The role of innate immune cells in tissue inflammation in spondyloarthritis
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GENERAL DISCUSSION AND SUMMARY

This chapter aims to put the research presented in this thesis in a broader perspective. First we will discuss our objectives and approaches. We will discuss our main conclusions, separated in sections on macrophages and on mast cells. And finally we will evaluate how the conclusions presented in this thesis fit in the current model of Spondyloarthritis (SpA) pathophysiology and will discuss future prospects.

INTRODUCTION
The pathophysiology of Spondyloarthritis
As discussed in chapter 1 and 2, SpA is the second most common form of chronic inflammatory arthritis. Since the major genetic risk factor for ankylosing spondylitis (AS), the prototypical form of SpA, is the major histocompatibility complex (MHC) class I molecule HLA-B27, the disease was considered for a long time as autoimmune. This autoimmune hypothesis, also known as the “arthritogenic peptide” hypothesis, suggested that a unique, potentially foreign, peptide presented by HLA-B27 would prime specific cytotoxic T cells, which could subsequently cross-react with self-peptides presented in the joints. A similar mechanism of HLA-restricted activation of self-reactive T lymphocytes has been postulated in MHC-class II associated inflammatory diseases such as rheumatoid arthritis, diabetes mellitus type 1 and coeliac’s disease. With exception of the HLA molecule itself, however, the crucial players of this hypothesis — a specific initiating peptide, autoreactive cytotoxic T cells, and a cross-reactive self-protein — were never identified in SpA or in any other inflammatory disease associated with HLA class I molecules, including psoriasis and Behçet’s disease. Moreover, T- and B-cell-targeted therapies are ineffective in AS [1,2] and HLA-B27 transgenic rats develop specific SpA-like features in the absence of cytotoxic T cells [3]. In chapter 2 we discuss alternative theories on the potential role of HLA-B27 in SpA pathophysiology that are independent of antigen presentation to cytotoxic T cells. We propose that SpA belongs to the group of inflammatory disorders [4], in which over-activation of innate inflammatory responses, by different types of cellular stress play a major role in the initiation and perpetuation of chronic tissue inflammation. Examples are the tumor necrosis factor (TNF) and interleukin 23 (IL-23)/interleukin 17 (IL-17) cytokine pathways, whose role in SpA is supported by genetic associations [5,6], by functional studies in human and animals [7,8] and, most importantly, by clinical intervention studies [9,10].

Tissue inflammation
The clinical phenotype of SpA is characterized by various forms of tissue inflammation such as synovitis, enthesitis, and osteitis. The study of tissue inflammation is challenging because of its intrinsic complexity with many versatile actors and continuously changing interactions. The cellular programming of many immune cells is controlled by the local environment, as evidenced in for example macrophage polarization [11] or innate lymphoid cell plasticity [12]. This context-dependent phenotype, molecular profiling, and function is not confined to well-studied migratory cells, but also resident cells can dramatically change their function.
depending on micro-environmental triggers [13–15]. Moreover, the dynamics of cellular migration, for example in and out of the tissue or patrolling within the tissue, leads to a continuous change of the cellular composition of specific tissue compartments. Descriptive studies have identified different cell types, soluble molecules, and cell-bound proteins as potentially important actors in chronic tissue inflammation [7,16,17] and are thus crucial for hypothesis generation. For the more detailed molecular and functional characterization of the proposed pathways, however, researchers usually fall back on in vitro assays using cells derived from human blood or on animal models. The validity of these approaches is limited by the fact that it is not clear to what extent findings can be extrapolated to human tissue inflammation. The reasons that human tissues are often not studied directly are the limited availability of disease-relevant human tissue biopsies and the laborious and technically challenging protocols to study cells directly ex vivo. The development of new, non-invasive biopsy methods and of cutting edge technologies such as flow-cytometric cell-sorting and single cell quantitative PCR analyses urged us to revisit the cellular pathophysiology of SpA by directly studying relevant innate cell populations in the inflamed synovial tissue.

OBJECTIVE

The main objective of this thesis was to delineate the role of innate cell populations, in particular macrophages and mast cells, in the key pro-inflammatory pathways (TNF and IL-23/IL-17) driving chronic inflammation in SpA, using peripheral synovitis as a model. The characterization of the cells that drive these pathways may not only help to understand for which disease manifestations blockade of these cytokines may be beneficial, but may also open new perspectives for more specific targeting of these pathways.

