Macrophage polarization in spondyloarthritis
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INTRODUCTION

The objective of this thesis was to investigate the characteristics and role of macrophages in spondyloarthritis (SpA) by using an in vitro model for macrophage polarization (Figure 1). As discussed in chapter 2, the current knowledge points towards an autoinflammatory pathogenesis of SpA [1]. Autoinflammatory disorders are caused by a malfunctioning innate immune system, in comparison to autoimmune disorders, such as rheumatoid arthritis (RA) of systemic lupus erythematosus (SLE), which are caused by aberrant self-reactive adaptive immune responses [2].

Macrophages are a key component of the innate immunity and play a pivotal role in chronic inflammatory conditions, such as SpA, RA, Crohn’s disease, atherosclerosis and diabetes [3,4]. Macrophage biology was extensively studied in the last decades in terms of phenotype, function, signalling pathways and epigenetic mechanisms. However, the exact in vivo features of this highly heterogeneous cell population in diverse disorders remain largely unknown.

In SpA, peripheral synovitis is characterized by similar levels of inflammation and infiltration with immune cells as in RA [5–8]. Interestingly, immunopathologic studies showed that synovial CD163-expressing macrophages are significantly higher in SpA compared to RA [5–8]. CD163 functions as a receptor for haemoglobin-haptoglobin complexes and was

Figure 1: Conceptual design of this thesis. In this thesis we analysed the correlation between the phenotype and function of in vitro polarized human macrophages and the phenotype and function of synovial macrophages in SpA. Ang-2, angiopoietin-2; CIA, collagen-induced arthritis; ER, endoplasmic reticulum; ICs, immune complexes; TEMs, Tie-2-expressing macrophages; TLR, Toll-like receptor.
also reported to bind Gram-positive and -negative bacteria [9]. Furthermore, CD163 is upregulated in vitro by IL-10, corticosteroids and M-CSF [10,11] and was shown to have an immunomodulatory role [12-14]. The predominance of CD163+ macrophages in SpA synovitis suggests that this macrophage subset plays an important, as yet unelucidated role in the disease pathogenesis.

The concept of macrophage polarization

Early studies reported that specific mediators were able to differentially shape macrophage morphology, phenotype and function [15,16]. The combination of phenotypic changes and functional skewing was called macrophage polarization and led – after activation – to two main subsets, based on their pro- versus anti-inflammatory functions: classically activated macrophages (M1) and alternatively activated macrophages (M2) (Table 1). M1 were mainly induced by IFN-γ (MΦ$_{	ext{IFN-γ}}$), while M2 were later subdivided into IL-4- (MΦ$_{	ext{IL-4}}$), as prototype for wound-healing macrophages, and IL-10-polarized macrophages (MΦ$_{	ext{IL-10}}$), which represented the regulatory macrophages [3,17-19]. Several other molecules besides cytokines, such as lipids [20] and apoptotic cells [21] were also described to modulate macrophage phenotype and function [22] (Figure 2). Importantly, early studies reported

![Diagram of macrophage polarization](image)

Figure 2: Integration of different in vitro models for the concept of macrophage polarization. IFN-γ, TNF, TLR ligands and GM-CSF were reported to induce classically activated macrophages (M1). IL-4, IL-10, TGF-β, corticosteroids, M-CSF, lipids, apoptotic cells, prostaglandins and cAMP analogues were reported to induce alternatively activated macrophages (M2) [3,17-21,27-29,88].
significant interspecies discrepancies in polarized macrophage subsets [23,24], thereby indicating that not all properties described in mouse macrophages can be extrapolated to human cells [25,26].

Macrophage phenotype in vitro

In chapter 3 of this thesis we validated phenotypic markers for in vitro polarized human macrophages. We showed that CD80 is the most robust phenotypic marker for MΦ_{IFN-γ}, CD200R was upregulated and CD14 was specifically downregulated on MΦ_{IL-4}, while CD163 and CD16 were specific markers for MΦ_{IL-10}.

