathogenese van RA. Th17 cellen worden gekarakteriseerd door de productie van IL-17A en door het tot expressie brengen van CCR6. Het wordt echter nog niet goed begrepen welke mechanismen ten grondslag liggen aan de pathogene effecten van CCR6+ Th cellen. Verder vormen CCR6+ Th cellen een zeer heterogene populatie die bestaat uit verschillende subpopulaties. Het is onduidelijk of al deze subpopulaties voorkomen in patiënten met RA, en welke van de subpopulaties bijdragen aan de pathologie van RA. Daarnaast is het onbekend hoe deze subpopulaties reageren op RA medicatie, en of hun aanwezigheid verschilt tussen verschillende vormen van RA, zoals autoantilichaam positieve en negatieve RA.

In **hoofdstuk 3** hebben we het mechanisme bestudeerd dat verantwoordelijk is voor autocriene IL-17A productie door CCR6+ Th cellen wanneer zij in contact worden gebracht met synoviale fibroblasten van RA patiënten (RASF). COX-2 en het product van dit enzym PGE_2 werden geïdentificeerd als kritieke factoren in het reguleren van IL-17A productie in CCR6+ Th cel/RASF kweken. Het remmen van COX-2 activiteit leidde ook tot een lagere productie van ontstekingsbevorderende producten zoals IL-6 en IL-8 en matrix-destructieve producten zoals metalloproteinases (MMPs). Gecombineerde COX-2 en TNFα remming leidde tot hogere suppressie van deze producten dan het remmen van TNFα alleen. Dit geeft aan dat er additionele waarde ligt voor de behandeling van RA patiënten in het gecombineerd remmen van TNFα en COX-2 of IL-17A vergeleken met het remmen van TNFα alleen.

CCR6+ Th cellen produceren naast IL-17A ook IL-22. CCR6+ Th cellen die hoge hoeveelheden IL-22 maar geen of weinig IL-17A produceren worden Th22 cellen genoemd. De rol van Th22 en IL-22 in RA is controversieel. In **hoofdstuk 4** hebben we zowel Th17 als Th22 cellen gevonden in de synoviale vloeistof van RA patiënten. Echter, synoviale en perifere Th22 cellen waren minder efficiënt dan Th17 cellen in het induceren van productie van ontstekingsbevorderende cytokines en MMPs door RASF. Het blokken van IL-22, in tegenstelling tot het blokken van IL-17A signalering, leidde niet tot een reductie van IL-6, IL-8 en MMP productie in CCR6+ Th cel/RASF kweken. De ernst van artritis, inclusief parameters van synoviale ontsteking, waren vergelijkbaar in IL-22 deficiënte en normale muizen in antigeen-geïnduceerde artritis (AIA). Deze bevindingen tonen aan dat Th17/IL-17A gemedieerde synoviale ontsteking onafhankelijk is van Th22/IL-22.

Naast Th17 en Th22 cellen zijn verschillende andere subpopulaties aanwezig in de CCR6+ Th cellen op basis van CXCR3 en CCR4 expressie. In **hoofdstuk 5** werden Th17, Th17.1, CXCR3/CCR4 dubbel positieve (DP) en CXCR3/CCR4 dubbel negatieve (DN) CCR6+ subpopulaties gevonden in perifeer bloed

en synoviale vloeistof van RA patiënten. De productie van IL-17A, IL-17F en IFNγ varieerde sterk tussen de subpopulaties. Desondanks waren alle subpopulaties erg effectief in het induceren van IL-6, IL-8 en MMPs productie vergeleken met Th1 cellen. Lymfocyten kunnen transporteiwitten, die actief medicijnen uit de cel pompen, tot expressie brengen op het celmembraan. Deze 'multidrug transporters' zijn geassocieerd met niet reageren op therapie (non-respons). Alle subpopulaties brachten multidrug transporters tot expressie, maar de Th17.1 en DN cellen waren het meest effectief in efflux. Deze CCR6+ Th subpopulaties zijn aanwezig in perifeer bloed en ontstoken synovium van RA patiënten, en lijken bijzonder pathogeen en therapie resistent te zijn.