APPROACH

Cross-sectional comparison

A frequently used approach to identify inflammatory pathways is a cross-sectional comparison of patients with healthy individuals. However, this approach is not feasible when studying inflammatory pathways in the tissue since the histological architecture of inflamed tissue is different between a healthy and an affected individual, precluding any meaningful comparison. As an example, the normal synovial membrane consists of a single layer of fibroblast-like synoviocytes and intimal macrophages overlaying loose connective tissue containing fibroblasts, fat cells, and a few resident macrophages. In actively inflamed joints, the synovial lining layer is strongly hyperplastic (up to 15 layers thick) and the submucosa is heavily infiltrated with immune cells and blood vessels. Moreover, synovial fluid can only be extracted for analysis from patients with active joint inflammation and cannot be obtained from healthy joints. An additional limitation of the use of ‘healthy’ control tissue is the inability to discriminate between disease-specific presumable ‘upstream’ immunopathology and secondary, non-specific, ‘downstream’ pathways of inflammation. In the context of selective therapeutic targeting, the former are obviously of major interest.

In order to perform our cross-sectional analysis, we therefore used as controls not healthy individuals but patients with other forms of chronic inflammatory arthritis, such
as rheumatoid arthritis (RA), Bechet’s disease and gout. While these control diseases have different pathophysiological mechanisms, they present with—at first glance—comparable clinical and histological synovitis with similar levels of clinical and histological inflammation. This approach allows us to discriminate between non-specific general pathways of inflammation and disease-specific processes. For example, overexpression of TNF is relevant to all chronic inflammatory arthritis and inhibition of TNF relieves symptoms in all mentioned disease. However, the rapid relapse of these diseases after cessation of treatment strongly suggests that the underlying primary pathological mechanisms are not affected. In our cross-sectional analysis we define unique traits as possibly related to primary pathophysiological mechanisms specific to SpA and shared traits as secondary inflammatory pathways. Since SpA is characterized by inflammation in multiple other tissues beside peripheral joints, we assessed if similar mechanisms drive all these disease manifestations, by performing additional cross-sectional analysis on other ‘accessible’ target tissues such as skin and gut.

**Longitudinal comparison**

Next to our cross-sectional tissue analyses, we have collected longitudinal data before and after treatment initiation. Many new therapies, for example monoclonal antibodies, are so specific for a certain molecule or pathway that, although initially designed to improve patient care, they became useful molecular ‘knock-down’ tools to study the immunopathology of human diseases *in vivo*. Cellular and molecular analysis of target tissue samples before and after treatment can define which pathways are and which are not affected by the selective neutralization of, for example, TNF and can thereby help to define the pathogenic hierarchy of the disease.

**Macrophages**

Macrophages are a group of innate effector cells that play an important role in inflammatory disorders. From our previous work [18,19] we know that synovial macrophages are key drivers of local tissue inflammation through the production of a variety of inflammatory mediators including, but not limited to, TNF. *In vitro* studies have shown that macrophages can be polarized by different cytokine cocktails towards phenotypically and functionally distinct subsets [11]. Human macrophages may exert many functions, yet in the context of immune mediated disease two prototypic subtypes are recognized. *In vitro* stimulation of peripheral blood monocytes with interferon gamma (INF-γ) induces macrophages that produce TNF and IL-1, mediators that drive inflammation. Historically and in chapter 3, these macrophages were labeled as “classically activated” or M1. Nowadays the same subset is referred to as Mφ-INF-γ. Other cytokines, like IL-4 and IL-10, can polarize monocytes to macrophages that were historically labeled as “alternatively activated”, also identified as M2. Since there is a considerable heterogeneity within this group, the identifiers Mφ-IL-4 and Mφ-IL-10 have replaced M2 in the literature. Here and after we will use the modern identifiers. Of special interest is the Mφ-IL-10 subset, which is characterized by the expression of surface marker CD163 and the production of the anti-inflammatory cytokine IL-10 [11,20]. A disrupted balance between pro-inflammatory “classically activated” and anti-inflammatory “alternatively
activated" macrophages and their respective cytokines may lead to exaggerated activation of inflammatory responses and thereby contribute to chronic tissue inflammation. We previously described augmented expression of the Mφ \(_{IL-10}\) marker CD163 in SpA compared to RA synovial tissue and concluded that this could be a reflection of differential macrophage polarization [18]. In chapter 3, we hypothesized that the cytokine milieu in the synovial fluid of SpA and RA is responsible for differential macrophage polarization. In order to test this hypothesis we assessed the polarization capacity of synovial fluid of SpA and RA patients \textit{in vitro} and observed that SpA fluid skewed macrophage polarization towards Mφ \(_{IL-10}\) whereas RA fluid skewed the cells towards Mφ \(_{INF-\gamma}\). However, analysis of the levels of the prototypical factors of polarization towards Mφ \(_{IL-10}\) in the respected fluids revealed, unexpectedly that IL-10 was significantly lower expressed in SpA synovial fluid. As polarization towards Mφ \(_{IL-4}\) and Mφ \(_{IL-10}\) may also occur through a lack of factors promoting Mφ \(_{INF-\gamma}\), we analyzed Mφ \(_{INF-\gamma}\) polarizing factors and observed that while GM-CSF was similarly expressed in SpA and RA fluid, INF-\gamma was undetectable in all samples. We can, however, not exclude that INF-\gamma has potent effects on polarization in very low concentrations in a paracrine manner.