Since GM-CSF and M-CSF were also described to induce M1 versus M2 polarization [27–30], we also compared the phenotype of MΦ_{IFN-γ}, MΦ_{IL-4} and MΦ_{IL-10} with that of GM-CSF.
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(MΦ_{GM-CSF}) and M-CSF-differentiated macrophages (MΦ_{M-CSF}). M-CSF failed to upregulate the MΦ_{IL-4} marker CD200R, while GM-CSF did not upregulate the MΦ_{IFN-γ} marker CD64 and was previously shown to induce the MΦ_{IL-4} marker CD206 [31]. These observations suggest that, despite sharing a functional M1 signature (IL-23^highIL-12^highIL-10^low) [27,32], MΦ_{GM-CSF} and MΦ_{IFN-γ} display clear phenotypic differences. Among the M2 cells (IL-23^lowIL-12^lowIL-10^high), MΦ_{M-CSF} seemed to phenotypically mimic MΦ_{IL-10} rather than MΦ_{IL-4}’ since both subsets specifically upregulated CD163 and CD16. We also reported phenotypic differences between human and mouse macrophages. For instance the mannose receptor (CD206), which was first described as a MΦ_{IL-4} marker in the mouse [16,33], was in our experiments more potently upregulated by GM-CSF than by IL-4. Concerning the human MΦ_{IL-4} marker CD200R, a previous report showed that CD200R expression in mice is not dependent on IL-4 [31]. Finally, we assessed in vitro whether polarization leads to an irreversible phenotypic skewing or is, at least partially, reversible when the cytokine environment changes. Since changes in macrophage phenotype after renewed exposure to cytokines in vitro were previously reported [34-37], in chapter 3 we addressed macrophage plasticity by ‘re-polarizing’ MΦ_{GM-CSF} and MΦ_{M-CSF} in the presence of IFN-γ, IL-4 and IL-10. We observed that IL-10-polarized MΦ_{GM-CSF} upregulated MΦ_{IL-10} markers CD163 and CD16, while IFN-γ-polarized MΦ_{M-CSF} selectively expressed MΦ_{IFN-γ} markers CD80 and CD64. Furthermore, the phenotypic plasticity of MΦ_{M-CSF} seemed to be higher than that of MΦ_{GM-CSF}’ which is in line with the hypothesis that MΦ_{M-CSF} represent homeostatic steady-state macrophages [38].

Soluble immune complexes (ICs) were also described to induce an M2 profile in mouse macrophages after TLR ligation [3,19,39-41]. As this was defined by the cytokine profile (IL-12^lowIL-10^high) of the cells, it remained unclear whether ICs really polarize macrophages or rather induce a specific activation of already polarized cells. This issue relates to the central question whether the function (studied in this thesis solely by assessing cytokine profiles) of macrophages is mainly determined by their polarization of by the exact type of activation. Therefore, in chapter 5 we investigated whether soluble ICs could modulate the phenotype and/or function of human macrophages. In our experiments, ICs alone or in combination with IFN-γ, IL-4, IL-10 or LPS did not alter the expression of the previously validated phenotypic markers for human polarized macrophage subsets. However, ICs and TLR ligands induced a distinct cytokine production profile in comparison with TLR ligands alone in different polarized cell subsets. These data thus question the existence of a separate M2 subtype induced by LPS and ICs in the human system.

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**Macrophage phenotype in vivo**

In chapter 4 we correlated the in vitro macrophage phenotype with the phenotype of synovial macrophages in SpA. We first investigated whether the preferential CD163 expression by SpA synovial macrophages was due to intrinsic properties of the myeloid cells or to extrinsic factors. Based on the lack of phenotypic differences in monocytes and monocyte-derived macrophages from SpA and RA patients and healthy donors, we find no evidence for intrinsic biases in macrophage polarization in SpA and propose that local mediators are probably responsible for the phenotypic differences between SpA and RA synovial macrophages.

