THE IL-23/IL-17 IMMUNE PATHWAY IN ARTHRITIS

Ferry Cornelissen

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De IL-23/IL-17 immuun route in artritis

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I

The IL-12/IL-23 axis and its role in Th17 cell development, pathology and plasticity in arthritis.

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ABSTRACT

Rheumatoid arthritis (RA) was originally thought to be a T-helper (Th)1- but not a Th2-associated disorder; however, it currently is unclear whether RA is a Th1- and/or Th17-mediated disease, and what the contributions of these T-cell subsets are in the pathogenesis of RA. Results from studies using different arthritis models have demonstrated that IL-17-producing T-cells are the dominant cell type in the development of arthritis. In addition, a critical role of the IL-23/IL-17 axis in the progression to chronic destructive arthritis has been demonstrated. Interestingly, Th1 and Th17 cells both may have unique pathogenic potential, and the recent insights into T-cell plasticity may change the understanding of the role of T-cell subsets in chronic autoimmune diseases.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the joint synovium by activated inflammatory leukocytes (such as T-helper [Th] lymphocytes and monocytes), synovial hyperplasia, neo-angiogenesis, and progressive destruction of cartilage and bone. In the Western world, the prevalence of RA is approximately 1% (1). The primary cause is unknown, but multiple factors seem to be involved, including genetic and environmental factors. The identification and diagnosis of RA early in the disease course is becoming increasingly important because early and intensive treatment has been demonstrated to prevent joint damage, to preserve joint function, and to improve work participation of the patient (2-5). The prognosis of patients with RA has improved considerably as a result of intensified treatment regimens and the availability of new drugs. Common treatments include general painkillers and anti-inflammatory drugs, but to prevent long-term damage, disease-modifying anti-rheumatic drugs (DMARDs) are often used. Biologicals also have become available for the treatment of RA, of which anti-TNFα therapy (eg, etanercept, infliximab and adalimumab) has been best studied. Other cytokine-targeting drugs include anakinra (anti-IL-1) and tocilizumab (anti-IL-6). Additionally, rituximab (anti-CD20) is used to target B-cells and abatacept can be used to block the T-cell costimulation receptor CD28 in RA (6). However, the efficacy of these strategies varies, both among patients and during the disease course in individual patients. Both traditional DMARDs and biologicals can have severe side effects, which underscore the need for effective, but also safer therapeutic strategies. The immunological mechanisms that are integral to the development of RA have not been fully established, and it will be necessary to understand these mechanisms in order to identify novel and safer therapeutic strategies.

RA was originally thought to be a Th1-, not Th2-, associated disorder (7). With the

discovery of the IL-23/Th17 pathway, diseases earlier classified as IL-12/Th1-mediated diseases, for example multiple sclerosis and RA, need to be reconsidered. In fact, a pivotal role for the IL-23/Th17 pathway in the development of most experimental autoimmune inflammatory diseases, such as collagen-induced arthritis (CIA) (8), experimental autoimmune encephalomyelitis (EAE) (9-10) and uveitis (EAU) (11), has been demonstrated.

Targeting IL-23 and IL-17 in human autoimmune diseases

The treatment of inflammatory diseases using anti-IL-12/23p40 therapy has been tested using ustekinumab (Stelara®, CNTO-1275, Centocor Inc.), briakinumab (ABT-874, Abbott Laboratories) and apilimod (STA-5326; Synta Pharmaceuticals Corp). Ustekinumab has been successfully tested in clinical trials for the treatment of (moderate-to-severe) plaque psoriasis (12-14), psoriatic arthritis (PsA) (15) and Crohn's disease (16), but not for multiple sclerosis, although the drug was well tolerated (17). Interestingly, it has recently been demonstrated that ustekinumab showed higher efficacy than that of high-dose etanercept (18). In addition, briakinumab was demonstrated to be effective in a phase II trial of patients with chronic plaque psoriasis (19). Apilimod has been tested in Crohn's disease but results from a phase I/IIa trial (20) and a phase II trial (21) were conflicting. A phase II trial of apilimod in patients with RA is currently underway (ClinicalTrials.gov identifier: NCT00642629).

Phase II anti-IL-17A therapies have been performed by Novartis AG (AIN-457) in patients with psoriasis, RA and non-infectious uveitis and showed that blocking IL-17A is clinically effective in these diseases (22). AIN-457 is now in phase III study for non-infectious uveitis (NCT01090310 and NCT01032915), and in phase II trials for Crohn's disease (NCT00584740 and NCT00936585), psoriasis (NCT00770965, NCT01071252 and NCT00941031), PsA (NCT01169844), ankylosing spondylitis (NCT00770965 and NCT01109940) and, finally, in a phase II study with RA patients taking methrotrexate (NCT00928512). Additionally, Eli Lilly and company have also tested anti-IL-17A (LY2439821) in RA in an phase I study and showed that the drug was well tolerated and significantly decreased DAS28 and ACR criteria for RA parameters (23) and is now being evaluated in phase II trials in RA (NCT00966875) and psoriasis (NCT01107457). The IL-23/Th17 pathway and its potential role in RA are discussed in this review.

IL-12 and IL-23 in autoimmune diseases

IL-12 is a heterodimeric cytokine composed of a p40 and a p35 subunit (24-25). However, the p40 subunit is not unique to IL-12; p40, together with a p19 subunit, forms the heterodimeric cytokine IL-23 (26). IL-12 binds to the IL-12 receptor, which is composed

of the heterodimeric chains IL-12Rβ1 and IL-12Rβ2 (27-28). Together with the IL-12Rβ1 receptor chain, the IL-23 receptor (IL-23R) chain forms a functional receptor for IL-23 (29).

It has been demonstrated that the IL-23R gene is associated with immune-mediated diseases, such as inflammatory bowel disease (IBD) (30), psoriasis (31), PsA (32-33), ankylosing spondylitis (34) and RA (35). IL-23R is expressed on T-cells, NK-cells, monocytes and dendritic cells (26, 29), and corresponds with the ability of these cells to respond to IL-23. However, IL-23R is not expressed on precursor T-cells, suggesting that IL-23 signaling is not involved in the primary differentiation of naïve T-cells (36). The signaling of IL-12 and IL-23 leads to the activation of both overlapping and divergent signal transduction pathways and pathological roles in experimental arthritis. Signal transduction by IL-12 and IL-23 is mediated by JAK and STAT proteins (29). Upon binding of IL-12 or IL-23 to their receptor, autophosphorylation and trans-phosphorylation of receptor-associated JAK proteins is initiated. JAK proteins then phosphorylate tyrosines located in the intracellular domain of the receptor subunits. These phosphorylated tyrosines serve as docking sites for STAT proteins and potentially other signaling molecules. While STAT1 and STAT4 are important for Th1 differentiation and function, STAT3 appears to have a major role in IL-23 signaling, as several IL-23-induced DNA-binding complexes contain this factor (29). In addition, STAT4 is required for the development of IL-23-primed IL-17 secreting cells, but not TGFβ1 plus IL-6-primed cells, and for the production of IL-17 in response to IL-23 plus IL-18 (37).

IL-23 is elevated in many autoimmune diseases, such as psoriasis, RA, multiple sclerosis (MS) and IBD (38-39). IL-23 transgenic mice develop systemic inflammation, including inflammation of the small and large intestine (40), highlighting the role of this pathway in promoting the activation of effector T-cells and perpetuation of inflammatory tissue responses. At least part of the effect of IL-23 in autoimmune diseases likely is mediated via IL-17-producing T-cells (10, 36, 41-43), and elevated IL-17 levels have been observed in different chronic inflammatory diseases (38), such as RA (44) and IBD (45).

Based on these observations, the modulation of the IL-23 signaling pathway would be a rational therapeutic strategy for chronic inflammatory diseases such as RA, PsA, MS, psoriasis and IBD. It has been postulated that specific targeting of the IL-23/IL-23R pathway may be effective in blocking chronic tissue inflammation, with less compromise of protective responses compared with blocking IL-12/IL-23p40 (46).

The role of IL-12 and IL-23 in experimental arthritis

In early studies, the role of IL-12 in experimental disease models was often investigated using IL-12p40-knockout mice, or neutralizing antibodies against p40. However, it is now understood that such approaches target IL-23 in addition to IL-12. Table 1 summarizes relevant studies in which the role of IL-12 or IL-23 in different experimental arthritis models was investigated. In most studies using the autoimmune CIA model, the effect of neutralizing p40 resulted in a reduction in disease score, independent of the stage of disease at which the treatment was initiated.

The results from these preclinical studies suggest that IL-12 and/or IL-23 have a role at multiple stages of arthritis. However, it is unclear whether IL-12 or IL-23 act alone, or additively or synergistically at different stages of disease. In CIA models, specifically targeting IL-12p35 did not ameliorate the disease (8). Conversely, IL-12 administered near the time of CIA onset exacerbated collagen arthritis (47). Overall, the exact role of IL-12 in CIA is not fully understood, moreover because there are no studies in which IL-12p35 is therapeutically targeted. In contrast to IL-12p35-/- mice, IL-12/23p40-/- and IL-23p19-/- mice did not develop CIA (8), which suggests that IL-23 has a major role in the development of CIA. Using a polyclonal antibody, it was demonstrated that neutralizing IL-23 after CIA onset reduced paw volume but not the arthritis score in rats (48). Additionally, we confirmed these data in DBA/1 mice in which no amelioration of CIA disease severity was observed when blocking IL-23 was initiated after disease onset. However, when IL-23 was neutralized before onset of CIA, disease scores were significantly lower compared to control mice demonstrating IL-23 dependent and independent stages during CIA (**chapter II**). In contrast to CIA in IL-23p19-/- mice, in the non-autoimmune antigen-induced arthritis (AIA) model, IL-23p19-/- mice did develop arthritis; however, disease severity was significantly lower than in wild-type mice and, unlike wild-type mice, these IL-23-deficient mice did not progress to full-blown arthritis. This observation was accompanied by a lower proportion of IL-17-expressing CD4+ T-cells in IL-23p19-deficient than in wild-type mice [(49), **chapter IV**]. Additionally, in two acute arthritis models induced with peptidoglycan or streptococcal cell wall fragments, IL-23p19-/- mice demonstrated a significantly milder joint inflammation compared with wild-type mice (**chapter V**). These data together suggest that IL-23 has an integral role in the development of arthritis affecting both the adaptive and the innate immune system.

Th17 cells and their specific transcription factors and cytokines

In 2000, Infante-Duarte and colleagues suggested that IL-17A-producing CD4+ T-cells might represent a unique Th cell subset that differed from the classical Th1 and Th2 cell

subsets (50). The incubation of naïve mouse T-cells with the lysate of *Borrelia burgdorferi* induced IL-17 production independently of Th1 or Th2 cytokine production, and it was suggested that IL-6 might have a role in the development of the IL-17A-producing Th cells (50). The potential link between IL-23 and the IL-17A-producing T-cells was established in vitro by demonstrating that IL-23 enhanced IL-17 production from memory CD4+ T-cells, but not from naïve CD4+ T-cells (43). This indicates that IL-23 is required for the effector function of Th17 cells, but not for their differentiation. This link was further confirmed by *in vivo* experiments conducted by Cua and colleagues, who demonstrated that IL-23-primed Th17 cells were more pathogenic in EAE and CIA than IL-12-primed Th1 cells (8-10). Based on these studies, Th17 cells have been recognized as a distinct Th cell population that have an integral role in CD4+ T-cell-mediated adaptive immunity.

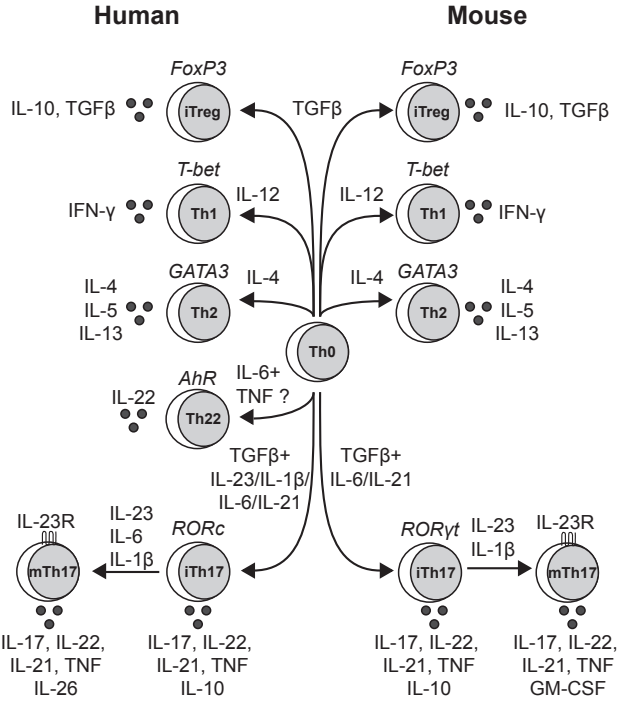

Figure 1. Human and mouse T helper (Th) cell differentiation. Naïve CD4+ T helper cells differentiate towards various Th subsets by means of cytokine-driven polarization. When a T cell receptor binds its cognate peptide-MHC complex in the presence of certain cytokines, the naïve T helper cell (Th0) starts to differentiate into any of the illustrated Th subset. Above each Th subset the master regulator of transcription for that specific subset is depicted (in italics). So far, no mouse equivalent to human Th22 cells have been described although also for human Th22 cells the essential polarizing cytokines have not been uniformly identified but proposed to be IL-6 and TNF-α. In case of Th17 cells, differentiation occurs in a two-step model first generating immature Th17 (iTh17) and in a second step generating mature Th17 (mTh17) cells. The cytokines required for these two steps are different and also differs for human and mouse. IL-23 is however an important driving factor for mTh17 differentiation for both human and mouse Th cells and its action is amplified by IL-1β.

Murine Th17 cells can be induced by a combination of cytokines including TGFβ, IL-6, IL-21 and IL-23, and are characterized by the production of IL-17A, IL-17F, IL-21, IL-22, TNFα and GM-CSF (36, 41, 51-56). The differentiation of naïve T-cells into Th17 cells occurs via a mechanism distinct from the signals driving the development of Th1 and Th2 cells. Transcription factors and signaling molecules that are important for the differentiation of Th1 or Th2 cells, including STAT1, STAT6 and T-bet, are not necessary for the development of Th17 cells (51-52). Moreover, it was demonstrated that Th1 cytokines (IFNγ and IL-12) and Th2 cytokines (IL-4 and IL-13) repress Th17 cell development (51-52). The neutralization of both IFNγ and IL-4 favors IL-23/TGFβ/IL-6-induced IL-17-producing cells. Retinoic acid-related orphan receptor (ROR)γt and RORα have been identified as novel Th17 transcription factors (57-58). In addition, the transcription factors IRF-4 and the aryl hydrocarbon receptor are involved in Th17 polarization (59-61) (Figure 1).

In humans, TGFβ, IL-6, IL-23, IL-21 and IL-1β have roles in Th17 polarization from naïve CD4+ T-cells (62-66), whereas IL-23, IL-1β and IL-6 induce the secretion of IL-17A by human central memory CD4+ T-cells (65). In addition to cytokines expressed by murine Th17 cells, human Th17 cells express IL-26 (Figure 1), which is present as a pseudogene in mice (66). IL-17-producing T-cells may be integral in the development of persistent arthritis. The neutralization of IL-17A after CIA onset has been demonstrated to be beneficial in a mouse model of CIA, reducing joint damage and joint inflammation, and preventing cartilage and bone destruction (67). Furthermore, the spontaneous development of arthritis observed in IL-1RA-/- mice was completely suppressed in IL-17A-/-IL-1RA-/- mice (68). Also, TNFα deficiency completely suppressed the development of arthritis in these IL-1RA-/- mice (69). The induction of antigen-specific Th17 cells, but not Th1 or Th2 cells, by immunization with antigens and adjuvants was abolished in IL-1 receptor type I-deficient mice (70). Furthermore, IL-23 alone, or in combination with IL-1 or IL-6, may have a role in the pathogenic function of human effector memory T-cells and in the secretion of IL-17A by these memory T-cells (65). Conversely, IL-17A can induce IL-6, TNFα and IL-1 cytokine expression in non-hematopoietic cells, such as fibroblasts and endothelial cells (71). This effect may create a positive stimulation loop that creates an environment in which an acute inflammation can shift to become persistent arthritis (72).

IL-23 and pathogenic Th17 cells

The role of IL-1, IL-6, IL-23, TGFβ and TNFα in the differentiation of mouse and human Th17 cells has been reported (83); however, the role of IL-23 in the effector function of Th17 cells is not understood. The essential role for IL-23 in the pathogenic function of Th17 cells was demonstrated in models of EAE (84). In this study, Th17 cells, which

were polarized under IL-23-polarizing conditions, were potent inducers of EAE, while the pathogenic function in TGFβ/IL-6-polarized Th17 cells was completely abolished (84). A possible explanation for this discrepancy could be that IL-6/TGFβ-polarized Th17 cells produced the anti-inflammatory cytokine IL-10, while IL-23-polarized Th17 cells produce little IL-10. Interestingly, IL-23/IL-6/TGFβ-polarized Th17 cells did not result in EAE, indicating that the suppressive effect of IL-6/TGFβ-polarized cells dominated over the pro-inflammatory effect of IL-23-polarized cells (84). These data suggest that IL-23 is essential for the pathogenic function of Th17 cells. Similarly, it has been demonstrated that IL-23 and TGFβ/IL-6 differentially regulate Th-specific transcription factors during Th17 development in autoimmune experimental arthritis; this finding may explain the differential pathogenic potential of IL-23 compared with IL-6/TGFβ [(85), **chapter III**]. In CD4+ T-cells from naïve and collagen-type II immunized DBA/1 mice, IL-23 increased the expression of IL-17A, IL-17F and RORγt. In contrast to TGFβ/IL-6, IL-23 inhibited the Th1 and regulatory T-cell-specific transcription factors T-bet and FoxP3, respectively (85). Recently, two individual research groups have identified GM-CSF as a key cytokine produced by T helper cells in the pathogenesis of EAE (55-56). GM-CSF appeared to be regulated by IL-23 (55-56) and it was proposed that T cell-derived GM-CSF induced the production of IL-23 by antigen-presenting cells thereby providing a positive feedback loop (56). Also, IL-23-skewed Th17 cells, by means of GM-CSF production, showed to be more pathogenic than IL-6/TGFβ induced Th17 cells (55), which is in line with our own observations [(85), **chapter III**]. In summary, IL-23-driven Th17 cells appear to have a larger pathogenic potential than Th17 cells driven only by IL-6/TGFβ (Figure 1).

IL-23-regulated TCRγδ T-cells as an important source of IL-17A

In addition to CD4+ TCRαβ T-cells, CD4-T-cell receptor (TCR)γδ (γδ) T-cells are able to produce IL-17 (86), which is regulated by IL-23 (49, 87-102). The production of IL-17 by γδ T cells might be an important mechanism during the early inflammatory response. In the lungs and skin, γδ T-cells are the largest RORγt+ population and produce high levels of IL-17, providing evidence for a role for γδ T-cells in the early inflammatory response (103). Furthermore, emerging evidence exists that IL-17 and IFNγ producing γδ cells are differentially programmed; whereas IL-17-producing γδ cells arose from thymic ligand-naïve γδ T-cells (104) and were MyD88 dependent (105), IFNγ-producing γδ cells were thymic ligand-experienced (104) and required TCR and CD27 mediated signals (105). Directly after immunization, these thymic-derived IL-17-producing γδ T-cells were located in the draining lymph nodes, suggesting that these cells might have a role in the onset of an inflammatory response (104). Interestingly, CD27 can used as a negative marker for both murine and human IL-17-producing γδ cells (100, 105-106). Additional phenotypical markers present on IL-17 producing γδ T cells are the chemokine receptor

CCR6 (91, 93, 100, 107-108), CD161 (100, 107, 109), IL-23R (92, 99, 108, 110), IL-1R1 (96), CD25 (111) and SCART2 (112), many of which are also found on Th17 cells. Besides IL-23 other factors that drive the production of IL-17 in γδ cells have been discovered and comprise several Th17-promoting cytokines as IL-1β (90, 92, 96-97, 100, 113), TGFβ (97-98, 100, 114-115) but also by a combination of IL-23 and caspase-1 processed IL-18 (102). This suggests that similar factors that drive Th17 expansion also drive IL-17 in γδ cells which in turn can rapidly produce IL-17 with no need for antigen-specific synapses. Interestingly, a synergistic role for TLR signaling and IL-23 has been shown (91, 116). Identified transcription factors that drive the production of IL-17 in γδ cells are Tyk2 (89) and B lymphoid kinase (Blk) (117). Very recently, the NFkB-associates RelA and RelB in an lymphotoxin-β-receptor dependent manner (118) and the Notch-Hes1 pathway (119) were described to drive IL-17 production in γδ cells. In contrast, IL-17 production has been shown to be suppressed by type I IFN signaling (120) and, interestingly, by CD4+ Treg cells (121-122) although Treg function might be controlled by IL-17 producing γδ T cells as well (99) showing complex regulatory networks between innate IL-17 production and adaptive immunity.

Although γδ T-cells are able to produce IL-17, the role of these cells in autoimmune diseases is not well understood. Increased numbers of γδ T-cells are found in the synovium in RA (123-124). The role of γδ T-cells in experimental arthritis has been investigated in several studies, and the role of these cells in arthritis etiology and pathogenesis differs. The role of γδ T-cells in experimental arthritis was investigated using TCRδ-/- mice, which lack γδ T-cells. These mice demonstrated no difference in CIA incidence and severity, suggesting a minor role for γδ T-cells in the development of CIA (125). However, treatment with an anti-TCRγδ mAb (UC7-13D5) before an injection of type II collagen delayed both the onset and the severity of CIA (126). In contrast, the treatment of mice with CIA led to a more severe form of arthritis. These opposing effects may be caused by the ability of the antibody to deplete or block non-activated γδ T-cells in non-diseased mice, while but to induce activated γδ T-cells in diseased mice (126). In addition, in rat adjuvant arthritis, the depletion of γδ T-cells resulted in an aggravation of joint destruction (127); however, treatment with UC7-13D5 and GL3 (anti-TCRδ) led to the internalization of the γδ TCR, making γδ T-cells 'invisible' for assessment by flow cytometry, which is a frequently used method to evaluate the efficacy of antibody-based depletion (128). This might imply that although significant biological effects were observed (126), the role of the targeted γδ T-cells remains in question. At least in antigen-induced arthritis (AIA), relatively high proportions of IL-17-producing γδ T-cells were present in the spleen and joints compared to CD4+ T cells and this was correlated with a higher expression of RORγt in γδ cells than in CD4+ cells (49).

Table 1. Overview of the in vivo role of IL-12 and IL-23 cytokines in different models of arthritis.

Model	Disease model	Target/treatment	Disease phase at administration of treatment	Effect on disease state	Clinical observation	Reference
DBA/1 mice	CIA	recIL-12 (50 to 1000 ng)	With immunization (CII/IFA) and with booster (CII/PBS)	+	↑ incidence, ↑ severity	(73)
DBA/1 mice	CIA	recIL-12 (1 μg)	With immunization (CII/IFA) and with booster (CII/PBS)	-	↓ incidence, ↓ severity	(74)
C57BL/6 or B10.Q mice	CIA	recIL-12 (40 to 1000 ng)	With immunization (CII/IFA) and with booster (CII/PBS)	No effect	No effect	(75)
DBA/1 mice	CIA	IL-12p40-/-	Knockout	-	↓ incidence, ↓ severity	(76)
DBA/1 mice	CIA	recIL-12 (100 ng)	Before onset (day 28 to 32)	+	↑ incidence, ↑ severity, ↑onset	(47)
DBA/1 mice		LPS/anti-IL-12 Ab (polyclonal, 200μg)	Before onset (days 28, 30 and 32)	-	↑ incidence, ↑ severity	
DBA/1 mice		anti-IL-12 Ab (polyclonal, 200 μg)	Before onset (days 28, 30 and 32)	-	↓ incidence, ↓ onset	
DBA/1 mice		anti-IL-12 Ab (polyclonal, 200 μg)	Approximately at onset (days 35, 37 and 39)	+	↑ severity	
DBA/1 mice		recIL-12 (100 ng)	Approximately at onset (days 35 to 41)	-	↓ severity	
DBA/1 mice	CIA	Anti-IL-12p40 Ab (10F6, 500 μg)	At immunization to onset (twice per week)	-	↓ severity, = incidence to controls	(77)
DBA/1 mice	CIA	AdIL-12p70	Before onset (day 25)	+	↑ severity	(78)
		Anti-IL-12 Ab (antiserum)	Before onset (days 26, 28, 30 and 32)	-	↓ severity, ↓ onset	
IFNγ-/- DBA/1 mice	CIA	Anti-IL-12p40 Ab in wild-type controls (C17.8, 200 μg)	After immunization (days 0, 7 and 14)	Full protection from arthritis	No onset	(79)
		Anti-IL-12p40 Ab in IFNγ-/- (C17.8, 200 μg)	After immunization (days 0, 7 and 14)	Full protection from arthritis	No onset	
	CIA	Anti-IL-12p40 Ab in IFNγ-/- (C17.8, 200μg)	After onset (days 24, 31 and 28)	Full protection from arthritis	↓ severity, ↓ progression	

Model	Disease model	Target/treatment	Disease phase at administration of treatment	Effect on disease state	Clinical observation	Reference
DBA/1 mice	CIA	recIL-12 (5 ng)	Approximately at onset (days 24 to 33)	+	↑ severity	(80)
		recIL-12 (500 ng)	Approximately at onset (days 24 to 33)	-	↓ severity, ↓ onset	
DBA/1 mice	CIA	CII-specific CD4+ T-cells transduced with IL-12p40	Before booster (day 20)	-	↓ severity	(81)
C57BL/6 mice	CIA	IL-12p35-/-	Knockout	No effect	Similar to wild-type controls	(8)
		IL-12p40-/-	Knockout	Full protection from arthritis	No onset	
		IL-23p19-/-	Knockout	Full protection from arthritis	No onset	
DA/Slc rats	CIA	Anti-IL-23 Ab (polyclonal)	After onset (days 14, 16, 18, 20 and 22)	Full protection from arthritis	↓ severity	(48)
DBA/1	CIA	Anti-IL-23 Ab (monoclonal)	Before onset (days 15, 22, 29)	-	↓ severity	**chapter II**
			After onset	No effect	No effect	
BALB/c mice	IL-1Ra-/-	AdIL-23p40p19 in IL-1Ra-/-	At 8 weeks of age	+	Arthritis acceleration, ↑ pannus formation	(82)
C57BL/6 mice	PG/SCW (acute)	IL-23p19-/-	Knockout	-	↓ joint swelling	**chapter V**
C57BL/6 mice	AIA (primary)	IL-23p19-/-	Knockout	-	↓ joint swelling, ↓ inflammation	(49), **chapter IV**
C57BL/6 mice	AIA (flare)	IL-23p19-/-	Knockout	-	↓ joint swelling	Cornelissen et al., unpublished data
C57BL/6 mice	AIA (flare)	Anti-IL-23p19 Ab	Before flare induction	-	↓ joint swelling	**chapter II**

– decreased arthritis; + increased arthritis; Ab antibody; Ad adenovirus (transduced with the specified gene); AIA antigen-induced arthritis (methylated BSA); CII collagen type 2; CIA collagen-induced arthritis; IFA incomplete Freund's adjuvant; PG peptidoglycan; Rec recombinant; SCW streptococcal cell wall fragments.

AIA was induced in IL-23p19-/-. In these mice, the proportion and numbers of IL-17-producing γδ T-cells were severely reduced in the spleen and arthritic joints compared to wildtype mice, while IFNγ-producing γδ T-cells were unaffected [(49); **chapter IV**]. It also has been demonstrated that γδ T-cells are able to produce high levels of IL-17 during CIA, and that the numbers of IL-17-producing γδ T-cells were equal to those of CD4+ IL-17-producing cells (129). The IL-17-producing γδ T-cells in CIA preferentially expressed the Vγ4/Vδ4 γδ TCR and, upon treatment with a depleting anti-Vγ4 antibody, it was demonstrated that the Vγ4+ γδ T-cells contributed to CIA as evidenced by the lower disease severity and incidence of disease observed in Vγ4 γδ-treated mice compared with untreated mice (129). This finding indicates that a specific subtype of γδ T-cells, which produces IL-17, is involved in the development of CIA (129-130). Later, it was published that γδ T cells provided the first wave of IL-17 production in the joint of CIA mice and that CCR6+ IL-17-producing γδ cells could worsen disease in immunized mice (90). However, additional data showed that virtually no IL-17 producing γδ cells were present in human RA joint, where Th1 cells dominated (90). Finally, Pöllinger and colleagues (131) demonstrated that CIA joint-derived γδ could induce osteoclastogenesis in vitro but not in vivo and, in fact, were differentially located with a greater fraction of CD4+ versus γδ+T cells being located in bone-area in the CIA model but also in human RA suggesting that rather CD4+ T cells than γδ cells promote bone destruction (131). However, these data do not show the (relative) contribution of the IL-17A-producing Vγ4+ subset of γδ cells (129).

IL-12/Th1- and IL-23/Th17-driven pathology

Th17 cells are recognized as important contributors to inflammatory processes; however, evidence also suggests the potential of Th1 cells as pathological mediators. Although IL-17 had a dominant role in EAU and the neutralization of this cytokine prevented or reversed disease, using a transfer model, polarized Th1 cells could induce severe EAU independently of host IL-17 (11). Kroenke et al have demonstrated that IL-12- and IL-23-modulated T-cells induce distinct types of EAE based on histology, CNS chemokine profile and response to cytokine inhibition (132). IL-12p70-driven disease was characterized by macrophage-rich infiltrates and prominent nitric oxide synthase 2 upregulation, whereas neutrophils and G-CSF were prominent in IL-23-driven lesions (132). Treatment with anti-IL-17 or anti-G-CSF inhibited EAE induced by the transfer of IL-23-polarized, but not IL-12p70-polarized, cells (132). Notably, anti-IFNγ exacerbated not only IL-23-driven lesions, but also IL-12p70-driven lesions, and anti-TNFα therapy was beneficial under both conditions (132). In another study, the adoptive transfer of both Th1- and Th17-polarized hen egg lysozyme specific T-cells into eyes of mice expressing hen egg lysozyme induced ocular inflammation, with differences in histological pathology (133). Three different key activities of Th1 and Th17 cells were noted: (i) Th17 cells were inferior

to Th1 cells in their capacity to trigger massive lymphoid expansion and splenomegaly; (ii) the proportion of Th1 cells among infiltrating cells in inflamed recipient eyes declined rapidly, becoming a minority by day 7, whereas Th17 cells remained in the majority throughout this period; and (iii) differences were noted between Th1 and Th17 cells in their expression of certain surface markers (133). No neutralizing studies of IL-17 or IFNγ were conducted. Furthermore, Th17 cells promoted pancreatic inflammation, but only induced diabetes efficiently in hosts with lymphopenia after conversion into Th1 cells (134).

There is evidence from different arthritis models that IL-17-producing T-cells are the dominant cell type in the development and progression of arthritis. IL-17+IFNγ+ 'double-positive' cells have been observed in different experimental arthritis models (135). This finding suggests that arthritis can develop in the context of either a Th17 or a Th1 effector response, depending on the model, and that the dominant effector phenotype may be determined, at least in part, by conditions present during initial exposure to antigen (11). However, transfer studies with antigen-specific polarized Th1 and Th17 cells in arthritis have not been conducted. From earlier studies, it was understood that the absence of IFNγ or IFNγ receptor signaling accelerated the onset of CIA. Importantly, this effect was accompanied by lower serum levels of anti-collagen type II antibodies (136-137). Anti-IL-12 antibody prevented the development and progression of CIA in IFNγ receptor-deficient mice, suggesting that, in the absence of a functional IFNγ system, endogenous IL-12 exerts its disease-promoting effect by favoring the production of TNFα, inhibiting the development of IL-4- and IL-5-producing T-cells, and stimulating the production of anti-collagen auto-antibodies (79). It has since became clear that the sensitivity of IFNγ-deficient B6 mice to experimental arthritis is associated with high IL-17 production, and blocking IL-17 almost completely prevented the development of arthritis (138-139).
In addition, the role of IL-23 in promoting IFNγ-producing Th1 cells is not well studied. IL-23 influenced Th1 responses because IL-23 deficiency or neutralization by antibodies reduced not only IL-17, but also the IFNγ response to antigen (132). Mono-arthritis induction in IL-23-deficient mice did not result in a lower proportion of IFNγ-positive CD4+ T-cells in the spleen or lymph node, in contrast to a lower proportion of IFNγ-positive cells in the target organ, the joint [(49), **chapter IV**]. Therefore, IL-23 could be required to promote Th1 and Th17 effector responses, especially at the target organ/tissue, and further studies are needed to understand this effect.

The IL-12/IL-23 axis in T-helper subset plasticity

Classically, autoimmune diseases such as RA were thought to be a Th1- and not a Th2-associated disorder (7). It currently is unclear whether RA is a Th1- and/or Th17-mediated disease (140). It has been demonstrated that IL-17A is produced by proinflammatory

Th1/Th0 cells isolated from synovial membranes and from the synovial fluid of patients with RA (141). In the joints of patients with established RA, predominantly Th1, but not Th17, cells were observed (142). Notably, the Th1 and Th17 cytokines IFNγ and IL-17, respectively, are often co-expressed in human memory CD4+CD45RO+ T-cells from treatment-naïve patients with early RA (143).

Interestingly, Th17 cells appear to be associated with regulatory T-cells (Treg cells). This association is indicated by the use of a common inducer (TGFβ), an overlapping chemokine receptor profile, and the expression of the Th17-associated transcription factor RORγt (57, 144). In mice, it has been demonstrated that Treg cells can be converted to IL-17-producing T-cells (58, 145-146). Additionally, human Treg cells, defined as CD4+CD25highFoxP3+CD127-CD27+, were reported to differentiate to IL-17-producing T-cells in vitro (147). This differentiation was accompanied by an upregulation of RORγt and CCR6 expression (147). In addition to plasticity between Treg and Th17 cells, data have been published suggesting that Th17 cells are closely related to Th1 cells. In mice, differentiated Th17 cells rapidly responded in vitro to IL-12, by upregulating the expression of IFNγ and downregulating IL-17 expression (148). Also, the development of IFNγ-producing effector T-cells from IL-17-producing progenitor cells is inhibited in the presence of TGFβ, a cytokine that is important for the maintenance of IL-17 expression by Th17-polarized cells (134, 148-149). A role for epigenetic regulation of effector T-cell plasticity has been identified. For example, the gene encoding T-bet, the master regulator of Th1 differentiation, is in an active state, according to histone methylation marks, in both Th17 and Treg cells. This finding implies that Th17 and Treg cells have the potential to upregulate the expression of T-bet and to differentiate toward Th1 cells (150).

Associations between T-cell subsets, such as Th17 and Th1 cells, have been observed in inflammation and the progression of RA. In joint inflammation in patients with RA, high levels of IL-17 and almost no IFNγ expression can be detected in the joint synovium (151-152). This finding correlated with studies in mice, which indicate the importance of IL-17 in the induction of arthritis (153). In a rat model of adjuvant arthritis, IL-17 was induced shortly after initiation of disease, and IL-17 levels declined over time (154). In contrast, IFNγ cytokine levels were detected in the joint synovium of patients in later stages of RA, and IFNγ expression was detected in joint-infiltrating CD4 T-cells and in CD4 T-cell clones obtained from inflamed synovium (141, 155-156). In agreement with these findings, in the rat model of adjuvant arthritis, increased levels of IFNγ were detected in lymph nodes and synovium at later stages of RA (154). In a 2-year predictive study in patients with RA (n = 60), it was demonstrated that IFNγ had a role in reducing joint damage progression (157). Interestingly, the inhibition of IFNγ in later stages of disease exacerbated the disease (157).

