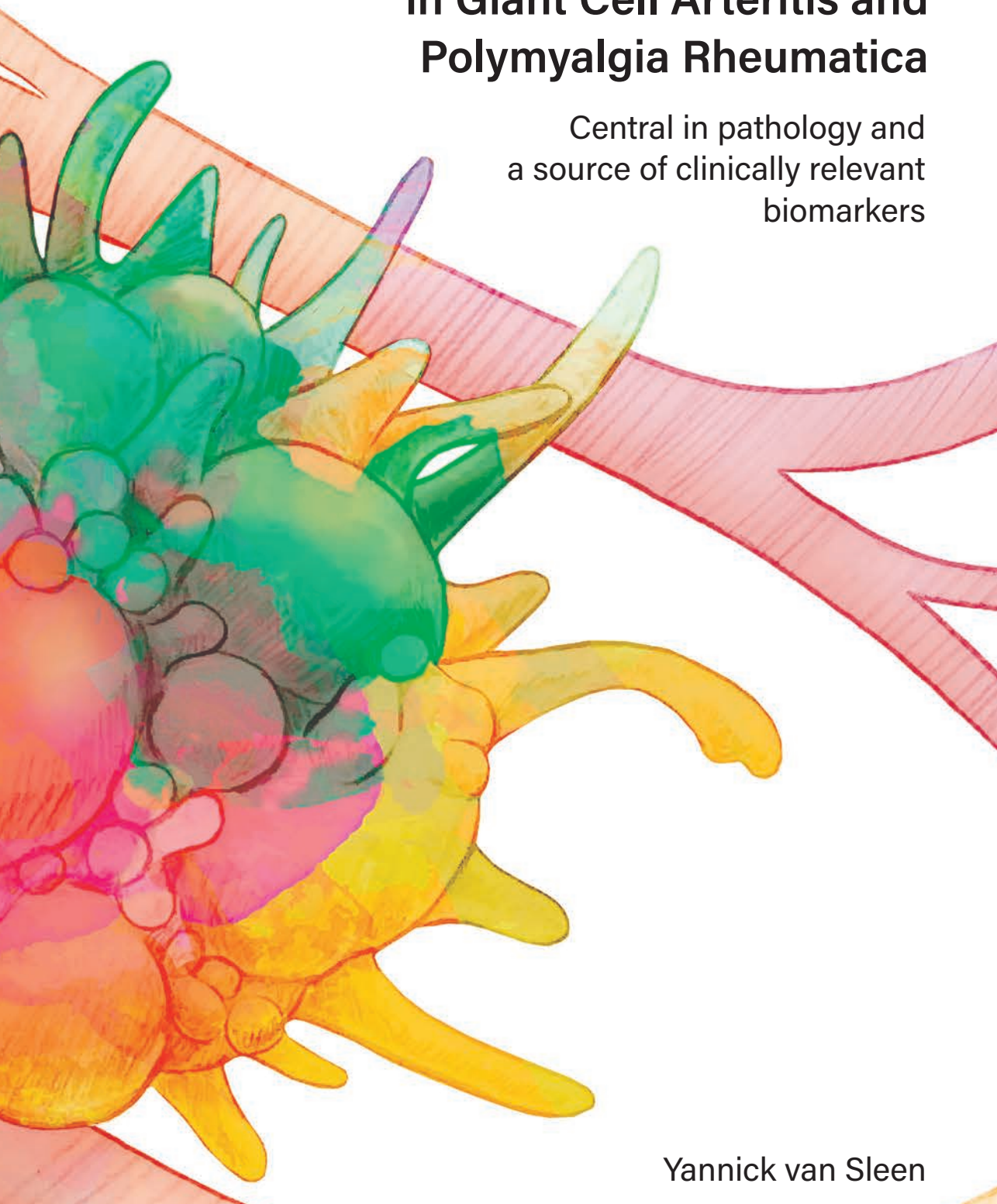
Monocyte and macrophage heterogenity in Giant Cell Arteritis and Polymyalgia Rheumatica

Central in pathology and a source of clinically relevant biomarkers



Yannick van Sleen

MONOCYTE AND MACROPHAGE HETEROGENEITY IN GIANT CELL ARTERITIS AND POLYMYALGIA RHEUMATICA

Central in Pathology and a Source of Clinically Relevant Biomarkers

Yannick van Sleen

Printing of this thesis was kindly supported by:

The research presented in this thesis was supported by Reuma Nederland and de Stichting De Cock –Hadders

978-94-034-2415-6 (printed version)
978-94-034-2414-9 (e-book)

Cover: Sieben Medical Art
Layout & printing: Off Page, Amsterdam

rijksuniversiteit
groningen

Monocyte and Macrophage Heterogeneity in Giant Cell Arteritis and Polymyalgia Rheumatica

Central in Pathology and
a Source of Clinically Relevant Biomarkers

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. C. Wijmenga
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 26 februari 2020 om 14.30 uur

door

Yannick van Sleen

geboren op 9 augustus 1990
te Smilde

Promotor

Prof. dr. A.M.H. Boots

Copromotores

Dr. E. Brouwer
Dr. W.H. Abdulahad
Dr. K.S.M. van der Geest

Beoordelingscommissie

Prof. dr. C.A.H.H. Daemen
Prof. dr. E.M. Hauge
Prof. dr. J.M. Van Laar

TABLE OF CONTENTS

ONE

Thesis introduction

GIANT CELL ARTERITIS AND POLYMYALGIA RHEUMATICA

Giant cell arteritis (GCA) is an inflammatory disease that occurs exclusively in older people. It was first described more than 100 years ago, but it took until the 1940s for it to be recognized as a specific disease [1]. Patients with GCA suffer from inflammation of their medium- and large-sized arteries. The size of the affected vessels distinguishes this disease from other types of vasculitides, such as granulomatosis with polyangiitis (GPA) and microscopic polyangiitis [2]. Like GCA, Takayasu arteritis also affects the large arteries, but this disease only affects younger people and is limited to the aorta and its branches [3].

Typically, clinicians have viewed GCA as a 'headache disease', even though GCA commonly leads to systemic symptoms as well, depending on which arteries are affected [4]. Cranial symptoms of GCA (C-GCA) include temporal headache, jaw or tongue claudication and scalp tenderness. The most feared complication of C-GCA is vision loss, which can be transient or permanent. Fortunately, fast track clinics and early initiation of treatment have led to a substantial decrease in visual complications [5]. Symptoms of large-vessel GCA (LV-GCA) are more difficult to identify, as they are not disease-specific [6]. These symptoms include night sweats, malaise, weight loss, fever and arm claudication. However, patients with LV-GCA are at risk of aortic complications such as development of aneurysms, dissections and stenosis [7]. A majority of GCA patients present with overlapping cranial and systemic symptoms (**Figure 1**).

In the 1960s, it became accepted that GCA can be found associated with another inflammatory disease, polymyalgia rheumatica (PMR, **Figure 1**) [1]. At the time the disease was also called polymyalgia arteriitica. PMR is characterized by (peri)articular inflammation, occurring mostly in the shoulders and hip girdle. PMR patients suffer from morning stiffness and pain in these regions [8]. The incidence of GCA among PMR patients is reported to be between 16 and 21%, while PMR is observed in approximately half of the GCA patients. This is due to the higher incidence of PMR compared to GCA: 41-113 cases per 100.000 people aged ≥50 years for PMR and 18-29 for GCA (among populations of Northern European ancestry) [4]. Epidemiology of GCA and PMR is similar as well, as both diseases occur more frequently in women (two-fold higher than in men) and peak at a similar median age (72 years in the GCA/PMR cohort at the UMCG).

A suspicion of GCA or PMR is primarily based on clinical signs and symptoms in combination with acute-phase markers in the blood. The golden standard for diagnosis of GCA remains a temporal artery biopsy (TAB), which is particularly useful in the diagnosis of C-GCA. However, diagnosis of C-GCA based on TAB may be missed, as GCA is typically 'patchy' leaving parts of the arteries unaffected. Indeed, in temporal arteries, massively infiltrated parts of arteries are intermixed with uninvolved regions (known as skip lesions) [9]. Therefore, other diagnostic tools are needed, including ultrasound and 18F-fluorodeoxyglucose positron emission tomography- computed tomography (FDG-PET-CT). These techniques can detect LV-GCA without cranial involvement. Imaging is also useful in diagnosing synovial and bursal inflammation in PMR. Both GCA and PMR are characterized by a strong acute-phase response, which is reflected by an elevated C-reactive protein (CRP) and/or erythrocyte sedimentation rate (ESR) in the vast majority of the patients [10].

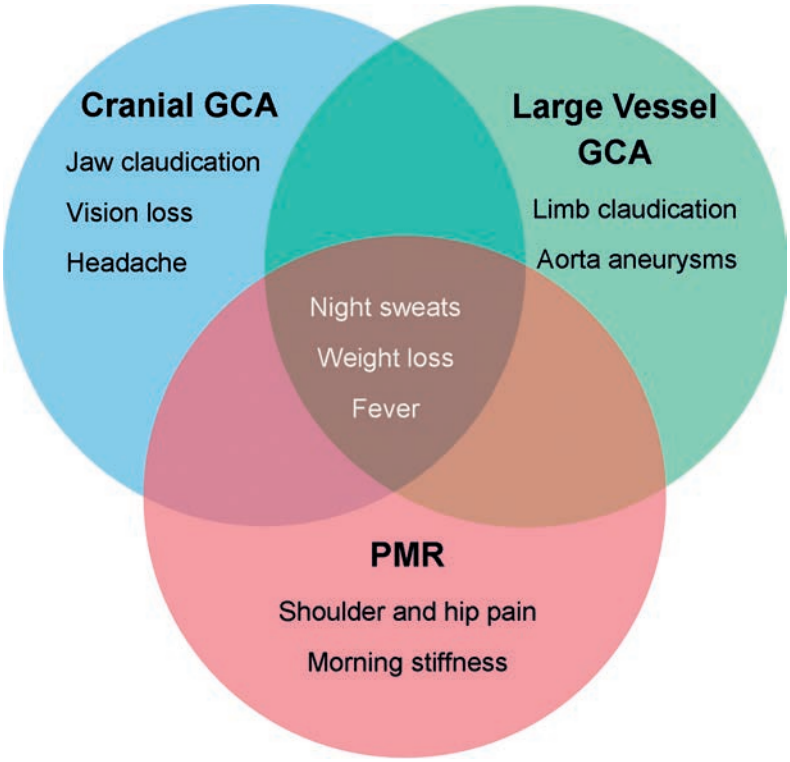


Figure 1. GCA symptoms depend on which vessels are affected. Diagnosis of overlapping PMR may occur at the same moment as the diagnosis of GCA, but can also precede or follow GCA. C-GCA, LV-GCA and PMR can all be associated with systemic symptoms such as night sweats, weight loss and fever due to systemic inflammation (e.g. elevated inflammatory markers and IL-6 levels).

As of today, glucocorticoids (prednisolone), remain the main treatment option for GCA and PMR patients a situation that has not changed since the 1950s [1]. Glucocorticoids are efficient at relieving symptoms of GCA and PMR, especially those linked to the acute-phase response (fever, night sweats, malaise, weigh loss) [10, 11]. However, long-term glucocorticoid use is accompanied by severe side-effects, such as development of type II diabetes, hypertension, weight gain, increased infection risk and decreased bone density [12, 13]. Moreover, the majority of patients treated with glucocorticoids experience relapses [14]. To suppress these relapses, the glucocorticoid dose is increased, amplifying the glucocorticoid-associated morbidity. Even though the acute-phase response is strongly suppressed in glucocorticoid treated patients, recent evidence showed that tissue inflammation is not sufficiently controlled in GCA [15, 16]. For these patients, extended tissue inflammation may be dangerous, as it could lead to complications such as occlusion and development of aortic aneurysms [7]. Alternative treatment options have been introduced as well. Methotrexate, a conventional synthetic DMARD, has especially been used in relapsing patients, with varying results [17, 18]. More recently, tocilizumab, an interleukin (IL)-6 receptor blocker, has been shown to lower the relapse risk and reduce glucocorticoid use in GCA patients [19].

DISEASE PATHOLOGY AT THE INFLAMMATORY SITE

The pathology of GCA is not completely understood. It is generally thought that GCA starts in the adventitia (the outer vessel wall layer), where dendritic cells (DCs) are activated by stimulation of their pattern recognition receptors, including Toll-like receptors (TLRs) [20]. These receptors are capable of sensing pathogen- or danger-associated molecular patterns (PAMPs and DAMPs) [21]. This is important, as development of GCA is often seen after seasonal infections, albeit that the culprit(s) remains to be identified. Recent studies have demonstrated that the vessel wall is not sterile, but rather contains an extensive microbiome [22]. This microbiome in GCA TABs is distinct from healthy controls. In 2015, it was suggested that GCA was caused by varicella zoster infection, as it was detected in most TABs of GCA patients [23]. However, this hypothesis has now largely been debunked [24, 25] as modest associations with many infectious agent were found for GCA [26].

Most knowledge on the pathogenesis of GCA has been obtained from the TAB, which shows a granulomatous infiltrate consisting mostly of macrophages and CD4+ T-cells. This is in sharp contrast to non-inflamed arteries, which are comprised of smooth muscle cells, endothelium and some occasional DCs (**Figure 2**). Activation of DCs leads to migration of CD4+ T-cells, monocytes, and to some level CD8+ T-cells, B-cells and neutrophils to the vessel wall [20]. These cells produce

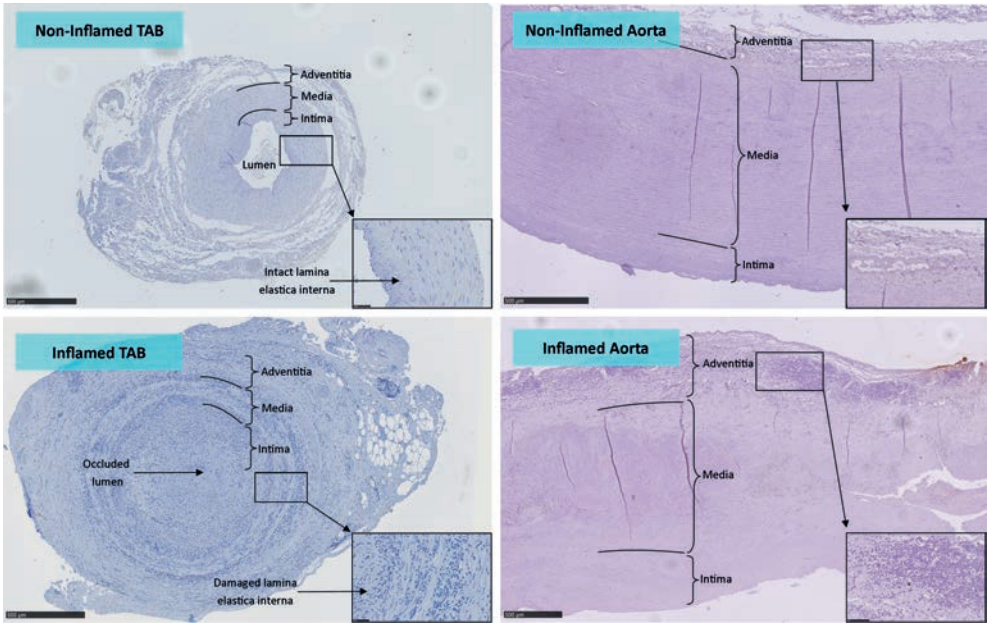


Figure 2. Vascular histology in healthy vessels and GCA vessels. Shown here are healthy (age-matched) and GCA-affected vessels stained with hematoxylin. Compared to non-inflamed TAB, the inflamed TAB is characterized by occlusion of the lumen, expansion of the intima layer and a large infiltrate of leukocytes within all three layers. The GCA-affected aorta is also characterized by infiltrating leukocytes, but luminal occlusion does not occur. In addition, this tissue is characterized by massive accumulation of leukocytes in the adventitia.

chemokines and cytokines, including the key cytokine IL-6, that further fuel the infiltration and inflammation in the vessel wall [27].

Besides inflammation, key processes in GCA pathogenesis are tissue destruction and remodeling. Characteristic is the damaged lamina elastica interna, which forms the border between the intima and the media. Macrophages produce proteins such as matrix metalloproteinases that are able to break down the lamina elastica interna, thereby facilitating the infiltration of T-cells, myofibroblasts and monocytes into the intima [28]. Infiltration and proliferation in the intimal layer ultimately leads to occlusion of mostly the cranial arteries, a process that is responsible for ischemic symptoms including vision loss [27]. Vessel occlusion does not occur in inflamed aortas (LV-GCA); rather, breakdown of extracellular matrixes and the smooth muscle layer in the media leads to loss of vessel integrity, causing aortic aneurysms, dissections and stenosis [29]. New small vessel formation (angiogenesis) occurs within the vessel wall to deliver nutrients and oxygen [30]. This process is important in fueling the ongoing inflammatory process.

Very little is known on PMR pathogenesis, as synovial biopsies are rarely performed in these patients, because these are not needed for the diagnosis. Studies from the early 2000s, however, showed, similar to GCA, an inflammatory infiltrate mostly consisting of monocytes/macrophages and CD4+ T-cells [31]. Also, elevated production of the pro-angiogenic mediator vascular endothelial growth factor (VEGF) was described in the inflamed tissue and in the blood, hinting at new small vessel formation in the inflamed shoulders and hips [32].

IMPORTANT CELLULAR PLAYERS IN GCA AND PMR PATHOGENESIS

The pathogenesis of GCA and PMR is thus characterized by a granulomatous infiltrate of both innate (monocytes/macrophages/DCs) and adaptive immune cells at the tissue level. Remarkably, GCA and PMR show overlapping profiles in the blood regarding immune cells and inflammatory markers. Monocytes comprise approximately 2-10% of the circulating leukocytes. They are derived from the bone-marrow and are characterized by their ability to present antigens, phagocytize and produce cytokines. Monocytes display heterogeneity, as there are three monocyte subsets, based on expression of surface markers CD14 and CD16. The most common subset are the classical monocytes ($CD14^{bright}CD16^{neg}$), while the intermediate ($CD14^{bright}CD16^{+}$) and non-classical ($CD14^{dim}CD16^{+}$) monocytes both comprise less than 10% of the total monocyte population [33]. The fraction of $CD16^{+}$ monocytes is shown to increase with aging [34]. Moreover, an expansion or a decrease of a certain subset of monocytes has been observed and was linked to pathogenesis in various disorders including autoimmune diseases [35, 36]. Monocytes respond to chemokine gradients (such as CCL2 and CX3CL1) and can leave the blood stream to enter the tissue [37].

In tissue, monocytes undergo differentiation depending on the local environment to become macrophages or DCs [38]. Both macrophages and DCs have the capability to present antigens, but macrophages are specialized in phagocytosis and the production of tissue destructive and pro-angiogenic proteins [39]. Previously, these inflammatory, tissue destructive and angiogenic proteins have been studied as potential biomarkers in numerous inflammatory diseases.

The adaptive immune cells are comprised of CD4+ and CD8+ T-cells, and B-cells, which provide highly effective immunity against pathogens and dysfunctional cells. These cells have all been found

in TABs of GCA patients, but CD4+ T-cells are the most prominent [40, 41]. CD4+ T-cells have numerous functions, such as stimulation or inhibition of other cells by cytokines and via cell-cell contact, and the promotion of B-cell maturation. Naive T-cells ($CD45RA^+$) can recognize their cognate antigens presented by professional antigen presenting cells. If properly co-stimulated, T-cells become activated and start to proliferate. The naive T-cells become effector and memory T-cells ($CD45RO^+$) which can respond rapidly to reinfection. Importantly, the differentiation to specialized CD4+ effector T cells depends on the cytokine environment. Cytokine skewed differentiation of CD4+ T-cells leads to a specialized immune response against intracellular pathogens (Th1), extracellular parasites (Th2) and extracellular bacteria and fungi (Th17). T-cells activated in the presence of IL-12 and IL-18 develop into IFNγ producing Th1 cells, IL-4 skews T-cells to an IL-4 and IL-13 producing Th2 phenotype, and TGFβ, IL-6 and IL-23 lead to Th17 development, characterized by IL-17 production [42]. In addition, regulatory T-cells (Tregs) are induced in the presence of TGFβ and IL-10. These cells are required to maintain self-tolerance [42]. Dysfunctional regulation of Th-skewing is thought to be essential in the development of autoimmune diseases [42, 43]. Importantly, GCA and PMR patients were reported to have altered CD4+ subset frequencies (changes in Th1 and Th17) in the blood compared to healthy controls [44-47]. Moreover, inflamed TABs of GCA patients also display a mixed population of Th1 and Th17 cells, but almost no Th2 cells or Tregs [27]. CD8+ T-cells are also present in TABs, where they produce cytotoxic molecules such as TiA1 and granzyme B [41].

Genetics may play a role in GCA and PMR pathology, especially as the frequency of HLA-DRB1*04 is higher in these patients [48]. Moreover, the incidence of GCA and PMR varies substantially depending on genetic background, with the highest incidence in Scandinavia and in populations of Scandinavian descent [4].

THE GPS COHORT

In 2010, the prospective follow-up cohort study of newly diagnosed GCA and PMR patients was initiated at the University Medical Center Groningen. In parallel, an age-stratified cohort of healthy young and elderly volunteers was started, the SENEX cohort. Collectively, these cohorts are referred to as the GCA-PMR-SENEX (GPS) cohort. The GCA and PMR patients were asked to participate before start of treatment and requested to visit again at fixed time points thereafter. Next to clinical and standard laboratory data, biological samples were prospectively collected in the form of serum, plasma and cells. The thorough follow-up regimen in this cohort allows to record relapses, time to relapse and the duration of therapy. So far, this unique GPS cohort includes 50 GCA patients and 38 PMR patients who were followed since baseline and who were treatment-naive at inclusion. In addition, 116 healthy participants have visited at least once to participate in the SENEX cohort.

UNANSWERED QUESTIONS AND MEDICAL NEEDS IN GCA AND PMR

As the world's population is aging, the prevalence of GCA and PMR is expected to increase substantially [49]. At this moment, PMR is already the second most common rheumatic disease with a lifetime risk of 2.4% for woman and 1.7% for men [50]. The morbidity of the diseases, and their treatment, pose a substantial burden on the health care system and the society as a whole [51].

Therefore, it is essential to increase knowledge about how and why these diseases happen as they do, to potentially discover new targets for treatment. In addition, it is yet unclear what the effects are of glucocorticoids at the tissue level, and whether long-term treated patients are still at risk for developing sudden ischemic symptoms or aortic complications.

Currently, all patients with GCA, and all patients with PMR, start with the same treatment strategy, regardless of the diverse clinical symptoms, serological and immunological features displayed by patients suffering from these diseases. Accumulating evidence indicates that GCA is a heterogeneous disease. The extent of the local and systemic inflammatory response may differ among patients [52]. Immunologic heterogeneity in GCA is further suggested by outcomes of recent trials with an anti–IL-6 receptor antibody, because this targeted treatment is not effective in all GCA patients [53]. Recognition of distinct disease subsets is important, because it may eventually help to implement precision medicine for GCA and PMR. Ideally, biomarkers should be implemented in the daily clinical practice predicting whether a GCA/PMR patient is easy or difficult to treat with glucocorticoids, or rather a more targeted treatment should be considered (personalized medicine).

Another clinical demand is a simple biomarker that can detect whether a patient with PMR also has overlapping GCA. Many of the GCA symptoms are not disease-specific and are therefore easily overlooked in patients with PMR [54, 55]. Moreover, PMR is often treated by the general practitioner, who has little to no means for excluding GCA. An easy to use serum marker test that can identify PMR patients who are at risk for having overlapping GCA is highly needed. These at-risk patients should subsequently be more thoroughly screened for vascular inflammation, for instance by imaging tools.

Finally, as the diseases occur exclusively in the elderly, GCA and PMR can be considered as ideal model diseases to study the aging immune system and aging vessels.

AIMS AND OUTLINE OF THE THESIS

So far, no comprehensive analysis has been performed on the immune composition in peripheral blood of treatment-naive patients compared with age-matched controls, nor the changes induced after treatment. Therefore, this thesis starts with an overview of all major immunological players in the blood of GCA and PMR patients. It continues by focusing on the most abundantly present cells at the inflammatory site and the interaction between these cells: T-cells, macrophages and their precursors, monocytes. The aim of these chapters is to increase knowledge on key pathogenic processes that underlie these diseases and the processes that mediate their chronic and persistent nature. Finally, this thesis aims to identify important products produced by macrophages in the inflamed tissues, and to study whether these products are clinically relevant in GCA and PMR.

Chapter 2 aims to evaluate whether blood leukocyte counts can be used as cellular markers of inflammation. The counts of the six major leukocyte subsets in the blood of GCA and PMR patients before, during and after glucocorticoid treatment will be documented. We ask whether patients have an altered leukocyte composition at baseline and we explore the fluctuations in peripheral blood cell counts caused by glucocorticoid treatment. It is yet unknown whether patients in treatment-free remission are truly cured and if leukocyte subsets counts and inflammatory markers return to normal levels.

As monocytes are important in the immunopathology of GCA, we aim to assess the distribution of monocyte subsets in the blood of baseline GCA and PMR patients in **Chapter 3**. To study whether subsets of monocytes preferentially migrate to the inflamed tissues in GCA, we focus on the importance of chemokines and their receptors in monocyte migration.

Chapter 4 studies the interaction between monocytes and CD4+ T-cells in GCA and PMR patients. We investigate whether Th-skewing in patients is linked to expansion of a specific monocyte subset. We also enumerate other circulating antigen presenting cells in GCA and PMR patients: myeloid DCs and plasmacytoid DCs. In addition, we assess the expression of pattern recognition receptors on monocyte and DC subsets, as activation mediated by these receptors is important in Th-skewing and thought to initiate GCA and PMR pathology.

Chapter 5 provides a large-scale phenotype analysis of macrophages in TABs and aortas of GCA patients. The aim of this chapter is to identify different spatial heterogeneity of macrophages in the tissue, employing both TABs from C-GCA and aortas from LV-GCA. Macrophages are known to display considerable heterogeneity in response to cues from the environment. To address this, in vitro experiments using monocyte-derived macrophages from GCA patients are employed to reveal the mechanism behind these distinct macrophage phenotypes and functions.

Chapter 6 focuses on YKL-40, a well-known marker of inflammation and tissue remodeling. We aim to determine the cellular source of YKL-40 in GCA tissues, and to reveal whether YKL-40 can instigate angiogenesis in GCA. We first assess whether YKL-40 co-localizes with a distinct macrophage subset in specific regions of the inflamed TAB. Next, we determine whether YKL-40 is a candidate marker of vascular inflammation in the aorta. Finally, we aim to confirm that YKL-40 promotes angiogenesis, and assess expression of IL-13Rα2, the YKL-40 receptor, in inflamed vessels.

Chapter 7 and **Chapter 8** study whether markers produced by monocytes and macrophages can be used as biomarkers in GCA (chapter 7) or PMR (chapter 8). These products, measured in serum of treatment-naive GCA and PMR patients, are thereafter linked to data on treatment response. In both diseases, we assess if serum markers can predict whether a patient is easy or difficult to treat. In GCA patients, we aim to identify candidate markers that reflect vascular inflammation. For PMR patients, we aim for a diagnostic serum marker of concomitant GCA.

Finally, in **Chapter 9**, the findings in this thesis are summarized and discussed within a wider context.

REFERENCES

1. Hunder Gene G GG. 2006-8. The early history of giant cell arteritis and polymyalgia rheumatica: first descriptions to 1970. *Mayo Clin. Proc. 81: 1071-83.*
2. Jennette, J.C. 2013. Overview of the 2012 revised International Chapel Hill Consensus Conference nomenclature of vasculitides. *J Clin Exp Nephrol 17: 603-606.*
3. Kermani Tanaz A TA. 2019-1. Takayasu arteritis and giant cell arteritis: are they a spectrum of the same disease? *Int J Rheum Dis 22: 41-48.*
4. Dejaco, C., Brouwer, E., Mason, J.C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2017. Giant cell arteritis and polymyalgia rheumatica: current challenges and opportunities. *Nat. Rev. Rheumatol. 13(10):578-592.*
5. Singh, A.G., Kermani, T.A., Crowson, C.S., Weyand, C.M., Matteson, E.L., and Warrington, K.J. 2015. Visual manifestations in giant cell arteritis: trend over 5 decades in a population-based cohort. *J. Rheumatol. 42: 309-315.*
6. Koster, M.J., Matteson, E.L., and Warrington, K.J. 2018. Large-vessel giant cell arteritis: diagnosis, monitoring and management. *Rheumatology (Oxford) 57: ii32-ii42.*
7. Nuenninghoff, D.M., Hunder, G.G., Christianson, T.J., McClelland, R.L., and Matteson, E.L. 2003. Incidence and predictors of large-artery complication (aortic aneurysm, aortic dissection, and/or large-artery stenosis) in patients with giant cell arteritis: a population-based study over 50 years. *Arthritis Rheumatol. 48: 3522-3531.*
8. Buttgereit, F., Dejaco, C., Matteson, E.L., and Dasgupta, B. 2016. Polymyalgia Rheumatica and Giant Cell Arteritis: A Systematic Review. *JAMA 315: 2442-2458.*
9. Poller, D.N., van Wyk, Q., and Jeffrey, M.J. 2000. The importance of skip lesions in temporal arteritis. *J. Clin. Pathol. 53: 137-139.*
10. Kermani, T.A., Schmidt, J., Crowson, C.S., Ytterberg, S.R., Hunder, G.G., Matteson, E.L., and Warrington, K.J. 2012. Utility of erythrocyte sedimentation rate and C-reactive protein for the diagnosis of giant cell arteritis. *Semin. Arthritis Rheum. 41: 866-871.*
11. Salvarani C. 2005. Acute-phase reactants and the risk of relapse/recurrence in polymyalgia rheumatica: a prospective followup study. *Arthritis Rheumatol. 53: 33-8.*
12. Buttgereit F., Matteson E.L., Dejaco C., Dasgupta B. 2018. Prevention of glucocorticoid morbidity in giant cell arteritis. *Rheumatology (Oxford) 57(suppl_2): ii11-ii21.*
13. Broder, M.S., Sarsour, K., Chang, E., Collinson, N., Tuckwell, K., Napalkov, P., and Klearman, M. 2016. Corticosteroid-related adverse events in patients with giant cell arteritis: A claims-based analysis. *Semin. Arthritis Rheum. 46: 246-252.*
14. Restuccia, G., Boiardi, L., Cavazza, A., Catanoso, M., Macchioni, P., Muratore, F., Cimino, L., Aldigeri, R., Crescentini, F., Pipitone, N., and Salvarani, C. 2016. Flares in Biopsy-Proven Giant Cell Arteritis in Northern Italy: Characteristics and Predictors in a Long-Term Follow-Up Study. *Medicine (Baltimore) 95: e3524.*
15. Maleszewski, J.J., Younge, B.R., Fritzlen, J.T., Hunder, G.G., Goronzy, J.J., Warrington, K.J., and Weyand, C.M. 2017. Clinical and pathological evolution of giant cell arteritis: a prospective study of follow-up temporal artery biopsies in 40 treated patients. *Mod. Pathol. 30(6):788-796.*
16. Schmidt, W.A. 2018. Ultrasound in the diagnosis and management of giant cell arteritis. *Rheumatology (Oxford) 57: ii22-ii31.*
17. Jover, J.A., Hernández-García, C., Morado, I.C., Vargas, E., Bañares, A., and Fernández-Gutiérrez, B. 2001. Combined treatment of giant-cell arteritis with methotrexate and prednisone: a randomized, double-blind, placebo-controlled trial. *Ann. Intern. Med. 134: 106-114.*
18. Hoffman, G.S., Cid, M.C., Hellmann, D.B., Guillevin, L., Stone, J.H., Schousboe, J., Cohen, P., Calabrese, L.H., Dickler, H., and Merkel, P.A. 2002. A multicenter, randomized, double-blind, placebo-controlled trial of adjuvant methotrexate treatment for giant cell arteritis. *Arthritis Rheumatol. 46: 1309-1318.*
19. Stone, J.H., Tuckwell, K., Dimonaco, S., Klearman, M., Aringer, M., Blockmans, D.,

Brouwer, E., Cid, M.C., Dasgupta, B., Rech, J., Salvarani, C., Schett, G., Schulze-Koops, H., Spiera, R., Unizony, S.H., and Collinson, N. 2017. Trial of Tocilizumab in Giant-Cell Arteritis. *N. Engl. J. Med. 377: 317-328.*

20. Samson, M., Corbera-Bellalta, M., Audia, S., Planas-Rigol, E., Martin, L., Cid, M.C., and Bonnotte, B. 2017. Recent advances in our understanding of giant cell arteritis pathogenesis. *Autoimmun. Rev. 16: 833-844.*

21. O'neill, L. and Molloy, E.S. 2016. The role of toll like receptors in giant cell arteritis. *Rheumatology (Oxford) 55: 1921-1931.*

22. Hoffman, G.S., Getz, T.M., Padmanabhan, R., Villa-Forte, A., Clifford, A.H., Funchain, P., Sankunny, M., Perry, J.D., Blandford, A., and Kosmorsky, G. 2019. The Microbiome of Temporal Arteries. *Pathogens & immunity 4: 21.*

23. Gilden, D., White, T., Khmeleva, N., Heintzman, A., Choe, A., Boyer, P.J., Grose, C., Carpenter, J.E., Rempel, A., Bos, N., Kandasamy, B., Lear-Kaul, K., Holmes, D.B., Bennett, J.L., Cohrs, R.J., Mahalingam, R., Mandava, N., Eberhart, C.G., Bockelman, B., Poppiti, R.J., Tamhankar, M.A., Fogt, F., Amato, M., Wood, E., Durairaj, V., Rasmussen, S., Petursdottir, V., Pollak, L., Mendlovic, S., Chatelain, D., Keyvani, K., Brueck, W., and Nagel, M.A. 2015. Prevalence and distribution of VZV in temporal arteries of patients with giant cell arteritis. *Neurology 84: 1948-1955.*

24. Bhatt, A.S., Manzo, V.E., Pedamallu, C.S., Duke, F., Cai, D., Bienfang, D.C., Padera, R.F., Meyerson, M., and Docken, W.P. 2014. Brief Report: In Search of a Candidate Pathogen for Giant Cell Arteritis: Sequencing-Based Characterization of the Giant Cell Arteritis Microbiome. *Arthritis Rheumatol. 66: 1939-1944.*

25. Rondaan, C., van der Geest, Kornelis SM, Eelsing, E., Boots, A.M., Bos, N.A., Westra, J., and Brouwer, E. 2017. Decreased immunity to Varicella Zoster Virus in giant cell arteritis. *Front Immunol. 8: 1377.*

26. Rhee, R.L., Grayson, P.C., Merkel, P.A., and Tomasson, G. 2017. Infections and the risk of incident giant cell arteritis: a population-based, case-control study. *Ann. Rheum. Dis. 76: 1031-1035.*

27. Watanabe R., Maeda T., zhang H., Berry G.J., Zeisbrich M., Brockett R., Greenstein A.E., Tian L., Goronzy J.J., Weyand C.M. 2018. Matrix Metalloprotease-9 (MMP-9)-Producing Monocytes Enable T Cells to Invade the Vessel Wall and Cause Vasculitis. *Circ. Res. 123(6):700-715.*

28. Gonzalez-Gay, M.A., Garcia-Porrua, C., Piñeiro, A., Pego-Reigosa, R., Llorca, J., and Hunder, G.G. 2004. Aortic aneurysm and dissection in patients with biopsy-proven giant cell arteritis from northwestern Spain: a population-based study. *Medicine 83: 335-341.*

29. Kaiser, M., Younge, B., Bjornsson, J., Goronzy, J.J., and Weyand, C.M. 1999. Formation of new vasa vasorum in vasculitis. Production of angiogenic cytokines by multinucleated giant cells. *Am. J. Pathol. 155: 765-774.*

30. Meliconi, R., Pulsatelli, L., Uguccioni, M., Salvarani, C., Macchioni, P., Melchiorri, C., Focherini, M.C., Frizziero, L., and Facchini, A. 1996. Leukocyte infiltration in synovial tissue from the shoulder of patients with polymyalgia rheumatica. Quantitative analysis and influence of corticosteroid treatment. *Arthritis Rheumatol. 39: 1199-1207.*

31. Meliconi, R., Pulsatelli, L., Dolzani, P., Boiardi, L., Macchioni, P., Salvarani, C., Silvestri, T., Frizziero, L., and Facchini, A. 2000. Vascular endothelial growth factor production in polymyalgia rheumatica. *Arthritis Rheumatol. 43: 2472-2480.*

32. Ziegler-Heitbrock, L. 2015. Blood Monocytes and Their Subsets: Established Features and Open Questions. *Front. Immunol. 6: 423.*

33. Rogacev, K.S., Zawada, A.M., Hundsdorfer, J., Achenbach, M., Held, G., Fliser, D., and Heine, G.H. 2015. Immunosuppression and monocyte subsets. *Nephrol. Dial. Transplant. 30: 143-153.*

34. Mukherjee, R., Kanti Barman, P., Kumar Thatoi, P., Tripathy, R., Kumar Das, B., and Ravindran, B. 2015. Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous. *Sci. Rep. 5: 13886.*

35. Rossol, M., Kraus, S., Pierer, M., Baerwald, C., and Wagner, U. 2012. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheum. 64: 671-677.*

36. Liaskou, E., Zimmermann, H.W., Li, K.K., Oo, Y.H., Suresh, S., Stamataki, Z., Qureshi, O., Lalor, P.F., Shaw, J., Syn, W.K., Curbishley, S.M., and Adams,

D.H. 2013. Monocyte subsets in human liver disease show distinct phenotypic and functional characteristics. *Hepatology 57: 385-398*.

37. Boyette, L.B., Macedo, C., Hadi, K., Elinoff, B.D., Walters, J.T., Ramaswami, B., Chalasani, G., Taboas, J.M., Lakkis, F.G., and Metes, D.M. 2017. Phenotype, function, and differentiation potential of human monocyte subsets. *PLoS One 12: e0176460*.

38. Laskin, D.L., Sunil, V.R., Gardner, C.R., and Laskin, J.D. 2011. Macrophages and tissue injury: agents of defense or destruction? *Annu. Rev. Pharmacol. Toxicol. 51: 267-288*.

39. van der Geest, K.S., Abdulahad, W.H., Chalan, P., Rutgers, A., Horst, G., Huitema, M.G., Roffel, M.P., Roozendaal, C., Kluin, P.M., Bos, N.A., Boots, A.M., and Brouwer, E. 2014. Disturbed B cell homeostasis in newly diagnosed giant cell arteritis and polymyalgia rheumatica. *Arthritis Rheumatol. 66: 1927-1938*.

40. Samson, M., Ly, K.H., Tournier, B., Janikashvili, N., Trad, M., Ciudad, M., Gautheron, A., Devilliers, H., Quipourt, V., and Maurier, F. 2016. Involvement and prognosis value of CD8 T cells in giant cell arteritis. *J. Autoimmun. 72: 73-83*.

41. Geginat, J., Paroni, M., Maglie, S., Alfen, J.S., Kastirr, I., Gruarin, P., De Simone, M., Pagani, M., and Abrignani, S. 2014. Plasticity of human CD4 T cell subsets. *Front Immunol. 5: 630*.

42. Rossol, M., Kraus, S., Pierer, M., Baerwald, C., and Wagner, U. 2012. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheumatol. 64: 671-677*.

43. Samson, M., Audia, S., Fraszczak, J., Trad, M., Ornetti, P., Lakomy, D., Ciudad, M., Leguy, V., Berthier, S., and Vinit, J. 2012. Th1 and Th17 lymphocytes expressing CD161 are implicated in giant cell arteritis and polymyalgia rheumatica pathogenesis. *Arthritis Rheumatol. 64: 3788-3798*.

44. Deng, J., Younge, B.R., Olshen, R.A., Goronzy, J.J., and Weyand, C.M. 2010. Th17 and Th1 T-cell responses in giant cell arteritis. *Circulation 121: 906-915*.

45. Terrier, B., Geri, G., Chaara, W., Allenbach, Y., Rosenzwajg, M., Costedoat-Chalumeau, N., Fouret, P., Musset, L., Benveniste, O., and Six, A. 2012. Interleukin-21 modulates Th1 and Th17 responses in giant cell arteritis. *Arthritis Rheumatol. 64: 2001-2011*.

46. Saadoun, D., Garrido, M., Comarmond, C., Desbois, A., Domont, F., Savey, L., Terrier, B., Geri, G., Rosenzwajg, M., and Klatzmann, D. 2015. Th1 and Th17 cytokines drive inflammation in Takayasu arteritis. *Arthritis Rheumatol. 67: 1353-1360*.