RA patiënten die positief testen voor de aanwezigheid van antilichamen gericht tegen gecitrullineerde eiwitten (ACPAs) hebben een ernstiger ziekteverloop dan ACPA- patiënten. Er zijn aanwijzingen dat Th cellen betrokken zijn bij ACPA productie door B cellen. In **hoofdstuk 6** vonden we hogere proporties CCR6+ Th cellen in perifeer bloed van vroege, onbehandelde ACPA+ dan in ACPA- RA patiënten. Deze toename werd geobserveerd voor de subpopulaties Th22, Th17.1 en DP cellen. De proportie CCR6+ Th cellen was omgekeerd gecorreleerd met ziekteduur in ACPA-, maar niet in ACPA+ patiënten. Dit geeft aan dat CCR6+ Th cellen betrokken kunnen zijn bij het ernstiger ziekteverloop in ACPA+ patiënten.

In hoofdstuk 5 vonden we dat de respons op therapie geassocieerd is met het tot expressie brengen van multidrug transporters op het membraan van CCR6+ Th cellen. In **hoofdstuk 7** hebben we hogere proporties naïeve CD8+ T cellen die multidrug resistentie eiwit (MDR)1 tot expressie brengen voor aanvang van de therapie gevonden in responders dan in non-responders. Na 6 maanden therapie werd hogere expressie van multidrug resistentie-geassocieerd eiwit (MRP)1 geobserveerd in beide patiëntgroepen. De MRP1 efflux activiteit was echter gereduceerd in responders maar niet in non-responders. Deze bevindingen geven aan dat MDR1 en MRP1 expressie gebruikt kunnen worden om therapie respons te voorspellen en monitoren.

De resultaten gepresenteerd in dit proefschrift dragen bij aan de kennis over de pathogene rol en modulatie van CCR6+ Th cellen in RA. Toekomstig onderzoek zou zich moeten richten op het implementeren van deze resultaten in op CCR6+ Th cellen gerichte therapie en in gepersonaliseerde RA behandelingsstrategieën.

Dankwoord

Het zit erop, mijn proefschrift is af! Vier jaar zwoegen samengevat in 184 bladzijden. Maar natuurlijk is er veel meer gebeurd in die vier jaar dan de resultaten in dit boekje, en dat is vooral alles wat ik heb meegemaakt met de mensen die ik tijdens mijn promotie heb ontmoet, en met wie ik heb mogen samenwerken. Zij zijn overal tussen de regels door aanwezig, en deze mensen wil ik graag bedanken.

Allereerst natuurlijk mijn promotor, professor Hazes. Mieke, bedankt dat je me de kans en het vertrouwen hebt gegeven om op onze afdeling te promoveren. Jij zoekt altijd de meerwaarde voor de reumapatiënt in ons basale onderzoek, ik denk dat dit de kwaliteit en de relevantie van ons onderzoek zeer ten goede komt.

Dan wil ik natuurlijk ook graag mijn co-promotor bedanken. Erik, ook jou wil ik graag bedanken dat je me de kans en het vertrouwen hebt gegeven om in het reumalab te promoveren. Ik heb ontzettend veel geleerd in de afgelopen vier jaar. Ik ben er vanaf het begin duidelijk over geweest dat mijn toekomst niet in het onderzoek ligt, en ik waardeer het echt dat je mij toch hebt aangenomen, en me ook de kans en de tijd hebt gegeven om mezelf op andere vlakken te ontwikkelen. Jij gaf me altijd de tijd om cursussen te volgen en carrièredagen en congressen te bezoeken. Mede hierdoor heeft promoveren in ons lab erg gunstig uitgepakt voor mij!

Ook wil ik graag professor Hendriks, professor Katsikis and professor Taams, de kleine commissie, bedanken voor het kritisch lezen van mijn manuscript. Professor Baeten, professor Baan en Dr. Jansen, bedankt dat jullie toegezegd hebben zitting nemen in de grote commissie.

Ik heb het geluk gehad om in een lab te promoveren met superleuke collega's! Nadine, Nadientje, wat had ik zonder jou gemoeten? Het was in ieder geval veel minder leuk geweest. Jij hebt een ongelofelijke dosis geduld en begrip, en dan ook nog eens een flinke scheut optimisme en vrolijkheid! Ik zeg het niet vaak genoeg, maar enorm bedankt daarvoor. En daarnaast natuurlijk ook bedankt voor de gigantische inzet en hulp! Er zijn maar weinig proeven in dit manuscript waar je niet bij betrokken was. Afscheid nemen van ons dreamteam is een van de grootste nadelen van gepromoveerd zijn. Ik ga je missen, maar niet al te veel want ik kom gewoon langs;-). Uiteraard ben jij een van mijn paranimfen, en dat vind ik super!

