Thesis summary and discussion
THESIS SUMMARY

Giant cell arteritis (GCA) and polymyalgia rheumatica (PMR) are inflammatory diseases occurring in the elderly. GCA patients suffer from inflammation of medium- and large arteries, leading to symptoms such as headache, jaw claudication, fever and weight loss. Severe complications can occur, including blindness and aneurysms. GCA frequently overlaps with PMR. The latter disease is characterized by synovial inflammation in the shoulders and hips, with typical complaints of pain and morning stiffness.

Both diseases are characterized by systemic inflammation and local infiltration of CD4+ T-cells and macrophages at the inflammatory site. GCA and PMR are mainly treated with glucocorticoids (GCs). However, relapses are common, hence the long duration of GC treatment, which inevitably comes with severe side effects. The pathology of GCA and PMR is not completely understood. In this thesis, we first aimed to increase knowledge on immune pathways leading to GCA and PMR pathology, with a focus on macrophages, as instigators of inflammation and tissue destruction, and on their monocyte precursors in the blood (Chapter 2-6).

Because there are no accurate disease specific and prognostic biomarkers for GCA and PMR, the second aim of this thesis was to translate knowledge on the immunopathology of GCA/PMR to the clinic. Here, we studied the utility of macrophage-derived factors and markers of angiogenesis as diagnostic, monitoring and prognostic tools in GCA and PMR patients (Chapter 7 and 8).

In Chapter 2, we provide a comprehensive long-term study comparing leukocyte subset counts before, during and after GC treatment in peripheral blood of GCA and PMR patients. Compared to healthy controls, newly-diagnosed GCA and PMR patients display a change in leukocyte composition with a shift towards the myeloid lineage, evidenced by elevated monocyte and neutrophil counts, and reduced B-cell and NK-cell counts. GC treatment affected leukocyte subset counts, but did not normalize this inflammation-induced shift to the myeloid lineage. Rather, GCs boosted the myeloid profile even further. Moreover, this myeloid profile was retained well into treatment-free remission, possibly due to ongoing subclinical disease. Alternatively, this myeloid bias could point at an aged immune system (inflammaging) in these individuals thereby predisposing to the development of GCA and PMR.

Monocyte counts were consistently high in peripheral blood of GCA and PMR patients. Nowadays, three monocyte subsets can be identified based on CD14 and CD16 expression: classical (CD14^{high}CD16^{-}; the most common subset, specialized in phagocytosis), intermediate (CD14^{high}CD16^{+}; the most pro-inflammatory subset), and non-classical (CD14^{dim}CD16^{+}; the most mature subset) monocytes. In Chapter 3, we observed that the elevated monocyte counts in GCA and PMR were due to an increase in the classical subset. Non-classical (aged) monocyte counts were not elevated in GCA and PMR, and their proportions as part of the monocyte composition were found to be reduced as a result of the increase in classical monocytes. Moreover, only non-classical monocytes were sensitive to GC treatment. In this study, we also observed that all macrophages in the temporal artery biopsy (TAB) were CD16^{+}. In addition, we showed involvement of two migratory pathways in the recruitment of tissue infiltrating monocytes: the CCR2/CCL2 chemotaxis pathway for classical monocytes and the CX3CR1/CX3CL1 pathway for non-classical monocytes. As CCR2 was
infrequently expressed in TAB, but CX3CR1 expression by macrophages was abundant, we concluded that macrophages in TAB phenotypically resembled non-classical monocytes. Our data thus suggest that non-classical monocytes are the precursors of the tissue infiltrated macrophages in GCA.

Macrophages, dendritic cells (DCs) and pathogenic CD4+ T-cells (Th1 and Th17) are localized in granulomatous structures in GCA and PMR lesions. As GCA is a systemic disease, we hypothesized that counts of monocyte subsets, as precursors of macrophages, were linked to expanded Th1 and Th17 cell counts in the blood. In Chapter 4, we thus assessed counts of monocyte subsets and Th1 (IFNy producing) and Th17 (IL-17 producing) cells. We performed experiments in two small GCA cohorts, but in contrast to previous studies by others, we did not detect differences in levels of circulating Th1 and Th17 cells between treatment-naïve patients and age-matched healthy controls. As such, we did not detect correlations between monocyte subsets and Th1 and Th17 cells. Possibly, interactions between monocyte derived cells and CD4+ T cells mainly occur in tissue, and are therefore less conspicuous in the periphery. Next, we measured counts of circulating DCs (myeloid (mDCs) and plasmacytoid (pDCs)). We observed lower counts of mDCs in both treatment-naïve GCA and PMR than in healthy controls. As pattern recognition receptors are key in the initiation of immune responses, we assessed the expression of several pattern recognition receptors by monocyte and DC subsets. Expression of toll-like receptor (TLR)2 on mDCs was found to be elevated in GCA and PMR. Taken together, the reduced numbers of mDC and higher per cell expression of TLR2 may suggest that mDC migrate to the site of inflammation and are prone to activation by TLR2 ligands.

It is well known that macrophages in tissue may display considerable heterogeneity in response to cues from the environment. So far, there is little knowledge on macrophage heterogeneity in GCA. We hypothesized different spatial identities of macrophages, governed by local expression of growth and differentiation factors, associated with tissue destruction and intimal proliferation. In Chapter 5, we identified a distinct spatial distribution pattern of macrophage phenotypes in the TAB: CD206-expressing, matrix metalloprotease (MMP)-9 producing macrophages at the media borders, the sites of tissue destruction, and folate receptor (FRβ)–expressing macrophages at the site of intimal proliferation. Of note, this distinct pattern could also be observed in macrophage-rich areas in GCA aortas, but not in atherosclerotic aortas. We showed that CD206 was upregulated following granulocyte-macrophage colony-stimulating factor (GM-CSF) skewed macrophage differentiation, whereas FRβ was higher after macrophage colony-stimulating factor (M-CSF) differentiation. Therefore, the spatial distribution of macrophage subsets could be explained by sequential GM-CSF and M-CSF skewing in GCA tissues, which indeed was found to correspond with the staining patterns of GM-CSF and M-CSF in tissue. This study provides new clues for therapies targeting macrophage subsets, such as GM-CSF receptor blockade. Additionally new macrophage tracers could be designed to replace 18F-fluorodeoxyglucose (FDG) as a more cell specific tracer in PET-CT scans.