We next measured the expression of earlier validated phenotypic markers for MΦ_{IFN-γ}, MΦ_{IL-4} and MΦ_{IL-10} on synovial macrophages and confirmed the higher expression of CD163 in SpA compared to RA. However, the expression of M1 and other M2 (MΦ_{IL-4} or MΦ_{IL-10}) markers was similar in both diseases. Furthermore, intimal lining layer macrophages specifically expressed the MΦ_{IL-10} markers CD163 and CD32 in both SpA and RA, whereas synovial sublining macrophages co-expressed MΦ_{IFN-γ} and MΦ_{IL-10} markers. These findings do not support an overall M2 versus M1 polarization in SpA synovitis, but rather a specific up-regulation of CD163 on synovial macrophages in SpA. Moreover, the synovial sublining data indicate that the phenotype of tissue macrophages can not easily be classified according to the in vitro polarization models. These data are in agreement with immunopathologic studies in adipose tissue, atherosclerotic plaque and tumors revealing a similar overlap in phenotypic but also functional properties of tissue macrophages [42-46]. These observations raise a number of questions. A first question is whether in vitro defined phenotypic markers can be used to discriminate between macrophage subsets in vivo [4]. In vivo polarization models and transcriptional profiling were suggested to be more accurate in capturing the complexity of tissue macrophage polarization [32,47,48]. A next question is whether tissue macrophages are composed of distinct but stable subpopulations or represent a homogenous population, which rapidly changes its phenotype and function in response to polarizing or activating signals. The latter hypothesis is supported by the in vivo phenotypic switch that accompanies functional modulation of macrophages in inflammatory and malignant diseases [34,43,49,50]. Finally, it still needs to be elucidated whether the phenotypic differences between intimal lining layer and synovial sublining macrophages in chronic arthritis are related to different origins and functions of these cells. Previous studies hypothesized the preferential localization of mature resident macrophages in the intimal lining layer, and the infiltration of synovial sublining with monocyte-derived macrophages, which are associated with disease activity and decrease in number as response to therapy [51-54].

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Macrophage function in vitro

In chapter 5 we studied the cytokine production of $\Phi_{\text{IFN-}}$ and $\Phi_{\text{IL-10}}$ and the in vitro effect of ICs on human macrophages, since previous publications reported an enhanced production of IL-10 and prostaglandins after co-stimulation of mouse macrophages with ICs and TLR ligands [3,19,39-41]. As indicated before, we could not find any phenotypic evidence that ICs induce a distinct polarization and thus hypothesized that ICs rather induced a distinct type of macrophage activation. In the absence of TLR ligands polarized macrophages produced very low levels of cytokines, which is consistent with the concept that macrophage polarization represents mere priming, in the absence of activation. Upon TLR binding, $\Phi_{\text{IFN-}}$ secreted significant amounts of TNF and IL-6, $\Phi_{\text{IL-10}}$ did not respond to activation, and $\Phi_{\text{IL-4}}$ showed an intermediate cytokine production pattern, depending on the particular TLR stimulation. Soluble ICs represented by heat-aggregated gammaglobulins (HAGGs) did not induce cytokine production of polarized macrophages in the absence of TLR ligation. However, co-stimulation with HAGGs and different TLR ligands lead to an increased production of IL-10 by both $\Phi_{\text{IFN-}}$ and $\Phi_{\text{IL-10}}$. There was no increase in TNF and IL-6 production, or in IL-12 and IL-23 mRNA levels. The anti-inflammatory role of soluble ICs was recently also highlighted by studies reporting induction of IL-10 producing macrophages by Fc receptor-dependent binding of anti-TNF antibodies, such as infliximab [55]. Interestingly, we observed that immobilized IgG induced a robust increase in TNF, IL-6 and IL-10 production even in the absence of TLR co-stimulation, which confirms previous reports on the induction of pro-inflammatory cytokines by immobilized HAGGs of IgG [56-58]. The importance of these results consists in the observation that cytokine production is not only determined by macrophage polarization and activation by TLRs, but that other factors act as co-activators and thereby determine the cytokine profile of the macrophages. The exact type of co-activator, in this case soluble versus immobilized ICs, seems to be crucial for the functional outcome as, for example, so-called pro-inflammatory $\Phi_{\text{IFN-}}$ can be turned into strong IL-10 producers.