In addition to the association of Th17 cells with Th1 cells, an inverse correlation between IL-17-producing cells and FoxP3+ Treg cells has been identified. In children with juvenile idiopathic arthritis, the balance between IL-17+ cells and Treg cells may be essential to predict disease outcome (158). In addition, Treg cells are present in higher numbers in inflamed joints in patients with a mild RA phenotype compared with patients with more severe disease (159-160).

These observations suggest that plasticity between Treg, Th1 and Th17 subsets exist, and that the differentiation of these subsets is not completely restricted to separate lineages. Together, these studies provide a completely new concept for T-cell plasticity.

CONCLUSION

RA is an autoimmune disease mainly characterized by chronic inflammation of the joints, which may lead to the progressive destruction of cartilage and bone. With respect to treatment, many anti-inflammatory drugs and DMARDs are available. However, with the introduction of biologicals targeting different mediators of the disease, much progress has been made in the suppression of symptoms. As new and promising drugs are being tested in clinical trials, an improvement in the quality of life of patients with RA is expected. Still, the recognition of early symptoms is of vital importance in the treatment of RA because this, together with intensive treatment, may prevent joint damage. Although the immunological mechanisms involved in the development of RA are not fully understood, with the discovery of the IL-23/Th17 immune pathway the classical understanding of IL-12/Th1-mediated diseases needs to be reconsidered. In fact, the IL-23/Th17 immune pathway has been demonstrated to be of significant importance in most autoimmune animal disease models (8-10).

Studies predominantly using adoptive transfer with polarized Th1 and Th17 cells demonstrated that Th17 and Th1 cells are pathogenic (11, 133). In some diseases, Th17- and Th1-polarized cells induced distinct types of inflammatory cells, with a more macrophage-mediated infiltrate by Th1 cells and a granulocyte-mediated infiltrate by Th17 cells, which is in agreement with the ability of IL-17 to recruit neutrophils (132). Recent discoveries of T-cell plasticity provide a new hypothesis for T-cell subset changes during chronic inflammatory responses. Because of the central role of effector T-cells and proinflammatory cytokines in the development and progression of RA, it will be essential to understand the role of T-cell plasticity that may occur at different stages of the disease. Interestingly, IL-6 blockade in RA might involve a therapeutic mechanism distinct from that of TNFα blockade because the protective effects of IL-6 blockade, but

not TNF-α blockade, in CIA have been demonstrated to correlate with the inhibition of Th17 differentiation (161). Although it has been demonstrated that anti-TNFα treatment improves Treg function (162), the relative resistance of Th17 cells, compared with Th1 cells, to suppression by Treg may be relevant to RA pathology (163). Therefore, it will be important to understand the effects of T-cell plasticity on the efficacy of different therapies for RA. This understanding may help to further refine treatment toward personalized therapy for RA and to help classify different stages of the disease.

AIM OF THE THESIS

Understanding the immunological processes that drive joint inflammation are of vital importance to unravel arthritis etiology and to develop new therapeutic agents. With the discovery of the IL-23/IL-17 immune pathway as a major player in arthritis, detailed knowledge of this pathway during different arthritic settings is required. Therefore, we aimed in this thesis to investigate the role of IL-23 during various experimental arthritis models with a strong focus on the IL-23/IL-17 immune pathway. Additionally, we showed that IL-23, in concert with IL-1β, modulates the expression of IL-17A and IL-22 by human Th17 and Th22 cells although the clinical implications of this finding are unknown.

As shown by Murphy and co-workers (8), mice deficient for IL-23 did not develop any clinical symptoms of collagen-induced arthritis (CIA), a mouse arthritis model resembling many immunopathological aspects with human rheumatoid arthritis (RA). It has also been shown that neutralization of IL-23 activity could reduce paw volume when given after onset to CIA rats. However, the IL-23-dependency of CIA during different stages of the disease, as well as the downstream effects of blocking IL-23 remained elusive in this study. Therefore, in **chapter II**, we addressed the research question whether IL-23 acts at various stages of CIA. To this end, IL-23 was neutralized before clinical manifestation but after immunization to investigate IL-23-dependence during CIA development. Alternatively, IL-23 was blocked after the first clinical signs of CIA were expressed to explore IL-23-dependence during CIA progression. In addition, this chapter describes the role of IL-23 during antigen-driven reactivation of arthritis (flare-up) which is strongly memory T cell mediated.

Murphy *et al*. (8) also showed that CIA-resistant IL-23-deficient mice had reduced frequencies of IL-17 producing CD4+ T cells compared to control mice suggesting an in vivo contribution for IL-23 in the regulation of Th17 cells in arthritis. Therefore, we hypothesized in **chapter III**, that IL-23 promotes Th17 subset polarization during CIA.

Previous data indicated that IL-23 contributed to arthritis predominantly by acting on T helper cells. To investigate this more directly, antigen-induced arthritis (AIA) model was used because this model is strongly T cell dependent. So, in **chapter IV** it was investigated whether IL-23 drives T cell mediated arthritis.

Apart from conventional CD4+ T helper cells, early responder TCRγδ T cells are also able to produce IL-17A in response to environmental changes. Interestingly, IL-17A-producing TCRγδ T cells have been shown to contribute to CIA progression. However, whether IL-23 *in vivo* drives IL-17A production from TCRγδ T cells during arthritis has not been addressed previously. Therefore, the second research question we addressed in **chapter IV** was if IL-23 promotes IL-17A-production from TCRγδ T cells during arthritis.
The IL-23 receptor is mainly expressed on activated and memory CD4+ T helper cells, but data implies an additional function for IL-23 receptor ligation on myeloid-derived innate immune cells.

Macrophages normally residing in the joint rapidly respond to antigen by the production of pro-inflammatory mediators and as such contribute to joint inflammation. Experimental data suggests that macrophages respond to IL-23 stimulation but no functional role for this has been shown in joint inflammation. Therefore, we set out to explore in **chapter V** the hypothesis that IL-23 drives macrophage-driven acute joint inflammation and describe a novel IL-23/TNF-α immune axis during this inflammatory process.

Human IL-17-producing CD4+ memory T helper cells have been described to preferentially express the chemokine receptor CCR6 and these cells are found at higher frequencies in RA patients compared to healthy controls. Recently, these CCR6+ Th17 cells have been implicated to participate in the chronicity of joint inflammation. In addition, a subset of CCR6+ memory T cells co-express CCR10 and produce IL-22 but no IL-17 and are therefore referred to as Th22 cells, but their involvement in RA is not known. Because IL-23 and IL-1β, together with other cytokines, drive the differentiation of Th17 cells and because IL-23 is also important for IL-22 induction, we hypothesized **chapter VI** that IL-23, together with IL-1β, regulates the balance of CCR6+ Th17 and CCR6+CCR10+ Th22 memory cells.

Finally, the results described in these chapters are discussed in **chapter VII**.

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II

IL-23-dependent and independent stages of experimental arthritis

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ABSTRACT

IL-23 has been shown to be critical for the development of experimental autoimmune diseases, such as collagen-induced arthritis (CIA). Neutralizing IL-23 after onset of CIA in rats has been shown to reduce paw volume, but the effect on synovial inflammation and the immunological autoimmune response is not clear. In this study, we examined the role of IL-23 at different stages of CIA and during T cell memory mediated flare-up arthritis with focus on changes in B cell activity and Th1/Th17 modulation. Anti-IL-23p19 antibody (anti-IL23p19) treatment, starting 15 days after the type II collagen (CII)-immunization but before clinical signs of disease onset, significantly suppressed the severity of CIA. This was accompanied by significantly lower CII-specific IgG1 levels and lower IgG2a levels in the anti-IL-23p19 treated mice compared to the control group. Surprisingly, in the spleen, significantly enhanced CD4+ Th1 and Th17 cell numbers and IL-17A were found in the anti-IL-23p19 treated mice, implying decreased egression of lymphocytes. Importantly, neutralizing IL-23 after the first signs of CIA did not ameliorate the disease. This was in contrast to arthritic mice that underwent an arthritis flare-up since a significantly lower disease score was observed in the IL-23p19 treated mice compared to the control group, accompanied by lower synovial IL-17A and IL-22 expression in the knee joints of these mice.

These data show IL-23-dependent and IL-23-independent stages during autoimmune CIA; whereas early intervention with anti-IL-23p19 prevented full-blown CIA and antigen-induced flare-up, blocking IL-23 activity after CIA onset did not significantly suppress disease severity. This suggests that although IL-23 is important for the development of RA, targeting IL-23 in human RA may not significantly reduce disease severity although it might have beneficial effects in reactive arthritis.

INTRODUCTION

IL-23 is a heterodimeric cytokine consisting of a p40 subunit, shared with IL-12, and a p19 subunit that is unique to IL-23 (1). Together with the IL-12Rβ1 receptor, the IL-23 receptor (IL-23R) chain forms a functional receptor for IL-23 (2) that is expressed on T cells, NK cells, monocytes and dendritic cells (1, 2). However, IL-23R is not expressed on precursor T cells, suggesting that IL-23 signaling is not involved in the primary differentiation of naïve T cells (3-4). The signaling of IL-12 and IL-23 leads to the activation of both overlapping and divergent signal transduction pathways and pathological roles in experimental arthritis (2). IL-23 is elevated in many autoimmune diseases, such as psoriasis, rheumatoid arthritis (RA) and multiple sclerosis (5, 6). IL-

23 transgenic mice develop systemic inflammation, including inflammation of the skin and small and large intestine (7), highlighting the role of this pathway in promoting the activation of effector T cells and sustaining of inflammatory tissue responses.

The role of IL-23 in the development of autoimmune collagen-induced arthritis (CIA) has been shown using IL-23p19 knockout mice. These mice did not develop CIA compared to IL-23 sufficient controls (5). In these IL-23p19 deficient mice, no IL-17 producing cells were detected while the proportion of IFN-γ producing cells was unaltered (5). This indicates that IL-23 is involved in the generation of IL-17 producing T cells in vivo. Furthermore, neutralizing IL-23 after onset of CIA in rats has been shown to reduce paw volume (6), but the effect on synovial inflammation and the immunological autoimmune response need to be elucidated.

Here, we investigated the role of IL-23 during different stages of autoimmune CIA by utilizing an IL-23p19 specific antibody. When anti-IL-23p19 was given after CIA onset, arthritis severity was not ameliorated. However, when anti-IL-23p19 was administrated after type II collagen (CII)-immunization but before clinical CIA onset, significantly less severe disease was observed. Finally, we show in a memory T cell dependent antigen-induced arthritis model that IL-23 is essential for the development of full blown flare-up arthritis. In this model, synovial expression of IL-17A and IL-22 but not IFN-γ was markedly lower in the anti-IL-23p19 group compared to control, highlighting the role of IL-23 in memory T cell driven flare-up arthritis. Together, these data show IL-23 dependent and independent stages during CIA. Additionally, we showed that antigen-induced reactivation of memory T cell driven joint inflammation is IL-23 dependent.

MATERIAL AND METHODS

Collagen-induced arthritis

Male DBA/1 mice (Harlan, Horst, the Netherlands) were immunized intradermally with (100 μL of 1 mg/mL) bovine collagen type 2 (CII) (Chondrex) emulsified in complete Freund's adjuvant (CFA). On day 21, mice were given an intra-peritoneal (ip) injection of 100 μg CII in PBS. CIA severity was assessed as previously described (10).

Mice were given 100 μg neutralizing murine anti-murine IL-23p19 or murine IgG1 isotype control antibody ip on days 15 (before disease onset), 22 and 29. Alternatively, mice were observed daily and as soon as an arthritis score of 0.5-1.5 was observed, 100 μg anti-IL-23p19 or isotype control antibody was injected ip weekly for 3 or 6 weeks. All experiments were approved by the Dutch Animal ethics committee (DEC).

Antigen-induced arthritis

To induce antigen-induced arthritis (AIA), methylated BSA (mBSA, 8 mg/mL) was emulsified in an equal volume of CFA containing 1 mg/mL heat-killed *M. tuberculosis* (H37Ra; Difco). C57BL/6 Mice were immunized intradermally with 100 μL mBSA/CFA. One week later, 30 μg mBSA was injected intra-articular to induce mono-arthritis. Four weeks after the induction of the mono-arthritis, 100 μg anti-IL-23p19 or control antibody was injected ip, weekly for 5 weeks. Finally, one day after the last injection of anti-IL-23p19, a local arthritic flare was induced by injecting 2 μg mBSA intra-articular. One day later, mice were sacrificed and the severity of arthritis in the knee joint was scored macroscopically on a scale of 0–2 after removing the skin (0, no inflammation; 1, mild inflammation; 2, severe inflammation)

Anti-collagen antibody ELISAs

To determine anti-CII-specific antibodies, wells of microtiter plates were coated with 1 μg/mL CII, washed and blocked with 10% FCS. Samples and a reference-sample were serially diluted. Wells were washed, incubated with goat-anti-mouse IgG1, IgG2a or IgG2b-biotinylated antibodies (0.5 μg/mL) (Southern Biotechnology Associated, Inc.) and optical density at 450 nm was measured.

Levels of IL-17A (R&D Systems, Minneapolis, MN, USA) and IFNγ (BD OptEIA™, BD Biosciences, Sunnyvale, CA, USA) were measured by ELISA according to the manufacturers' instructions.

Isolation of ankle infiltrating cells

Ankles were cut into smaller pieces and put into 5 mL serum-free medium containing 0.65 Wünsch units Liberase TM Research Grade (Roche Applied Science, Indianapolis, In, USA). After 2 hours, cells were pushed through a 100 μm filter and these ankle-cells were processed for further use.

Flow cytrometric analyses

For intracellular detection of cytokines, cells were stimulated with PMA (0.05 μg/mL) and Ionomycin (0.5 μg/mL) in the presence of GolgiStop™ (BD Biosciences, Sunnyvale, CA, USA) for 4h. Cells were fixed using 2% PFA and permeabilized with 0.5% saponin. Samples were acquired on a BD FACS CANTO II flow cytometer and analyzed using FlowJo (Tree Star, Inc.) software.

Quantitative PCR

Total RNA of splenocytes was extracted, and DNaseI-treated RNA was used for cDNA synthesis (8). PCR primers were designed manually or using ProbeFinder software (Roche

Applied Science, Indianapolis, In, USA) and probes were chosen from the Universal probe library (Roche Applied Science). Quantitative realtime PCR was performed using the ABI Prism 7900HT sequence-detection system (Applied Biosystems, Foster City, CA, USA) and analyzed using SDS v2.3 software (Applied Biosystems). The Ct values obtained were normalized to those of glycereraldehyde-3-phosphate dehydrogenase (GAPDH).

In vitro restimulation of splenocytes

DBA/1 and C57BL/6 (WT and IL-23p19-deficient) mice were immunized with bovine CII and chicken CII respectively (in CFA) and splenocytes were isolated 10 days post-immunization. For antigen-specific restimulation, splenocytes from DBA/1 mice were cultured for 3 days in the presence or absence of 50 μg/mL bovine CII. To both DBA/1 and C57BL/6 splenocyte-cultures, either no antibody; IgG1 isotype control (1 μg/mL) or anti-IL-23p19 (1 μg/mL) was added. After culture, cytokines were measured in supernatant by ELISA.

Statistical analyses

Differences between groups were tested with the Mann-Whitney U test. P values less than 0.05 were considered significant.

RESULTS

IL-23 is not required for CII-primed splenocytes to produce IL-17A upon CII-specific restimulation

To profile the kinetics of Th1 and Th17 cells during collagen-induced arthritis (CIA), splenocytes were isolated from type II collagen (CII)-immunized DBA/1 mice at various time points post-immunization (p.i.) and assessed for intracellular cytokines by flow cytometry. At day 10 p.i. the highest proportions of total IL-17A+ and IL-17A+IFNγ-CD4+ T cells were observed as compared to naïve (non-immunized) mice as well as to mice 25 days p.i and CIA-diseased mice (Figure 1A). This shows the generation of CD4+ IL-17A-expressing T cells during CIA.

The role of IL-23 on CII-specific cytokine production was evaluated by stimulating splenocytes taken from naïve mice or from mice 10 days p.i. with CII or anti-CD3/anti-CD28 in absence of antibody (-) or in the presence of IgG control or anti-IL-23p19. Although CII-specific restimulation led to a significant enhancement of IL-17A secretion compared to unstimulated CII-primed splenocytes, adding anti-IL-23p19 did not change the production of IL-17A (Figure 1B). CII-specific restimulation did not enhance the

secretion of IFN-γ but anti-IL-23p19 enhanced the production of IFN-γ under non-antigen-specific conditions (Figure 1B). Additionally, no change in IL-17A or IFN-γ levels was observed when splenocytes were activated with anti-CD3/anti-CD28 in the presence of anti-IL-23p19 compared to controls (Figure 1B). This suggests that IL-23 is not required for CII-primed splenocytes to produce IL-17A or IFN-γ upon antigen-specific restimulation. Next, we tested in vivo whether IL-23 is required early for the generation of CII-specific IL-17A-producing cells. Splenocytes from naïve or CII/CFA-immunized wild-type (WT) and IL-23p19-deficient C57BL/6 mice were isolated and in vitro activated with anti-CD3/anti-CD28 to boost cytokine production. Significantly lower levels of IL-17A were secreted by splenocytes from IL-23p19-deficient mice compared to splenocytes from WT mice (Figure 1C). Together these data suggest that although IL-23 seems redundant for the maintenance of CII-specific IL-17A-production, IL-23 is essential in the generation of CII-primed IL-17A-producing cells.

Lower severity of CIA after neutralizing IL-23 during arthritis onset

To investigate the role of IL-23 during different stages of CIA, CII/CFA-immunized DBA/1 mice were randomly separated into two groups and treated with a murine anti-murine IL-23p19 antibody or isotype control antibody at days 15, 22 and 29 p.i. At day 21 p.i., a booster-injection was given according to our CIA protocol. Significantly less severe CIA developed in the anti-IL-23p19 treated group compared to control when evaluated using macroscopic scoring and statistically tested at each individual time point assessed or over time by using the area under the curve (AUC; Figure 1D). The disease incidence was not significantly different between the two groups; however, there was a trend for the anti-IL23p19 treated mice to have a lower incidence of disease (Figure 1D).

Reduced levels of CII-specific antibodies in anti-IL-23p19 treated mice

To determine the effect of anti-IL-23p19 treatment on auto-antibody production, serum was collected at day 35 and anti-CII-specific IgG antibodies were measured. As shown in Figure 2, IgG1 levels were significantly lower in the anti-IL-23p19 treated group compared to controls. Additionally, IgG2a and IgG2b levels were also markedly lower but no statistically significant difference was reached.

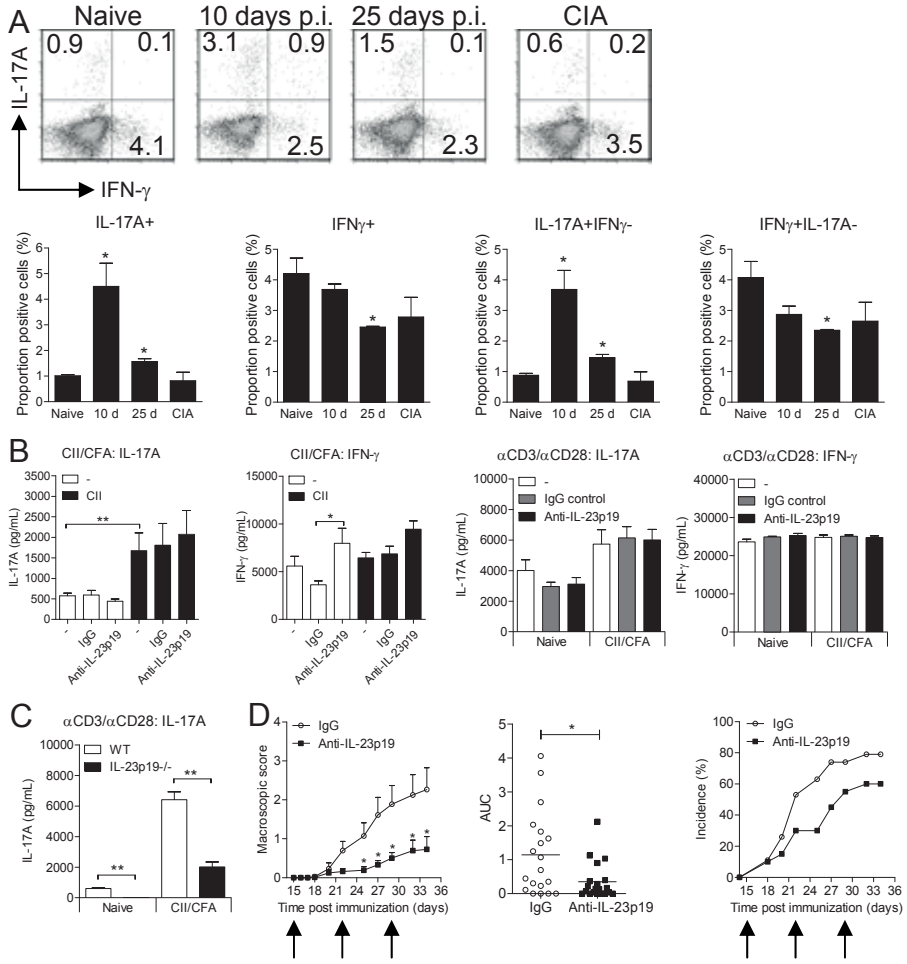

Figure 1. Administration of anti-IL-23p19 before onset prevents full-blown CIA. (A) DBA/1 mice were immunized with CII/CFA or left untreated. At days 10 and 25 days post-immunization (p.i.) and from mild-positive (macroscopic score in range of 0.25 – 0.75) CIA mice (CIA), splenocytes were isolated. Intracellular production of IL-17A and IFN-γ was assessed by flow cytometry after 4 hrs of stimulation with PMA/Ionomycin by gating on CD4+ T cells. Numbers in quadrant indicate percentage positive cells in that quadrant. Plots are representative of n=3 per group; a summary of the data is given in the histograms beneath the plots. *P<0.05 compared to naïve as calculated by unpaired student t-test. (B) IL-17A and IFNγ ELISA measurements from supernatants of splenocytes (from naïve and CII/CFA-immunized mice) in vitro stimulated with CII or with anti-CD3/anti-CD28, or left unstimulated, in complete absence of antibody (-) or with IgG control or anti-IL-23p19. (C) IL-17A and IFNγ ELISA measurements from supernatants of wild-type (WT) or IL-23p19-/- C57BL/6 splenocytes from naïve or CII/CFA-immunized mice were stimulated with anti-CD3/anti-CD28. (B-C) Data are the mean +SEM from n=3 mice per group and *P<0.05; **P<0.01; ***P<0.001 as calculated by Mann-Whitney U test. (D) DBA/1 mice were immunized with CII/CFA and three weeks later mice received a booster-injection. On days 15, 22 and 29 either anti-IL-23p19 (filled squares) or control antibody (open circles) was given intra-peritoneally. Macroscopic score (+SEM) and the area-under-the-curve (AUC; average macroscopic score per mouse of all time points assessed, assessed by student t-test) of n=20 mice per group from 2 independent experiments is shown, as well as the incidence (percentage positive mice).

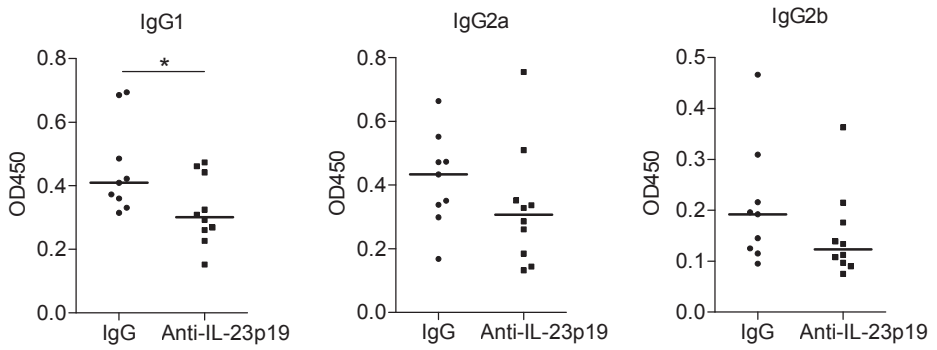

Figure 2. Decreased production of anti-CII antibodies in mice treated with anti-IL-23p19. At day 35, at the end of the experiment as shown in Figure 1D, serum was collected and anti-CII antibody levels of the IgG1, IgG2a and IgG2b subclasses were determined in sera. Data are shown for each individual mouse or as the mean +SEM of n=10 mice per group and *P<0.05; **P<0.01; ***P<0.001 as calculated by Mann-Whitney U test.

Increased CD4+ T cells in the spleen after anti-IL-23p19 therapy

We next investigated the effects of anti-IL-23p19 on T cells. At day 35, the absolute numbers of splenocytes was significantly higher (~1.4 fold) in mice treated with anti-IL-23p19, as well as the numbers of CD4+ T cells (~ 2 fold) compared to control (Figure 3A) suggesting accumulation of splenic T cells after IL-23 neutralization. To further investigate this, we calculated the absolute numbers of Th17 (CD4+IL-17A+IFNγ-) and Th1 (CD4+IFNγ+IL-17A-) cells in spleen and ankles of these mice. The absolute numbers of Th17 and Th1 cells in spleen was significantly higher at day 35 p.i. in the anti-IL-23p19 treatment-group compared to control (Figure 3A). Conversely, we did not observe significant differences in cell numbers in the ankles of control mice compared to anti-IL-23p19 treated mice (Figure 3A). These data suggest that neutralizing IL-23 before disease-onset prevents egression of T lymphocytes from the spleen.

To investigate the cytokine-production profile of these splenocytes, RNA was isolated and subjected to quantitative RT-PCR. Surprisingly, the T cell-associated cytokines IL-17A, IL-22, IL-21 and IL-1β were significantly upregulated in the anti-IL-23p19 treated group compared to control (Figure 3B). The expression of IFN-γ and TNF-α was however similar between mice treated with anti-IL-23p19 and control (Figure 3B). Additionally, a trend towards increased expression of the chemokine receptor CCR7 was observed in the anti-IL-23p19 treated group compared to control (Figure 3C). These data collectively imply decreased lymphocyte-migration after anti-IL-23p19 treatment leading to relatively higher T cell numbers in the spleen and increased expression of pro-inflammatory cytokines.

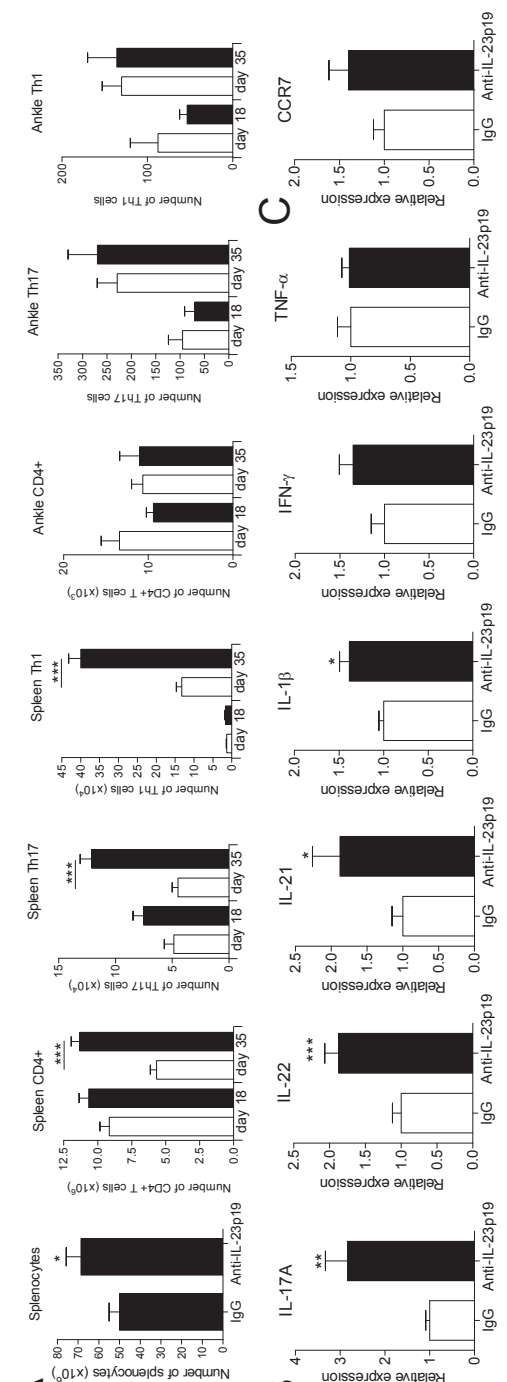

Figure 3. Increased CD4+ T cells in the spleens of anti-IL-23p19 treated mice. (b) CII/CFA-immunized mice were given anti-IL-23p19 or control antibody at days 15, 22 and 29. At day 21, a booster-injection was given. At day 35, cells from ankles and spleens were isolated and subjected to flow cytometry after 4 hrs of stimulation with PMA/Ionomycin. (A) The number of total of splenocytes is shown for day 35 p.i. The absolute number of CD4+, CD4+IL-17A+IFN-γ- (Th17) and of CD4+IFN-γ+IL-17A- (Th1) cells were calculated. (B-C) Gene expression of pro-inflammatory cytokines (B) and CCR7 (C) was quantified by RT-PCR using mRNA isolated from spleen at day 35 p.i. Data are the mean ± SEM of n=10 mice per group and *P<0.05; **P<0.01; ***P<0.001 as calculated by Mann-Whitney U test.

No therapeutic effect of neutralizing IL-23 after onset of CIA

Next, we asked whether anti-IL-23p19 has a therapeutic effect in CIA. To evaluate this, mice were treated with anti-IL-23p19 or control antibody weekly for 3 weeks after the first signs of CIA. Anti-IL-23p19 did not significantly suppress the macroscopic score, compared with isotype control-treated mice (Figure 4A), and no differences in anti-CII specific IgG antibody levels were noted between the two groups (Figure 4B). However, the anti-IL-23p19 mice trended toward a lower macroscopic score from days 15 – 20. To determine if longer treatment with anti-IL-23p19 would lead to a significant improvement in the macroscopic score, anti-IL-23p19 or control antibody was administrated for a period of 6 weeks after disease onset. However, no ameliorative effect was observed (Figure 4C) after blockade of IL-23 compared to control and this also did not affect the production of anti-CII antibodies (Figure 4B). Once again, there was a trend for improvement in the macroscopic score from approximately days 15 – 20 but the differences did not reach statistical significance. Together, these data indicate that specific neutralization of IL-23 after CIA onset has no beneficial effect on the CII-antibody titer or clinical score of CIA.

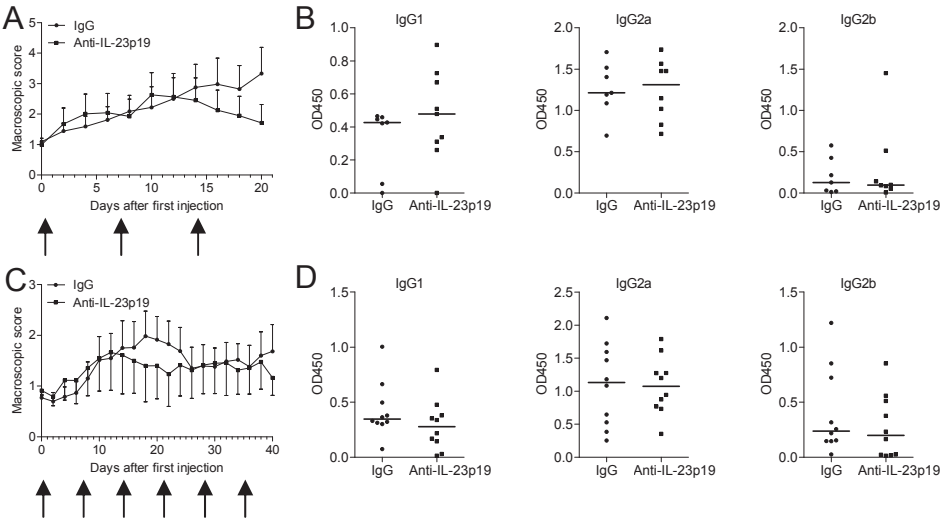

Figure 4. Neutralizing IL-23 after onset of CIA does not ameliorate disease activity. CIA was induced in DBA/1 mice and directly after the first signs of CIA anti-IL-23p19 or control antibody was injected when an arthritis score between 0.5-1.5 was observed. (A and C) Arthritis severity was scored macroscopically. Numbers on the x-axis indicate the day after the first injection with either antibody. Data are from (A) n=8 and (B) n= 10 mice per group. (B and D) At the end of each experiment, serum was collected and anti-CII antibody production of the IgG1, IgG2a and IgG2b subclasses was measured.

IL-23 is critically involved in memory T cell mediated arthritic flare

CIA pathology is initiated by CII-specific helper T and B cells which in turn lead to the generation of anti-CII-specific IgG producing plasma cells and the formation of pathogenic immune-complexes (ICs) (11). To further dissect the role of IL-23 on reactivation of memory T cell mediated pathology, we utilized the mBSA-driven antigen-induced arthritis (AIA) flare-up model. Mice were immunized with mBSA/CFA and one week later an intra-articular injection with mBSA was given to induce a primary mono-arthritis which typically lasts for 3 weeks. After recovery from the primary arthritis, mice received five weekly injections with a murine anti-murine IL-23p19 antibody or isotype control antibody. One day after the last antibody injection, a local arthritic flare-up was induced by a second intra-articular injection with mBSA and the following day mice were sacrificed and the arthritis severity was assessed macroscopically. Mice treated with anti-IL-23p19 developed significantly lower disease scores compared with control (Figure 5A). To gain insight in the effect of IL-23 neutralization, RNA from knee-infiltrating cells was isolated and gene transcription was determined by quantitative RT-PCR. Synovial IL-17A and IL-22 expression was markedly lower in anti-IL-23p19 treated mice compared to control (Figure 5B). In contrast, synovial IFN-γ expression was similar between the two groups (Figure 5B). These data show that anti-IL-23p19 treatment prevented full-blown flare-up arthritis by specific suppression of the Th17 cytokines IL-17A and IL-22 in the synovium.