47. Carmona, F.D., Mackie, S.L., Martín, J., Taylor, J.C., Vaglio, A., Eyre, S., Bossini-Castillo, L., Castañeda, S., Cid, M.C., and Hernández-Rodríguez, J. 2015. A large-scale genetic analysis reveals a strong contribution of the HLA class II region to giant cell arteritis susceptibility. Am J Hum Genet. *96: 565-580*.

48. De Smit, E., Palmer, A.J., and Hewitt, A.W. 2015. Projected worldwide disease burden from giant cell arteritis by 2050. *J. Rheumatol. 42: 119-125*.

49. Crowson Cynthia S CS. 2011-3. The lifetime risk of adult-onset rheumatoid arthritis and other inflammatory autoimmune rheumatic diseases. *Arthritis Care Res 63: 633-9*.

50. Mounié, M., Costa, N., Sailler, L., Lapeyre-Mestre, M., Bourrel, R., Savy, N., Molinier, L., and Pugnet, G. 2018. Incremental costs in giant cell arteritis. *Arthritis Care Res 70: 1074-1081*.

51. Salvarani, C., Cantini, F., and Hunder, G.G. 2008. Polymyalgia rheumatica and giant-cell arteritis. *Lancet 372: 234-245*.

52. Stone J.H., Tuckwell K., Dimonaco S., Klearman M., Aringer M., Blockmans D., Brouwer E., Cid M.C., Dasgupta B., Rech J., Salvarani C., Schulze-Koops H., Schett G., Spiera R., Unizony S.H., Collinson N. Glucocorticoid Doses and Acute-Phase Reactants at Giant Cell Arteritis Flare in a Randomized Trial of Tocilizumab. *Arthritis Rheumatol. 71:1329-1338*.

53. J. Narváez, P. Estrada, L. López-Vives, M. Ricse, A. Zacarías, S. Heredia, C. Gómez-Vaquero, J.M. Nolla. Prevalence of ischemic complications in patients with giant cell arteritis presenting with apparently isolated polymyalgia rheumatica. *Semin Arthritis Rheum. 45(3):328-33*.

54. Dejaco, C., Duftner, C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2016. The spectrum of giant cell arteritis and polymyalgia rheumatica: revisiting the concept of the disease. *Rheumatology (Oxford) 56(4):506-515*.

van Sleen Y*,

Graver JC*,

Abdulahad WH,

van der Geest KSM,

Boots AMH,

Sandovici M,

Brouwer E.

* Shared first author

TWO

Leukocyte Dynamics Reveal a Persistent Myeloid Dominance in Giant Cell Arteritis and Polymyalgia Rheumatica

Frontiers in Immunology. 2019 10;1981

ABSTRACT

Giant cell arteritis (GCA) and polymyalgia rheumatica (PMR) are inflammatory diseases requiring long-term glucocorticoid treatment. Limited data on dynamics in leukocyte counts before, during and after treatment are available. Leukocyte counts were measured, as cellular markers of inflammation, at fixed time points in our prospectively studied cohort of pre-treatment glucocorticoid-naive GCA (N=42) and PMR (N=31) patients. Values were compared with age-matched healthy controls (HCs; N=51) and infection controls (N=16). We report that before start of treatment monocyte and neutrophil counts were higher in GCA and PMR patients than in HCs, while NK- and B-cell counts were lower. C-reactive protein (CRP) levels correlated positively with monocyte counts in GCA, and negatively with B-cell and NK-cell counts in PMR. During glucocorticoid treatment, myeloid subsets remained elevated whereas lymphoid subsets tended to fluctuate. Interestingly, erythrocyte sedimentation rate (ESR) outperformed CRP as marker for relapses in GCA. We defined stable treatment-free remission groups in both GCA and PMR. GCA patients in treatment-free remission still demonstrated elevated monocytes, neutrophils, ESR and platelets. PMR patients in treatment-free remission had normalized levels of inflammation markers, but did have elevated monocytes, lowered CD8+ T-cell counts and lowered NK-cell counts. Finally, we showed that low hemoglobin level was predictive for long-term GC treatment in PMR. Overall, leukocyte composition shifts towards the myeloid lineage in GCA and PMR. This myeloid profile, likely induced by effects of inflammation on hematopoietic stem cell differentiation, persisted during glucocorticoid treatment. Surprisingly, the myeloid profile was retained in treatment-free remission, which may reflect ongoing subclinical inflammation.

INTRODUCTION

Giant cell arteritis (GCA) and polymyalgia rheumatica (PMR) are ageing-related inflammatory diseases that frequently overlap [1]. GCA and Takayasu arteritis both belong to the large vessel vasculitides, but GCA only occurs in the elderly and can also affect cranial arteries. Involvement of cranial arteries (C-GCA) is associated with cranial symptoms including headache, jaw claudication and vision loss. Large vessel GCA (LV-GCA) is more difficult to diagnose due to non-specific symptoms such as weight loss and low-grade fever. PMR is characterized by bursitis and synovitis leading to pain and stiffness mainly in the shoulder and hip girdle [2]. PMR is diagnosed in up to 60% of GCA patients [3], implying overlapping pathogenic pathways.

The pathogenesis of GCA, and especially PMR, are incompletely understood [4]. In GCA, temporal artery biopsies (TABs) reveal a granulomatous infiltrate of macrophages and CD4+ T-cells in the vessel wall [5, 6]. Infiltrating B-cells and neutrophils have been found in lower numbers [7-10]. The vast majority of newly diagnosed GCA and PMR patients display elevated interleukin (IL)-6-dependent acute-phase markers such as ESR and CRP [11, 12].

From the 1950s until now, glucocorticoids (GCs) have remained the cornerstone of treatment in GCA and PMR [13]. GC treatment, however, is accompanied by side-effects, and relapses during GC treatment are common [14, 15]. More recently, progress has been made regarding GC-sparing therapies in GCA and PMR [16-18].

The effects of GC-mediated immunosuppression are pleiotropic and not yet completely understood [19]. GCs strongly repress the acute-phase response [17], and therewith repress the utility of CRP, ESR, and other inflammatory markers in monitoring patients during treatment. Furthermore, recent evidence suggests that GCA patients on GC treatment with a normal CRP/ESR and absence of symptoms can still have persistent vessel wall inflammation [20-22]. Consequently, it is unknown whether patients who reached treatment-free remission are truly in remission or are suffering from ongoing subclinical disease. This is important, as GCA patients with subclinical vasculitis are at risk of aneurysm development and aortic dissection [1, 23].

In search for cellular markers of inflammation in GCA and PMR, we documented leukocyte dynamics during the entire disease course. Previously, altered monocyte, neutrophil, and B-cell blood counts have been reported at diagnosis [9, 24, 25]. In addition, small and mostly short-term studies have addressed the effect of GCs on blood leukocyte subset counts in GCA and PMR patients. Both myeloid (monocytes [25, 26], neutrophils [10, 24]) and lymphoid (CD4+/CD8+ T-cells [26-28], B-cells [9], NK-cells [26, 29]) cell counts appear to be affected by GCs. However, to the best of our knowledge, a comprehensive long-term study comparing leukocyte subset counts before, during and after GC treatment in GCA and PMR patients has not been performed.

The current study was conducted in our prospective cohort in which glucocorticoid-naive GCA and PMR patients were requested to participate at diagnosis and were followed for up to seven years. At fixed time points, leukocyte counts and other inflammatory markers were determined. We investigated the effects of disease on leukocyte subsets by comparison to healthy and infection controls. Next, we analysed the effects of short- and long-term treatment on leukocyte subsets in GCA and PMR patients and extended our investigation to patients who had reached stable treatment-

free remission. In addition, we evaluated the usefulness of leukocyte subsets and inflammatory markers in identifying relapses and assessed their prognostic value before start of treatment.

MATERIALS & METHODS

Patient population

Characteristics of newly-diagnosed patients before start of treatment and characteristics of controls are displayed in table 1. Forty-two GCA and 31 PMR patients participated in our cohort study and were seen at the Rheumatology and Clinical Immunology outpatient clinic of the University Medical Center Groningen between 2010 and 2018. These patients did not use GCs or other disease modifying anti-rheumatic drugs (DMARDs) at pre-treatment assessment. GCA patients were diagnosed based on a positive temporal artery biopsy (TAB) and/or a positive 18F-fluorodeoxyglucose-positron emission tomography-computed tomography (FDG-PET-CT) for LV-GCA. In GCA 29 of the 42 patients fulfilled the 1990 ACR criteria, as these criteria are mainly useful in diagnosis of C-GCA rather than LV-GCA. Diagnosis of PMR patients was based on a positive FDG-PET-CT scan, or based

Table 1. Pre-treatment characteristics of newly diagnosed, treatment-naive giant cell arteritis and polymyalgia rheumatica patients, aged healthy controls, and aged infection controls. *n for IL-6 is as follows HC=17, GCA=40, PMR=29, INF=13.

	HC	GCA	PMR	INF	p-value HC vs GCA	p-value HC vs PMR	p-value HC vs INF
n	51	42	31	16	-	-	-
Age in years; median (range)	**72** (57-91)	**72** (52-89)	**73** (54-84)	**74** (47-97)	NS	NS	NS
Females (%)	32 (63)	28 (67)	20 (65)	5 (31)	NS	NS	0.043
Smoking status; smoking / non smoking	9 / 42	13 / 29	3 / 28	4 / 10	NS	NS	NS
TAB positive / performed	NA	23 / 29	0 / 6	NA	-	-	-
FDG-PET-CT positive for GCA / PMR / GCA+PMR	NA	19 / 0 / 10	0 / 23 / 0	NA	-	-	-
IL-6 pg/mL; median (range)*	**1.5** (0.9-4.2)	**11.5** (1.4-233.6)	**19.8** (2-117)	**22.1** (0.9-152.7)	<0.0001	<0.0001	<0.0001
CRP mg/L; median (range)	**5** (0-7)	**47** (2.2-215)	**42** (3.2-186)	**70** (10-339)	<0.0001	<0.0001	<0.0001
ESR mm/hr; median (range)	**10** (1-28)	**81** (7-121)	**57** (8-109)	**60** (10-118)	<0.0001	<0.0001	<0.0001
Hb mmol/L; median (range)	**9.0** (7.2-10.1)	**7.4** (5.5-8.5)	**7.5** (5.6-9.3)	**7.4** (5.2-9.8)	<0.0001	<0.0001	0.0038
Platelets 10^9/mL; median (range)	**239** (121-345)	**358** (222-523)	**331** (170-562)	**275** (161-665)	<0.0001	<0.0001	NS

HC: healthy control, INF: infection control, TAB: temporal artery biopsy, FDG-PET-CT: 18F-fluorodeoxyglucose-positron emission tomography-computed tomography, IL-6: interleukin-6, Hb: hemoglobin, NS: not significant.

on clinical signs and symptoms if no FDG-PET-CT could be performed. Twenty-five out of 31 PMR patients fulfilled the Chuang criteria. Three PMR patients without a FDG-PET-CT scan, did not fulfil the Chuang criteria due to ESR levels below 40 mm/hr, but did have elevated CRP levels (>10 mg/L). All but one PMR patient fulfilled the preliminary ACR/EULAR 2012 classification criteria [30]. This patient did fulfil the Chuang criteria and had a positive FDG-PET-CT for PMR.

This study included cross-sectional data of 51 age- and sex-matched HCs and also 16 age-matched INFs. HCs were screened for past and present morbidities. Hospitalized INFs who suffered from urinary tract infection (n=10) or pneumonia (n=6) were requested to participate. All INFs were recruited during active infection, up to seven days after admission to the hospital. Volunteers in both control groups did not take any immunosuppressive drugs nor had comorbid diseases. Written informed consent was obtained from all study participants. All procedures were in compliance with the declaration of Helsinki. The study was approved by the institutional review board of the University Medical Center Groningen (METc2012/375 for HC and METc2010/222 for GCA, PMR and INF).

Follow-up and treatment

GCA and PMR patients were prospectively followed for a median period of 30 (range 0-71) and 46 months (range 0-75), respectively. For nine patients, only a pre-treatment visit could be included. We did not exclude these patients to make the pre-treatment data stronger. The number of GCA patients followed for one year was 31 (72%) and the number of GCA patients followed for two years was 23 (54%). For PMR the number of patients followed for one year was 24 (83%) and the number of patients followed for two years was 20 (69%). Patient visits were planned according to a fixed protocol. For analysis, follow-up visits were stratified into three groups: treatment phase I (two weeks, six weeks and three months), treatment phase II (six and nine months) and treatment phase III (twelve months and thereafter every six months). All patients in treatment phase I, II and III still receive treatment.

GCA patients started with a higher daily GC dose than PMR patients (median 60 mg in GCA, 15 mg in PMR). GCs were tapered upon remission according to BSR guidelines for GCA [31] and for PMR [32]. In this study, a relapse is defined by GCA- or PMR-specific signs and symptoms. In case of a relapse, an extra visit to the outpatient clinic was scheduled; daily GC dose was increased and/or a conventional synthetic DMARD (methotrexate or leflunomide) was added to the treatment regimen. None of the patients in this study used IL-6 receptor blockade (e.g. tocilizumab). In patients that remained in remission, GC and/or DMARD treatment was tapered until treatment-free remission was achieved. In order to analyse stable treatment-free remission, we excluded samples from the first three months of treatment-free remission and hereafter only included samples of patients who did not show return of signs and symptoms for at least six months.

Laboratory measurements

Basic laboratory measurements of CRP, ESR, Hb and platelets as well as blood leukocyte counts were collected at all available time points. CRP levels were determined using the Cobas 8000 modular analyser (Roche, Basel, Switzerland). ESR (Westergren method) and Hb were determined by the XN-9000 (Sysmex, Kobe, Japan). Platelets, monocytes and neutrophil counts were also

determined by the XN-9000, based on size and granularity (diff). Levels of serum IL-6 (standard curve range 4.8 - 1154; sensitivity 1.7 pg/ml) were measured with Human premix Magnetic Luminex screening assay kits (R&D Systems, Abingdon, UK) only pre-treatment (see previous study [12]).

Absolute counts of lymphocyte subsets were measured in EDTA blood by the BD (San Jose, CA, USA) MultiTest TruCount method, as described by the manufacturer. Lymphocytes were gated by size and positivity for CD45, after which the subsets were defined: CD4+ T-cells (CD3+CD4+), CD8+ T-cells (CD3+CD8+), B-cells (CD19+) and NK-cells (CD56+ and/or CD16+). TruCount measurements were performed on a FACS Canto-II (BD) and subsequently analysed with FACSCanto Clinical Software.

Monocyte counts were also determined by the TruCount method, which is based on size, granularity, and CD45 expression. We determined that counts of monocytes were 22% higher when measured by the XN-9000 method compared with the TruCount method. This factor was stable throughout all samples. Comparison of 20 samples measured by both methods and corrected (x 1.22), showed a strong correlation (r=0.87, p=<0.0001) and good agreement on a Bland-Altman plot (supplemental Figure 1). For this reason we applied this correction to all monocyte count measurements assessed by the TruCount method.

Statistical analysis

To analyse differences between groups and over time, 2-tailed non-parametric tests were performed. Fisher's exact test, Kruskal Wallis and Mann Whitney U tests were used when comparing patients with controls. Strength and statistical significance of correlations between measurements was tested using Spearman's rank correlation. The log rank test was used to compare the time to GC-free remission between patients with low or high inflammatory markers or cell counts pre-treatment. Analyses were performed with IBM SPSS 23 and GraphPad Prism 7.02 software.

RESULTS

Pre-treatment: altered levels of IL-6, CRP, ESR, Hb and platelets in GCA and PMR patients

IL-6, CRP, ESR and platelet counts were significantly higher whereas Hb levels were significantly lower for pre-treatment GCA and PMR patients compared to HCs (table 1). IL-6, CRP, ESR and Hb did not differ between GCA or PMR patients and infection controls (INFs), but platelet counts were significantly higher in GCA (p=0.008) and PMR (p=0.033) than in INFs. Smoking status did not differ between patients groups.

In GCA, CRP and ESR correlated positively (Rho= 0.80), whereas no correlation was observed in PMR (Rho= 0.36, NS; supplementary Figure 2). In addition, hemoglobin (Hb) correlated negatively with ESR in both patient populations (GCA Rho= -0.51, PMR Rho= -0.65). In GCA patients, platelet counts correlated positively with CRP and ESR (Rho= 0.49 and Rho= 0.54, respectively), and negatively with Hb (Rho= -0.39).

Leukocyte subsets in pre-treatment GCA and PMR patients: shift to the myeloid lineage

Absolute counts of leukocyte subsets measured in GCA and PMR patients before start of treatment were compared with counts in HCs and INFs (Figure 1A). Counts of neutrophils and monocytes were significantly higher, while NK-cells were significantly lower in GCA and PMR patients compared to HCs. Counts of these subsets in patients were similar to those in INF. B-cell counts were also significantly lower in PMR while for GCA patients a trend towards reduction of B-cells was observed (p=0.06). In contrast, T-cells (both CD4+ and CD8+) in GCA and PMR patients were not significantly

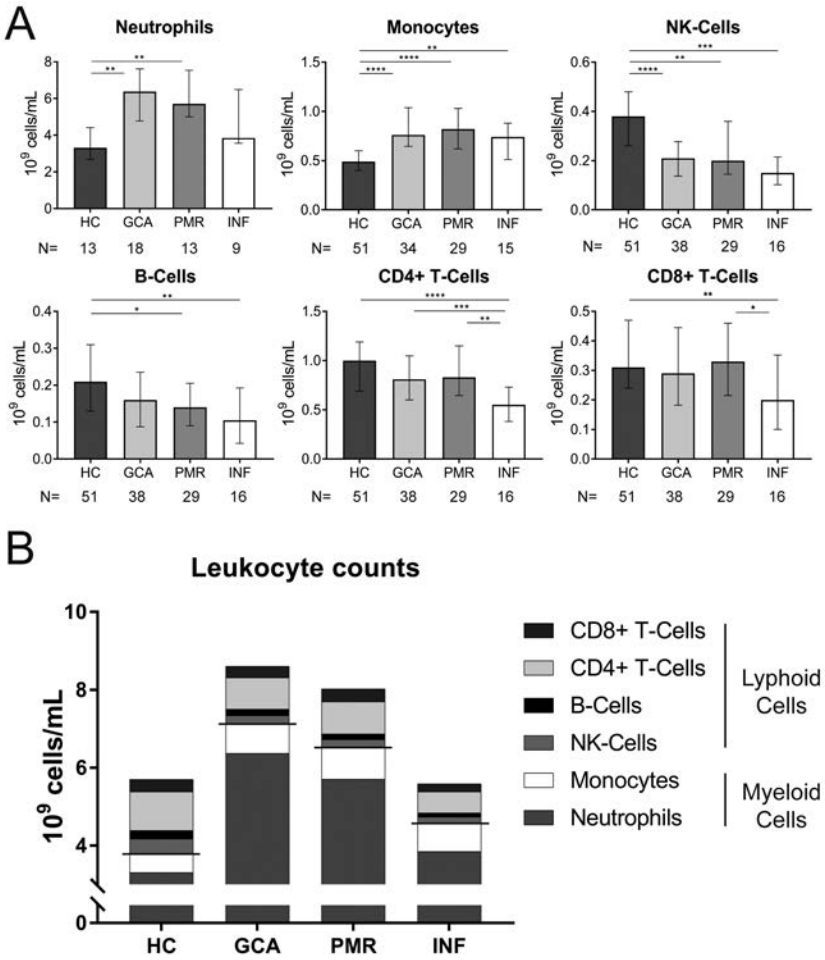


Figure 1. Pre-treatment measurements in newly-diagnosed, treatment-naive GCA and PMR patients. (**A**), Leukocyte counts in the blood for GCA and PMR as well as two control groups: HC and INF. The n is depicted in the figure and indicates the number of samples measured in the different groups. Data is expressed as median and interquartile range. Statistical differences by Mann Whitney U between groups are displayed if Kruskal Wallis testing indicated significant differences: * p<0.05, ** p<0.01, *** p<0.001**** p<0.0001. (**B**), Stacked leukocyte subset counts show a clear shift to the myeloid lineage in GCA and PMR pre-treatment.

different from HC, even though T-cell counts were lower in INFs. Overall, we observed a shift in leukocyte counts towards the myeloid lineage in both GCA and PMR patients as myeloid cell counts were elevated, while lymphoid cell counts were reduced or unchanged (Figure 1B).

To determine a possible involvement of leukocyte subsets in disease activity, we correlated numbers of circulating leukocyte subsets with CRP levels determined at the same visit (Figure 2). A significant positive correlation was observed between monocyte counts and CRP in GCA-patients only (Rho= 0.58), whereas CRP from PMR patients correlated negatively with numbers of circulating B-cells (Rho= -0.55) and NK-cells (Rho= -0.52). In addition, CRP from INFs showed a strong correlation with neutrophil counts (Rho= 0.70). For all correlations, see supplementary Figure 2.

During treatment: myeloid subsets remain elevated whereas lymphoid subsets fluctuate

After diagnosis and pre-treatment sampling, all patients started with GC treatment. To visualize fluctuations in absolute leukocyte counts during follow-up in both GCA and PMR, a (smoothed) median of 20 consecutive measurements over time was calculated and depicted in Figure 3. To apply an appropriate statistical analysis of treatment effects over time and to compare it with the HC group, the follow-up time was split into three treatment phases as depicted in Figure 3 and 4. Median daily GC dose successively decreased for GCA patients in treatment phase I, II and III: 40 mg, 10 mg and 5 mg, respectively. In PMR patients this was 15 mg, 7.5 mg and 5 mg.

In both GCA and PMR patients on treatment, myeloid cell counts (monocytes and neutrophils) remained higher over time compared to HCs (Figure 3, Figure 4). Neutrophils increased further during treatment phase I and II when compared to pre-treatment.

Lymphoid cells (NK-, T- and B-cells) were also affected by treatment. B-cells showed most fluctuations over time: during treatment phase I, we observed an increase compared to pre-treatment which was followed by a progressive decrease in treatment phase II and III. T-cell (CD4+ and CD8+) counts were low during treatment compared to pre-treatment and to HC counts. Interestingly, T-cell counts dropped significantly during treatment phase I in GCA patients, while this was not observed in PMR patients where T-cells were only lowered in phase II and III. NK-cells remained significantly lower throughout all phases compared to HCs.

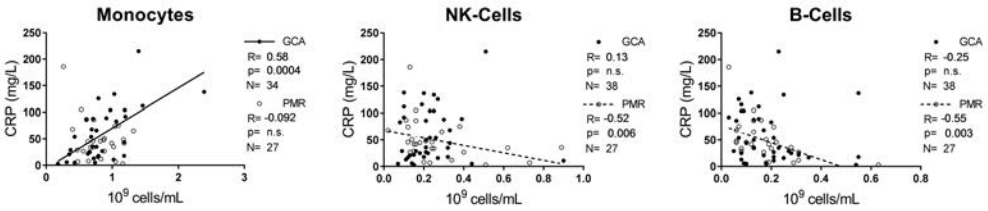


Figure 2. Correlations between three leukocyte subsets and the inflammatory marker CRP. Correlations between CRP and the leukocyte subset in pre-treatment GCA (closed circles) and PMR (open circles) patients. Spearman's R, the p-value of the correlation and the N are indicated in each graph for GCA and PMR. Regression line for GCA is shown as an uninterrupted line, for PMR as a dotted line. Correlations for neutrophils, CD4+ T-cells and CD8+ T-cells are displayed in supplemental Figure 2.

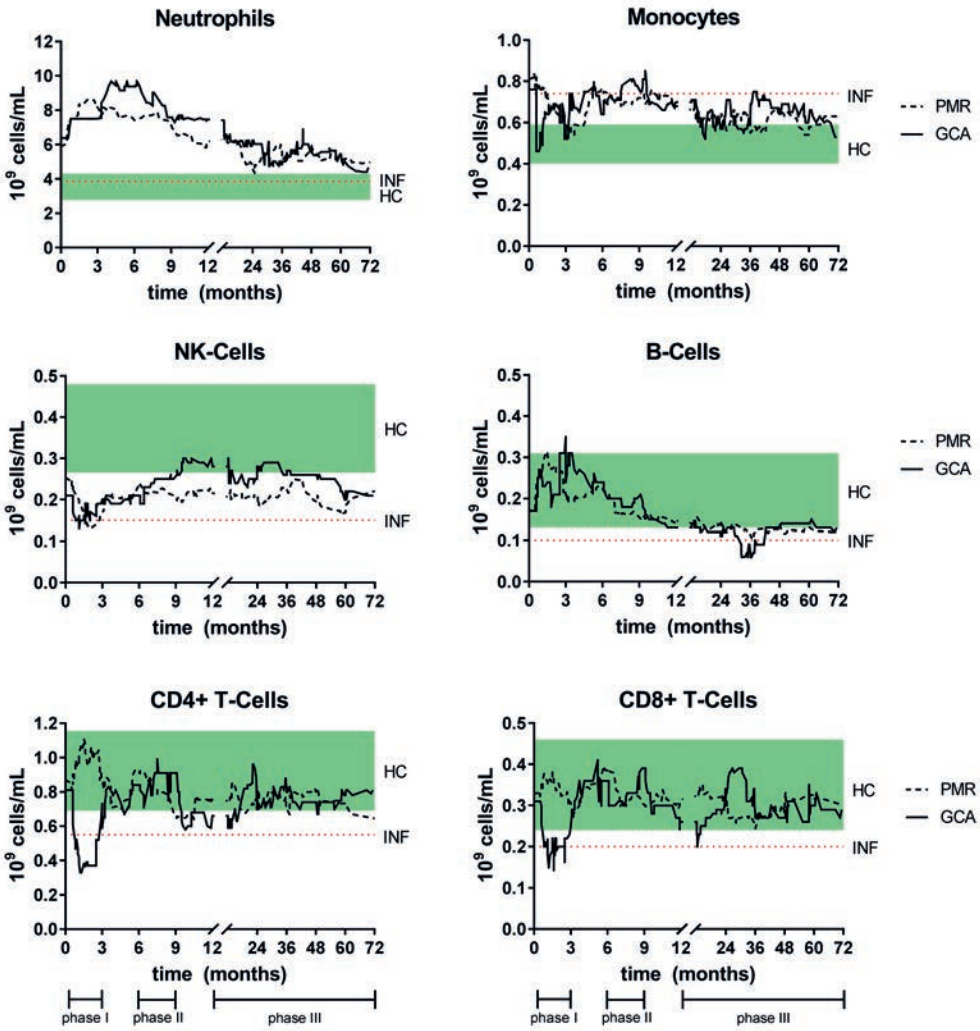


Figure 3. Smoothed median of leukocyte counts for GCA and PMR patients over time while on GC treatment. The smoothed median is calculated by taking the median of each new measurement and that of the 19 measurements before that point. This method enables to distinguish patterns over time that would be unnoticeable if each point is plotted separately. For interpretation the interquartile range of HC (green box, cross-sectional measurement) and the median of the INF (dotted line, cross-sectional) were added to the figures. Time point 0 indicates the pre-treatment sample. Also, the three different treatment phases are indicated.

Platelets, CRP and ESR all decreased from pre-treatment levels during the entire treatment but mostly remained elevated when compared to HCs (supplemental Figure 3). In GCA patients platelets were elevated in all phases, but CRP and ESR were elevated only in phase II and III. In PMR patients platelets were elevated in phase I, CRP in phase I and II and ESR in phase II and III. Hb increased from pre-treatment levels in both GCA and PMR but remained decreased compared to HC levels during all phases.

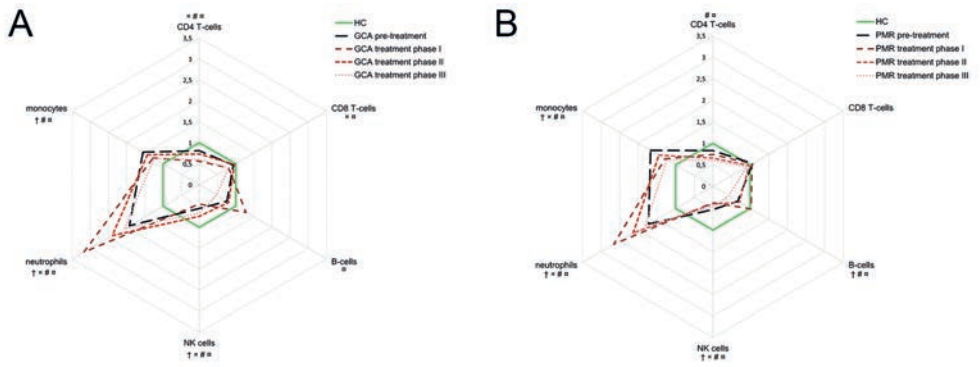


Figure 4. Dynamics in cell population counts during follow-up in GCA (**A**) and PMR (**B**) patients. Counts are expressed in radar plots as median fold-change compared to healthy controls (n=51) for the following groups: pre-treatment (GCA n=42, PMR n=31), treatment phase I (GCA n=38, 69 measurements; PMR n=25, 54 measurements), phase II (GCA n=32, 43 measurements; PMR n=23, 33 measurements) and phase III (GCA n=29, 65 measurements; PMR n=19, 56 measurements). Pre-treatment only includes the visit before start of treatment; treatment phase I includes follow-up visits at two weeks, six weeks and three months; treatment phase II includes six and nine months; treatment phase III includes twelve months and beyond. †: sign difference between HC and baseline, ×: sign difference between HC and treatment phase I, #: significant differences between HC and phase II and ¤: significant differences between HC and phase III (Mann-Whitney U test, P<0.05).

ESR outperforms CRP, Hb and platelets as marker for relapses in GCA during treatment

In order to determine the GC dose at which patients experienced a relapse, the daily GC dose of patients who were followed for at least two years was recorded (Table 2). Of the 24 GCA patients fulfilling this criterion, 79% experienced at least one relapse during these two years. In addition, 57% of the 21 PMR patients developed at least one relapse during this period. The median daily dose at relapse was 5 mg for GCA and 7.5 mg for PMR. Six GCA relapses and five PMR relapses occurred in patients who were not taking GCs anymore.

Next, we determined whether levels of inflammatory markers and leukocyte counts were different in relapsing patients compared to patients in treatment-induced remission (Figure 5). CRP levels did not reflect relapses in treatment phase I for both GCA and PMR patients when compared to remission patients. In PMR this was also true for treatment phase II. ESR, however, did discriminate GCA patients experiencing a relapse from remission patients in all treatment phases. In PMR patients this was only the case for treatment phase III. Lower Hb and higher platelets were observed in relapsing GCA patients during phase II and in relapsing PMR patients during phase III.

There were also differences in leukocyte counts between relapsing and remission patients in PMR. During relapses, patients displayed higher CD4+ T-cells in treatment phase I, lower NK-cells in phase II and higher neutrophils in phase II and III (data not shown).

Table 2. Daily glucocorticoid dose use at the time of relapse for GCA (N=24) and PMR (N=21) patients. In patients that were followed for two years, we registered the daily GC dose at which they relapsed. Relapses were defined by clinical signs and symptoms only.

GC dose (mg/day) at relapse	GCA relapses (%)	PMR relapses (%)
0	6 (21)	5 (25)
1-5	10 (35)	3 (15)
6-10	7 (24)	6 (30)
11-20	6 (21)	5 (25)
>20	0	1 (5)
Total	29	20

GC: glucocorticoid, GCA: giant cell arteritis, PMR: polymyalgia rheumatica.

The myeloid and inflammatory profiles persist in treatment-free remission patients

To determine whether leukocyte counts and inflammatory markers of GCA and PMR patients are truly normalized after treatment cessation, we investigated patients in treatment-free remission (defined as three months treatment-free and in stable remission for the next six months). So far, 13 GCA and 15 PMR patients have reached treatment-free remission and were included in the analysis.

GCA patients in treatment-free remission showed persistently elevated myeloid cell counts compared to HCs. Note that, compared to pre-treatment levels, neutrophil counts were found reduced (Figure 6A). PMR patients in treatment-free remission also still demonstrated significantly elevated myeloid cell counts although monocyte counts had decreased since pre-treatment levels (Figure 6B). Furthermore, NK-cell and CD8+ T-cell counts were lower in PMR treatment-free remission patients than in HCs. In GCA, there was a strong trend towards lower NK-cells in treatment-free remission compared to HCs (p=0.05).

Inflammatory markers normalized to HC levels in PMR patients in treatment-free remission (Figure 6C). In contrast, in treatment-free remission GCA elevated ESR and platelet counts and lowered Hb remained, whereas CRP was normal. We further investigated whether the elevated ESR in GCA patients was linked to changes in leukocyte subsets. We found a strong negative correlation between B-cell counts and ESR in treatment-free remission GCA patients (Figure 6D).

Pre-treatment low Hb predicts longer GC requirement in PMR

Finally, we assessed whether leukocyte subset counts and inflammatory markers, assessed before start of treatment, could predict time to GC-free remission. A predictive factor was found in PMR patients, only. Pre-treatment Hb level higher than the median, predicted a short time to GC-free remission (i.e. a favorable disease course) compared to patients with a low Hb before start of treatment (Figure 7, p=0.025). As the Hb is typically higher in males, we checked the sex distribution between PMR patients with low and high Hb and found an exactly equal distribution. The other inflammatory markers, CRP and ESR, were not prognostic for GC requirement, nor were any of the leukocyte subset counts.

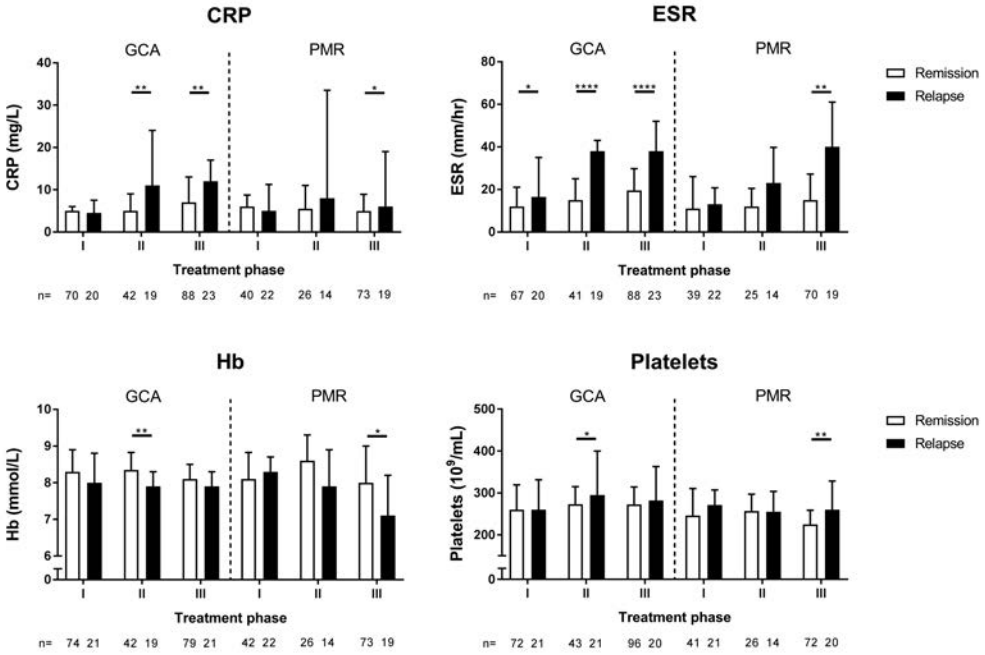


Figure 5. Levels of inflammatory markers during treatment phase I, II, and III for GCA and PMR patients in remission and during relapse. The definition of remission and relapse was based solely on clinical signs and symptoms. Data is expressed as median plus interquartile range. The number of measurements is indicated by n. Statistical significance is expressed as follows: * $p<0.05$, ** $p<0.01$, **** $p<0.0001$ (Mann Whitney U test).

DISCUSSION

This prospective study provides 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 glucocorticoid treatment as well as in stable treatment-free remission. Our main finding is that leukocyte counts shift to the myeloid lineage in both GCA and PMR and that this myeloid bias persists in spite of GC treatment and extends well into treatment-free remission.

Counts of myeloid leukocyte subsets were elevated in pre-treatment GCA and PMR patients. This may be explained by the actions of IL-6, a key pro-inflammatory cytokine in GCA and PMR, promoting monocyte and neutrophil production in the bone marrow [33]. In contrast to myeloid subset counts, pre-treatment lymphoid subset counts were either lowered (NK- and B-cells) or unchanged (CD4 and CD8 T-cells). In INF all lymphoid cell counts were lowered. These findings are in accordance with the notion that inflammation shifts the development of hematopoietic stem cells towards the myeloid lineage [34]. Previous studies have mostly documented similar findings [10, 24-29, 35], albeit that some reported on lowered monocyte counts [26] and lowered CD8+ T-cells [26, 27].

Typical acute phase markers are elevated in both GCA and PMR patients, at pre-treatment analysis, even though the ESR is significantly lower in PMR than in GCA patients. In contrast to

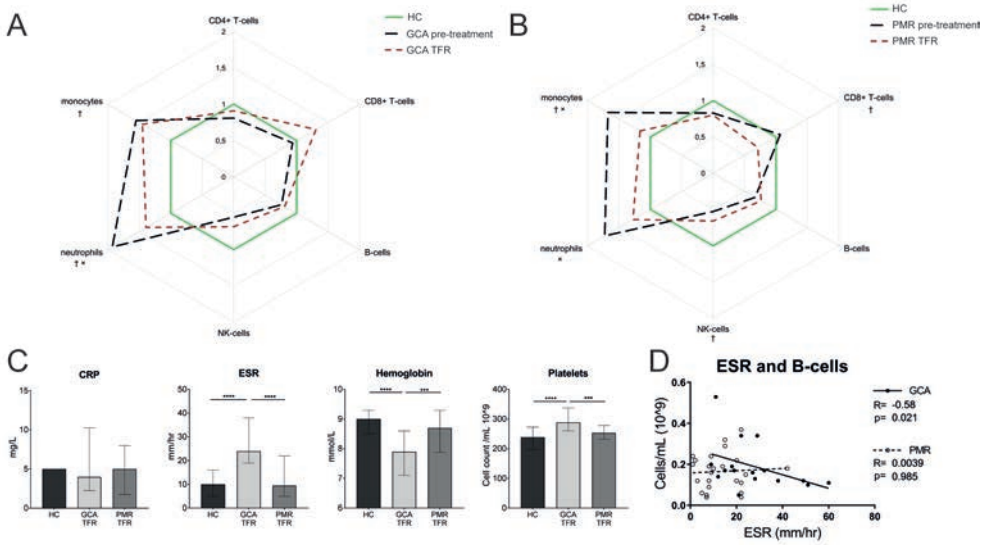


Figure 6. Different leukocyte subset counts and inflammatory markers in treatment- free remission. Leukocyte subset counts pre-treatment and in treatment-free remission (**A:** GCA n=13 patients, 17 samples and **B:** PMR n=15 patients, 25 samples) were expressed as median fold-change compared to healthy controls. †: sign difference between HC and treatment-free remission. ×: sign difference between pre-treatment and treatment-free remission (Mann-Whitney U test p<0.05). (**C):** Inflammatory markers in HC, GCA treatment-free remission and PMR treatment-free remission (Mann-Whitney U test: *** p<0.001**** p<0.0001). Data is expressed as median and interquartile range. (**D):** Correlation between B-cell counts and ESR in treatment-free remission patients.

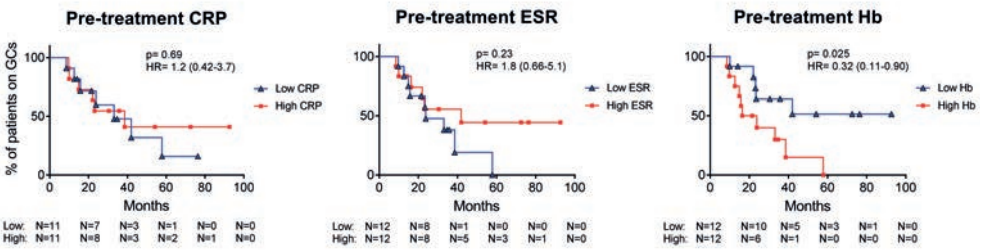


Figure 7. In PMR patients, long-term GC requirement is predicted by pre-treatment Hb levels, but not by CRP and ESR. The CRP, ESR and Hb of PMR patients before treatment were split into low or high levels (based on the median) and were plotted in a Kaplan-Meier curve against time to GC-free remission. p-value and hazard ratio (HR; including 95% confidence interval) of the log rank test are depicted in the graphs.

GCA, CRP and ESR are not correlated with each other in PMR patients. CRP is considered a more acute marker of inflammation, while ESR is more associated with longer-term chronic inflammation [36]. The ESR is a composition of several proteins, including fibrinogen, Hb and immunoglobulin levels [37]. Whether there is a discrepancy in the ESR of GCA patients compared to PMR patients, remains to be investigated. Remarkably, platelet counts were found even higher than INF. Whether platelet counts are useful as disease-specific biomarker, needs to be evaluated in a larger cohort.