Another example of co-activation is the effect of angiopoietins (Ang) on the production of cytokines and angiogenic factors by in vitro differentiated human macrophages. In chapter 6 we showed that Ang-1 or Ang-2 alone failed to modulate macrophage IL-6, IL-8 or MIP-1α production. Interestingly, both angiopoietins significantly upregulated the TNF-induced but not the LPS-induced IL-6 production, while they both synergized with TNF and LPS for the production of MIP-1α. This example emphasizes that the panel of extracellular co-activators extends beyond FcR ligation and that the functional effect is specifically dependent on the combination of stimuli.

In chapter 7 we studied whether not only extra-cellular signals (such as TLR, FcR and Tie-2...
ligation) but also intracellular signals can modulate the function of polarized macrophages and thus act as co-activators. In particular, we focused on endoplasmic reticulum (ER) stress in human macrophages as a) ER stress was reported to increase IL-23 production by dendritic cells and b) the induction of an unfolded protein response (UPR) as a result of HLA-B27 misfolding in the ER was hypothesized to underlie SpA pathogenesis [59,60]. Chemical induction of ER stress by thapsigargin (TG) impaired macrophage polarization towards a \( \text{M\Phi}_{\text{IL-10}} \) phenotype but did not modulate the cytokine production of \( \text{M\Phi}_{\text{IFN-\gamma}} \) and \( \text{M\Phi}_{\text{IL-10}} \). In combination with TLR ligation, however, TG impaired the production of TNF as well as other cytokines by \( \text{M\Phi}_{\text{IFN-\gamma}} \) but strongly increased the TNF production of \( \text{M\Phi}_{\text{IL-10}} \). These data illustrate the complexity of macrophage function as additional stimuli (in this case ER stress) can strongly bias the response of polarized macrophages to TLR ligands, whereas the same stimuli (TG + LPS) can have opposite effects on different polarized macrophage subsets (impairing TNF production in \( \text{M\Phi}_{\text{IFN-\gamma}} \) versus upregulating TNF production in \( \text{M\Phi}_{\text{IL-10}} \)). In conclusion, it appears that the functional cytokine profile of macrophages is determined by polarization as well as by the combination of different types of activation (extracellular or intracellular).

In contrast with the in vitro data, ER stress markers were not increased in polarized macrophages from HLA-B27+ SpA patients compared to controls, even after LPS stimulation. Furthermore, LPS-stimulated \( \text{M\Phi}_{\text{IFN-\gamma}} \) from HLA-B27+ SpA patients tended to express more IL-23p19 versus IL-10 than \( \text{M\Phi}_{\text{IFN-\gamma}} \) from HLA-B27- SpA and RA patients. On the contrary, LPS-stimulated \( \text{M\Phi}_{\text{IL-10}} \) expressed less IL-10 in HLA-B27+ versus HLA-B27- SpA patients. These findings do not support HLA-B27-induced ER stress as a mechanism for the aberrant cytokine production in SpA and are in line with previous reports on enhanced IL-23 production by macrophages from AS patients, in the absence of enhanced ER stress [61].