YKL-40 is a macrophage-derived factor that serves as a well-known serum marker of inflammation and tissue remodeling, and was our main focus in Chapter 6. We showed that YKL-40 was abundantly expressed in GCA TABs and aortas. YKL-40 expression in the aorta provides further evidence that YKL-40 qualifies as a candidate biomarker of vessel inflammation. We showed that

the CD206+ macrophage subset skewed by local GM-CSF signals is the main producer of YKL-40 in GCA. YKL-40 is implicated in new vessel formation, i.e. angiogenesis, an essential process fueling the pathology of GCA. Here, we confirmed the angiogenic capacity of YKL-40 by a tube formation assay with human microvascular endothelial cells. These angiogenic effects are likely governed by the receptor of YKL-40, IL-13 receptor α2 (IL-13Rα2) as our preliminary data showed expression of this receptor by endothelial cells and infiltrated cells in GCA TABs.

In an effort to translate this knowledge into clinical utility and in line with our second aim, chapters 7 and 8 show the potential of macrophage and angiogenesis markers as clinical biomarkers. Here, we aimed to find a solution for a number of clinical needs: there are no disease-specific diagnostic markers for GCA and PMR, no reliable markers to monitor relapses and tissue inflammation during treatment, and no markers that confidently predict GCA/PMR disease course. Our biomarker studies are unique in the world as we took advantage of our prospective GPS cohort at the UMCG. Participating patients are requested to donate blood regularly in a long-term (7 years) longitudinal set up: at diagnosis (before start of treatment), during treatment, and well into treatment free remission.

Based on clues from GCA characteristic pathogenic processes, we hypothesized in Chapter 7 a role for macrophage products and markers of angiogenesis as novel candidate biomarkers. Most of the acute-phase, macrophage and angiogenesis markers were found elevated compared to healthy controls, but similar to infection controls. GC treatment suppressed most markers, but calprotectin and YKL-40 levels remained high, possibly reflecting ongoing vascular inflammation. The study also included samples from patients in treatment-free remission, showing that several inflammatory and angiogenesis markers did not normalize but remained elevated in these patients. The most important finding of this study is the predictive value of a profile of angiogenesis markers: VEGF, YKL-40, and angiopoietin-1 and 2. This profile predicted a long GC therapy duration in GCA patients, most likely due to a relapsing disease course.

Chapter 8 was aimed at identifying biomarkers in PMR, based on markers reflecting inflammatory and angiogenic processes. It included baseline measurements of serum markers in patients with isolated PMR, patients with overlapping PMR and GCA, and control groups. Similar to GCA, most inflammatory and angiogenesis biomarkers were found elevated in patients with isolated PMR. However, erythrocyte sedimentation rate (ESR), soluble Tie2 and angiopoietin-2 levels were lower in isolated PMR than in PMR/GCA overlap. Of these markers, angiopoietin-2 had a superior receiver operating characteristic (ROC) curve; thus demonstrating a superior ability to discriminate between patients with isolated PMR and patients with overlapping PMR/GCA. This is important for patients as GCA is associated with severe complications. Moreover, angiopoietin-2 levels assessed at baseline are also promising markers in the prediction of a favorable or non-favorable disease course in isolated PMR patients. Validation of the findings in Chapter 7 and 8 in a second prospective cohort is essential.
DISCUSSION

The immunopathology of GCA and PMR is a complex process involving interactions between tissue resident cells and infiltrating immune cells in an aged patient. Macrophages are the most abundant cells in the infiltrated regions of vascular and synovial tissues of GCA and PMR, respectively. Nevertheless, scarce data are available on macrophages, or their monocyte precursors, in GCA. In addition, insight in the local pathobiology of PMR is very limited due to the lack of biopsy studies. Monocytes and macrophages are innate immune cells equipped to sense changes in the environment, to respond quickly to damage, to eliminate endogenous and foreign substances and to interact with both innate and adaptive immune cells to steer their functions. This thesis is dedicated to further characterize monocyte and macrophage heterogeneity in GCA and PMR, and to explore their role in disease pathophysiology. In this context, we also studied CD4+ T-cells, DCs, and endothelial cells. Part I of this chapter discusses the involvement of monocytes and macrophages in the pathogenic model of GCA and PMR. Part II discusses how we translated this knowledge for potential clinical relevance and assessed monocyte and macrophage products as diagnostic, prognostic and monitoring biomarkers in GCA and PMR. Finally, part III discusses possible future directions for research in the field of GCA and PMR.

PART I: MONOCYTES AND MACROPHAGES ARE CENTRAL IN GCA AND PMR PATHOLOGY

Monocytes are circulating myeloid cells that are part of the innate immune system. They are the precursors of mDCs and macrophages in the tissues. Monocytes, macrophages and mDCs have a number of functions, including phagocytosis, cytokine production, tissue destruction and remodeling, and are capable of shaping the adaptive immune response. Functioning and phenotype of these myeloid cells are influenced by aging [1]. All three cell types play an important role in autoimmunity, including GCA and PMR.

How to best study GCA and PMR pathology?