In GCA, systemic symptoms (e.g. fever, weight loss) are linked to the IL-6-dependent acute-phase response [38]. Previously, we indeed observed a strong positive correlation between IL-6 and CRP in our cohort [12]. In the current study, we also found pre-treatment CRP to be positively correlated with monocyte counts in GCA patients. Monocytes are important in the immunopathogenesis of GCA and work in tandem with CD4+ T-cells to promote granulomatous inflammation, angiogenesis and destruction of the vessel wall [5, 39]. Monocytes, as part of the innate immune system, sense pathogens and danger signals by pattern recognition receptors, including toll-like receptors (TLRs) [40]. Previously, TLR7 expression on monocytes of GCA and PMR patients was found elevated, hinting at a higher responsiveness to viral antigens [41]. The chemokine CCL2 is important for monocyte migration, and its levels were found lower in the blood of GCA patients [25]. This could be explained by the usage of CCL2 by monocytes migrating from the bone marrow to the blood.

In pre-treatment PMR, we found a negative correlation of CRP with B-cells and NK-cells, hinting that these cell types are important in the maintenance of immune homeostasis. This could be through immune regulatory functions as described before for both subsets (38,39) [42, 43]. Alternatively, low B-cell and NK-cell counts may reflect tissue migration. It is currently unknown if B-cells or NK-cells infiltrate PMR synovia but B-cell counts were found to be decreased in GCA and B-cells and are present in GCA vessels [8, 9, 44], implying migration. In GCA, NK-cells are not frequently found in the TABs, arguing against migration[25].

In this study, we also chartered effects of treatment on leukocyte subsets and inflammatory markers in GCA and PMR patients over time. Blood counts of monocytes and neutrophils remained elevated in patients compared to controls throughout the entire treatment period. GC-induced leukocytosis is a well-known phenomenon [45] and is mainly due to the effect of GCs on neutrophils. GC treatment causes the release of neutrophils from the marginal pool by decreasing the expression of adhesion molecules Mac-1 and L-selectin needed to bind to the endothelium [46, 47]. GCs mainly increase counts of mature neutrophils in the blood, as influx of infection-related, 'non-segmented' neutrophils from the bone marrow is minimal [45, 47]. While monocyte counts remained elevated compared to HC levels, they were lowered by GC treatment. This is likely due to a decrease in non-classical monocytes which are sensitive to GC-induced apoptosis [25, 48].

GC-treatment affected lymphoid leukocyte counts as well. Interestingly, a difference between GCA and PMR in CD4+ T-cell counts was observed in the first months of treatment, as these counts were markedly decreased in GCA patients only. This is likely caused by GCA patients receiving a higher GC dose. Indeed, high- but not low-dose GCs, have a strong apoptotic effect on CD4+ T-cells in-vitro and in-vivo and this decrease is associated with inhibition of IL-2 signalling [49]. Also noticeable is the pattern of B-cells during treatment in GCA patients; early treatment led to an increase in B-cell counts. This is likely caused by B-cells returning to the circulation from peripheral

sites [9]. In our cohort, patients on long-term GC treatment became lymphopenic, as especially their CD4+ T-cell and B-cell counts gradually lowered over time. In addition, NK-cell counts, that were already lower pre-treatment, were not found to reduce further on treatment. This is in accordance with previous reports on NK-cell counts in GCA and PMR and in line with NK-cells being resistant to GC-induced apoptosis [26, 29, 49]. Overall, long-term GC use significantly changes the composition of the peripheral leukocyte pool as well as the function of leukocyte subsets [14, 15], thereby making GCA and PMR patients susceptible to infections [50].

Our data are in congruence with the notion that GCs manage to actively suppress symptoms of the disease but have only a partial effect on tissue inflammation. This is based on the inflammation-induced myeloid dominance observed before treatment that persisted during treatment, despite a suppressed CRP and ESR. Indeed, a too rapid tapering of GCs will in most cases lead to a return of signs and symptoms [50, 51]. The observed GC dose at which patients experience their first relapse is in line with previous reports [52]. Moreover, a study investigating sequential TABs revealed that at least 44% of GCA patients have persistent inflammation in spite of treatment-induced remission [22]. Furthermore, recent studies on tissue inflammation markers during tocilizumab treatment raise caution for ongoing inflammation despite absence of symptoms [21, 53].

The strong suppressive effect of GCs on the acute-phase response makes the classic inflammatory markers, CRP and ESR, less trustworthy for monitoring disease activity. In the first months of treatment, solely ESR discriminated between relapsing and remission GCA patients and the difference in ESR between these groups were found to become significantly stronger at later phases and thus at lower GC doses. Overall, ESR appeared more suited than CRP in identifying relapses in GCA rendering ESR more useful in monitoring disease activity. This is in accordance with a previous study on monitoring biomarkers in GCA [54]. However, as described before [55], CRP and ESR are frequently normal at time of clinical relapse. This unsatisfactory use of CRP/ESR during GC treatment, and the fact that tocilizumab treatment suppresses these markers even more [21], raises the need for new inflammatory markers to aid in monitoring of GCA and PMR patients.

Peripheral blood cells of GCA and PMR patients in treatment-free remission were found to retain the myeloid bias. This may be explained by a long-lasting imprint of inflammation on peripheral blood cell composition. Yet, whereas markers of inflammation normalized in PMR, these markers (ESR, Hb and platelet counts, but not CRP) remained altered in GCA patients that have reached treatment-free remission. The combined data suggest that subclinical vessel wall inflammation may still be ongoing in GCA. Alternatively, this retained myeloid dominance could point towards cellular senescence of the immune system which had predisposed the patients to develop these diseases. Indeed, aging of the immune system has been linked to development of disease [56].

Interestingly, this ongoing response (ESR) is negatively correlated with B-cell counts. B-cells might be important in preventing a return of disease and/or B-cells might aggravate disease by tracking to the site of inflammation. Migration of B-cells towards the inflamed vessel has been documented in GCA [7, 8, 44] but their role in the tissue (either anti-or pro-inflammatory) remains to be established. Thus the question remains whether symptom treatment of GCA (and PMR) is sufficient. Persistence of the myeloid and inflammatory profile suggests ongoing inflammation eventually leading to vascular damage and associated morbidity and mortality [57].

Additionally, we discovered a prognostic value of pre-treatment Hb levels on disease course in PMR patients. Our data show that patients with a low Hb have a higher risk for an unfavorable long-term disease course. No such prognostic value was seen for ESR and CRP. The low pre-treatment level of Hb in PMR patients is a secondary effect of long-term inflammation [58]. We thus hypothesize that a low Hb better reflects the inflammatory load over a longer period of time than ESR and especially CRP. The latter inflammatory markers are indeed more prone to fluctuate over time [36]. The clinical utility of our finding is that low Hb levels may predict long-term GC requirement in PMR patients.

The major strength of this study is our well-defined, prospectively followed, long-term cohort of GCA and PMR patients who joined the study when they were treatment-naive, allowing to assess pre-treatment values. Often, GCA and PMR patients are included in cohorts after start of GCs. The strict follow-up regimen allowed us to investigate the immune status of patients during relapses and in treatment-free remission. Because of the clinical overlap between the two diseases, the drawn comparisons in this study are useful. The inclusion of the INF group helped to discriminate between disease specific and non-specific features. Another strength of the study is that we documented changes in six major peripheral blood leukocyte subsets using assays that are readily available in the clinical setting. Our study is limited by sole analysis of peripheral blood markers in both these systemic diseases which may only partly mirror the immunological processes at the sites of inflammation such as the vessel wall and the synovium in GCA and PMR, respectively.

In conclusion, we observed a clear shift towards the myeloid lineage in pre-treatment GCA and PMR patients. This myeloid bias was associated with inflammatory markers and persisted during glucocorticoid treatment and in treatment-free remission. Persistence of the myeloid and inflammatory profile during the entire disease course may reflect ongoing subclinical vasculitis, implying that current glucocorticoid-based treatment is unsatisfactory. Future studies using sensitive imaging techniques should address if these profiles indeed coincide with tissue inflammation. Also, treatment could aim at targeting the myeloid shift in GCA and PMR patients. Blocking the granulocyte macrophage colony stimulating factor (GM-CSF) receptor could prove to be beneficial in influencing this shift. Trials with this type of treatment are currently ongoing (NCT03827018).

REFERENCES

1. Dejaco, C., Duftner, C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2016. The spectrum of giant cell arteritis and polymyalgia rheumatica: revisiting the concept of the disease. *Rheumatology (Oxford) 56(4):506-515.*
2. Salvarani, C., Cantini, F., and Hunder, G.G. 2008. Polymyalgia rheumatica and giant-cell arteritis. *Lancet 372: 234-245.*
3. Salvarani, C., Gabriel, S.E., O'fallon, W.M., and Hunder, G.G. 1995. The incidence of giant cell arteritis in Olmsted County, Minnesota: apparent fluctuations in a cyclic pattern. *Ann. Intern. Med. 123: 192-194.*
4. Guggino G G. 2018. Pathogenesis of polymyalgia rheumatica. *Reumatismo 70: 10-17.*
5. Samson, M., Corbera-Bellalta, M., Audia, S., Planas-Rigol, E., Martin, L., Cid, M.C., and Bonnotte, B. 2017. Recent advances in our understanding of giant cell arteritis pathogenesis. *Autoimmun. Rev. 16: 833-844.*
6. Weyand, C.M. and Goronzy, J.J. 2013. Immune mechanisms in medium and large-vessel vasculitis. *Nat. Rev. Rheumatol. 9: 731-740.*
7. Ciccia, F., Rizzo, A., Maugeri, R., Alessandro, R., Croci, S., Guggino, G., Cavazza, A., Raimondo, S., Cannizzaro, A., Iacopino, D.G., Salvarani, C., and Triolo, G. 2017. Ectopic expression of CXCL13, BAFF, APRIL and LT-beta is associated with artery tertiary lymphoid organs in giant cell arteritis. *Ann. Rheum. Dis. 76: 235-243.*
8. Graver, J.C., Sandovici, M., Diepstra, A., Boots, A.M.H., and Brouwer, E. 2018. Artery tertiary lymphoid organs in giant cell arteritis are not exclusively located in the media of temporal arteries. *Ann. Rheum. Dis. 77: e16-2017-211860.*
9. van der Geest, K.S., Abdulahad, W.H., Chalan, P., Rutgers, A., Horst, G., Huitema, M.G., Roffel, M.P., Roozendaal, C., Kluin, P.M., Bos, N.A., Boots, A.M., and Brouwer, E. 2014. Disturbed B cell homeostasis in newly diagnosed giant cell arteritis and polymyalgia rheumatica. *Arthritis Rheumatol. 66: 1927-1938.*
10. Nadkarni, S., Dalli, J., Hollywood, J., Mason, J.C., Dasgupta, B., and Perretti, M. 2014. Investigational analysis reveals a potential role for neutrophils in giant-cell arteritis disease progression. *Circ. Res. 114: 242-248.*
11. Salvarani Carlo C. 2005-2-15. Acute-phase reactants and the risk of relapse/recurrence in polymyalgia rheumatica: a prospective followup study. *Arthritis Rheumatol.53: 33-8.*
12. van Sleen, Y., Sandovici, M., Abdulahad, W., Bijzet, J., van der Geest, K., Boots, A.M., and van der Geest, Kornelis SM. 2019. Markers of Angiogenesis and Macrophage Products for Predicting Disease Course and Monitoring Vascular Inflammation in Giant Cell Arteritis. *Rheumatology (Oxford) 58: 1383–1392.*
13. Hunder Gene G GG. 2006. The early history of giant cell arteritis and polymyalgia rheumatica: first descriptions to 1970. *Mayo Clin. Proc. 81: 1071-83.*
14. Broder, M.S., Sarsour, K., Chang, E., Collinson, N., Tuckwell, K., Napalkov, P., and Klearman, M. 2016. Corticosteroid-related adverse events in patients with giant cell arteritis: A claims-based analysis. *Semin. Arthritis Rheum. 46: 246-252.*
15. Buttgereit F., Matteson E.L., Dejaco C., Dasgupta B. 2018. Prevention of glucocorticoid morbidity in giant cell arteritis. *Rheumatology (Oxford) 57(suppl_2): ii11-ii21.*
16. Lally, L., Forbess, L., Hatzis, C., and Spiera, R. 2016. Brief Report: A Prospective Open-Label Phase IIa Trial of Tocilizumab in the Treatment of Polymyalgia Rheumatica. *Arthritis Rheumatol. 68: 2550-2554.*
17. Stone, J.H., Tuckwell, K., Dimonaco, S., Klearman, M., Aringer, M., Blockmans, D., Brouwer, E., Cid, M.C., Dasgupta, B., Rech, J., Salvarani, C., Schett, G., Schulze-Koops, H., Spiera, R., Unizony, S.H., and Collinson, N. 2017. Trial of Tocilizumab in Giant-Cell Arteritis. *N. Engl. J. Med. 377: 317-328.*
18. Leon Leticia L. 2018 Mar-Apr. Treatment with methotrexate and risk of relapses in patients with giant cell arteritis in clinical practice. *Clin. Exp. Rheumatol. 36: 121-128.*
19. Zen, M., Canova, M., Campana, C., Bettio, S., Nalotto, L., Rampudda, M., Ramonda, R., Iaccarino, L., and Doria, A. 2011. The kaleidoscope of glucorticoid effects on immune system. *Autoimmun.Rev. 10: 305-310.*

20. Reichenbach, S., Adler, S., Bonel, H., Cullmann, J.L., Kuchen, S., Bütikofer, L., Seitz, M., and Villiger, P.M. 2018. Magnetic resonance angiography in giant cell arteritis: results of a randomized controlled trial of tocilizumab in giant cell arteritis. *Rheumatology (Oxford) 57: 982-986.*

21. Gloor A.D., Yerly D., Adler S., Reichenbach S., Kuchen S., Seitz M., Villiger P.M. 2018. Immuno-monitoring reveals an extended subclinical disease activity in tocilizumab-treated giant cell arteritis. *Rheumatology (Oxford) 57:1795-1801*

22. Maleszewski, J.J., Younge, B.R., Fritzlen, J.T., Hunder, G.G., Goronzy, J.J., Warrington, K.J., and Weyand, C.M. 2017. Clinical and pathological evolution of giant cell arteritis: a prospective study of follow-up temporal artery biopsies in 40 treated patients. *Mod. Pathol. 30(6):788-796.*

23. Koster, M.J., Matteson, E.L., and Warrington, K.J. 2018. Large-vessel giant cell arteritis: diagnosis, monitoring and management. *Rheumatology (Oxford) 57: ii32-ii42.*

24. Oh, L.J., Wong, E., Andrici, J., McCluskey, P., Smith, J.E.H., and Gill, A.J. 2017. The full blood count as an ancillary test to support the diagnosis of giant cell arteritis. *Intern. Med. J. 48:408-413*

25. van Sleen, Y., Wang, Q., van der Geest, K.S.M., Westra, J., Abdulahad, W.H., Heeringa, P., Boots, A.M.H., and Brouwer, E. 2017. Involvement of Monocyte Subsets in the Immunopathology of Giant Cell Arteritis. *Sci. Rep. 7: 6553*

26. Macchioni, P., Boiardi, L., Salvarani, C., Rossi, F., Casadei-Maldini, M., Mancini, R., Beltrandi, E., and Portioli, I. 1993. Lymphocyte subpopulations analysis in peripheral blood in polymyalgia rheumatica/giant cell arteritis. *Br. J. Rheumatol. 32: 666-670.*

27. Dasgupta, B., Duke, O., Timms, A.M., Pitzalis, C., and Panayi, G.S. 1989. Selective depletion and activation of CD8+ lymphocytes from peripheral blood of patients with polymyalgia rheumatica and giant cell arteritis. *Ann. Rheum. Dis. 48: 307-311.*

28. Pountain, G.D., Keogan, M.T., Brown, D.L., and Hazleman, B.L. 1993. Circulating T cell subtypes in polymyalgia rheumatica and giant cell arteritis: variation in the percentage of CD8+ cells with prednisolone treatment. *Ann. Rheum. Dis. 52: 730-733.*

29. Uddhammar, A., Roos, G., Nasman, B., and Dahlqvist, S.R. 1995. Peripheral blood lymphocyte subsets in polymyalgia rheumatica. *Clin. Rheumatol. 14: 62-67.*

30. Dasgupta, B., Cimmino, M.A., Kremers, H.M., Schmidt, W.A., Schirmer, M., Salvarani, C., Bachta, A., Dejaco, C., Duftner, C., and Jensen, H.S. 2012. 2012 provisional classification criteria for polymyalgia rheumatica: a European League Against Rheumatism/American College of Rheumatology collaborative initiative. *Arthritis Rheumatol. 64: 943-954.*

31. Dasgupta, B., Borg, F.A., Hassan, N., Alexander, L., Barraclough, K., Bourke, B., Fulcher, J., Hollywood, J., Hutchings, A., and James, P. 2010. BSR and BHPR guidelines for the management of giant cell arteritis. *Rheumatology (Oxford) 49: 1594-1597.*

32. Dasgupta, B., Borg, F.A., Hassan, N., Barraclough, K., Bourke, B., Fulcher, J., Hollywood, J., Hutchings, A., Kyle, V., and Nott, J. 2009. BSR and BHPR guidelines for the management of polymyalgia rheumatica. *Rheumatology (Oxford) 49: 186-190.*

33. Walker, F., Zhang, H.H., Matthews, V., Weinstock, J., Nice, E.C., Ernst, M., Rose-John, S., and Burgess, A.W. 2008. IL6/sIL6R complex contributes to emergency granulopoietic responses in G-CSF- and GM-CSF-deficient mice. *Blood 111: 3978-3985.*

34. Kovtonyuk, L.V., Fritsch, K., Feng, X., Manz, M.G., and Takizawa, H. 2016. Inflamm-aging of hematopoiesis, hematopoietic stem cells, and the bone marrow microenvironment. *Front Immunol. 7: 502.*

35. Carvajal Alegria, G., Devauchelle-Pensec, V., Renaudineau, Y., Saraux, A., Pers, J., and Cornec, D. 2017. Correction of abnormal B-cell subset distribution by interleukin-6 receptor blockade in polymyalgia rheumatica. *Rheumatology (Oxford) 56: 1401-1406.*

36. Sbong, S. and Feldman, M. 2015. Frequency and causes of C-reactive protein and erythrocyte sedimentation rate disagreements in adults. *Int J Rheum Dis 18: 29-32.*

37. Talstad, I. and Haugen, H.F. 1979. The relationship between the erythrocyte sedimentation rate (ESR) and plasma proteins in clinical materials and models. *Scand. J. Clin. Lab. Invest. 39: 519-524.*

38. Kermani, T.A., Schmidt, J., Crowson, C.S., Ytterberg, S.R., Hunder, G.G., Matteson, E.L., and Warrington, K.J. 2012. Utility of erythrocyte sedimentation rate and C-reactive protein for the diagnosis of giant cell arteritis. *Semin. Arthritis Rheum. 41: 866-871.*

39. Watanabe R., Maeda T., zhang H., Berry G.J., Zeisbrich M., Brockett R., Greenstein A.E., Tian L., Goronzy J.J., Weyand C.M. 2018. Matrix Metalloprotease-9 (MMP-9)-Producing Monocytes Enable T Cells to Invade the Vessel Wall and Cause Vasculitis. *Circ. Res. 123(6):700-715.*

40. O'neill, L. and Molloy, E.S. 2016. The role of toll like receptors in giant cell arteritis. *Rheumatology (Oxford) 55: 1921-1931.*

41. Alvarez Rodriguez, L., Lopez-Hoyos, M., Mata, C., Fontalba, A., Calvo Alen, J., Marin, M.J., Fernandez-Luna, J.L., Aguero Balbin, J., Aranzamendi Zaldunbide, M., Blanco, R., and Martinez-Taboada, V.M. 2011. Expression and function of toll-like receptors in peripheral blood mononuclear cells of patients with polymyalgia rheumatica and giant cell arteritis. *Ann. Rheum. Dis. 70: 1677-1683.*

42. Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. 2008. Functions of natural killer cells. *Nat. Immunol. 9: 503.*

43. Mauri, C. and Blair, P.A. 2010. Regulatory B cells in autoimmunity: developments and controversies. *Nature Rev. Rheum. 6: 636.*

44. Graver, J.C., Boots, A.M., Haacke, E.A., Diepstra, A., Brouwer, E., and Sandovici, M. 2019. Massive B-cell infiltration and Organization into Artery Tertiary Lymphoid Organs in the Aorta of Large Vessel Giant Cell Arteritis. *Front. Immunol. 10: 83.*

45. Shoenfeld, Y., Gurewich, Y., Gallant, L., and Pinkhas, J. 1981. Prednisone-induced leukocytosis: influence of dosage, method and duration of administration on the degree of leukocytosis. *Am. J. Med. 71: 773-778.*

46. Crockard, A., Boylan, M., McMillan, S., Droogan, A., and Hawkins, S. 1998. Methylprednisolone-induced neutrophil leukocytosis—down-modulation of neutrophil L-selectin and Mac-1 expression and induction of granulocyte-colony stimulating factor. *Int. J. Clin. Lab. Res. 28: 110-115.*

47. Nakagawa, M., Terashima, T., D'yachkova, Y., Bondy, G.P., Hogg, J.C., and van Eeden, S.F. 1998. Glucocorticoid-induced granulocytosis: contribution of marrow release and demargination of intravascular granulocytes. *Circulation 98: 2307-2313.*

48. Dayyani, F., Belge, K.U., Frankenberger, M., Mack, M., Berki, T., and Ziegler-Heitbrock, L. 2003. Mechanism of glucocorticoid-induced depletion of human CD14+CD16+ monocytes. *J. Leukoc. Biol. 74: 33-39.*

49. Leussink, V.I., Jung, S., Merschdorf, U., Toyka, K.V., and Gold, R. 2001. High-dose methylprednisolone therapy in multiple sclerosis induces apoptosis in peripheral blood leukocytes. *Arch. Neurol. 58: 91-97.*

50. Proven, A., Gabriel, S.E., Orces, C., O'fallon, W.M., and Hunder, G.G. 2003. Glucocorticoid therapy in giant cell arteritis: duration and adverse outcomes. *Arthritis Care Res 49: 703-708.*

51. Hachulla, E., Boivin, V., Pasturel-Michon, U., Fauchais, A., Bouroz-Joly, J., Perez-Cousin, M., Hatron, P., and Devulder, B. 2001. Prognostic factors and long term evolution in a cohort of 133 patients with giant cell arteritis. *Clin. Exp. Rheumatol. 19: 171-176.*

52. Stone J.H., Tuckwell K., Dimonaco S., Klearman M., Aringer M., Blockmans D., Brouwer E., Cid M.C., Dasgupta B., Rech J., Salvarani C., Schulze-Koops H., Schett G., Spiera R., Unizony S.H., Collinson N. Glucocorticoid Doses and Acute-Phase Reactants at Giant Cell Arteritis Flare in a Randomized Trial of Tocilizumab. *Arthritis Rheumatol. 71:1329-1338.*

53. Camellino D., Solano S., Cutolo M., Cimmino M.A. 2018. Dissecting the inflammatory response in polymyalgia rheumatica: the relative role of IL-6 and its inhibition. *Rheumatol. Int. 38:1699-1704*

54. Visvanathan, S., Rahman, M.U., Hoffman, G.S., Xu, S., García-Martínez, A., Segarra, M., Lozano, E., Espígol-Frigolé, G., Hernández-Rodríguez, J., and Cid, M.C. 2011. Tissue and serum markers of

inflammation during the follow-up of patients with giant-cell arteritis—a prospective longitudinal study. *Rheumatology (Oxford) 50: 2061-2070.*

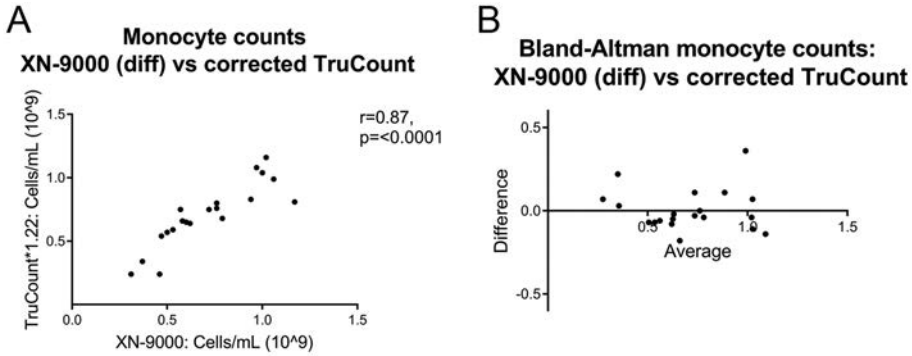
55. Kermani, T.A., Warrington, K.J., Cuthbertson, D., Carette, S., Hoffman, G.S., Khalidi, N.A., Koening, C.L., Langford, C.A., Maksimowicz-McKinnon, K., McAlear, C.A., Monach, P.A., Seo, P., Merkel, P.A., Ytterberg, S.R., and Vasculitis Clinical Research Consortium. 2015. Disease Relapses among Patients with Giant Cell Arteritis: A Prospective, Longitudinal Cohort Study. *J. Rheumatol. 42: 1213-1217.*

56. Mohan, S.V., Liao, Y.J., Kim, J.W., Goronzy, J.J., and Weyand, C.M. 2011. Giant cell arteritis: immune and vascular aging as disease risk factors. *Arthritis Res. Ther. 13: 231.*

57. Lensen, K., Voskuyl, A., Comans, E., van der Laken, C., and Smulders, Y. 2016. Extracranial giant cell arteritis: a narrative review. *Neth. J. Med. 74: 182-192.*

58. Kotzé, S., Pedersen, O., Petersen, M., Sørensen, E., Thørner, L., Sørensen, C., Rigas, A., Hjalgrim, H., Rostgaard, K., and Ullum, H. 2016. Low-grade inflammation is associated with lower haemoglobin levels in healthy individuals: results from the Danish blood donor study. *Vox Sang. 111: 144-150.*

SUPPLEMENTARY DATA



Supplementary Figure 1. Comparison between two methods of measuring monocyte counts. The first method is the XN-9000 (diff) and the second method is the corrected Trucount values presented were calculated by multiplying with 1.22. **A**, Correlation between the two measurement methods for 20 samples. **B**, Bland-Altman plot showing the agreement between the two different methods for 20 samples.

A

GCA	Neutro-phils	Mono-cytes	NK-cells	B-cells	CD4+ T-cells	CD8+ T-cells	CRP	ESR	Hb	N
Neutrophils										18
Monocytes	0,15									34
NK-cells	-0,46	0,21								38
B-cells	0,20	-0,07	0,10							38
CD4+ T-cells	0,32	-0,06	-0,18	0,60**						38
CD8+ T-cells	-0,05	0,35*	0,15	0,54**	0,21					38
CRP	0,17	0,57**	0,12	-0,25	-0,02	-0,22				42
ESR	0,05	0,39*	0,25	-0,13	-0,08	-0,26	0,80**			41
Hb	0,10	-0,14	0,06	0,28	0,10	0,26	-0,39*	-0,51**		42
Platelets	0,08	0,29	0,09	-0,09	0,20	-0,10	0,49**	0,54**	-0,39*	40

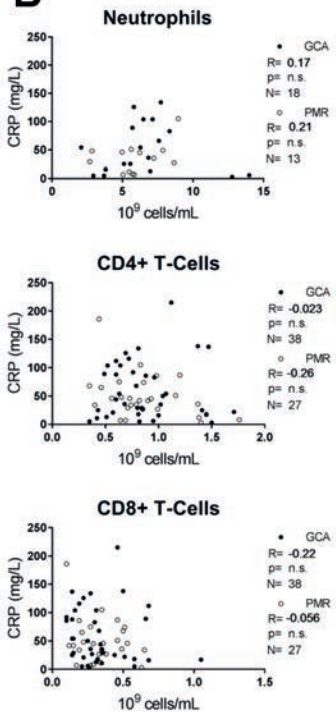
PMR	Neutro-phils	Mono-cytes	NK-cells	B-cells	CD4+ T-cells	CD8+ T-cells	CRP	ESR	Hb	N
Neutrophils										13
Monocytes	0,19									29
NK-cells	0,23	0,33								29
B-cells	0,17	0,29	0,41*							29
CD4+ T-cells	0,41	0,11	0,18	0,59**						29
CD8+ T-cells	0,11	-0,17	-0,03	0,23	0,14					29
CRP	0,21	-0,10	-0,52**	-0,55**	-0,26	-0,06				29
ESR	0,64*	-0,40*	-0,22	-0,19	-0,25	0,02	0,36			30
Hb	-0,49	0,45*	0,21	0,23	0,01	-0,16	-0,23	-0,65**		30
Platelets	0,43	-0,01	-0,26	-0,04	0,41*	-0,01	0,35	0,13	-0,29	30

INF	Neutro-phils	Mono-cytes	NK-cells	CD19+	CD4+ T-cells	CD8+ T-cells	CRP	ESR	Hb	N
Neutrophils										9
Monocytes	0,40									15
NK-cells	-0,18	0,20								16
CD19+	0,16	0,01	0,07							16
CD4+ T-cells	0,24	0,08	0,21	0,74**						16
CD8+ T-cells	-0,08	0,28	0,28	-0,05	0,30					16
CRP	0,70*	-0,13	-0,47	-0,20	-0,19	-0,42				16
ESR	0,34	0,12	-0,73*	-0,39	-0,25	-0,13	0,83**			9
Hb	-0,15	0,01	0,42	0,32	0,38	0,02	-0,62**	-0,79*		16
Platelets	0,51	0,24	-0,33	0,40	0,44	0,35	0,31	0,55	-0,31	15

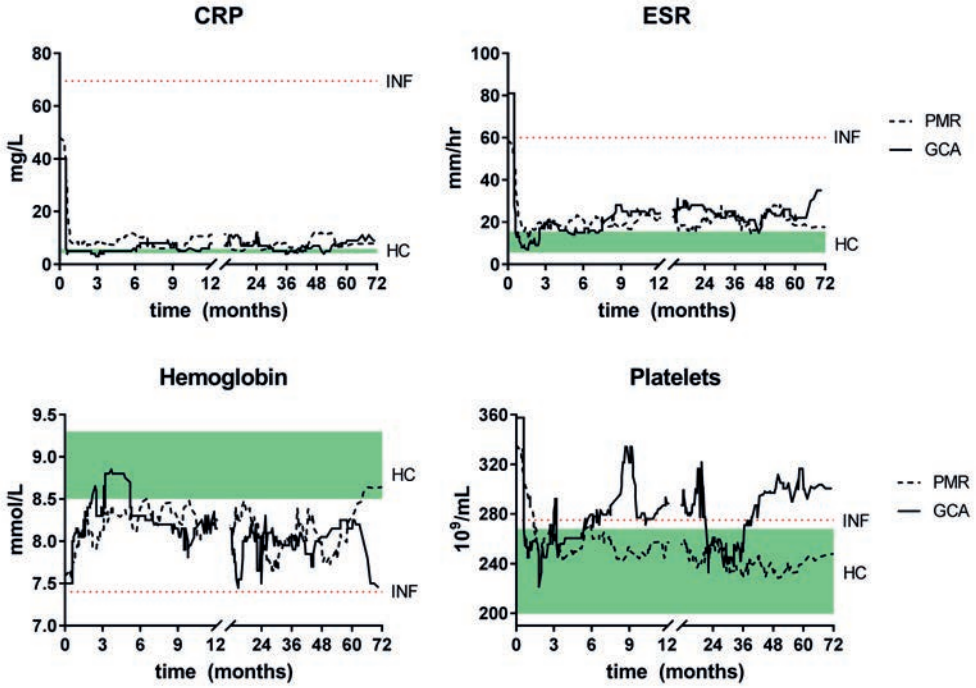
Legend:

Spearman R =
- >0,75
- >0,5
- >0,25
- < -0,25
- < -0,5
- < -0,75

B



Supplementary Figure 2. A: Spearman's correlation coefficients for leukocyte subset counts and disease activity parameters in pre-treatment GCA (N=42) and PMR (N=31) patients, and in INF (N=16). The N in the figure depicts the number of measurements. Strength of the correlation is indicated by the cell colors. Significant correlations are flagged by * ($p<0.05$) and ** ($p<0.01$). **B:** Correlations between three leukocyte subsets and the inflammatory marker CRP. Correlations between CRP and the leukocyte subset in pre-treatment GCA (closed circles) and PMR (open circles) patients. Spearman's R, the p-value of the correlation and the N are indicated in each graph for GCA and PMR. Regression line for GCA is shown as an uninterrupted line, for PMR as a dotted line.



Supplementary Figure 3. Moving median of disease activity markers for GCA and PMR patients over time while on GC treatment. For interpretation of the data the interquartile range of HC (green box, cross sectional measurement) and the median of the INF (dotted red line, cross sectional measurement) were added to the figures. Time point 0 indicates the pre-treatment sample. GCA: giant cell arteritis, PMR: polymyalgia rheumatica, HC: healthy control, INF: infection control.

vvan Sleen Y*,

Wang Q*,

van der Geest KSM,

Westra J, Abdulahad WH,

Heeringa P,

Boots AMH#,

Brouwer E#

* Shared first author
# Shared last author

THREE

Involvement of Monocyte Subsets in the Immunopathology of Giant Cell Arteritis

Scientific Reports. 2017 26;7(1):6553

ABSTRACT

Monocytes/macrophages are critical in systemic and local inflammation in giant cell arteritis (GCA) and possibly in clinically overlapping polymyalgia rheumatica (PMR). Therefore, we aimed to understand the contribution of monocyte subsets and the CX3CR1-CX3CL1 and CCR2-CCL2 migratory pathways, to the pathology of GCA.

Peripheral blood monocytes were enumerated in samples from newly-diagnosed, untreated GCA and PMR patients and after prednisone-induced remission. The distribution of classical (CD14brightCD16neg) and the more pro-inflammatory, intermediate (CD14brightCD16+) and non-classical (CD14dimCD16+) monocyte subsets was analysed by flow cytometry. The phenotype of macrophages in temporal artery biopsies (TABs) from GCA patients was studied by immunohistochemistry and immunofluorescence.

A clear monocytosis was seen in newly diagnosed GCA and PMR patients caused by elevated numbers of classical monocytes. Prednisone treatment suppressed numbers of non-classical monocytes. Both chemokine CX3CL1 and CCL2 were highly expressed in the TAB. Most macrophages in the TAB of GCA patients expressed non-classical monocyte markers CD16 and CX3CR1 whereas co-localization of CD16 with classical monocyte marker CCR2 was infrequent.

In conclusion, we report an altered distribution of monocyte subsets in both GCA and PMR patients. The majority of macrophages in TABs of GCA patients were CD68+CD16+CX3CR1+CCR2- and thereby resembled the phenotype of non-classical monocytes.

INTRODUCTION

Giant cell arteritis (GCA) is an immune mediated vasculitis characterized by granulomatous infiltrates in the vascular wall of medium and large arteries causing vascular occlusion leading to blindness or stroke. GCA is not solely a ''headache disease" (cranial GCA (C-GCA)) but can present with systemic vessel inflammation (large vessel GCA (LV-GCA)). Both C-GCA and LV-GCA patients can have signs and symptoms of polymyalgia rheumatica (PMR), which is characterized by pain and stiffness of both shoulders and hips and by systemic inflammation. PMR is observed in 50% of GCA patients and 15% of patients with PMR may develop GCA when left untreated. As GCA and PMR develop in persons over 50 years of age, with a median age of 70 at onset, it has been suggested that ageing-associated changes of the immune system may be involved [1-3]. Glucocorticoid treatment is currently the first choice for clinical management of GCA and PMR, but long-term glucocorticoid treatment is associated with severe side effects [4]. An improved understanding of the immunopathogenesis of GCA and PMR may eventually lead to highly needed alternative treatment options for GCA and PMR patients.

The immunopathogenesis of both GCA and PMR is not yet well understood. There is consensus, however, that GCA pathology is initiated by local dendritic cell activation followed by infiltration of the vessel wall by CD4+ T-cells and monocytes/macrophages via the vasa vasorum [5]. Within the vessel wall, migrated monocytes/macrophages produce pro-inflammatory cytokines and matrix metalloproteases causing severe vascular damage. Monocytes, the precursors of tissue infiltrating macrophages, are phagocytes generated in the bone marrow from which they are released into the bloodstream where they circulate for several days [6]. Three monocyte subsets can be distinguished by phenotypic and functional characteristics: classical monocytes (CD14brightCD16neg), intermediate monocytes (CD14brightCD16+) and non-classical monocytes (CD14dimCD16+) [7]. CD14brightCD16neg classical monocytes represent the most abundant subset in the peripheral blood whereas the pro-inflammatory CD16+ subsets (both intermediate and non-classical) are less frequent [8]. CD16+ monocytes are the more mature cells compared to the classical monocytes; a developmental relationship has been established, and their numbers increase with age [9]. Importantly, increased proportions of CD16+ monocytes have been associated with numerous vascular and inflammatory diseases like RA [10], sarcoidosis [11, 12], SLE [8] and ANCA-associated vasculitis [13, 14].

To study the contribution of monocytes/macrophages to the immunopathogenesis of GCA, it is crucial to understand the monocyte subsets as precursors of the tissue macrophages and their chemokine directed migration in this disease. Tissue migration of different monocyte subsets is determined by differential expression of chemokine receptors [15]. Classical monocytes show a marked CCR2brightCX3CR1dim expression whereas non-classical monocytes show CCR2negCX3CR1bright expression [16]. Also, CD16+ monocytes show an increased capacity to adhere to endothelial cells and thereby more readily migrate across the endothelium when compared to CD16neg monocytes [17, 18]. Migration of CD16+ monocytes is guided by fractalkine (CX3CL1) – CX3CR1 interaction and inhibition of this interaction reduces transmigration [19, 20].

So far, the distribution of the three monocyte subsets in GCA and PMR patients has not been studied. Moreover, as CD16+ monocytes are pro-inflammatory and increase with age, we hypothesized that these monocytes preferentially migrate to the vascular wall and contribute to GCA pathogenesis. We therefore studied monocyte subset distribution in newly diagnosed GCA and PMR patients and effects of prednisone treatment on these subsets. Next, we assessed whether CD16 was expressed by macrophages in temporal artery biopsies (TABs) of GCA patients. Lastly, we investigated expression of defined chemokine receptors and their ligands in peripheral blood of GCA and PMR patients and in temporal arteries of GCA patients.

MATERIALS AND METHODS

Study populations

Peripheral blood (PB) analysis: in a prospective study design, 42 patients who were newly diagnosed as having GCA (n = 22) or PMR (n = 20) were consecutively enrolled (Table 1, supplementary Table 1AB). None of the patients were receiving glucocorticoids (prednisone) or disease-modifying anti-rheumatic drugs (DMARDs) at the time of blood withdrawal. Blood samples were obtained before noon and all donors were non-fasted. nGCA patients either had a positive TAB and/or positive 18F-fluorodeoxyglucose-positron emission tomography-computed tomography (FDG PET-CT). nPMR patients fulfilled the Chuang/Hunder criteria or showed a positive FDG PET-CT scan for PMR [21]. Diagnosis of the PMR patients did not change to GCA during a follow-up period of at least 6 months. As controls, we obtained blood samples from 24 age-matched, healthy controls (HC) who were screened for past or actual morbidities (Table 1 and supplementary Table 1C).

We obtained 30 follow-up samples of GCA (n=15) and PMR (n=15) patients, who were in remission after 3 months of prednisone treatment. Remission was defined as absence of clinical signs and symptoms and a normal erythrocyte sedimentation rate (ESR) (<30 mm/hr) and/ or c-reactive protein (CRP) <5 mg/L.

TAB immunohistochemistry study: TAB were obtained from a total of 16 biopsy positive GCA patients. Eight biopsy positive TAB were included from the 11 TAB available from the PB cohort. In addition, 8 nGCA patients who had a positive TAB were included in the study (supplementary Table 1A).

Written informed consent was obtained from all study participants. All procedures were in compliance with the declaration of Helsinki. The study was approved by the institutional review board of the UMCG (METc2012/375 for HC and METc2010/222 for GCA and PMR patients).

Patients treatment

GCA patients were initially treated with 40-60 mg/day (median dose; range 30-60) and PMR patients with 15-20 mg/day (median dose; range 10-40) of prednisone, respectively. Tapering of prednisone treatment was started after 2-4 weeks, based on normalization of clinical signs and symptoms together with normalization of the ESR and/or CRP. After 3 months, the median prednisone dose was 25 mg/day (range 15-50) in GCA patients and 15mg/day (range 5-17.5) in PMR patients.