**Macrophage function in vivo**

In chapter 6 we did not only demonstrated that Ang-2 promoted a pro-inflammatory macrophage profile in vitro, as discussed previously, but also studied the role of Ang-2 in vivo in experimental arthritis. Tie-2-expressing macrophages (TEMs) were described to be the primary targets of Ang-2 in the synovium [62] and blocking Tie-2 signaling in a collagen-induced arthritis (CIA) model significantly ameliorated clinical disease [63,64]. We used a CIA model to demonstrate that Ang-2 neutralization significantly reduces disease severity, synovial inflammation, neo-vascularization and synovial macrophage infiltration. These experiments suggest that, despite major differences between in vitro polarized macrophages and tissue macrophages, clear in vitro pro-inflammatory effects, as observed here with Ang-2, do correlate with disease-promoting effects in vivo in experimental arthritis.
Finally, in chapter 8 we attempted to study macrophage polarization and function directly in the human disease by assessing the local macrophage-derived cytokine milieu in SpA and RA. We found lower levels of M1-derived cytokines (TNF, IL-1β, IL-12 and IFN-γ-inducible protein 10), but similar levels of M2-derived cytokines (IL-10, IL-4 and IL-13) in SpA compared to RA synovial fluid. Furthermore, the synovial fluid levels of these M1-derived mediators correlated with joint inflammation in RA, but not in SpA. Accordingly, adding pooled synovial fluid to healthy donor peripheral blood-derived macrophages resulted in an up-regulation of CD163 and CD200R in the presence of SpA compared to RA synovial fluid. These findings suggest the absence of a M1-signature in SpA compared to RA synovitis. Surprisingly, despite similar levels of M2-derived cytokines, SpA but not RA synovial fluid induced upregulation of MΦ_{IL-4} and MΦ_{IL-10} markers on healthy macrophages, which suggests either the existence of other polarizing mediators besides IL-4 or IL-10 in the SpA inflammatory milieu and/or the presence of M1 polarizing factors in RA synovial fluid which counteract a ‘default’ M2 polarization. The latter hypothesis would fit with the reverse IFN signature described in SpA peripheral blood monocytes [65].

Concluding remarks and future directions
In this thesis we showed that CD163 is indeed a specific marker for MΦ_{IL-10} in vitro but that the predominance of CD163+ macrophages in the inflamed SpA synovium does not solely reflect altered polarization, since these CD163+ macrophages did not display other phenotypic characteristics of MΦ_{IL-10}. Moreover, we demonstrated in vitro that polarization is reversible and that macrophage cytokine production is only partially dependent on the polarization status. Indeed, a major conclusion of this thesis is that cytokine production by in vitro polarized human macrophage subsets is highly dependent on so-called co-activators (ICs, Ang-2, ER stress) in combination with TLR ligands. Our ex vivo data indicate that macrophage polarization is not intrinsically biased in SpA but suggest that local factors may lead to altered macrophage cytokine production. Taken together, the data presented here illustrate that macrophage polarization and activation in vivo is a complex and dynamic process, which often exceeds the limitations of in vitro models.

It is crucial, however, to realize that we studied only a small part of the macrophage functional roles. Firstly, in this thesis we analysed a limited selection of well-known pro- and anti-inflammatory cytokines, which have been involved in the pathogenesis of arthritis. Obviously, many more macrophage-derived cytokines may be involved in SpA pathogenesis. An important example is the IL-12 family member IL-27, which was shown to induce Th1. At the same time IL-27 stimulates IL-10 production by Th2 cells and suppresses Th17 differentiation, hereby counteracting the effect of IL-23 [66]. Furthermore, IL-27
ameliorated inflammation in CIA [67,68] and represents a potential therapeutic agent in arthritis. Among the cytokines studied in this thesis, TNF deserves a special attention, since genetic association studies revealed a link between SpA and molecules involved in TNF signalling [69,70]. Furthermore, TNF blocking agents are very effective for the treatment of SpA. However, it is still not known which type of TNF (soluble versus transmembrane) and which TNF receptor is crucial in the disease process, or how TNF-mediated inflammation can lead to distinct structural phenotypes in SpA compared to RA. As both CD163 and TNF can be cleaved from the cell membrane by TNF-alpha-converting enzyme (TACE), we hypothesized that increased CD163 expression on synovial tissue macrophages is paralleled by an increased transmembrane (tmTNF) versus soluble TNF (sTNF) ratio in SpA. Recently, we found support for an altered balance between tmTNF and sTNF on polarized macrophages in vitro and in the inflamed SpA joint in vivo. Additionally, we demonstrated the spontaneous development of SpA-like axial and peripheral joint inflammation and new bone formation in tmTNF transgenic mice (manuscript in preparation).