Next, we investigated if the differential macrophage polarization in SpA versus RA was reflected by differences in macrophage-derived cytokines in the synovial fluid of these disorders. We found that prototypical Mφ \(_{INF-\gamma}\) cytokines such as TNF, IL1-\beta, IL-12 and IP-10 were 2 to 5-fold lower expressed in SpA than in RA. Thus, this study reveals that first, the local environment of joints in SpA promotes differentiation towards Mφ \(_{IL-10}\) macrophages and that second, soluble ‘Mφ \(_{INF-\gamma}\) cytokines’ are unlikely to drive the immunopathology of peripheral synovitis in SpA. Both conclusions point towards a shifted balance in macrophage polarization/differentiation favoring Mφ \(_{IL-10}\) macrophages in SpA (Figure 1).

In chapter 4 we aimed to extend the conclusions of chapter 3 by direct analysis of synovial tissue macrophages. First, we found that the differences in polarization between the diseases were not intrinsic but fully related to the local inflammatory environment. Second, we confirmed that the Mφ \(_{IL-10}\) marker CD163 was more frequently expressed on intimal lining macrophages in SpA than in RA. Additional Mφ \(_{IL-10}\) as well as Mφ \(_{INF-\gamma}\) markers were unexpectedly not differentially expressed. Based upon this data we reject our hypothesis that the balance between pro- and anti-inflammatory macrophages is shifted in SpA (Figure 1). Whereas the Mφ \(_{INF-\gamma}\) and Mφ \(_{IL-10}\) model describes two extremes as seen in cells under artificial conditions \textit{in vitro}, we show \textit{in vivo} that most macrophages display a mixed phenotype. The lining macrophages, however, are phenotypically clearly more closely related to Mφ \(_{IL-10}\). Our double immunostainings indicated that these Mφ \(_{IL-10}\)-like cells infiltrate the lining similarly in SpA, RA, and gout and are thus a general feature of chronic synovitis. In conclusion, the lower levels of Mφ \(_{INF-\gamma}\) cytokines in SpA compared to RA synovial fluid cannot be explained by a clear difference in macrophage polarization. The CD163-expression was enhanced without the support of additional Mφ \(_{IL-10}\) markers, a situation that is not coherent with the data from \textit{in vitro} experiments. This indicates that the \textit{in vivo} situation is more complex than \textit{in vitro} and leaves the significance of the CD163 expression unclear. The current data weakens the link between the expression of CD163 and the lower levels of ‘Mφ \(_{INF-\gamma}\) cytokines’ in SpA. The study is limited by the fact that we
Rejected hypothesis:
A shifted balance between $\text{M}\Phi\text{IL-10}$ and $\text{M}\Phi\text{INFy}$ in SpA synovium may explain:

1. Enhanced expression of $\text{M}\Phi\text{IL-10}$ marker CD163 in SpA synovial tissue
2. Reduced levels of pro-inflammatory ($\text{M}\Phi\text{INFy}$) cytokines in SpA synovial fluid

New hypothesis:
A difference in posttranslational modification of TNF and CD163, for example mediated by the extracellular proteolytic enzyme ADAM-17, may explain:

In SpA synovial fluid compared to RA: $\text{sCD163} \downarrow$  $\text{sTNF} \downarrow$
In SpA synovial tissue compared to RA: $\text{tmCD163} \uparrow$  $\text{tmTNF} \uparrow$

Figure 1. Hypotheses explaining the differences in macrophage phenotype and function between SpA and RA. Upper panel: representation of the rejected hypothesis that SpA and RA macrophage polarization balance is different and results in different local cytokine profiles and expression of the tissue marker CD163. Lower panel: representation of the new hypothesis, that SpA and RA differ in the cleavage activity (represented by the size of the scissors) of ADAM-17 that releases $\text{sTNF}$ and $\text{sCD163}$ from the cell membrane. Synovial tissue macrophages residing in the sublining layer display a mixed phenotype in vivo, intimal lining macrophages resemble $\text{M}\Phi\text{IL-10}$ in SpA and in RA.
had to use phenotypic characterizations in order to approximate the actual cytokine production. Our phenotypic markers were not optimized for in vivo macrophages and no direct translation was possible from our in vitro models. Our analysis was based on cell number and not on other dimensions, like activation state or amount of cytokine produced per cell. Still other local cell types, beside macrophages, may produce the same cytokines and can thus be responsible for the lower levels of ‘MϕINF-γ’ cytokines’ that we discovered in chapter 3. At last, the cytokines can be regulated on another level than linear production, like degradation, sequestration, post-translational modification. An example is TNF, which is produced in a membrane spanning form, which can be cleaved by extracellular enzymes to form the soluble TNF molecule. Our measurements of soluble TNF can thus relate to other mechanisms than solely production by macrophages, namely the efficiency of the cleaving. Interestingly, the enzyme responsible for cleaving transmembrane TNF from the membrane, ADAM-17 (TACE), cleaves the transmembrane form of CD163 in a similar manner from the cell surface. This leads to the novel hypothesis that both increased expression of CD163 on the plasma membrane and the decreased presence of soluble TNF in SpA synovitis could be related to alterations in ADAM-17 expression or activity (Figure 1). This hypothesis will be further discussed at the end of this chapter.