Table 1. Baseline characteristics of patients and controls in the peripheral blood study. Shown are characteristics of newly diagnosed glucocorticoid/DMARD-free GCA (nGCA) and PMR patients (nPMR) and of healthy controls (HC). Five out of 22 GCA patients also had PMR. In 14 out of 20 PMR patients LV-GCA was excluded based on PET-CT. Importantly, the diagnosis of PMR did not change into GCA during a minimal follow-up of 6 months. The Kruskal-Wallis test was performed to compare data among the three study groups. The Mann-Whitney U test was used to compare each patient group with HC. P-values of less than 0.05 (2-tailed) were considered statistically significant..

	nGCA (N = 22)	**nPMR (N = 20)**	**HC (N = 24)**
Age (yr); Median (range)	71 (52-81)	75 (54-84)	72 (53-83)
Females (%)	73	70	75
GCA diagnosis:			
PET-CT/TAB/PET-CT+TAB	11/5/6	NA	NA
PMR diagnosis:			
PET-CT/Chuang/ PET-CT+Chuang	1/0/4	1/6/13	NA
Leukocytes (10^9/L); Median (Range)	9.2 (5.0-18.4) p<0.0001	8.7 (4.5-14.4) p=0.0011	6.0 (4.2-9.6)
Hb (mmol/L); Median (Range)	7.0 (5.5-8.5) p<0.0001	7,5 (5.6-9.3) p<0.0001	8,7 (7.2-9.9)
ESR (mm/h); Median (Range)	65 (31-118) p<0.0001	52 (30-124) p<0.0001	12 (2-30)
CRP (mg/L); Median (Range)	47 (11-138) p<0.0001	44 (7-186) p<0.0001	2 (2-5)

yr = years. PET-CT = positron emission tomography-computed tomography. TAB = temporal artery biopsy. Hb = haemoglobin, ESR = erythrocyte sedimentation rate. CRP = C-reactive protein. NA = not applicable

Flow cytometry

Absolute numbers of monocytes in freshly drawn PB samples were determined by BD MultiTest TruCount, as described by the manufacturer. Data were acquired on a FACS Canto-II (BD Biosciences) and analysed with FACSCanto Clinical Software (BD). PB mononuclear cells (PBMC) were isolated from fresh heparinized blood with Lymphoprep (Axis-Shield), frozen in medium with 10% DMSO/ FCS, and stored in liquid Nitrogen for analysis at a later date. Thawed PBMCs were stained with the following mAb to quantify monocyte subsets and their chemokine receptor expression: CD3, CD14, CD16, CD19, CD56, CD66b, CCR2 and CX3CR1 (supplementary Table 3). Proper isotype controls were included. Cells were fixed and analysed using a LSR-II (BD) flow cytometer. Kaluza software (BD) was used for analysis. Classical (CD14brightCD16neg), intermediate (CD14brightCD16+) and non-classical (CD14dimCD16+) monocytes subsets were gated as previously described [22].

Immunohistochemistry (IHC)

TAB were obtained from 16 biopsy confirmed GCA patients (Supplementary Table 1A). The tissue was fixed in formalin and paraffin embedded. Tissue sections of 3 μm were deparaffinized and rehydrated. After antigen retrieval and endogenous peroxidase blocking, sections were incubated with anti-human primary antibodies detecting cellular markers CD16, CD68, CD56, CCR2 and

CX3CR1 and the chemokines CCL2 and CX3CL1 (product information in supplementary Table 4). Proper isotype controls were included. Next, slides were incubated with secondary antibody rabbit anti-mouse HRP (DAKO P0260) or goat anti-rabbit HRP (DAKO P0448). Following washing, slides were incubated with peroxidase (DAKO, Carpinteria, CA, USA, P0448 and DAKO P0260). After detection of peroxidase activity with 3-amino-9-ethylcarbazole, slides were counterstained with haematoxylin. Since the temporal artery vessel walls of GCA contain skip lesions, detection of CD16-, CD68-, CD56-, CCR2-, CX3CR1- expressing cells (irrespective of intensity) in affected areas was semi-quantitatively scored on a five-point scale (0-4): 0 = no positive cells, 1 = occasional positive cells (0-1% estimated positive), 2 = low numbers of positive cells (>1-20%), 3 = moderate numbers of positive cells (>20-50%), 4 = high numbers of positive cells (more than 50%). Affected regions containing infiltrating cells were scored. Scoring was performed by two independent investigators, trained by a pathologist and average scores were calculated.

For quantification of tissue chemokine expression, stained sections were scanned using a Nanozoomer Digital Pathology Scanner (NDP Scan U10074-01, Hamamatsu Photonics K.K., Hamamatsu, Japan). Positivity of the staining was quantified (positive pixels/total number of pixels) for representative areas of the three vessel layers (total area>1x104μm2) within infiltrated areas using software of Aperio ImageScope (V11.2.0.780 Aperio Technologies, CA, USA).

Immunofluorescence

To investigate if CD68 and CD16, CD16 and CX3CR1 or CD16 and CCR2 are co-expressed by cells in situ, double-labelling immunofluorescence stainings were performed. Formalin-fixed paraffin-embedded TAB tissue was deparaffinized and antigen retrieval was performed. Dilutions (1:50) of anti-CD68, anti-CD16, anti-CCR2, or anti-CX3CR1 antibodies were added and incubated overnight. Following washing, FITC-labelled goat anti-rabbit IgG (A11008, Lifetechnologies, Carlsbad, CA, USA) and AF555-labeled donkey anti-mouse IgG (A31570, Lifetechnologies) were used as the secondary antibodies, respectively. DAPI (10236276001, Roche Life Science, Penzberg, Upper Bavaria, Germany) was performed to stain nuclei. Images were taken using Leica DFC345 FX.

Serum chemokine measurements

Serum levels of CCL2 and CCL11 (ligands of CCR2) and CX3CL1 and CCL26 (ligands of CX3CR1) were measured by Human premix Magnetic Luminex screening assay kit (R&D system, Minneapolis, MN, USA) according to the manufacturers' instructions. The assay was read by the Luminex LX100™ (Luminex, Austin, TX, USA) multiplex assay detection system. Raw data were analysed using Star Station V2.3. Lower and upper detection limits for the chemokine assays are 3-7936 pg/mL for CCL2, 8-384218 pg/mL for CX3CL1, 2-5963 pg/mL for CCL26 and 8-30043 pg/mL for CCL11.

Statistical analysis

Since flow cytometry data and serum cytokine data are not normally distributed, non-parametric tests were used for data analysis. To compare data among more than two study groups the non-parametric Kruskal-Wallis test was performed. The Mann-Whitney U test was used to compare data

of patient groups with HC. Paired samples (patients at diagnosis and after 3 months of treatment) were compared with the Wilcoxon signed rank test. Analyses were performed with GraphPad Prism 5.0 software. Correlations were assessed using Spearman's rank correlation coefficient. P-values of less than 0.05 (2-tailed) were considered statistically significant.

RESULTS

Monocyte counts are elevated in newly diagnosed GCA and PMR patients

Numbers of circulating monocytes as determined in a standard hematology counter were higher in newly diagnosed GCA (nGCA) and newly diagnosed PMR (nPMR) patients compared to HC (Fig. 1, Table 1). In addition, we assessed the effects of glucocorticoids after 3 months on treatment. Treatment led to normalization of monocyte numbers in remission PMR patients (rPMR) but this was not the case for monocyte numbers in remission GCA (rGCA) patients.

Altered distribution of circulating monocyte subsets in GCA and PMR

As a clear monocytosis was observed in nGCA/nPMR, we next analysed the distribution of the three different monocyte populations defined by CD14 and CD16 expression (Fig. 2A). Our analysis revealed numerical increases of classical monocytes in nGCA and nPMR patients when compared to HC, whereas numbers of intermediate and non-classical monocytes were largely comparable (Fig. 2B, supplementary Fig. 1). The increase in classical monocytes led to proportional decreases of non-classical monocytes in both nGCA and nPMR patients, but did not alter intermediate

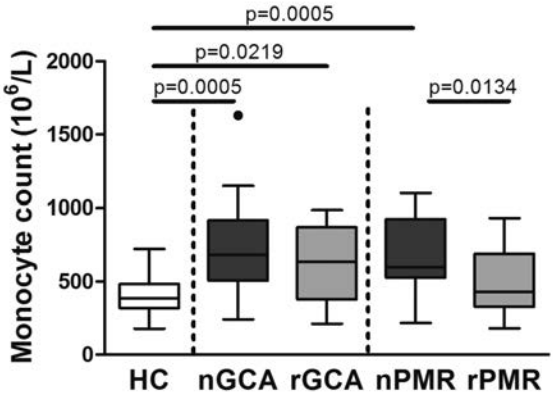


Figure 1. Total monocyte counts are elevated in newly diagnosed GCA and PMR patients. Absolute numbers of monocytes in freshly drawn whole blood obtained from healthy controls (HC, n=20), newly-diagnosed patients with GCA (nGCA; n=21), PMR (nPMR; n=19) and in follow-up samples from patients in remission after 3 months of treatment (rGCA; n=14, rPMR; n=15). Data are expressed as Tukey box and whisker plots. The bottom and top of the box represent the first and third quartiles, and the band inside the box represents the second quartile (the median). The whiskers represent the 1.5 interquartile range (IQR) of the lower and the upper quartiles. The outlier is plotted as a dot. The Mann-Whitney U test was used to compare each patient group with HC. Paired samples (e.g. nGCA vs rGCA and nPMR vs rPMR) were compared with the Wilcoxon signed rank test. P-values of less than 0.05 (2-tailed) were considered statistically significant. P values are indicated in the graph.

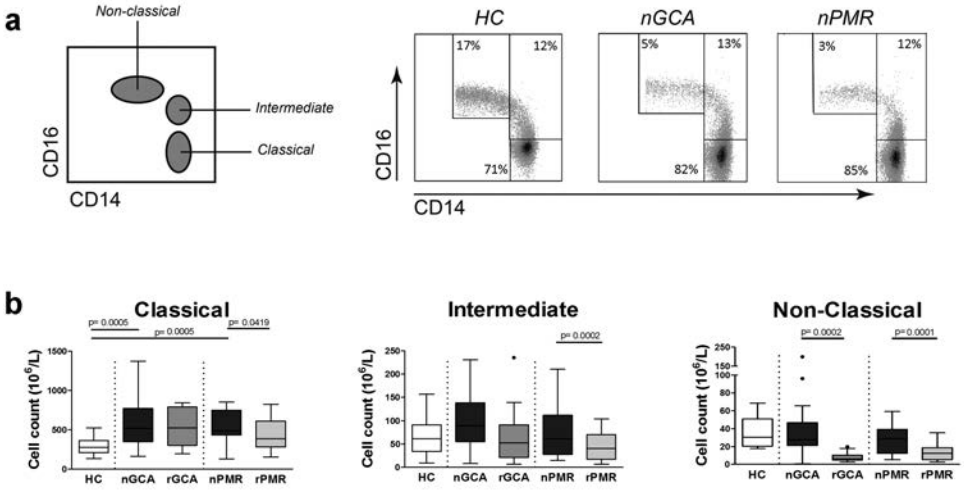


Figure 2. Altered distribution of monocyte subsets in GCA and PMR. **a**, Flow cytometry gating strategy based on CD14 and CD16 expression to distinguish classical (CD14brightCD16neg), intermediate (CD14brightCD16+) and non-classical (CD14dimCD16+) monocytes subsets (left panel). Flow cytometry dot plots showing representative samples (equal numbers of events) from a healthy control (HC), a newly diagnosed GCA patient (nGCA) and a newly diagnosed PMR patient (nPMR) (right panel). **b**, Absolute numbers of classical, intermediate, and non-classical monocytes in HC (HC, n=20), newly-diagnosed patients with GCA (nGCA; n=21) and PMR (nPMR; n=19) and in the follow-up samples of GCA (rGCA; n=14) and PMR (rPMR; n=15) patients in remission after 3 months of glucocorticoid treatment. Data are expressed as Tukey box and whisker plots. The bottom and top of the box represent the first and third quartiles, and the band inside the box represents the second quartile (the median). The whiskers represent the 1.5 IQR of the lower and the upper quartiles. Outliers are plotted as dots. The Kruskal-Wallis test was performed to compare data among study groups. The Mann-Whitney U test was used to compare each patient group with HC. Paired samples were compared with the Wilcoxon signed rank test. P-values of less than 0.05 (2-tailed) were considered statistically significant.

type monocyte proportions (Fig. 2A, supplementary Fig. 1). Glucocorticoid treatment reduced the numbers of all three monocyte subsets in rPMR patients (Fig. 2B). Interestingly, in rGCA patients, 3 months of glucocorticoid treatment reduced numbers of non-classical monocytes but had no effect on numbers of classical and intermediate monocytes. Thus, both nGCA and nPMR patients are characterized by higher numbers of classical monocytes leading to proportional reductions of non-classical monocytes. Remarkably, clinical remission in GCA patients was associated with a clear reduction of non-classical monocytes.

CD16+ macrophages are readily detected in GCA TABs

As monocytes are recruited from the blood to peripheral sites of inflammation, where they differentiate into macrophages, we next investigated the phenotype of tissue macrophages in TABs with findings diagnostic of GCA and evaluated expression of CD16 and of macrophage marker CD68 (Fig. 3A, supplementary Fig. 2 for isotype control stainings). CD68 and CD16 were both abundantly expressed within the infiltrates of the adventitia, media and intima layer of the vessel wall

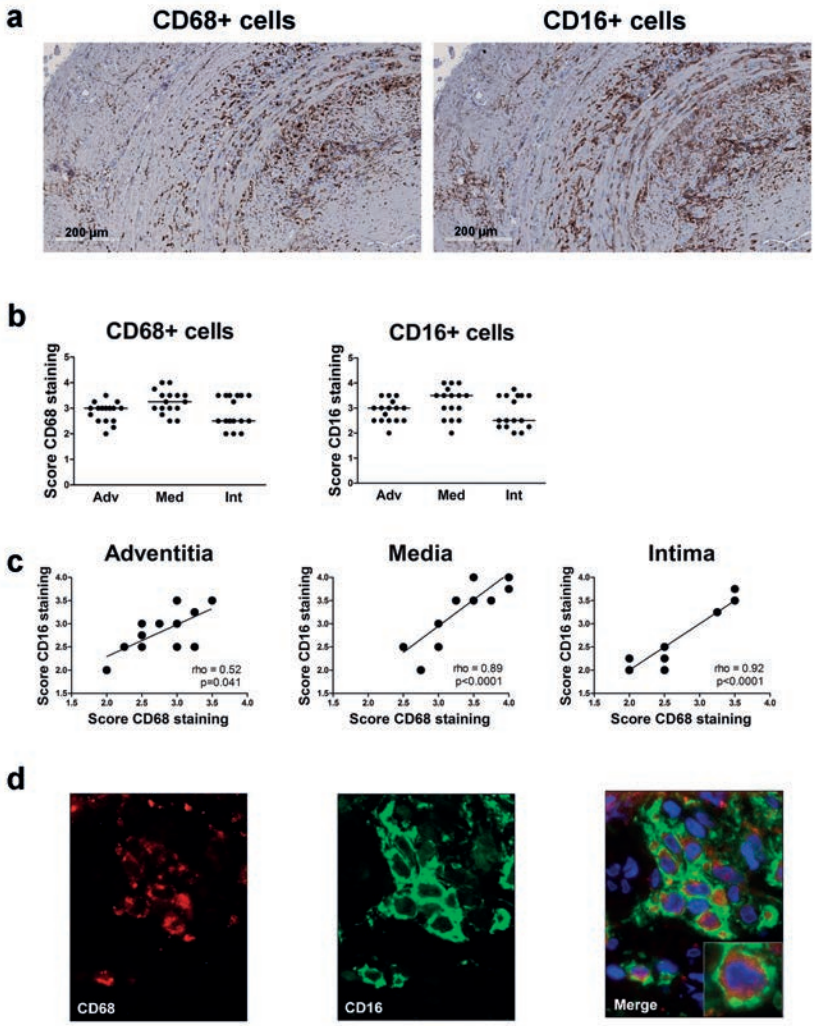


Figure 3. CD16+ cells co-localize with CD68+ macrophages in GCA temporal artery biopsies. **a**, Immunohistochemical staining for CD68 and CD16 in a representative temporal artery biopsy (TAB) diagnostic of GCA. Note that consecutive sections of the same tissue were used in Fig. 3 and Fig. 5. **b**, Semi-quantitative mean scores of CD68+ cells and CD16+ cells in inflammatory areas of GCA TABs (n =16). Scores are given for the adventitia (Adv), media (Med, infiltrating cells only) and intima (Int). Data are presented as scatter plots. The horizontal line indicates the median. c, Positive correlation between CD16 and CD68 scores in intima, media and adventitia in inflammatory areas of GCA TABs (n =16), as determined by Spearman's rank correlation coefficient. Due to overlap in semi-quantitative scores not all 16 scores can be appreciated. d, Single staining for CD68, CD16 and double staining for CD16 and CD68, respectively, from left to right in the inflammatory area of a TAB section. An example of a magnified merged picture is shown for clarity. Blue= DAPI staining of nuclei; Green (FITC)= CD16 expression, Red (AF555) = CD68 expression; a macrophage cytoplasmic granules marker.

(Fig. 3B). CD16 expression was found to overlap with macrophage-rich areas as evidenced by CD68 expression. Indeed, scores of CD16+ and CD68+ cells in adventitia, media and intima were positively correlated (Fig. 3C). To rule out an involvement of NK cells expressing CD16, we stained for CD56 and found this marker to be virtually absent in the vessel wall. To further confirm the co-localization of CD68 and CD16, double immunofluorescence staining was performed. Indeed, within infiltrated regions, substantial co-localization of CD16 and CD68 was found, confirming that the majority of macrophages in the vascular wall express CD16 (Fig. 3D).

Monocyte CCR2 and CX3CR1 expression in GCA and PMR patients

Recruitment of monocytes to tissues is driven by specific chemokine and chemokine receptor interactions. As the three different monocyte subsets can also be distinguished by differences in their chemokine receptor expression profiles, we assessed expression of CCR2 and CX3CR1 on classical, intermediate and non-classical monocytes. In accordance with previous studies [23], classical monocytes demonstrated a high per cell expression (Mean Fluorescence Intensity) of CCR2 with low expression of CX3CR1, while non-classical monocytes demonstrated high per cell expression of CX3CR1 and a lack of CCR2 expression (Fig. 4A, B). Following glucocorticoid treatment, CX3CR1 expression was down-modulated by all monocyte subsets in rGCA and rPMR patients. In contrast, CCR2 expression by monocyte subsets was not sensitive to glucocorticoid treatment (Fig. 4C,D).

Systemic expression of CCR2 and CX3CR1 ligands in GCA and PMR patients

Next, we assessed if serum levels of the relevant chemokines were altered in the patient groups (Supplementary Table 2). Systemic levels of CCL2 and CCL11, the CCR2 receptor ligands, were lower in nGCA patients but normalized after glucocorticoid treatment. A similar pattern was observed in PMR patients but this did not reach statistical significance. In contrast, levels of both the CX3CR1 receptor ligands CX3CL1 (fractalkine) and CCL26 were not altered in GCA and PMR patients when compared to HC. Glucocorticoid treatment upregulated serum levels of CX3CL1 in rGCA patients. Thus, our combined data show a down modulation of CX3CR1 expression by monocyte subsets and a concomitant increase of the soluble form of CX3CL1 in rGCA patients. Also, CCR2 expression by monocyte subsets was not altered in nGCA and nPMR patients when compared to HC (data not shown), but reduced levels of the CCR2 ligands were noted. The latter may be explained by high consumption/binding to increased numbers of circulating CCR2-positive monocytes in newly diagnosed GCA and PMR patients.

Expression of CCR2, CX3CR1 and their ligands in GCA TABs

Next, we investigated expression of CCR2, CX3CR1 and their ligands in TABs of GCA patients. In the inflamed vascular wall, high numbers (median score 3-4) of CX3CR1-positive cells were detected (Fig. 5AB). CX3CR1-positive cells were clearly detected in macrophage-rich areas of adventitia, media and intima. Double staining for CD16 and CX3CR1 showed co-localization of these markers, although different patterns were observed (Fig. 5C). In contrast, only few to moderate numbers of CCR2-positive cells (median scores 2-3) were found in these same areas (Fig. 5A,B). Double staining for CD16 and CCR2 confirmed that CD16+ cells rarely co-localize with CCR2 (Fig. 5D).

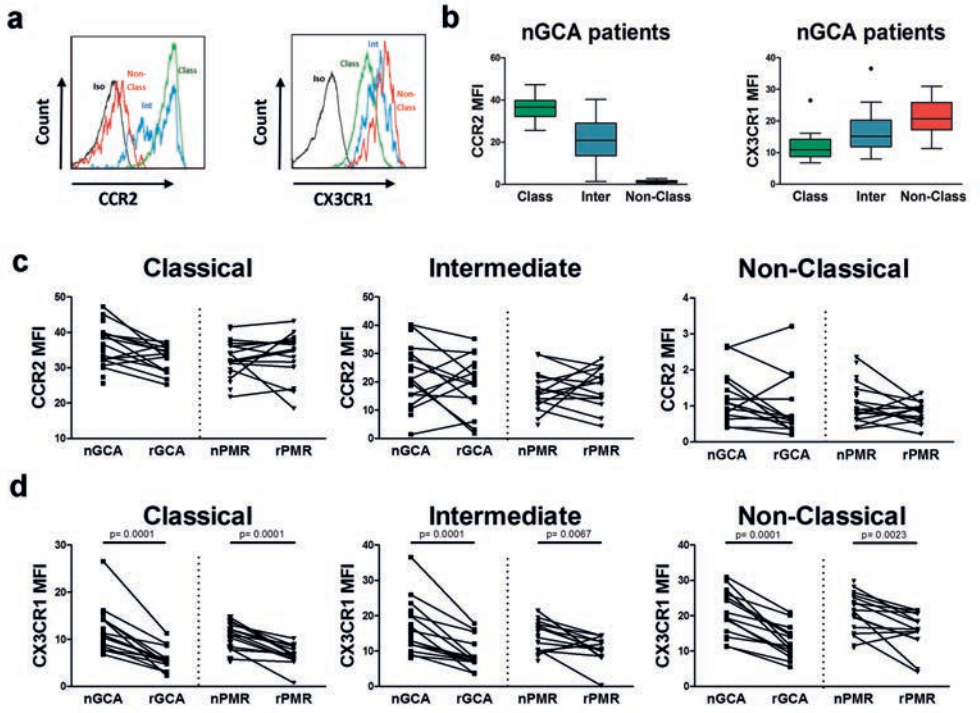


Figure 4. Expression of CCR2 and CX3CR1 by monocyte subsets in peripheral blood of GCA and PMR patients and effects of glucocorticoid treatment. **a**, Representative flow cytometry dot plots of CCR2 and CX3CR1 by classical, intermediate and non-classical monocytes in a GCA patient. **b**, Mean fluorescence intensity (MFI) of CCR2 and CX3CR1 on classical (Class), intermediate (Inter) and non-classical (Non-Class) monocytes in newly diagnosed GCA patients (nGCA; n = 21). Data are expressed as Tukey box and whisker plots. The bottom and top of the box represent the first and third quartiles, and the band inside the box represents the second quartile (the median). The whiskers represent the 1.5 IQR of the lower and the upper quartiles. Outliers are plotted as dots. **c**, Mean fluorescence intensity (MFI) of CCR2 and CX3CR1 (d) on classical, intermediate and non-classical monocytes in newly diagnosed GCA (nGCA; n=21) and PMR (nPMR; n=20) patients and in the follow-up samples of GCA (rGCA; n=15) and PMR (rPMR; n=14) patients in remission after 3 months of glucocorticoid treatment. Data are expressed as dot plots linking individual paired data. The Wilcoxon signed rank test was used to compare paired samples. P values are indicated in the graph.

Next, we analysed tissue expression of chemokines CCL2 and CX3CL1. Both in inflamed and non-inflamed TAB we detected expression of CCL2 and CX3CL1 by VSMCs (Fig. 5E, Supplementary Fig. 3). Quantification of CCL2 and CX3CL1 in inflamed temporal arteries demonstrated expression of both chemokine receptor ligands in all three vessel layers with highest expression in the media (Fig. 5E,F). Thus, the phenotype of tissue macrophages in GCA TABs (CD68+CD16+CX3CR1+CCR2-) resembles the phenotype of the non-classical monocytes in blood.

DISCUSSION

Monocytes/macrophages are critical contributors to inflammatory diseases. Improved understanding of the role of monocyte/macrophages in systemic and local inflammation as seen

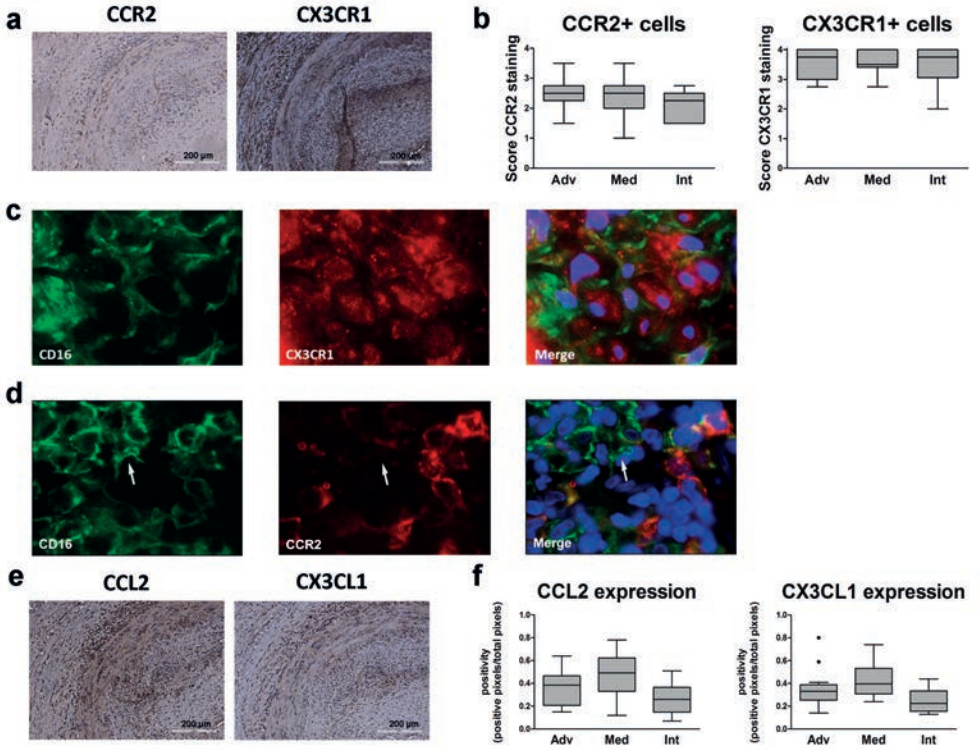


Figure 5. Expression of CCR2, CX3CR1 and their ligands in GCA temporal artery biopsies. **a**, Immunohistochemical (IHC) staining for CCR2 and CX3CR1 in a representative temporal artery biopsy diagnostic of GCA. Note that consecutive sections of the same tissue were used in Fig. 3 and Fig. 4. **b**, Semi-quantitative score of CCR2-, CX3CR1-postive cells in the inflammatory area of GCA TAB (n =15 and n =16, respectively). Intensity of staining was not taken into account. Scores are given for the adventitia (Adv), media (Med, infiltrating cells only) and intima (Int). Data are presented as Tukey box and whisker plots. The bottom and top of the box represent the first and third quartiles, and the band inside the box represents the second quartile (the median). The whiskers represent the 1.5 IQR of the lower and the upper quartiles. **c**, Co-localization of CD16 and CX3CR1 in TAB from GCA patients. Single staining for CD16, CX3CR1 and double staining for CD16 and CX3CR1, respectively from left to right in the inflammatory area of a TAB section. Blue= DAPI staining of nuclei; Green(FITC)= CD16 expression, Red(AF555)= CX3CR1 expression. **d**, CCR2 and CD16 rarely co-localize in TAB from GCA patients. Single staining for CD16, CCR2 and double staining for CD16 and CCR2, respectively from left to right in the inflammatory area of a TAB section. Blue= DAPI staining of nuclei; Green(FITC)= CD16 expression, Red(AF555)= CCR2 expression. **e**, IHC staining for CCL2, CX3CL1 in a representative TAB. **f**, Quantification of staining intensity (positive pixels/ total number of pixels) of CCL2 and CX3CL1 in the inflammatory vessel wall (n = 16 for both markers). Data are presented as Tukey box and whisker plots. The bottom and top of the box represent the first and third quartiles, and the band inside the box represents the second quartile (the median). The whiskers represent the 1.5 IQR of the lower and the upper quartiles. Outliers are plotted as dots.

in GCA and the closely related PMR may provide a rational for novel, prednisone-sparing treatment strategies. Our studies revealed a clear monocytosis in newly diagnosed GCA and PMR patients which is consistent with the notion of inflammation-induced monocyte recruitment from the bone marrow as observed in other inflammatory diseases [24, 25]. When analysing the contribution of the different monocyte subsets to the rise in total blood monocytes, we found numbers of classical monocytes to be substantially increased in both GCA and PMR patients. The increase in classical monocytes led to a relative decrease of non-classical monocytes in these patient groups. Interestingly, glucocorticoid treatment further suppressed non-classical monocytes in GCA and PMR.

The increase of classical monocytes in newly-diagnosed GCA and PMR is likely due to bone marrow production of new monocytes in response to inflammation. Although all three monocyte subsets can be found in the bone marrow [26, 27], monocyte recruitment from the bone marrow is mainly driven by the CCL2/CCR2 pathway which would explain preferential recruitment of classical monocytes expressing high levels of CCR2 [28]. In line with this, we found systemic CCL2 levels decreased in newly diagnosed GCA patients, suggesting binding of this ligand to increased numbers of classical monocytes. Glucocorticoid treatment normalized serum levels of CCL2. A similar pattern was observed in PMR patients.

Although monocytosis is a characteristic of many inflammatory diseases such as RA [24], Crohn's disease [25] and sepsis [29], several further studies in these indications report on elevated numbers or proportions of CD16+ monocytes [30-32]. In contrast, we did not detect an increase in numbers and proportions of CD16+ monocytes in newly diagnosed GCA and PMR patients. Rather, a proportional decrease of non-classical monocytes particularly, was noted. This is remarkable as classical monocytes are expected to develop into intermediate and non-classical monocytes. The lack of an increase in non-classical monocytes could be due to blunted (cause unknown) differentiation of intermediate monocytes to non-classical monocytes [9, 33]. M-CSF and GM-CSF are thought to be the most potent inducers of differentiation towards the non-classical phenotype [12, 34, 35]. Better understanding of M-CSF and GM-CSF signalling in GCA and PMR patients may elucidate whether differentiation to non-classical monocytes is blunted in GCA and PMR. Alternatively, a selective loss of non-classical monocytes through spontaneous apoptosis, to which the CD16+ monocytes are more susceptible [36] may underlie the proportional decrease of non-classical monocytes, a notion to be further explored.

Previous studies on the role of non-classical monocytes in homeostasis and in inflammation have revealed that non-classical monocytes actively patrol the vascular endothelium and are preferentially found in the marginal pool [37, 38]. Indeed, non-classical monocytes demonstrate an increased capacity to adhere to endothelial cells by virtue of the adhesion-related CX3CR1 which binds to the membrane-bound form of fractalkine (CX3CL1) expressed by endothelial cells [38, 39]. In the resting state, they clear damaged cells and debris and in infection or inflammation they are thought to be important in resolution of inflammation. Yet, in several disease conditions, non-classical monocytes may aggravate disease, possibly due to their pro-inflammatory potential and following in situ reprogramming [8, 30]. More recently, Mukherjee et al showed that untouched non-classical monocytes indeed become pro-inflammatory upon activation [8, 40]. In line with

this, non-classical monocytes are potent producers of the pro-inflammatory cytokine IL-6, which is a key molecule in the immunopathogenesis of GCA and PMR [41]. Indeed, elevated levels of IL-6 are found in the serum of newly diagnosed patients [5, 42]. Taken together, the relative decrease of non-classical monocytes in GCA and PMR may be explained by an enhanced accumulation of non-classical monocytes in the marginal pool [37], thereby facilitating preferential migration of non-classical monocytes to sites of inflammation in GCA and PMR. The latter notion was studied here in GCA TABs only.

We are the first to describe a massive accumulation of CD16+ macrophages in TAB of GCA patients. CD16+ macrophages were found in all layers of the vascular wall. As the adventitia is considered to be the site of primary immunological activation in GCA [43], this transmural inflammation is likely achieved following entry via the vasa vasorum in the adventitial layer, progressing to the medial and intimal layers of the vascular wall. Accumulation of CX3CR1+CD16+ macrophages, rarely co-localizing with CCR2, indicates that most of the macrophages in the GCA TAB resemble the phenotype of non-classical monocytes in blood. Still, we cannot exclude the possibility that both CD16 and CX3CR1 expression can be acquired after migration of classical monocytes to tissue. Also, classical monocytes may gain CX3CR1 and lose CCR2 upon migration to tissue [44]. Thus, it remains to be established if non classical monocytes are the only precursors of tissue macrophages in GCA.

Previously, it was proposed that local proliferation of tissue-resident precursors may give rise to macrophage expansion at local sites rather than infiltration by monocytes [45]. We investigated this option but found that staining for the proliferation marker ki-67 was largely negative in TABs of GCA patients (data not shown), thereby excluding this notion.

The strong local expression of CX3CL1 in the GCA TAB is consistent with massive expression of CX3CR1. The presence of CD16+ monocytes in the tissue of GCA may thus be guided by the CX3CR1-CX3CL1 chemokine axis. Although CCL2 is also expressed in lesions of temporal artery tissue, it seems that CCL2 is less important for the migration of CD16+ monocytes. In early in vitro migration studies, it was noted that non-classical monocytes failed to migrate in response to CCL2, consistent with the absence of CCR2 on these cells [46]. It was also evidenced that in the absence of CCL2 action, i.e., in CCR2-/- mice, monocytes can still traffic into sites of infection [28]. Importantly, many studies described the importance for chemokine receptor CX3CR1 for tissue migration [19, 47, 48].

Our study illustrated that glucocorticoid treatment led to clear reductions of non-classical monocyte counts in both GCA and PMR patients. Glucocorticoid treatment has been described to induce apoptosis of non-classical monocytes via a caspase-dependent mechanism [49, 50]. Selective glucocorticoid -induced apoptosis of non-classical monocytes may be explained by high expression of the glucocorticoid receptor in this monocyte subset [49]. Whether glucocorticoids also deplete the CD16+ macrophages in the GCA temporal artery, or in PMR synovial tissues, awaits further studies.

Glucocorticoid treatment led to clear reduction of CX3CR1 per cell expression by peripheral blood monocytes and an up regulation of serum CX3CL1. Thus, glucocorticoid treatment may have a dual effect. Induction of apoptosis on the one hand and reduced adhesion to endothelium by non-classical monocytes through suppression of CX3CR1 expression on the other hand; both effects will hamper the influx of new CD16+ monocytes to the tissue. Interestingly, we found a differential

response of monocyte subsets to glucocorticoid treatment in PMR patients. All three subsets were down modulated in PMR patients in remission at 3 months. This is an unexpected finding as the accumulated glucocorticoid dose is substantially lower in PMR patients. An unexplored possibility is that classical monocytes in PMR are more sensitive to glucocorticoid treatment due to their altered characteristics; a previous study showed an altered functionality of monocytes from PMR patients when compared with monocytes from GCA patients and controls [51].

GCA and PMR are clinically closely related. Evidence for a common immunopathology, however, is lacking due to a paucity of histological data on TAB and synovial tissues obtained from PMR patients. Although our study showed similar changes in circulating monocyte subsets in both diseases, the local involvement of CD16+ monocytes/macrophages in PMR tissues remains to be established.

Other limitations, imposed by logistical constraints, involve the use of thawed PBMCs for the determination of monocyte subset numbers. Whole blood monocyte counts were established using a hematology counter and monocyte subsets were analysed on a later date using thawed samples of liquid nitrogen stored PBMCs (isolated on the day of blood withdrawal). Percentages of monocyte subsets were related to the whole blood monocyte counts to calculate the numbers of the monocyte subsets. We cannot exclude differential loss of cells during PBMC isolation or due to the freezing/thawing procedure, although this would not be expected to differ between patients and controls.

We show an altered monocyte subset distribution in GCA and PMR with a relative decrease of non-classical monocytes. Moreover, macrophages in temporal arteries of GCA patients were found to resemble the phenotype of non-classical monocytes. The data can be taken to suggest that driven by the CX3CL1 chemokine non-classical monocytes infiltrate the arterial wall and develop into an inflammatory population of CD68+CD16+CX3CR1+CCR2- macrophages in GCA. Glucocorticoids reduce the number of non-classical monocytes in blood and their expression of the CX3CR1 receptor, supporting a concept that the influx of new monocytes into the vessel wall is decreased by glucocorticoids leading to the eventual resolution of local inflammation. New insights into the role of monocytes/macrophages in systemic and local inflammation as seen in GCA and the clinically overlapping PMR may provide a rational towards novel treatment strategies and help the identification of highly awaited, disease-specific biomarkers.

ACKNOWLEDGEMENTS

We thank Johan Bijzet, Berber Doornbos-van der Meer, Henk Moorlag, Mirjam P. Roffel and Gerda Horst for their technical support. We thank Arjan Diepstra, pathologist, for his expert advice in examining and scoring GCA pathology.

REFERENCES

1. Salvarani, C., Cantini, F., and Hunder, G.G. 2008. Polymyalgia rheumatica and giant-cell arteritis. *Lancet 372: 234-245*.
2. Dejaco, C., Duftner, C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2016. The spectrum of giant cell arteritis and polymyalgia rheumatica: revisiting the concept of the disease. *Rheumatology (Oxford) 56(4):506-515*.
3. Mohan, S.V., Liao, Y.J., Kim, J.W., Goronzy, J.J., and Weyand, C.M. 2011. Giant cell arteritis: immune and vascular aging as disease risk factors. *Arthritis Res. Ther. 13: 231*.
4. Buttgereit, F., Dejaco, C., Matteson, E.L., and Dasgupta, B. 2016. Polymyalgia Rheumatica and Giant Cell Arteritis: A Systematic Review. *JAMA 315: 2442-2458*.
5. Weyand, C.M. and Goronzy, J.J. 2013. Immune mechanisms in medium and large-vessel vasculitis. *Nat. Rev. Rheumatol. 9: 731-740*.
6. Varol, C., Landsman, L., Fogg, D.K., Greenshtein, L., Gildor, B., Margalit, R., Kalchenko, V., Geissmann, F., and Jung, S. 2007. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J. Exp. Med. 204: 171-180*.
7. Ziegler-Heitbrock, L. and Hofer, T.P. 2013. Toward a refined definition of monocyte subsets. *Front. Immunol. 4: 23*.
8. Mukherjee, R., Kanti Barman, P., Kumar Thatoi, P., Tripathy, R., Kumar Das, B., and Ravindran, B. 2015. Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous. *Sci. Rep. 5: 13886*.
9. Rogacev, K.S., Zawada, A.M., Hundsdorfer, J., Achenbach, M., Held, G., Fliser, D., and Heine, G.H. 2015. Immunosuppression and monocyte subsets. *Nephrol. Dial. Transplant. 30: 143-153*.
10. Rossol, M., Kraus, S., Pierer, M., Baerwald, C., and Wagner, U. 2012. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheumatol. 64: 671-677*.
11. Dubaniewicz, A., Typiak, M., Wybieralska, M., Szadurska, M., Nowakowski, S., Staniewicz-Panasik, A., Rogoza, K., Sternau, A., Deeg, P., and Trzonkowski, P. 2012. Changed phagocytic activity and pattern of Fcgamma and complement receptors on blood monocytes in sarcoidosis. *Hum. Immunol. 73: 788-794*.
12. Hofer, T.P., Zawada, A.M., Frankenberger, M., Skokann, K., Satzl, A.A., Gesierich, W., Schuberth, M., Levin, J., Danek, A., Rotter, B., Heine, G.H., and Ziegler-Heitbrock, L. 2015. slan-defined subsets of CD16-positive monocytes: impact of granulomatous inflammation and M-CSF receptor mutation. *Blood 126: 2601-2610*.
13. Tarzi, R.M., Liu, J., Schneiter, S., Hill, N.R., Page, T.H., Cook, H.T., Pusey, C.D., and Woollard, K.J. 2015. CD14 expression is increased on monocytes in patients with anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitis and correlates with the expression of ANCA autoantigens. *Clin. Exp. Immunol. 181: 65-75*.
14. O'Brien, E.C., Abdulahad, W.H., Rutgers, A., Huitema, M.G., O'Reilly, V.P., Coughlan, A.M., Harrington, M., Heeringa, P., Little, M.A., and Hickey, F.B. 2015. Intermediate monocytes in ANCA vasculitis: increased surface expression of ANCA autoantigens and IL-1beta secretion in response to anti-MPO antibodies. *Sci. Rep. 5: 11888*.
15. Zimmermann, H.W., Trautwein, C., and Tacke, F. 2012. Functional role of monocytes and macrophages for the inflammatory response in acute liver injury. *Front. Physiol. 3: 56*.
16. Zawada, A.M., Rogacev, K.S., Rotter, B., Winter, P., Marell, R.R., Fliser, D., and Heine, G.H. 2011. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. *Blood 118: e50-61*.
17. Merino, A., Buendia, P., Martin-Malo, A., Aljama, P., Ramirez, R., and Carracedo, J. 2011. Senescent CD14+CD16+ monocytes exhibit proinflammatory and proatherosclerotic activity. *J. Immunol. 186: 1809-1815*.
18. Zimmermann, H.W., Bruns, T., Weston, C.J., Curbishley, S.M., Liaskou, E., Li, K.K., Resheq, Y.J., Badenhorst, P.W., and Adams, D.H. 2016.