The immune pathology of systemic diseases like GCA/PMR can be studied both in the blood and at the site of inflammation. An advantage of peripheral blood studies is that it can be easily and continuously sampled, allowing to study effects of disease and treatment over time. A disadvantage of blood studies is that their relationship with the events at the site of pathology, the vessel wall and synovium in GCA and PMR, respectively, is less clear.

Chapter 2 is a prospective study, providing a comprehensive overview of peripheral blood leukocyte dynamics and inflammatory markers in GCA and PMR during the entire disease course: before and after start of GC treatment as well as in stable treatment-free remission. The main finding of this chapter was a persistent shift of the leukocyte subset composition towards the myeloid lineage. Specifically, this means that counts of neutrophils and monocytes are elevated and counts of lymphocytes (B/T/NK-cells) are reduced. At baseline, this finding may not be very remarkable, as the myeloid shift is observed in a wide range of inflammatory conditions [2]. However, this study notably shows that GC treatment does not normalize the peripheral blood composition, but rather contributes to a further myeloid bias. Moreover, GCA and PMR patients well into treatment-free remission, defined as a lack of signs of disease symptoms during a longer period, still have a myeloid-biased leukocyte subset composition. Persistence of the myeloid profile during the entire disease course may reflect ongoing subclinical vasculitis, implying that current GC-based treatment is unsatisfactory. This is important knowledge and may aid the optimization of therapeutic regimens in these patient groups.

The second means to learn about GCA/PMR pathology are tissue studies. For GCA, this is a sensible and accessible method, as temporal artery biopsies (TABs) are routinely taken for diagnostic purposes. For PMR, tissues studies are scarce; our department has recently started taking synovial PMR biopsies guided by ultrasound imaging. The advantage of tissue studies is that the actual site of inflammation is studied at the time of disease activity. Disadvantages of tissue studies are that they are invasive, limited as amount and reflect only one moment in time and place. Additionally, interventional (treatment) studies are hard to perform. The most commonly used technique for tissue studies is immunohistochemistry, which provides little information on the protein quantity. Nevertheless it delivers qualitative and localized information on the markers investigated. Other techniques such as qPCR and single cell sequencing of tissues will deliver more quantitative data but are generally at the expense of tissue morphology. Ideally, quantitative single cell data should be obtained in combination with preserved tissue morphology. These techniques such as imaging mass cytometry, which are able to detect multiple overlapping markers in tissue, are becoming more and more state of the art and would be highly useful especially when studying scarce patient tissues. More conventional tissue studies by immunohistochemistry and immunofluorescence for assessing co-localization of markers are part of Chapter 3, 6 and 7 and are the backbone of Chapter 5. In chapter 5, we performed a thorough characterization of macrophages throughout the three layers of the vessel wall (both in GCA TABs as well as in aortas) characterizing distinct spatial identities of macrophage subsets governed by local expression of growth factors. This characterization may aid in developing new targets for treatment as well as new tracers for PET-CT imaging.

What is lacking in GCA and PMR research are studies bridging blood and tissue. It is difficult to translate changes in the blood to pathology at the inflammatory site, and vice versa. This is mainly due to the lack of proper animal models for GCA, as the anatomical build-up and vessel size is very different in commonly used animal models such as mice and rats [3]. As an example, the vasa vasmorum are lacking in large vessels of mice. Some functional studies have been performed on explanted TABs, which aided our understanding of the disease substantially [4, 5]. However, these studies remain rather artificial, as the tissue is dismembered from a functioning immune system, and thus only partly resembles the situation in the human body. Future studies could make use of cell-specific tracers in combination with whole body imaging, which could provide insight in the migration of leukocyte subpopulations towards the inflammatory site. These tracer studies could for example elucidate whether non-classical monocytes indeed preferentially migrate to the inflammatory site in GCA and PMR, as proposed in Chapter 3.

Another issue is that GCA and PMR patients present with a well-developed disease pathology at diagnosis, thereby obscuring early-stage processes that have initiated the disease. Patients generally
visit their general practitioner when they experience disease symptoms, which only develop when the pathology has progressed to large scale inflammation of the target tissues. Recently, cases of GCA and PMR have been described to develop in cancer patients treated with checkpoint inhibitors (CTLA-4 and PD-1) [6, 7]. These cases are excellent opportunities to study earlier stages of GCA/PMR, as they sometimes develop very quickly after initiation of checkpoint inhibitor treatment. It remains to be studied, however, whether these patients immunologically and clinically present with a similar disease phenotype as ‘regular’ GCA and PMR patients. Alternatively, large scale prospective population studies, such as the Groningen Lifelines cohort, could identify immunological and other factors that predispose elderly participants to the development of GCA/PMR [8].

Macrophage heterogeneity in GCA: spatially distributed macrophages specialized in tissue destruction

Macrophages can have various roles in tissues, such as phagocytosis, promoting inflammation (cytokine production), tissue destruction (release of reactive oxygen species and MMPs) and angiogenesis (production of VEGF, angiopoietin-2) [9-11]. We asked the question if a single macrophage subset is involved in these processes or whether these are mediated by distinct or evolving macrophage subsets with different spatial identities in tissue. In Chapter 5 we indeed identified different macrophage subsets in inflamed tissues of GCA patients with specialized functions. CD206+ macrophages were found at the sites of MMP-9-driven tissue destruction and the FRβ macrophage subset at the site of intimal proliferation. Previously, macrophages producing transforming growth factor (TGF)β were reported to reside mainly in the adventitia, whereas MMP-2 and inducible nitric oxide synthases expression was observed in the intima [12]. Our study, however, is the first to assign these different functions to distinct macrophage subsets, defined by surface markers CD206 and FRβ, located at different sites within the tissue.