Mast cells as the cellular source of IL-17A

Historically, the discovery of IL-17A as pro-inflammatory cytokine in arthritis preceded the description of the T-helper 17 cells by many years, but it was the latter that allowed to demonstrate a major role for this axis in host defense as well as in chronic tissue inflammation [21,22]. In the translation of these concepts to human immune-mediated inflammatory diseases, one of the essential questions was to confirm that, also in humans, T cells are the major cellular source of IL-17A. Studies performed on peripheral blood cells consistently found that a fraction of CD4+ T cells produces IL-17A upon ex vivo restimulation [23,24]. The importance of these cells for mounting a proper response to mucocutaneous candida infections, became apparent from syndromes in patients that lack IL-17-IL-17R signaling (ACT1, IL-17F, IL-17RA, IL-17RC), signal transduction downstream of IL-23R (STAT3, Tyk2) and pattern recognition receptors that are involved in T<sub>H</sub>17 cell development (Dectin1, Dectin2). Accordingly, inhibition of these targets in murine studies showed likewise problems with host defense against candida species. However, in contrast to the human syndromes these mice show defects in the defense against much more pathogens, including several pathogenic bacteria [25–28]. Later less prevalent cell populations, which are able to produce IL-17A, were described. These populations include other T cell populations (like Tc17, NKT cell, iNKT cells, γδ T cells [29] and mucosal-associated invariant T (MAIT) cells [30]) and innate lymphoid cells [31]. γδ T cells, MAIT cells and innate lymphoid cells are members of the innate immune system, as they function independently of antigen-specific receptors and are of particular interest since the concept that the IL-17 axis can be driven by the innate immune system fits better to our current view of SpA pathophysiology (see chapter 1 and 2).

Between 2013 and 2014, the first trials demonstrated clinical efficacy of IL-17A blockade in Pso and SpA. This clinical breakthrough has put characterization of the pathophysiological
mechanisms that drive the IL-17 axis high on the global research agenda. Until now, however, there is no consensus on the pathological source of IL-17A in Pso or SpA, nor in other conditions. Systemic elevation of IL-17A is conflictingly reported by various research groups: Melis at al [32] found similar serum levels between SpA patients and healthy individuals, whereas other groups reported a marginal increase of IL-17A in SpA [33–35]. Melis et al also reported that the synovial fluid is not enriched for IL-17A over the serum in spondyloarthritis patients, arguing against strong local production of IL-17A in the synovial tissue [32]. Our own unpublished work on synovial mRNA expression confirms that very little IL-17A and IL-17F is produced locally, in contrast to other pro-inflammatory cytokines like TNF, IL-8 and IL-6. This is in line with another study comparing affected skin and synovial tissue obtained from patients with psoriatic arthritis, which reported 16 times lower expression of IL-17A and IL-17F in the joint as compared to skin, whereas similar expression levels between skin and joint were observed for TNF [36]. This data suggest that local production of IL-17A may be a differentiating factor between the pathophysiology of psoriatic skin inflammation and SpA synovitis (Figure 4). The relatively low levels of IL-17A and IL-17F mRNA in tissue biopsies, indicates local production in small amounts and suggests that these cytokines may act in a paracrine manner or even via immunological synapses, may synergize with other cytokines such as TNF and/or that the sensitivity of IL-17-responsive cells is greatly enhanced in SpA synovium. Therefore better understanding how the IL-23/IL-17 axis operates in the joint, including identification of the cellular source of IL-17A may be relevant to the pathophysiology and proper targeting of SpA. The most obvious candidate for production of pathological IL-17A in SpA are T cells, since they comprise 30%-50% of all immune cells in the inflamed synovial tissue and various T-cell subtypes are able to produce IL-17A. In chapter 7 we have investigated synovial T cells and demonstrated that these cells express the transcription factor RORC and can be stimulated ex vivo by various compounds, including PMA/ionomycin, to produce IL-17A. However, directly ex vivo, we did not detect
Figure 3. Hypotheses on the role of IL-17A-positive mast cells in SpA. As we observed that mast cells contain IL-17 in SpA (chapter 5) we hypothesized that these cells may contribute to the disease pathophysiology by production of pathogenic IL-17A. This form of uncontrolled overproduction could have been caused by an intrinsic defect in mast cells or faulty regulation from the local environment. This hypothesis was rejected, since we discovered that in SpA synovitis very little IL-17A is produced de novo and that mast cells are not equipped with the molecular machinery for IL-17A production (IL-23R and transcription factor RORC). Alternatively, we demonstrated that mast cells take up, store and release exogenous IL-17A. We revisited our original research question; if not production of IL-17A, what is the role of mast cells in SpA? This led to two hypotheses: 1) mast cells provide a major source of IL-17A, independent of the direct control of IL-23. 2) misregulation of the newly discovered pathways of uptake, storage or release leads to inappropriate control of IL-17A. Upper scheme shows IL-23-dependent pathological IL-17A production by mast cells and other cells. The lower scheme shows that mast cells are not equipped with canonical IL-17A production machinery, like the transcription factor RORC and the IL-23 receptor. Uptake and release pathways replaced the arrow representing IL-17A production.
The IL-23/IL-17 axis in tissue inflammation