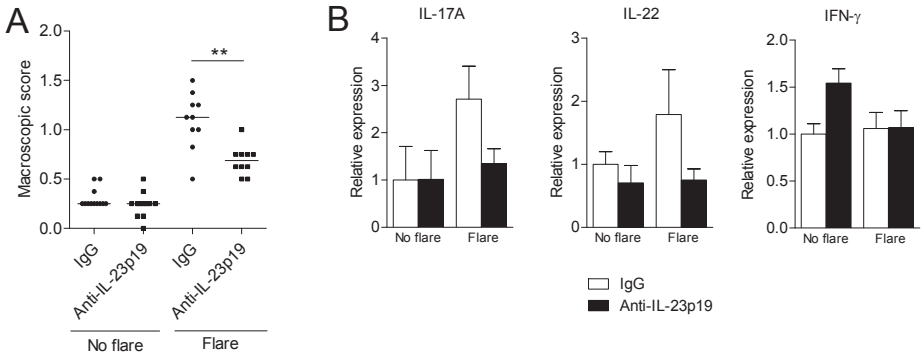

Figure 5. IL-23 plays a critical role in the arthritis flare-up reaction. In C57BL/6 mice, antigen-induced arthritis (AIA) was induced. Four weeks after the induction of AIA either IgG control or anti-IL-23p19 was administrated intra-peritoneally weekly for 5 weeks. One day after the final injection mice were given an intra-articular injection with mBSA to induce a flare-up or were left untreated. (A) Twenty-four hours later, knee joints were assessed macroscopically on a 0-2 scale. Each symbol represents data from an individual knee joint and the horizontal line depicts the median. Data are from n=5 mice per group assessing both knee joints. *P<0.05; **P<0.01; ***P<0.001 by Mann-Whitney U test. (B) Cells from the knee-joints were isolated, RNA was extracted and gene expression for the indicated genes was quantified by quantitative RT-PCR and normalized for GAPDH. Data are the mean +SEM from 5 mice per group.

DISCUSSION

In the present study, we showed that anti-IL-23p19 antibody treatment, started after the onset of CIA, did not ameliorate arthritis severity. However, when anti-IL-23p19 antibody was administrated after type II collagen (CII)-immunization but before clinical signs of CIA onset, significantly less severe disease was observed. Finally, we showed in a memory T cell dependent antigen-induced arthritis model that IL-23 is essential for the development of full blown flare-up arthritis. In this model, synovial expression of IL-17A and IL-22 but not IFN-γ was markedly lower in the anti-IL-23p19 group compared to control, highlighting the role of IL-23 in memory T cell driven flare-up arthritis.
In contrast to our observation that neutralizing IL-23p19 after onset of CIA was not effective, Yago et al have reported that anti-IL-23 antibody administration after onset of CIA in rats significantly reduced paw volume and attenuated CIA (6). However, these authors did not find a significant difference in arthritis score between anti-IL-23 treated rats and controls rats when they started treatment after onset of CIA and no hard data regarding the degree of bone erosion was reported (6). Here, in the present study we clearly showed in repeated independent experiments no beneficial clinical effect of CIA when anti-IL-23 was administered after CIA onset. This indicates that IL-23 is not critical during this stage of CIA. However, it has been shown that IL-17A is still involved in the pathophysiology of CIA at this stage of the disease (10). This may indicate uncoupling of the IL-23/IL-17A axis during this stage of CIA and further studies will be needed to examine this hypothesis.

In the present study, we showed that IL-23 is redundant for the maintenance of CII-specific IL-17A production in autoimmune arthritis. We found the highest proportion of splenic IL-17A-expressing CD4+ T cells 10 days after CII/CFA-immunization, showing that immunization with type II collagen (CII) induced the generation of Th17 cells. In line with the critical role of IL-23 in the formation of CD4+ IL-17A+ T cells (5), we found that the formation of IL-17A-producing cells in vivo is depend on IL-23 since substantial lower levels of IL-17A were secreted upon stimulation of CII-primed IL-23p19-deficient splenocytes compared with wild-type control. However, using antigen specific re-stimulation in vitro, neutralizing IL-23 after the formation of CII-specific IL-17A-producing cells did not reduce IL-17A levels. Together, we confirmed that IL-23 is important in the generation of CII-specific IL-17A-producing cells but also showed that IL-23 is not required for IL-17A secretion by CII-specific effector T cells.

We then examined the in vivo role of IL-23 during the stage of CIA where CII-specific CD4+ IL-17+ T cells were already formed which is immunological quite different compared to the IL-23p19 deficient mice, since these mice lack IL-17A-expressing CD4+

T cells from the start (see discussion above, (5)). Neutralizing IL-23p19 after the induction of CII-specific of IL-17A-expressing CD4+ T cells did not prevent the onset of CIA as was described for the IL-23p19 deficient mice (5), and no difference was noted in disease incidence between anti-IL-23p19 treated mice and the control group. However, mice that developed CIA showed a significantly lower severity than control mice, indicating that IL-23 is involved in the regulation of disease progression during this stage of CIA (Figure 1D).

A surprising observation was the fact that the absolute numbers of CD4+ T cells, as well as several prominent pro-inflammatory cytokines, were increased in the spleens of DBA/1 mice treated with anti-IL-23p19 compared to isotype control treated mice, suggesting accumulation of T cells in secondary lymphoid organs as a consequence of decreased lymphocyte-migration. Notably, CCR7, reported to be involved in the migration of pathogenic T cells (12) and in the regulation of the IL-23/Th17 axis (7), was moderately increased after IL-23 neutralization. We next tested the hypothesis of decreased lymphocyte-migration by calculating the absolute numbers of CD4+, Th17 and Th1 cells spleen and ankles of mice treated before onset of CIA. Neutralization of IL-23 led to an increase of these T cell populations in spleen but not in ankles suggesting that the kinetics of T cells are not within the time points we investigated or that they act from a location other than the ankle. Nevertheless, the data is concurrent with other reports suggesting IL-23 involvement in lymphocyte migration (8-11). However, whether the decreased ankle/spleen ratio in anti-IL-23p19 treated mice was truly a matter of decreased egression or resulted from a disturbed proliferation/apoptosis balance remains to be elucidated.

Another plausible explanation for the reduced disease severity in mice treated with anti-IL-23p19 before CIA onset is the role of IL-23 in the generation of pathogenic Th17 cells (9, 11-12). IL-23 suppresses IL-10 production by IL-6/TGFβ-differentiated Th17 cells (13) as well as from ex-vivo isolated CII-primed CD4+ T cells (Lubberts et al., unpublished observations). Additionally, exogenous IL-23 enhances the production of IL-17A and IL-22 by CD4+ CII-primed T cells by down-regulating T-bet and FoxP3 and up-regulating RORγt (20). Altogether, this strongly suggests that IL-23 drives full maturation of pathogenic Th17 cells in vivo. Whether the Th17 cells have less pathogenic behavior after neutralizing IL-23p19 is at present under investigation.

The levels of CII-specific IgG's were influenced after neutralizing IL-23 during onset of CIA. Both IgG1 and IgG2a/2b were lowered indicating no specific regulation of B cell activity. From this study it is not clear whether IL-23 had a direct effect on B cell activity and CII-specific IgG formation or indirectly. No difference in expression of B

cell activation factors such as BAFF and APRIL were noted (data not shown). In contrast, no difference in the levels of CII-specific IgG's was noted after blocking IL-23 after onset of CIA which was accompanied with no clinical improvement of the disease during this stage of CIA. Thus, neutralizing IL-23 before disease onset effectively suppressed disease severity whereas anti-IL-23p19 treatment of mice after CIA onset did not ameliorate disease. This strongly suggests IL-23-dependent and -independent stages of CIA. In EAE (a mouse model for human multiple sclerosis), however, anti-IL-23p19 effectively ameliorated EAE when treatment was started at disease onset, peak of disease, or even during the first remission (Kikly et al., unpublished data). This difference in therapeutic efficacy of anti-IL-23p19 may be explained by the fact that the later stages of EAE autoimmunity are highly T cell dependent and less so in CIA. Indeed, our current data suggests that neutralizing IL-23 mainly dampens T cell activity and the lower anti-CII antibodies in mice treated with anti-IL-23p19 before, but not after onset of CIA highlighting the role of IL-23 on T cell dependent B cell activation (14).

To further dissect the role of IL-23 on memory T cell dependent pathology, we used an antigen-induced arthritic (AIA) flare-up model in which memory T cells are responsible for joint inflammation (15). Previously, we have shown that the primary antigen-induced arthritis (AIA) model is Th17 mediated, since IL-23p19 deficient mice were protected from progressive joint inflammation (16, 23). Here, we started treatment of anti-IL-23p19 antibody after the primary AIA was declined and no clinally joint inflammation was observed. Persistent blocking of IL-23 activity before inducing the AIA flare-up significantly prevented the expression of severe joint inflammation with clearly lower synovial mRNA expression of IL-17A and IL-22. This shows that memory T cell driven joint pathology is IL-23-dependent, a phenomenon comparable to the prevention of EAE relapses with anti-IL-23p19 or anti-IL-23R (8, 11).

In summary, we have shown IL-23-dependent and IL-23-independent stages during autoimmune CIA. Furthermore, the flare-up reaction of antigen-induced arthritis is IL-23-mediated. These data suggest that specific neutralization of IL-23 using an anti-IL-23p19 antibody after onset of autoimmune arthritis will not be beneficial as a therapeutic therapy for RA patients. However, antigen-driven memory T cell mediated joint inflammation seems to benefit from anti-IL-23 treatment.

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III

IL-23 promotes Th17 differentiation by inhibiting T-bet and FoxP3 and is required for IL-22 but not IL-21 in autoimmune experimental arthritis

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ABSTRACT

Objective

To unravel the role of IL-23 in subgroup polarization of IL-17A+ and/or IFN-γ+ T-cells in the prone autoimmune DBA-1 mice with-and-without collagen-induced arthritis.

Methods

CD4+ T-cells were isolated using the MACS system from the spleen of naïve and type II collagen (CII) immunized DBA-1 mice. These CD4+ T-cells were stimulated in vitro under Th0, Th1 or different Th17 conditions. Intracellular staining for IL-17A and IFN-γ was evaluated by flow cytometry. In addition, Th17 cytokines and T-helper-specific transcription factors were analyzed by ELISA and/or Q-PCR.

Results

In CD4+ T-cells from naïve DBA-1 mice, IL-23 alone hardly induced RORγt, Th17 polarization and Th17 cytokines but inhibited T-bet expression. In contrast, TGF-β/IL-6 was a potent inducer of RORγt, RORα, IL-17A, IL-17F, IL-21, and FoxP3 in these cells. IL-23 in contrast to TGF-β/IL-6 was critical for the induction of IL-22 in CD4+ T-cells from both naïve and CII-immunized DBA-1 mice. In line with these findings, IL-23 was more pronounced in inducing the IL-17A+IFN-γ- subset in CD4+ T-cells from CII-immunized mice. However, under naïve condition, IL-23 significantly increased the TGF-β/IL-6 induced Th17 polarization including elevated IL-17A and IL-17F levels and decreased T-bet and FoxP3 expression. Of note, the IL-23-induced increase of IL-17A and IL-17F was prevented in T-bet-deficient mice.

Conclusion

IL-23 promotes Th17 differentiation by inhibiting T-bet and FoxP3 and is required for elevation of IL-22 but not IL-21 in autoimmune arthritis. These data indicate different mechanisms for IL-23 and TGF-β/IL-6 at the transcription factor level during Th17 differentiation in autoimmune experimental arthritis.

INTRODUCTION

A novel pathogenic T cell population (Th17 cells) has been identified which induces autoimmune inflammation in mice (1). These Th17 cells are far more efficient at inducing Th1-mediated autoimmune inflammation in mice than classical Th1 cells (IFN-γ), although the pathogenic potential of Th17 as well as Th1 cells has been demonstrated (2-3). It was shown that Th17 cells were induced by IL-23 (p19/p40), a novel member of the IL-12 (p35/p40) family. Dendritic cells (DC) secrete cytokines of the IL-12 family such as IL-12 and IL-27 (p28/EBI3) and play a role in Th1 cell proliferation and their activation results in the production of IFN-γ. IL-23 induces the differentiation of naive T cells into

Th17 cells through a mechanism distinct from the signals driving the development of Th1 cells and Th2 cells. Neutralization of both IFN-γ and IL-4 has been found to favor IL-23-induced IL-17-producing cells (4-5). However, despite a requirement for IL-23 *in vivo* in the development of Th17 cell-mediated disease such as EAE and CIA (1, 6-7), IL-23 cannot drive Th17 cell differentiation from naïve CD4+ T cells (8) in vitro. This in vitro Th17 cell differentiation is driven by the combination of IL-6 and transforming growth factor-β1 (TGF-β1) (8-10). During Th17 development induced by IL-6 and TGF-β, Th17 cells become responsive to IL-23, which subsequently serves as a survival factor for committed Th17 cells (8). More recently, it was shown that IL-21 is an autocrine cytokine that is sufficient and necessary for Th17 differentiation (11-12).

Th17 cells are characterized by the expression of IL-17 (IL-17A) and also reportedly express IL-17F, IL-6, tumor necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-21, and IL-22 but neither interferon-γ (IFN-γ) nor IL-4 (1, 4-5, 11-16). In mice, Th17 cells have been established as a separate lineage of T helper cells distinct from conventional Th1 and Th2 cells (4-5). Transcription factors and signaling molecules that are important for the differentiation of Th1 or Th2 cells, including STAT-1, -4, -6 (signal transducers and activators of transcription) and T-bet, are dispensable for the development of Th17 cells (4-5). Recently, RORγt and RORalpha were discovered as novel Th17 transcription factors (17-18). Moreover, Th1 cytokines (IFN-γ and IL-12) and Th2 cytokines (IL-4, IL-13) repress Th17 cell development (4-5, 19).

IL-23 is required for the development of IL-17-producing T cells in collagen arthritic C57BL/6 mice since IL-23p19 deficient C57BL/6 mice showed a lack of these cells (7). However, the role of IL-23 in subgroup polarization of IL-17A+IFN-γ- (Th17), IL-17A+IFN-γ+ (‘double positive’), and IL-17A-IFN-γ+ (Th1) in the prone autoimmune DBA-1 mice with or without collagen arthritis is still unknown.

In the present paper, we demonstrated that IL-23 promoted Th17 differentiation by inhibiting T-bet and FoxP3 expression. Furthermore, IL-23 is required for IL-22 but not IL-21 in autoimmune experimental arthritis. These data indicate different mechanisms for IL-23 and TGF-β/IL-6 at the transcription factor level during Th17 differentiation in autoimmune experimental arthritis.

MATERIALS AND METHODS

Mice

DBA-1 mice were purchased from Harlan. B6.129S6-Tbx21tm1Glm/J (T-bet deficient) were purchased from The Jackson Laboratory, Bar Harbor, Maine, USA. Mice were kept under specific pathogen free conditions and provided with food and water *ad libithum*. Mice between 8-12 weeks of age were used for experiments. All experiments were approved by the Dutch Animal ethics committee (DEC).

CIA induction

CIA was induced by an intradermal injection of bovine type II collagen (CII) (Chondrex) emulsified in complete Freund's adjuvant (CFA) (Difco), as described (20). The animals were sacrificed 10 days after immunization.

Purification of T cells and in vitro T cell cultures

CD4+ T cells were purified from spleens from naive and CII-immunized DBA-1 mice and naïve T-bet deficient mice by negative MACS isolation. Purity of obtained fractions was typically >98%. CD4+ T cell fractions were cultured at concentrations of 1 x 10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) (BioWhittaker, Walkersville, MD), containing 10% heat-inactivated fetal calf serum (FCS) (Sigma, St. Louis, MO), 5 x 10^{-5} M β-mercaptoethanol (Merck, Darmstadt, Germany), supplemented with various cytokines (R&D Systems), as described below. Plates were coated with anti-CD3 and anti-CD28 (145-2C11; 37.51, BD Biosciences) at a concentration of 10 μg/ml each in PBS at 4°C overnight. For Th1 polarizing conditions, IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml; 11B11, Bioceros B.V., The Netherlands) were added. Th17 polarizing conditions included anti-IL-4 and anti-IFN-γ (5 μg/ml; XMG1.2, Bioceros B.V.) and additionally contained TGF-β (3 ng/ml), IL-6 (20 ng/ml), and/or IL-23 (50 ng/ml). At day 3, anti-CD3/CD28 activation was stopped. T cell cultures were expanded in the presence of the indicated cytokines, supplemented with IL-2 (5 ng/ml) for up to 7 days. Supernatant was collected, cells were collected for RNA isolation and the expanded cells were stained for intracellular cytokine detection.

Flow cytometric analyses

Single-cell suspensions from the spleen were prepared and incubated with mAb's for four-color flow cytometry as previously described (21). Monoclonal antibodies were purchased from BD Biosciences. For intracellular detection of cytokines, cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiStop (BD Biosciences) for 4h. Cells were harvested, extracellularly stained with anti-CD4 mAbs, followed by intracellular staining (IL-17A and IFN-γ, BD bioscience) using 2%

paraformaldehyde and 0.5% saponin. Samples were acquired on a FACSCalibur™ flow cytometer and analyzed using CELLQuest™ (Becton Dickinson, Sunnyvale, CA) and FlowJo (Tree Star, Inc., Ashland, CA). Live events were collected based on forward and side scatter.

ELISA

Cytokines in culture supernatants were measured by IL-17A and IL-21 ELISA (R&D systems); IFN-γ ELISA (OPTIEA BD Bioscience), IL-22 (Antigenix, USA), according to the manufacturer's instructions. IL-17F ELISA was performed using the R&D systems protocol using recombinant mouse IL-17F and coating IL-17F Ab which were kindly provided by Dr. J. Wright, Wyeth, Boston, MA, USA.

Quantitative PCR analyses

Total RNA was extracted using the GeneElute mammalian total RNA miniprep system (Sigma) and one μg was used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers. PCR Primers spanning at least one intron-exon junction were designed manually or using ProbeFinder software (Roche Applied Science, Indianapolis, In) and probes were chosen from the universal probe library (Roche Applied Science) or designed manually and purchased from Eurogentec (Seraing, Belgium). Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). To confirm the specificity of the amplification products, samples were analyzed by standard agarose gel electrophoresis. Threshold levels were set and further analysis was performed using the SDS v1.9 software (Applied Biosystems). The obtained Ct values were normalized to those of glycereraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

The paired Student-t-test was used to test differences between two groups within the naive or within the collagen population. P values less than 0.05 were considered significant.

RESULTS

IL-23 is more prominent in inducing the IL-17+IFN-γ- subset in CD4+ T cells from CII-immunized versus naïve DBA-1 mice

IL-23 did not markedly induce the IL-17+IFN-γ- subset (Th17) in vitro in CD4+ T cells isolated from the spleen of naïve DBA-1 mice in contrast to a marked induction of IL-17+IFN-γ- CD4+ T cells from collagen-type II (CII) immunized DBA-1 mice (Figure 1).

In contrast, TGF-β/IL-6 induced a relatively high percentage of the IL-17+IFN-γ- CD4+ T subset from naïve animals which was more prominent under CII-immunized condition (Figure 1). Under these CII-immunized condition, a lower percentage of TGF-β/IL-6 induced IL-17-IFN-γ+ (Th1) subset was noted compared to IL-23 alone (Figure 1). Furthermore, the percentage of IL-17+IFN-γ+ 'double positive' cells induced by TGF-β/IL-6 was higher compared to IL-23 alone. Adding IL-23 to TGF-β/IL-6 stimulated CD4+ T cells markedly increase the percentage of Th17, but not of Th1 and 'double positive' cells under naïve condition compared to TGF-β/IL-6 alone and this increase was less prominent under CII-immunized condition (Figure 1).

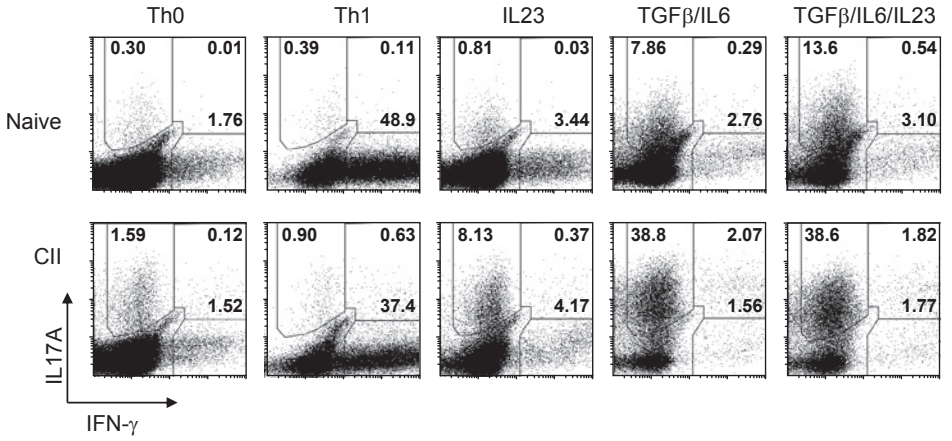

Figure 1. IL-23 markedly increase the TGFβ/IL-6 induced IL-17+IFN-γ- subset in CD4+ T cells from naïve DBA-1 mice. Flow cytometric analysis for intracellular IL-17A and IFN-γ expression in CD4+ T cells isolated from the spleen of naïve DBA-1 mice or ten days after immunization with type II collagen (CII). CD4+ T cells were activated with anti-CD3/anti-CD28 and cultured for seven days under Th0, Th1 (IL-12/anti-IL-4 antibody) or different Th17 (IL-23, TGF-β and IL-6, or IL-23, TGF-β and IL-6) condition. Numbers indicate the percentage of cells within a quadrant. Dates are representatives of 3 separate experiments wherein CD4+ T cells from a total 5 mice per group (naïve versus CII-immunized DBA-1 mice) were cultured and analyzed.

IL-23 decreases the expression of the Th1 specific transcription factor T-bet in CD4+ T cells

The Th1 specific T-bet expression was induced under Th1 culture condition as expected ($P<0.05$) (Figure 2A). IL-23 suppressed T-bet expression in CD4+ T cells isolated from the spleen from naïve mice compared to Th0 and Th1 conditions (both conditions $P<0.01$). Adding IL-23 to TGF-β/IL-6 resulted in a reduction of T-bet expression in CD4+ T cells from naïve DBA-1 mice compared to TGF-β/IL-6 alone, although not reaching a statistical significant difference ($P=0.07$) (Figure 2A). Of note, T-bet expression was significantly lower in TGF-β/IL-6 stimulated CD4+ T cells compared to Th0 condition from CII-immunized DBA-1 mice ($P<0.05$) (Figure 2A). In contrast to the naive condition,

IL-23 alone did not significantly suppressed T-bet expression in CD4+ T cells from CII-immunized DBA/1 mice compared to the CII/Th0 condition. However, compared to the CII/Th1 condition, under all three Th17 conditions T-bet expression was significantly suppressed (for all three conditions $P<0.05$) (Figure 2A). These data indicate that IL-23 directly or indirectly decreased the expression of T-bet in CD4+ T cells from both naïve and CII-immunized DBA-1 mice.

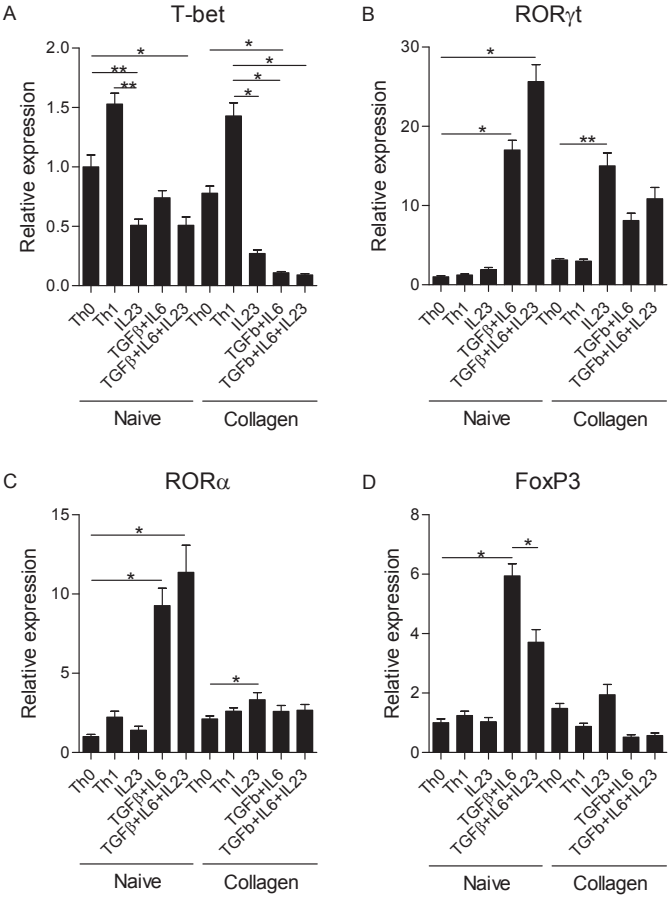

Figure 2. IL-23 suppressed T-bet expression and TGF-β/IL-6 induced FoxP3 expression. Quantitative RT-PCR analysis of: (A) T-bet; (B) RORγt; (C) RORα; (D) FoxP3 expression in seven days cultures of activated CD4+ T cells from Th0, Th1 (IL-12/anti-IL-4 antibody) or different Th17 condition (IL-23, TGF-β and IL-6, or IL-23, TGF-β and IL-6). Expression levels were normalized for GAPDH and values of activated CD4+ T cells from naïve DBA-1 mice were set to one. Values are the mean and SEM of 3 separate experiments wherein CD4+ T cells from a total of 5 mice per group (naïve versus CII-immunized DBA-1 mice) were cultured and analyzed. *P<0.05, **P<0.01, ***P<0.001, by paired Student-t-test.

IL-23 induces the Th17 transcription factor RORγt and RORα expression in CD4+ T cells from CII-immunized but not naïve DBA-1 mice

IL-23 hardly induced RORγt and RORα expression in CD4+ T cells from naïve DBA-1 mice when compared to TGF-β/IL-6 (Figure 2B,C). Adding IL-23 to TGF-β/IL-6 further increased RORγt (P=0.05) and RORα expression in CD4+ T cells from naïve DBA-1 mice, although not reaching a statistical significant difference (Figure 2B,C). Moreover, compared to the naïve situation, IL-23 significantly increased RORγt and RORα expression in CD4+ T cells from CII-immunized DBA-1 mice (P<0.001 and P<0.05, respectively) (Figure 2B,C). These data suggest that IL-23 influenced RORγt and RORα expression and that the increase in IL-17+IFN-γ- CD4+ T cells from naive DBA-1 mice after adding IL-23 to TGF-β/IL-6 (Figure 1) is accompanied by lower T-bet and elevated RORγt expression.

The TGF-β/IL-6 induced FoxP3 expression is inhibited by IL-23 in CD4+ T cells from naive DBA-1 mice

TGF-β/IL-6 but not IL-23 increased the regulatory T cell (Treg) transcription factor FoxP3 in CD4+ T cells from naive DBA-1 mice compared to Th0 condition (P<0.05) (Figure 2D). Interestingly, the presence of IL-23 significantly suppressed the TGF-β/IL-6 induced expression of FoxP3 in these cells (P<0.05) (Figure 2D). In contrast to CD4+ T cells from naive mice, FoxP3 expression was low under TGF-β/IL-6 condition tested in CD4+ T cells from CII-immunized DBA-1 mice (Figure 2D).

The expression of the Th2 transcription factor GATA-3 was significantly suppressed under all Th17 conditions in CD4+ T cells from naive and CII-immunized DBA/1 mice compared to the Th0 culture conditions (P<0.05) (data not shown).

These data indicate that in addition to the induction of RORγt and RORα, TGF-β/IL-6 also induces the Treg transcription factor FoxP3 and the expression of this transcription factor is suppressed by IL-23.

IL-23 has an additive effect on the IL-17A and IL-17F levels induced by TGF-β/IL-6

IL-23 alone is less potent in inducing IL-17A and IL-17F in CD4+ T cells isolated from naive DBA-1 mice compared to TGF-β/IL-6 (Figure 3A,B). Significantly higher levels of IL-17A and IL-17F were detected in the presence of TGFβ/IL-6 compared to IL-23 alone (both P<0.01). Although IL-23 alone induced a significant increase in IL-17A and IL-17F in CD4+ T cells from naïve DBA-1 mice (IL-17A: P<0.05 and IL-17F: P<0.01), the IL-23 induced IL-17A and IL-17F was more pronounced in CD4+ T cells isolated from CII-immunized DBA-1 mice (both P<0.01) (Figure 3A,B). In addition, when CD4+ T cells of both naïve and CII-immunized DBA-1 mice were incubated with the combination of IL-23 and TGF-β/IL-6 a significant increase in the levels of IL-17A (for both naive and CII

P<0.01) and IL-17F (for naive P<0.01 and CII P<0.05, respectively) was noted compared to TGF-β/IL-6 alone (Figure 3A,B).

To further examine the importance of suppressing T-bet expression by IL-23 for the increase in IL-17A and IL-17F by the combination of IL-23 and TGF-β/IL-6 compared to TGF-β/IL-6 alone, we used T-bet deficient mice. Interestingly, the lack of T-bet expression prevented the IL-23-induced increase in IL-17A and IL-17F under the triple combination (Figure 3C,D), underscoring the functional link between IL-23 and T-bet under the Th17 condition.

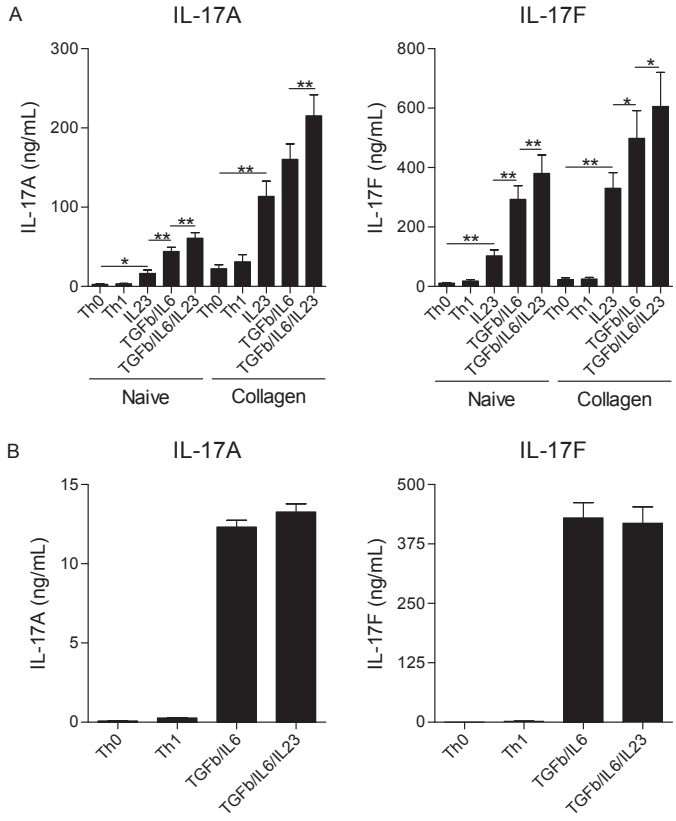

Figure 3. IL-23 increased the induction of IL-17A and IL-17F induced by TGFβ/IL-6 in CD4+ T cells from CII-immunized DBA-1 mice. ELISA analysis of IL-17A (A) and IL-17F (B) in seven days culture supernatants of activated CD4+ T cells from Th0, Th1 (IL-12/anti-IL-4 antibody) or different Th17 (IL-23, TGF-β and IL-6, or IL-23, TGF-β and IL-6) condition. Values are the mean and SEM of 3 separate experiments wherein CD4+ T cells from a total of 5 mice per group (naïve versus CII-immunized DBA-1 mice) were cultured and analyzed. *P<0.05, **P<0.01, by paired Student-t-test. ELISA analysis of IL-17A (C) and IL-17F (D) in seven days culture supernatants of activated CD4+ T cells from Th0, Th1 or Th17 (TGFbeta/IL-6) and Th17 (TGFbeta/IL-6) + IL-23 conditions. Values are the mean and SEM of CD4+ T cells from a total of 4 T-bet deficient mice per group.

TGF-β/IL-6 and not IL-23 is critical for the induction of IL-21

Incubation of CD4+ T cells isolated from naïve DBA-1 mice with TGF-β/IL-6 resulted in a significant increase in IL-21 level compared to Th0 condition (P<0.05) (Figure 4). In contrast, incubation of CD4+ cells from naïve mice with IL-23 showed a significantly lower IL-21 level compared to the Th0 condition (P<0.01) (Figure 4). These data show that IL-23 alone is not a potent inducer of IL-21, and no significant increase in IL-21 level was found after incubation of CD4+ T cells from naïve or CII-immunized DBA-1 mice with the combination IL-23/TGF-β/IL-6 compared to TGF-β/IL-6 alone (Figure 4). These data indicate that IL-23 is not critical for the increase of IL-21 in autoimmune arthritis.

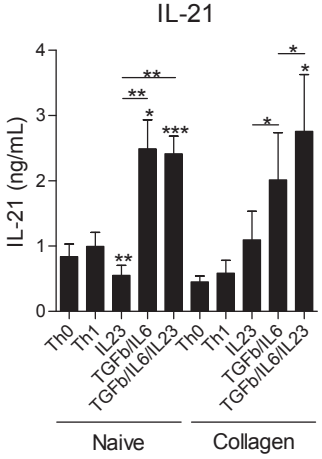

Figure 4. TGFβ/IL-6 and not IL-23 is critical for the induction of IL-21. ELISA analysis of IL-21 in seven days culture supernatants of activated CD4+ T cells from Th0, Th1 (IL-12/anti-IL-4 antibody) or different Th17 (IL-23, TGF-β and IL-6, or IL-23, TGF-β and IL-6) condition. Values are the mean and SEM of 3 separate experiments wherein CD4+ T cells from a total of 5 mice per group (naïve versus CII-immunized DBA-1 mice) were cultured and analyzed. *P<0.05, **P<0.01, ***P<0.001, by paired Student-t-test.

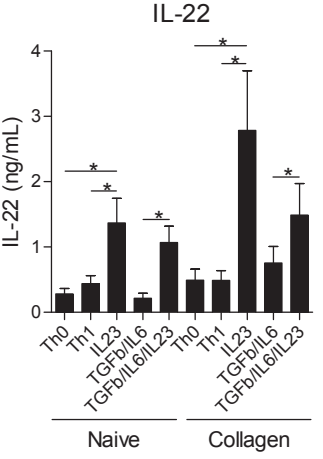

Figure 5. IL-23 and not TGF□/IL-6 is critical for the induction of IL-22. ELISA analysis of IL-22 in seven days culture supernatants of activated CD4+ T cells from Th0, Th1 (IL-12/anti-IL-4 antibody) or different Th17 (IL-23, TGF-β and IL-6, or IL-23, TGF-β and IL-6) condition. Values are the mean and SEM of 3 separate experiments wherein CD4+ T cells from a total of 5 mice per group (naïve versus CII-immunized DBA-1 mice) were cultured and analyzed. *P<0.05, by paired Student-t-test.

IL-23 is critical for the induction of IL-22

Th1 as well as Th17 cells can produce IL-22. Therefore, we examined the expression of IL-22 under both culture conditions using CD4+ T cells from naïve and CII-immunized DBA-1 mice. IL-23 is a potent inducer of IL-22 and significantly higher levels of this cytokine were found under Th17/IL-23 condition compared to Th0 and Th1 condition in CD4+ T cells from both naïve and CII-immunized DBA-1 mice (for all these conditions $P<0.05$) (Figure 5). Incubation with TGF-β/IL-6 alone did not result in a significant increase of IL-22 compared to the Th0 and Th1 conditions and this accounts for both CD4+ T cells isolated from naïve and CII-immunized DBA-1 mice (Figure 5). IL-23 was required to significantly increase IL-22 levels under TGF-β/IL-6 condition (naïve and CII both $P<0.05$) (Figure 5). These data indicate the requirement of IL-23 for elevated IL-22 induction in autoimmune arthritis.