The macrophage tissue heterogeneity is likely caused by a distinct spatial production of GM-CSF and M-CSF. Classically, macrophages have been subclassified by the M1/M2 paradigm (pro-inflammatory/anti-inflammatory), which is mostly based on observations from in vitro differentiation [13]. A more recently adopted subclassification of macrophages is based on GM-CSF and M-CSF skewing [14]. In GCA TABS, GM-CSF signals seem to generate the phenotype of a CD206 expressing macrophage subset present at the media borders (Chapter 5). These cells have characteristics of both M1 (pro-inflammatory cytokine production) and M2 macrophages (tissue remodeling and angiogenesis) [15]. GM-CSF is most strongly expressed in the adventitia, most likely produced by CDA4+ T-cells. Recently, a CDA4+ T-cell subset has been identified that specializes in GM-CSF production, rather than IFNγ or IL-17 [16]. In addition, B-cells may also be a source of GM-CSF in GCA. In multiple sclerosis, a higher number of B-cells have been described that are not limited to producing antibodies, but rather produce high levels of GM-CSF [17]. B-cells are present in most TABS, albeit in lower numbers than T-cells [18]. However, in aorta biopsies of GCA patients, large accumulations of B-cells are observed in the adventitia [19]. These biopsies are obtained from GCA patients suffering from an aneurysm, a late stage GCA complication. B-cell-derived GM-CSF may be of importance in late-stage GCA. Further studies are needed to identify the cellular source of GM-CSF in early- and late-stage GCA. These findings on GM-CSF in GCA are important, as a clinical trial is currently ongoing with the GM-CSF receptor blocker Mavrilimumab (NCT03827018).

Macrophages in GCA are the main drivers of tissue damaging processes such as the destruction of the lamina elasticas. We showed that CD206+ macrophages in GCA TABS and aortas have overlapping expression of MMP-9 at distinct locations within the tissue. MMPs are involved in both physiological and pathological tissue reshaping, for example the degradation of collagen, one of the components of the extracellular matrix [20]. In GCA, MMP-9 production by monocytes/macrophages was deemed essential for T-cell infiltration in the vessel wall [14]. Moreover, the degradation of extracellular matrix by MMP-9 is essential to facilitate invading endothelial cells during angiogenesis [21]. Previous studies discovered that MMP-9 expression in macrophages can be induced by YKL-40, a pro-angiogenic protein (9,13). This is further corroborated by concomitant expression of YKL-40, its receptor IL-13Ra2, and MMP-9 at the media borders in GCA TABs (Chapter 6). Future experiments using YKL-40 stimulation of TAB explants could provide further evidence that YKL-40 instigates MMP-9 production in GCA.

We thus established clear phenotypic and functional heterogeneity of macrophage subsets at distinct sites in the vessel wall which are governed by local GM-CSF and M-CSF. It is yet unclear whether the macrophage heterogeneity observed in GCA tissues is caused by macrophage plasticity, or the infiltration of new monocytes [11]. Possibly, tissue infiltrating monocytes progressively differentiate from pro-inflammatory macrophages into tissue-destructive and/or pro-fibrotic macrophages depending on signals from the local microenvironment. Alternatively, the pro-inflammatory macrophages disappear once the inflammatory trigger has been cleared. A second wave of monocytes then enters the tissue which can differentiate into tissue-destructive and/or pro-fibrotic macrophages in response to cues from the microenvironment. Monocyte subsets have inherently different capacities, and may retain some of these capacities upon differentiation into tissue macrophages [22]. Thus, we asked the question if the distinct macrophage phenotypes would already be visible in peripheral blood monocytes of GCA/PMR patients.

Non-classical monocytes: tissue destructive cells in GCA and PMR pathology?

The involvement of monocytes in GCA and PMR immunopathology is underexplored. In Chapter 2, we describe persistently elevated monocyte counts in the blood of GCA and PMR patients compared to healthy controls, a finding consistent with the myeloid shift observed in inflammatory conditions. Moreover, monocyte counts were correlated with CRP levels in baseline GCA patients. These findings put monocytes and their subsets in the center of attention in the pathology of GCA and PMR. The three monocyte subsets defined by CD14 and CD16 expression have distinct functional characteristics [23]. In Chapter 3, we observed a disturbed monocyte subset distribution in peripheral blood of GCA and PMR patients, with a relative decrease of non-classical monocytes. We propose that this is due to preferential migration of this monocyte subset to GCA and PMR lesions, where they contribute to disease pathogenesis. Alternatively, the non-classical monocyte proportions are reduced due to a developmental block.
DISCUSSION

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Chapter 4

We did not observe a proportional increase of $A$ and $B$, we did not identify presence in tissue would add to slan+ macrophages in GCA/PMR lesions has not yet been assessed, but once established, their appearance in complement-associated responses [26, 31].

CD16+slan+ monocytes. This was indeed confirmed, as shown in Figure 1.

Interestingly, a fraction of the non-classical monocyte subset expresses ‘slan’, a carbohydrate residue present on the cell surface [24]. This marker is expressed exclusively on non-classical monocytes, but not on intermediate monocytes (Figure 1). Slan+ cells have wrongly been described as DCs [25], but now there is consensus that slan+ cells are monocytes [24, 26]. It has been proposed to identify true non-classical monocytes as CD16+slan+, and intermediate monocytes as CD16+slan-. The gating strategy to define the two CD16+ monocyte subsets (i.e. intermediate and non-classical) based on arbitrary CD14 expression is thus no longer needed. The slan-based strategy results in a smaller proportion of non-classical monocytes, matching the gene expression pattern of this subset [24]. As we observed a lower proportion of non-classical monocytes in the blood of newly-diagnosed GCA and PMR patients, we also expected to see lower proportions of CD16+slan- monocytes. This was indeed confirmed, as shown in Figure 1.