**Psoriasis skin**

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<tr>
<th>IL-17 production in situ</th>
<th>IL-17 import/storage</th>
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<td>IL-23 independent?</td>
<td>mast cell</td>
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<tr>
<td>IL-23</td>
<td>IL-17</td>
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<td>IL-17</td>
<td>neutrophil</td>
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<td>inflammation</td>
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Response to therapy:
- IL-23 blockade: YES
- IL-17 blockade: YES

Source of IL-17:
- production in situ: YES
- import/storage: mast cell and neutrophil extracellular traps contain IL-17A

Source of IL-17:
- production: in situ
- import/storage: mast cell and neutrophil extracellular traps contain IL-17A

**Crohn’s disease gut**

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<th>IL-17 production in situ</th>
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<td>IL-23 independent?</td>
<td>mast cell</td>
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<tr>
<td>IL-23</td>
<td>IL-17</td>
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<tr>
<td>epithelial barrier</td>
<td>neutrophil</td>
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<tr>
<td>homeostasis</td>
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<td>inflammation</td>
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Response to therapy:
- IL-23 blockade: NO/Worsening

Source of IL-17:
- production in situ: LOW
- import/storage: unknown

Paradox:
- effect of IL-17A on homeostasis of epithelial barrier
- IL-23 mediated, IL-17A independent inflammation

**Spondyloarthritis synovium**

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<td>neutrophil</td>
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<td>inflammation</td>
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Response to therapy:
- IL-23 blockade: no data
- IL-17 blockade: YES

Source of IL-17:
- production in situ: LOW
- import/storage: IL-17A-containing mast cells contribute to pathophysiology

although not conclusive for the axis; secukinumab (IL-17A) superior to ustekinumab (IL-12/IL-23p40) in PsA:
- IL-12 or IL-23 dependent — joint protective — cytokines?
- IL-23 independent source of IL-17A?

**Figure 4.** Uncoupling of IL-23/IL-17 pathway suggests existence of a source of IL-17A that is independent of IL-23 and de novo IL-17A synthesis in situ in SpA. Is there a role for mast cells? In psoriasis, blocking IL-23p19 and blocking IL-17A/IL-17RA are similarly efficacious. This leads to a model with a linear axis, IL-23 = IL-17 = inflammation. As IL-17 is produced locally (IL-17A and IL-17F mRNA expression is increased in lesional vs normal skin [36,72]), the main source of pathological IL-17A is an IL-23-responsive cell in the skin. In Crohn’s disease, blocking IL-23p19 and blocking IL-17A/IL17RA have opposite effects. As a result the axis cannot be linear, IL-23 ≠ IL-17. Paradoxical inflammation upon IL-17A blockade may be explained by an alternative role of IL-17A in the homeostasis of the epithelial barrier [64,65]. In the discussion we address several mechanisms explaining how blockade of IL-23 reduces inflammation, other than via the control of IL-17A. In spondyloarthritis, clinical trial of blocking IL-23 is ongoing, so the axis cannot be reliably modelled. However, since blockade of IL-17A is superior to blockade of the IL-23p40 subunit [70], the axis may be partially uncoupled. In the discussion we address several mechanisms suggesting how p40 inhibition may be less effective than IL-17A inhibition in PsA. These mechanisms include the production of joint protective cytokines and an IL-23-independent source of IL-17A. Additionally, there is no indication for local production, since very low amounts of IL-17A and IL-17F mRNA are measured in inflamed synovium. As being both independent of direct IL-23 signaling and de novo synthesis of IL-17A, mast cells may be implicated as the major source of IL-17A in SpA joints.
any mRNA for IL-17A and IL-17F in isolated T cells from SpA synovium. Additionally, we have recently performed single cell qPCR analysis of sorted T cells from SpA synovial tissue and again we were unable to detect expression of IL-17A and IL-17F in unstimulated conditions, despite adequate expression of other pro-inflammatory mediators such as TNF and IFNγ (unpublished observations). Next to gene expression we assessed capacity of synovial T cells to secrete IL-17A protein \textit{ex vivo}. The protein data was in line with mRNA data: isolated synovial T cells, after polyclonal stimulation, secreted in all samples IL-17A protein in substantial quantities, where IL-17A produced by unstimulated T cells consistently fell below the detection limit in our experimental set-up.