Wendy, toen jij kwam zat ik er pas een paar maanden, en wat was ik blij dat je kwam! Ik had bedacht dat het heel leuk zou zijn, omdat jij ook nieuw was en dat we dan samen alles zouden ontdekken. En dat was ook zo! Wij zijn samen naar een hoop cursussen en congressen gegaan, en in sommige gevallen (denk aan bijv. de statistiekcursus) was jouw aanwezigheid het enige dat het draaglijk maakte. Toppunten waren de cursus waarbij we er bijna uitgezet zijn wegens kletsen (jouw schuld, maar dat gelooft dus niemand), en de girls only EWRR in Praag. Bedankt dat je niet op mijn gezicht geschreven hebt toen je de kans had. En ook bedankt voor alle gezelligheid en het verleden en de toekomst, want we blijven afspreken natuurlijk! Superleuk dat je mijn paranimf gaat zijn samen met Nadientje!

En dan natuurlijk alle huidige en ex-collega's van het reumalab! Allereerst mijn kamergenoten. Odilia, Odiel, toen ik net begon keek ik enorm naar je op (en terecht hoor, dat doe ik nog steeds wel een beetje), maar gelukkig kwam ik er ook al snel achter dat je ook heel gezellig bent. Jij was niet alleen heel

lang mijn kamergenootje, maar vooral toch ook mijn partner in crime in de Boudewijn. Ik vond het zo erg dat je vertrok bij ons, maar ben heel blij dat we elkaar nog zo vaak zien! Bedankt voor alle Boudewijn-sessies, whiskyclubjes, avondjes in Delft en etentjes. Dat er nog vele mogen volgen! Henrique, you are the nicest Portuguese guy I know, and I'm not just saying that because you're the only one. No seriously, I think you're a very dedicated scientist, and also a very cool person to share an office with. We've had fun! Wida, wat was het gezellig om naast jou te zitten! Met jou kon ik het altijd over typische meidendingen hebben, met als enorme bonus Henriques gezicht als we elkaar weer eens waren aan het complimenten met onze fantastische kledingsmaak. Je bent super en ik mis jou ook!

Alle andere collega's van het reumalab, Anne-Marie, Patrick, Marlieke, Inge en Eddy, ook jullie wil ik graag bedanken voor alle gezelligheid. Anne-Marie en Patrick, bedankt voor al jullie hulp in het lab en het regelen van kleine en grote zaken, en natuurlijk ook voor de gezelligheid de laatste vier jaar.

Verder wil ik ook graag alle collega's van de afdeling reumatologie bedanken. We hebben een erg leuke afdeling en er zijn veel gezellige uitjes geweest in de afgelopen vier jaar. Bedankt Radboud, Philomine, Marijn, Sylvia, Edgar, Angelique, Jolanda, Joyce, Ron, Martijn, Pascal, David, Florentien, Myrthe, Annelieke, Maren, Lonneke, Hilal, Jenny, Albert, Anne-Marie de Vroed, Margot, Hetty en Anke, voor alle gezelligheid en goede gesprekken. Joyce, als 'afdelingsmama' speel jij een belangrijke rol in de sociale aspecten van de afdeling, en dat doe je heel goed. Bedankt daarvoor! Albert, bedankt voor de gezelligheid (vooral tijdens congressen), je droge humor, en de leuke gesprekken. Altijd leuk als je langskomt! Pascal, bedankt voor de prettige samenwerking, die nog tot een mooie publicatie heeft geleid. Voor mij is wat jij met de database en statistiek doet pure magie!

Het reumalab hoort ook deels bij de afdeling immunologie, waar ik niet alleen voor de wetenschap maar ook voor de gezelligheid regelmatig was te vinden. Bedankt alle collega's van de afdeling immunologie, met name Naomi, Alice, Prisca, Zana, Chris, Matti, Karin, Jeroen, Wouter, Willem-Jan, Christina, Ann, Wouter, Marjan, Annet en René. Ook met jullie was het altijd erg gezellig.

Ik heb met een aantal fantastische mensen samengewerkt tijdens mijn promotie, wat al tot een mooie co-publicatie heeft geleid en hopelijk snel nog meer co-publicaties gaat opleveren. Ik vond het erg prettig om met jullie samen te werken, Caroline, Naomi en Zana.

Tijdens mijn promotie heb ik een fantastische student mogen begeleiden, Heleen. Begeleiden moet je in dit geval met een korreltje zout nemen want Heleen wist eigenlijk alles al en heeft me vooral heel veel geholpen. Heleen, de periode dat jij, Nadine en ik zo veel proeven gedaan hebben met z'n drieën was de leukste periode van mijn promotie, en dat komt zeker voor een groot deel door jouw enthousiasme, vrolijkheid en inzet. Ik vond het superleuk met jou te werken, en ik ben er heel blij mee dat je een promotieplek hebt gevonden binnen het Erasmus. Gaan we snel weer koffie drinken?