Slan is also expressed on macrophages in tissue, with concomitant expression of CD16 [28]. Slan-expressing macrophages are observed in inflamed tissues in RA and in HIV infected tissues [29, 30]. Their phenotype is typically pro-inflammatory: they produce TNFα and IL-12 upon TLR stimulation and they appear to be important in complement-associated responses [26, 31]. The presence of slan+ macrophages in GCA/PMR lesions has not yet been assessed, but once established, their presence in tissue would add to the notion of non-classical monocyte migration to tissue in GCA.

Non-classical monocytes are likely derived from CD14+ classical monocytes. Elegant isotope labeling studies have shown that classical monocytes are the first monocyte subset in the blood, after migrating from the bone marrow [32]. Additionally, hematopoietic stem cell transplantation after kidney transplantation shows a similar pattern: firstly, classical monocytes appear in the blood [33]. Subsequently, intermediate and finally non-classical monocytes develop. Both M-CSF and GM-CSF signaling are thought to stimulate monocyte maturation towards the non-classical phenotype. Individuals lacking the M-CSF receptor due to a genetic mutation have no CD16+ monocytes [24]. In vitro studies by Duterte et al showed that expression of slan can also be upregulated by GM-CSF stimulation [34]. This is interesting, as we showed in Chapter 5 that non-classical monocytes have very little GM-CSF receptor expression compared to the other monocyte subsets. Possibly, monocytes lose GM-CSF receptor expression after stimulation with GM-CSF through a negative feedback loop.

Interestingly, non-classical monocytes display similarities with GM-CSF skewed macrophages (Chapter 5 and 6), as they have the ability to produce the highest levels of MMP-9 and YKL-40 of all circulating leukocytes [35, 36]. Our additional data showed that serum levels of YKL-40 negatively correlated with the proportions of non-classical monocytes ($N=27$, $R=-0.51$, $p=0.007$). This counterintuitive finding may be explained by migration of YKL-40 producing non-classical monocytes to the inflammatory site. Indeed, this tissue remodeling role for non-classical monocytes has been implicated in a mouse model for rheumatoid arthritis (RA) [37]. Taken together these findings suggest that non-classical monocytes are the precursors of the CD206+, MMP9+, YKL-40+ macrophage subset and that they constitute the ‘second wave’ of infiltrating monocytes in affected GCA and PMR tissues as promoters of tissue damage [11]. This wave of monocytes infiltrate the tissue after the first wave of pro-inflammatory cytokine producing monocytes have entered the tissue.

No evidence for altered proportions of pro-inflammatory intermediate monocytes in GCA/PMR

In many inflammatory diseases, the proportions as well as absolute counts of circulating intermediate monocytes are found to be elevated. Examples are RA [38], sarcoidosis [24], ANCA-associated vasculitis [39] and cardiovascular disease [40]. Intermediate monocytes are thought to be the most pro-inflammatory monocyte subset, and express the highest level of TLR2 and TLR4 of all monocyte and DC subsets (Chapter 4). They may therefore constitute the ‘first wave’ of infiltrating pro-inflammatory monocytes. Intermediate monocytes have high HLA-DR expression and their proportions are strongly associated with expansion of Th17 cells in RA as shown by Rossol et al [41]. In contrast to these studies, in Chapter 3, we did not observe an proportional increase of the intermediate monocyte subset in GCA/PMR, but rather an expansion of classical monocytes at the expense of non-classical monocytes. Moreover, in Chapter 4, we did not identify a meaningful correlation between intermediate monocytes and Th17 cells.

Intermediate monocytes, in contrast to non-classical monocytes, express Tie-2, which is the receptor for angiopoietin-1 and -2 [27, 42]. Tie-2+ monocytes have also been dubbed ‘angiogenic’ monocytes. They were found to have angiogenic properties, as they can adhere to injured endothelium, and instigate vascular growth [43]. Tie-2 monocytes are able to respond to

Figure 1. The proportions of non-classical monocytes, as defined by the slan approach, is lower in both GCA and PMR patients compared to healthy controls. Thawed peripheral blood mononuclear cells were stained for CD14 and HLA-DR to identify monocytes. Slan expression (identified by the M-DC8 antibody, Miltenyi) was present only on a subset of CD16+ monocytes (A). Classical and intermediate monocytes are displayed in red, slan+ non-classical monocytes in green and slan+ monocytes in purple. This indicates that only a subpopulation of non-classical monocytes (gated on CD14 expression) is slan positive. The proportion of monocytes positive for both CD16 and slan in treatment-naïve, newly-diagnosed GCA and PMR patients were compared to age- and sex-matched controls (B, $N=7$ for each group).

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DISCUSSION

We observed lower tissue resident DCs are γ and β. Macrophages are derived from infiltrating monocytes. However, we did not see expansion of Th1 γ and β. PDGF production (4). YKL-40+, VEGF+ cells (3). M-CSF signaling leads to FR macrophages upregulate CD206 expression, and develop in tissue destructive (MMP-9+) and angiogenic expressing macrophages, thought to be involved in intimal proliferation by PDGF production (4).

Myeloid dendritic cells sensitized to TLR2 ligands may initiate and fuel GCA and PMR

Another myeloid cell subset important to GCA/PMR pathology are mDCs. Tissue resident DCs are in a resting state, until they become activated by stimulation via pattern recognition receptors, including TLRs (5, 45). In TABs of GCA patients, frequencies of DCs are expanded (46), but DC counts and phenotypes in the blood have not yet been enumerated. In Chapter 4, we observed lower counts of circulating mDCs in GCA and PMR patients. Additionally, the higher TLR2 expression on mDCs suggests an increased sensing capacity. Activation of pattern recognition receptors (i.e. TLR2) expressed by tissue residing mDCs is thought to be the trigger for GCA/PMR pathology.