Secondly, in this thesis we did not discuss other macrophage-derived products than cytokines. As an example, S100 calcium-binding protein A8/A9 (calprotectin) produced by macrophages and in particular by MΦ IL-10 [Eikmans, unpublished observations] was shown to play an important regulatory role in many inflammatory diseases [71]. Calprotectin is already used as a biomarker for IBD [72] and seems to be a better marker for inflammation in SpA than CRP [51,73 and Turina, unpublished observations].

Thirdly, besides the production of various mediators, macrophages are involved in phagocytosis of pathogens and debris, antigen presentation and matrix remodelling. Among these functions, regulation of matrix metalloproteinases and production of profibrotic mediators and chemokines by macrophages requires further research in the light of the disease-specific stromal signature that we recently demonstrated in SpA synovitis [Yeremenko et al, Arthritis Rheum, in press].

A fourth issue is the involvement of other innate immune cells in SpA pathogenesis. As discussed in chapter 2, the IL-23/STAT3/IL-17 axis plays a key role in the pathogenesis of SpA [74]. It was recently shown that not Th17 cells but mast cells and neutrophils were the main IL-17-expressing cells in both peripheral and axial SpA [8,75-77]. Macrophages, mast cells and neutrophils have common functions, such as phagocytosis, release of toxic molecules, cytokines and chemokines, or matrix remodelling, but it is the complex crosstalk between these innate immune cells which modulates the functions of adaptive immune cells, fibroblasts, smooth muscle cells or osteoblasts in the inflamed joint. Further fundamental and clinical research on the role of mast cells in SpA synovitis is ongoing.

Finally, due to the difficulty of obtaining samples from the inflamed spine or sacroiliac
joints, we focused our research on peripheral arthritis in SpA. A systematic comparison between axial and peripheral inflammation represents an important challenge for future studies. Furthermore, there is a very limited number of studies which address enthesitis by collecting biopsies from inflamed entheses of SpA patients and more research is needed to unravel the relationship between inflammation and bone formation in SpA [78,79]. 

Another essential feature of SpA, which is not analysed in this thesis, is the link with gut inflammation. Besides the shared genetic background, such as the association with IL-23R polymorphisms [80-82], histopathologic studies showed that gut mucosa of both SpA and Crohn’s disease patients displays an increased number of CD163+ macrophages [5,83], which points towards common pathogenic pathways.

The studies included in this thesis are relevant for the concept of macrophages as a highly dynamic innate immune cell population, which can be reversibly polarized and differentially activated by a large variety of mediators (Figure 3). Taking into account the limitations of

**Figure 3: Macrophage polarization and activation.** Monocytes are recruited from the blood into the inflamed tissue, where they differentiate into macrophages and are polarized by various mediators, such as IFN-γ, IL-4 or IL-10. Subsequently, macrophages can be activated by TLR ligands, in the absence or presence of co-activators, such as immune complexes (ICs) or angiopoietins (Ang). There is not much known about the differential involvement of monocyte-derived and resident macrophages in this process.
in vitro and animal models, one future line of research is the transcriptional profiling of human macrophages sorted from the inflamed tissue. Furthermore, the already existing single-cell microarray technology would allow us to investigate macrophage heterogeneity at tissue level [84]. Since macrophages are characterized by a high plasticity, and not by a default functional program, modulating macrophage polarization and activation could bring more therapeutic benefits than depleting macrophage number or blocking the signaling of one specific cytokine. A number of drugs, which are currently used for the treatment of arthritis, diabetes, atherosclerosis, asthma and different types of cancer, were already shown to target macrophage polarization [55,85].

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