Layal, you came to our lab three years a row, each time a bit longer, to learn about doing science in a lab. You are incredibly ambitious and smart, and also a lot of fun to have around. I loved showing you around and I'm very happy to hear that you've found a very good promotion place in such a short time. Catch up when you're in the Netherlands again?

Hoyan, je was niet mijn student maar ik vond het wel altijd erg leuk met jou. Top dat je nog steeds langs komt om koffie te drinken!

Frankapanka!!!!!! Ik ken weinig mensen die zo enthousiast/vrolijk /melig/ongeremd/prettig gestoord zijn als jij. Zelfs op m'n zuurste dagen weet jij me nog vrolijk te maken. Jij versterkt mijn beste en mijn slechtste eigenschappen (dezelfde dus) en maakt van mij ook een stuiterbal. Wat hebben wij arme Odiel aangedaan met z'n tweeën. Never change, maar laten we toch maar nooit meer een kantoor delen, dat komt niet goed;-). Maar gelukkig zijn we niet Bram M……

En dan natuurlijk Brem, Maarten. Jij begon tegelijkertijd met mij (of een week later?) als student, en bent gelukkig altijd in de buurt gebleven. Met jou klikte het vanaf dag 1 en volgens mij was het al in onze eerste maand dat we na de Dinch nog 'even een drankje gingen doen' met collega's en we allebei de dag erna met een kater op het werk verschenen (ik ben het helemaal met je eens dat het Jan Piets schuld was). De toon was goed gezet, en alhoewel we daarna nooit meer zo ver zijn gegaan (gelukkig) zijn er wel vele mooie avonden in de Boudewijn op gevolgd. Wij blijven zeker vrienden, en er gaan nog vele Boudewijn-sessies en kopjes koffie volgen!

Naast de collega's zijn er ook andere mensen die ik graag wil bedanken voor alle steun de laatste paar jaar. Mijn fantastische vrienden, die er altijd begrip voor hebben dat ik wat minder tijd heb, en niet altijd even vrolijk was, vooral Eveline, Glenn en Esmee. Ook mijn schoonfamilie heeft mij veel gesteund en afgeleid van de promotiestress de laatste jaren. Verder heb ik aan beide kanten een geweldige familie, die ik hier niet allemaal apart ga noemen, maar iedereen: ik vind het super dat we allemaal zo'n goede band hebben, en weinig dingen vrolijken me zo op als iets met de familie doen. Het familieweekend was fantastisch, en ik verheug me alweer op het volgende kerstfeest. Ik vind het geweldig dat jullie er allemaal bij zijn bij mijn verdediging.

Mam en pap, Bart en Ruud, jullie hebben me gesteund in alle keuzes die ik gemaakt heb, en zijn altijd zo trots op mij. Ik kan me geen betere ouders en broertjes wensen. Thuiskomen bij jullie is altijd een feest!

Dan als allerlaatste mijn lieve Jan Piet. Jij hebt zo ongelofelijk veel gedaan aan dit boekje, zonder jou zou het er niet hetzelfde uit zien, als het überhaupt al af was nu. Zowel op het werk als thuis ben je er altijd voor mij, en de afgelopen vier jaar hebben soms veel van je gevraagd. Toch vond ik vond het super om met je samen te werken, en zou ik het eigenlijk niet anders gewild hebben. Daarnaast hebben we gelukkig ook een privéleven, en wij hebben het zo leuk en zo goed samen! Met jou op vakantie gaan is fantastisch, of we nu naar Duitsland of naar Bali gaan, met jou is het altijd super. Wat ik het leukst vind aan 'ons', is dat we na een hele dag in het lab gezeten te hebben met elkaar, we met z'n tweeën naar de Ierse pub of de Boudewijn kunnen gaan en dan nóg de hele avond dingen aan elkaar te vertellen hebben. Ik zou me geen leuker vriendje kunnen indenken. Daarom ben jij de nummer 1 reden dat ik dolblij ben dat ik bij het reumalab ben gaan promoveren!