DISCUSSION

In the present study, we analyzed the effects of IL-23 and/or TGF-β/IL-6 on the subgroup polarization of IL-17+IFN-γ- (Th17), IL-17+IFN-γ+ ('double positive' cells), and IL-17-IFN-γ+ (Th1) in the prone autoimmune DBA-1 mice with and without collagen-induced arthritis. We demonstrated that IL-23 and TGF-β/IL-6 differentially contributed to the induction of Th17 cytokines in the autoimmune collagen-induced arthritis model by regulating differently the expression of T helper transcription factors. We detected under Th17 polarizing conditions a higher percentage of Th17 cells in CD4+ T cells from CII-immunized compared to naïve DBA-1 mice. Also, 4 hours stimulated splenic CD4+ T cells isolated from CII-immunized DBA-1 mice ten days after immunization showed an increase in the percentage of IL-17+IFN-γ- CD4+ T cells (Th17) compared to splenic CD4+ T cells from naïve DBA-1 mice (data not shown). IL-23 was more pronounced in inducing IL-17+IFN-γ- (Th17) cells under CII-immunized conditions. Furthermore, we demonstrated that IL-23 promoted Th17 differentiation by inhibiting T-bet and FoxP3 expression. Moreover, IL-23 is required for IL-22 but not IL-21 in autoimmune experimental arthritis. Whether an environment with IL-23 or TGF-β/IL-6, or the combination of IL-23 and TGF-β/IL-6 will influence the pathogenic potential of these Th17 cells in experimental arthritis is at present under investigation.

It has been shown that IL-23 is essential for IL-17-producing CD4+ T cell function in collagen-induced arthritis (7). IL-23 is considered as a survival factor for Th17 cells and could also be involved in Th17 proliferation but is not essential for early Th17

polarization since naïve T cells do not express the IL-23 receptor (8-10, 22). Of note, T-bet expression was reduced in CD4+ T cells from naïve DBA-1 mice under IL-23 condition. However, these cells were not triggered by IL-23 to significantly increase their RORγt and RORα expression, although a small but significant increase in IL-17A and IL-17F levels was noted. In line with the idea that IL-23 is not essential for early Th17 polarization, in the present study we found that IL-23 was more pronounced in inducing Th17 cells under CII-immunized conditions. On the other hand, TGF-β, IL-6 and recently IL-21 has been recognized as key cytokines for the differentiation of naïve T helper cells into Th17 cells (11-12). Here, we showed that the combination TGF-β/IL-6 and not IL-23 was critical for IL-21 which is in line with the idea that TGF-β/IL-6 is important in early Th17 differentiation.

TGF-β is a potent inducer of the Treg transcritption factor FoxP3. In the present study, we found that TGF-β together with IL-6 significantly induced FoxP3 expression as well as RORγt. It has been reported that the transcription factor FoxP3 can bind to RORγt and therefore FoxP3 may directly regulate RORγt activity (23). Our data reveal that IL-23 markedly influenced the capacity of TGF-β/IL-6 to induce FoxP3 expression. In fact, TGF-β/IL-6 induced FoxP3 was significantly suppressed by IL-23 whereas RORγt expression was increased. These data suggest that IL-23 promotes Th17 polarization and activity by influencing the balance between FoxP3 and RORγt expression in CD4+ T cells. Moreover, this change in balance may also be important for the pathogenic potential of these Th17 cells and plasticity between Treg and Th17 cells in arthritis and this is at present under investigation (24).

IL-23 has an important role in regulating IL-22 expression (16, 19). Here we showed that IL-23 and not TGF-β/IL-6 was critical for IL-22 expression in CII-immunized DBA-1 mice. IL-22 is a member of the IL-10 family and can function as a pro-inflammatory cytokine. It has been shown that IL-22 mediated IL-23-induced acanthosis and dermal inflammation (19). Of interest, IL-22 together with IL-17A and/or IL-17F synergistically regulated the expression of β-defensin-2 and S100 calcium-binding protein A9 (S100A9) (16). However, IL-22 deficiency did not protect mice against EAE (25), but decreased the severity of CIA although the CIA incidence was enhanced ((26), Lubberts E et al., unpublished observations). Further studies may better elucidate the role of IL-22 in experimental arthritis and to evaluate the importance of the interaction and co-expression between IL-23 and IL-22 in particular.

IL-23 alone compared to TGF-β/IL-6 is not a strong inducer of RORγt, IL-17A and IL-17F in CD4+ T cells from naïve DBA/1 mice. Of note, IL-23 markedly increased the TGF-β/

IL-6 induced expression of IL-17A and IL-17F in CD4+ T cells from both naïve and CII-immunized mice. Also a trend of an increase in RORγt was found. Whether IL-23 truly induced RORγt or just increased the percentage of cells expressing RORγt is unclear. We observed that the cell numbers are not quite different between the groups, indicating that IL-23 stimulation does not result in a marked T cell proliferation. This suggests that IL-23 may increase truly RORγt expression in our CD4+ T cells although we can not exclude the possibility that IL-23 in particular increases the number of RORγt positive cells. The role of IL-17A in experimental arthritis has been well described (27). Recently, it was shown that IL-17F is an important regulator of inflammatory responses that seems to function differently than IL-17A in immune responses and diseases (28-29). It has been shown that the development of the spontaneous IL-1ra-/- arthritis was considerably, but only partially, suppressed in IL-17F-/-IL-1ra-/- mice compared to littermate controls (29). However, CIA developed normally in IL-17F-/- mice (29). However, further studies may better elucidate the role of IL-17F in experimental arthritis and the interaction of IL-17F with other Th17 cytokines.

IL-23 was able to suppress T-bet expression under Th17 conditions. However, IL-23 did not repress T-bet expression in Th0 or Th1 cells (data not shown). Interestingly, IL-23 was not able to increase IL-17A and IL-17F in TGF-β/IL-6 treated CD4+ T cells from T-bet deficient mice. These data are the first to our knowledge to show a functional link between IL-23 and the Th1 transcription factor T-bet under a Th17 condition.

In conclusion, this study revealed that IL-23 promotes Th17 differentiation by inhibiting T-bet and FoxP3 and is required for elevation of IL-22 but not IL-21 in autoimmune arthritis. These data indicate different mechanisms for IL-23 and TGF-β/IL-6 at the transcription factor level during Th17 differentiation in autoimmune experimental arthritis. Further studies are needed to unravel the pathogenic potential of IL-23 or TGF-β/IL-6, or the combination of IL-23 and TGF-β/IL-6 induced Th17 cells in experimental arthritis.

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IV

Interleukin-23 is critical for full-blown expression of a non-autoimmune destructive arthritis and regulates interleukin-17A and RORγt in γδ T cells

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ABSTRACT

Introduction

Interleukin (IL)-23 is essential for the development of various experimental autoimmune models. However, the role of IL-23 in non-autoimmune experimental arthritis remains unclear. Here, we examined the role of IL-23 in the non-autoimmune antigen-induced arthritis (AIA) model. In addition, the regulatory potential of IL-23 in IL-17A and retinoic acid-related orphan receptor gamma t (RORγt) expression in CD4+ and TCRγδ+ T cells was evaluated systemically as well as at the site of inflammation.

Methods

Antigen-induced arthritis was induced in wild-type, IL-23p19-deficient and IL-17 Receptor A – knockout mice. At different time points, synovial cytokine and chemokine expression was measured. At days 1 and 7 of AIA, splenocytes and joint-infiltrating cells were isolated and analyzed for intracellular IL-17A and interferon (IFN)-γ *ex-vivo* by flow cytometry. In Splenic $CD4^+$ and $TCR\gamma\delta^+$ T cells gene expression was quantified by flow cytometry and quantitative PCR.

Results

IL-23 was critical for full-blown AIA. Lack of IL-23 did not prevent the onset of joint inflammation but stopped the progression to a destructive synovitis. IL-23 regulated IL-17A expression in CD4+ T cells in the spleen. Of note, IL-17A and IFN-γ expression was reduced in $CD4^+$ T cells in the inflamed joints of IL-23p19-deficient mice. Interestingly, IL-23 was also critical for the induction of IL-17A and RORγt but not IFN-γ in $TCR\gamma\delta^+$ T cells in the inflamed joints. The importance of the IL-23/IL-17 axis was further confirmed using IL-17 Receptor A knockout mice showing significantly milder AIA compared to control mice, with a disease course comparable to that of IL-23p19-deficient mice.

Conclusion

These data show that IL-23 is critical for full-blown expression of a non-autoimmune destructive arthritis and regulates the proportion of IL-17A and IFN-γ-positive $CD4^+$ T cells at the site of inflammation. Furthermore, IL-23 regulates IL-17A and RORγt expression in TCRγδ T cells in arthritis. These findings indicate that regulating the IL-23 pathway may have therapeutic potential in non-autoimmune arthritis.

INTRODUCTION

Interleukin (IL)-23 is a member of the IL-12 family and consists of both an IL-23-specific p19 subunit, and of a p40 subunit which is shared with IL-12 (1). IL-23 is elevated in many autoimmune diseases such as psoriasis, rheumatoid arthritis (RA), multiple sclerosis (MS), and inflammatory bowel disease (IBD) (2). It has been shown in animal

models that IL-23, and not IL-12, is critical in the induction of autoimmunity (3-7). Mice deficient for IL-23p19 were fully protected against collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune uveitis (EAU), in contrast to IL-12p35 deficient mice (3-4, 6). Although initial studies show that the development of autoimmunity was through Th17 cells, recent data show that also Th1 cells are able to induce pathology (6, 8). Thus, the role of Th17 and Th1 cells and their interaction in these models needs further clarification (9). However, in all these autoimmune models it is evident that IL-23 is essential in their development. Still, it has not been elucidated whether IL-23 is critical for the progression of arthritis into a non-autoimmune destructive arthritis.

In collagen-immunized IL-23p19 knockout mice, no IL-17A-producing $CD4^+$ T cells were noted although no difference in IFN-γ-producing $CD4^+$ T cells was observed (4). This indicates that IL-17A plays an important role in the early phase of CIA which is in line with earlier observations (10-11). Apart from Th17 cells, other cells such as $CD8^+$, NKT, and $TCR\gamma\delta^+$ T cells are able to produce IL-17A (12-13) and it has been shown that $TCR\gamma\delta^+$ T cells produced relatively high levels of IL-17A in CIA (14) and, in fact are the predominant source of IL-17-producing cells in the CIA joint (15). Depletion of IL-17A-producing $V\gamma4^+$ $TCR\gamma\delta^+$ T cells resulted in a significant reduction of the clinical disease score although mice were not fully protected (14). However, the role of IL-23 in regulating IL-17A production in these $TCR\gamma\delta^+$ T cells is unknown.

Here, our results revealed that IL-23 is essential for the development of full-blown antigen-induced arthritis. We used IL-23p19 knockout (IL-23p19KO) mice to demonstrate that lack of IL-23 did not prevent the onset of joint inflammation but stopped the progression to a destructive synovitis. In the joints of IL-23p19KO mice, the proportions of IL-17A and IFN-γ-positive $CD4^+$ T cells were reduced. $TCR\gamma\delta^+$ T cells also required IL-23 for IL-17A but not for IFN-γ production in the inflamed joints. Of note, the transcription levels of RORγt were significantly higher in $TCR\gamma\delta^+$ T cells than in $CD4^+$ T cells from wild type mice. The importance of the IL-23/IL-17 axis was further confirmed using IL-17 Receptor A knockout (IL-17RAKO) mice showing a similar arthritis expression as IL-23p19KO mice. Thus, IL-23 is critical for full-blown expression of a non-autoimmune destructive arthritis. Furthermore, IL-23 regulates IL-17A and RORγt expression in $TCR\gamma\delta^+$ T cells during joint inflammation.

MATERIALS AND METHODS

Antigen-induced arthritis

IL-23p19 knockout mice were kindly provided by Dr. N. Ghilardi, Genentech Inc., San Francisco, CA, USA (16), and IL-17 Receptor A knockout mice by Dr. J. Tocker, Amgen Inc., Seattle, WA, USA (17). Both strains were backcrossed on the C57BL/6 background for at least 10 generations. Mice were kept under specific pathogen free conditions and provided with food and water *ad libitum*. Mice between 8 and 12 weeks of age were used for experiments. All experiments were approved by the Dutch Animal Ethics Committee (DEC).

To induce AIA, methylated Bovine Serum Albumin (mBSA, 8 mg/mL) was emulsified in an equal volume of CFA containing 1 mg/mL heat-killed *M. tuberculosis* (H37Ra; Difco). At day -7, mice were immunized intradermal with 100 μL mBSA/CFA. One week later, 60 μg mBSA was injected intra-articular to induce mono-arthritis. The severity of arthritis in the knee joint was scored macroscopically on a scale of 0 to 2 after removing the skin. At different time points during AIA, rear limbs were hematoxylin and eosin stained as previously described (18), and the severity of joint infiltration and bone erosion was determined on a scale from 0 to 3 (0 = no infiltration, 1 = mild infiltration, 2 = moderate infiltration, 3 = maximal infiltration; 0 = no erosion, 1 = mild erosion, 2 = moderate erosion and 3 = maximal erosion).

Synovial cytokine levels

To measure synovial cytokine levels, patellae with adjacent synovium was isolated from knee joints as described earlier (18). MCP-1, TNF-α, IL-6, IFN-γ, IL-12p70 and IL-10 were measured using the cytometric bead array using the mouse inflammation kit with a detection limit of 10 pg/mL (BD Biosciences, Sunnyvale, CA, USA). IL-17A was measured by ELISA (R&D Systems, Minneapolis, MN, USA).

Single-cell isolation and flow-cytometric analyses

With Blendzyme3 (60 μg/mL, Roche Diagnostics, Mannheim, Germany), cells from inflamed joints were isolated (18). For intracellular detection of cytokines, we stimulated splenocytes or cells isolated from the joints with phorbol myristate acetate (PMA) (0.05 μg/mL) and Ionomycin (0.5 μg/mL) in the presence of GolgiStop (BD Biosciences) for four hours. For intracellular cytokine staining, cells were fixed using 2% PFA and permeabilized in 0.5% saponin. For intracellular staining of RORγt, T-bet and GATA-3, a Fixation and Permeabilization kit (eBioscience, San Diego, CA, USA) was used. Samples were acquired on a FACS Calibur or on a FACS CANTO flow cytometer and analyzed using FlowJo (Tree Star, Inc., Ashland, OR, USA) software.

Purification of effector T cells and in vitro T cell stimulation

$CD3^+CD4^+$ and $CD3^+TCR\gamma\delta^+$ T cells were FACS-sorted from spleens obtained at day 7 of AIA using a FACS Aria cell sorting system and BD FACS Diva software (BD Bioscience). Purity of obtained fractions was >98%.

Quantitative PCR analyses

Total RNA of sorted $CD3^+CD4^+$ and $CD3^+TCR\gamma\delta^+$ T cells was extracted, and DNaseI-treated RNA was used for cDNA synthesis (18). PCR primers were designed manually or using ProbeFinder software (Roche Applied Science, Indianapolis, In, USA) and probes were chosen from the universal probe library (Roche Applied Science). Quantitative realtime PCR was performed using the ABI Prism 7900 HT sequence-detection system (Applied Biosystems, Foster City, CA) and analyzed using SDS v2.3 software (Applied Biosystems). The Ct values obtained were normalized to those of glycereraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Differences between groups were tested with the Mann-Whitney *U* test or the unpaired student t-test. P values less than 0.05 were considered significant.

RESULTS

IL-23 has a critical role in the progression of destructive arthritis

To investigate the role of IL-23 in the progression of a T cell mediated destructive inflammatory arthritis, we induced antigen-induced arthritis (AIA) in WT and IL-23p19 knockout (IL-23p19KO) mice by immunization with methylated BSA (mBSA) in Complete Freund's Adjuvant (CFA) and induced mono-arthritis one week later by a single intra-articular injection of mBSA into the knee joint. Maximum arthritis score was observed at day 4, which stayed high until day 10 (Figure 1A). In contrast, in IL-23p19KO mice the onset of arthritis was not prevented although significantly milder joint inflammation was noted which decreased rapidly to almost normal at day 10. Seven days after the induction of mono-arthritis, histological analyses revealed significantly less joint infiltration (Figure 1B) and bone erosion (Figure 1C) in IL-23p19KO mice than in WT mice. These data show that IL-23 is required for the progression of AIA into destructive synovitis.

To gain insight in the local chemokine and cytokine expression, synovial washouts from WT and IL-23p19KO mice were taken at different time points during AIA. The highest expression of MCP-1 was measured at day 1 which was significantly lower in

IL-23p19KO compared to WT mice (Figure 1D). TNF-α, was significantly lower in IL-23p19KO than in WT mice at days 1 and 2, and undetectable at day 7 (Figure 1E). Also IL-6 was measured in synovial washouts but no statistical significant differences of this cytokine were observed between WT and IL-23p19KO mice at days 1 and 2 (Figure 1F). Of high interest, IL-17A and IFN-γ levels were significantly lower in IL-23p19KO than in WT mice at day 1 of AIA (Figure 1G and 1H). Of note, no IL-12p70 and IL-10 could be detected (data not shown).

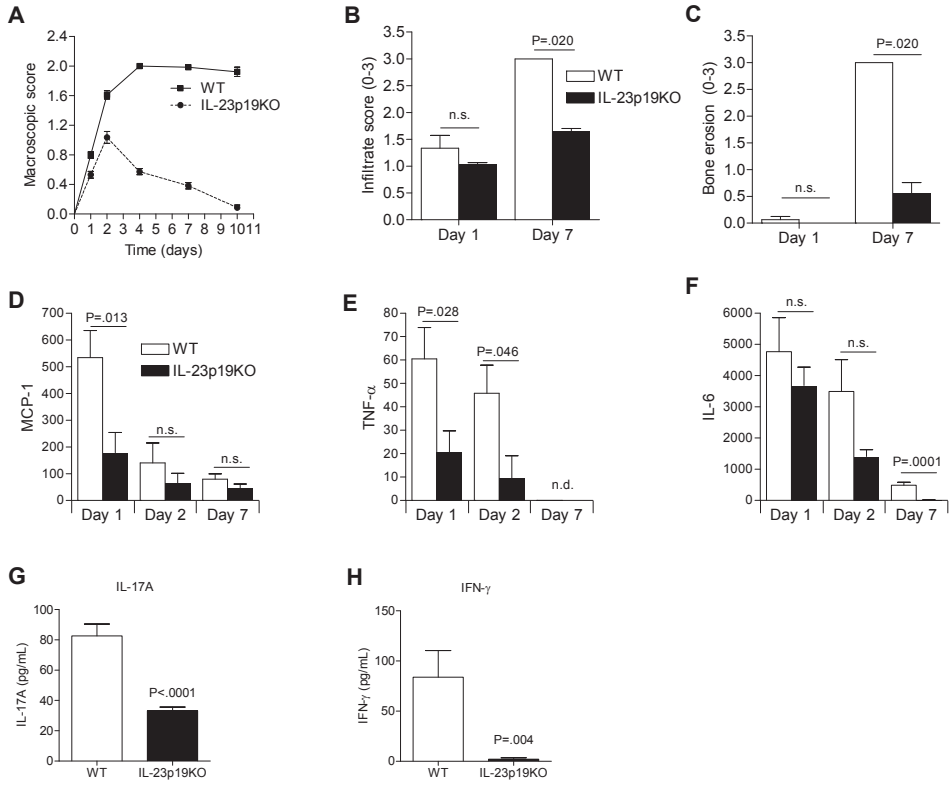

Figure 1. IL-23 has a critical role in the progression of antigen-induced arthritis. WT and IL-23p19KO mice were immunized with mBSA/CFA and 1 week later mono-arthritis was induced by injecting mBSA directly into the knee joints. (A) Arthritis score was determined macroscopically at different time points. Mean values and SEM are given for 8-31 mice per group. Data are obtained from 3 separate experiments. * $p<0.001$, WT vs IL-23p19KO. (B) Histological analyses of joint inflammation and, (C) bone erosion after H&E staining. (B-C) Mean values and SEM are given from two separate experiments with a total of 20 knee joints per group. (D-F) Cytokine levels in synovial washouts taken at days 1, 2 and 7. (G-H) IL-17A and IFN-γ levels in synovial washouts taken at day 1. Mean values and SEM are given for 5-10 washouts obtained from two separate experiments.

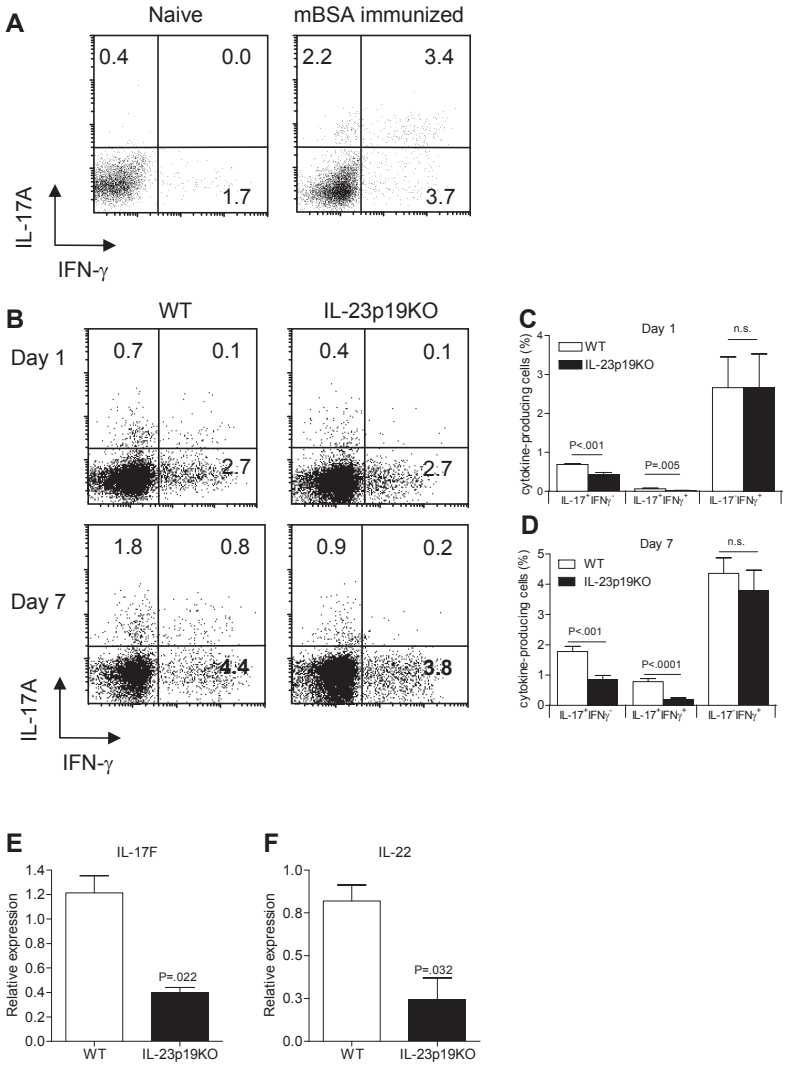

Figure 2. The induction of Th17 cells in AIA is IL-23 mediated. (A) WT mice were immunized with mBSA/CFA and 10 days later splenocytes were isolated and stimulated for 4h with PMA/Ionomycin, gated for $CD4^+$ T cells and analyzed for intracellular IL-17A and IFN-γ expression. Numbers indicate percentage of positive cells within each quadrant. (B-D) Antigen-induced arthritis was induced in WT and IL-23p19KO mice and at days 1 and 7 after i.a. mBSA injection the splenocytes were isolated and stimulated for 4h with PMA/Ionomycin and analyzed for intracellular IL-17A and IFN-γ expression on a $CD4^+$ T cell gate. (B) Numbers indicate percentage of positive cells within each quadrant. (C-D) Quantification of flow cytrometric analyses from (B); mean values and SEM are given for 6-12 mice per group from 2-4 independent experiments. (E-F) On day 7 of AIA, splenic $CD3^+CD4^+$ T cells were FACS-sorted and gene expression was analyzed by quantitative RT-PCR for IL-17F and IL-22 respectively. Mean values and SEM are given for 3 mice. P-values were calculated using the student t-test.

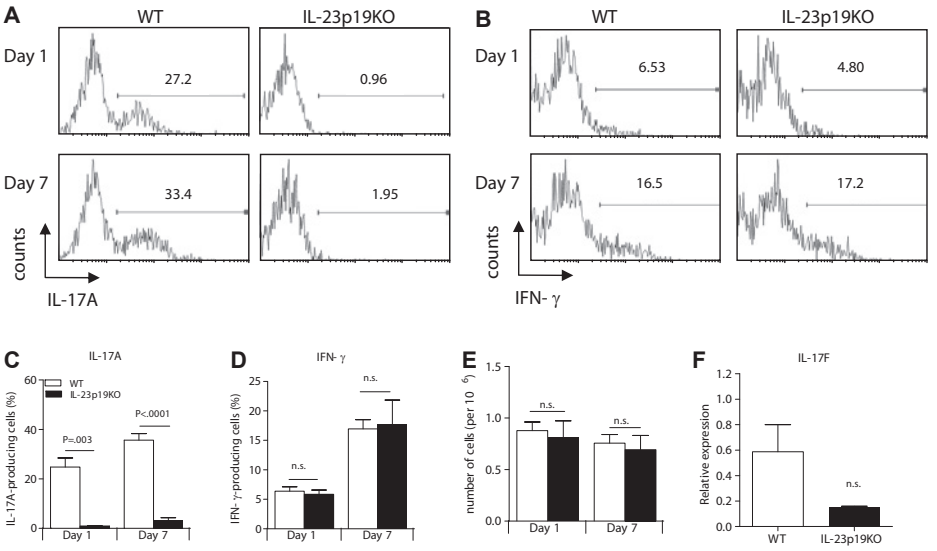

Figure 3. IL-23 is critical for the induction of IL-17A and RORγt in TCRγδ T cells. (A-B) At days 1 and 7 of AIA, splenocytes were isolated and stimulated for 4h with PMA/Ionomycin, gated for $CD3^+TCR\gamma\delta^+$ T cells and analyzed for intracellular IL-17A and IFN-γ respectively. Numbers in quadrants indicate percentage of cytokine-positive cells. (C-D) Quantification of flow cytrometric analyses from *A* and *B* for IL-17A and IFN-γ; mean values and SEM are given for 3-6 mice for day 1 and for 9 mice for day 7. (E) Cell counts of total amount of $TCR\gamma\delta^+$ T cells present in spleen on day 1 and 7 of AIA in WT and IL-23p19KO mice. (F) On day 7 of AIA, splenic $CD3^+TCR\gamma\delta^+$ T cells were FACS-sorted and IL-17F gene expression was analyzed by quantitative RT-PCR. Mean values and SEM are given for 3 mice. P-values were calculated using the student's t-test.

IL-23 is essential for the induction of IL-17A and RORγt in $TCR\gamma\delta^+$ T cells during arthritis

To investigate whether $TCR\gamma\delta^+$ T cells were present during AIA and able to produce IL-17A as was described for CIA [14], we isolated splenocytes at days 1 and 7 of AIA. On day 1, a relatively high proportion of $TCR\gamma\delta^+IL\text{-}17A^+$ T cells was detected in the spleen of WT mice, and this proportion was elevated at day 7 (Figure 3A and 3C). Interestingly, on these time points, a significantly lower proportion of $TCR\gamma\delta^+IL\text{-}17A^+$ T cells was present in IL-23p19KO than in WT mice (Figure 3A and 3C), which was not associated with lower numbers of splenic $TCR\gamma\delta^+$ T cells (Figure 3E). Of note, the proportion of $TCR\gamma\delta^+IL\text{-}17A^+$ T cells was 15- to 27-fold reduced in IL-23p19KO mice versus WT mice (Figure 3A and 3C) compared to a two-fold reduction of $CD4^+IL\text{-}17A^+$ T cells (Figure 2B-D). The proportions of both IL-17A-positive $TCR\gamma\delta^+$ and $CD4^+$ T cells isolated from the draining lymph-nodes were substantially reduced in IL-23p19KO mice versus WT mice (Figure S3 in Additional file 1). Notably, since the proportion of IL-17A-positive $CD4^+$ and $TCR\gamma\delta^+$ T cells were not decreased in splenocytes taken from naïve IL-23p19KO compared to WT mice, we can exclude that there is an intrinsic deficiency of PMA/

Ionomycin-induced IL-17A-production in IL-23p19KO cells (Figure S2 in Additional file 1). These data indicate that the IL-17A production in TCRγδ$^+$ T cells during AIA is highly IL-23 dependent.

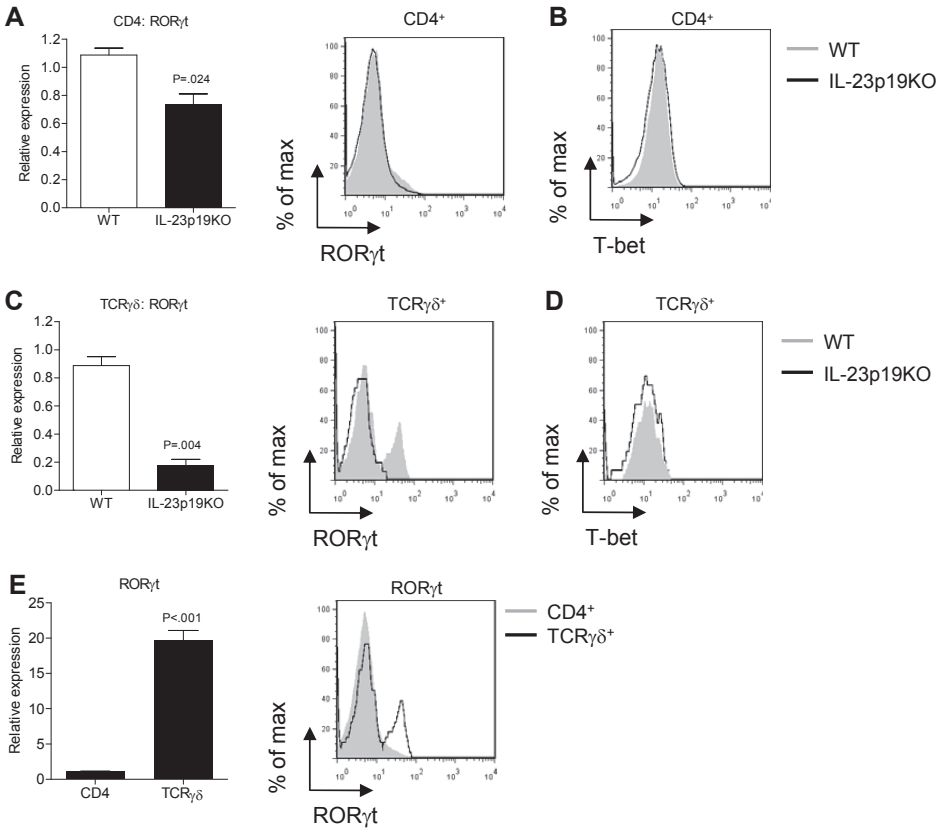

Figure 4. IL-23 is essential for RORγt expression in TCRγδ$^+$ T cells. At day 7 of AIA, WT and IL-23p19KO mice were sacrificed and splenocytes were isolated. (A) CD3$^+$CD4$^+$ T cells were FACS-sorted and RORγt gene expression was analyzed by quantitative RT-PCR (left panel); RORγt was measured intracellular and CD4$^+$ T cells were gated (right panel). (B) T-bet expression was measured intracellular and CD4$^+$ T cells were gated. (C) CD3$^+$TCRγδ$^+$ T cells were FACS-sorted and RORγt gene expression was analyzed by quantitative RT-PCR (left panel); RORγt was measured intracellular and γδ$^+$ T cells were gated (right panel). (D) T-bet expression was measured intracellular and γδ$^+$ T cells were gated. (E) *C*omparison of the mRNA quantification of RORγt expression between FACS-sorted CD4$^+$ and γδ$^+$ T cells. Mean values and SEM are given for 3 mice and P-values were calculated using the student's t-test.

IL-23 regulates RORγt, but not T-bet, in TCRγδ$^+$ T cells

Since the proportion of CD4$^+$ IL-17A$^+$IFN-γ$^-$ but not IL-17A$^-$IFN-γ$^+$ T cells was significantly lower in IL-23p19KO than in WT mice, we measured RORγt mRNA expression in FACS-sorted splenic CD4$^+$ and TCRγδ$^+$ T cells isolated at day 7 of AIA.

In addition, RORγt, T-bet and GATA3 expression was measured by intracellular protein-stainings. No GATA3 expression by flow cytometry was found in neither CD4$^+$ nor in TCRγδ$^+$ T cells (data not shown). Figure 4A shows that RORγt was decreased in CD4$^+$ T cells from IL-23p19KO mice compared to WT mice, while T-bet expression was similar (Figure 4B). In TCRγδ$^+$ T cells, the expression of RORγt was also significantly reduced by lack of IL-23p19 (Figure 4C), while T-bet expression was similar between WT and IL-23p19KO γδ T cells (Figure 4D). Interestingly, RORγt was expressed at higher levels in WT TCRγδ$^+$ T cells than in WT CD4$^+$ T cells (Figure 4E).

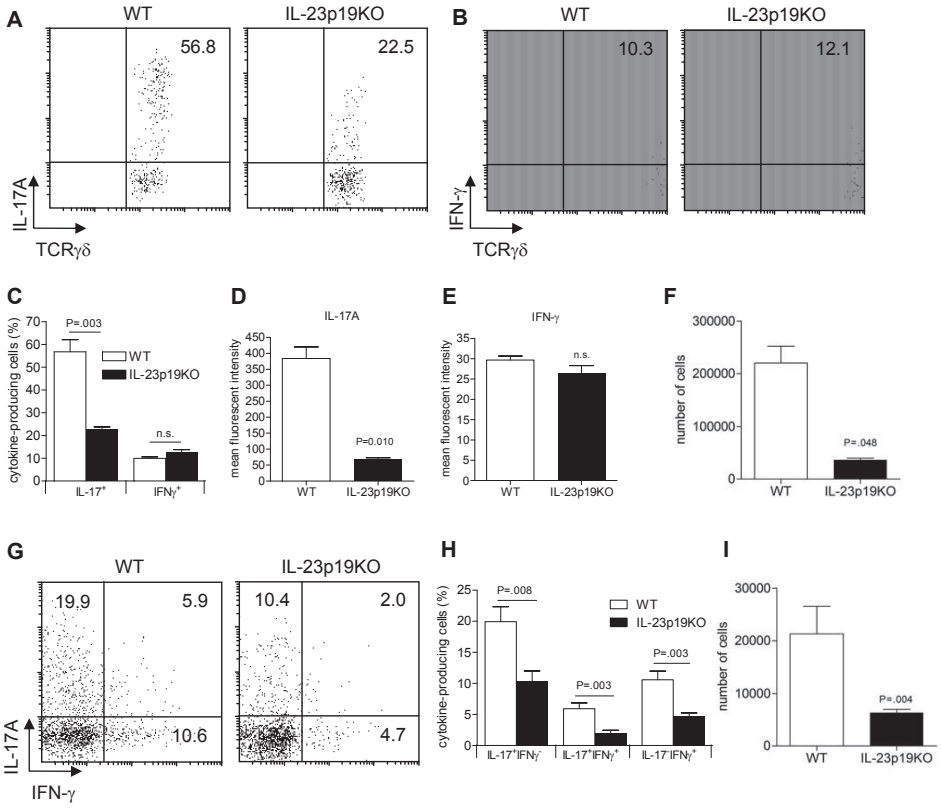

Figure 5. IL-23 deficiency results in less IL-17 production in the inflamed joint. At day 7 of AIA, cells from the arthritic joints of WT and IL-23p19KO mice were isolated and stimulated for 4h with PMA/Ionomycin. (A) Intracellular cytokine staining of TCRγδ$^+$ T cells for IL-17. (B) Intracellular cytokine staining of TCRγδ$^+$ T cells for IFN-γ; data from (A-B) are representatives from 3 mice per group. (C) Quantification of flow cytrometric analyses from (A-B). (D) MFI was calculated for TCRγδ$^+$ IL-17$^+$ T cells. (E) MFI was calculated for TCRγδ$^+$ IFN-γ$^+$ T cells. (F) Total numbers of TCRγδ$^+$ T cells in the joints of arthritic WT and IL-23p19KO mice. (C-F) Mean values and SEM are given for 3 mice and P-values were calculated using the student t-test. (G) Intracellular cytokine staining of CD4$^+$ T cells for IL-17A and IFN-γ. Data are representatives of 6 mice per group. (H) Quantification of flow cytrometric analyses from (G). (I) Total numbers of CD4$^+$ T cells present in arthritic joints from WT and IL-23p19KO mice.