Furthermore, in agreement with these data, none of the IL-17A producing cell subsets identified by studies on peripheral blood can be detected by immunostainings of IL-17A protein of the tissue. Instead, we and others have described that IL-17A protein is exclusively found inside mast cells and neutrophils in a variety of healthy and inflamed tissues including skin [37–39], gut, synovial tissue [40,41], lung, and kidney [42,43]. The discrepancy between the cellular source of IL-17A in the tissue and the blood can depend on the characteristics of these environments, as, for example, mast cells are absent in the bloodstream. It is, however, not excluded that part of the discrepancy is related to technical issues. First, in most cases protein expression within cells from blood was measured after artificial activation with potent stimuli. Tissue cells, however, are considered to be intrinsically activated by the local inflammatory environment and thus do not require artificial stimuli. Second, blood is usually analyzed fresh and the cells are viable, where tissue is usually frozen or chemically fixated before analysis. This can be of relevance because viable cells are frequently treated with protein transport inhibitors and accumulate cytokines otherwise excreted. Also freezing and fixation may change the antibody specific epitope. And third, the method of protein detection varies: for blood most researchers use flow-cytometry whereas immunostainings is the standard approach for tissue. In chapter 6 and 7 we have evaluated these forms of bias. First, we have tested whether the signal for IL-17 obtained by immunostainings was generated from genuine IL-17A protein. We could show that multiple antibodies, including the goat polyclonal one broadly used for immunohistochemistry, were able to detect IL-17A protein by Western blotting in tissue mast cells. Moreover, also in mouse skin we can detect IL-17A in mast cells by immunostaining and Western blot (see figure 2B and chapter 7). The ultimate guarantee would be to detect IL-17A using an antibody-independent technique such as peptide mass fingerprinting. However, this was not feasible in our setup due to limited amount of cellular material. Second, the subcellular localization of IL-17A in mast cells was demonstrated by high resolution fluorescence microscopy (chapter 7) and by electron microscopy [44]. A role for unspecific binding of IL-17A to cell membrane receptors, sugars or extracellular matrix components was excluded by washing off extracellular protein with acidic buffer, which did not alter detection of IL-17A.

The concept that the main source of IL-17A in the synovium of spondyloarthritis patients are mast cells and neutrophils was enhanced by similar findings reported for rheumatoid synovitis [45]. Moreover, the same study demonstrated that hematopoietic progenitor-derived mast cells, a common model for human mast cells, are able to produce IL-17A in
a RORC dependent manner when triggered with inflammatory signals such as TNF or LPS. Based on these data the hypothesis that innate cells, like mast cells and neutrophils, rather than cells of the acquired immune system, like T\textsubscript{H}17, are the drivers of IL-17-mediated tissue inflammation was broadly accepted [46,47].

As at that time we observed a marked increase in mast cells in SpA synovium, we proposed a model for SpA pathophysiology in which local IL-17A production by influx of overactive mast cells is a central driver of disease (Figure 3). In order to confirm our hypothesis we investigated intrinsic capacity of IL-17A production by \textit{bona fide} freshly isolated synovial mast cells (chapter 6 and 7). Strikingly, we were not able to detect any IL-17A expression as measured on mRNA level. Moreover, expression of T\textsubscript{H}17-associated molecules such as the IL-23 receptor and the transcription factor RORC was not detected in these cells. Meanwhile emerging animal studies argued against production of IL-17A by mast cells. Thus, in mice, tissue mast cells did not express IL-17A, IL-17F, ROR\textgamma{t} or IL-23R [48–52] corroborating human data. Trying to explain the discrepancy between the presence of intracellular IL-17A protein and absence of its active transcriptional machinery, we demonstrated in a model system that human mast cells can acquire IL-17A from the milieu and store it in specialized granules. The ultimate fate of this IL-17A is not yet sufficiently clear but our data suggest that mast cells can, at least partly, excrete IL-17A as a functional cytokine. Our findings change the paradigm by rejecting the hypothesis on overactive IL-17-producing mast cells and introducing instead a novel, potentially pathogenic, mechanism. An intrinsic or acquired defect in uptake, storage, or release of IL-17 can influence local levels of IL-17A, and thus tissue inflammation in SpA. Release of IL-17A in an active form is of particular interest since further study revealed (chapter 6), that IL-17A+ mast cells are not only found in inflamed environments such as synovium, skin or gut but also in normal healthy tissues. This suggests that IL-17-positive mast cells play a role under homeostatic conditions, rather than that their appearance is restricted to inflamed tissue or a particular disorder and while uptake of IL-17 by mast cells may be a physiological phenomenon, its release may be triggered by a particular inflammatory signal.