Curriculum vitae

Sandra Paulissen was born on September 25th 1986 in Geleen. She finished her secondary education at Groenewald in Stein, where she graduated *cum laude* in 2004. She then studied Nutrition and Health at the Wageningen University and Research, where she obtained her bachelor's degree in 2008 and her master's degree in 2010. During her studies, she specialized in molecular nutrition, and graduated on the research project "The effect of cannabinoids on nitric oxide production and regulation during inflammation in macrophages". She followed additional internships at Kinesis Pharma in Breda (department nutraceuticals) and Maastricht University (department surgery).

In 2011, she started the work described in this thesis under the supervision of Dr. E. Lubberts and prof. Dr. J.M.W. Hazes. In 2013, she worked two months at the UCSD (University of California, San Diego) in the lab of Prof. Dr. Cornelis Murre to learn novel deep sequencing technology.

In august 2015, she started working as scientific advisor immunoscience at Bristol-Myers Squibb.

Portfolio

Name PhD student: Sandra Maria Johanna Paulissen
Erasmus MC Department: Rheumatology Research School: Molecular Medicine
PhD period: April 2011 – May 2015
Promotor(s): Prof. Dr. J.M.W. Hazes
Supervisor: Dr. E. Lubberts

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- BROK ('Basiscursus Regelgeving Klinisch Onderzoek'	2011	0
- NIBI course time management for PhDs and Postdocs	2011	1
- Biomedical English Writing and Communication	2012	2
- Biostatistical methods I: basic principles	2012	5.7
- Photoshop and Illustrator CS5 workshop	2013	0.3
- Laboratory animal science	2014	3
Specific courses (e.g. Research school, Medical Training)		
- Course on molecular medicine	2012	0.7
- Molecular Immunology	2012	3
Seminars and workshops		
Presentations		
- NVR annual meeting	2012	1
- NVVI annual meeting	2012	1
(Inter)national conferences		
- NVR annual meeting	2011	1
- NVVI annual meeting	2011	1
- EWRR annual meeting	2012	1
- MolMed day	2012	1
- NVVI Summer School	2012	1
- Dublin Cytokines meeting	2012	1
- Molmed Day	2013	1
- EWRR annual meeting	2013	1
- EULAR annual meeting	2013	1
- Lymphoid Tissue meeting	2013	1
- ACR annual meeting	2013	1
- NVVI annual meeting	2013	1
- EULAR annual meeting	2014	1
- NVR annual meeting	2014	1
- NVVI annual meeting	2014	1
- EWRR annual meeting	2015	1
Other		

2. Teaching

	Year	Workload (Hours/ECTS)
Lecturing		
Supervising practicals and excursions, Tutoring		
- Supervising laboratory rotations students master Infection and Immunity	2012 & 2013	1
Supervising Master's theses		
- Master student Biology of Disease Utrecht University (6 months)	2012	10
Other		

Publications

This thesis

IL-17/Th17 mediated synovial inflammation is IL-22 independent.
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Arthritis Research and Therapy, in press.

Multidrug resistant lymphocytes of patients with rheumatoid arthritis are predictive for DMARD and glucocorticoid treatment response.
Sandra M.J. Paulissen*, Jan Piet van Hamburg, Nadine Davelaar, Johanna M.W. Hazes and Erik Lubberts.*
Manuscript in preparation.

IL-17A-low CCR6+ Th cell populations of patients with rheumatoid arthritis are pathogenic, multidrug resistant and associated with DMARD and glucocorticoid treatment response.
Sandra M.J. Paulissen*, Jan Piet van Hamburg, Nadine Davelaar, Johanna M.W. Hazes and Erik Lubberts.*
Manuscript in preparation.

Other

T-helper 17 cell cytokines and interferon type I: partners in crime in systemic lupus erythematosus?
Zana Brkic, Odilia B.J. Corneth, Cornelia G. van Helden-Meeuwsen, Radboud J.E.M. Dolhain, Naomi I. Maria, ***Sandra M.J. Paulissen****, Nadine Davelaar, Jan Piet van Hamburg, Paul L. van Daele, Virgil A. Dalm, P. Martin van Hagen, Johanna M.W. Hazes, Marjan A. Versnel and Erik Lubberts.*
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Manuscript submitted.

Specific induction of CCR6^{+} Th17.1, and not CCR6^{-} Th1 cells, in sarcoidosis.
Caroline E. Broos, Menno van Nimwegen, Johannes C.C.M. in 't Veen, ***Sandra M.J. Paulissen****, Jan Piet van Hamburg, Jouke T. Annema, Roxane Heller-Baan, Henk C. Hoogsteden, Rudi W. Hendriks, Bernt van den Blink and Mirjam Kool*
Manuscript submitted.