Bidirectional transendothelial migration of monocytes across hepatic sinusoidal endothelium shapes monocyte differentiation and regulates the balance between immunity and tolerance in liver. *Hepatology 63: 233-246.*

19. Aspinall, A.I., Curbishley, S.M., Lalor, P.F., Weston, C.J., Blahova, M., Liaskou, E., Adams, R.M., Holt, A.P., and Adams, D.H. 2010. CX(3)CR1 and vascular adhesion protein-1-dependent recruitment of CD16(+) monocytes across human liver sinusoidal endothelium. *Hepatology 51: 2030-2039.*
20. Yano, R., Yamamura, M., Sunahori, K., Takasugi, K., Yamana, J., Kawashima, M., and Makino, H. 2007. Recruitment of CD16+ monocytes into synovial tissues is mediated by fractalkine and CX3CR1 in rheumatoid arthritis patients. *Acta Med. Okayama 61: 89-98.*
21. Chuang, T.Y., Hunder, G.G., Ilstrup, D.M., and Kurland, L.T. 1982. Polymyalgia rheumatica: a 10-year epidemiologic and clinical study. *Ann. Intern. Med. 97: 672-680.*
22. Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D.N., Leenen, P.J., Liu, Y.J., MacPherson, G., Randolph, G.J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J.M., and Lutz, M.B. 2010. Nomenclature of monocytes and dendritic cells in blood. *Blood 116: e74-80.*
23. Gautier, E.L., Jakubzick, C., and Randolph, G.J. 2009. Regulation of the migration and survival of monocyte subsets by chemokine receptors and its relevance to atherosclerosis. *Arterioscler. Thromb. Vasc. Biol. 29: 1412-1418.*
24. Hirohata, S., Yanagida, T., Itoh, K., Nakamura, H., Yoshino, S., Tomita, T., and Ochi, T. 1996. Accelerated generation of CD14+ monocyte-lineage cells from the bone marrow of rheumatoid arthritis patients. *Arthritis Rheumatol. 39: 836-843.*
25. Mee, A.S., Berney, J., and Jewell, D.P. 1980. Monocytes in inflammatory bowel disease: absolute monocyte counts. *J. Clin. Pathol. 33: 917-920.*
26. Mandl, M., Schmitz, S., Weber, C., and Hristov, M. 2014. Characterization of the CD14++CD16+ monocyte population in human bone marrow. *PLoS One 9: e112140.*
27. Shantsila, E., Wrigley, B., Tapp, L., Apostolakis, S., Montoro-Garcia, S., Drayson, M.T., and Lip, G.Y. 2011. Immunophenotypic characterization of human monocyte subsets: possible implications for cardiovascular disease pathophysiology. *J. Thromb. Haemost. 9: 1056-1066.*
28. Serbina, N.V. and Pamer, E.G. 2006. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol. 7: 311-317.*
29. Ziegler-Heitbrock, L. 2015. Blood Monocytes and Their Subsets: Established Features and Open Questions. *Front. Immunol. 6: 423.*
30. Kawanaka, N., Yamamura, M., Aita, T., Morita, Y., Okamoto, A., Kawashima, M., Iwahashi, M., Ueno, A., Ohmoto, Y., and Makino, H. 2002. CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis Rheumatol. 46: 2578-2586.*
31. Fingerle, G., Pforte, A., Passlick, B., Blumenstein, M., Strobel, M., and Ziegler-Heitbrock, H.W. 1993. The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. *Blood 82: 3170-3176.*
32. Koch, S., Kucharzik, T., Heidemann, J., Nusrat, A., and Luegering, A. 2010. Investigating the role of proinflammatory CD16+ monocytes in the pathogenesis of inflammatory bowel disease. *Clin. Exp. Immunol. 161: 332-341.*
33. Yona, S., Kim, K.W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M., Misharin, A., Hume, D.A., Perlman, H., Malissen, B., Zelzer, E., and Jung, S. 2013. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity 38: 79-91.*
34. Huang, Y., Yin, H., Wang, J., Liu, Q., Wu, C., and Chen, K. 2012. Aberrant expression of FcgammaRIIIA (CD16) contributes to the development of atherosclerosis. *Gene 498: 91-95.*
35. Dutertre, C.A., Amraoui, S., DeRosa, A., Jourdain, J.P., Vimeux, L., Goguet, M., Degrelle, S., Feuillet, V., Liovat, A.S., Muller-Trutwin, M., Decroix, N., Deveau, C., Meyer, L., Goujard, C., Loulergue, P., Launay, O., Richard, Y., and Hosmalin, A. 2012. Pivotal role of M-DC8(+) monocytes from viremic HIV-infected patients

in TNFalpha overproduction in response to microbial products. *Blood 120: 2259-2268*.

36. Zhao, C., Tan, Y.C., Wong, W.C., Sem, X., Zhang, H., Han, H., Ong, S.M., Wong, K.L., Yeap, W.H., Sze, S.K., Kourilsky, P., and Wong, S.C. 2010. The CD14(+/low)CD16(+) monocyte subset is more susceptible to spontaneous and oxidant-induced apoptosis than the CD14(+)CD16(-) subset. *Cell. Death Dis. 1: e95*.

37. Frankenberger, M., Hofer, T.P., Marei, A., Dayyani, F., Schewe, S., Strasser, C., Aldraihim, A., Stanzel, F., Lang, R., Hoffmann, R., Prazeres da Costa, O., Buch, T., and Ziegler-Heitbrock, L. 2012. Transcript profiling of CD16-positive monocytes reveals a unique molecular fingerprint. *Eur. J. Immunol. 42: 957-974*.

38. Collison, J.L., Carlin, L.M., Eichmann, M., Geissmann, F., and Peakman, M. 2015. Heterogeneity in the Locomotory Behavior of Human Monocyte Subsets over Human Vascular Endothelium In Vitro. *J. Immunol. 195: 1162-1170*.

39. Rennert, K., Heisig, K., Groeger, M., Wallert, M., Funke, H., Lorkowski, S., Huber, O., and Mosig, A.S. 2016. Recruitment of CD16 monocytes to endothelial cells in response to LPS-treatment and concomitant TNF release is regulated by CX3CR1 and interfered by soluble fractalkine. *Cytokine 83: 41-52*.

40. Gonzalez-Dominguez, E., Dominguez-Soto, A., Nieto, C., Flores-Sevilla, J.L., Pacheco-Blanco, M., Campos-Pena, V., Meraz-Rios, M.A., Vega, M.A., Corbi, A.L., and Sanchez-Torres, C. 2016. Atypical Activin A and IL-10 Production Impairs Human CD16+ Monocyte Differentiation into Anti-Inflammatory Macrophages. *J. Immunol. 196: 1327-1337*.

41. Thaler, B., Hohensinner, P.J., Krychtiuk, K.A., Matzneller, P., Koller, L., Brekalo, M., Maurer, G., Huber, K., Zeitlinger, M., Jilma, B., Wojta, J., and Speidl, W.S. 2016. Differential in vivo activation of monocyte subsets during low-grade inflammation through experimental endotoxemia in humans. *Sci. Rep. 6: 30162*.

42. van der Geest, K.S., Abdulahad, W.H., Rutgers, A., Horst, G., Bijzet, J., Arends, S., Roffel, M.P., Boots, A.M., and Brouwer, E. 2015. Serum markers associated with disease activity in giant cell arteritis and polymyalgia rheumatica. *Rheumatology (Oxford) 54: 1397-1402*.

43. Weyand, C.M., Ma-Krupa, W., and Goronzy, J.J. 2004. Immunopathways in giant cell arteritis and polymyalgia rheumatica. *Autoimmun. Rev. 3: 46-53*.

44. Dal-Secco, D., Wang, J., Zeng, Z., Kolaczkowska, E., Wong, C.H., Petri, B., Ransohoff, R.M., Charo, I.F., Jenne, C.N., and Kubes, P. 2015. A dynamic spectrum of monocytes arising from the in situ reprogramming of CCR2+ monocytes at a site of sterile injury. *J. Exp. Med. 212: 447-456*.

45. Khmelewski, E., Becker, A., Meinertz, T., and Ito, W.D. 2004. Tissue resident cells play a dominant role in arteriogenesis and concomitant macrophage accumulation. *Circ. Res. 95: E56-64*.

46. Weber, C., Belge, K.U., von Hundelshausen, P., Draude, G., Steppich, B., Mack, M., Frankenberger, M., Weber, K.S., and Ziegler-Heitbrock, H.W. 2000. Differential chemokine receptor expression and function in human monocyte subpopulations. *J. Leukoc. Biol. 67: 699-704*.

47. Ancuta, P., Rao, R., Moses, A., Mehle, A., Shaw, S.K., Luscinskas, F.W., and Gabuzda, D. 2003. Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. *J. Exp. Med. 197: 1701-1707*.

48. Zhang, J. and Patel, J.M. 2010. Role of the CX3CL1-CX3CR1 axis in chronic inflammatory lung diseases. *Int. J. Clin. Exp. Med. 3: 233-244*.

49. Dayyani, F., Belge, K.U., Frankenberger, M., Mack, M., Berki, T., and Ziegler-Heitbrock, L. 2003. Mechanism of glucocorticoid-induced depletion of human CD14+CD16+ monocytes. *J. Leukoc. Biol. 74: 33-39*.

50. Heimbeck, I., Hofer, T.P., Eder, C., Wright, A.K., Frankenberger, M., Marei, A., Boghdadi, G., Scherberich, J., and Ziegler-Heitbrock, L. 2010. Standardized single-platform assay for human monocyte subpopulations: Lower CD14+CD16++ monocytes in females. *Cytometry A. 77: 823-830*.

51. Alvarez-Rodriguez, L., Lopez-Hoyos, M., Calvo-Alen, J., Aurrecoechea, E., Villa, I., and Martinez-Taboada, V.M. 2013. Phagocyte dysfunction in polymyalgia rheumatica and other age-related, chronic, inflammatory conditions. *J. Leukoc. Biol. 94: 1071-1078*.

SUPPLEMENTARY DATA

Supplementary Table 1A. Characteristics of GCA patient cohort and overview of sample inclusion per patient.

Patient ID	Sex	Age	Diagnosis	Cell count PB	Monocyte Subsets (%) PB	Chemokine Receptors PB	Chemo-kines Serum	Follow-up Cell count PB	Follow-up %Monocytes Subsets PB	Follow-up Chemokine Receptors PB	Follow-up Chemokines Serum	IHC study	GC before biopsy
GCA7	M	65	PET-CT	X	X		X	X	X	X			
GCA17*	F	81	PET-CT	X	X	X		X	X	X			
GCA13*	M	56	PET-CT	X	X	X			X	X			
GCA2	F	52	PET-CT	X	X	X	X	X	X	X			
GCA10	F	60	PET-CT	X	X	X	X	X	X	X			
GCA9	F	78	PET-CT	X	X	X	X	X	X	X	X		
GCA15	F	69	TAB	X	X	X	X	X	X	X	X		
GCA11	M	79	PET-CT	X	X	X	X	X	X	X	X		
GCA4*	F	68	PET-CT		X	X	X	X	X	X	X		
GCA18	F	62	PET-CT	X	X	X							
GCA16	M	62	TAB	X	X	X	X						
GCA23*	F	76	PET-CT	X	X	X	X						
GCA3	F	68	PET-CT	X	X	X	X						
GCA1	F	79	TAB	X	X	X	X						
GCA8	F	59	PET-CT + TAB	X	X	X	X	X	X	X	X	X	N
GCA12	F	73	TAB	X	X	X	X	X	X	X	X	X	N
GCA6	F	74	PET-CT + TAB	X	X	X	X	X	X	X	X	X	N
GCA14*	F	73	PET-CT + TAB	X	X	X	X	X	X	X		X	N
GCA20	F	79	TAB	X	X	X	X	X	X	X	X	X	N
GCA21	M	77	PET-CT + TAB	X	X	X	X	X	X	X	X	X	N
GCA5	F	73	PET-CT + TAB	X	X	X	X					X	N
GCA22	M	66	PET-CT + TAB	X	X	X	X					X	N
GCA1010	F	76	TAB									X	Y
GCA25	M	60	PET-CT + TAB									X	Y
GCA26	M	65	PET-CT + TAB									X	Y
GCA1001	F	78	TAB									X	Y

Supplementary Table 1A. (continued)

Patient ID	Sex	Age	Diagnosis	Cell count PB	Monocyte Subsets (%) PB	Chemokine Receptors PB	Chemo-kines Serum	Follow-up Cell count PB	Follow-up %Monocytes Subsets PB	Follow-up Chemokine Receptors PB	Follow-up Chemokines Serum	IHC study	GC before biopsy
GCA1012	F	73	TAB									X	N
GCA1002	M	71	TAB									X	N
GCA000	M	71	TAB									X	N
GCA1009	F	72	TAB									X	N
Figures				1,2, S1A	S1B	4		1,2, S1A	S1B	4		3,5, S2,S3	
Tables							S2				S2	S3, S4	

GCA = Giant cell arteritis. * GCA patients with concomitant PMR. PB: peripheral blood; M: male; F: Female; PET-CT: positron emission tomography-computer tomography; TAB: temporal artery biopsy; GC: glucocorticoids; X: sample was included; N: no GC therapy; Y: GC treatment started before biopsy was taken.

Supplementary Table 1B. PMR patient cohort and overview of sample inclusion per patient. PB: peripheral blood;

Patient ID	Sex	Age	Diagnosis	Cell count PB	Monocyte Subsets (%) PB	Chemokine Receptors PB	Chemokines Serum	Follow-up Cell count PB	Follow-up %Monocytes Subsets PB	Follow-up Chemokine Receptors PB	Follow-up Chemokines Serum
PMR501	M	62	Chuang + PET-CT	X	X	X	X	X	X	X	X
PMR504	M	58	Chuang	X	X	X	X	X	X	X	X
PMR505	F	74	Chuang + PET-CT	X	X	X	X	X	X	X	X
PMR512	F	81	Chuang + PET-CT	X	X	X	X	X	X	X	X
PMR518	F	64	Chuang + PET-CT	X	X	X	X	X	X	X	X
PMR515	F	77	Chuang + PET-CT	X	X	X	X	X	X	X	X
PMR517	F	79	Chuang	X	X	X	X	X	X	X	X
PMR517	F	79	Chuang	X	X	X	X	X	X	X	X
PMR502	F	75	Chuang + PET-CT	X	X	X		X	X	X	
PMR503	M	82	PET-CT	X	X	X	X	X	X	X	
PMR516	F	75	Chuang + PET-CT	X	X	X	X	X	X	X	
PMR513	F	76	Chuang	X	X	X	X	X	X	X	
PMR520	F	65	Chuang + PET-CT	X	X	X	X	X	X	X	
PMR514	M	54	Chuang + PET-CT	X	X	X	X	X	X		X
PMR507	F	68	Chuang + PET-CT		X	X	X	X	X	X	X
PMR000	F	61	Chuang	X	X	X	X				
PMR509	F	69	Chuang + PET-CT	X	X	X	X				
PMR511	M	84	Chuang	X	X	X	X				
PMR506	M	82	Chuang + PET-CT	X	X	X	X				
PMR510	F	62	Chuang + PET-CT	X	X	X					
Figures				1,2, S1A	S1B	4		1,2, S1A	S1B	4	
Tables							S2				S2

M: male; F: Female; PET-CT: positron emission tomography-computed tomography; TAB: temporal artery biopsy; GC: glucocorticoids; X: sample was included.

Supplementary Table 1C. HC cohort and overview of sample inclusion per donor.

ID	Sex	Age	Cell count PB	% Monocyte Subsets PB	Chemokine Serum
SEN5	M	65	X	X	X
SEN14	F	75	X	X	X
SEN501	F	53	X	X	X
SEN55	F	67	X	X	X
SEN57	F	66	X	X	X
SEN75	F	75	X	X	X
SEN79	F	79	X	X	X
SEN61	F	72	X	X	X
SEN66	M	72	X	X	X
SEN62	M	73	X	X	X
SEN3	F	71	X	X	X
SEN6	F	62	X	X	X
SEN58	F	62	X	X	X
SEN10	M	71	X	X	X
SEN32	M	80	X	X	X
SEN39	F	73	X	X	X
SEN47	F	63	X	X	X
SEN59	M	61	X	X	
SEN60	F	59	X	X	
SEN30	F	64	X	X	
SEN54	F	74		X	X
SEN25	F	75		X	
SEN26	F	79		X	
SEN45	F	83		X	
Figures			1,2, S1A	S1B	
Tables					S2

HC: Healthy control; PB: peripheral blood; M: male; F: Female; X: sample was included.

Supplementary Table 2. Serum chemokine concentrations in healthy controls, GCA and PMR patients before and after glucocorticoid treatment. Systemic concentrations of CCL2 and CCL11 (CCR2 receptor ligands) and CX3CL1 and CCL26 (CX3CR1 ligands) were measured in healthy controls (HC, n=18), newly-diagnosed patients with GCA (nGCA; n=19) and PMR (nPMR; n=18) and in the follow-up samples of GCA (rGCA; n=9) and PMR (rPMR; n=10) patients in remission after 3 months of glucocorticoid treatment. Results are expressed as median values and range in pg/mL. The Kruskal-Wallis test was performed to compare data among study groups. The Mann-Whitney U test was used to compare each patient group with HC and is indicated with *. Paired samples (rGCA vs nGCA and rPMR vs nPMR) were compared with the Wilcoxon signed rank test and is indicated with ^. P-values of less than 0.05 (2-tailed) were considered statistically significant.

pg/mL	HC	nGCA	rGCA	nPMR	rPMR
CCL2	409 (187-589)	281 (42-595) *p= 0.032**	400 (221-893) *p=0.0078^*	322 (172-673)	475 (146-725)
CCL11	114 (68-201)	68 (30-356) *p=0.0057**	178 (127-873) *p=0.0078^*	99 (50-178)	140 (68-277)
CX3CL1	956 (728-1789)	924 (526-1969)	1177 (794-19175) *p=0.0391^*	891 (594-1789)	956 (661-2265)
CCL26	4 (4-48)	4 (4-109)	10 (4-29)	4 (4-10)	4 (4-10)

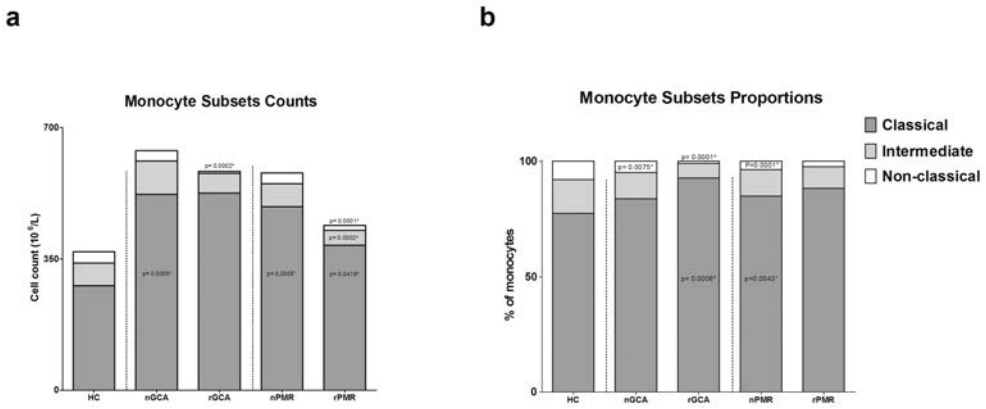
Supplementary Table 3. Primary antibody information used in flow cytometry

Antibody	Conjugation	Clone	Supplier
Anti-CD2	PE-Cy7	RPA-2.10	eBioscience, San Diego, CA, USA
Anti-CD3	APC	UCHT1	BD Biosciences Franklin Lakes, NJ, USA
Anti-CD3	EF605	OKT3	eBioscience
Anti-CD14	PE	M5E2	Biolegend, San Diego, CA, USA
Anti-CD16	V450	3G8	BD Biosciences
Anti-CD16	AF700	3G8	Biolegend
Anti-CD19	APC-eFluor780	HIB19	eBioscience
Anti-CD19	EF605	HIB19	eBioscience
Anti-CD56	BV510	HCD56	Biolegend
Anti-CD56	FITC	MEM188	eBioscience
Anti-CD66b	PE-Cy7	G10F5	eBioscience
Anti-CCR2	PerCP-Cy5.5	K036C2	Biolegend
Anti-CX3CR1	FITC	2A9-1	Biolegend
Anti-IL-6	APC	MQ2-13A5	eBioscience

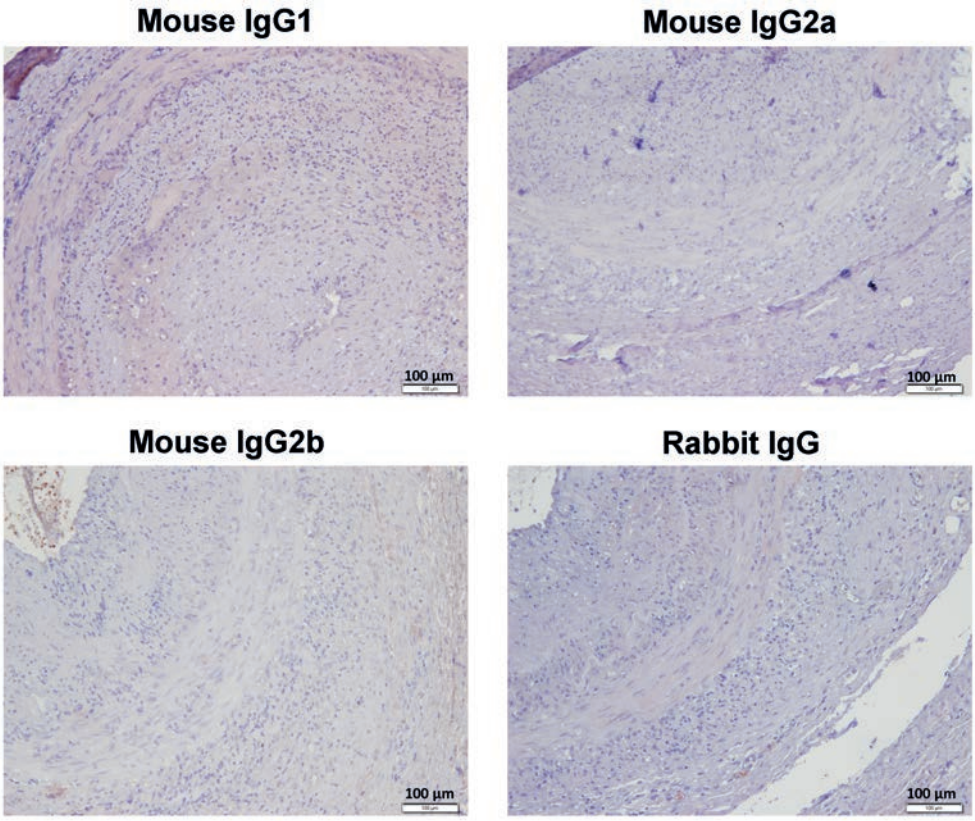
Supplementary Table 4. Primary antibody information used in immunohistochemistry.

Antibody	Isotype	Clone	Supplier / Cat #	Dilution	Antigen retrieval
Anti-CD16	Rabbit IgG	SP175	Abcam (Cambridge, UK) ab183354	1:50	10mM tris-HCL+ 1mM EDTA pH=9
Anti-CD68	Mouse IgG3κ	PG-M1	DAKO (Troy, Mich, USA), M0876	1:50	idem
Anti-CD56	Mouse IgG2b	MEM-188	Abcam, ab8233	1:100	idem
Anti-CCR2	Mouse IgG2a	7A7	Abcam, ab176390	1:50	idem
Anti-CX3CR1	Mouse IgG1	8E10.D9	Biolegend, 824001	1:50	idem
Anti-CCL2	Mouse IgG2b	23002	R&D (Minneapolis, Minn, USA), MAB679	1:5	idem
Anti-CX3CL1	Rabbit IgG#,	NA	Abcam, ab25088	1:100	idem

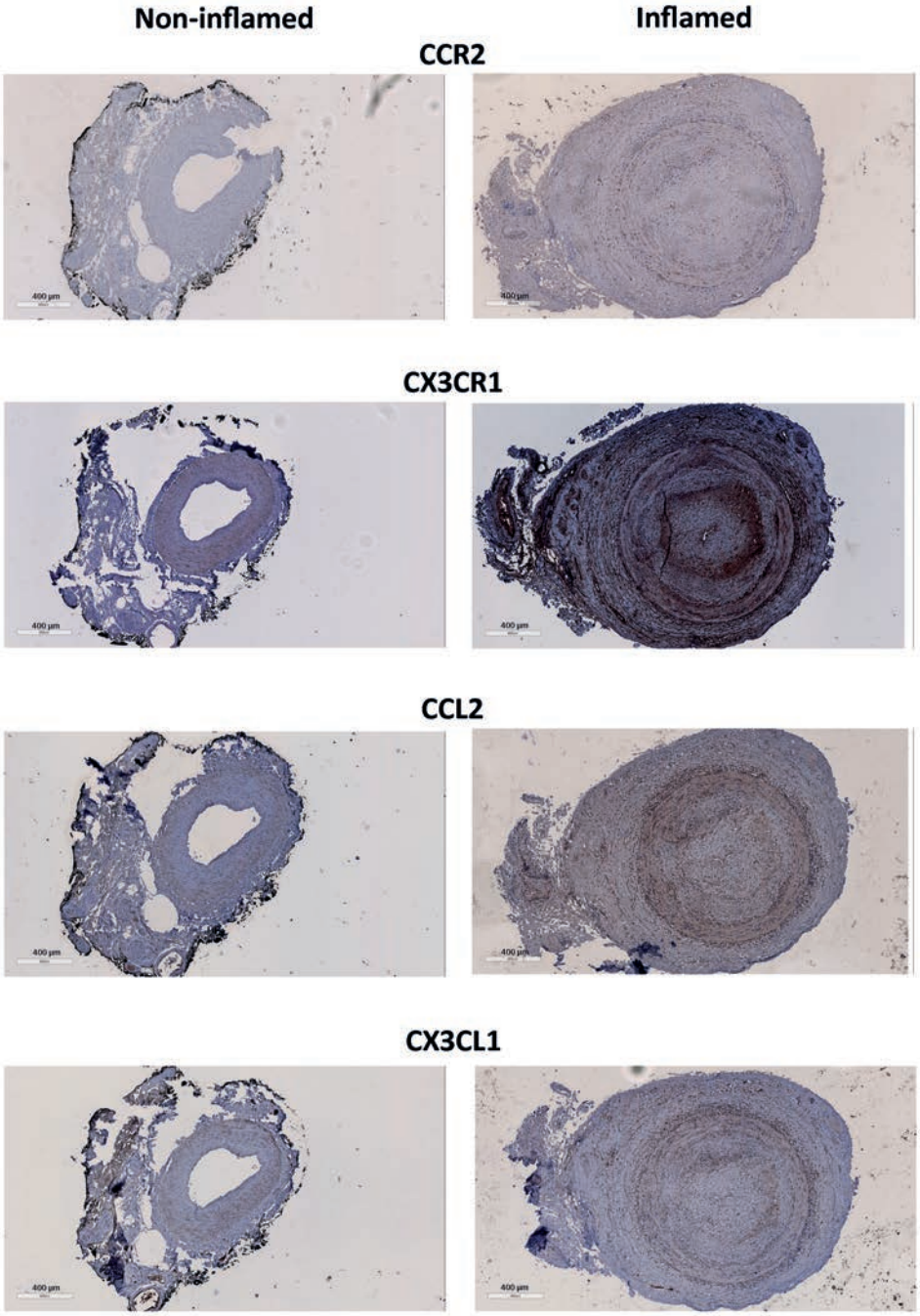
protein A purified polyclonal antibodies. NA = not applicable



Supplementary Figure 1. Cumulative schematic overview of the three monocyte subsets in PBMC from GCA/PMR. **a**, A cumulative schematic overview of the median values of absolute numbers of classical, intermediate, and non-classical monocytes in healthy controls (HCs, n=20), newly-diagnosed patients with GCA (nGCA; n=21) and PMR (nPMR; n=19) and in the follow-up samples of GCA (rGCA; n=14) and PMR (rPMR; n=15) patients in remission after 3 months of prednisone treatment. **b**, A cumulative schematic overview of the proportions (percentages of total monocytes) of classical, intermediate, and non-classical monocytes in the study groups. The Kruskal-Wallis test was performed to compare data among study groups (HCs, GCA and PMR). The Mann-Whitney U test was used to compare nGCA and nPMR with HCs and is indicated with *. Paired samples were compared with the Wilcoxon signed rank test and is indicated with ^.



Supplementary Figure 2. Isotype controls for immunohistochemical staining. Immunohistochemical staining for isotype controls e.g. mouse IgG1, mouse IgG2a, mouse IgG2b, Rabbit IgG in a representative inflamed temporal artery biopsy specimen from GCA patients using equivalent antibody concentrations.



Supplementary Figure 3. Comparison immunohistochemical staining of non-inflamed and inflamed temporal artery biopsies from GCA patients. Left panels show non-inflamed TABs and right panels show GCA TABs. Representative staining for CCR2, CX3CR1, CCL2 or CX3CL1 is shown using equivalent antibody concentrations. Non-inflamed TABs lack infiltrates and tissue remodelling typical of GCA and consist mainly of vascular smooth muscle cells.

van Sleen Y,

Brouwer E,

Huitema MG,

Abdulahad WH,

Boots AMH,

van der Geest KSM

FOUR

Numerical Decline of Circulating Myeloid Dendritic Cells with High Toll-Like Receptor 2 Expression in Treatment-Naive Giant Cell Arteritis and Polymyalgia Rheumatica

Work in progress.

ABSTRACT

Monocytes, CD4+ T-cells and dendritic cells (DCs) are the main contributors to the immunopathology of giant cell arteritis (GCA) and polymyalgia rheumatica (PMR). Previously, altered counts of monocyte subsets and activated T-helper (Th)1 and Th17 cells were reported. However, data on counts of circulating myeloid DCs (mDCs) and plasmacytoid DCs (pDC) in GCA and PMR are lacking. Monocytes and DCs are activated through pattern recognition receptors, a process thought to initiate GCA and PMR pathology and important in Th lineage differentiation. Here, we assessed if numbers of circulating monocytes are correlated with Th1 and Th17 frequencies in GCA and PMR patients. In addition, we enumerated DC subsets and determined the expression of pattern recognition receptors on monocyte and DC subsets.

Counts of circulating monocyte and DC subsets were assessed by flow cytometry in treatment-naive GCA and PMR patients, as well as in healthy controls (HCs). Also, counts of CD4+ T cells with the capacity to produce interferon γ (Th1) and IL-17 (Th17) were documented by flow cytometry. Expression of pattern recognition receptors toll-like receptor (TLR)2, TLR4, TLR7, TLR8 and absent in melanoma (AIM)2 by monocyte and DC subsets was measured.

Enumeration of Th1 and Th17 cells did not reveal differences between groups. Counts of Th1 and Th17 did not correlate with monocyte subset counts. Counts of circulating mDCs were reduced in both GCA and PMR patients compared to HCs, whereas counts of pDC were similar. Expression of TLR2 by mDCs was higher in GCA and PMR patients than in HCs.

Reduced numbers of circulating mDCs in GCA and PMR may suggest their migration to the inflammatory site in GCA and PMR. Elevated TLR expression may render these cells prone to activation due to their increased sensing capacity. Our findings suggest that skewing of CD4+ T-cells towards pathogenic Th1 and Th17 phenotypes occurs mainly at the inflammatory site, a notion to be further investigated.

INTRODUCTION

Giant cell arteritis (GCA) is an inflammatory disease of medium and large-sized arteries [1]. Symptoms of GCA include headache, jaw claudication and vision loss [2]. GCA is frequently associated with polymyalgia rheumatica (PMR). Approximately 50% of GCA patients have overlapping PMR, whereas the incidence of GCA among PMR patients is between 16 and 21% [2]. PMR is characterized by bursal and synovial inflammation, leading to pain and stiffness in the shoulders and hips. Both GCA and PMR occur exclusively in the elderly and are characterized by an acute-phase response in the blood.

The pathology of GCA and PMR is not completely understood. In GCA, it is generally thought that the disease starts in the adventitia (i.e. the outer vessel wall layer), where dendritic cells (DCs) become activated via binding of an unknown ligand to their pattern recognition receptors, e.g. Toll-like receptors (TLRs) [1]. In GCA, DCs may be prone to activation, due to a defect in PD-L1 expression, leading to chemokine production and recruitment of CD4+ T-cells and monocytes to the arterial wall [1, 3]. The temporal artery biopsy (TAB) of GCA patients shows a granulomatous infiltrate consisting of macrophages, DCs and CD4+ T-cells. The infiltrated cells in turn produce chemokines and cytokines, such as IL-6, that may further fuel the infiltration and inflammation in the vessel wall [1, 4]. As GCA and PMR are systemic diseases, we here investigate the peripheral blood compartment of patients with these conditions.

Infiltrated CD4+ T-cells display a dysregulated Th cell distribution, that may contribute to the development of GCA [1, 4, 5]. Inflamed arteries of GCA patients contain pro-inflammatory T-helper 1 (Th1) and Th17 cells, but almost no Th2 cells or regulatory T-cells. This is likely instigated by local cytokines such as IL-12 and IL-18 that favour the development of Th1 cells, and IL-1-, IL-6 and IL-23 that favour the development of Th17 cells [5]. Moreover, GCA and PMR patients were reported to have higher proportions of Th1 and Th17 cells in the blood than healthy controls [6-8], although one study documented lower Th1 proportions in GCA and PMR [9]. Th17 cells are derived from precursor CD161+ CD4+ T-cells [10] and CD161 expression is more abundant in the GCA TAB than in the blood [11].

Monocytes are thought to be crucial in GCA and PMR pathology as they migrate to the inflammatory site, guided by chemokines, where they develop in macrophages and myeloid DCs (mDCs) [12, 13]. Monocytes, macrophages and mDCs produce a vast array of pro-inflammatory cytokines, many of which are important in Th-skewing [5, 6]. We previously revealed a clear monocytosis in treatment-naive GCA and PMR patients due to an expansion of the classical monocyte subset [13]. It is currently unknown whether counts of monocyte subsets, defined by CD14 and CD16 expression, are linked to numbers of Th subsets. Both mDCs and plasmacytoid DCs (pDCs) have been described in GCA TABs. Even though DCs may play a key role in the early pathogenesis, it is not yet known if numbers of DC subsets are altered in the blood of GCA and PMR patients [3, 14-16].

Pattern recognition receptors, and TLRs in particular, are likely critical for activation of monocytes and dendritic cells in GCA [17-19]. These receptors are essential for sensing pathogen associated molecular patterns (PAMPs), expressed by bacteria and viruses, but also damage associated molecular patterns (DAMPs), which for instance are released by necrotic cells [17]. The different pattern recognition receptors recognize distinct PAMPs and DAMPs. In healthy

arteries, DCs variably express TLRs, dependent on the type of artery [20]. In vitro and ex vivo experiments revealed that pattern recognition receptor stimulation, with most evidence for TLR2 and TLR4, can activate and mature vessel-wall embedded DCs [3, 14-16, 21]. Furthermore, elevated expression of TLR7 was found on B-cells and monocytes in blood of GCA and PMR patients [19]. Per cell expression of these pattern recognition receptors may vary among monocyte and DC subsets, but this has not been assessed specifically in GCA and PMR.

In the current study, we thus investigated if numbers of circulating monocytes and DCs are linked to Th1 and Th17 frequencies in GCA and PMR patients [12, 22, 23]. In addition, we determined the expression of pattern recognition receptors on monocyte and DC subsets, as signalling via these receptors might impact Th1 or Th17 skewing. Our studies did not reveal correlations of monocyte subsets with Th1 or Th17 skewing. We did reveal, however, a numerical decline of mDCs with elevated TLR2 expression, warranting further investigation.

MATERIALS AND METHODS

Patient inclusion

This study entails flow cytometry experiments on peripheral blood mononuclear cells (PBMCs) from patients with GCA, PMR and age- and sex-matched healthy controls (HCs). GCA and PMR patients were treatment-naive and newly-diagnosed. GCA patients with involvement of cranial and/or systemic arteries were included. Therefore, not all GCA patients fulfilled the 1990 ACR criteria for GCA, which is biased towards cranial GCA. The diagnosis of GCA was confirmed by a positive temporal artery biopsy (TAB) and/or a positive 18F-fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET-CT). All PMR patients, except three, were diagnosed based on clinical signs and symptoms, and a positive FDG-PET-CT for PMR. The remaining three PMR patients that did not undergo a FDG-PET-CT, but fulfilled the Chuang criteria and the provisional ACR-EULAR criteria for PMR [24, 25]. HCs had no morbidities and were without any immunomodulatory medication. The study was approved by the institutional review board of the University Medical Center Groningen (METc2010/222). Written informed consent was obtained from all study participants. All procedures were in compliance with the Declaration of Helsinki.

Two cohorts

We studied monocytes, DCs and CD4+ T-cells in thawed PBMCs of two patient and HC control cohorts (Table 1). The first set of experiments included 11 GCA patients, 9 PMR patients and 9 HCs (cohort A). The second set of experiments included another 10 GCA patients, 10 PMR patients and 10 HCs (cohort B). Both cohorts consisted of patients and HCs included in the prospective cohort at the University Medical Center Groningen. Experiments on cohort A were performed in 2013, whereas experiments on cohort B were performed in 2019. Patients in cohort A and B did not significantly differ in age, sex, clinical manifestations or biochemical features (CRP, ESR, monocyte and CD4+ T-cell counts).

Table 1. Clinical characteristics of patients and controls included.

	Cohort A			Cohort B		
	GCA	PMR	Healthy control	GCA	PMR	Healthy control
N	11	9	9	10	10	10
Age: **median**, (range) in years	**72**	**69**	**66**	**69**	**74**	**72**
	(52-79)	(58-82)	(58-74)	(56-79)	(63-82)	(59-78)
Sex (% female)	82	67	89	80	50	70
Fulfilled ACR criteria (yes/no)	7/4	0/9	NA	8/2	0/10	NA
Jaw, tongue or limb claudication (yes/no)	5/6	0/9	NA	4/6	0/10	NA
Visual ischemia (yes/no)	4/7	0/9	NA	1/9	0/10	NA
PMR diagnosis (yes/no)	1/10	9/0	NA	1/9	10/0	NA
CRP: **median,** (range) in mg/L	**50**	**42**	**<5**	**45**	**36**	**1.2**
	(11-126)	(16-87)		(5-134)	(3-127)	(0.3-3.2)
ESR: **median,** (range) in mm/hr	**71**	**52**	**13**	**81**	**60**	**10**
	(31-106)	(32-88)	(2-21)	(28-107)	(30-109)	(1-36)
Monocyte counts: **median,** (range) in 10^9 cells/mL	**0.83**	**0.67**	**0.36**	**0.72**	**0.86**	**0.42**
	(0.35-1.46)	(0.4-1.32)	(0.31-0.69)	(0.39-1.18)	(0.51-1.16)	(0.31-1.00)
CD4+ T-cell counts **median,** (range) in 10^9 cells/mL	**0.69**	**0.85**	**1.11**	**1.05**	**0.81**	**0.96**
	(0.41-1.71)	(0.35-1.39)	(0.61-1.61)	(0.35-1.45)	(0.51-1.40)	(0.68-1.57)

GCA: giant cell arteritis, TAB: temporal artery biopsy, PMR: polymyalgia rheumatica, ACR: American college of rheumatology, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate.