Since the tissue of SpA patients contains significantly more IL-17A positive mast cells than RA patients, we have concluded in chapter 5, that IL-17+ mast cells may contribute to the pathophysiology SpA. To pre-clinically assess if mast cells are pathognomonic in SpA, we have first used the selective tyrosine kinase inhibitor imatinib to target mast cells in fresh synovial tissue biopsies explants. This \textit{ex vivo} treatment resulted in the reduction of inflammatory mediators such as IL-6, IL-8 and IL-17A produced by the biopsies. Translating these findings to the clinic, we have initiated a phase II randomized placebo-controlled trial in a proof of concept setup in order to test clinical efficacy of nilotinib and to assess the role of mast cells in SpA. For the trial, we have chosen nilotinib, the successor of imatinib, a drug with a similar target list and mode of action, but with a better safety profile. In chapter 8 we have described the results of this trial. The treatment was well-tolerated and a positive signal, including subjective scores like patient and physician global assessment, but also objective values like CRP, was seen when compared to placebo, being in line with our hypothesis that mast cells may play a pathogenic role in SpA pathophysiology. An important
note here is that treatment was ineffective in the subgroup of patients that was not affected
by peripheral arthritis, but had isolated axial complaints. Differences in the exact working
of the inflammatory axis between axial and peripheral disease may explain these findings.
For example, as we know that IL-17-expressing cells are differently distributed in axial
disease compared to peripheral tissues [41], an appealing thought is that IL-17A positive
mast cells are intrinsically different or differentially instructed in the various tissue locations.
This warrants further investigation. An important conceptual limitation of this study is that
the study compounds imatinib and nilotinib are small molecule tyrosine kinase inhibitors
that block multiple targets. Amongst these targets is c-kit, a receptor important for mast cell
survival, which is, however, also expressed on innate lymphoid cells. Other targets include
PDGF-R, a receptor expressed on myofibroblasts, and c-FMS, which is expressed on CD163-
positive macrophages. Since all these cell types have been implicated in the pathophysiology
of SpA is not clear at the moment via which mechanism of action nilotinib reduces
synovial inflammation.

In conclusion, since the inhibition of IL-17A is an efficacious treatment for SpA,
identification of the cellular source of IL-17A in synovium may help us to understand how
this pathway operates and, by consequence, how the pathway can be targeted other than
by blockade of IL-17A. Low levels of IL-17A and IL-17F mRNA argue against de novo synthesis
locally in the tissue and demand an alternative explanation to local overproduction models.
Mast cells, as well as neutrophils, contain IL-17A in the tissue and may provide the cellular
source of IL-17A by excretion. Mast cells may be pathognomonic for SpA, since they are
specifically infiltrated in SpA- compared to RA synovial tissue and since mast cell targeting is
clinically effective in peripheral SpA.

SCIENTIFIC PROGRESS AND FUTURE PROSPECTS

This work highlights the complexity of SpA pathophysiology and demonstrates a) how
application of properly chosen approaches as cross-sectional and longitudinal analysis
of tissue inflammation may lead to the identification of new molecular players in SpA
pathogenesis and b) how consequent functional testing in vitro and in vivo may define
the relevance of these players. We illustrated this with two cytokine pathways highly relevant
for SpA, TNF and IL-23/IL-17. Despite that blocking of TNF is efficacious for patients with
SpA, the exact mechanism of action of TNF inhibitors remains largely unknown. In order to
investigate how this pathway operates in the inflamed SpA joint, we compared SpA synovitis
to RA synovitis. Analysis of the synovial fluid of SpA patients demonstrated significantly lower
levels of soluble TNF as compared to synovial fluid of RA patients. However, the production
of TNF in the synovial tissue, as measured by mRNA expression, was comparable in both
diseases. Since TNF is initially produced as a transmembrane protein, we assessed levels of
the membrane-bound form of TNF (tmTNF) and discovered that tmTNF is enhanced in SpA
synovial tissue compared to RA synovial tissue. Since the proteolytic enzyme ADAM-17 (also
known as TNF converting enzyme or TACE) is responsible for the release of the soluble form
of TNF (sTNF), we hypothesized that impaired expression and/or activity of ADAM-17 may
be implicated in the relative overexpression of tmTNF over sTNF in SpA joint. Of interest, we
previously reported a similar ratio of transmembrane over soluble expression of CD163 in SpA versus RA joints (chapter 3 and [18,53]). Since CD163 is another target of ADAM-17, both findings may be explained by the same mechanism (Figure 1). The functional relevance of soluble versus transmembrane forms of TNF in the pathophysiology of chronic inflammatory disease, like SpA and RA, can be illustrated by comparing mouse models overexpressing TNF. In the first model, overexpression of human TNF leads to severe systemic disease and destructive polyarthritis, phenocopying human RA, rather than human SpA [54]. Although blockade of DKK-1, an inhibitor of the Wnt-pathway, reverses this phenotype to a less destructive/more remodeling phenotype, characterized by new bone formation [55,56], human SpA is not fully recapitulated since mice do not develop enthesitis or spondylitis. In the second model, overexpression of the wildtype murine TNF gene, via deletion of AU-rich elements in the TNF locus, also leads to destructive polyarthritis, but now including sacroiliitis and gut inflammation, two features related to human SpA [57]. However, also this model does not display hallmark SpA features like spondylitis and new bone formation. Both models are characterized by the presence of the sTNF form. In sharp contrast, selective overexpression of the tmTNF form in mice (via mutation in the cleavage site of TNF) leads to a convincing SpA-like phenotype. In the absence of severe systemic inflammation, these mice developed axial and peripheral joint inflammation, enthesitis, osteitis and new bone formation, ultimately leading to ankyloses [7] (reviewed by us in chapter 2 and in [58]). Cumulatively these data suggest, that the membrane-bound form, rather than the soluble form of TNF is responsible for the key features of SpA. Although future studies have to answer via which mechanism tmTNF leads to pathology in SpA, this example illustrates how systematic comparison of inflamed synovial tissue resulted in the identification of a new pathway (Figure 1).