Monocytes/macrophages are key in skewing CD4+ T-cells in tissue, but not in peripheral blood

Macrophages and DCs play a major role in shaping the CD4+ T-cell response in GCA and PMR pathology (51). Naive CD4+ T-cells, when properly stimulated, can develop into different T-helper lineages producing different cytokine profiles, dependent on skewing signals from the microenvironment (52). These cytokine signals are produced by neighboring macrophages, such as IL-1β, IL-6, IL-23 (Th17-skewing) or IL-12 (Th1 skewing) (53), as also shown in Chapter 5. In GCA/PMR tissues, Th1 and Th17 cells are frequently observed, as evidenced by local IFNγ and IL-17 production (54). Moreover, IL-12 and IL-23 are promising therapeutic targets in GCA, by inhibiting Th1 and Th17 cells (55, 56). Currently, a randomized controlled trial targeting IL-12 and IL-23 is ongoing (ustekinumab, NCT03711448).

Figure 2. Monocytes and macrophages in GCA. Monocytes are derived from infiltrating monocytes. Monocytes can enter the vessel wall guided by CCL2, for classical and intermediate monocytes, and CX3CL1, for CD16+ intermediate and non-classical monocytes (1). Macrophages in the vessel wall of GCA patients express CD16 and are capable producers of pro-inflammatory cytokines (2). Under the influence of GM-CSF, macrophages upregulate CD206 expression, and develop in tissue destructive (MMP-9+) and angiogenic (YKL-40+, VEGF+) cells (3). M-CSF signaling leads to FRβ expressing macrophages, thought to be involved in intimal proliferation by PDGF production (4).
with local macrophages may reveal more insight into the molecular processes underlying vascular inflammation and tissue remodeling in GCA.

**PART II: CLINICAL OUTLOOKS FOR GCA AND PMR PATIENTS**

Relatively little has changed over the last decades in the management of GCA and PMR patients. Only recently, the IL-6 receptor blocker tocilizumab showed promising results in a large randomized controlled trial in GCA. GCs thus remain the cornerstone of the treatment strategy, and virtually all patients with GCA and all patients with PMR start with the same drug, prednisolone. Moreover, PMR patients are commonly treated by their general practitioner, who typically has limited, if any, means to exclude overlapping GCA. This thesis has provided a number of clues that are potentially relevant for changing the management of GCA and PMR in daily clinical practice.

So far, many biomarkers (e.g. C-reactive protein (CRP), ESR, SAA) have been found that can distinguish GCA and PMR from healthy controls [61, 62] (Figure 3). This makes sense, as GCA and PMR are characterized by systemic inflammation with an acute-phase response, which is strongly dependent on IL-6 (Chapter 7). Indeed, the vast majority of treatment-naive GCA and PMR patients has an elevated CRP. Using additional markers such as ESR or angiopoietin-2, practically all patients can be discriminated from healthy controls. The utility of acute-phase markers drops substantially once patients are on GC treatment as markers of inflammation are typically suppressed [63].

An important clinical problem however, is that GCA and PMR are difficult to distinguish from patients with infections. It is for example challenging to determine whether a high CRP can be attributed to GCA disease activity or to a urinary tract infection; in both cases, CRP levels can be elevated. This is true for all biomarkers studied in Chapter 7 and 8, as none of the serum markers were specifically elevated in GCA or PMR patients. In Chapter 2 however, we observed higher plateau counts in GCA and PMR patients (Figure 3), which is, to the best of our knowledge, the only biomarker found to date that may distinguish GCA/PMR from infections. Future studies would benefit from including a control group of age-matched infection controls. In addition, future studies are needed to identify biomarkers separating PMR patients from seronegative rheumatoid arthritis patients, as symptoms and most biomarker levels of these diseases can overlap [64].

Another important issue at the time of PMR diagnosis, is whether the patient has overlapping GCA. This is important, as complications of GCA can be dangerous, such as blindness and aneurysms. GCA patients also require treatment with local macrophages may reveal more insight into the molecular processes underlying vascular inflammation and tissue remodeling in GCA.

Figure 4. Markers during the disease course of GCA/PMR patients. Patients in our studies are all treated with GCs that are tapered over time, unless a patient experiences a relapse. If a patient relapses, the GC dose is increased, and a DMARD can be added (in our study methotrexate or leflunomide). In patients that remained in remission, GC treatment was tapered until GC-free remission was achieved. We used the time to GC-free remission as a measurement of an (un)favorable disease course. Additionally, there is also a need for markers that aid clinicians in identifying a GCA or PMR relapse. Finally, markers are shown that are still altered in GCA/PMR patients that reached stable treatment-free remission compared to healthy controls. Markers in bold have been studied in this thesis (Chapter 2, 7, 8). Markers in red have been studied in the context of this thesis, but are not part of the chapters in this thesis.

Figure 3. Diagnostic biomarkers in GCA and PMR. Numerous markers (serum markers, cell counts or other) are altered in GCA and/or PMR compared to healthy controls. Scarcce studies have looked at markers that are altered in GCA/PMR versus patients with an infection, and so far only platelet counts were found to be higher in GCA and PMR (Chapter 2). Some studies have tried to differentiate GCA patients from patients with isolated PMR. In chapter 8, we show that angiopoietin-2 has a high diagnostic accuracy in identifying GCA from isolated PMR. Markers in bold have been studied in this thesis (Chapter 2, 7, 8).
DISCUSSION

Chapter 6 and we show that CRP and ESR perform poorly in monitoring shows that two macrophage-produced proteins remain 195),

However, this biological is not a panacea as about 50% of patients failed to achieve sustained GC-free remission at the opposite site of the primary biopsy, inflamed tissues may be missed due to skip lesions and vascular inflammation may be present in the aorta and its branches (which may be even more GC resistant [75]). Interestingly, Chapter 7 shows that two macrophage-produced proteins remain elevated in serum of GCA patients during the first year of treatment. Calprotectin and YKL-40, released by infiltrating phagocytes and CD206+ macrophages respectively, thus possibly reflect tissue inflammation. Calprotectin and YKL-40 are still expressed in aorta biopsies of patients with GCA-caused aneurysm (Chapter 6), a complication typically representing late-stage disease. This is in accordance with leukocyte subset data in Chapter 2, showing that the myeloid bias is not corrected by GCs and is still apparent in treatment-free GCA and PMR patients. Numerous other inflammatory and angiogenic biomarkers were found to be higher in treatment-free remission than in healthy controls (Figure 6).