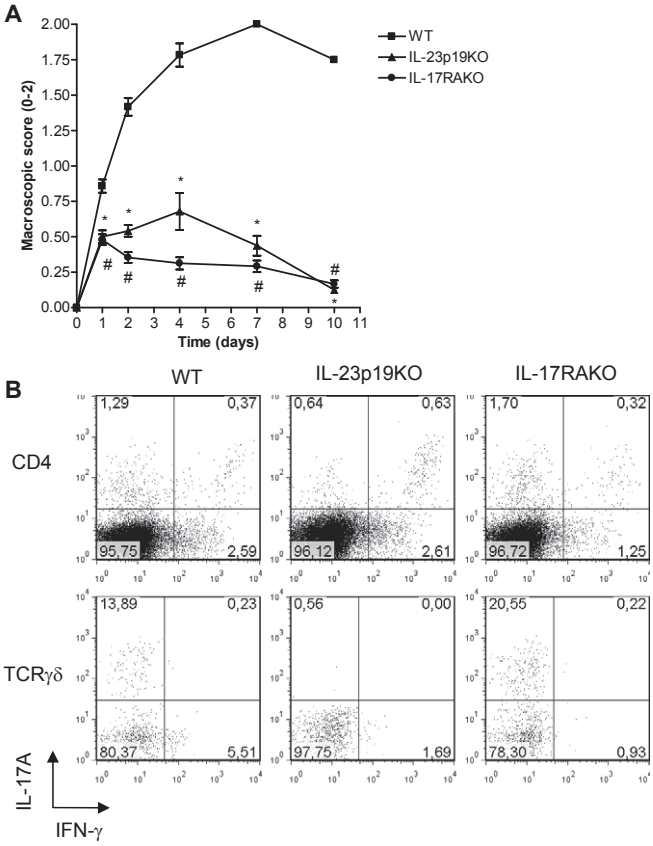

Figure 6. IL-17 receptor signaling is critical for the progression of AIA. (A) WT, IL-23p19KO and IL-17RAKO mice were immunized with mBSA/CFA and mono-arthritis was induced 1 week later by an i.a. injection of mBSA. At days 1, 2, 4, 7 and 10 macroscopic scores were assessed. Mean values and SEM are given for 6 mice per group. *, P<0.01 WT vs IL-23P19KO and #, P<0.01 WT vs IL-17RAKO. (B) At day 7 of AIA, splenocytes from WT, IL-23p19KO and IL-17RAKO were isolated and stimulated for 4h with PMA/Ionomycin and analyzed for intracellular IL-17A and IFN-γ expression on a $CD4^+$ and $TCR\gamma\delta^+$ T cell gate. Numbers indicate percentage of positive cells within each quadrant and each dot plot shows a representative mouse from 3 mice per group analyzed.

IL-23 is essential for local IL-17A-production by $TCR\gamma\delta^+$ and $CD4^+$ T cells

Since severe joint inflammation was observed in WT but not in IL-23p19KO mice at day 7 of AIA (Figure 1A), we wondered whether the proportions of IL-17A-producing cells in the inflamed joints of WT and IL-23p19KO mice were different. A significantly lower proportion of IL-17A, but not of IFN-γ-producing $TCR\gamma\delta^+$ T cells was observed in the inflamed joints of IL-23p19KO mice compared to WT mice (Figure 5A-C; Figure S4 in Additional file 1 shows the gating-strategy we used to plot $TCR\gamma\delta^+$ T cells isolated from the joint). Interestingly, the mean-fluorescent intensity (MFI) of WT IL-17A-producing

TCRγδ$^+$ T cells was significantly higher than the MFI of these cells in IL-23p19KO (Figure 5D). In contrast, the MFI of IFN-γ in the IFN-γ-producing TCRγδ$^+$ T cells was similar between IL-23p19KO and WT controls (Figure 5E). In line with the reduced level of inflammation observed in IL-23p19KO mice (Figure 1B), the total number of TCRγδ$^+$ T cells was lower in the arthritic joints from IL-23p19KO mice than from WT mice (Figure 5F).
At the site of inflammation, the proportions of CD4$^+$ IL-17A$^+$IFN-γ$^-$, IL-17A$^+$IFN-γ$^+$ *double positive* and IL-17A$^-$IFN-γ$^+$ T cells were markedly elevated compared to the spleen (Figures 2B-D and 5G-H). In addition, in IL-23p19KO mice, the absolute numbers of CD4$^+$ T cells were significantly lower compared to WT mice (Figure 5I).

Critical role for the IL-23/IL-17 immune pathway in the progression of destructive arthritis
Since the proportions and numbers of IL-17A but also of IFN-γ-producing CD4$^+$ T cells in the inflamed joint were lower in IL-23p19KO than in WT mice (Figure 5G-I), we investigated the importance of IL-17A signaling using IL-17 receptor A knockout (IL-17RAKO) mice. IL-17RAKO mice showed a similar pattern of arthritis as IL-23p19KO mice did, and this was significantly suppressed in both mouse knockout strains compared to WT mice (Figure 6A). The lack of IL-17R-signalling did however not lead to a reduced proportion of IL-17A-positive CD4$^+$ or TCRγδ$^+$ T cells (Figure 6B).

DISCUSSION

Our findings show for the first time that IL-23 is critical for full-blown non-autoimmune antigen-induced arthritis. IL-23 deficiency did not prevent the onset of joint inflammation but stopped the progression to a destructive synovitis. In the inflamed joint, IL-17A and IFN-γ-producing CD4$^+$ T cells were reduced under IL-23-deficient conditions. In addition, IL-23 was also essential for the induction of IL-17A and RORγt, but not for IFN-γ, in TCRγδ$^+$ T cells both systemically and locally at the site of inflammation. The importance of the IL-23/IL-17 immune pathway was confirmed by using IL-17RAKO mice which developed arthritis similar to IL-23p19KO mice. These data show that IL-23 is critical in the regulation of a non-autoimmune inflammatory arthritis and that it regulates IL-17A and RORγt expression in CD4$^+$ and TCRγδ$^+$ T cells.

IL-23 is required for Th17 function in vivo and is considered to be a survival factor for these T cells (19-20). TGF-β and IL-6 in contrast to IL-23 are critical in Th17 polarization from naïve T cells (21-23). Interestingly, TGF-β and IL-6 drive the production of IL-17A and IL-10 by T cells and restrain Th17 cell-mediated pathology (24). In addition, IL-23 promotes the proinflammatory Th17 profile induced by TGF-β and IL-6 and is required

for IL-22 expression [(25-26) and Lubberts et al. unpublished observations]. In the present study, we found that IL-23 deficiency did not prevent the onset of antigen-induced arthritis. Although MCP-1, TNF-α, IL-17A and IFN-γ as well as joint inflammation were suppressed in the early phase of arthritis in IL-23p19KO compared to WT mice, these data indicate that the early joint inflammatory responses in this non-autoimmune T cell mediated arthritis-model is at least partly IL-23 independent. In contrast, further progression of arthritis into a chronic destructive arthritis is IL-23 dependent. In this stage of the disease effector T cells migrate from the lymphoid tissues to the inflamed joint and play an important role in the progression of arthritis. Whether this is solely a matter of Th17 cells or that other IL-17A producing cells are involved as well needs further investigation, especially since we now found that IL-23 regulates IL-17A production in TCRγδ$^+$ T cells.

In addition to Th17 cells there are other IL-17A-producing T cells such as CD8$^+$, NKT and TCRγδ$^+$ T cells (27-31). A direct role for TCRγδ$^+$ T cells in the pathogenesis of collagen-induced arthritis has been demonstrated (32). Interestingly, these cells produced relatively high levels of IL-17A (14). In fact it has been shown that the number of IL-17-producing Vγ4$^+$ TCRγδ$^+$ T cells in the draining lymph nodes was equal to the number of CD4$^+$ TCRαβ$^+$ Th17 cells (14) and, most recently, it has been shown that the predominant IL-17-producing T cells in the joint of CIA-mice are TCRγδ$^+$ and not CD4$^+$ (15). Depletion of Vγ4$^+$ TCRγδ$^+$ T cells significantly reduced clinical disease scores and incidence of disease (14). On the other hand, depletion of TCRγδ$^+$ T cells did not prevent or ameliorate but rather aggravate rat adjuvant arthritis (33). Here, we found a relatively high percentage of IL-17-producing TCRγδ$^+$ T cells in the spleen and inflamed joints of arthritic wild type mice. Of note, in contrast to IL-17A and IL-17F, no expression of IL-22 was detected in FACS-sorted splenic TCRγδ$^+$ T cells from WT arthritic mice. Of high interest, IL-23 regulates IL-17A production in TCRγδ$^+$ T cells in spleen, lymph nodes as well as in the inflamed joints. In contrast, no reduction in the proportion of IFN-γ-producing TCRγδ$^+$ T cells was noted in IL-23p19KO mice compared to WT mice. These data show for the first time that the formation of IL-17A and RORγt-expressing TCRγδ$^+$ T cells is IL-23 dependent *in vivo*. However, this study does not reveal the contribution of IL-17A-producing TCRγδ$^+$ T cells in the arthritis process.

Th17 differentiation is driven by the orphan nuclear receptor RORγt (34) and RORα (35). We found that, compared to CD4$^+$ T cells, the expression of RORγt was substantially higher in TCRγδ$^+$ T cells, which corresponds with the higher proportion of IL-17A-producing cells in the TCRγδ$^+$ fraction than in the CD4$^+$ T cell pool. This suggests that in TCRγδ$^+$ T cells, as in Th17 cells, the production of IL-17A and perhaps also of IL-17F is under control of RORγt, which is in line with earlier observations in which it has been

shown that in lung and skin the largest population of RORγt$^+$ T cells express the γδ TCR and produce the highest levels of IL-17A (36). In the present study, a clear reduction of RORγt levels in IL-23p19KO mice under arthritic conditions was observed. This suggests that IL-23 regulates the induction of RORγt in γδ T cells.

Next to regulation of CD4$^+$ IL-17$^+$IFN-γ$^-$ T cells by IL-23, data from the present study also show marked reduction of CD4$^+$ IL-17$^+$IFN-γ$^+$ *double positive* cells in the absence of IL-23 during arthritis. The role and function of these *double positive* cells in the inflammatory process is unknown. Since these cells express both IL-17 and IFN-γ reflecting both Th17 and Th1 cytokine activity, these cells might be even more pathogenic than single IL-17$^+$ or IFN-γ$^+$ T cells. On the other hand, these cells may represent the transition phase of Th17 into Th1 cells. It has been shown that IFN-γ is protective against bone erosion and the expression of IFN-γ in human arthritis is accompanied with less joint destruction (37-38). This might indicate that the IL-17$^+$IFN-γ$^+$ *double positive* cells might be less pathogenic compared to IL-17$^+$IFN-γ$^-$ T cells in arthritis. Although Th17 cells are more pathogenic in inducing autoimmune diseases than IFN-γ producing Th1 cells in mice (3-4, 6), it was shown that both Th1 and Th17 cells are able to induce EAE after adaptive T cell transfer of these specific Th subsets into nude mice although Th1 and Th17 cells induced different pathology (8). In this study we showed that the IL-23/IL-17A immune pathway is critical for the progression of arthritis into a chronic destructive synovitis in a non-autoimmune arthritis model, which is in line with the critical pathogenic role for IL-17A in the arthritis process (39). Elevated levels of the pro-inflammatory T cell cytokine IL-17A have been detected in synovia of RA patients (40-41). IL-17A contributes to the pathogenesis of destructive arthritis (42-43). Important sources of IL-17A in this disease are Th17 cells and potentially TCRγδ$^+$ T cells. This latter cell type was elevated in synovia from RA patients with active synovitis and those RA patients with increased synovial TCRγδ$^+$ T cells had an increased tissue inflammation score compared to RA synovia with few TCRγδ$^+$ T cells (44). However, this study does not show the dominance of TCRγδ$^+$ T cells compared with conventional TCRαβ T cells. In collagen-induced arthritis, it has been shown that a relatively high proportion of TCRγδ$^+$ T cells are able to produce IL-17A (14) and are present at relatively high cell numbers in CIA joints (15).
In contrast, in the synovia of patients with established RA, only few IL-17-producing TCRγδ T cells were present (15). Therefore, studies on early RA patient materials including synovial tissue infiltrates are needed to evaluate the presence of IL-17-producing TCRγδ T cells. Interestingly, CD4$^+$CD45RO$^+$IL-17A$^+$ T cells were found in treatment-naïve early RA patients with active disease [45]. The present observation that IL-23 regulates IL-17A production in both Th17 cells and TCRγδ T cells in experimental arthritis underscores the need for further studies to unravel the potential of IL-23 as a therapeutic target in the pathogenesis of human destructive arthritis.

In conclusion, this study shows that IL-23 is critical for full-blown expression of a non-autoimmune destructive arthritis. Furthermore, IL-23 regulates the formation of both $CD4^+$ and $TCR\gamma\delta^+$ IL-17A-producing T cells. These data add new insight to the role of IL-23 in the regulation of a non-autoimmune inflammatory arthritis. Furthermore, these data show that IL-23 regulates IL-17A and RORγt expression in $TCR\gamma\delta^+$ T cells during joint inflammation. These findings may be relevant to other chronic inflammatory conditions and infectious diseases as well.

CONCLUSIONS

The aim of our study was to examine the role of IL-23 in the non-autoimmune antigen-induced arthritis model. Additionally, we investigated the regulatory potential of IL-23 in IL-17A and RORγt expression in CD4+ and TCRγδ+ T cells from the spleen and joints of arthritic mice. In the present study we showed that IL-23 is essential for the development of full-blown antigen-induced arthritis; IL-23p19-deficiency did not prevent the onset of joint inflammation but stopped the progression to a destructive synovitis. Furthermore, in the inflamed joints of IL-23p19KO mice, the proportion of IL-17A and IFN-γ-expressing $CD4^+$ T cells were reduced whereas IL-23 was also required for IL-17A but not for IFN-γ-producing $TCR\gamma\delta^+$ T cells in the inflamed joints. The transcription levels of RORγt were significantly higher in $TCR\gamma\delta^+$ T cells than in $CD4^+$ T cells from wild type arthritic mice. Finally, since $CD4^+IFN\text{-}\gamma^+$ cells were lower in the inflamed joints of IL-23p19-deficient mice, we confirmed the importance of the IL-23/IL-17 axis using IL-17RA deficient mice showing a similar arthritis expression as IL-23p19KO mice.

Thus, this study adds new insight to the role of IL-23 in the regulation of non-autoimmune arthritis. Furthermore, although it is suggested that IL-23 is involved in IL-17A-production by $TCR\gamma\delta^+$ T cells, we show for the first time a direct *in vivo* role for IL-23 in regulating IL-17A and RORγt expression by $TCR\gamma\delta^+$ cells. These findings indicate that regulating the IL-23 pathway may have therapeutic potential in non-autoimmune arthritis.

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SUPPLEMENTAL FIGURES

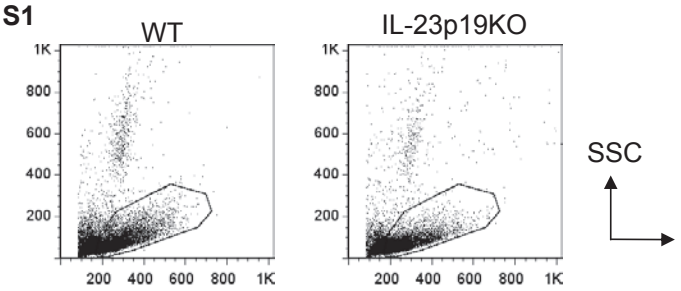

Figure S1 Antigen-induced arthritis was induced in WT and IL-23p19KO mice and at day 7 after i.a. mBSA injection the splenocytes were isolated and stimulated for 4h with PMA/Ionomycin and analyzed by flow cytometry. Shown are total ungated cells.

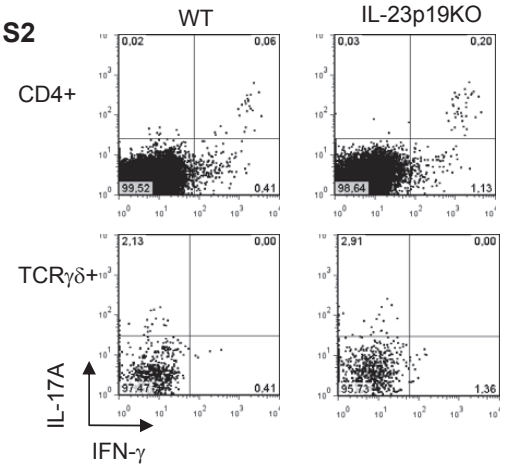

Figure S2 Splenocytes were isolated from naïve WT and IL-23p19KO mice and stimulated for 4h with PMA/Ionomycin and analyzed by flow cytometry for intracellular expression of IL-17A and IFN-γ. $CD4^+$ (top panel) and $CD3^+TCR\gamma\delta^+$ (lower panel) T cells were gated. Numbers indicate percentage of cytokine-positive cells within each quadrant.

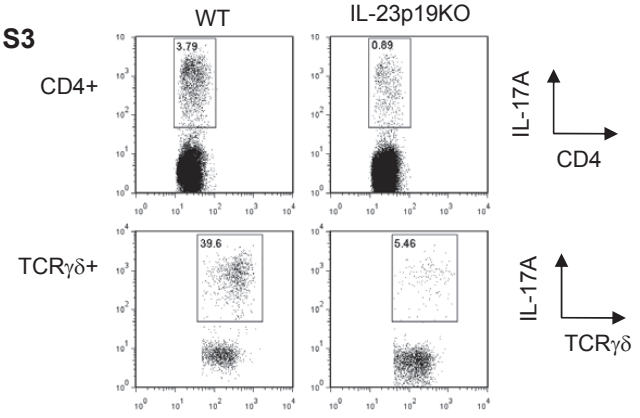

Figure S3 Antigen-induced arthritis was induced in WT and IL-23p19KO mice and at day 7 after i.a. mBSA injection cells from the draining lymph-nodes were isolated and stimulated for 4h with PMA/Ionomycin and analyzed by flow cytometry. $CD4^+$ (top panel) and $CD3^+TCR\gamma\delta^+$ (lower panel) T cells were gated. Numbers indicate percentage of positive cells within each gate.

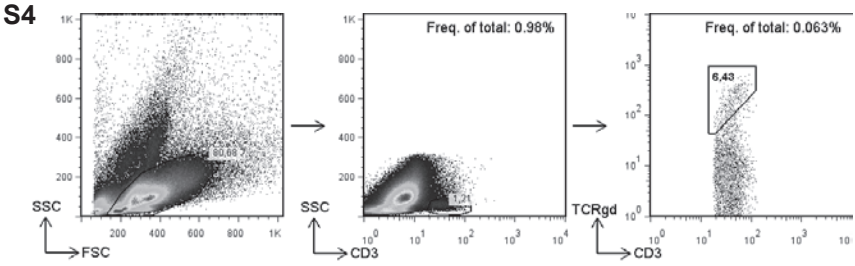

Figure S4 Pseudocolor plot of cells isolated from the joint of a representative WT mouse at day 7 of AIA. Shown is the FSC/SSC of all cells (left figure), and subsequent gating-steps for plotting TCRγδ$^+$ T cells. Numbers adjacent to gates indicate the percentage of cells in that specific gate and the numbers in the top-right corner indicate the percentage of cells in the gate relative to all cells.

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V

Critical role for an IL-23/TNF-α axis during acute joint inflammation

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ABSTRACT

Objective

IL-23 is essential in the development of chronic autoimmune diseases and supports the maturation of pathogenic Th17 cells. Although the role of IL-23 during adaptive immunity in arthritis has been studied extensively, its contribution during acute joint inflammation is unknown. Here, we investigated the function of IL-23 in the development of acute innate-driven arthritis.

Methods

Wildtype and IL-23p19 deficient mice were intra-articularly injected with peptidoglycan or streptococcal cell wall (SCW) fragments to induce acute innate-driven joint inflammation, and macroscopic scores were assessed over time. Kinetics of IL-23p19 expression was determined by quantitative RT-PCR. Local production of cytokines was measured in ex-vivo short-term synovial cultures and after in vitro stimulation of synovial explants with SCW.

Results

Synovial IL-23p19 transcripts were detected in wildtype mice at 1.5 and 4 h after intra-articular injection of peptidoglycan (PG) and were further increased 1 day after PG injection with a peak at day 2. PG and streptococcal cell wall (SCW) induced acute joint inflammation was IL-23 dependent because IL-23p19-deficiency resulted in a profound reduction of the macroscopical score compared to control mice. Interestingly, IL-23p19-/- mice showed a significant reduction in synovial TNF-α, but not IL-6 levels 4 h after induction of arthritis. In line with this, reduced TNF-α levels were detected in the culture supernatant of SCW-stimulated synovial explants from IL-23p19-/- mice compared to control mice.

Conclusion

These data show a critical role for IL-23 in the development of acute innate-driven joint inflammation and reveal a novel IL-23/TNF-α axis in this process.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder in which the synovial microenvironment is heavily infiltrated by activated immune cells producing pro-inflammatory cytokines, chemokines, and matrix metalloproteinases causing hyperplasia of the synovial membrane and eventually irreversible degradation of cartilage and bone. Acute (reactive) arthritis can be mimicked experimentally by a single intra-articular injection of Streptococcal cells wall fragments (SCW) from *Streptococcus pyogenes* or with peptidoglycan (PG) from *Streptococcus aureus* (1-3). Importantly, cells

containing PG were shown to be present in RA patients' synovia (4). SCW and PG are agonists of the toll-like (TLR) and/or nucleotide oligomerization domain (NOD)-like (NLR) receptors that are highly expressed by macrophages residing in the knee joint composing the synovial lining together with synovial fibroblasts. The importance of the SCW/PG-TLR2 pathway has been shown using TLR2-/- mice in which acute joint inflammation was reduced after local injection with SCW (5-7) or PG (8). In addition to TLR2, NOD2-deficient mice also had less severe joint inflammation after injection with PG (8) or SCW (7). This shows that acute joint inflammation induced by SCW and PG is mediated through TLR2 and NOD2 signaling. Of note, TLR2 and NOD2 did not depend on each other during PG-induced acute joint inflammation (8). Signaling through the TLR2 and NOD2 pathways is mediated by the NF-κB signaling cascade leading to the induction of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 (9). The roles for TNF-α and IL-1β have been addressed in SCW-induced arthritis and both are important mediators of the disease. Whereas anti-TNF-α blocked joint swelling, anti-IL-1β inhibited cartilage destruction and cellular influx (10).

IL-23 is produced by activated dendritic cells, monocytes and macrophages (11-12). IL-23 is a heterodimeric cytokine composed of a p19 subunit, and of a p40 subunit which is shared with IL-12 (11). The receptor for IL-23 (IL-23R) is expressed on various cell types including (memory) T cells, monocytes, macrophages, dendritic cells (12-13), synovial fibroblasts (14), γδ T cells and innate-like lymphocytes (13) allowing these cells to respond to IL-23 signaling. IL-23 plays an important role in the development of chronic autoimmunity since mice deficient for IL-23 completely fail to develop collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) (15-16). Because the IL-23R is expressed on innate cells, IL-23 might also play a role during early inflammation mediated by myeloid cells (16). Indeed, peritoneal macrophages harvested shortly after intraperitoneal injection of IL-23 expressed high levels of TNF-α and IL-1β transcripts (16). In addition, Hue and co-workers demonstrated in RAG-/- mice that during acute intestinal inflammation IL-23 was of vital importance since blocking IL-23 greatly reduced the inflammation score (17). Interestingly, IL-23 acts synergistically with TLR2 in the promotion of IL-17 production by γδ (18-19), αβ (19) T cells and, as recently shown, by human innate lymphoid cells (20). However, the role of IL-23 in acute joint inflammation has not been clarified yet.

Here, we determined the role of IL-23 in the development of acute joint inflammation induced by SCW and PG. We show that IL-23-deficiency significantly reduced the development of severe arthritis and identify a novel IL-23/TNF-α axis in this innate-driven joint inflammatory process.

MATERIALS AND METHODS

Mice and experimental arthritis models

IL-23p19 knockout (IL-23p19-/-) mice were kindly provided by Dr. N. Ghilardi, Genentech Inc., San Francisco, CA (21) and bred in-house. Mice were kept under specified pathogen free conditions (SPF) and provided with food and water ad libithum. Male and female mice between 8 and 12 weeks of age were used for experiments. All experiments were approved by the Dutch Animal Ethics Committee (DEC) of the Erasmus MC, University Medical Center.

Insoluble peptidoglycan (PG) isolated from *Staphylococcus aureus* (22) was injected intra-articular to induce acute mono-arthritis (15 μg per knee-joint unless stated otherwise). Alternatively, 25 μg of streptococcal cell wall fragments (SCW) from *Streptococcus pyogenes* (PG-PS 100P, BD Lee Laboratories, Grayson, GA) was injected intra-articular per knee joint. At several time points after injection, mice were sacrificed and joint inflammation was assessed macroscopically based on a scale of 0 to 2 after removing the skin. Alternatively, joint swelling was measured over time using a digital caliper (average of three measurements per knee joint). To quantify synovial cytokine levels, patellae with adjacent synovium was isolated from knee joints as described earlier (23). Briefly, patellae with adjacent synovium was incubated in RPMI/BSA (1% m/v) for 1 h after which cytokines were measured in the supernatant.

Synovium culture assay

Synovial explants from naïve wildtype and IL-23p19-/- female mice were cultured in RPMI/BSA (1% m/v) for 24 h in the presence or absence of 100 ng/mL SCW after which cytokines were measured in the supernatant.

Measurement of cytokines

Cytokines were measured by ELISA (R&D Systems, Minneapolis, MN) or by multiplex cytokine bead assay (eBioscience, San Diego, CA) following the manufacturer's protocol.

Quantitative real-time PCR

Total RNA was isolated from single-cell suspensions from knee-infiltrating cells using Blendzyme3, Roche Diagnostics, Mannheim, Germany, as previously described (23), or from synovial tissue biopsies. Total RNA was isolated using the GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and DNaseI-treated RNA was converted to cDNA with SuperScript® II reverse transcriptase (Invitrogen) (23). PCR primers were designed using ProbeFinder software (Roche Applied Science, Indianapolis, IN) and probes were chosen from the universal probe library (Roche). Quantitative real-time PCR

was performed on a ABI Prism 7300 or 7900 HT sequence-detection system (Applied Biosystems, Foster City, CA) and analyzed using SDS software (Applied Biosystems). The Ct values obtained were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression was calculated using the 2-ΔCt method.

Statistical analyses

Statistical differences between experimental groups were tested with the unpaired Student t-test using Prism software v5 (Graphpad Software Inc., La Jolla, CA). P values less than 0.05 were considered significant.

RESULTS

IL-23p19 deficient mice have normal expression of TLRs

In the present study, we investigated the contribution of IL-23 in the development of TLR2/NOD2-mediated acute joint inflammation. However, to first exclude that the IL-23p19-/- mice expressed aberrant levels of TLRs, splenocytes were isolated from naïve wildtype and IL-23p19-/- mice and expression levels were determined by flow cytometry. Importantly, no difference in the expression of TLR2 was observed between wildtype and IL-23p19-/- mice indicating that IL-23p19-/- mice have no intrinsic TLR2-signaling aberrancies. Additionally, TLR4 expression was similar between wildtype and IL-23p19-/- mice (Figure 1).

Induction of IL-23 during acute joint inflammation

PG and SCW induce arthritis when injected into the knee joint of naïve recipient wildtype mice. However, whether IL-23 is involved in this process is currently unknown. Prior to addressing this question, we first evaluated the optimal dose of PG to use for in vivo experiments. Different quantities of PG were injected intra-articular into naïve wildtype mice and joint inflammation was assessed macroscopically (Figure 2A). Doses ranging from 10 – 25 μg induced arthritis which was macroscopically visible at day 1, peaked at day 2 and then gradually decreased to almost normal at day 7. However, a dose of 50 μg PG induced a type of arthritis that persisted until at least day 7 indicating that prolonged inflammation can be achieved by high-dose PG. From these experiments, the dose of 15 μg PG was chosen for future in vivo experiments since it induced the most predictable acute joint inflammation with the lowest variation in macroscopical scores between mice (Figure 2A).

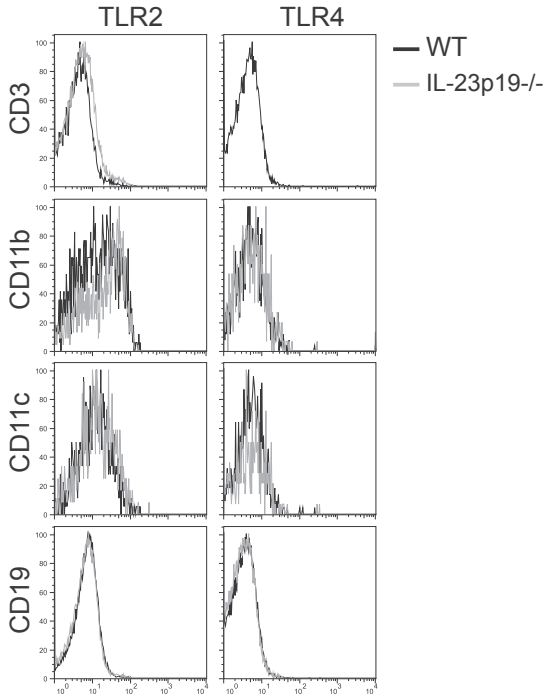

Figure 1. IL-23p19-/- mice have normal TLR-expression. Splenocytes from wildtype and IL-23p19-/- mice were isolated and surface expression of TLR2 and TLR4 were measured by flow cytometry. Shown are histograms of cells gated for the indicated CD marker. Data shown are from a representative mouse from 3 mice analyzed per group.

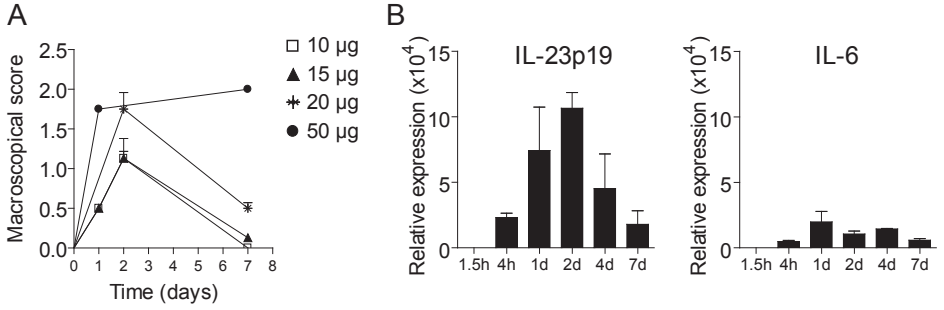

Figure 2. IL-23p19 production during acute joint inflammation. (A) Normal wildtype mice were injected at day 0 with various doses of PG (10 – 50 µg per knee joint) to induce acute joint inflammation. At different time points, mice were sacrificed and joint inflammation was assessed macroscopically. Data is average score (+SD) from at least 5 mice per group. (B) Normal wildtype mice were injected with 15 µg PG as selected as optimal dose (see A) and at different time point the synovium was removed and subjected to qRT-PCR. Data is average (+SEM) from 6 (1.5-24 h) or 3 (2-7 days) mice per group.

Next, the expression kinetics of IL-23 during acute PG-induced arthritis was monitored. IL-23p19 expression could be detected as early as 1.5 and 4 h after PG injection, increased profoundly on day 1 and peaked at day 2. Thereafter, IL-23p19 expression declined as measured at day 4 and reached baseline levels at day 7 (Figure 2B). We also measured the expression of IL-6 which was expressed at clearly lower levels than IL-23p19. Together, this shows that intra-articular injection of PG induces IL-23p19 expression in normal recipient mice suggesting that IL-23 is involved in acute joint inflammation.

Important role for IL-23 during acute joint inflammation

To investigate the contribution of IL-23 during the development of acute innate-driven joint inflammation, wildtype and IL-23p19-/- mice were injected with PG and macroscopic scores were assessed over time (Figure 3A). Although all wildtype and IL-23p19-/- developed arthritis, significantly lower macroscopical scores were observed in IL-23p19-/- mice compared to wildtype mice. PGs are the major components of the bacterial cell wall which also can induce acute experimental arthritis. To study the IL-23 dependence during cell wall induced arthritis, streptococcal cell wall fragments (SCW) were used in a similar setting as with PG. Again, all wildtype and IL-23p19-/- mice developed acute joint inflammation. However, IL-23p19-/- mice showed significantly less severe arthritis (Figures 3B and 3C). This shows that IL-23 is an important mediator of acute joint inflammation.

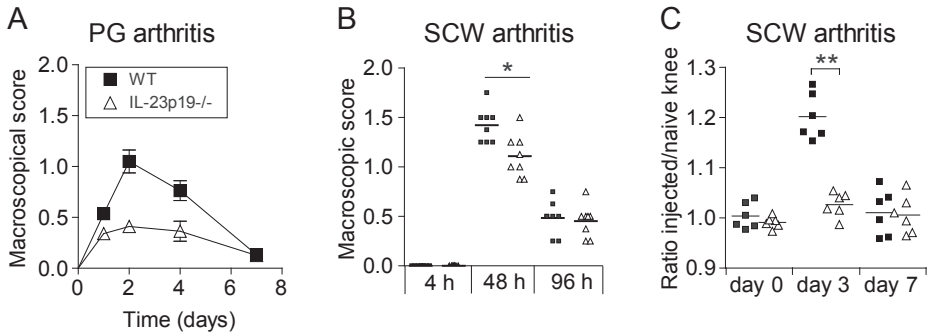

Figure 3. IL-23 is critical in the development of acute joint inflammation. (A) wildtype and IL-23p19-/- mice were injected in both knees with PG at day 0 and at days 1, 2, 4 and 7 joint inflammation was macroscopically assessed. Data is average score (+SEM) of 8-10 mice per group. (B) Wildtype and IL-23p19-/- mice were injected in both knees with SCW at day 0 and at 4, 48 and 96 h joint inflammation was macroscopically assessed. Data is average score (+SEM) of 4 mice per group. (C) Wildtype and IL-23p19-/- mice were injected with SCW at day 0, and at days 3 and 7 the level of joint inflammation was assessed by measuring the thickness of a knee-joint left untreated versus the thickness of an injected knee joint. Data is average (+SEM) of 3 mice per group.