Flow cytometry of monocytes and DCs

Monocyte subset proportions and absolute counts for samples from cohort A were determined as previously described [13]. In short, monocytes were stained for negative selection markers CD56, CD66b and CD3, and positive selection markers CD14 and CD16. Classical $CD14^{high}CD16^{-}$, intermediate $CD14^{high}CD16^{+}$ and non-classical $CD14^{dim}CD16^{+}$ monocytes were thereafter defined. For cohort B, a flow cytometry panel was used to study the expression of pattern recognition receptors by monocyte and DC subsets. Reagents for cohort A and cohort B are shown in Supplementary Table 1.PBMCs of cohort B were stained for surface markers CD14, CD16, HLA-DR, TLR2, TLR4, CD11c and CD303. Next, the cells were incubated with Fix/Perm solution (Thermo Fisher), and washed with Permeabilization buffer (Thermo Fisher). After incubation with mouse and rat normal serum, the PBMCs were incubated with intracellular antibodies detecting TLR7, TLR8 and absent in melanoma (AIM2). The cells were then measured on a LSR-II (BD, San Jose, CA, USA) flow cytometer. We compared the mean fluorescence intensity (MFI) between experiments by using FACSDiva CS&T research beads (BD) to calibrate the flow cytometer. Cell populations were defined by a fixed gating strategy (Figure 1). Monocytes were first gated based on size and granularity, followed by exclusion of doublets. Contaminating CD16+ cells (NK-cell or granulocytes) were excluded by gating for CD14 and HLA-DR, and monocyte subsets were defined. To calculate absolute counts per subset, the total monocyte counts were multiplied with the percentages of monocyte subsets.

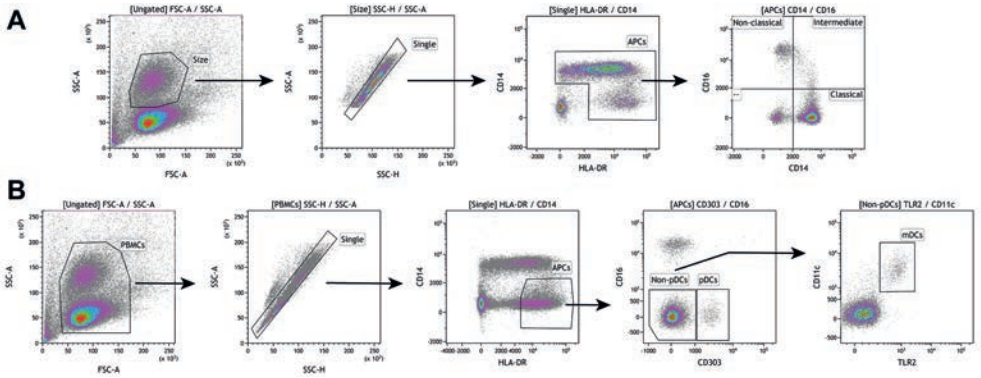


Figure 1. Gating strategy for monocyte subsets (**A**) and DC subsets (**B**) performed in cohort B.

Due to their variable size/granularity, DCs were identified in the total PBMC fraction. After doublets exclusion, cells were gated for CD14 negativity and high HLA-DR expression. Next, pDCs were gated based on CD303 expression [12]. Finally, mDCs were gated for CD16 negativity, to exclude CD16+ monocytes, and positivity for CD11c and TLR2. Absolute counts of circulating DC subsets were determined by multiplying their percentages with total PBMC counts.

Flow cytometry of CD4+ T-cells

The induced production of lineage cytokines by circulating CD4+ T-cells was measured in both cohorts. PBMCs were cultured in culture medium (RPMI) containing 5% fetal calf serum (FCS), in the presence of 50 ng/mL phorbol myristate acetate (PMA), 1.6 µg/mL calcium ionophore and 10µg/mL brefeldin A. As controls, PBMCs were cultured in culture medium containing 5% FCS, but only in the presence of brefeldin A. After 4 hours, cells were stained for surface antibodies and for dead cells (Supplementary Table 2). Cells were subsequently washed, fixed and permeablized (FIX & PERM™ Cell Permeabilization Kit, Thermo Fisher). The PBMCs were then intracellularly stained for the cytokines. For both cohorts, expression of interferon-γ (IFNγ) and IL-17 was determined using the same fluorochrome labeled antibodies (supplementary Table 2). In addition, IL-2 and IL-22 expression was measured in cohort A, and IL-4 expression in cohort B. Cytokine production by CD4+ T-cells was measured using the LSR-II flow cytometer and data were analyzed by Kaluza software (BD).Identification of CD4+ T-cells was performed in single cells within the lymphocyte gate. Next, as these cells had been stimulated for 4 hours in vitro, dead cells were excluded. To detect CD4+ tT-cells in cohort A, intracellular staining for CD4 was performed. In cohort B, CD4+ T-cells were defined as CD3+CD8- T cells. Percentages of CD4+ T-cells producing IFNγ, IL-17, IL-4, IL-22 or IL-2 were multiplied with total CD4+ T-cell counts to generate absolute counts of these subpopulations.

Absolute counts

Monocyte and CD4+ T-cell counts were measured in EDTA blood by the MultiTest TruCount method, as described by the manufacturer (BD). Monocytes and lymphocytes were gated based on their side and forward scatter properties and the expression of the pan-leukocyte marker CD45. Further, Th

cells were determined among the lymphocyte gate according to their surface expression of CD3 and CD4 lineage markers. The absolute numbers of Monocytes and Th cells were determined by comparing cellular events with beads events. In addition, total PBMC counts were measured to determine the absolute count for mDCs and pDCs. TruCount measurements were performed on a FACS Canto-II (BD) and subsequently analyzed with FACSCanto Clinical Software.

Statistics

As the data were non-normally distributed, non-parametric testing was performed to compare groups. Shown are p-values of the Mann Whitney U test; these were considered significant only if the Kruskal Wallis test p-value was lower than 0.05. Strength and significance of correlations was also non-parametrically tested by the Spearman R. Statistical significance was defined as $p<0.05$.

RESULTS

Altered counts of monocyte subsets in peripheral blood of GCA and PMR patients

Absolute counts (and proportions) of monocyte subsets are shown in supplementary Figure 1. As we previously published [13], the monocytosis in GCA and PMR is mainly attributed to an expansion of the classical monocyte subset. Additionally, intermediate monocyte counts are significantly higher in GCA patients than in HCs, but not in PMR versus HCs. Together, this translates to lower proportions of non-classical monocytes in GCA and PMR, mainly due to the expansion of classical monocytes.

No evidence for altered distribution of Th-cell subsets in the blood of GCA and PMR patients

We observed no differences in the proportions or the absolute counts of IFNγ producing Th1 cells or IL-17 producing Th17 cells in GCA and PMR patients, when compared to HCs (Figure 2). We next compared whether proportions of Th1 and Th17 differed between cohort A and cohort B (supplementary Figure 2). Overall, we did observe higher proportions of Th1 cells and lower proportions of Th17 cells in cohort A, possibly due to differences in the gating strategy, or in patient selection. However, the per cohort analysis did not reveal statistical differences between GCA/PMR and HC. Additionally, we measured IL-4 producing Th2 cells, and IL-22 and IL-2 production by CD4+ T-cells (supplementary Figure 3). The proportions of Th2 cells and IL-22 and IL-2 positive cells among CD4+ T-cells was not significantly different between the groups. We did, however, observe a trend ($p=0.06$), suggesting lower numbers of IL-2 producing Th cells in GCA compared to HC.

As CD161 defines Th17 lineage cells and CD161+ CD4+ T cells have been detected in arteries of GCA patients, we measured CD161 expression by total CD4+ T-cells (unstimulated). We observed no differences in the CD161 positivity in GCA or PMR compared to HC (supplementary Figure 4). Next, we assessed the CD161 expression by Th1, Th1/Th17 and Th17 cells. As expected, CD161 positivity is highest for Th17 cells, followed by Th1/Th17 cells and Th1 cells. Similar patterns were observed in GCA and PMR patients as well as in HCs, and no differences were observed between the groups.

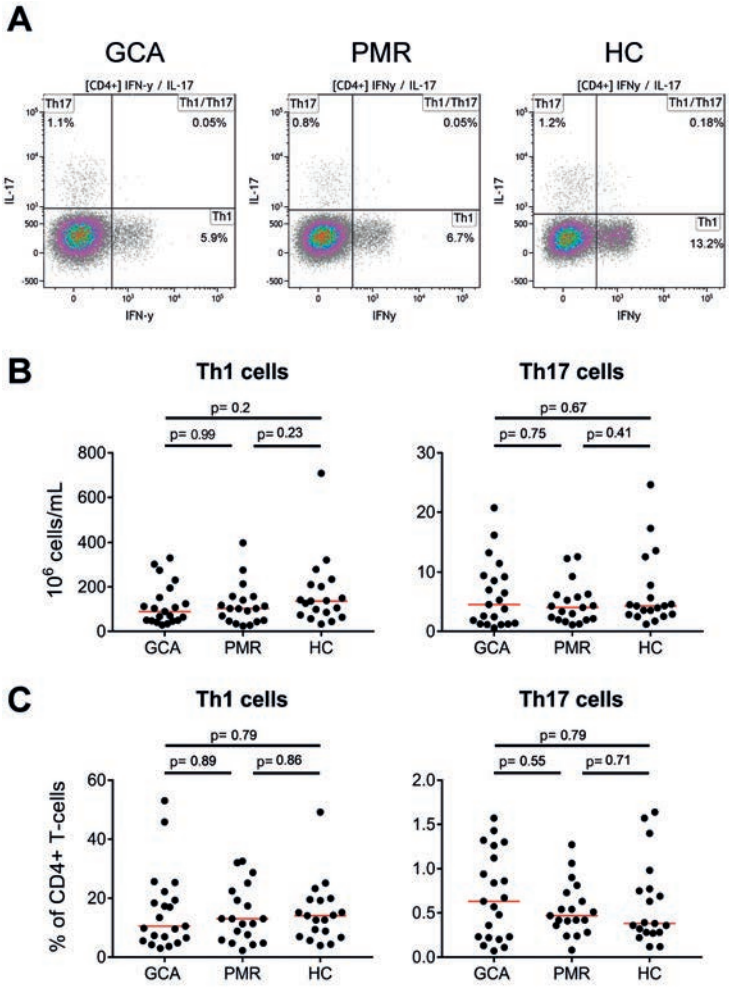


Figure 2. Counts and proportions of peripheral Th1 cells and Th17 cells do not differ between GCA/PMR and controls. Cells were gated based on cytokine expression in unstimulated CD4+ T-cells, double positive populations were not included within the population of Th1 and Th17 (representative dot plots in **A**). Total Th1 and Th17 counts are shown in **B**, the percentage Th1 and Th17 cells of CD4+ T-cells is shown in **C**. N=21 for GCA and N=19 for PMR and HC. P-values of the Mann Whitney U test are shown in the graphs.

Correlations between monocyte subsets, CD4+ subsets and inflammatory markers

Th17 frequencies were linked to intermediate subsets in rheumatoid arthritis. Here, we investigated if numbers of the three different monocyte subsets were correlated with Th1 and Th17 counts in GCA and PMR patients (Figure 3A). Spearman's correlation coefficient with R> 0.50 or R< -0.50 together with a p value < 0.05 were considered significant. We did not observe significant correlations between monocyte subset counts and numbers of Th1 and Th17 cells in GCA and PMR. In addition,

the expression of HLA-DR on monocyte subsets, important in antigen presentation, was not correlated with Th1 and Th17 counts (data not shown).

We next assessed which subset best correlated with disease activity markers CRP and ESR (Figure 3A and B). In GCA patients, ESR correlated positively with counts of intermediate monocytes (R= 0.63), but this was not observed in PMR patients.

Reduced counts of circulating mDCs in GCA and PMR patients

Subsequently, we assessed the counts of circulating mDCs and pDCs. Remarkably, absolute counts of mDCs were significantly lower in newly-diagnosed, treatment-naive GCA (p=0.002) and PMR (p=0.01) patients compared to age-matched HCs (Figure 4). In addition, proportions of mDCs

A

GCA	Classical Monocytes	Intermediate monocytes	Non-classical monocytes	Th1 cells	Th17 cells
Th1 cells	-0.19	-0.15	-0.22		
Th17 cells	-0.29	0.16	0.01		
CRP	0.18	0.39	0.07	-0.25	-0.19
ESR	0.14	0.62**	0.21	-0.25	-0.02

Legend: R> 0.50

** p< 0.01

PMR	Classical Monocytes	Intermediate monocytes	Non-classical monocytes	Th1 cells	Th17 cells
Th1 cells	0.38	-0.04	0.13		
Th17 cells	0.44	0.46*	0.42		
CRP	-0.15	-0.21	-0.25	-0.39	-0.06
ESR	-0.29	0.05	-0.08	-0.17	-0.22

B

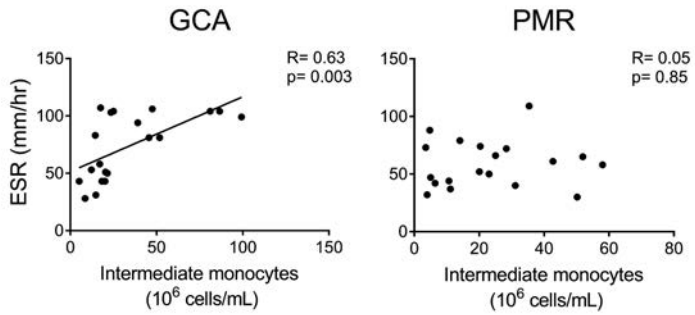


Figure 3. Relation between monocyte, Th subsets and inflammatory markers. Correlations as determined by Spearman's correlation coefficient are displayed for monocyte subsets (N=21 for GCA, N=19 for PMR), Th1 and Th17 cells (N=21 for GCA and N=19 for PMR), CRP and ESR (N=21 for GCA and N=19 for PMR) in **A**. In **B**, individual scatter plots are shown for the correlation of the ESR and the counts of intermediate monocytes.

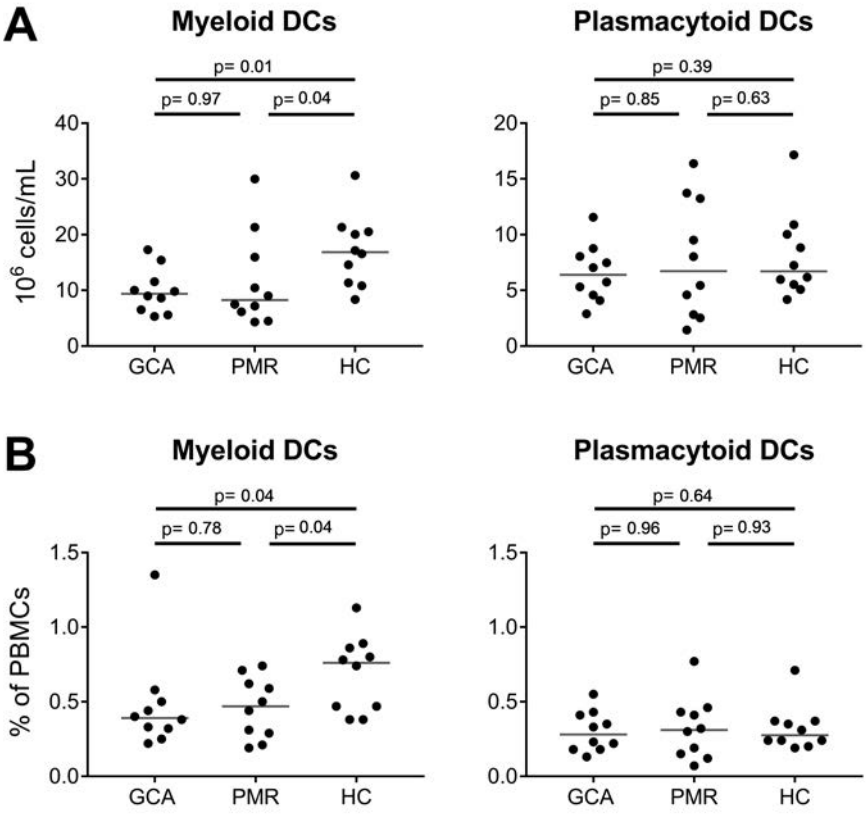


Figure 4. Absolute counts and proportions of mDCs are reduced in GCA and PMR patients. In **A**, the absolute counts of the circulating DC subsets are shown. Shown in **B** are proportions of mDCs and pDCs within total PBMCs. P-values of the Mann Whitney U test are shown in the graphs.

calculated as percentages of the total PBMC fraction, were lower as well. In contrast, absolute counts (and proportions) of circulating pDCs were unchanged in both GCA and PMR. Interestingly, counts of pDCs correlated negatively with Th1 cells in GCA (R=-0.71).

Elevated TLR2 expression by circulating mDCs in GCA and PMR

To obtain clues on sensing capabilities of monocytes and DCs, we measured the per cell expression of pattern recognition receptors TLR2, TLR4, TLR7, TLR8 and AIM2 (Figure 5). These pattern recognition receptors are specialized in detecting exogenous (PAMP) or endogenous (DAMP) ligands. Here, we show that TLR2 expression on mDCs is significantly higher in GCA (p=0.002) and PMR patients (p=0.01) than in HCs. No other significant differences between the groups were found by the Kruskal Wallis test. However, a strong trend for altered AIM2 expression on classical monocytes was observed (Kruskal Wallis p=0.05), with higher AIM2 per cell expression in classical monocytes of GCA patients than in HC (Mann Whitney U p=0.009). A heat map comparing the per cell expression of pattern recognition receptors between monocyte and DC subsets is shown in

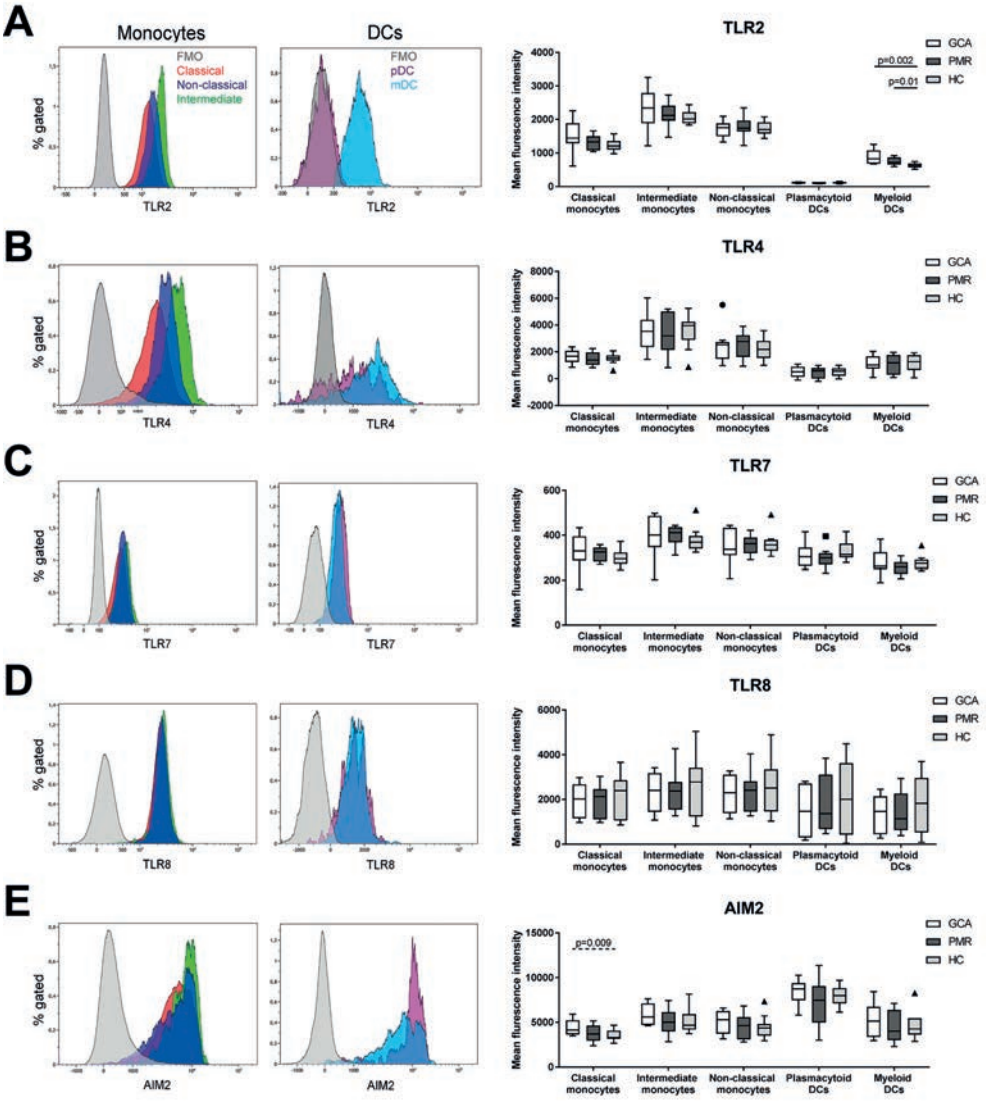


Figure 5. Expression of pattern recognition receptors on monocyte subsets and DC subsets. Shown are representative histogram and mean fluorescent intensities per group for TLR2 (**A**), TLR4 (**B**), TLR7 (**C**), TLR8 (**D**) and AIM2 (**E**). The histograms display expression for monocytes subsets and DC subsets. In the monocyte plot, classical monocytes are depicted in red, intermediate monocytes in green and non-classical monocytes in dark blue. In the DC plot, mDCs are depicted light blue and pDCs purple. The fluorescence minus one (FMO) control is depicted in grey. Kruskal Wallis and Mann Whitney U tests identified significant differences in TLR2 expression on mDCs between GCA, PMR and HC (N=10 for each group). The Kruskal Wallis test identified a trend (p=0.05) for differences in AIM2 expression on monocytes, with a significant difference between GCA and HC by Mann Whitney U. N=10 for each group.

supplementary Figure 5. Overall, intermediate monocytes clearly showed the highest expression of TLR2 and TLR4. Expression of AIM2 was highest on pDCs, while these cells had the lowest TLR2 and TLR4 expression.Expression of HLA-DR was also measured on monocyte and DC subsets as a measure of antigen presentation capacity (supplementary Figure 6). This capacity is suggested to be the highest for mDCs. Indeed, we found the highest per-cell HLA-DR expression on mDCs. Remarkably, also intermediate monocytes showed a relatively high HLA-DR expression. As expected, classical monocytes displayed the lowest levels. Expression of HLA-DR by all subsets was not different for GCA and PMR when compared to HC.

DISCUSSION

This is the first study simultaneously measuring the frequencies of circulating antigen presenting cells and Th subsets in treatment naive GCA and PMR patients. In this study, we show an altered composition of circulating monocyte subsets in GCA and PMR, but normal numbers of circulating Th1 and Th17 cells compared to HC. Overall, no relation was found between monocyte subset counts and Th1 or Th17 cell counts. Interestingly, our study revealed reduced numbers of circulating mDCs, but not pDCs, in both GCA and PMR. Finally, higher expression of TLR2 on circulating mDCs of GCA and PMR patients implies higher TLR2 mediated PAMP and DAMP sensing capacity for these cells.Myeloid DCs likely migrate from blood to inflamed tissue sites in GCA and PMR. Here, we show reduced numbers of mDCs, but not pDCs, in the blood of treatment-naive GCA and PMR patients. Although we have previously shown that classical monocyte counts are increased in GCA/PMR [13], we are not aware of previous investigations of circulating mDCs and pDCs in GCA and PMR. Previously, lower counts of circulating mDCs have also been described in inflammatory conditions such as Sjögren's syndrome [26], and other types of vascular inflammation, such as coronary artery disease [27]. In healthy arteries, a myeloid origin has been ascribed to resident DCs [3, 16]. Frequencies of DCs are 5-10 times higher in GCA TABs, suggesting massive recruitment of mDCs to the vessel wall during active disease [14]. These cells express CCR7 and are thought to be retained in the vessel wall due to high local production of CCR7 ligands CCL19 and CCL21. Similar findings in PMR suggest that mDCs might migrate to the inflamed synovium in these patients. Alternatively, enhanced apoptosis of mDCs or a reduced generation of mDCs could underlie their lower numbers in blood of GCA and PMR patients. Growth factors GM-CSF and M-CSF, locally produced in inflamed arteries of GCA patients [28], are important in the survival and generation of DC subsets [12]. Future studies should further characterize and phenotype the circulating DC subsets, for example by measuring CCR7 subset expression.

Different functions and phenotypes have been ascribed to the CD11c+ mDCs and the CD303+ pDCs [12]. DCs detect PAMPs and DAMPs through pattern recognition receptors, leading to activation and maturation, including the upregulation of CD83, CD86 and MHC-II molecules (e.g. HLA-DR) [3, 12]. Lymphoid progenitors are thought to be the precursors of pDCs, in contrast to the myeloid origin of mDCs [12]. mDCs are important for priming of naive T cells and skewing Th lineage differentiation. Indeed, we here found the highest expression of HLA-DR by mDCs. In contrast, pDCs have lower antigen presentation capabilities, but produce large amounts of type

I IFN and pro-inflammatory cytokines in response to pathogens. Compared to mDCs, pDCs have a drastically impaired capacity to migrate to the inflammatory site in response to inflammatory chemotactic chemokines [29]. Circulating pDCs may induce an anergic state of CD4+ T-cells [30], an observation that may explain the negative correlation we observed between pDCs and Th1 cells in GCA patients. The elevated expression of TLR2 on mDCs in GCA and PMR suggests an increased capacity for sensing TLR2 ligands, which may lead to DC activation. TLR2 and TLR4 on the cell surface, can recognize bacterial ligands (gram+ and gram-, respectively) as well as a variety of endogenous patterns [17]. One of the endogenous TLR2 ligands is serum amyloid A, which is locally produced in GCA TABs, and is reported to have pro-inflammatory and pro-angiogenic effects in an ex vivo GCA model [18, 31]. Pattern recognition receptors are important contributors to innate pathways acting as the first line of defence, and in shaping the adaptive immune response [17, 23]. Monocytes and DCs are able to specifically skew CD4+ T-cells depending on which pattern recognition receptor is activated [12, 22, 23]. PAMP stimulation of TLR2 was shown to skew CD4+ T-cells towards a Th17 phenotype, rather than towards Th1, in an experimental arthritis model [32]. As source of PAMPs, infectious pathogens have been implicated to be the trigger in the development of GCA and PMR, although so far no specific pathogen has been identified [33]. It would be interesting to investigate the responsiveness of mDCs to various pattern recognition receptor ligands in GCA and PMR patients, in particular TLR2 ligands such as varicella zoster and serum amyloid A [18, 34]. Expression of AIM2 appeared to be elevated in GCA classical monocytes. AIM2 is a cytosolic DNA sensor detecting double stranded DNA of certain bacteria and viruses (e.g. varicella zoster virus) as well as endogenous exposed DNA [35, 36]. AIM2 activation triggers inflammasome formation, leading to the cleavage of the pro-forms of IL-1-and IL-18, pro-inflammatory cytokines that are abundantly expressed in GCA TABs [14, 28]. AIM2 expression decreases with aging, suggesting increased vulnerability to infections in the elderly [35]. In contrast, hypomethylation of the AIM2 gene was observed in TABs of patients with GCA [37]. Our findings indicate that this potential upregulation of AIM2 in the inflamed artery is paralleled by increased expression of AIM2 by peripheral blood monocytes. Other pattern recognition receptors were not altered on monocytes and DCs of GCA and PMR patients. In contrast to Alvarez Rodriguez et al. [38], we did not observe a significantly higher expression of RNA sensor TLR7 on monocytes in GCA, even though we observed a trend ($p=0.07$ by Mann Whitney) for higher per cell TLR7 on GCA classical monocytes.

No correlation was found between monocyte subsets and Th subsets in blood of GCA and PMR patients. Monocytes, macrophages and DCs play an important role in Th-skewing by producing pro-inflammatory cytokines [5, 6]. Intermediate monocytes (CD14++CD16+) are the most pro-inflammatory monocyte subset and have been implicated in Th17 expansion in rheumatoid arthritis [39]. Here, we show that intermediate monocytes correlate with the inflammatory marker ESR in GCA patients, but not in PMR. This is in congruence with our previous study in which total monocyte counts were correlated with the systemic inflammatory response in GCA only [40]. As we did not observe elevated Th17 counts in this study, a possible link with intermediate monocyte counts may be hard to detect. Previous studies did report an expansion of circulating Th1 and Th17 subpopulations in GCA [6-8]. Although we documented similar proportions of Th1 and Th17 cells in HCs, our study documents lower Th1 and Th17 proportions in GCA patients than in these

prior studies. Interestingly, Samson et al, reported even on reduced counts of Th1 cells in GCA/PMR patients [9]. As our protocol for defining Th1 and Th17 cells appears to be similar to the other studies, we propose differences in patient selection. Alternatively, Th1 and Th17 skewing should be studied at the site of inflammation since Th1 and Th17 skewing cytokines are all highly expressed by macrophages and DCs at the inflammatory site [28, 41, 42].

Strengths of this study include the thorough characterization of the patient and control populations, including criteria that excluded individuals with other morbidities. Patients and controls did not take immunomodulatory medication, thereby excluding drug effects on cell numbers and phenotypes. Moreover, this study is the first to document absolute counts of DC and Th subsets. Limitations of this study include the relatively small number of patients for some of the analyses, implying that results should be interpreted with caution. However, the similar findings in GCA and PMR patients strengthen confidence in our data.

In conclusion, we confirm our previous findings on altered distribution of monocyte subsets in blood of GCA and PMR patients. We also found reduced circulating mDC counts and elevated per cell expression of TLR2 by mDCs in GCA/PMR. Future studies should address if higher pattern recognition receptor expression by mDCs of GCA and PMR patients translates into a higher sensing activity for TLR2 ligands. Moreover, studies tracking circulating mDCs could reveal if these cells indeed migrate to the inflammatory site in GCA and PMR.

REFERENCES

1. Samson, M., Corbera-Bellalta, M., Audia, S., Planas-Rigol, E., Martin, L., Cid, M.C., and Bonnotte, B. 2017. Recent advances in our understanding of giant cell arteritis pathogenesis. *Autoimmun. Rev. 16: 833-844.*
2. Dejaco, C., Duftner, C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2016. The spectrum of giant cell arteritis and polymyalgia rheumatica: revisiting the concept of the disease. *Rheumatology (Oxford) 56(4):506-515.*
3. Ma-Krupa, W., Jeon, M.S., Spoerl, S., Tedder, T.F., Goronzy, J.J., and Weyand, C.M. 2004. Activation of arterial wall dendritic cells and breakdown of self-tolerance in giant cell arteritis. *J. Exp. Med. 199: 173-183.*
4. Weyand, C.M., Hicok, K.C., Hunder, G.G., and Goronzy, J.J. 1994. Tissue cytokine patterns in patients with polymyalgia rheumatica and giant cell arteritis. *Ann. Intern. Med. 121: 484-491.*
5. Geginat, J., Paroni, M., Maglie, S., Alfen, J.S., Kastirr, I., Gruarin, P., De Simone, M., Pagani, M., and Abrignani, S. 2014. Plasticity of human CD4 T cell subsets. *Front. Immunol. 5: 630.*
6. Deng, J., Younge, B.R., Olshen, R.A., Goronzy, J.J., and Weyand, C.M. 2010. Th17 and Th1 T-cell responses in giant cell arteritis. *Circulation 121: 906-915.*
7. Terrier, B., Geri, G., Chaara, W., Allenbach, Y., Rosenzwajg, M., Costedoat-Chalumeau, N., Fouret, P., Musset, L., Benveniste, O., and Six, A. 2012. Interleukin-21 modulates Th1 and Th17 responses in giant cell arteritis. *Arthritis Rheumatol. 64: 2001-2011.*
8. Saadoun, D., Garrido, M., Comarmond, C., Desbois, A., Domont, F., Savey, L., Terrier, B., Geri, G., Rosenzwajg, M., and Klatzmann, D. 2015. Th1 and Th17 cytokines drive inflammation in Takayasu arteritis. *Arthritis Rheumatol. 67: 1353-1360.*
9. Samson, M., Audia, S., Fraszczak, J., Trad, M., Ornetti, P., Lakomy, D., Ciudad, M., Leguy, V., Berthier, S., and Vinit, J. 2012. Th1 and Th17 lymphocytes expressing CD161 are implicated in giant cell arteritis and polymyalgia rheumatica pathogenesis. *Arthritis Rheumatol. 64: 3788-3798.*
10. Cosmi, L., De Palma, R., Santarlasci, V., Maggi, L., Capone, M., Frosali, F., Rodolico, G., Querci, V., Abbate, G., Angeli, R., Berrino, L., Fambrini, M., Caproni, M., Tonelli, F., Lazzeri, E., Parronchi, P., Liotta, F., Maggi, E., Romagnani, S., and Annunziato, F. 2008. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J. Exp. Med. 205: 1903-1916.*
11. Dress, R.J., Wong, A.Y., and Ginhoux, F. 2018. Homeostatic control of dendritic cell numbers and differentiation. *Immunol. Cell Biol. 96: 463-476.*
12. van Sleen, Y., Wang, Q., van der Geest, K.S.M., Westra, J., Abdulahad, W.H., Heeringa, P., Boots, A.M.H., and Brouwer, E. 2017. Involvement of Monocyte Subsets in the Immunopathology of Giant Cell Arteritis. *Sci. Rep. 7: 6553.*
13. Krupa, W.M., Dewan, M., Jeon, M., Kurtin, P.J., Younge, B.R., Goronzy, J.J., and Weyand, C.M. 2002. Trapping of misdirected dendritic cells in the granulomatous lesions of giant cell arteritis. *Am. J. Pathol. 161: 1815-1823.*
14. Wagner, A., Wittkop, U., Prahst, A., Schmidt, W., Gromnica-Ihle, E., Vorpahl, K., Hudson, A., and Zeidler, H. 2003. Dendritic cells co-localize with activated CD4 T cells in giant cell arteritis. *Clin. Exp. Rheumatol. 21: 185-192.*
15. Han, J.W., Shimada, K., Ma-Krupa, W., Johnson, T.L., Nerem, R.M., Goronzy, J.J., and Weyand, C.M. 2008. Vessel wall–embedded dendritic cells induce T-cell autoreactivity and initiate vascular inflammation. *Circ. Res. 102: 546-553.*
16. O'neill, L. and Molloy, E.S. 2016. The role of toll like receptors in giant cell arteritis. *Rheumatology (Oxford) 55: 1921-1931.*
17. O'Neill, L., Rooney, P., Molloy, D., Connolly, M., McCormick, J., McCarthy, G., Veale, D.J., Murphy, C.C., Fearon, U., and Molloy, E. 2015. Regulation of Inflammation and Angiogenesis in Giant Cell Arteritis by Acute-Phase Serum Amyloid A. *Arthritis Rheumatol. 67: 2447-2456.*
18. Alvarez Rodriguez, L., Lopez-Hoyos, M., Mata, C., Fontalba, A., Calvo Alen, J., Marin, M.J., Fernandez-Luna, J.L., Aguero Balbin, J., Aranzamendi Zaldunbide, M., Blanco, R., and Martinez-Taboada, V.M. 2011. Expression and function of toll-like receptors in peripheral blood mononuclear cells of patients with

polymyalgia rheumatica and giant cell arteritis. *Ann. Rheum. Dis. 70: 1677-1683.*

19. Pryshchep, O., Ma-Krupa, W., Younge, B.R., Goronzy, J.J., and Weyand, C.M. 2008. Vessel-specific Toll-like receptor profiles in human medium and large arteries. *Circulation 118: 1276-1284.*

20. Deng, J., Ma-Krupa, W., Gewirtz, A.T., Younge, B.R., Goronzy, J.J., and Weyand, C.M. 2009. Toll-like receptors 4 and 5 induce distinct types of vasculitis. *Circ. Res. 104: 488-495.*

21. Rossol, M., Kraus, S., Pierer, M., Baerwald, C., and Wagner, U. 2012. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheumatol. 64: 671-677.*

22. Wenink, M.H., Santegoets, K.C., Broen, J.C., van Bon, L., Abdollahi-Roodsaz, S., Popa, C., Huijbens, R., Remijn, T., Lubberts, E., van Riel, P.L., van den Berg, W.B., and Radstake, T.R. 2009. TLR2 promotes Th2/Th17 responses via TLR4 and TLR7/8 by abrogating the type I IFN amplification loop. *J. Immunol. 183: 6960-6970.*

23. Dasgupta, B., Cimmino, M.A., Kremers, H.M., Schmidt, W.A., Schirmer, M., Salvarani, C., Bachta, A., Dejaco, C., Duftner, C., and Jensen, H.S. 2012. 2012 provisional classification criteria for polymyalgia rheumatica: a European League Against Rheumatism/American College of Rheumatology collaborative initiative. *Arthritis Rheumatol. 64: 943-954.*

24. Chuang, T.Y., Hunder, G.G., Ilstrup, D.M., and Kurland, L.T. 1982. Polymyalgia rheumatica: a 10-year epidemiologic and clinical study. *Ann. Intern. Med. 97: 672-680.*

25. Ozaki, Y., Amakawa, R., Ito, T., Iwai, H., Tajima, K., Uehira, K., Kagawa, H., Uemura, Y., Yamashita, T., and Fukuhara, S. 2001. Alteration of peripheral blood dendritic cells in patients with primary Sjögren's syndrome. *Arthritis Rheumatol. 44: 419-431.*

26. Yilmaz, A., Weber, J., Cicha, I., Stumpf, C., Klein, M., Raithel, D., Daniel, W.G., and Garlichs, C.D. 2006. Decrease in circulating myeloid dendritic cell precursors in coronary artery disease. *J. Am. Coll. Cardiol. 48: 70-80.*

27. Jiemy, W.F., van Sleen, Y., van der Geest, K.S.M., ten Berge, H.A., Abdulahad, W.H., Sandovici, M., Boots, A.M.H., Heeringa, P., and Brouwer, E. 2019. Distinct Macrophage Subsets Dictated by Local GM-CSF and M-CSF expression are Associated with Tissue Destruction and Intimal Hyperplasia in Giant Cell Arteritis. *Submitted.*

28. de la Rosa, G., Longo, N., Rodríguez-Fernández, J.L., Puig-Kroger, A., Pineda, A., Corbí, ÁL., and Sánchez-Mateos, P. 2003. Migration of human blood dendritic cells across endothelial cell monolayers: adhesion molecules and chemokines involved in subset-specific transmigration. *J. Leukoc. Biol. 73: 639-649.*

29. Kuwana, M. 2002. Induction of anergic and regulatory T cells by plasmacytoid dendritic cells and other dendritic cell subsets. *Hum. Immunol. 63: 1156-1163.*

30. van Sleen, Y., Sandovici, M., Abdulahad, W., Bijzet, J., van der Geest, K., Boots, A.M., and van der Geest, Kornelis SM. 2019. Markers of Angiogenesis and Macrophage Products for Predicting Disease Course and Monitoring Vascular Inflammation in Giant Cell Arteritis. *Rheumatology (Oxford) 2019 58;8: 1383-1392*

31. de Aquino, S.G., Abdollahi-Roodsaz, S., Koenders, M.I., van de Loo, F.A., Pruijn, G.J., Marijnissen, R.J., Walgreen, B., Helsen, M.M., van den Bersselaar, L.A., de Molon, R.S., Avila Campos, M.J., Cunha, F.Q., Cirelli, J.A., and van den Berg, W.B. 2014. Periodontal pathogens directly promote autoimmune experimental arthritis by inducing a TLR2- and IL-1-driven Th17 response. *J. Immunol. 192: 4103-4111.*

32. Rhee, R.L., Grayson, P.C., Merkel, P.A., and Tomasson, G. 2017. Infections and the risk of incident giant cell arteritis: a population-based, case-control study. *Ann. Rheum. Dis. 76: 1031-1035.*

33. Wang, J.P., Kurt-Jones, E.A., Shin, O.S., Manchak, M.D., Levin, M.J., and Finberg, R.W. 2005. Varicella-zoster virus activates inflammatory cytokines in human monocytes and macrophages via Toll-like receptor 2. *J. Virol. 79: 12658-12666.*

34. Wang, Q., Westra, J., van der Geest, Kornelis SM, Moser, J., Bijzet, J., Kuiper, T., Lorencetti, P.G., Joosten, L.A., Netea, M.G., and Heeringa, P. 2016. Reduced levels of cytosolic DNA sensor AIM2 are associated with impaired cytokine responses in healthy elderly. Exp. Gerontol. 78: 39-46.