Persistent tissue inflammation is likely not only an issue in GC-treated patients, but also for add-on tocilizumab treated patients. MRI evidence shows signs of inflammation in large vessels of GCA patients on tocilizumab treatment [79]. Studies on tocilizumab treated patients, comparable to GC-treated patients in Chapters 7 and 8 of this thesis, showed persistently altered tissue

Highly awaited are predictive biomarkers that can stratify patients qualifying for tocilizumab treatment, treatment with other biologicals (e.g. GM-CSF receptor blockade), or patients for whom GC treatment is sufficient [72]. In Chapter 2, 7 and 8 we have investigated the prognostic utility of leukocyte subsets, serum markers and other parameters, measured before the start of treatment (Figure 4). We chose to use the time to GC-free remission as measurement of a favorable disease course. Most of the other studies have used the number of relapses, or the time to the first relapse, but a relapse of GCA and PMR may be hard to define. We chose the time to GC-free remission as this reflects a sum of the number of relapses, their timing and duration, but also because it is a very important patient-related outcome. GC-treatment has toxic effects on patients and this becomes increasingly worse with the duration of the treatment [73, 74]. In Chapter 7 and 8 we identified that markers important in angiogenesis outperform acute-phase markers as predictors of time to GC-free remission. Remarkably, some of these markers behave similarly in GCA and PMR: high VEGF at baseline is protective whereas high angiopoietin-2 and YKL-40 levels are hazardous. This implies that in both diseases, angiogenic processes might be important determining the GC sensitivity of individual patients. How these markers compare to other proposed prognostic markers [62, 75, 76] needs further evaluation, although VEGF (in GCA) and angiopoietin-2 (in PMR) outperformed the commonly used markers CRP and ESR. Using these markers, patients may be selected that would benefit from a short-term GC treatment only, with rapid tapering.

In addition, there is increasing evidence that GCA and PMR symptoms can readily return due to persistent inflammation at the tissue level. Measures of systemic inflammation are suppressed in treated patients, but do not necessarily reflect an ongoing tissue inflammation. Observations by ultrasound imaging show that vessel-wall thickening persists for years in GC-treated GCA patients [77]. Malezewski et al performed a follow-up temporal artery biopsy in GCA patients that had biopsy-proven GCA [78]. A majority of patients showed persistent vascular inflammation with macrophages and T-cells after up to one year of GC treatment. This number of patients with persistent inflammation is likely even an underestimation, as the follow-up biopsy was taken on the opposite site of the primary biopsy, inflamed tissues may be missed due to skip lesions and vascular inflammation may be present in the aorta and its branches (which may be even more GC resistant [75]).

GCs are known to have a wide range of effects on the immune system, including macrophages and CD4+ T-cells [68]. GCs specifically prevent IL-6 production and signaling, a process that is essential for instigating the acute-phase response and systemic inflammation. GCA/PMR complaints such as fever, night sweats, weight loss and malaise are directly or indirectly related to this response [69]. It is therefore not unexpected that patients experience almost instant relief after initiation of GC treatment. Unfortunately, the disease often relapses, especially when the GC dose is tapered. As stated before, commonly used disease activity markers CRP and ESR lose accuracy during GC treatment. In Chapter 2 we show that CRP and ESR perform poorly in monitoring of GCA and PMR relapses, even though ESR appears to be more useful in flagging GCA relapses than the more frequently used CRP. Other biomarkers have been studied as relapse markers, but generally lack sensitivity and specificity [70]. Recently, the combination of GCs with IL-6 receptor blocker tocilizumab was shown to be superior to monotherapy with GCs, with regard to sustained GC-free remission in patients with GCA [71]. Besides, tocilizumab had a strong GC-sparing effect. However, this biological is not a panacea as about 50% of the patients failed to achieve sustained GC-free remission at the end of the study (week 52) [63]. Moreover, tocilizumab is very expensive. In addition, CRP and ESR are completely unreliable as markers of inflammation during treatment with tocilizumab, making monitoring of disease activity even more difficult.

Figure 5. IL-6 signaling in CD4+ T-cells. In A, we show serum levels of IL-6 (Chapter 7), soluble IL-6 receptor (sIL-6R) and soluble gp130 (sgp130) in treatment naive GCA and healthy control (HC). Levels of sIL-6R and sgp130 were assessed by ELISA. Next, we show the percentage of membrane-bound IL-6R (B) and pSTAT3 (C) positive immune cells in 3 HCs after IL-6 stimulation (0, 50 and 100 ng for 30 minutes). Data are expressed as median and range. D: Percentages of induced pSTAT3 in naive CD4+ T cells from healthy controls (HC, n=3) and infection controls (INF, n=3) following stimulation with IL-6.
inflammation markers [80]. It is currently unknown whether treatment should be intensified in patients who are without symptoms or complaints, but who have subclinical vascular inflammation.

PART III: FUTURE PERSPECTIVES IN THE FIELD OF GCA AND PMR

In this final section, two potential future directions for GCA and PMR research are proposed.

Does cellular senescence underlie the age-dependence in GCA and PMR?