Another important finding presented in this thesis is related to the IL-23/IL-17 cytokine pathway. Consistent evidence indicates that this immune axis plays a pivotal role in the pathophysiology of SpA, psoriasis (PsO) and Crohn's disease (CD), yet our current understanding of IL-23-responsive cells and other possible sources of IL-17A is limited and, consequently, our rationales for choice of therapeutic targets are still very restricted. Systematic comparison of the currently available results of clinical trials in PsO, CD, psoriatic arthritis (PsA) and ankylosing spondylitis (AS) indicates that the functioning of the IL-23/IL-17 axis in these pathologies is slightly different. Inhibition of IL-23p19, IL-17A and IL-17RA is comparably effective in PsO clinical trials [59], suggesting linear mode of IL-23/IL-17 axis in this disease. The situation is different for CD. The data from clinical trials demonstrate that IL-17A blockade was not successful [60] and anti-IL-17RA treatment induced flares over placebo [61]. In contrast, CD patients can be effectively treated with IL-23 inhibitors [62,63]. Further studies are required to understand these results, however there are a number of possible explanations. First of all it is plausible to assume that in the gut IL-17A preserves the intestinal epithelial barrier and its neutralization affects protective effects against pathogens, as has been shown in rodent models [64,65]. Since IL-23 blockade maintains basal levels of IL-17A, the protective effects may only be interrupted by blocking IL-17A signaling [25]. An alternative possibility is that IL-23 acts via other routes than induction of
IL-17A only and may affect the production of other pathogenic cytokines in CD, whereby blocking of IL-17A alone is not sufficient to stop gut inflammation. In spondyloarthritis, both in AS and PsA, inhibition of IL-17A is highly efficient [10,66]. We still lack clear data for IL-23 blocking therapies; results of clinical trials with anti-IL-23p19 antibodies are not available yet and blockade of the p40 subunit, which targets both IL-23 and IL-12, is efficacious in PsA [67,68], however it was only tested in an open label setting in AS [69]. Yet, clinical trials in PsA suggest that anti-IL-17A therapy is superior to anti-p40 therapy in this disease [70]. This could lead to different hypotheses including that anti-p40 treatment, next to blocking pathogenic IL-17A, also blocks cytokines that are protective against joint inflammation.

These protective cytokines may belong to the IL-12/T<sub>H</sub>1 pathway or to the IL-23/T<sub>H</sub>17 pathway, such as IL-22 [71]. Another option is the existence of an IL-23-independent route for IL-17 production. Such expression of IL-17A by γδ T cells in an IL-23-independent manner was recently demonstrated in a murine colitis model [64]. As to the strikingly low amounts of IL-17A mRNA in peripheral synovitis in SpA, sources of IL-17A other than de novo production may be present in the synovium. A special niche in this route could be reserved for IL-17A-containing mast cells, which due to their unique capacity to accumulate and release IL-17A may control local levels of IL-17A independently of IL-23 and de novo IL-17A production (Figure 3 and 4). Therefore detailed molecular characterization of this pathway, including investigation of putative IL-17A uptake receptor and establishment of triggers that drive release of IL-17A from mast cells, is required for better understanding of the contribution of this route to IL-23/IL-17 immune axis in SpA tissue inflammation and for selection of novel clear-cut targets for disease interference.

Taking together, data from clinical trials demonstrate that IL-23/IL-17 immune axis is certainly not linear, but rather that IL-23 and IL-17 have a partially overlapping, but also partially distinct biological effect and this may differ from one tissue/disease to another. Hence direct in-depth tissue analysis is necessary for optimal targeting of this axis in these conditions.
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