35. Rathinam, V.A., Jiang, Z., Waggoner, S.N., Sharma, S., Cole, L.E., Waggoner, L., Vanaja, S.K., Monks, B.G., Ganesan, S., and Latz, E. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat. Immunol. 11: 395.*

36. Coit, P., De Lott, L.B., Nan, B., Elner, V.M., and Sawalha, A.H. 2015. DNA methylation analysis of the temporal artery microenvironment in giant cell arteritis. *Ann. Rheum. Dis. 75:1196-202.*

37. Alvarez Rodriguez, L., Lopez-Hoyos, M., Mata, C., Fontalba, A., Calvo Alen, J., Marin, M.J., Fernandez-Luna, J.L., Aguero Balbin, J., Aranzamendi Zaldunbide, M., Blanco, R., and Martinez-Taboada, V.M. 2011. Expression and function of toll-like receptors in peripheral blood mononuclear cells of patients with polymyalgia rheumatica and giant cell arteritis. *Ann. Rheum. Dis. 70: 1677-1683.*

38. Rossol, M., Kraus, S., Pierer, M., Baerwald, C., and Wagner, U. 2012. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheumatol. 64: 671-677.*

39. van Sleen, Y., Graver, J.C., Abdulahad, W.H., van der Geest, K., Boots, A.M., Sandovici, M., and Brouwer, E. 2019. Leukocyte Dynamics Reveal a Persistent Myeloid Dominance in Giant Cell Arteritis and Polymyalgia Rheumatica. *Front. Immunol. 10: 1981.*

40. van der Geest, K.S., Abdulahad, W.H., Rutgers, A., Horst, G., Bijzet, J., Arends, S., Roffel, M.P., Boots, A.M., and Brouwer, E. 2015. Serum markers associated with disease activity in giant cell arteritis and polymyalgia rheumatica. *Rheumatology (Oxford) 54: 1397-1402.*

41. Espigol-Frigole, G., Planas-Rigol, E., Ohnuki, H., Salvucci, O., Kwak, H., Ravichandran, S., Luke, B., Cid, M.C., and Tosato, G. 2016. Identification of IL-23p19 as an endothelial proinflammatory peptide that promotes gp130-STAT3 signaling. *Sci. Signal. 9: ra28.*

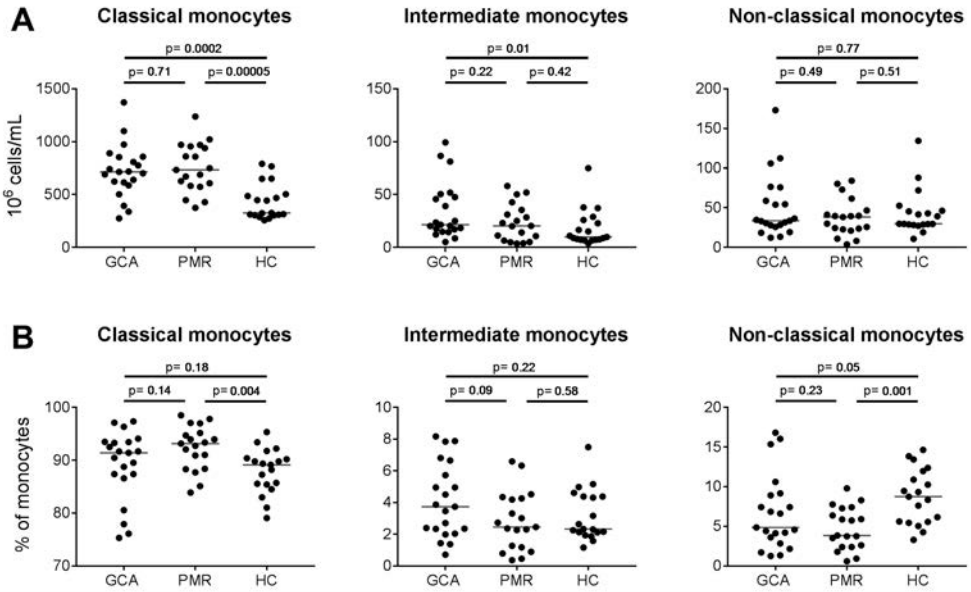
SUPPLEMENTARY DATA

Supplementary Table 1. Antibodies for flow cytometry used for monocytes and DCs in cohort A and B.

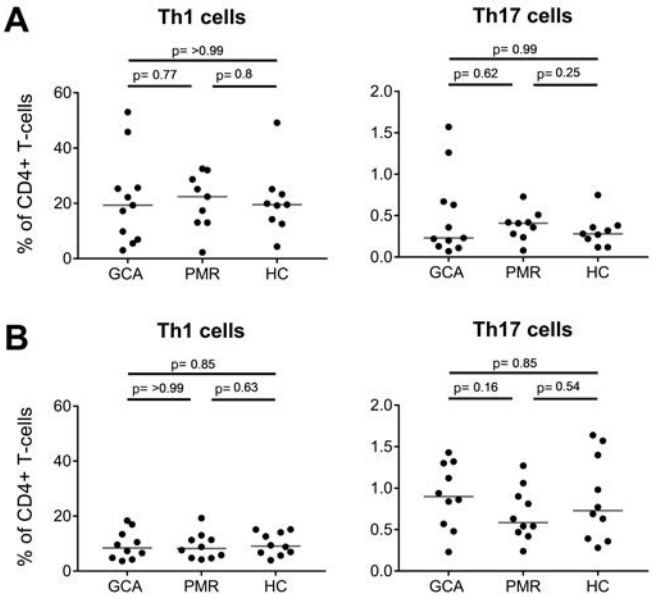
	Marker	Fluorochrome	Clone	Company
Cohort A	CD14	PE	OKT3	Biolegend, San Diego, CA, USA
	CD16	V450	3G8	BD biosciences, San Jose, CA, USA
	CD56	BV510	HCD56	Biolegend
	CD66b	PE-cy7	G10F5	Thermo Fisher, Waltham, MA, USA
	CD3	APC	UCHT1	BD
Cohort B	CD14	Pacific Orange	TuK4	Thermo Fisher
	CD16	BUV737	3G8	BD
	HLA-DR	Percp-cy5.5	L243	Sony, Tokio, Japan
	CD11c	APC-efluor780	BU15	Thermo Fisher
	CD303	BV785	201A	Biolegend
	TLR2	FITC	TL2.1	Thermo Fisher
	TLR4	BV711	TF901	BD
	TLR7	Alexa Fluor 594	533707	R&D Systems, Minneapolis, MN, USA
	TLR8	DyLight 405	44C143	R&D Systems
	AIM2	Alexa Fluor 647	10M5G5	R&D Systems

Supplementary Table 2. Antibodies for flow cytometry used for CD4+ T-cells in cohort A and B.

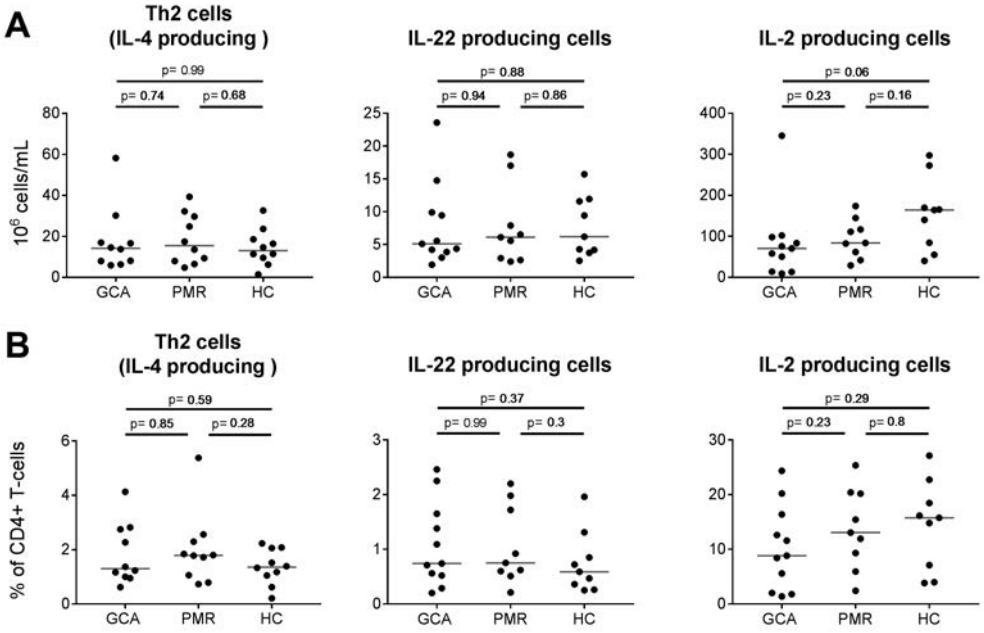
	Marker	Fluorochrome	Clone	Company
Cohort A	CD3	APC	UCHT1	BD
	CD4	APC-H7	RPA-T4	BD
	IL-17	Alexa Fluor 488	eBio64DEC17	Thermo Fisher
	IFNγ	Percp-cy5.5	4S.B3	Biolegend
	IL-22	PE-cy7	22URTI	Thermo Fisher
	IL-2	Alexa Fluor 700	MQ1-17H12	Biolegend
	Viability	Dye450		Thermo Fisher
Cohort B	CD3	Alexa Fluor 700	UCHT1	Biolegend
	CD8	APC-H7	SK-1	BD
	CD161	PE	191B8	Miltenyi, Cologne, Germany
	IL-17	Alexa Fluor 488	eBio64DEC17	Thermo Fisher
	IFNγ	Percp-cy5.5	4S.B3	Biolegend
	IL-4	PE-cy7	MP4-25D2	Biolegend
	Viability	Dye450		Thermo Fisher



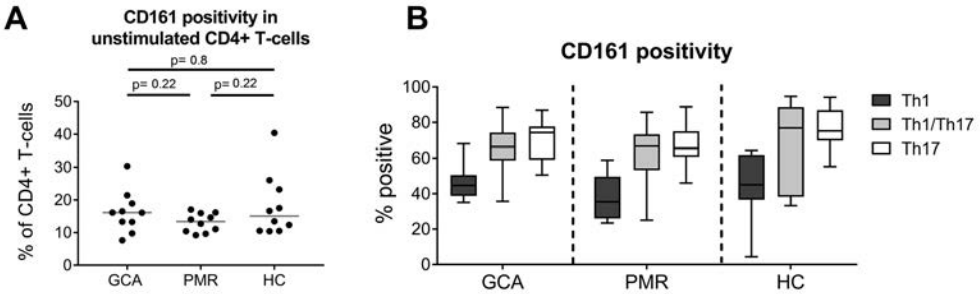
Supplementary Figure 1. Absolute counts (**A**) and proportions (**B**) of circulating monocyte subsets. N= 21 for GCA, N=19 for PMR and HC. P-values of the Mann Whitney U test are shown in the graphs.



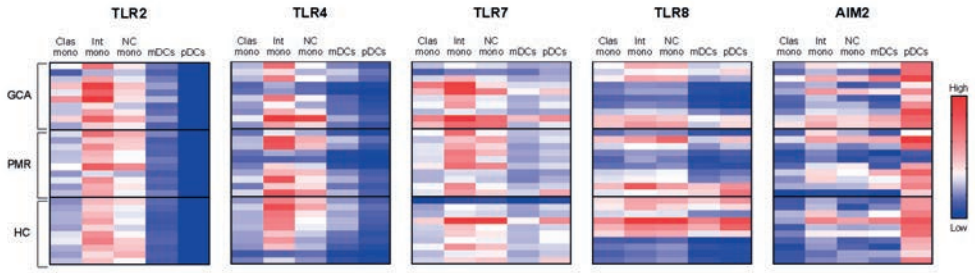
Supplementary Figure 2. Comparison of the proportions of Th1 and Th17 cells in cohort A (**A**) and cohort B (**B**). The percentage of Th1 cells is higher in cohort A than in cohort B, whereas Th17 cells are higher in cohort B than in cohort A. Cohort A: N=11 for GCA, N=9 for PMR and HC. Cohort B: N=10 for all groups. P-values of the Mann Whitney U test are shown in the graphs.



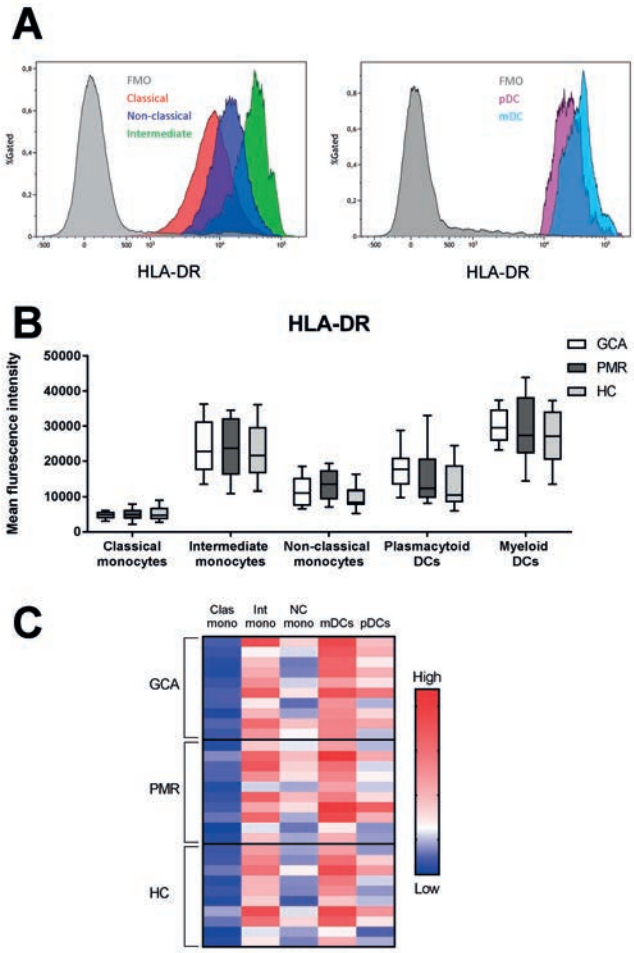
Supplementary Figure 3. Absolute counts (**A**) and proportions (**B**) of IL-4 (Th2 cells), IL-22 and IL-2 producing CD4+ T-cells. IL-4: N=10 for each group. IL-22/IL-2: N=11 for GCA, N=9 for PMR and HC. P-values of the Mann Whitney U test are shown in the graphs.



Supplementary Figure 4. Expression of CD161 on unstimulated CD4+ T-cells (**A**) and on activated CD4+ T-cell subsets (**B**). N=10 for each group.



Supplementary Figure 5. Heat maps of relative expression of pattern recognition receptors on monocyte and DC subsets. Cell colors represent relative expression of each receptor in GCA/PMR patients and HCs (N=10).



Supplementary Figure 6. HLA-DR expression on monocyte and DC subsets. Of the monocyte subsets, HLA-DR expression was highest on the intermediate subset (**A**). HLA-DR expression on mDCs was higher than on pDCs. Kruskal Wallis and Mann Whitney U tests identified no significant differences between GCA, PMR and HC (**B**, N=10 for each group). A heat map of individual HLA-DR expression per subset is depicted in **C**.

Jiemy WF*,

van Sleen Y*,

ten Berge HA,

van der Geest KSM,

Abdulahad WH,

Sandovici M, Boots AMH,

Heeringa P,

Brouwer E.

* Shared first author

FIVE

Distinct Macrophage Subsets Dictated by Local GM-CSF and M-CSF Expression are Associated with Tissue Destruction and Intimal Hyperplasia in Giant Cell Arteritis

Submitted

ABSTRACT

Giant cell arteritis (GCA) is a form of vasculitis that affects medium-sized (i.e., the temporal artery) and large-sized arteries (i.e., the aorta) and is characterized by massive infiltration of T-cells and macrophages. It is well known that macrophages in tissue may display considerable heterogeneity in their responses to cues from the environment. To date, little is known about macrophage heterogeneity in GCA. We hypothesized that the spatial distribution of different macrophage phenotypes, governed by local GM-CSF and M-CSF expression, is associated with tissue destruction and intimal proliferation.

Temporal artery biopsies (TABs, n=11) from treatment-naive GCA patients, aorta samples from GCA-related aneurysms (n=10) and atherosclerotic aorta samples (n=10) were included. To assess macrophage phenotypes, we employed immunohistochemistry targeting selected macrophage phenotypic markers (CD64, CD206, CD86, FRβ), cytokines, matrix metalloproteinases (MMPs), and growth factors (GM-CSF and M-CSF). Expression by macrophages was established by double-staining with PU.1. In vitro macrophage differentiation was performed to assess whether GM-CSF and M-CSF are crucial drivers of macrophage phenotypic heterogeneity. Macrophage marker expression was determined by flow cytometry, and soluble products (IL-6, IL-10, IL-23, MMP-9) were measured by Luminex assay.

A distinct spatial distribution pattern of macrophage phenotypes in TABs was identified. CD206-expressing, MMP-9-producing macrophages were located at the site of tissue destruction, whereas FRβ-expressing macrophages were located in the inner intima of arteries with a high degree of intimal hyperplasia. Notably, this distinct pattern could also be observed in macrophage-rich areas in GCA aortas but not in atherosclerotic aortas. In vitro, CD206 was highly upregulated by GM-CSF treatment, while FRβ expression was observed only on M-CSF-skewed macrophages. In line with this finding, localized GM-CSF and M-CSF expression that could contribute to macrophage heterogeneity in the tissue was detected.

Our characterization of macrophage phenotypes in GCA lesions documents a distinct spatial distribution pattern of CD206+MMP-9+ macrophages involved in tissue destruction and FRβ+ macrophages associated with intimal proliferation. We suggest that these macrophages are phenotypically skewed by sequential GM-CSF and M-CSF signals. Based on this study, therapies targeting specific macrophage phenotypes could be designed. These macrophage markers may also prove useful for imaging.

INTRODUCTION

Giant cell arteritis (GCA) is the most frequent form of vasculitis; it often affects medium and large vessels, and it occurs exclusively in elderly individuals [1]. Patients with GCA present with various symptoms, depending on which arteries are affected [2]. Inflammation of cranial arteries (e.g., the temporal artery) often leads to headache but can also cause ischemic symptoms (such as jaw claudication and vision loss) that are related to narrowing of the vascular wall as a result of inflammation. Large arteries such as the aorta can also be affected, although symptoms of large-vessel GCA are often nonspecific, which may lead to diagnostic delay [3]. Without proper treatment, large-vessel GCA can cause aortic aneurysm and dissection [4] due to chronic damage to the vascular wall. Glucocorticoids (GCs) remain the main treatment option for GCA patients, although novel GC-sparing therapies have recently become available, such as tocilizumab (IL-6 receptor blockade) [5].

The pathology of GCA is characterized by a granulomatous infiltrate in the vessel wall, which mainly consists of T-cells and macrophages [1]. Some of the macrophages fuse and develop into multinucleated giant cells [6]. Macrophages in GCA lesions are derived from circulating monocytes, of which three subsets have been identified: classical CD14highCD16- cells, intermediate CD14highCD16+ cells and non-classical CD14dimCD16+ cells. Monocytes migrate to the inflamed vessel wall guided by chemokines such as CCL2 and CX3CL1 [7].

Macrophages are the main producers of proinflammatory cytokines, growth factors and tissue-destructive molecules, including matrix metalloproteinases (MMPs) [8], which enhance inflammation, cause damage to the lamina elastica [9] and contribute to vessel wall remodeling and intimal hyperplasia. Infiltration and proliferation in the intimal layer of the artery ultimately leads to occlusion, a process responsible for ischemic symptoms including vision loss [1]. Recently, macrophage-derived MMP-9 was reported to be essential in T-cell infiltration into the vessel wall [10]. Macrophages also play a major role in the skewing of T-cells in the vessel wall by producing polarizing cytokines. T-cells activated in the presence of IL-12 and IL-18 develop into IFN-γ-producing Th1 cells, whereas TGF-β, IL-6 and IL-23 lead to Th17 activation [11]. GCA tissue displays a mixed population of proinflammatory Th1 and Th17 cells but essentially lacks Th2 cells or Tregs [1].Macrophages are incredibly plastic cells that can switch phenotypes and functions depending on environmental cues. Recently, the growth factors granulocyte macrophage stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) were shown to skew macrophages into different phenotypes [12]. CD206 (mannose receptor), a macrophage marker associated with tissue remodeling, was recently found to be highly upregulated on GM-CSF-primed macrophages [13]. Folate receptor β (FRβ), on the other hand, has been described as a marker of M-CSF differentiation [14]. FRβ+ macrophages have been associated with fibroblast activation and proliferation in rheumatoid arthritis [15]. Fibroblast proliferation is also a key finding in the intimal layer in GCA and eventually leads to occlusion of the vessel. Whether macrophages from GCA patients respond similarly to GM-CSF and M-CSF skewing signals needs to be elucidated.

The prominence and function of macrophages in GCA pathology make them a promising target for new treatment strategies. New therapies have targeted cytokines produced by macrophages: tocilizumab (IL-6 receptor [5]) and ustekinumab (IL-12 and IL-23; NCT03711448). Another current

trial is assessing the efficacy of targeting macrophages with mavrilimumab (NCT03827018), an antibody directed toward the GM-CSF receptor. However, the expression of GM-CSF and M-CSF in GCA lesions and their relation to macrophage phenotypes has not yet been assessed.

Although macrophages are one of the dominant inflammatory cellular infiltrates in GCA lesions, little is known about their phenotypic heterogeneity and spatial distribution within the affected vessel wall. The purpose of this study was to determine the presence and spatial distribution of macrophage subsets in GCA lesions relative to the morphological features of tissue destruction and intimal hyperplasia. We hypothesized that within GCA lesions, distinct macrophage subsets are associated with distinct functions and lesion morphology dictated by local GM-CSF and M-CSF production. To address this hypothesis, we first comprehensively characterized macrophage phenotypes in affected temporal artery biopsies (TABs) and aortic samples from GCA patients using a panel of established macrophage polarization markers and inflammatory factors in relation to lesion morphology. Next, to investigate whether GM-CSF and M-CSF signals are crucial drivers of macrophage polarization, their effects on macrophage differentiation and phenotypes were determined in vitro in conjunction with assessment of the expression of these growth factors in GCA lesions.

MATERIALS AND METHODS

Patients

Eleven TAB tissue samples of histologically proven GCA collected before the start of GC treatment were studied (Table 1). In addition, 15 noninflamed TAB tissue samples were included as controls: five from patients who had (PET-CT proven) GCA, five from isolated PMR patients, and five from individuals who had neither GCA nor PMR. The diagnosis of GCA was based on positive 18F-fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET-CT) for GCA and/or a positive TAB, based on a pathologist's assessment. PMR was diagnosed by a positive PET-CT for PMR. Visual ischemia was scored if a patient suffered from either permanent vision loss or amaurosis fugax. Other ischemic symptoms were scored under claudication: jaw/tongue claudication, transient ischemic attack (TIA), cerebrovascular accident (CVA), and arm/leg claudication. Clinical and laboratory data for these patients were collected as part of our prospective cohort study. The study was approved by the institutional review board of University Medical Center Groningen (METc2010/222). Written informed consent was obtained from all study participants. All procedures were in compliance with the Declaration of Helsinki.

Aorta tissues from GCA patients (n=10) and age-matched atherosclerotic controls (n=10) were retrospectively obtained after aortic aneurysm surgery (Table 1). None of the patients used GCs at the time of surgery. GCA was diagnosed by pathologists after surgery was performed. The patients' clinical and laboratory data at the time of surgery were extracted from medical records. Consent from the Internal Review Board and written patient consent were not required under Dutch law for human medical research (WMO) since the tissue was obtained during necessary surgery. The patients were informed about the study and agreed that the obtained medical data could be used for research purposes in accordance with privacy rules.

Table 1. Clinical characteristics of patients and controls included in the tissue study and the *in vitro* study.

	GCA positive TAB	GCA aorta	Arthero-sclerosis aorta	GCA PBMCs	Healthy control PBMCs
N	11	10	10	10	10
Age (median; years)	74	66	65	72	72
Sex (% female)	70	70	50	70	70
Fulfilled ACR criteria (yes/no)	11/0	NA	NA	8/2	NA
Claudication (yes/no)	9/2	NA	NA	4/6	NA
Visual ischemia (yes/no)	4/7	NA	NA	1/9	NA
PMR clinic (yes/no)	1/10	NA	NA	2/8	NA
CRP (mg/L; median)	66	7	10	38	1,5
ESR (mm/hr; median)	83	13	15	73	9

GCA: giant cell arteritis, TAB: temporal artery biopsy, PMR: polymyalgia rheumatica, PBMCs: peripheral blood mononuclear cells, ACR: American College of Rheumatology, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate.

Frozen peripheral blood mononuclear cells (PBMCs) from treatment-naive GCA patients (n=10) and age- and sex-matched healthy controls (HCs, n=10) participating in the prospective cohort study were used for in vitro studies (Table 1). Additionally, for these patients, the GCA diagnosis was confirmed by TAB and/or PET-CT. HCs were screened by health assessment questionnaires, physical examination and laboratory tests for past and actual morbidities and excluded when they were not healthy according to the Senieur criteria [16].

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissues were cut into sections of 3 μm. The sections were deparaffinized and rehydrated, followed by antigen retrieval in a 95°C water bath (for buffers, see Supplementary Table 1). For single staining, tissues were incubated with primary anti-human antibodies (Supplementary Table 1), followed by endogenous peroxidase blocking. The tissues were subsequently incubated with secondary antibodies (Supplementary Table 1), 3-amino-9-ethylcarbazole for peroxidase activity detection, and finally hematoxylin as a counter stain. Matching isotype controls were also included (Supplementary Figure 2). For double staining with the macrophage transcription factor PU.1, tissues were simultaneously incubated with two primary antibodies (Supplementary Table 1). A MultiVision alkaline phosphatase and horseradish peroxidase double-staining kit was used. Positive control tissues were also included. For most staining experiments, the positive control was reactive tonsil tissue, but for IL-10, it was lymph node tissue. All tissues were scanned using a Nanozoomer Digital Pathology Scanner (NDP Scan U 10074-01, Hamamatsu Photonics K.K.).

The tissues were stained for a selection of proinflammatory and tissue remodeling markers (Supplementary Table 1). Three layers, namely, the adventitia, media-intima and inner intima, were scored for each TAB, and the media-intima layer was defined as the media layer plus the area with dense infiltrating cells surrounding the internal lamina elastica. The inner intima layer was defined as the intimal proliferation area with a lower density of infiltrating leucocytes (Supplementary

Figure 1). Occlusion scores were calculated as a percentage of the intima thickness calculated from the intima-media border to the center of the lumen. Occlusion scores above 70% were categorized as massive occlusion.

GCA-positive TABs, GCA-positive aortas and atherosclerotic aortas were semiquantitatively scored on a five-point scale (0–4): 0 = no positive cells, 1 = occasional positive cells (0–1% estimated positive), 2 = small numbers of positive cells (>1–20%), 3 = moderate numbers of positive cells (>20–50%), 4 = large numbers of positive cells (more than 50%). An average score was calculated from assessments by two independent investigators. Tissues were scored in representative areas that contained infiltrating cells, as GCA can contain skip lesions.

Generation of monocyte-derived macrophages *in vitro*

PBMC-derived monocytes from GCA patients and HCs were differentiated for 7 days in vitro in the presence of GM-CSF and M-CSF to generate GM-CSF-differentiated macrophages (GM-MØs) and M-CSF-differentiated macrophages (M-MØs), respectively. GCA and HC monocytes were isolated from thawed peripheral blood mononuclear cells (PBMCs) by negative selection using the EasySep monocyte enrichment kit (Stemcell, Vancouver, BC, Canada), which does not deplete CD16+ monocytes. Isolated monocytes were analyzed by flow cytometry or cultured for seven days in DMEM containing 2 mM glutamine, 60 µg/mL penicillin-streptomycin and 10% FCS in the presence of 100 ng/mL GM-CSF (Peprotech, Rocky Hill, NJ, USA) to generate GM-MØs or 100 ng/mL M-CSF (Peprotech) to generate M-MØs. The medium was replaced on the second and fourth days. On day 7, after collecting the supernatants, monocyte-derived macrophages were harvested using citrate saline (135 mM potassium chloride, 15 mM sodium citrate and 1 mM EDTA) for 15 minutes at 37°C.

Flow cytometry

Phenotyping of monocytes and monocyte-derived macrophages was performed by flow cytometry using fluorochrome-conjugated monoclonal antibodies specific for HLA-DR (FITC, BD Biosciences Franklin Lakes, NJ, USA), CD14 (Pacific Orange, Thermo Fisher Scientific, Waltham, MA, USA), CD16 (BUV737, BD), CD64 (APC-Cy7, Biolegend, San Diego, CA, USA), CD86 (BV711, BD), CD206 (PE-Cy7, Biolegend) and FRβ (APC, Biolegend). The expression of the GM-CSF receptor (BV650, BD) and the M-CSF receptor (PE-Cy7, Biolegend) on monocyte subsets was analyzed by a separate flow cytometry panel (including the aforementioned CD14, CD16 and HLA-DR antibodies). Cells were measured on an LSR-II (BD) flow cytometer. For comparisons of the mean fluorescence intensity (MFI) between experiments, the LSR-II flow cytometer was calibrated for each run using FACSDiva CS&T research beads (BD). Data were analyzed using Kaluza software (BD). Monocytes and macrophages were gated by FSC/SSC, doublets were excluded, and dead cells were excluded using Zombie dye (Biolegend). To exclude contaminating lymphocytes in the monocyte gate, cells negative for both HLA-DR and CD14 were gated out. Monocyte subsets were gated based on CD14 and CD16 expression.

Luminex assay

Supernatants from the GM-MØ and M-MØ cultures were collected and stored at -20°C until further use. Levels of IL-1β, IL-6, IL-10, IL-12, IL-23, and MMP-9 were measured with Human premix Magnetic Luminex screening assay kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions and read on a Luminex Magpix instrument (Luminex, Austin, TX, USA). Data were analyzed with xPONENT 4.2 software (Luminex). Supernatant levels were corrected for the macrophage cell count at the time of harvesting and are expressed as ng/mL per 50,000 cells.

RNA extraction and qPCR

Total RNA was extracted from healthy donor-derived GM-MØs and M-MØs using the RNeasy® Mini Kit (Qiagen). Total RNA was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) with random hexamers (Promega). Real-time qPCR was conducted with a ViiA™ 7 Real-Time PCR System with TaqManTM probes (Thermo Fisher) targeting M-CSF (CSF1, Hs00174164_m1) and GM-CSF (CSF2, Hs00929873_m1). Amplification plots were analyzed with QuantStudioTM Real-Time PCR software v1.3. Relative gene expression was normalized to β-actin (ACTB, Hs99999903_m1) as an internal control.

Statistics

Differences in FRβ expression scores for the inner intima between patients with low and high vessel occlusion scores were assessed by nonparametric Mann–Whitney U tests (two-tailed). To analyze the differences between HC and GCA in the in vitro study, Mann–Whitney U tests were also used.

RESULTS

Leukocyte infiltrates are located in different compartments of the arterial wall in GCA-affected temporal arteries, GCA-affected aortas and atherosclerotic aortas

Transmural inflammation was found in all GCA-positive TABs, whereas no leukocyte infiltrates were found in the non-inflamed TABs from GCA/PMR patients and controls (Supplementary Figure 1A and 1B). GCA-positive TABs presented with a high degree of intimal hyperplasia and luminal occlusion, whereas TABs that were negative for GCA presented with no or minimal intimal hyperplasia. In the aortas from patients diagnosed with GCA, infiltrating leukocytes were found mainly in the adventitial and medial layers of the vessel wall (Supplementary Figure 1C). The infiltrates in the media of the GCA aorta often formed a granulomatous rim around necrotic areas. This granulomatous infiltration pattern was not found in atherosclerotic aortas. In atherosclerotic aortas, however, adventitial infiltrates and massive intimal infiltrates surrounding plaques with minimal medial infiltration were found (Supplementary Figure 1D).

Different phenotypes of infiltrating macrophages were found in specific compartments of the GCA-affected vessel wall

CD64, CD86, IL-12, IL-23, IL-1β and IL-6 were stained as proinflammatory markers, while CD206, FRβ, MMP-2, MMP-9, MMP-12 and IL-10 were used as tissue remodeling markers (see Supplementary Figure 2 for isotype controls). CD64, CD206, FRβ, MMP-9, IL-12 and IL-23 were subsequently double

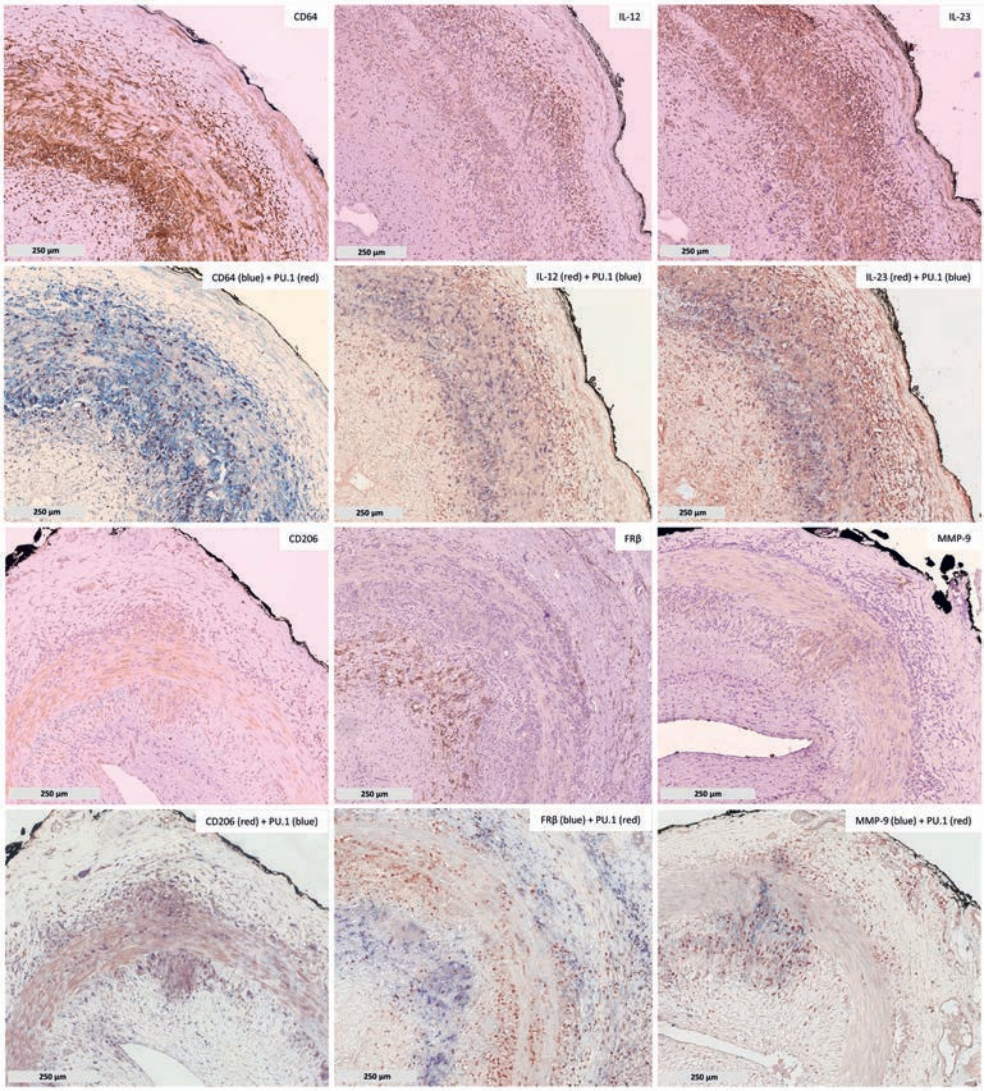


Figure 1. Macrophage phenotypic markers, proinflammatory cytokines and MMP-9 expression in GCA TABs. Single-staining immunohistochemistry showed the expression of CD64, IL-12, IL-23, CD206, FRβ, and MMP-9 in GCA TAB. Double-staining immunohistochemistry with the transcription factor PU.1 (in either blue or red) showed that these cells were macrophages. GCA: giant cell arteritis, TAB: temporal artery biopsy.

stained with the macrophage transcription factor PU.1 to confirm expression by macrophages. The proinflammatory markers CD64, IL-12, IL-23 (Figure 1), CD86, IL-1, and IL-6 (Supplementary Figure 3) were strongly expressed in all three layers of the vessel wall but most prominently in the adventitia. Double staining of CD64, IL-12 and IL-23 with PU.1 (Figure 1) showed that all positive cells were also PU.1 positive, indicating that these cells were indeed macrophages. IL-6 was expressed primarily by macrophages in the adventitia but also by endothelial cells of the vasa vasorum.

Markers of tissue remodeling macrophages were expressed in different compartments of the vessel wall. CD206 positivity was found mainly in the adventitia-media border, media and media-intima border (Figure 1). In contrast, FRβ-positive cells were mainly found in the adventitia and inner intima but rarely in the media (Figure 1). IL-10 was weakly expressed throughout the vessel wall (Supplementary Figure 3). Of the matrix metalloproteinases, MMP-2 was detected mainly in the adventitia and the media (Supplementary Figure 3), while MMP-9 was detected mainly in macrophages of the media and media borders (Figure 1). MMP-12, on the other hand, was scarcely detected in the vessel wall (Supplementary Figure 3). Semiquantitative analysis of the tissue staining experiments further corroborated the distinct distribution pattern of these macrophages

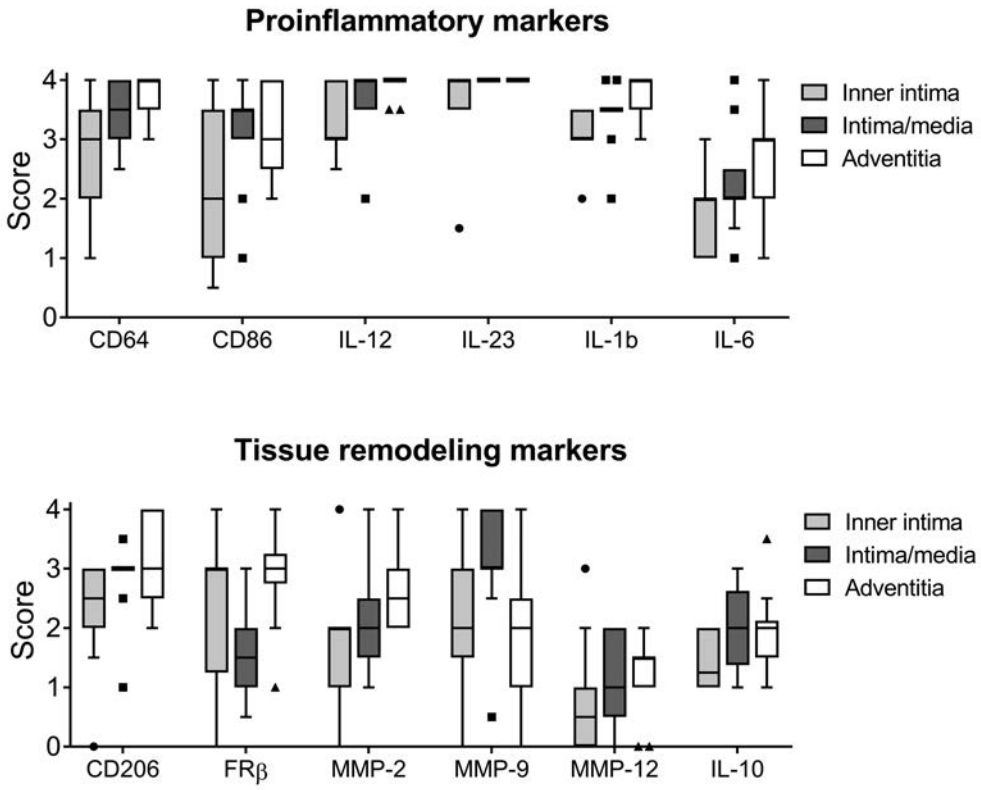


Figure 2. Localization of proinflammatory and tissue remodeling markers in GCA TABs. Expression of surface markers, cytokines, and matrix metalloproteinases (MMPs) in GCA-affected TABs (n=11) was semiquantitatively scored. Data are presented as Tukey boxplots. GCA: giant cell arteritis, TAB: temporal artery biopsy.

(Figure 2). Taken together, the results indicated that proinflammatory markers scored high throughout the vessel wall but most prominently in the adventitia. Of the tissue remodeling markers, CD206 scored highest in the adventitia and media, whereas FRβ scored highest in the adventitia and inner intima region, with the lowest scores in the media.

We also studied GCA-affected aortas and atherosclerotic aortas. Among the different areas of leukocyte infiltration, GCA aortas exhibited infiltration in the adventitia and media layers, while atherosclerotic aortas exhibited infiltration in the adventitia and intimal layers. All surface markers and cytokines were found to be abundantly expressed in both GCA and atherosclerotic aortas, albeit in different layers (Supplementary Figure 4).