As GCA and PMR occur exclusively in the elderly, the aging of the immune system and the target tissues have been placed in the center of the pathogenesis of these diseases [81]. The aging of an individual is paralleled by aging at the cellular level [81, 82]. An essential process in aging is cellular senescence. Cellular senescence is a cell fate involving extensive changes to the functioning of the cell, including proliferative arrest [83]. Indeed, numbers of senescent cells increase with aging and these cells can be found at pathologic sites in chronic diseases.

The process of acquiring a senescent state is complex. There are different molecular pathways involved, depending on the cause of senescence. Senescence can be initiated by DNA damage (telomere shortening, radiation exposure, release of mitochondrial DNA after cell damage) and amplified by pro-inflammatory cytokines [84]. These processes trigger DNA damage sensors, that initiate the DNA damage response involving transcription factors that are responsible for the senescent phenotype [83-85]. Importantly, although senescent cells do not proliferate, they are not innocent bystanders. Senescent cells release pro-inflammatory cytokines, chemokines and tissue destructive proteins [82]. This senescent associated secretory phenotype (SASP) is able to induce senescence of the surrounding healthy cells.

Although cellular senescence has not yet been assessed in affected tissues of GCA (and PMR) patients, literature suggests its probable salient role in GCA pathology. A number of microRNAs that can be induced by cellular senescence have been found highly expressed in GCA TABs [86]. In Chapter 3, we implicated aged monocytes in disease pathogenesis. These non-classical monocytes increase with age, have short telomeres, have a clear inflammatory phenotype and show signs of senescence [87, 88]. Additionally, the cytosolic DNA damage sensor absent in melanoma (AIM)2 was recently suggested to be upregulated in GCA TABs [89]. DNA damage sensing by AIM2 may lead to senescence-like features of vascular smooth muscle cells and/or endothelial cells [90]. This is in congruence with the elevated expression of AIM2 observed in classical monocytes of GCA and PMR patients in Chapter 4.

Considering that senescent cells secrete potentially damaging proinflammatory mediators, and additionally promote development of senescence in their immediate environment, removal or ablation of senescent cells is a therapeutically interesting concept. The field of senolytics aims to develop new drugs targeting senescent cells. Senolytics target the apoptosis resistance of senescent cells, thereby promoting cell death [91]. Senolytics may represent an interesting therapeutic angle in GCA, as targets for treatment are highly awaited, but in order to do this, a thorough characterization of the prevalence of senescent cells in tissue is necessary.

Can the IL-6-mediated signaling cascade serve as a prognostic biomarker for predicting response to tocilizumab in GCA patients?

IL-6 signaling is an important treatment target in GCA, and one of the important cytokines in Th17 skewing [71]. Binding of IL-6 to the membrane-bound IL-6 receptor leads to dimerization of gp130, the common signal transducing subunit for the IL-6 family of cytokines [92]. Downstream signaling induces transcription of a STAT3-associated gene signature. Previously, IL-6-mediated STAT3 signaling in CD4 T cells was identified as a candidate biomarker of seronegative rheumatoid arthritis [93]. The trial with tocilizumab finally made substantial changes to the treatment regimen of GCA patients [71]. However, it still needs to be elucidated which patients benefit most of this new treatment, as 50% of GCA patients still fail on tocilizumab. There is currently no means to predict the response to tocilizumab.

Assessing STAT3 phosphorylation (pSTAT3) in CD4+ T-cells may be used as a reflection of exposure to IL-6 signaling. In Chapter 7, we showed significantly elevated levels of IL-6 in patient serum at the group level. However, the excess of soluble IL-6 receptor (and soluble gp130, sgp130), precludes to assess the extent of IL-6 stimulation at the individual patient’s level (Figure 5). Therefore, we analyzed intracellular expression of pSTAT3 in CD4+ T-cells as a more direct marker of prior IL-6 stimulation in vivo. Interestingly, our data shows high expression of IL-6 receptor on both naïve CD4+ T-cells and memory CD4+ T-cells, as opposed to CD8 T-cells, B-cell and NK-cells (Figure 5). Naïve CD4+ T-cells were consequently the most sensitive cells to detect IL-6-induced pSTAT3. Chronic in vivo exposure to inflammation (e.g. IL-6) may lead to exhaustion of the IL-6 signaling pathway and reduced induction of pSTAT3 [94]. We confirmed that in vivo exposure to inflammatory cytokines as seen in infection controls reduces IL-6-induced pSTAT3 responses in naïve CD4+ T cells from peripheral blood. Continuous exposure to IL-6 in GCA and PMR is likely associated with a T-cell STAT3 signature. Evaluation of this pathway in patients may be an important first step to help recognition of GCA patients who will benefit from tocilizumab therapy.

OVERALL CONCLUSION

There is still much to gain in the clinical management of GCA and PMR patients. GCs remain the cornerstone for treating these diseases, despite their lack of efficacy in a large subset of patients and their side effects. A better understanding of interactions between innate immune cells, adaptive immune cells and tissue resident cells is needed to pinpoint relevant targets and develop new treatment regimens. With this thesis, we extended our knowledge on heterogeneity of monocytes and macrophages, as monocyte/macrophage subsets have distinct pathogenic functions in GCA and PMR pathology.

Despite the different disease subsets in GCA and PMR patients, there are no validated means for personalized treatment. To allow for stratification of different treatment strategies, GCA and PMR patients need to be clinically, immunologically and biochemically characterized at diagnosis. Given the central role of monocytes and macrophages in disease pathology, we aimed to use their
products in serum as candidate biomarkers to improve patient characterization. This thesis indicates that serum markers such as VEGF, YKL-40 and angiopoietin-2 may be clinically relevant markers for GCA and/or PMR patients and can aid in patient stratification at diagnosis. Future studies will need to validate if these markers are indeed useful for designing a personalized treatment regimen.

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