IL-23/TNF-α axis during acute joint inflammation

To investigate if pro-inflammatory cytokines were reduced in IL-23-deficient mice, synovial washouts were taken shortly after PG injection. Interestingly, TNF-α levels were significantly lower in IL-23p19-/- mice compared to wildtype mice at 4 h (Figure 4A) while IL-6 levels were similar (Figure 4B). This suggests that an IL-23/TNFα axis drives acute arthritis.

To directly investigate a putative IL-23/TNF-α axis in synoviocytes during acute inflammation, synovial explants were taken from naïve wildtype and IL-23p19-/- mice and cultured for 24 h in absence or presence of SCW after which TNF-α levels were measured in the culture supernatant. In this system, SCW induced TNF-α production in synovial explant cultures from both wildtype and IL-23p19-/- mice although significantly lower levels were measured in IL-23p19-/- explant cultures (Figure 4C). This shows that IL-23 is essential for optimal TNF-α production during acute joint inflammation.

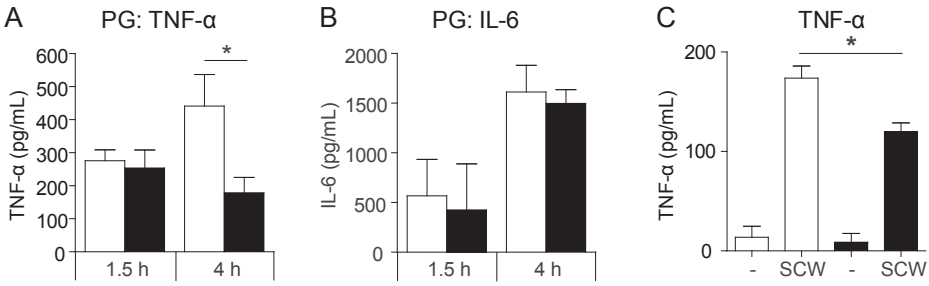

Figure 4. IL-23/TNF-α axis in TLR2/NOD2-mediated acute joint inflammation. (A-B) Mice were treated as in figure 3A. 1.5 and 4 h after PG-injection, mice were sacrificed and TNF-α (A) and IL-6 (B) levels were measured in 1 h-synovial cultures (see material and methods for details). Data is average (+SEM) from 3 mice per group. (C) Synovial explants were taken from naïve wildtype and IL-23p19-/- mice and cultured for 24 h in absence or presence of 100 ng/mL SCW. TNF-α was measured in the culture supernatant.

DISCUSSION

In the present study, we show that IL-23 plays an important role during acute joint inflammation induced by PG or SCW. After intra-articular injection with PG, increased IL-23p19 transcripts were detected at day 1, peaked at day 2 and reached baseline levels at day 7. IL-23 was critical in the development of acute arthritis since IL-23p19-/- mice developed significantly less severe joint inflammation than wildtype mice. Additionally, in the synovium of PG-injected IL-23p19-/- mice and in the culture supernatant of SCW-stimulated synovial explants of IL-23p19-/- mice, TNF-α levels were significantly lower compared to wildtype mice showing an IL-23/TNF-α axis during acute joint inflammation.

TNF-α is a major driver of acute innate-driven joint inflammation (10, 24). TLRs are the major trigger for this early increase of TNF-α (5). Kinetic studies showed that TNF-α expression preceded IL-1β expression. TNF-α peaked at 1.5 h whereas IL-1β peaked at 3 – 6 h after local SCW injection (10). IL-1β has been linked to cartilage damage and can be expressed and function independently of TNF-α during acute joint inflammation (10). Interestingly, this study suggested that the pro-inflammatory cytokine IL-6 is not able to replace TNF-α activity since lower TNF-α but similar levels of IL-6 resulted in lower joint inflammation. Furthermore, although our data showed that TNF-α expression is upstream of IL-23 expression, lack of IL-23 prevented full expression of joint inflammation and lower expression of TNF-α was noted. This suggests an interrelation of these two cytokines for full expression of joint inflammation. Further research is needed to exclude whether TNF-α and IL-23 target different cell types in this process or whether IL-23 can induce joint inflammation independent of TNF-α.

Recently, it has been proposed that IL-23 acts synergistically with TLR2 in innate and adaptive T cells (18-20) in their capacity to produce cytokines and to induce cell proliferation. Here, we found a similar TLR2/IL-23 axis with regard to TNF-α production. This TLR2-driven IL23/TNF-α axis may act in a synergistic manner since TNF-α alone and in synergy with IL-1β (14) or IL-17 (25) can enhance IL-23 production. However, no reduced IL-1β levels were found in IL-23p19-/- mice (data not shown) suggesting that IL-23 does not regulate IL-1β during experimental acute arthritis. Also, IL-17 receptor-deficient mice showed no reduced acute SCW-induced joint inflammation compared to controls (26-27) showing that IL-17 does not have a prominent role in acute joint inflammation. Activated macrophages are a major source of TNF-α during this TLR2-induced acute joint inflammation. The IL-23/TNF-α axis may involve in particular the (synovial) macrophages but innate T cells or other TNF-α producing cells in this acute joint inflammatory process can not be excluded.

In this study, we revealed a novel role of IL-23 in the development of acute joint inflammation that is linked to the IL-23/TNF-α axis. Therefore, our data suggest that IL-23 is a promising target for therapeutic treatment during acute (reactive) joint inflammation, although additional research is needed to validate its role in acute inflammatory arthritis.

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VI

IL-23 inhibits IL-22 and AhR signaling and with IL-1β regulates the human peripheral Th17/Th22 balance

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ABSTRACT

Human memory Th17 cells express CCR6 and a minor subset co-expressing CCR10 was named Th22 cells and is characterized by the production of IL-22 but almost no IL-17. Although it is well documented that maximal production of IL-17A by Th17 cells requires IL-23 and IL-1β, the role of these cytokines on memory Th22 cells remains unclear. Therefore, we isolated memory Th17 and Th22 cells based on chemokine expression and cultured these cells in absence or presence of IL-23 and IL-1β and, unexpectedly, found that IL-23 inhibits IL-22 production by both T helper subsets while promoting the secretion of IL-17A when IL-1β is present. Interestingly, IL-1β had no influence on the production of IL-22 but did induce high levels of IL-17 in Th22 cells showing that these cells still have the potential to produce IL-17. In Th17 cells but not in Th22 cells, IL-23 suppressed aryl hydrocarbon receptor (AhR) expression, a transcription factor believed to be essential for IL-22 transcription. FICZ, a tryptophan-derivative, induced profound expression of the AhR-target gene CYP1A1 which was significantly inhibited by IL-23 supporting the idea that IL-23 controls AhR signaling thereby regulating IL-22 production in Th22 cells. Further analysis revealed that Th22 express less IL-23R transcripts than Th17 cells possibly accounting for the lack of AhR gene expression by IL-23 stimulation. Together these data show that IL-23 and IL-1β co-operate in the regulation of IL-17A and IL-22 in Th17 and Th22 cells thereby controlling the balance of human memory Th17 and Th22 cells in the periphery. These data add new insight in the role of IL-23 and IL-1β in the regulation of different T helper subsets.

INTRODUCTION

IL-23 is involved in the expansion and survival of murine Th17 cells (1) and may regulate the pathogenic potential of these cells (2). In fact, mice deficient for IL-23 are devoid of IL-17A-producing T cells and do not develop autoimmunity as shown in several experimental models (3-6), and work from our group demonstrated that IL-23 promotes Th17 differentiation in experimental arthritis by regulating IL-17 and IL-22 by inhibiting T-bet and FoxP3 expression (7). Additionally, IL-23, together with IL-1β, IL-6 and TGFβ, drives the differentiation of human Th17 cells (8-10). Different studies reported that human IL-17A-expressing T cells in peripheral blood can be recognized by the expression of the chemokine receptor CCR6 as well as the IL-23 receptor (11-14). In different patient-cohorts, elevated numbers of memory CCR6+ Th17 cells were found (15-18) suggesting a pathogenic potential of these cells. Recently, it was shown that these CCR6+ Th17 cells but not Th1 cells are potent inducers of metalloproteinase's, IL-6, and IL-8 upon rheumatoid arthritis synovial fibroblast interaction suggesting that these

CCR6+ memory Th17 cells have a functional role in driving chronic destructive arthritis (19).

Besides IL-17A+ memory CCR6+ T cells, a minor population produces IL-22 (13) and based on the expression of CCR10, IL-22-producing CCR6+ T cells can be distinguished from IL-17A-producing CCR6+ T cells (20-21). These CCR6+CCR10+ memory cells produced IL-22, little IL-17A but no IFN-γ (20-21). Furthermore, it seemed that the aryl hydrocarbon receptor (AhR) positively controls IL-22 expression (21-23). However, the role of these CCR10+ Th22 cells is still unclear, but since CCR10 is involved in skin-homing Th22 cells might have a direct contribution to skin pathology and perhaps in other organ-specific autoimmune disorders as well. In fact, IL-22 was found to be involved in arthritis (17, 24-25) and in skin inflammation (26-28).

Several studies have suggested that IL-23 positively regulates IL-22 production during in vitro differentiation of naïve T cells (10, 28-31) or during stimulation of effector memory cells (7, 13, 29). However, with the recent acknowledgement of Th22 cells as a distinct subset, the role of the T cell differentiating cytokines IL-23 and IL-1β in the regulation of Th22 cells requires further attention, especially since these cytokines might regulate the balance of the Th17 and Th22 subsets.

In the present study, we show the potential of IL-23 and IL-1β in the regulation of IL-17A and IL-22 by human memory CCR6+ Th17 and CCR6+CCR10+ Th22 cells. Whereas IL-1β strongly enhanced the proportion of CCR6+ Th17 cells and IL-17A secretion, IL-23 significantly suppressed IL-22 production. When combined, IL-23 and IL-1β regulated the balance of IL-17A+ and IL-22+ CCR6+ cells in favor of IL-17A. The regulation of IL-17A and IL-22 by IL-23 and IL-1β went further than CCR6+ Th17 cells since in CCR6+CCR10+ Th22 cells IL-23 clearly inhibited AhR-signaling and IL-22 production and IL-1β induced high levels of IL-17A in these CCR10+ Th22 cells. Together, these data demonstrate that IL-23 cooperates with IL-1β to regulate the balance of memory Th17 and Th22 cells in favor of Th17. These findings add new insight to the role of IL-23 and IL-1β in the regulation of Th17 and Th22, in particular. Furthermore, these data have important therapeutic relevance since Th17 and Th22 are emerging as important target cells in autoimmune disorders.

MATERIALS AND METHODS

Flow cytometry antibodies and cell sorting

Monoclonal antibody preparations, intracellular cytokine detection and flow cytometry were described previously (24). For intracellular cytokine detection, cells were stimulated for 4 hours with 50 ng/mL PMA and 500 ng/mL Ionomycin in the presence of Golgistop (BD Biosciences, San Diego, CA, USA). The following Abs were used; Fitc-anti-CD45RO, PE-anti-CCR6, PerCP-Cy5.5 or APC-H7-conjugated anti-CD4, Pe-Cy7-anti-IFN-γ (BD Biosciences), APC-anti-CCR10 (R&D systems, Minneapolis, MN, USA), PE-anti-IL-17A and Alexa-Fluor 647-conjugated anti-IL-22 (eBioscience, San Diego, CA, USA). Cells were acquired on a FACS Canto II (BD Biosciences) and analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA) software. Purified T cell populations were isolated from buffy coats using a FACS Aria cell sorting system (BD Biosciences). Purity of the obtained T cell populations was > 98%.

Cell cultures

T cells were cultured for three days in 96-wells round-bottom plates at a concentration of 1.25 x 10^5 cells per mL in IMDM (BioWhittaker), supplemented with 10% FCS, 100 U/mL penicillin/streptomycin, L-glutamine and 5 x 10^{-5} M β-mercapto-ethanol (Merck, Darmstadt, Germany). Cells were activated with soluble anti-CD3 and anti-CD28 (0.3 μg/mL and 0.4 μg/mL resp., Sanquin, Amsterdam, the Netherlands). Recombinant IL-23, IL-1β, IL-6 (10 ng/mL, R&D systems), 6-formylindolo[3,2-b]carbazole (FICZ; 2 μM, Enzo Life Sciences Inc, Farmingdale, NY, USA) or combinations were added.

Quantitative real-time PCR analysis

RNA extraction and cDNA synthesis were described previously (32-33). PCR primers were designed using ProbeFinder software and probes were chosen from the universal probe library (Roche Applied Science, Indianapolis, IN, USA). Quantitative real-time PCR was performed using the ABI Prism 7300 or 7900HT sequence detection systems (Applied Biosystems, Foster City, CA, USA) and analyzed using SDS software (Applied Biosystems). The obtained Ct values were normalized to those of GAPDH.

Cytokine measurements

IFN-γ and IL-10 expression levels were determined using ELISA (Invitrogen). IL-17A and IL-22 production was measured using the DuoSet ELISA development kit (R&D systems). ELISAs were performed according to the manufacturers' instructions.

Statistical analysis

Statistical differences between experimental groups were tested with a paired or unpaired student t-test as indicated in the figure legends using Prism software v5 (Graphpad Software Inc., La Jolla, CA, USA). P values less than 0.05 were considered significant.

RESULTS

IL-23 and IL-1β cooperatively regulate the balance of IL-17A and IL-22 production in circulating memory CCR6+ Th17 cells

Naïve human T cells can be differentiated in vitro to Th17 cells in the presence of IL-6, TGFβ, IL-23 and IL-1β (8-9) and can be identified in the periphery based on the expression of the chemokine receptor CCR6+ on CD4+CD45RO+ (memory) T cells (11-14). To investigate the role of Th17 inducing cytokines on peripheral human memory Th17 cells, we activated (anti-CD3 plus anti-CD28) FACS-sorted memory CCR6+ and CCR6- T cells for 3 days in the presence of different combinations of Th17-inducing cytokines. However, since we used TGFβ-containing IMDM culture medium (8-9) we did not add exogenous TGFβ and no role for IL-6 in the regulation of IL-17A, IL-22, IL-10 or IFN-γ production was observed under the tested conditions (Supplemental figure S1).

In contrast, IL-1β significantly enhanced the proportion of IL-17A producing CCR6+ Th17 cells and, when combined with IL-23, the proportion of IL-17A expressing cells was significantly higher compared to IL-23 or IL-1β alone (Figures 1A-B). As measured in culture supernatant, IL-1β alone and in conjunction with IL-23 enhanced the production of IL-17A (Figure 1C). Besides IL-17A, Th17 cells have been reported to produce IL-22. However, whether IL-22 is regulated by IL-23 in these peripheral human memory CCR6+ Th17 cells has not been previously addressed. To resolve this issue, we quantified the percentage of IL-22+ CCR6+ T cells and IL-22 secretion in our experiments and, surprisingly, found that IL-23 alone and in the presence of IL-1β significantly suppressed the proportion of IL-22+ CCR6+ T cells (Figure 1A-B) and IL-22 production by these cells (Figure 1C). Interestingly, compared to the control condition, IL-23 also reduced the proportion of IL-22+IL-17A+ double positive cells (Figure 1A). IL-23 nor IL-1β induced high levels of IL-17A or IL-22 in CCR6- cells, although a profound IFN-γ secretion was noted induced by the combination of IL-23/IL-1β (Figure 1C).

We also observed a slight increase of IL-10 production after IL-23 stimulation as reported earlier (34) and, in contrast, IL-1β decreased IL-10 production compared to control and IL-23 alone. Noteworthy, IL-10 was produced at higher levels by CCR6- T cells than by CCR6+ T cells (Figure 1C). Together, these data show that IL-23 in the presence of IL-

1β shifts the percentage of IL-17A+ and IL-22+ CCR6+ Th17 cells in favor of IL-17A (Figure 1D).

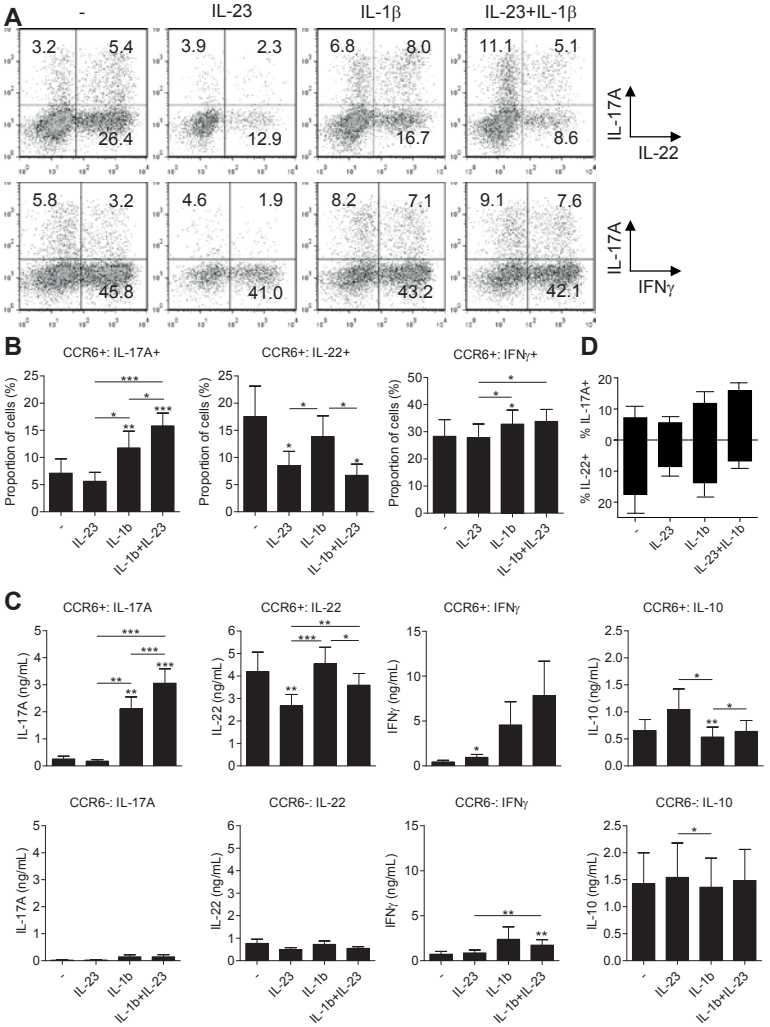

Figure 1. IL-23 and IL-1β act together to enhance IL-17A expression by human CCR6+ memory T cells. (A) Flow cytometry of IL-17A and IL-22 (top panel) and IL-17A and IFN-γ (lower panel) of FACS-sorted CD4+CD45RO+CCR6+ (CCR6+) T cells after 3 days of stimulation with anti-CD3 plus anti-CD28 in the absence (-) or presence of indicated cytokines. Numbers in quadrants indicate the percentage of cells in that quadrant. Data shown is from a representative donor. (B) Quantification of the flow cytometric analyses; mean values and SEM are given for 5 different donors from two independent experiments. (C) ELISA of cytokine production in culture supernatants of CCR6+ and CCR6- T cells after 3 days of culture; data are mean and SEM of 8 (CCR6+) and 7 (CCR6-) different donors from 3 and 4 independent experiments respectively. (D) Histogram showing the proportion (%) of IL-17A+ versus IL-22+ CCR6+ T cells from (B). Asterisk(s) (*) depicts statistical difference compared to control (-). *P<0.05; **P<0.01; ***P<0.001 as analyzed by the paired student t-test.

IL-23 suppresses the transcription of AhR and FoxP3

To verify that the CCR6+ T cells express a stable Th17-like transcription profile in culture, we compared the expression of Th17-related genes between unstimulated CCR6+ and CCR6- T cells. In comparison to CCR6- T cells, CCR6+ T cells expressed significantly more RORc2 and IL-23R mRNA (Figure 2A). Notably, similar mRNA quantities of IRF4, AhR and FoxP3 were expressed in CCR6- and CCR6+ cells (Figure 2A).

Next, we investigated the effect of IL-23 and IL-1β on these Th17-related genes in CCR6+ Th17 cells. Transcription of FoxP3 and AhR were both negatively regulated by IL-23 compared to the control condition. However, the presence of IL-1β had no effect on FoxP3 and AhR transcription, since similar levels were observed between IL-1β and control, and between IL-23/IL-1β and IL-23 alone (Figure 2B). In contrast, the expression levels of RORc2 were not influenced by either IL-23 or IL-1β and no regulation of the IL-23 receptor (IL-23R) was observed under the tested conditions (Figure 2B).

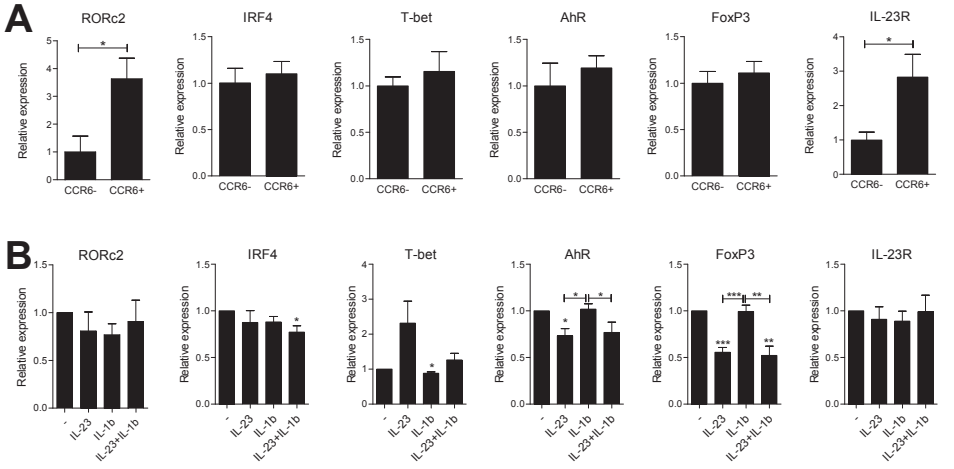

Figure 2. IL-23 suppresses the transcription of the aryl hydrocarbon receptor and of FoxP3 in CCR6+ T cells. (A) Quantitative RT-PCR of indicated genes in CCR6- and CCR6+ T cells after 3 days of culture with anti-CD3 plus anti-CD28; normalized (gapdh) Ct values are calculated by the ddCt method and are presented as relative expression compared to those in CCR6- cells. (B) Quantitative RT-PCR in CCR6+ T cells after 3 days of culture with anti-CD3 plus anti-CD28 and IL-23, IL-1β or IL-23+IL-1β; normalized Ct values are shows as relative to unstimulated (-) cells. (A-B) Data are the mean and SEM of 5-7 donors from 2-3 independent experiments for CCR6- and CCR6+ cells respectively. *P<0.05; **P<0.01; ***P<0.001 as analyzed by the (A) unpaired and (B) paired student t-test.

IL-23 negatively regulates IL-22 in memory Th22 cells

Because we observed that IL-23 plus IL-1β skews the balance of IL-17A+ and IL-22+ CCR6+ T cells in the direction of IL-17A (Figure 1D), we aimed to investigate

the role of IL-23 and IL-1β on memory Th22 cells, which were recently described to preferentially express the chemokine receptors CCR6 and CCR10 (20-21). Highly purified CCR6+CCR10+ memory T cells (shortly CCR10+) and their CCR6+CCR10- counterparts were cultured in the absence or presence of IL-23 and/or IL-1β. To first determine the molecular differences between CCR10+ and CCR10- T cells, we compared mRNA levels of key transcription factors. As expected, CCR10+ Th22 cells transcribed more AhR mRNA than CCR10- T cells and also more CYP1A1 mRNA (Figure 3A) which is a direct target of AhR signaling. Surprisingly, CCR10+ T cells also expressed significantly more FoxP3. In contrast to the IL-1R (IL-1R1) which was expressed at similar levels between the two subsets, CCR10+ T cells expressed significantly less IL-23R mRNA than CCR10- T cells.

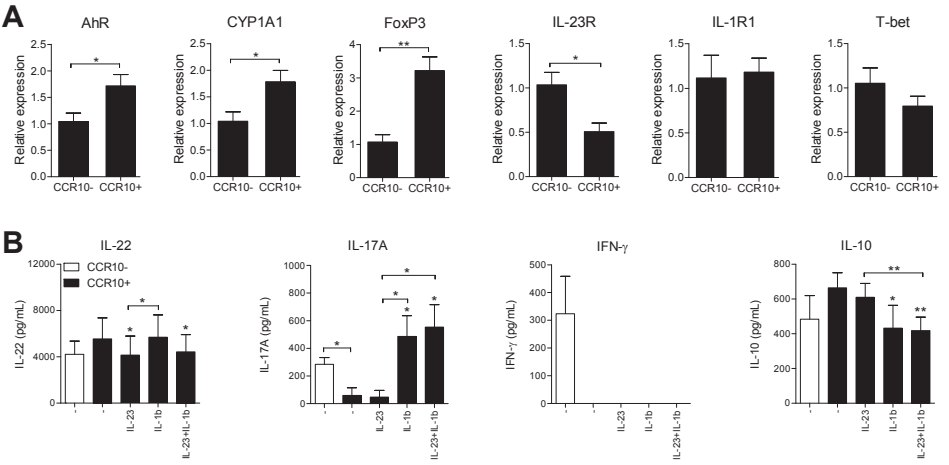

Figure 3. IL-23 inhibits IL-22 production by CCR10+ Th22 cells. (A) Comparison of gene expression between CCR10- and CCR10+ cells in absence of cytokines; statistical differences were calculated with the unpaired student t-test. (B) ELISA of cytokine production in culture supernatants of CD4+CD45RO+CCR6+CCR10+ (CCR10+) and CCR6+CCR10- T cells after 3 days of stimulation with anti-CD3 plus anti-CD28 in the absence (-) or presence of indicated cytokines; statistical differences between CCR10- and CCR10+ cells were calculated with the unpaired student t-test; differences between different cytokine-conditions within the CCR10+ T cell population were tested with the paired student t-test. (A-B) Mean values and SEM are given for 6 different donors from two independent experiments. *P<0.05; **P<0.01; ***P<0.001.

Although CCR10+ T cells did not produce substantially higher levels of IL-22 than CCR10- T cells, CCR10+ T cells produced significantly lower levels of IL-17A and no IFN-γ secretion could be detected from CCR10+ T cells (Figure 3B), which resembles the cytokine-profile described for Th22 cells (20-21). In these CCR10+ Th22 cells, IL-23 significantly suppressed IL-22 but did not enhance IL-17A production (Figure 3B). Inversely, IL-1β did not regulate IL-22 but significantly enhanced IL-17A. Interestingly, in the presence of both IL-23 and IL-1β, IL-22 was suppressed and IL-17A was induced

(Figure 3B). Notably, none of the tested conditions induced IFN-γ production by Th22 cells. Additionally, we show in Supplemental figure S2 that in CCR10- T cells, IL-23 did not statistically suppress IL-22 and that IL-1β alone or in the presence with IL-23 strongly induced IL-17A and IFN-γ in these cells.

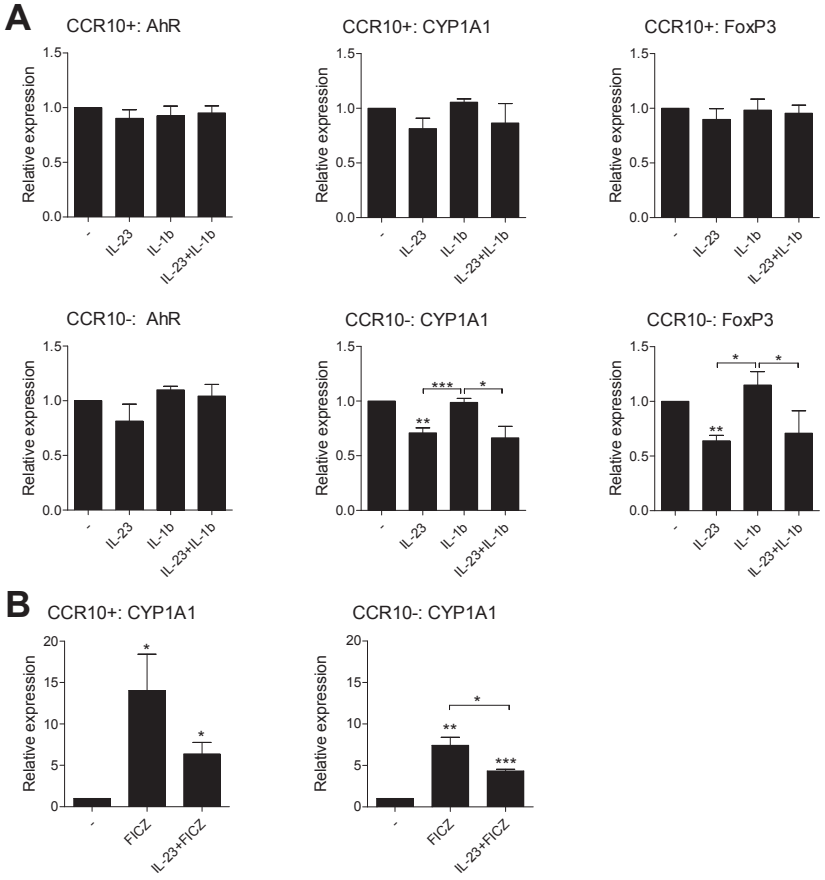

Figure 4. IL-23 inhibits AhR signaling. (A) Quantitative RT-PCR of indicated genes in CCR10+ (top panel) and CCR10- (lower panel) T cells after 3 days of culture with anti-CD3 plus anti-CD28 and IL-23, IL-1β or IL-23+IL-1β; normalized (gapdh) Ct values are calculated by the ddCt method and are presented as relative expression compared to those in unstimulated (-) cells. (B) Quantitative RT-PCR of CYP1A1 in CCR10+ and CCR10- T cells after 3 days of culture with 6-formylindolo[3,2-b]carbazole (FICZ) and/or IL-23 in the presence of anti-CD3 plus anti-CD28; normalized Ct values are shows as relative to unstimulated (-) cells. (A-B) Mean values and SEM are given for 6 different donors from two independent experiments. *P<0.05; **P<0.01; ***P<0.001 as analyzed with the paired student t-test.

IL-23 suppresses FICZ-induced CYP1A1 expression

We next investigated the regulation of these key transcription factors by IL-23 and IL-1β in the CCR10+ and CCR10- subsets. Since AhR is proposed to be a master regulator of

IL-22, we expected that IL-23 stimulation would down-regulate AhR expression but, AhR and CYP1A1 mRNA expression was not different between IL-23 stimulated CCR10+ T cells and control (Figure 4A). This prompted us to investigate if, instead, IL-23 could interfere with AhR signaling. For this, CCR6+CCR10+ Th22 cells were stimulated with the AhR agonist 6-formylindolo[3,2-b]carbazole (FICZ) in the absence or presence of IL-23. As a functional read-out, we quantified the relative expression of CYP1A1, a direct target of AhR signaling (35). As expected, stimulation with FICZ induced high levels of CYP1A1 expression which was more prominent in CCR10+ T cells than in CCR10- T cells (Figure 4B). Interestingly, IL-23 significantly suppressed FICZ-induced CYP1A1 expression in both CCR10+ and CCR10- T cells suggesting that IL-23 modulates AhR signaling.

DISCUSSION

In the present study, we showed that IL-23 cooperated with IL-1β to regulate the balance of peripheral human memory Th17 and Th22 cells. IL-23 suppressed IL-22 whereas IL-1β strongly induced IL-17A in both CCR6+ Th17 and CCR6+CCR10+ Th22 cells. When combined, IL-23 and IL-1β stimulation led to Th17 polarization while inhibiting Th22 polarization.

This is the first study in which IL-23 is found to negatively regulate IL-22 production in human T cells. Earlier studies recognized enhanced IL-22 levels after IL-23 stimulation. However, these studies used either differentiating naïve T cells (10, 28-31) and/or total memory effector T cells (7, 13, 29), the latter of which include various T helper subsets that may mask the effect on individual T cell subsets as shown in this study. Our findings indicate that the role of IL-23 on IL-22 expressions differs between naïve T cells differentiating towards Th17 compared with the role of IL-23 on IL-22 expression in effector memory cells.

No role for IL-6, either alone or in combination with IL-23 and/or IL-1β, in the modulation of Th17-cytokines in CCR6+ Th17 cells was observed. This suggests that IL-6, although essential for the differentiation of Th17 (8-10) and Th22 cells (20), does not vitally contribute to the regulation of IL-17 and IL-22 expression in peripheral memory CCR6+ Th17 cells. In line with this, in total effector memory T cells, no prominent role for IL-6 alone in the induction of IL-17A was found, although IL-6 might act together with IL-23 or IL-1β here (13). However, since IFNγ and IL-4 were neutralized in these cultures there was a bias for Th17 differentiation and/or expansion (36-37). Neutralizing IL-4/IFNγ resulted in enhanced IL-17A production by CCR6+ Th17 cells, while IL-22 levels were slightly decreased (data not shown).

We confirmed that our purified CCR6+CCR10+ T cells represent the Th22 phenotype (20-21) since these cells were enriched for AhR and CYP1A1 transcripts and expressed IL-22, little IL-17A and no IFNγ. Additionally, relatively high levels of FoxP3 were found in Th22 cells compared to CCR6+CCR10- Th17-like cells. The implication of this is as yet not clear but it was recently described that AhR in combination with TGFβ induces FoxP3+ iTreg cells (38). In the CCR6+ Th17 and CCR6+CCR10- Th17-like cells, IL-23 suppressed FoxP3 which might function to help the IL-1β-enhanced induction of IL-17A expression since FoxP3 acts as a repressor for IL-17A (39).

Parallel to the suppression of IL-22 by IL-23, we found that IL-23 reduced AhR expression in CCR6+ Th17 cells. Since AhR is involved in the regulation of IL-22 (21-22), this suggests that IL-23 regulates IL-22 expression through interfering with the AhR signaling cascade. However, in CCR6+CCR10+ Th22 cells, no suppression of AhR transcription was noted after IL-23 stimulation, although reduced IL-22 transcription levels were found. In fact, Th22 cells stably expressed AhR since none of the tested conditions with polarizing cytokines led to altered AhR expression. We further investigated this discrepancy of IL-23-dependent AhR expression in Th17 versus Th22 cells and found that IL-23 was able to impair FICZ-induced CYP1A1 expression in Th22 cells, a process that depends on AhR signaling (35, 40). This showed that IL-23 regulated AhR activity in both Th17 and Th22 cells and thereby modulated the expression of AhR targets including CYP1A1 and IL-22. Importantly, Th22 cells expressed lower IL-23 receptor mRNA than CCR6+CCR10- Th17-like cells, indicating that Th22 might require higher IL-23 concentrations to downregulate AhR transcripts.
In conclusion, the present work demonstrates that IL-23 and IL-1β work in a co-operative manner to regulate the production of IL-17A and IL-22 in CCR10+ Th22 cells as they do in CCR6+ Th17 cells thereby controlling the balance of memory Th17 and Th22 cells (Figure 5).

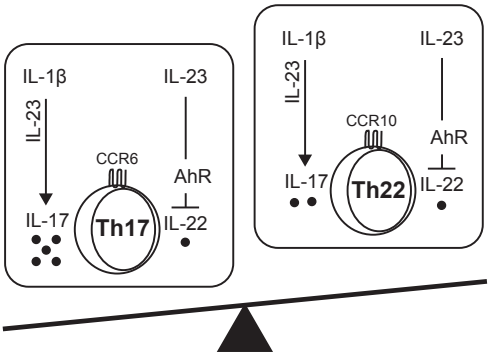

Figure 5. Proposed model of the IL-23/IL-1β-regulated balance of human peripheral memory Th17 and Th22 cells.

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SUPPLEMENTAL FIGURES

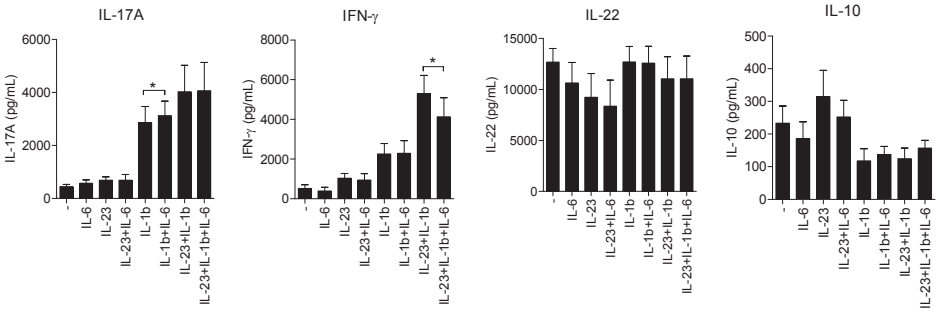

Figure S1. IL-6 does not contribute to the production of cytokines by CCR6+ T cells. ELISA of cytokine production in culture supernatants of CCR6+ T cells after 3 days of culture in the absence (-) or presence of indicated cytokines; data are mean and SEM of 3 different donors. *P<0.05; **P<0.01; ***P<0.001 as analyzed by the paired student t-test.

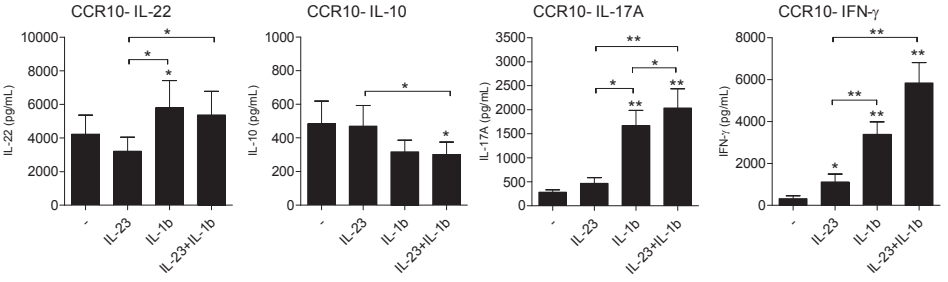

Figure S2. This figure is supplemental to Figure 4. ELISA of cytokine production in culture supernatants of CD4+CD45RO+CCR6+CCR10- (CCR10-) T cells after 3 days of stimulation with anti-CD3 plus anti-CD28 in the absence (-) or presence of indicated cytokines. Mean values and SEM are given for 6 different donors from two independent experiments. Asterisk(s) (*) directly above a histogram shows statistical difference of that particular condition compared to control (-). *P<0.05; **P<0.01; ***P<0.001 as analyzed with the paired student t-test.

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VII

General discussion

This thesis describes the function of IL-23 in different experimental arthritis models (**chapters II-V**), with a strong focus on the IL-23/IL-17 immune pathway in these models (**chapters II-IV**). Additionally, as the contribution of Th22 cells during arthritis is as yet unknown, the regulation of IL-23 and IL-1β on IL-17 and IL-22 production by human Th22 in addition to Th17 cells was investigated (**chapter VI**). By these experiments, we have shown that IL-23 acts at various stages of the arthritic process through the regulation of distinctive immunological and cellular pathways (Figure 1). This final chapter discusses the findings described in this thesis by comparing the IL-23-dependencies during the different experimental arthritis models utilized in these studies and discusses the function of IL-23 on the cytokine network during the immunopathological processes in these models with a strong focus on the cytokines IL-17 and IL-22. Lastly, some final considerations and future perspectives will be given.

THE ROLE OF IL-23 DURING EXPERIMENTAL ARTHRITIS

IL-23p19-/- mice did not develop collagen-induced arthritis (CIA) whereas IL-12p35-/- mice did, suggesting that IL-23 but not IL-12 is essential for the development of chronic autoimmune arthritis (1). This was the first study that directly compared the roles for IL-12 and IL-23 in experimental arthritis and suggested that IL-17A-producing CD4+ T cells but not collagen-specific Th1 cells drove arthritis since no IL-17A producing CD4+ T cells but normal IFNγ+ Th1 cells were found in IL-23p19-/- mice (1). Later, it was shown by others that blocking endogenous IL-23 activity after CIA onset reduced paw volume but not the arthritis score in rats (2). However, no clues were given concerning joint inflammation nor on the immunological response. So, it was still not clear what the function of IL-23 was during different stages of CIA. Therefore, we conducted a murine CIA study using a mouse-anti-mouse monoclonal anti-IL-23p19 specific antibody and showed that CIA possesses IL-23 dependent and independent stages since neutralizing IL-23 significantly prevented the development of full-blown CIA when administered after immunization of normal DBA/1 mice but no effect was observed when anti-IL-23p19 was given after disease onset (**chapter II**). This suggests that IL-23 is critical for the development of CIA but that late stages of CIA do not critically depend on IL-23.

In mice treated with anti-IL-23p19 antibody before CIA onset, the lower disease severity was accompanied by lower serum IgG antibodies, which was not observed in mice treated with anti-IL-23p19 after onset. This suggests that IL-23 hampers B cell activation. Indeed, it has been demonstrated that IL-23 is important for T-cell dependent B cell activation but not for T cell independent B cell activation (3). CIA pathology is initiated by CII-specific T helper cells and B cells, leading to the generation of antigen-specific IgG and

pathogenic immune-complexes (4). Therefore, blocking IL-23 activity before CIA onset likely impairs full activation and/or maturation of CII-specific T helper cells and thereby prevents the formation of auto-reactive plasma cells and auto-antibodies.

The results obtained from the studies performed in the CIA model suggest that IL-23 acts mainly on T cells (**chapter II**). To investigate the role of IL-23 on T cell mediated arthritis directly, we used the antigen-induced arthritis (AIA) model. AIA is a model par excellence to study the role of T cells in arthritis because this model is strongly CD4+ (not CD8+) T cell dependent (5). In AIA, IL-23p19-/- mice developed significantly lower arthritis severity than control mice (**chapter IV**). We also observed significantly less T cell infiltration and bone erosion in the knee joints of IL-23p19-/- mice than of WT mice. In the synovium of these mice, protein levels of MCP-1 (CCL2), TNF-α, IL-17A and IFN-γ were significantly lower in IL-23p19-/- mice demonstrating that IL-23 regulates the expression of these cytokines.

AIA typically lasts for 3 weeks after which joint inflammation returns to almost normal. However, during this primary mBSA-induced arthritis, memory T cells are formed which, upon reactivation with low quantities of mBSA injected intra-articularly, drive a rapid flare-up arthritis. We exploited this AIA flare-up model to investigate the role of IL-23 in memory T cell reactivation. After the primary AIA disease score returned to normal levels, IL-23 activity was neutralized during a period of 4 weeks with an anti-IL-23p19 antibody prior to the induction of the antigen-induced flare-up (**chapter II**). Mice that received anti-IL-23p19 developed significantly lower disease scores compared with controls suggesting that anti-IL-23 prevents full re-activation of antigen specific arthritogenic memory T cells in vivo. In the synovium, IL-17A and IL-22 expression were lower in those mice that received anti-IL-23p19 mAb compared to control mice suggesting that IL-23 has a role in the regulation of the expression of Th17-related cytokines in this model (**chapter II**). Collectively, these data show that IL-23 is important in the initial development but also in the process of antigen-induced T cell mediated flare-up arthritis.

In addition to T cells, the IL-23R is expressed by myeloid cells (6-7) making these cells responsive to IL-23 stimulation. In the EAE model is was already suggested that although IL-23p19-/- mice developed no disease, IL-23 might participate in innate, early inflammatory processes (8). Indeed, recombinant IL-23 could induce TNF-α and IL-1β transcripts in peritoneal macrophages (8). Later, Hue et al. demonstrated an important role for IL-23 in the development of acute *H. hepaticus*–induced intestinal inflammation (9). However, whether IL-23 could also contribute to acute inflammation of the joints remained elusive. To investigate this, acute joint inflammation was induced in wildtype

and IL-23p19-/- mice by a single intra-articular injection with peptidoglycan (PG) or streptococcal cell wall fragments (SCW). This revealed that mice lacking IL-23 developed clearly less severe arthritis showing that IL-23 is needed for acute inflammation of the joints. Moreover, significantly lower TNF-α levels were observed in the joints of arthritic IL-23p19-/- mice (Figure 1), and in *in vitro* SCW-stimulated synovial explants from IL-23p19-/- mice compared to wildtype mice.

In conclusion, using different animal arthritis models this thesis identifies IL-23 to be an important mediator in the developmental stages of arthritis by regulating effector cytokine production from various cell types.

THE IL-23/IL-17 IMMUNE PATHWAY IN ARTHRITIS

The dimeric receptor for IL-23 is composed of the IL-12Rβ1 subunit (also essential for IL-12 signaling) and of an IL-23R chain and is expressed on activated but not naïve T helper cells but also on some myeloid and innate-like lymphocytes. Upon ligation of the IL-23R complex, several signal molecules and activators of transcription (STATs) are phosphorylated, of which STAT3 is the most prominent one (7). However, since the IL-23R is not expressed on naïve T cells (10-12), IL-23 likely does not play a role in the early phases of T helper differentiation (10, 12). Differentiation of naïve T helper cells towards Th17 cells requires TGFβ and IL-6 or IL-21 instead leading to the upregulation of the IL-23R and RORγt in a STAT3-dependent manner (13-14). In addition, although TGFβ, IL-6 and IL-21 drive Th17 differentiation (10, 12, 14-16), IL-23 is essential for further maturation of Th17 cells by enhancing transcription of RORγt, IL-23R and the hallmark cytokines IL-17A and IL-22 (10, 12, 16-17), while suppressing IL-10 (16), the latter of which is a negative regulator of Th17 differentiation (16). More recently, IL-23-driven, Th17-derived GM-CSF has been shown to be essential for driving neuroinflammation in the EAE model (18-19). In turn, GM-CSF promoted IL-23 secretion from APCs providing a positive feedback loop in neuronal inflammation (19). Whether Th17-derived GM-CSF is also important for arthritis development remains however elusive but it does show that IL-23 drives the effector function of Th17 cells.

In CIA-resistant IL-23p19-/- mice, IL-17A-producing CD4+ T cells (but not IFNγ-producing cells) were absent (1) suggesting a role for IL-23 in the formation of IL-17A producing cells. However, whether IL-23 is also required for the maintenance of IL-17A expression during CIA was not addressed. In **chapter II**, we showed that the highest proportion of splenic IL-17A-producing CD4+ T cells was present 10 days after CII-immunization demonstrating Th17 cells are formed during CIA development. Next,

the role of IL-23 in the maintenance of IL-17A production by CII-primed T cells was investigated. Splenocytes were taken from CII-immunized DBA/1 mice and cultured in absence or presence of anti-IL-23p19 antibodies. Although CII-specific restimulation in these cultures significantly enhanced the production of IL-17A (but not IFNγ), blocking IL-23 did not suppress IL-17A secretion. In contrast, IL-23p19-/- splenocytes from CII-immunized C57BL/6 mice produced significantly less IL-17A than splenocytes from wild type mice. Together, this suggests that IL-23 is essential in the *de novo* generation of IL-17A-producing T cells during CIA development but not for their survival. Additionally, we showed in **chapter III** that the addition of exogenous IL-23 to CD4+ T cells isolated from CII-immunized mice cultured under Th17-polarizing conditions (blocking IL-4 and IFNγ) clearly enhanced the proportion of IL-17A-producing cells (20). Interestingly, as this was not the case in CD4+ T cells taken from naïve mice, it suggests that CII-immunization induces the expression of the IL-23R. Indeed, naïve T cells do not yet express the IL-23R (11) and CII/CFA immunization induces production of pro-inflammatory cytokines including IL-6 which, in concert with TGFβ and/or IL-21 induces IL-23R expression (11, 14, 21). This was also observed in our studies (**chapter III**), in which IL-6+TGFβ markedly enhanced the proportion of IL-17A expressing CD4+ T cells from both naïve and CII-immunized mice albeit the induction was more prominent in the CD4+ T cell fraction taken from CII-immunized mice. Additionally, IL-23 further enhanced the proportion of IL-17A+ CD4+ T cells induced by TGFβ+IL-6. This shows that during CIA development, CII/CFA-immunization, possibly through the induction of IL-6, induces naïve T cells to express the IL-23 receptor allowing these cells to respond to IL-23 and to produce IL-17A at relatively high quantities. IL-23 enhanced IL-17A expression in this study by inhibiting T-bet in CD4+ T cells from both naïve and CII-immunized mice and this was functionally relevant since IL-23, in contrast to wild-type T cells, could not enhance IL-6+TGFβ-induced IL-17A production from T-bet deficient CD4+ T cells. IL-23 also enhanced the expression of RORγt (and RORα) induced by IL-6+TGFβ in T cells from non-immunized mice. In contrast, in CII-primed T cells, IL-23 alone (no IL-6+TGFβ) was sufficient to enhance RORγt expression. Together, this shows that IL-23 enhances IL-6+TGFβ-induced IL-17A production and RORγt expression by inhibiting T-bet suggesting that IL-23 does not initiate but rather amplifies the induction of potentially pathogenic Th17 cells (Figure 1). Interestingly, it has been shown that T-bet regulates the expression of RORγt indirectly through Runx1 (22) providing even more complexity to the regulation of IL-17A transcription.

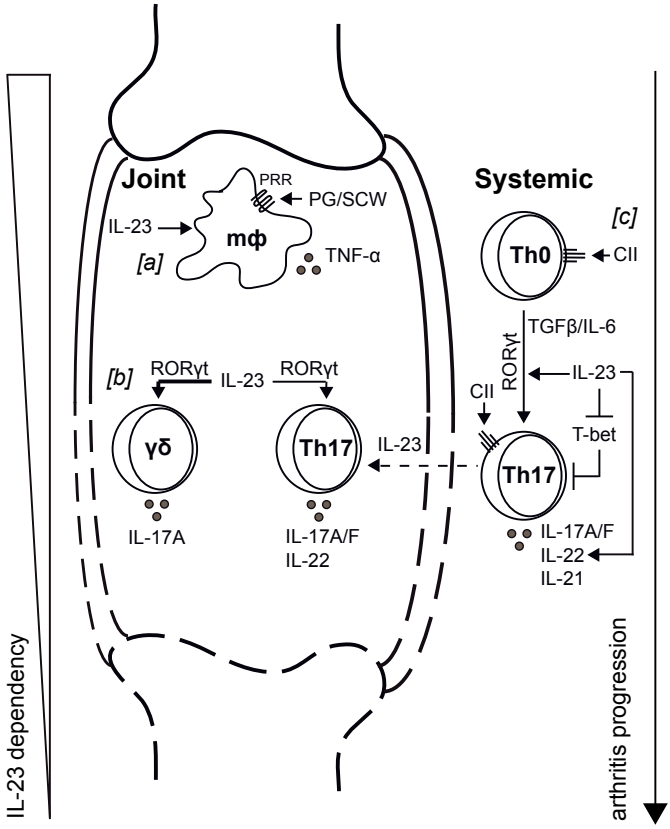

Figure 1. The IL-23/IL-17 immune pathway acts at various stages during arthritis. [a] Macrophages, together with fibroblasts are the main cell types found in the non-diseased joint making up the synovial layer but become hyperplastic and are a source of pro-inflammatory cytokines including TNF-α during arthritis. Activation of macrophages during arthritis development might occur by ligation of their innate pattern-recognition receptors (PRR) with bacterial-derived components such as peptidoglycan (PG) or streptococcal cell wall fragments (SCW). In this pathway, IL-23 has now been shown to be important since lower TNF-α were measured in IL-23p19-/- mice upon an intra-articular challenge with either PG or SCW, and in *in vitro* cultures of synovial explants stimulated with SCW (chapter V). [b] In the mBSA-induced arthritis model, both CD4+ and CD3+TCRγδ+ (γδ) T cells were reduced in number and in their capacity to produce IL-17A and IL-17F systemically (not illustrated) and in the knee joints of IL-23p19-/- mice. It is assumable that similar to peripheral CD4+ and γδ T cells (not illustrated) in which a strong reduction in the proportion of RORγt+ cells in IL-23p19-/- mice was observed, IL-23 also controls RORγt expression in these cells found in the knee joints (chapter IV). [c] In the periphery during the development of chronic autoimmune experimental arthritis (CIA), the highest proportion of IL-17+ CD4+ T cells (Th17) cells were found 10 days after immunization. When taken from IL-23p19-/- mice, these cells secreted significantly less IL-17 compared to wildtype mice after polyclonal stimulation *in vitro* (chapter II). Under Th17-polarizing conditions, IL-23 was more potent to induce IL-17 production from CD4+ T cells from *in vivo* type II collagen (CII)-primed mice than from naïve mice. This shows that under arthritic conditions IL-23 promotes Th17 development. The combination of TGFβ/IL-6, compared to IL-23, induced a larger population of CD4+IL-17+ T cells under arthritis conditions but IL-23 further enhanced the secretion of IL-17A and IL-17F in these cells by inhibiting T-bet transcription (chapter III). Finally, based on the contribution of IL-23 during CIA pathogenesis (chapter II) it seems that the IL-23 dependency decreases as arthritis progresses.

In addition to CII/CFA immunization, mBSA/CFA immunization induced the formation of IL-17A+IFNγ- (Th17) but also of IFNγ+IL-17A- (Th1) CD4+ T cells (**chapter IV**). IL-23p19 deficiency did not lead to a reduced proportion of IFNγ+IL-17A- but did reduce the IL-17A+IFNγ- subset. Additionally, transcripts for IL-17F, IL-22, and RORγt were reduced in IL-23p19-/- FACS-purified CD4+ T cells compared to their wildtype counterparts. This shows that during the T cell driven AIA model, IL-23 regulates the formation of IL-17A-producing CD4+ T cells. We also found a profound reduced expression in IL-23p19-/- mice of IL-17A and RORγt by TCRγδ T cells in this model (discussed below). At day 7 of AIA, a respectable number of CD4+ T cells could be isolated from the joints of WT mice of which the majority produced IL-17A but not IFNγ. Whether these cells had migrated from the periphery or had undergone local proliferation was not investigated. In accordance with the lower AIA disease severity, the amount of CD4+ T cells was lower in IL-23p19-/- mice demonstrating that IL-23 is involved in controlling the (T helper) cellular inflammation during AIA. Interestingly, the proportions of IL-17A+ and IFNγ+ CD4+ T cells were significantly lower in the joints of arthritic IL-23p19-/- mice compared with wildtype mice (Figure 1). Furthermore, by using IL-17 receptor A deficient (no IL-17A/F signaling) mice, it was shown that IL-17 receptor signaling is crucial for progression of AIA since these mice developed significantly less joint inflammation than wildtype mice which was in fact comparable with IL-23p19-/- mice. Together, this shows that IL-23 is importantly involved in the generation, cytokine production and localization of arthritogenic CD4+ T cells during T cells driven AIA and that IL-17RA signaling is of crucial importance in the IL-23/IL-17 immune pathway in this arthritic model.

COMPEX REGULATION OF IL-22 BY IL-23

IL-22 is an IL-10 cytokine family member (23) expressed by activated T cells including human and mouse Th17 cells. The main effector function of IL-22 is the induction of antimicrobial agents by epithelial cells resulting in innate antimicrobial defenses although there might be a shift towards a more pathogenic function for IL-22 when IL-17 is present as well (24). The role of IL-22 in experimental arthritis has been shown to be pro-inflammatory since IL-22-/- mice developed less severe CIA than control mice (25). However, the exact mechanism by which IL-22 elicited this effect was not investigated. Recently, a novel T helper subset was described in humans that produces IL-22, no IFNγ and only little IL-17A (26-27) and has been named Th22 (28). However, Th22 cells have only been recognized thus far in humans and have been described to be present in skin from psoriasis patients (28).

Studies performed in experimental arthritis models described in this thesis recognized a positive (**chapters II-IV**) but also a negative (**chapter VI**) regulatory role for IL-23 in the regulation of IL-22, showing a complex regulation of IL-22 by IL-23. When CD4+ T cells were taken from naïve or CII/CFA immunized animals and cultured under Th17-polarizing conditions (neutralizing IFNγ and IL-4) in the presence of IL-23, IL-22 production was significantly enhanced compared to IL-23-deficient conditions showing that IL-23 stimulates IL-22 production in developing Th17 cells (**chapter III**). This is line with studies performed by others describing a prominent IL-23/IL-22 axis in murine Th17 cells (29-31). During AIA, IL-23 promoted T cell derived IL-22 since lower IL-22 transcript levels were found in CD4+ T cells isolated from IL-23p19-/- mice 7 days after intra-articular antigen recall (**chapter IV**) and in mice prophylactic treated with neutralizing anti-IL-23p19 antibodies before the induction of an antigen-driven flare-up reaction (**chapter II**). Together, these data suggest that during experimental arthritis IL-23 stimulates the production of T cell derived IL-22 (Figure 1).

In agreement with the mouse system, a positive regulatory role for IL-23 on IL-22 production during human Th17 polarization has been described (31-34). In addition, IL-23 induces IL-22 in unfractionated human memory CD4+ T cell (32, 35). However, since the latter contains various T helper subsets the effect on individual T cell subsets may be masked. In contrast, when highly purified human memory CCR6+ Th17 or CCR6+CCR10+ Th22 cells were cultured with IL-23 under non-polarizing conditions, IL-22 was significantly suppressed thereby stabilizing the Th17 profile in Th17 cells and controlling the balance of Th17 and Th22 cells in favor of Th17 (**chapter VI**). Altogether, this suggests that IL-23 positively regulates IL-22 during Th17 differentiation and in unpolarized T cells, compared to a negative regulatory role in memory Th17 and Th22 cells. Whether this complexity also exists in mice is unclear and difficult to study since no murine IL-17A-IL-22+ Th22-like Th subset has been described.

IL-23 IN THE REGULATION OF IL-17 PRODUCTION BY ΓΔ T CELLS

As extensively discussed above, IL-23 is involved in the maturation and stabilization of the Th17 phenotype and is also crucially important for the maintenance of the Th17 cell pool. Additional to the expression of the IL-23R on effector and memory CD4+ TCRαβ T cells is the IL-23R expressed on other (innate) lymphoid cells, including CD3+TCRγδ (γδ) T cells (36) with the capacity to produce IL-17 in response to IL-23 (37-38).

In human RA, the role and contribution of γδ T cells is controversial although the identification of γδ subsets with the potential to produce IL-17 might help to unravel their role during arthritis. Indeed, Vγ4+ γδ T cells were enriched for IL-17 production and were shown to contribute to the exacerbation of CIA (39). A recent report suggested that although γδ cells isolated from CIA mice could promote osteoclastogenesis in vitro, they failed to do so in vivo and were in fact spatially apart from the bone-areas in the joint of CIA mice and human RA, in contrast to CD4+ T cells suggesting that not γδ cells but CD4+ T cells promoted bone destruction in arthritis (40). In spite of this, the authors did not differentiate between IL-17-producing and non-producing γδ+ cells or CD4+ cells in their osteoclastogesis-assays nor were co-stainings for γδ cells and IL-17 performed making it difficult to firmly exclude the contribution of IL-17-producing γδ cell subsets during CIA. Also, since γδ cells are a source of rapid IL-17A production, it might be that these cells already deposit IL-17 in the joints before the manifestation of clinical symptoms. Even more, it has been suggested that IL-17 producing γδ cells may amplify the Th17 response during organ inflammation (41). Therefore, it is of essence to investigate the possible pathogenic potential of IL-17 producing cells during early joint inflammation, especially since they might aggravate ongoing CIA when adoptively transferred (42).

Similar to the autoimmune CIA model, IL-17A-producing γδ T cells could be isolated from spleen, draining lymph nodes and knee joints during non-autoimmune antigen-induced arthritis (AIA) [(43), **chapter IV**]. In fact, the proportion of IL-17A+ γδ T cells was higher in the joints than in the spleen of AIA mice and this proportion was higher than the proportion of CD4+IL-17A+ T cells in spleen, lymph nodes and arthritic joints suggesting a role for γδ cells in this disease. However, absolute cell numbers of CD4+ T cells outnumbered that of γδ cells. In line with the reduced disease scores, the proportion of IL-17+ γδ cells and the mean IL-17A expression *per cell* (MFI) was severely reduced in IL-23p19-/- mice compared to controls (Figure 1). This shows that IL-23 regulates IL-17 expression in γδ T cells during arthritis although the immunopathological contribution of these cells was not directly shown. Interestingly, since IL-23 can act in synergy with TLR ligands (44-45) which are essential for optimal immunization in experimental (arthritis) models, this synergism might assist or even initiate subclinical IL-17-mediated bone degradation. Speculatively, joint damage might be continued by Th17 cells assisted by IL-17A producing γδ cells (41) towards macroscopically visible arthritis. Conversely, IL-17+ γδ cells might additionally promote Th17 mediated pathology by preventing the development of regulatory T cells, which was even IL-23-dependent (46). Together, these experimental data show a significant role for IL-17-producing γδ cells in (autoimmune) inflammatory diseases although their role might be most prominent during the early inflammatory processes.

THE IL-23/IL-17 IMMUNE PATHWAY IN HUMAN ARTHRITIS

Using various experimental arthritis models to study the role of IL-23 during different stages of the arthritis process, we have learned that IL-23 is critically important for the development of autoimmune (CIA; **chapters II-III**), non-autoimmune (AIA, **chapter IV**) and acute (PG/SCW, **chapter V**) arthritis. Blocking IL-23 before disease onset (CIA and AIA flare) significantly prevented full-blown arthritis. However, when IL-23 was blocked after onset of the disease (CIA), no therapeutic effect was observed. Extrapolating these findings to human disease predicts that IL-23 does not have great therapeutic potential to treat ongoing RA but might hamper the development of RA when given after early recognition of the disease and/or during flare-up (reactive) arthritis. Interestingly, several clinical trials showed that blocking IL-12/IL-23 (p40) activity was beneficial for patients suffering from e.g. psoriasis (47-51) or psoriatic arthritis (52) and a phase II trial with the anti-IL-12/23p40 drug apilimod is currently being conducted (ClinicalTrials.gov NCT00642629). Importantly, with anti-IL-12/23 (p40) therapy targeting both the IL-12 and the IL-23 pathways, the relative contribution of these cytokines in human RA remains unclear and it could be speculated based on experimental data (1) that IL-23 has a more prominent role during RA development than IL-12. Moreover, blocking IL-12/IL-23 activity in psoriasis appeared to be an even better clinical approach than blocking TNF-α activity (53). Since at least one-third of RA patients fail to respond to anti-TNF therapy (54-55), additional treatment regimens are required to be introduced in the clinic, with anti-IL12/23 therapy being an interesting target. Additional to the effect of anti-IL-12/23 on patients with active RA, it would be of high interest to evaluate the therapeutic efficacy of anti-IL-12/23 therapy in early RA patients, since data presented in this thesis predicts that especially RA patients in their earliest stages of disease are most likely to benefit from depleting IL-23 activity.

Mechanistically, work from our group has shown that CCR6+ memory T cells i) are found at an increased frequency in blood of treatment-naïve RA patients compared to healthy controls, ii) produced elevated levels of IL-17 and iii) co-operated with RA synovial fibroblasts in the induction of matrix metalloproteinases in a TNFα/IL-17A dependent manner, suggesting that these cells support RA chronicity through a pro-inflammatory feedback loop (56). Since these CCR6+ memory T cells expressed relatively high levels of the IL-23R (compared to CCR6- T cells) (56), we hypothesized that they might be regulated by IL-23. Indeed, as described in **chapter VI**, human peripheral blood-isolated memory CCR6+ Th17 cells respond to IL-23, in co-operation with IL-1β, to enhance their Th17-profile by inducing IL-17A and suppressing IL-22 production. This is interesting from a clinical perspective as well since in human RA synovial fluid it was documented that IL-23 could modulate IL-1β production (57), and vice versa (58-59). Also, IL-23 correlated with IL-17 levels in RA synovial fluid (60-61) and RA disease activity (62).

MAIN FINDINGS

With the discovery that not the IL-12/Th1 but rather the IL-23/Th17 immune axis is likely to be responsible for many autoimmune disorders, many cytokines, transcription factors and immune cells involved in this pathway have been identified. This has resulted in the discovery of new drug targets that hopefully will contribute to the treatment of autoimmune diseases including rheumatoid arthritis. In fact, anti-IL12/23 and anti-IL-17 are already being tested in the clinic and results seem promising. In this thesis, the IL-23/IL-17 immune pathway in various experimental arthritis models and human memory Th17 and Th22 cells was investigated and from this, the main findings are:

1. IL-23 drives the development of acute (chapter V) and chronic arthritis (chapters II-IV) but is not essential during ongoing chronic autoimmune arthritis (chapter II) predicting that patients with persistent RA will likely not benefit considerably from IL-23-targeting drugs although patients that are early diagnosed or have a reactive arthritic potentially will benefit from anti-IL-23 therapy.

2. IL-23 promotes the formation of Th17 cells (chapter II) and enhances their differentiation and effector cytokine production (chapter III) during collagen-induced arthritis (CIA).

3. IL-23 is essential for IL-17 and RORγt expression in TCRγδ T cells in addition to CD4+ T cells during arthritis (chapter V) showing that IL-23 controls IL-17 production from early (TCRγδ) and late (CD4+) acting responder T cells.

4. IL-23 promotes local TNF-α production during acute joint inflammation (chapter V).

5. IL-23 in conjunction with IL-1β promotes the Th17 profile in human memory CCR6+ Th17 and CCR6+CCR10+ Th22 cells by enhancing IL-17A but suppressing IL-22 (chapter VI).

RECOMMENDATIONS FOR FUTURE EXPERIMENTS

Does anti-IL-23 has a better therapeutic efficacy than anti-IL12?

With the finding that anti-IL-23 could not ameliorate CIA (chapter II) we proposed that anti-IL-23 might not have great therapeutic potential in human RA. However, anti-IL-12/23p40 (blocking both IL-12 and IL-23) had already been demonstrated to be beneficial for several autoimmune disorders (37). Although this might seem controversial, evidence exists that shows that late-stage disease is rather Th1 than Th17 mediated suggesting that the IL-23/Th17 dependency decreases during disease progression while the IL-12/Th1 dependency increases. Therefore, to directly compare IL-23 and IL-12 as therapeutic targets, neutralizing monoclonal antibodies against IL-12p35 should be generated and compared to IL-23p19 in experimental chronic autoimmune arthritis disease models (i.e. CIA).

Is the novel IL-23/TNF-α axis functionally involved in arthritis?

In chapters IV and IV we found that lack of IL-23 led to reduced synovial levels of TNF-α during experimental arthritis. However, whether the reduced joint inflammation observed in these IL-23p19-/- mice compared to wildtype controls was TNF-α mediated remained unclear but essential to investigate. Therefore, a model should be created in which IL-23 is uncoupled from TNF-α. One such approach could be to generate virus particles genetically modified to produce biological active IL-23 (p40p19) and to introduce these particles into TNF-α-/- mice versus wildtype mice during acute joint inflammation as performed in chapter V. Such an approach has shown to be useful to study the TNF-α dependency of IL-17 during arthritis (63).

What is the pathological potential of IL-17 producing γδ T cells in arthritis?

IL-23 was critical for IL-17 production by γδ T cells during antigen-induced arthritis and these cells were found to be increased in number in the joint of arthritic mice compared to naïve mice (chapter IV) suggesting that γδ cells play a role during joint inflammation in this model. However, whether γδ T cells directly contributed to the pathogenesis in this arthritis model was not investigated. Of note, in CIA it had been shown that Vγ4+ γδ T cells produced IL-17 and that CIA could be ameliorated when these cells were depleted (39). In contrast, a later study showed that not γδ but Th17 cells drove bone destruction in mice (40) but the authors did not target the IL-17A+ γδ T cells specifically. So, whether γδ T cells really contribute to joint inflammation remains unclear. Therefore, intra-articular injection of IL-17A-/- γδ cells versus wildtype γδ cells from antigen-immunized mice into naïve recipient mice that lack host-derived γδ cells [i.e. δ-/- mice (64)] should reveal the arthritogenic potential of IL-17-producing γδ T cells. Another elegant approach would be to use mice that express a fluorescent protein (e.g. GFP) under control of the IL-17A gene

promoter so that these cells, taken from immunized mice, can be easily isolated (FACS-sorting) and injected into TCRδ-/- mice in comparison with unfractionated γδ cells and untreated control mice.

Are human memory Th22 cells pro-inflammatory in RA, and does IL-23 control its effector function?

We described in chapter VI that IL-23 enhanced the Th17 profile in CCR6+ Th17 and in CCR6+CCR10+ Th22 cells by enhancing IL-1β-induced IL-17 and suppressing IL-22 production. However, since the role of IL-22 and Th22 cells in arthritis is still unclear, the role of this IL-23-mediated IL-22 suppression in arthritis should be addressed. Therefore, coculture-studies of synovial fibroblasts from RA patients can be utilized. This system has been used before to demonstrate the increased capacity of RA-derived CCR6+ Th17 as compared to healthy-control subjects-derived Th17 cells to induce pro-inflammatory cytokines and MMPs produced by the RASF (56). So, this coculture system could be used here in which RA fibroblasts are cocultured with CCR6+ Th17 or CCR6+CCR10+ Th22 cells in absence or presence of IL-23, IL-1β or both to provide insight into the role of the IL-23-mediated IL-22 suppression during immune-mediated pathology.

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SUMMARY

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting approximately 1% of the people in the Western community. The disease is characterized by chronic inflammation of the joint synovium resulting in swelling and accumulation of synovial fluid. The synovial layer which is 1-2 cell layers thick normally but during arthritis progression it becomes hyerplastic and pannus will form. At later stages, active and irreversible breakdown of cartilage and bone tissue occurs resulting in ankylosis and malformations of the affected joints. The joint inflammation is composed of various types of immune cells including monocytes, macrophages and fibroblasts. These innate immune cells participate in the inflammatory reaction by the production of matrix metalloproteinases (MMPs) which actively degrade extracellular matrix proteins leading to tissue remodeling. These cells also produce cytokines importantly including tumor necrosis factor alpha (TNF-α), interleukin (IL) -1β, IL-6, IL-8, IL-12 and IL-23. These cytokines are essential in the activation, proliferation, differentiation and recruitment of T and B lymphocytes and as such these cytokines contribute to the development of chronic inflammation. B lymphocytes produce IgM and IgG rheumafactor auto-antibodies and anti-citrullinated protein antibodies (ACPA). These auto-antibodies are important determinants in serological testing for RA. Besides these auto-antibodies, B and T lymphocytes produce cytokines, some of which acting additively or even synergistically. For instance, IL-17A synergistically acts with TNF-α in driving osteoclastogenesis.

For a long time the general idea was that IFNγ producing T helper 1 (Th1) cells, driven by IL-12, were responsible for driving chronic autoimmune arthritis since targeting IL-12 suppressed experimental arthritis. However, conflicting data existed concerning the role of IFN-γ. In fact, most studies showed that neutralizing IFN-γ enhanced rather than inhibited arthritis severity in animal models. This dichotomy began to be resolved with the discovery of a IL-12-like cytokine presently known as IL-23. Whereas IL-12 consists of the subunits p40 and p35, IL-23 consists of this same p40 subunit but comprises an IL-23 specific p19 peptide in addition. Since many original studies investigating the role of IL-12 or Th1 cells aimed at the p40 subunit to block IL-12 activity, these studies actually disrupted both IL-12 and IL-23 signaling. To unravel the relative contribution of IL-12 and IL-23 during arthritis development, Murphy et al. compared mice lacking either the p40, p35 or p19 subunits and showed that not IL-12 but rather IL-23 drives disease since both IL-12/23p40 and IL-23p19 but not IL-12p35 deficient mice did not develop collagen-induced arthritis (CIA) at all. Interestingly, it appeared that in IL-23 deficient mice the expression of IL-17A but not IFN-γ was lower than that of IL-12 deficient or control mice suggesting that IL-23 drives a novel T helper cell subset specialized to produce IL-17A. Further studies confirmed these findings and the current knowledge is that IL-23 stabilizes and further matures a population of IL-17-producing cells referred to

as Th17 cells which are induced by the combination of TGFβ and IL-6 or IL-21. In turn, Th17-derived IL-17A but also Th17 cells themselves have been shown to participate in chronic joint inflammation. From this, the current dogma is that not the IL-12/Th1/IFN-γ but rather the IL-23/Th17/IL-17A immune pathway drives chronic arthritis.

In the present thesis, we have investigated the role for IL-23 in different experimental arthritis models keeping a strong focus on the IL-23/IL-17 immune pathway in T lymphocytes. The study conducted by Murphy et al. already showed that IL-23p19-deficient mice were fully protected from CIA development demonstrating an essential role for IL-23 in autoimmune arthritis development. However, the study did not reveal a possible function for IL-23 during later stages of disease nor its possible therapeutic potential. Therefore, we neutralized IL-23 activity in vivo to study the role for IL-23 at different stages of CIA (**chapter II**). When IL-23 was neutralized after immunization but before disease onset, the disease severity was significantly lower compared to control animals, though the mice were not fully protected. This shows that IL-23 is important during CIA onset. Next, we showed that IL-23 did not have a crucial role during the clinical phase of CIA since blocking IL-23 after disease onset did not ameliorate disease symptoms. Together, these data show that autoimmune CIA possesses IL-23 dependent and independent stages and predicts that anti-IL-23 therapy does not have great therapeutic potential although early intervention may reduce arthritis progression.

To obtain more insight into the role of IL-23 during Th17 cell subgroup polarization during CIA, splenic CD4+ T cells were isolated from naïve and type II collagen (CII) immunized wildtype animals and cultured in the presence of various combinations of cytokines (**chapter III**). These experiments revealed that IL-23 was more potent to induce IL-17A from CII-primed CD4+ T cells than from naïve CD4+ T cells. Further investigation showed that IL-23 induced IL-17A production by suppressing T-bet expression. However, most IL-17A producing CD4+ T cells were generated when CD4+ T cells from CII-primed mice were cultured with IL-6/TGFβ. Strikingly, IL-23 clearly enhanced the proportion of IL-17A+ CD4+ T cells taken from naïve mice cultured with IL-6/TGFβ. This suggests that CII-primed CD4+ T cell respond better to IL-23 stimulation than non-primed cells possibly by enhanced IL-23 receptor expression on the former cells compared to naïve T cells as a result of the CII-immunization. It was also shown that CD4+ T cells cultured with IL-23 produced higher IL-22 levels compared to unconditioned or IL-6/TGFβ-stimulated by both naïve and CII-primed CD4+ T cells. Altogether, this chapter shows that IL-23 enhances the Th17 profile through suppression of T-bet and that IL-23 is required for Th17-derived IL-22 during CIA.

The IL-23 receptor (IL-23R) is expressed on activated but not on naïve CD4+ T helper cells and as a result, IL-23 does not signal in naïve T helper cells. However, using IL-23R-eGFP reporter mice, it was shown that the IL-23R is expressed on naïve TCRγδ

(γδ) T cells. In CIA mice, it was shown that a specific subset of γδ T cells produced relatively large amounts of IL-17A (as compared to CD4+ T cells) and contributed to CIA pathogenesis. However, whether IL-23 contributed in the IL-17A production from these γδ T cells during arthritis remained unclear. Therefore, we used the T cell driven antigen-induced arthritis (AIA) model in IL-23p19-/- and normal wildtype mice to study the IL-23 dependency of IL-17A producing CD4+ and γδ+ T cells (**chapter IV**). First, IL-23p19-/- animals expressed significantly less severe AIA and bone erosion than control mice although they were not fully protected. This shows an important function of IL-23 during AIA development. Interestingly, as compared to wildtype mice, γδ T cells isolated from spleen, draining lymph nodes and joint of IL-23p19-/- mice expressed significantly less IL-17A and RORγt showing that IL-23 is required for IL-17A and RORγt expression in γδ T cells during AIA. We also observed a significant reduction in the proportion of IL-17A-producing CD4+ T cells. Comparing CD4+ and γδ T cells showed that a higher proportion of γδ T cells produced IL-17A and RORγt mRNA than CD4+ T cells in this model. In conclusion, IL-23 critically regulates IL-17A and RORγt expression from γδ T cells in addition to conventional CD4+ T cells in a T cell mediated arthritis model.

In addition to αβ and γδ T cells, the IL-23R is expressed by myeloid cells and that IL-23 has a role in innate immunity. To investigate whether IL-23 contributes to the development of acute joint inflammation, peptidoglycan (PG) or streptococcal cell-wall fragments (SCW) were injected directly into the knee joints of wildtype and IL-23p19-/- mice (**chapter V**). In normal wildtype mice, IL-23p19 mRNA expression was monitored after PG injection and revealed that IL-23p19 was measurable as early as 1.5 h after injection, increased at day 1 and peaked at day 2 after which expression returned to baseline levels. Next, it was observed that IL-23p19-/- mice developed significantly less severe acute arthritis than control mice showing that the development of acute joint inflammation depends on IL-23. Interestingly, this was accompanied with lower TNF-α levels in synovial washouts and in the culture supernatant of SCW-stimulated synovial explants showing a novel IL-23/TNF-α axis during acute joint inflammation.

Human naïve T cells can be *in vitro* skewed towards the Th17 linage using a combination of the polarizing cytokines IL-1β, IL-6, IL-23 and TGFβ. In peripheral blood, Th17 cells manifest by the surface phenotype CD4+CD45RO+CCR6+. These memory Th17 cells produce besides IL-17 IL-22, and are thought to play a role in human RA since they are found at higher frequencies in RA patients than control subjects. Additionally, it was shown that they might promote chronicity in crosstalk with synovial fibroblasts. However, the role of Th17-promoting cytokines on peripheral memory Th17 cells remained uninvestigated. Using highly purified CCR6+ Th17 cells we show in **chapter VI** that IL-23, when combined with IL-1β, enhanced IL-17A production compared to IL-1β alone or unconditioned cells. Interestingly, IL-23 alone significantly suppressed IL-22

production from these memory Th17 cells. Within the CCR6+ Th17 cell pool, a minor fraction of cells co-expresses the chemokine receptor CCR10 and these cells express clearly lower levels of IL-17A than their CCR10- counterparts while IL-22 expression is comparable. Therefore, these CCR6+CCR10+ memory T cells were referred to as Th22 cells. Although their role in human RA is unclear, it is postulated that they might have a role in autoimmune skin diseases. Also, IL-22 deficient mice developed less severe CIA than control mice but no Th22 cells have been identified in mice so far. Still, it is unclear whether IL-23 also regulates IL-22 from Th22 cells and therefore this was investigated subsequently. IL-23 reduced the proportion of IL-22 producing Th22 cells compared to IL-1β or unconditioned cells. Interestingly, while under non-polarizing conditions IL-17A production from CCR10+ Th22 was minimal compared to CCR10- cells, IL-1β alone (not IL-23 alone) induced profound IL-17A production from CCR10+ Th22 cells. This shows that IL-23 and IL-1β promote the Th17-phenotype in both CCR6+ Th17 and CCR10+ Th22 cells thereby skewing the Th17/Th22 balance in favor of Th17 cells.

The research described in this thesis increases the current knowledge concerning the function of IL-23 and in particular the IL-23/IL-17 immune pathway in arthritis This might help the development and further testing of IL-23 as a clinical drug for RA and possibly other (auto-)immune diseases.

NEDERLANDSE SAMENVATTING

Reumatoïde artritis (RA) is een chronische auto-immuun ziekte waar ongeveer 1% van de mensen in de westerse wereld aan lijdt. De ziekte wordt gekenmerkt door chronische ontsteking van de gewrichten resulterend in zwelling en accumulatie van gewrichtsvocht (synoviaal vocht). In latere fase van de ziekte treedt er actieve en onomkeerbare kraakbeen- en botafbraak op van de gewrichten. Dit resulteert in de zo voor RA kenmerkende stijfheid en misvorming van de gewrichten.

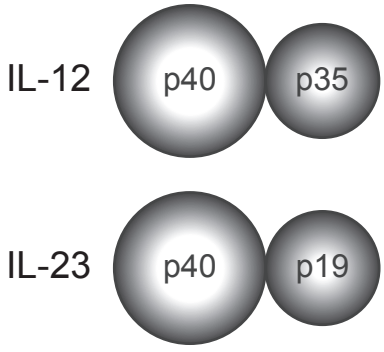
Bij gezonde mensen is de cellaag rond het gewrichtskapsel (synoviaalmembraan) 1 tot 2 cellagen dik. Echter, bij mensen met RA treedt er versnelde celdeling (hyperproliferatie) op en vormt er zich een dicht netwerk van bindweefsel (pannus) rond het synoviaal membraan. Deze gewrichtsontsteking (artritis) bestaat uit verschillende cellen van het afweersysteem met monocyten, macrofagen en fibroblasten als belangrijkste spelers van het aangeboren immuunsysteem en participeren in de ontstekingsreactie door het produceren van cytokinen (signaal-eiwitten) en kraakbeen-afbrekende enzymen (matrix metalloproteinases). De bekendste ontstekings-cytokinen zijn tumor necrose factor alpha (TNF-α), en interleukine (IL) 1β, IL-6, IL-8, IL-12 en IL-23. Deze cytokinen zijn essentieel voor activatie, proliferatie (celdeling), differentiatie (maturatie) en het aantrekken van T en B lymfocyten (kader 1). Bij mensen met RA kunnen B lymfocyten de auto-antistoffen reumafactor en anti-gecitrullineerde eiwitten (zogenaamde ACPAs) produceren. De aanwezigheid van deze ACPAs verhoogd de kans op (een ernstige vorm van) RA en het testen op deze auto-antistoffen maakt dan ook deel uit van het standaard onderzoek om de diagnose RA vast te kunnen stellen. Hiernaast produceren B en T cellen cytokinen waarvan sommige elkaar kunnen versterken. Zo is het aangetoond dat TNF-α en IL-17A samen meer bijdragen aan actieve botafbraak (als een gevolg van osteoclastogenese) dan de som der delen.

Kader 1: T en B lymfocyten

T en B lymfocyten behoren tot het verworven immuunsysteem en herkennen antigeen (eiwit-structuren aan het oppervlak van een cel of weefsel) zeer specifiek. T en B lymfocyten zijn essentieel voor de afweer tegen moeilijk te bestrijden pathogenen (bijv. bacteriën en virussen) wanneer het aangeboren afweersysteem (welke reageert op een niet erg antigeen-specifieke manier) tekortschiet. Elke T of B lymfocyt kan maar één type antigeen herkennen via een specifieke antigeen-receptor aan het celoppervlak. De formatie van een adequate T en B cel-gemediëerde specifieke afweer duurt enkele dagen tot een week waarbij een naïeve lymfocyt (een cel welke nog niet eerder antigeen heeft herkent) wordt geactiveerd en gaat delen (proliferatie). Tijdens deze proliferatie ondergaat de lymfocyt ook differentiatie en in het geval van T cellen groeit het uit tot

één van de T helper (Th) subsets Th1, Th2, Treg, Th17 of Th22 onder invloed van cytokinen (zie figuur 1 van hoofdstuk I voor een grafische weergave hiervan). Na een immuunreactie zullen enkele geactiveerde lymfocyten uitgroeien tot geheugen cellen welke decennia lang in het lichaam blijven en zeer snel reageren als 'zijn' antigeen zich een tweede maal aanbiedt. Echter, in sommige gevallen herkennen lymfocyten lichaamseigen antigeen als zijnde lichaamsvreemd en wordt er een immuunreactie gevormd tegen dit lichaamseigen weefsel of cellen. Men spreekt dan van autoreactieve lymfocyten. De antistoffen gericht tegen lichaamseigen cellen of weefsels noemt men auto (=zelf)-antistoffen. Autoreactieve T en B cellen en auto-antistoffen kunnen bijdragen tot het vormen van een auto-immuun ziekte zoals RA door het aanvallen van lichaamseigen cellen of weefsels.

Voor een lange tijd heeft men gedacht dat interferon-gamma (IFN-γ) producerende T helper 1 (Th1) cellen, aangestuurd door IL-12, verantwoordelijk waren voor het aansturen van RA omdat het remmen van de activiteit van IL-12 in diermodellen resulteerde in verlaagde ziekteactiviteit. Echter, wanneer IFN-γ werd geremd werd er doorgaans geen reductie van de ziekteactiviteit waargenomen. Deze discrepantie kon voor lange tijd niet worden uitgelegd. Echter, met de ontdekking van IL-23 werd het duidelijk waarom het remmen van IL-12 wel, maar het remmen van IFN-γ niet leidde tot suppressie van artritis. Het bleek namelijk dat een deel van de IL-12 en IL-23 eiwitten exact hetzelfde waren; waar IL-12 is opgebouwd uit een p40 en een p35 deel-eiwit (subunit), is IL-23 opgebouwd uit ditzelfde p40 subunit alsmede uit een IL-23-specifiek p19 subunit (Figuur 1).



Figuur 1. IL-12 en IL-23 tonen structurele overeenkomsten. Waar IL-12 is opgebouwd uit de subunits p40 en p35 is IL-23 ook opgebouwd uit de p40 subunit maar ook uit een IL-23-specifieke p19 subunit.

Omdat de meeste studies met betrekking tot de bijdrage van IL-12 in artritis gericht waren op de p40 subunit bleek nu, met de ontdekking van IL-23 (p40/p19) dat niet alleen IL-12 (p40/p35) maar ook de activiteit van IL-23 werd geremd. In een studie, uitgevoerd door Murphy en collega's, werd de relatieve contributie van IL-12 en IL-23 voor de ontwikkeling van artritis in collageen type II geïnduceerde artritis (CIA, kader 2), een diermodel voor humane RA vergeleken. Hieruit bleek dat muizen die het IL-12/IL23p40 eiwit niet konden maken (dus geen IL-12 en IL-23 activiteit) geen artritis ontwikkelde, evenals dieren die geen IL-23p19 (geen IL-23, wel IL-12) konden maken. Opvallend genoeg, dieren die het IL-12p35 eiwit miste (geen IL-12, wel IL-23) ontwikkelde wel degelijk artritis. Dus, muizen welke specifiek IL-23 maar niet IL-12 miste ontwikkelde geen CIA. Hieruit werd geconcludeerd dat niet IL-12 maar juist IL-23 essentieel is voor de ontwikkeling van CIA en mogelijk RA. Tevens observeerde deze wetenschappers in de IL-23p19-deficiënte dieren een sterk verlaagde productie van het cytokine IL-17A geproduceerd door T helper cellen, ten opzichte van IL-12p35 deficiënte of normale controle dieren. Opvallend genoeg bleef de productie van IFNγ (Th1) onveranderd in de IL-23p19 deficiënte dieren ten opzichte van IL-12p35 deficiënte en normale controle dieren. Dit suggereerde dat IL-23 de productie van IL-17 producerende T helper cellen aanstuurt. Vervolg studies bekrachtigde deze observaties en het is nu duidelijk dat IL-23 zorgt voor stabilisatie van T helper 17 (Th17; genoemd naar IL-17) cellen. De differentiatie van Th17 cellen vanuit naïeve (kader 1) T cellen wordt aangestuurd door tumor groei factor beta (TGFβ) en IL-6 of IL-21 (zie figuur 1, hoofdstuk I). In conclusie, waar men eerst dacht dat de IL-12/Th1/IFN-γ immuun route verantwoordelijk was voor de vorming van artritis is het nu duidelijk dat juist de IL-23/Th17/IL-17 immuun route hiervoor verantwoordelijk is.

Kader 2: Collageen geïnduceerde artritis

Collageen-geïnduceerde artritis (CIA) wordt geïnduceerd door muizen in te spuiten in de staartbasis (immuniseren) met type II collageen (CII, het hoofdbestanddeel van gewrichtskraakbeen) geïsoleerd uit runderen samen met een adjuvant (een emulsie welke de immuunreactie versterkt). Drie weken na immunisatie wordt er nogmaals een injectie gegeven met CII in de buikholte om het immuunsysteem een extra impuls te geven (booster). Het ingespoten rundercollageen wordt als lichaamsvreemd herkent en als zodanig door het immuunsysteem aangevallen. Echter, doordat de structuur van het rundercollageen sterk op het muis-eigen CII lijkt ontwikkelt zich na verloop van tijd een immunologische reactie gericht tegen het muis-eigen CII. Dit resulteert in collageen-afbraak in met name de gewrichten welke enkele dagen na de booster injectie klinisch waarneembaar is. Echter, het moment van klinische manifestatie alsmede de

mate van artritis (artritis score) varieert per dier. De CIA artritis score wordt bepaald aan de hand van de ernst van roodheid (ontsteking) en zwelling (oedeem) van voor- en achterpoten. Zowel T als B lymfocyten zijn verantwoordelijk voor het ontstaan van dit model. T lymfocyten spelen een belangrijke rol door de productie van cytokinen en sturen de B lymfocyten aan tot de productie van auto-antistoffen welke uiteindelijk verantwoordelijk zijn voor de ziekte.

In dit proefschrift is de rol van IL-23 in verschillende experimentele artritis modellen onderzocht met een sterke focus op de IL-23/IL-17 immuun route in T lymfocyten. In deze Nederlandse samenvatting zullen de belangrijkste bevindingen worden beschreven.

In de studie van Murphy en collega's was al bewezen dat IL-23 essentieel is in de ontwikkeling van CIA. Echter, de IL-23 afhankelijkheid van dit model tijdens verschillende stadia van de ziekte werd in die studie niet onderzocht. Gebruikmakend van een antilichaam welke de activiteit van IL-23 neutraliseert (anti-IL-23p19) hebben wij in **hoofdstuk II** het belang van IL-23 onderzocht in verschillende fasen van CIA. Wanneer de activiteit van IL-23 werd geneutraliseerd nádat de muizen waren geïmmuniseerd maar vóór de booster en dus vóór klinische verschijnselen, kon ernstige ziekte worden voorkomen. Echter, de muizen waren niet volledig beschermd tegen de vorming van gewrichtontsteking. Deze resultaten bewijzen dat IL-23 deels verantwoordelijk is voor het ontstaan van CIA. In deze studie werd ook aangetoond dat IL-23 niet essentieel bijdraagt aan het verloop van de ziekte wanneer er al klinische verschijnselen zijn omdat de artritis score niet significant afnam wanneer IL-23 werd geneutraliseerd in dieren waarin al artritis zichtbaar was. Tezamen laten deze resultaten zien dat CIA IL-23 afhankelijke en onafhankelijke fasen kent en voorspeld dat het neutraliseren van IL-23 in humane RA geen groot therapeutisch potentieel heeft al zou vroege interventie met anti-IL-23 de ziekte activiteit van RA wel kunnen remmen.

Om meer inzicht te krijgen in de rol van IL-23 met betrekking tot de formatie van Th17 cellen gedurende CIA werden T lymfocyten geïsoleerd uit de milt van normale en CII-geïmmuniseerde muizen en *in vitro* (in een petrischaal) gekweekt in de aan- of afwezigheid van verschillende cytokinen (**hoofdstuk III**). Uit deze experimenten bleek dat IL-23 potenter was in het induceren van IL-17A producerende T cellen geïsoleerd uit CII-geïmmuniseerde dieren dan uit naïeve dieren en het toevoegen van IL-23 aan TGFβ/IL-6-gestimuleerde cellen verhoogde de productie van IL-17. Deze resultaten suggereren dat IL-17A-producerende cellen geïsoleerd uit CIA dieren gevoeliger

zijn voor IL-23 stimulatie dan wanneer geïsoleerd uit naïeve muizen, waarschijnlijk doordat T cellen geïsoleerd uit geïmmuniseerde dieren meer IL-23 receptor (IL-23R) tot expressie brengen dan T cellen uit naïeve dieren. Tevens werd waargenomen dat IL-23 gestimuleerde T cellen meer IL-22 produceerde dan ongestimuleerde of IL-6+TGFβ-gestimuleerde T cellen. In conclusie, dit hoofdstuk beschrijft dat tijdens CIA, IL-23 het Th17 profiel verhoogt door het induceren van IL-17A en dat IL-23 essentieel is voor IL-22 productie in T lymfocyten.

De IL-23R komt tot expressie op geactiveerde, maar niet op naïeve T helper cellen en dus kunnen naïeve T helper cellen niet reageren op IL-23 stimulatie. Echter, de IL-23R komt wel tot expressie op naïeve gamma-delta (γδ) T cellen (kader 3). In muizen met CIA is aangetoond dat een specifieke subset van γδ T cellen IL-17A produceert en bijdraagt aan de ernst van de ziekte. Echter, of de IL-17A productie door deze γδ T cellen tijdens artritis IL-23 afhankelijk is, was niet onderzocht. Om dit nader te onderzoeken hebben wij gebruik gemaakt van het antigeen-geïnduceerde artritis (AIA) model (**hoofdstuk IV**). Dit model werd gekozen omdat in dit model T cellen verantwoordelijk zijn voor het ontstaan van artritis. Uit deze studie bleek dat IL-23p19-/- dieren significant minder AIA en botafbraak ontwikkelde dan normale controle muizen alhoewel IL-23p19-/- muizen niet volledig beschermd waren. Dit toont aan dat de ontwikkeling van AIA sterk afhankelijk is van IL-23. TCRγδ T cellen geïsoleerd uit de milt, drainerende lymfe knopen en uit de knieën van IL-23p19-/- muizen produceerde significant minder IL-17A en RORγt (kader 4) dan TCRγδ cellen geïsoleerd uit controle dieren wat aantoont dat IL-23 essentieel is voor IL-17A productie in γδ T cellen tijdens AIA. Tevens werd een significant kleinere populatie T helper cellen gemeten welke IL-17A produceert in de IL-23p19-/- ten opzichte van controle muizen. In conclusie, deze gegevens bewijzen dat IL-23 een essentiële factor is voor de productie van IL-17A en RORγt in TCRγδ T cellen in dit sterk T cel afhankelijke artritismodel.

Kader 3: TCRγδ T cellen

Elke T helper cel brengt een unieke receptor tot expressie aan de buitenkant van de cel, de zogenoemde T cel receptor (TCR). Deze receptor wordt gevormd tijdens de ontwikkeling van de T cel in de thymus (zwezerik door een ingewikkeld proces wat V(D)J-recombinatie wordt genoemd). Na een succesvolle V(D)J recombinatie is er een TCR gevormd welke is opgebouwd uit twee verschillende ketens; alpha en beta (TCRαβ, dit zijn de T helper cellen [kader 1] en beslaat ~95% van de totale hoeveelheid T cellen) of gamma en delta (TCRγδ). In het geval van de TCRαβ T helper cellen

herkent de T cel zijn specifieke antigeen welke wordt gepresenteerd door speciale antigeen-presenterende cellen middels een eiwit-complex (major histocompatibility complex). Voor TCRγδ T cellen (slechts 2% van de totale hoeveelheid T cellen) is de herkenning van antigeen door antigeen-presenterende cellen echter niet nodig waardoor zij minder beperkt (maar ook minder specifiek) dan T helper cellen antigeen herkennen. Hierdoor zijn de TCRγδ T cellen sneller in staat een immuunreactie tot stand te brengen dan T helper cellen.

Kader 4: RORγt

Transcriptie factoren zijn eiwitten binnen de cel welke belangrijk zijn voor het 'aan-' en 'uitzetten' van genen en zodoende de productie van het gen-corresponderende eiwit controleren (elk gen codeert namelijk voor één eiwit). Er zijn honderden transcriptie factoren welke elk hun 'eigen' gen of juist meerdere genen tezamen controleren, vaak in een cascade waarin meerdere transcriptie factoren of andere signaal moleculen betrokken zijn. De activiteit van transcriptie factoren is op hun beurt weer afhankelijk van andere interne en externe signalen, en voor bepaalde groepen transcriptie factoren zijn dit cytokinen. Specifiek voor IL-17A zijn er zodoende ook een aantal transcriptie factoren beschreven waarvoor RORγt en RORα de voornaamste zijn, en welke worden geïnduceerd door onder andere IL-23. Dus, de aanwezigheid van relatief hoge concentraties RORγt is een goede indicatie voor een hoge IL-23-afhankelijke IL-17 productie. Zo is bewezen dat muizen welke geen RORγt kunnen produceren minder Th17 cellen en een verminderde IL-17A productie hebben.

Naast T helper en γδ T cellen is aangetoond dat de IL-23R tot expressie komt op immuun cellen van het aangeboren immuunsysteem (monocyten, macrofagen, fibroblasten) en is gesuggereerd uit IL-23p19-/- studies in een muis-model voor humane multiple sclerose (MS) dat IL-23 deze cellen stimuleert. Macrofagen kunnen middels bepaalde receptoren moleculaire structuren herkennen welke voorkomen op de buitenwand van bacteriën. Door nu juist dit soort bacteriële componenten rechtstreeks in de knieën van normale muizen te injecteren worden deze macrofagen geactiveerd om cytokinen te gaan produceren. Dit leidt ertoe dat er snel een lokale (acute) gewrichtsontsteking ontstaat; een model voor reactieve artritis in de mens. In **hoofdstuk V** werd onderzocht of zo'n acute artritis IL-23 afhankelijk is. Al na 1,5 uur na injectie werd er lokaal IL-23 gemeten, en dit nam verder toe zoals gemeten 1 dag post-injectie. Echter, de hoogste IL-23 expressie werd gemeten op dag 2 waarna IL-23 weer afnam en achtergrond waarden bereikte op dag 7. Hierna werd de rol van IL-23 in de ontwikkeling van acute

artritis bepaald in IL-23p19-/- muizen; significant minder artritis werd waargenomen in IL-23p19-/- dieren ten opzichte van controle dieren. Deze observatie ging gepaard met significant minder TNF-α concentraties in het kniegewricht van IL-23p19-/- muizen. Dit suggereert dat tijdens een acuut macrofaag-gemediëerde gewrichtsontsteking er een IL-23/TNF-α immuun route bestaat die belangrijk is voor de ontwikkeling van een acute gewrichtsontsteking.

Humane naïeve T cellen kunnen *in vitro* differentieren tot Th17 cellen in aanwezigheid van een combinatie van IL-1β, IL-6, IL-23 en TGFβ. In het perifere bloed worden Th17 cellen gekenmerkt door aanwezigheid van de oppervlakte eiwitten (markers) CD4 (marker voor T helper cellen), CD45RO (marker voor geheugen cellen) en CCR6 (CCL20 chemokine-receptor). Deze geheugen Th17 cellen produceren naast IL-17A ook IL-22 en spelen mogelijk een belangrijke rol in humane RA omdat ze met een hogere frequentie worden gevonden in het bloed van RA patiënten dan bij gezonde mensen. Tevens is gesuggereerd dat deze CCR6+ Th17 cellen bijdragen aan het chronische karakter van RA door een interactie aan te gaan met fibroblasten geïsoleerd uit het synovium van RA patiënten. Echter, de IL-17 en IL-22 regulerende rol van IL-23 en andere Th17-polariserende cytokinen op deze cellen was nog niet eerder onderzocht. Gebruikmakend van uiterst zuiver geïsoleerde CCR6+ Th17 cellen wordt in **hoofdstuk VI** aangetoond dat IL-23 tezamen met IL-1β, IL-17A productie door CCR6+ Th17 cellen verhoogd ten opzichte van niet-gestimuleerde of IL-1β-gestimuleerde cellen. Een verrassende ontdekking was echter dat IL-23-stimulatie resulteerde in een verlaagde productie van IL-22 door CCR6+ Th17 cellen. Binnen de CCR6+ Th17 cel populatie bestaat er een fractie cellen welke additioneel de chemokine receptor CCR10 tot expressie brengt en duidelijk minder IL-17A maar vergelijkbare hoeveelheden IL-22 produceert vergeleken met CCR6+CCR10- cellen. Deze CCR6+CCR10+ T cellen worden daarom ook wel Th22 genoemd. Alhoewel Th22 cellen als zodanig nog niet zijn beschreven in de muis, is wel aangetoond dat IL-22 artritis promoot omdat IL-22-deficiënte dieren minder ernstige CIA ontwikkelde dan controle dieren. Echter, een eventuele regulerende functie voor IL-23 met betrekking tot IL-22 productie door humane Th22 was nog onbekend. Stimulatie met IL-23 met en zonder IL-1β, leidde tot een consistente en significante reductie van het aantal IL-22 producerende Th22 cellen ten opzichte van niet-gestimuleerde cellen. Tevens induceerde IL-1β met en zonder IL-23 de IL-17A productie door Th22 cellen, terwijl ongeconditioneerde Th22 cellen relatief weinig IL-22 produceerde. IL-22 productie staat onder controle van de transcriptie factor 'aryl hydrocarbon receptor' (AhR) welke direct de expressie van het cytochroom P450 gen CYP1A1 controleert. AhR activiteit kan worden geïnduceerd door het tryptofaan metaboliet FICZ. Door FICZ toe te voegen aan een Th22 celkweek met en zonder IL-23 kon er zodoende worden onderzocht of IL-23 via

AhR de productie van IL-22 controleerde door de expressie van CYP1A1 te meten. Ten opzichte van ongestimuleerde Th22 cellen verhoogde FICZ de expressie van CYP1A1 en leidde de combinatie FICZ+IL-23 tot verlaagde CYP1A1 expressie ten opzichte van FICZ alleen. In conclusie laat deze data zien dat IL-23 en IL-1β samen het Th17-fenotype versterkt in zowel Th17 als Th22 cellen door inductie van IL-17 enerzijds en suppressie van AhR-gemediëerde IL-22 expressie anderzijds. Dit kan leiden tot een veranderde Th17/Th22 balans in het voordeel van Th17 cellen en dus mogelijk verergering van Th17-afhankelijke RA.

Het onderzoek beschreven in dit proefschrift vergroot de huidige kennis met betrekking tot de rol van IL-23, en de IL-23/IL-17 immuun route in het bijzonder, in experimentele artritis en humane Th17 en Th22 cellen welke mogelijk belangrijk zijn in RA. Deze onderzoeksresultaten zullen een belangrijke bijdrage hebben voor de ontwikkeling en het verder testen van IL-23 als mogelijk therapeutisch doelwit in RA en andere auto-immuun ziekten.

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CURRICULUM VITAE

Ferry (Fredericus Hermanus Johannes) Cornelissen was born on March 9th 1981 in Huissen (NL). From 1999-2003 he studied Biology and Medical Laboratory Research (HLO, Bachelor of Applied Science) in Nijmegen (NL) and specialized in Biochemistry. During this study, he conducted research on the generation of a subunit vaccine at the department of Bacteriological R&D of Intervet BV, Boxmeer (NL). In 2003 he started to study Biotechnology, specialization Cellular and Molecular Biotechnology, at the Wageningen University in Wageningen (NL). During this study he contributes to the successful cloning and characterization of a toll-like receptor gene in *P. monodon* (supervised by Prof.dr. RJ Stet and Dr. JA Arts). In 2005 he obtained his Master of Science (MSc) degree and in this same year he started his PhD project at the departments of Rheumatology and Immunology at the Erasmus Medical Center, Rotterdam (NL). In September 2010 he started his postdoc project in the group of Dr. T. Cupedo (department of Hematology, Erasmus Medical Center) to investigate the regulation of cytokine signaling of RORC+ innate lymphoid cells.

PHD PORTFOLIO

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Departments of Rheumatology and Immunology

Research school: Postgraduate School of Molecular Medicine

PhD period:	December 2005 – December 2011
Promotor:	Prof.dr. J.M.W. Hazes
Copromotor:	Dr. E. Lubberts

Courses

2011	4th Symposium and master classes on Mucosal Immunology "Adaptive immune response in the mucosa: B cells and beyond"
	Photoshop and Illustrator CS5 Workshop
2010	Molmed course "Workshop on Basic data analysis on gene expression arrays (BAGE)
2009	NIBI Management course for PhD-students and Postdocs
2008	Scientific English Writing
2006	Laboratory Animal Science (art.9)
	KNAW symposium "The role of DNA polymorphisms in complex traits and diseases" Amsterdam, the Netherlands Molecular Immunology, Rotterdam, the Netherlands

Teaching activities

2007-2009	Supervising practicals 1st and 2nd years medical students (themes 1A the healthy person' and 2.1 'infection and immune diseases)
2008	Supervising Bachelor's thesis 'Monocytes and Th17 polarizing cytokines', J. Anber (8 months)

(Inter)national conferences, selected

2011

- EMBO congress "Signaling in the immune system; Lymphocyte signaling: translating membrane signals into differentiation programs", Siena, Italy, *poster presentation*
- European Workshop for Rheumatology Research (EWRR), Amsterdam, the Netherlands, *oral presentation*

2010

- European Workshop for Rheumatology Research (EWRR), Bamberg, Germany, *oral and poster presentation*
- Dutch Society for Immunology (NVVI) Annual Meeting, Noordwijkerhout, the Netherlands

2009

- Keystone Symposium "Th17 in health and disease", Vancouver, Canada, *poster presentation*
- Dutch Society for Rheumatology (NVR) Annual Meeting, Papendal, the Netherlands, *oral presentation*

2008

- American College of Rheumatology (ACR), San Francisco, USA, *oral and poster presentation*
- Dutch Society for Rheumatology (NVR) Annual Meeting, Papendal, the Netherlands, *oral presentation*
- European League Against Rheumatism (EULAR) Annual Meeting, Paris, France, *oral presentation, travel grant, registration waiver*
- Dutch Society for Immunology (NVVI) Symposium, Lunteren, the Netherlands
- European Workshop for Rheumatology Research (EWRR), Toulouse, France, *oral and poster presentation, travel grant*

2007

Dutch Society for Immunology (NVVI) Annual Meeting, Noordwijkerhout, the Netherlands

European League Against Rheumatism (EULAR) Annual Meeting, Barcelona, Spain, *poster presentation*

Dutch Society for Immunology (NVVI) Symposium, Lunteren, the Netherlands

2006

Dutch Society for Immunology (NVVI) Annual Meeting, Noordwijkerhout, the Netherlands

Dutch Society for Immunology (NVVI) Symposium, Lunteren, the Netherlands

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