CD206+ macrophages but not FRβ+ macrophages produce MMP-9

Immunohistochemical staining of consecutive tissue sections revealed different macrophage phenotypes in different compartments of GCA-affected TABs and aortas (Figure 3). CD64+ macrophages were abundant in all three layers of GCA-affected TABs. However, these CD64+ macrophages also showed concomitant expression of the tissue remodeling markers CD206 and FRβ in specific compartments of the lesions. More specifically, CD64+CD206+ macrophages were mainly found along the media borders. A similar pattern was observed for MMP-9 positive staining, suggesting that CD206+ macrophages express MMP-9, which would be in line with a tissue-invasive and proangiogenic phenotype. Interestingly, FRβ positivity was high in the adventitia and inner intima of TABs, thus showing a different pattern for the CD206+ macrophages. Indeed, FRβ was

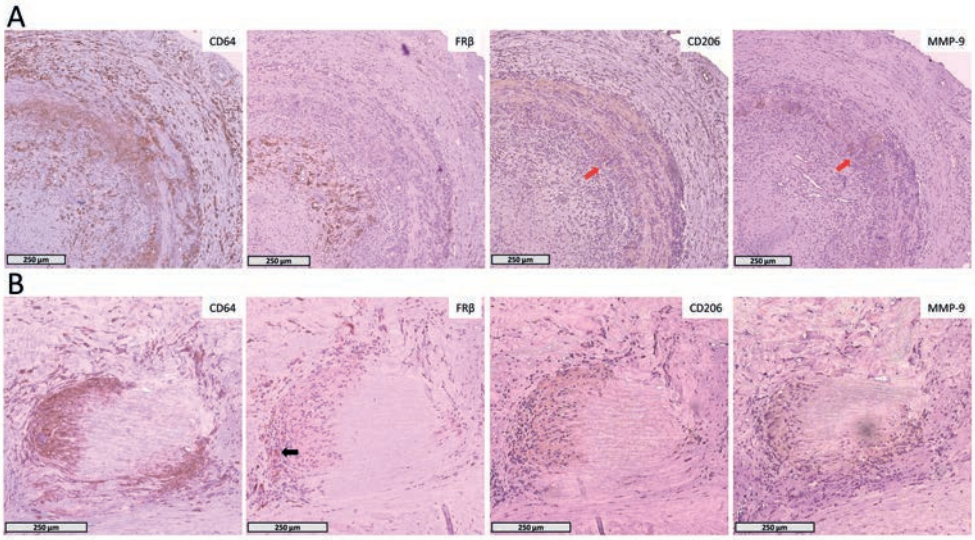


Figure 3. Macrophage phenotype is dependent on their location within the TAB and aorta. Shown are consecutive tissue staining experiments for CD64, FRβ, CD206 and MMP-9 in the GCA-affected TAB (**A**) and GCA-affected aorta (**B**). The red arrows indicate overlapping positivity for CD206 and MMP-9. The black arrow shows FRβ-positive cells adjacent to the CD206-positive rim. GCA: giant cell arteritis, TAB: temporal artery biopsy.

found to be typically expressed on macrophages adjacent to CD206+ macrophages. This particular pattern of different macrophage subsets was also found in the media of GCA-affected aortas (Figure 3B). In the granulomatous rim, around sites of tissue necrosis, CD206+MMP-9+ macrophages were surrounded by FRβ+ macrophages. This distinct pattern of macrophage subsets, however, was not found in atherosclerotic aortas (Supplementary Figure 5). Macrophages around atherosclerotic plaques showed a mixed phenotype, with overlapping expression of CD64, CD206, and FRβ+ without a distinct distribution pattern.

FRβ positivity in the inner intima is associated with intimal hyperplasia

FRβ positivity was found in the inner intima of the TAB; this is the region where intimal proliferation occurs. Interestingly, FRβ positivity increased with the degree of intimal hyperplasia. To determine whether the extent of FRβ positivity was associated with the severity of intimal hyperplasia, we divided luminal occlusion in GCA-affected TABs into mild or massive occlusion based on the intimal thickness score (Figure 4A) and related this factor to the extent of FRβ positivity. Indeed, FRβ expression was higher in the inner intima region of TABs with massive intimal hyperplasia than in that of TABs with mild intimal hyperplasia (p=0.011; Figure 4B).

GM-CSF and M-CSF contribute to macrophage phenotypic differences

As GM-CSF and M-CSF are known to influence macrophage phenotypes, we hypothesized that they play a key role in skewing macrophage phenotypes in GCA lesions. To test this hypothesis, we investigated the effects of GM-CSF and M-CSF on the phenotype of monocyte-derived macrophages in vitro. GM-MØs and M-MØs from healthy donors and GCA patients were analyzed for expression of CD64, CD86, CD206 and FRβ by flow cytometry. The culture supernatant was analyzed for IL-1β, IL-6, IL-12, IL-23, IL-10 and MMP-9.

Per-cell expression of CD64, CD86 and CD206 was upregulated in both GM-MØs and M-MØs compared to unstimulated monocytes (Figure 5A). CD206 expression was found to be significantly higher on GM-CSF-primed macrophages (HC p=<0,0001; GCA p=0.0002), while CD64 and CD86

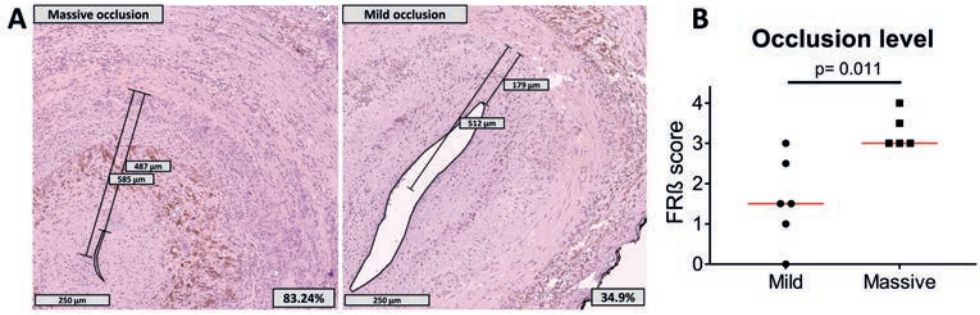


Figure 4. FRβ expression in the inner intima is associated with occlusion. Classification of massively and mildly occluded TABs was based on intimal thickness (**A**). The Mann–Whitney U test showed higher expression of FRβ in the inner intima of TABs with massive intimal hyperplasia (**B**). TAB: temporal artery biopsy.

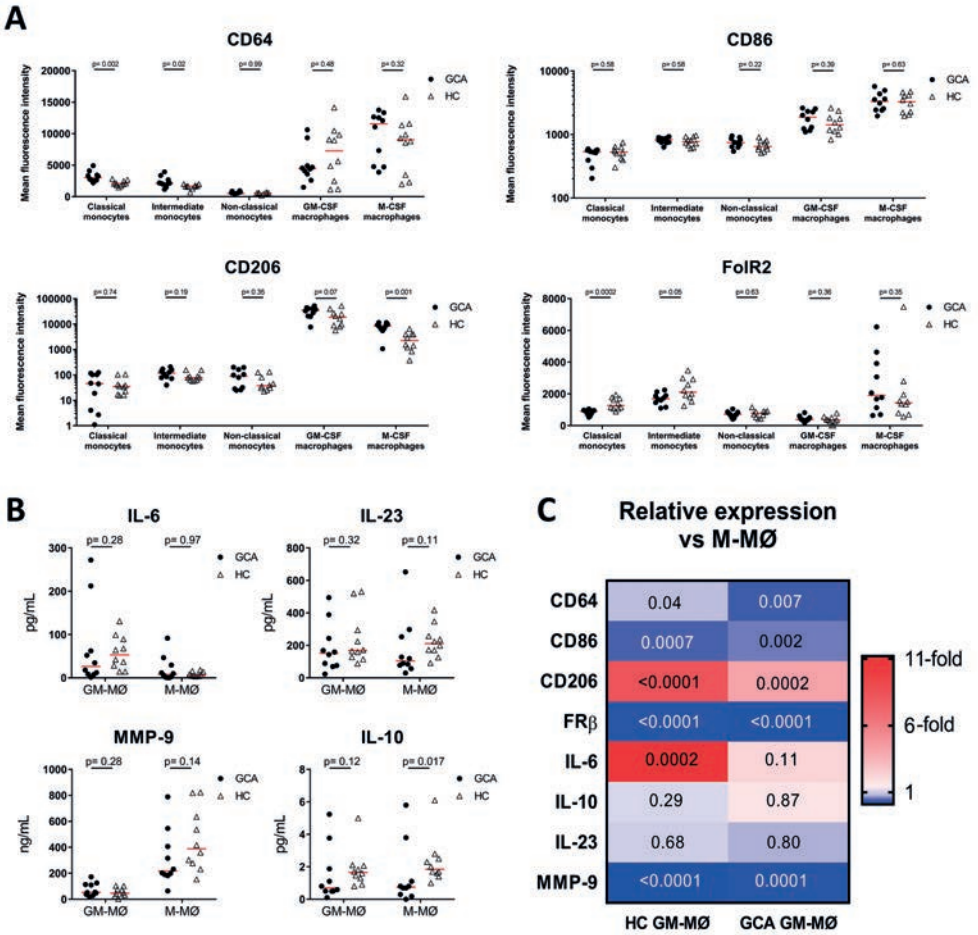


Figure 5. Macrophage surface marker expression and cytokine production depended on GM-CSF and M-CSF signals. Mean fluorescence intensity of CD64, CD86, CD206 and FRβ on monocyte subsets, GM-CSF-differentiated macrophages (GM-MØs) and M-CSF-differentiated macrophages (M-MØs) from GCA patients (n=10) and healthy controls (n=10) (**A**). Luminex assay (normalized per 50,000 cells) of IL-6, IL-12, IL-10 and MMP-9 in culture supernatants of GM-MØs and M-MØs from GCA patients (n=10) and healthy controls (n=10) (**B**). Heat map showing relative expression of the markers on GM-MØs compared to M-MØs (**C**). GCA: giant cell arteritis.

levels were higher on M-CSF-primed macrophages (Figure 5C). In addition, CD206 expression was higher in GCA GM-MØs (p=0.07) and GCA M-MØs (p=0.001) than in their counterparts from HCs. FRβ, however, was expressed only on M-MØs (Figure 5A&C). GM-CSF appeared to downregulate FRβ expression, as FRβ was expressed by monocytes but not by GM-MØs.

Although clear phenotypic differences were observed by flow cytometry, only minor differences in cytokine production were observed. While IL-6, IL-23, IL-10 and MMP-9 production was detected in supernatants (Figure 5B), IL-12 and IL-1β production was not (data not shown). IL-6 levels were found to be significantly higher in the HC GM-MØ supernatant than in the HC M-MØ supernatant

(Figure 5C, p=0.0002). However, this pattern was not observed for GCA GM-MØs vs. M-MØs, in which IL-6 levels were similar. IL-10 production was also found to be significantly lower in GCA M-MØs than in healthy donor M-MØs (Figure 5B). For both GCA and HCs, MMP-9 production was found to be significantly higher in M-MØs than in GM-MØs (Figure 5C).

Additionally, the phenotypic differences observed in macrophages from GCA patients may, to some extent, already be present in circulating monocytes. By flow cytometry, differences in the expression of these markers on subsets of monocytes, defined by CD14 and CD16 expression, were indeed observed (Figure 5A). We found significantly higher per-cell expression of the proinflammatory marker CD64 on classical (p=0.002) and intermediate (p=0.02) monocytes from GCA patients than on those from HCs. In contrast, FRβ expression on GCA classical (p=0.0002) and intermediate (p=0.05) monocytes was found to be significantly lower than that on monocytes from HCs. Overall, intermediate monocytes had the highest CD86, CD206 and FRβ expression, except for CD64, which was expressed most strongly on both classical and intermediate monocytes. Thus, monocytes from GCA patients already demonstrated a skewed phenotype, which appears to be similar to that of GM-CSF-stimulated macrophages.

GM-CSF and M-CSF may contribute to the distinct macrophage distribution pattern in GCA lesions

As we observed distinct effects of GM-CSF and M-CSF on macrophage surface marker expression, we performed IHC for GM-CSF and M-CSF on GCA-affected TABs and aortas. These experiments revealed that GM-CSF is dominantly expressed by infiltrating leukocytes and endothelial cells in the adventitial layer of GCA TABs (Figure 6A). M-CSF, on the other hand, was found to be abundantly expressed at the site of the CD206+MMP9+ macrophages at the intima-media borders in TABs. These findings were substantiated by semiquantitative scoring showing the highest M-CSF score in the media-intima of TABs (Figure 6B). In the aorta, GM-CSF was only weakly expressed in medial granulomas, whereas M-CSF was highly expressed by the CD206+ macrophages surrounding the necrotic areas.

To assess the production of GM-CSF and M-CSF by macrophages, we performed real-time qPCR on total mRNA from GM-MØs and M-MØs (derived from healthy donors). We observed significantly higher expression of M-CSF transcripts in GM-MØs than in M-MØs (p=0.0281, Figure 6C). GM-CSF transcripts, however, were not detected. This finding is in line with the tissue staining experiments, where CD206+ macrophages in the media and media borders were found to be the major producers of M-CSF.

As CD206 expression was observed to be higher in GCA GM-MØs than in HC GM-MØs, we reasoned that per-cell expression of the GM-CSF receptor might be upregulated in GCA monocytes. However, this did not appear to be the case in peripheral blood monocyte subsets from HCs and GCA patients, in which no differences were found (Figure 6D).

Taken together, our data suggest that the expression pattern of GM-CSF and M-CSF in GCA lesions may underlie the spatial distribution of macrophage phenotypes in GCA lesions. M-CSF produced by CD206+ macrophages is likely to prime adjacent macrophages to express FRβ.

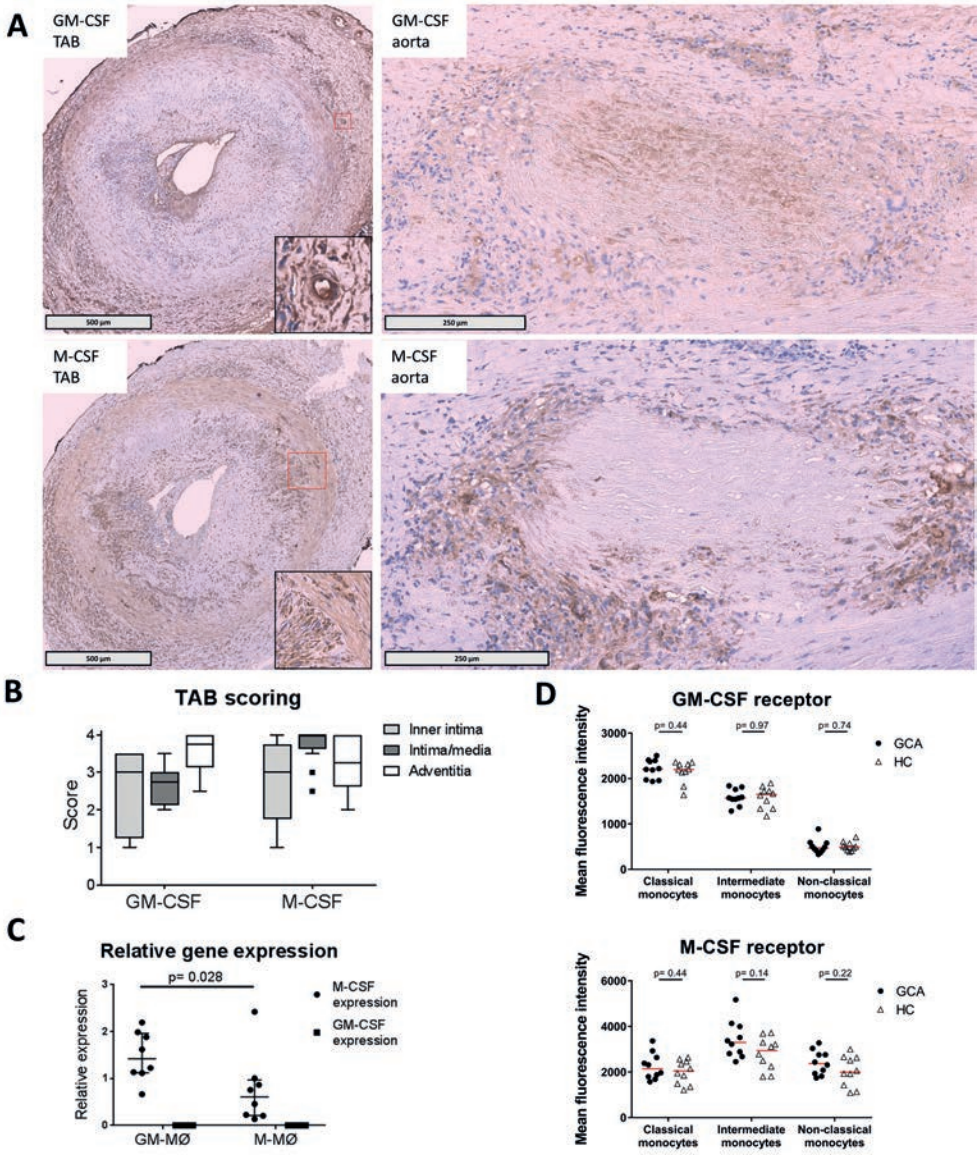


Figure 6. GM-CSF and M-CSF signaling in GCA tissues, macrophages and monocyte subsets. Tissue expression of GM-CSF and M-CSF in temporal artery (TAB) and aorta biopsy tissues from GCA patients (**A**). In the TABs, regions of interest (red) are magnified and shown in the lower right corner. In (**B**), semiquantitative scores for GM-CSF and M-CSF in GCA TABs (n=11) are displayed. The relative GM-CSF and M-CSF gene expression of healthy donor PBMC-derived GM-MØs and M-MØs (n=8 each) normalized to β-actin is shown in (**C**). In (**D**), the mean fluorescence intensity of the GM-CSF receptor and the M-CSF receptor in PBMC-derived monocytes from healthy controls (HC) and GCA patients (n=10 each) is shown. GCA: giant cell arteritis, TAB: temporal artery biopsy, GM-MØ: GM-CSF macrophages, M-MØ: M-CSF macrophages.

DISCUSSION

In this study, we revealed a distinct spatial distribution of macrophage phenotypes in GCA-affected vessel walls. We identified GM-CSF and M-CSF as the key contributors to the development of these distinct macrophage phenotypes. Moreover, distinct macrophage subsets were associated with tissue destruction and intimal hyperplasia. Macrophages are known as one of the major infiltrates in GCA lesions [7, 17-20]. Although it has been suggested previously that macrophages have different functions in different compartments of the inflamed vessel wall in GCA [18], our study is the first to assign different macrophage subsets to defined regions of the vessel wall based on a broad selection of macrophage markers.

Our data demonstrate that macrophages in GCA-affected TAB show a distinct expression pattern of surface markers, which is dependent on their location in the tissue. Macrophages with a proinflammatory phenotype, including expression of Th1- or Th17-skewing cytokines, were detected throughout the vessel wall. However, in specific compartments of the vessel wall, some of these macrophages also concomitantly expressed the tissue remodeling markers CD206 and FRβ. CD206 and MMP-9 positivity was mainly found in the media and media borders along the sites of lamina elastica degradation, in line with a previous report that MMP-9-producing macrophages are located in the media borders [8, 10]. FRβ-expressing macrophages, on the other hand, were mainly found in the adventitia and the inner intima adjacent to the CD206+ macrophages.

Our data suggest a role for GM-CSF and M-CSF in macrophage phenotypic heterogeneity in GCA lesions. In recent studies, CD206 and FRβ were found to be markers for GM-CSF- and M-CSF-differentiated macrophages, respectively [13, 14]. Our in vitro differentiation data confirmed that GM-MØs indeed have high expression of CD206 and lack expression of FRβ. In contrast, FRβ expression was upregulated in M-MØs. These findings suggest that CD206+ macrophages in the media and media borders are primed by GM-CSF, while FRβ+ macrophages are primed by M-CSF. This also implies a gradient of GM-CSF and M-CSF production in different layers of the vessel wall that may be responsible for the distinct macrophage subset distribution observed.

We therefore hypothesized that macrophage phenotypes in the vessel wall of GCA patients are particularly influenced by GM-CSF and M-CSF. GM-CSF expression was highest in the adventitia and was mainly expressed by endothelial cells and infiltrating leukocytes, presumably activated T-cells [21, 22]. M-CSF expression, on the other hand, was localized at the site of medial CD206+ macrophages. GM-CSF can induce M-CSF production, as previously demonstrated in monocytes [23] and confirmed by our qPCR data (Figure 6C). Recently, Watanabe et al. proposed two nonmutually exclusive pathways by which monocyte-derived macrophages contribute to tissue injury and repair [24]. In the first pathway, tissue-infiltrating monocytes progressively differentiate from proinflammatory macrophages into proresolving macrophages depending on signals that these macrophages encounter within the local microenvironment. In the second pathway, the proinflammatory macrophages disappear once the inflammatory trigger has been cleared. A second wave of monocytes then enters the tissue, which differentiates into proresolving macrophages in response to cues within the microenvironment. Based on our data, we propose a model (Figure 7) in which monocytes that enter the vessel wall are initially primed by GM-CSF, after which they differentiate

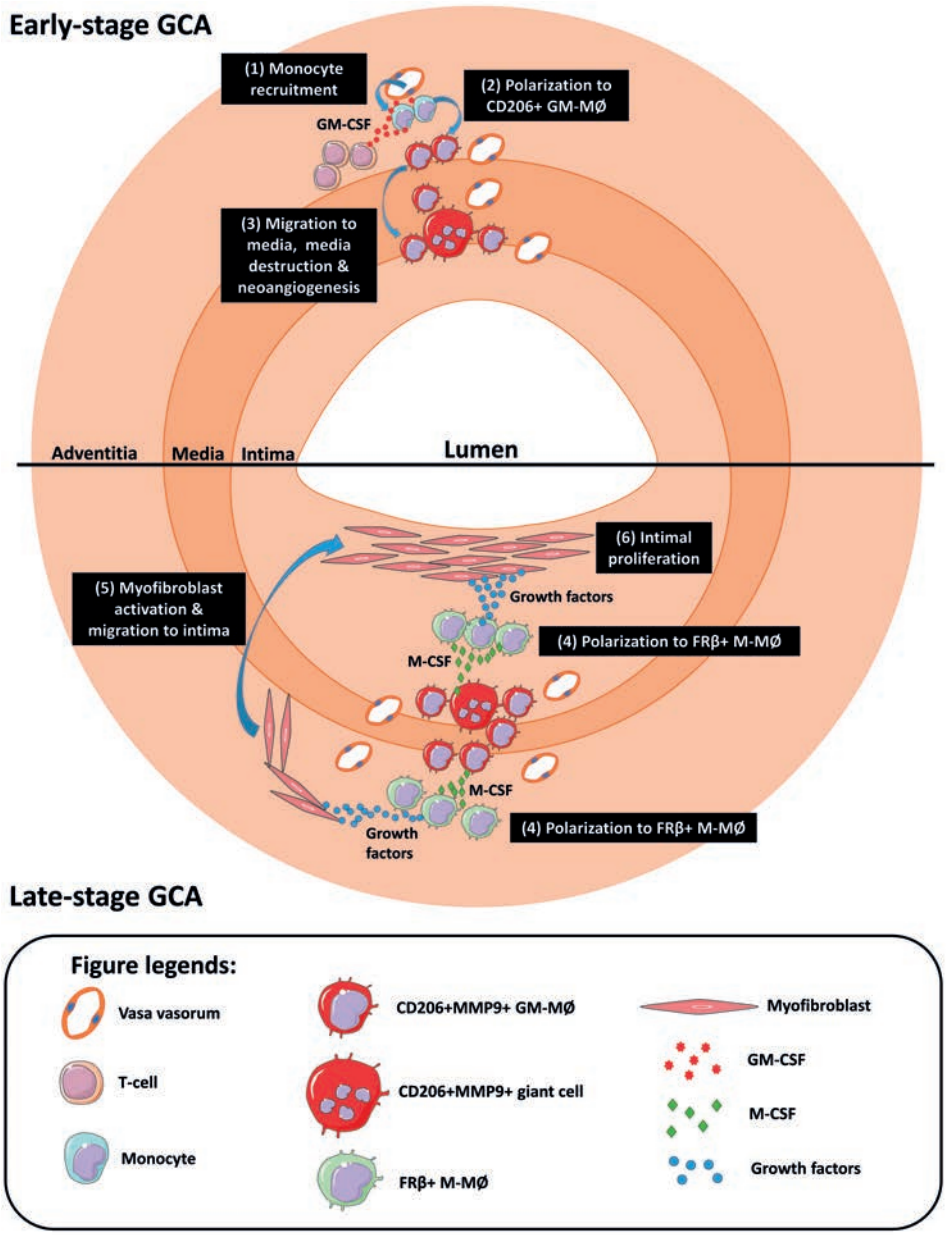


Figure 7. Model of step-by-step giant cell arteritis (GCA) pathogenesis in the temporal artery involving GM-CSF and M-CSF. The following steps occur in **early-stage GCA**: 1. Monocytes enter the vessel wall. 2. Infiltrating monocytes are primed by GM-CSF produced by T-cells and endothelial cells, after which they differentiate into CD206+ GM-MØs. 3. These CD206+ GM-MØs then migrate to the media and media borders, exerting their tissue-invasive, digestive and proangiogenic capabilities. In **late-stage GCA**, the following steps occur: 4. CD206+ GM-MØs release large amounts of M-CSF, which in turn primes the macrophages surrounding them to express FRβ. 5. FRβ+ macrophages release high concentrations of growth factors that activate myofibroblasts, promoting their migration to the intima 6. Induction of myofibroblast proliferation, which causes intimal hyperplasia and ultimately leads to luminal occlusion.

into CD206+ macrophages. These GM-MØs then migrate to the media borders, exerting their tissue-invasive, digestive and proangiogenic effects. Additionally, these CD206+ GM-MØs release large amounts of M-CSF, which in turn primes the macrophages surrounding them to express FRβ. These FRβ+ macrophages then initiate the activation of myofibroblasts, inducing their migration and proliferation, eventually leading to luminal occlusion.

Although our data imply that GM-CSF and M-CSF may contribute to the distinct spatial distribution of macrophage subsets in GCA, additional signals are needed for full activation. In contrast to our tissue staining results, MMP-9 production was found to be higher in M-MØ than in GM-MØ. This result may be explained by the fact that although GM-CSF and M-CSF differentiate monocytes into macrophages, they do not fully activate them. Indeed, the GCA tissue environment is much more complex and enriched with a multitude of cytokines. Cytokines such as IFNγ, which are highly expressed in GCA lesions [25], could further modulate the expression of surface markers, cytokines and MMPs. Indeed, it has been reported that IFNγ synergizes with GM-CSF to stimulate increased MMP-9, IL-12 and IL-1β production in macrophages [26-28].

Circulating monocytes and monocyte-derived macrophages from GCA patients display a GM-CSF signature compared to HCs. This finding was reflected by lower FRβ expression on monocytes and higher CD206 expression after differentiation into macrophages. This implies that monocytes from GCA patients have a stronger response to GM-CSF [13]. However, no difference in GM-CSF receptor expression was found between the groups, implying that other factors confer increased sensitivity of GCA monocytes to GM-CSF.

We found significantly higher expression of FRβ in the inner intima in TABs with a higher degree of intimal hyperplasia. This finding suggests that FRβ macrophages may play a role in myofibroblast activation, migration and proliferation, leading to intimal hyperplasia and ultimately, luminal occlusion. Indeed, improvement of pulmonary fibrosis was shown by depleting FRβ+ macrophages [29]. FRβ expression has previously been reported in the adventitia of GCA TABs [30]. Here, we showed that FRβ is also expressed in the inner intima. This discrepancy can be explained by differences in the degree of intimal hyperplasia in the TABs between studies. As we found that M-MØs are FRβ+, it is interesting that M-MØs have previously been reported to produce higher levels of TGF-β and PDGF-A [31], which are growth factors contributing to myofibroblast activation, migration and proliferation [32, 33]. Notably, these growth factors are expressed in GCA lesions [19], although their production by FRβ+ macrophages in GCA remains to be determined.

A distinct macrophage distribution pattern was also observed in GCA-affected aortas but not in atherosclerotic aortas. In contrast to TABs, a distinct macrophage distribution pattern was observed only within the media of the GCA aortas. The variation in this distribution pattern between TABs and the aorta may be caused by differences in vessel wall size and anatomical buildup, as aortas have thicker media with multiple lamina elastica layers. In contrast to GCA, but confirming previous reports, macrophages in atherosclerotic aortas were found mainly in the intima surrounding the atherosclerotic plaques [34]. These macrophages showed overlapping CD64, CD206 and FRβ expression. In line with our findings of FRβ positivity around atherosclerotic plaques, reports have shown that M-CSF is the dominant growth factor in atherogenesis. The heterozygous M-CSF null mouse model showed reduced atherogenesis, [35] and M-CSF-activated gene signatures are

dominant in early atherogenesis [36]. We demonstrated that CD206 did not colocalize with FRβ+ macrophages in GCA, whereas concomitant CD206/FRβ expression was shown in atherosclerosis macrophages. The Th2 cytokine IL-4, expressed in atherosclerosis but not in GCA, can upregulate CD206 expression on FRβ macrophages [25, 37, 38]. Importantly, GM-CSF was reported to be important in necrotic core formation in late-stage atherogenesis [39]. Overall, although macrophages are abundant in both diseases, the environmental cues governing macrophage phenotypes and function are different. For GCA, we propose a sequential evolution of macrophage polarization that is initially driven by GM-CSF followed by M-CSF signals, whereas the opposite sequence of events occurs in atherogenesis.

The major strength of our study is the comprehensive analysis of multiple markers of inflammation and tissue remodeling, which allows the identification of distinct macrophage phenotypes in different compartments of the lesion. Our biopsy tissues were obtained from treatment-naive patients to exclude potential effects of GCs on macrophage phenotypes. Future studies should, however, address the impact of GCs on the skewing of lesional macrophage phenotypes. Finally, we also included atherosclerotic aortas for comparison and found that the roles of macrophages in the pathogenic processes leading to these two diseases are indeed different. We identified a possible role for GM-CSF and M-CSF in the local skewing of macrophage subsets in GCA and substantiated this finding with in vitro differentiation studies. We are aware that our in vitro model does not fully capture the events in the tissue, as a plethora of cytokines that can lead to further skewing and activation of macrophages were not explored.

Our study may aid in expanding current GCA pathogenic models and identifying markers for targeted therapy. Currently, a GM-CSF receptor-blocking antibody, mavrilimumab (NCT03827018), is being evaluated in a phase 2 clinical trial for the treatment of GCA, and our findings add to the rationale for targeting the GM-CSF receptor in this disease. Additionally, reduced inflammation was shown in a rheumatoid arthritis cartilage explant model with a CD64-targeted immunotoxin [40]. Although further studies are still needed, targeting CD206 might also prove to be useful in reducing tissue destruction, while targeting FRβ might prevent luminal occlusion in GCA.

This study also implicates macrophage phenotypic markers as tracer targets for imaging. Currently, the most commonly used PET-CT tracer for detecting GCA is FDG [41]. FDG-PET-CT uptake and its associated diagnostic accuracy also decrease dramatically in patients undergoing GC treatment. Additionally, tissue inflammation can persist during treatment with GCs and IL-6 receptor blockade, as evidenced by biomarker levels, follow-up biopsies and MRI studies [17, 42-44]. Therefore, more specific imaging markers are needed. This study shows that CD64, FRβ and CD206 could be useful as PET-CT tracer targets. FRβ- and CD206-targeted radiotracers are currently being developed and may be useful in diagnosis and treatment follow-up for GCA [45].

CONCLUSION

The vascular lesions of GCA patients display a distinct spatial distribution pattern of polarized macrophage phenotypes that are (most likely) governed by local expression of M-CSF and GM-CSF. These findings contribute to improved insights into the pathogenesis of GCA and lay the foundation

for designing new macrophage-targeted therapies and novel markers for diagnostic and treatment follow-up imaging.

ACKNOWLEDGEMENTS

The authors thank Johan Bijzet, Theo Bijma and Geert Mesander for their technical Support in Luminex and flow cytometry. We also thank the patients and UMCG clinical center staff.

REFERENCES

1. Samson, M., Corbera-Bellalta, M., Audia, S., Planas-Rigol, E., Martin, L., Cid, M.C., and Bonnotte, B. 2017. Recent advances in our understanding of giant cell arteritis pathogenesis. *Autoimmun. Rev. 16: 833-844.*
2. Dejaco, C., Brouwer, E., Mason, J.C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2017. Giant cell arteritis and polymyalgia rheumatica: current challenges and opportunities. *Nat. Rev. Rheumatol. 13(10):578-592.*
3. Koster, M.J., Matteson, E.L., and Warrington, K.J. 2018. Large-vessel giant cell arteritis: diagnosis, monitoring and management. *Rheumatology (Oxford) 57: ii32-ii42.*
4. Nuenninghoff, D.M., Hunder, G.G., Christianson, T.J., McClelland, R.L., and Matteson, E.L. 2003. Incidence and predictors of large-artery complication (aortic aneurysm, aortic dissection, and/or large-artery stenosis) in patients with giant cell arteritis: a population-based study over 50 years. *Arthritis Rheumatol. 48: 3522-3531.*
5. Stone, J.H., Tuckwell, K., Dimonaco, S., Klearman, M., Aringer, M., Blockmans, D., Brouwer, E., Cid, M.C., Dasgupta, B., Rech, J., Salvarani, C., Schett, G., Schulze-Koops, H., Spiera, R., Unizony, S.H., and Collinson, N. 2017. Trial of Tocilizumab in Giant-Cell Arteritis. *N. Engl. J. Med. 377: 317-328.*
6. Anderson, J.M. 2000. Multinucleated giant cells. *Curr. Opin. Hematol. 7: 40-47.*
7. van Sleen, Y., Wang, Q., van der Geest, K.S.M., Westra, J., Abdulahad, W.H., Heeringa, P., Boots, A.M.H., and Brouwer, E. 2017. Involvement of Monocyte Subsets in the Immunopathology of Giant Cell Arteritis. *Sci. Rep. 7: 6553.*
8. Rodriguez-Pla, A., Bosch-Gil, J.A., Rossello-Urgell, J., Huguet-Redecilla, P., Stone, J.H., and Vilardell-Tarres, M. 2005. Metalloproteinase-2 and -9 in giant cell arteritis: involvement in vascular remodeling. *Circulation 112: 264-269.*
9. Nikkari, S.T., Höyhtyä, M., Isola, J., and Nikkari, T. 1996. Macrophages contain 92-kd gelatinase (MMP-9) at the site of degenerated internal elastic lamina in temporal arteritis. *Am. J. Path. 149: 1427.*
10. Watanabe R., Maeda T., zhang H., Berry G.J., Zeisbrich M., Brockett R., Greenstein A.E., Tian L., Goronzy J.J., Weyand C.M. 2018. Matrix Metalloprotease-9 (MMP-9)-Producing Monocytes Enable T Cells to Invade the Vessel Wall and Cause Vasculitis. *Circ. Res. 123(6):700-715.*
11. Geginat, J., Paroni, M., Maglie, S., Alfen, J.S., Kastirr, I., Gruarin, P., De Simone, M., Pagani, M., and Abrignani, S. 2014. Plasticity of human CD4 T cell subsets. *Front. Immunol. 5: 630.*
12. Lacey, D.C., Achuthan, A., Fleetwood, A.J., Dinh, H., Roiniotis, J., Scholz, G.M., Chang, M.W., Beckman, S.K., Cook, A.D., and Hamilton, J.A. 2012. Defining GM-CSF–and macrophage-CSF–dependent macrophage responses by in vitro models. *J. Immunol. 188: 5752-5765.*
13. Kittan, N.A., Allen, R.M., Dhaliwal, A., Cavassani, K.A., Schaller, M., Gallagher, K.A., Carson IV, W.F., Mukherjee, S., Grembecka, J., and Cierpicki, T. 2013. Cytokine induced phenotypic and epigenetic signatures are key to establishing specific macrophage phenotypes. *PloS one 8: e78045.*
14. Puig-Kröger, A., Sierra-Filardi, E., Domínguez-Soto, A., Samaniego, R., Corcuera, M.T., Gómez-Aguado, F., Ratnam, M., Sánchez-Mateos, P., and Corbí, A.L. 2009. Folate receptor β is expressed by tumor-associated macrophages and constitutes a marker for M2 anti-inflammatory/regulatory macrophages. *Cancer Res. 69: 9395-9403.*
15. Nagai, T., Tanaka, M., Tsuneyoshi, Y., Matsushita, K., Sunahara, N., Matsuda, T., Yoshida, H., Komiya, S., Onda, M., and Matsuyama, T. 2006. In vitro and in vivo efficacy of a recombinant immunotoxin against folate receptor β on the activation and proliferation of rheumatoid arthritis synovial cells. *Arthritis Rheumatol. 54: 3126-3134.*
16. Ligthart, G.J., Corberand, J.X., Fournier, C., Galanaud, P., Hijmans, W., Kennes, B., Müller-Hermelink, H.K., and Steinmann, G.G. 1984. Admission criteria for immunogerontological studies in man: the SENIEUR protocol. *Mech. Ageing Dev. 28: 47-55.*
17. van Sleen, Y., Sandovici, M., Abdulahad, W., Bijzet, J., van der Geest, K., Boots, A.M., and van der Geest, Kornelis SM. 2019. Markers of Angiogenesis and Macrophage Products for Predicting Disease Course

and Monitoring Vascular Inflammation in Giant Cell Arteritis. *Rheumatology (Oxford) 58: 1383–1392.*

18. Weyand, C.M., Wagner, A.D., Bjornsson, J., and Goronzy, J.J. 1996. Correlation of the topographical arrangement and the functional pattern of tissue-infiltrating macrophages in giant cell arteritis. *J. Clin. Invest. 98: 1642-1649.*
19. Kaiser, M., Weyand, C.M., Björnsson, J., and Goronzy, J.J. 1998. Platelet-derived growth factor, intimal hyperplasia, and ischemic complications in giant cell arteritis. *Arthritis Rheumatol. 41: 623-633.*
20. Rittner, H.L., Kaiser, M., Brack, A., Szweda, L.I., Goronzy, J.J., and Weyand, C.M. 1999. Tissue-destructive macrophages in giant cell arteritis. *Circ. Res. 84: 1050-1058.*
21. Burg, J., Krump-Konvalinkova, V., Bittinger, F., and Kirkpatrick, C.J. 2002. GM-CSF expression by human lung microvascular endothelial cells: in vitro and in vivo findings. *Am. J. Physiol. Lung Cell Mol. Physiol. 283: L460-L467.*
22. Sheng, W., PNG, C.W., Reynolds, J.M., and Zhang, Y. 2015. T cell-derived GM-CSF, regulation of expression and function. *Immunome Res. 11: 1.*
23. Gruber, M.F. and Gerrard, T.L. 1992. Production of macrophage colony-stimulating factor (M-CSF) by human monocytes is differentially regulated by GM-CSF, TNFα, and IFN-γ. *Cell. Immunol. 142: 361-369.*
24. Watanabe, S., Alexander, M., Misharin, A.V., and Budinger, G.S. 2019. The role of macrophages in the resolution of inflammation. *J. Clin. Invest. 129: 2619-2628*
25. Weyand, C.M., Hicok, K.C., Hunder, G.G., and Goronzy, J.J. 1994. Tissue cytokine patterns in patients with polymyalgia rheumatica and giant cell arteritis. *Ann. Intern. Med. 121: 484-491.*
26. Masters, S.L., Mielke, L.A., Cornish, A.L., Sutton, C.E., O'donnell, J., Cengia, L.H., Roberts, A.W., Wicks, I.P., Mills, K.H., and Croker, B.A. 2010. Regulation of interleukin-1β by interferon-γ is species specific, limited by suppressor of cytokine signalling 1 and influences interleukin-17 production. *EMBO Rep. 11: 640-646.*
27. Grohmann, U., Belladonna, M.L., Vacca, C., Bianchi, R., Fallarino, F., Orabona, C., Fioretti, M.C., and Puccetti, P. 2001. Positive regulatory role of IL-12 in macrophages and modulation by IFN-γ. J. *Immunol. 167: 221-227.*
28. Zhou, M., Zhang, Y., Ardans, J.A., and Wahl, L.M. 2003. Interferon-gamma differentially regulates monocyte matrix metalloproteinase-1 and -9 through tumor necrosis factor-alpha and caspase 8. *J. Biol. Chem. 278: 45406-45413.*
29. Nagai, T., Tanaka, M., Hasui, K., Shirahama, H., Kitajima, S., Yonezawa, S., Xu, B., and Matsuyama, T. 2010. Effect of an immunotoxin to folate receptor β on bleomycin-induced experimental pulmonary fibrosis. *Clin. Exp. Immunol. 161: 348-356.*
30. Albano-Aluquin, S., Malysz, J., Aluquin, V.R., Ratnam, M., and Olsen, N. 2017. An immunohistochemical analysis of folate receptor beta expression and distribution in giant cell arteritis - a pilot study. *Am. J. Clin. Exp. Immunol. 6: 107-114.*
31. Preisser, L., Miot, C., Le Guillou-Guillemette, H., Beaumont, E., Foucher, E.D., Garo, E., Blanchard, S., Frémaux, I., Croué, A., and Fouchard, I. 2014. IL-34 and macrophage colony-stimulating factor are overexpressed in hepatitis C virus fibrosis and induce profibrotic macrophages that promote collagen synthesis by hepatic stellate cells. *Hepatology 60: 1879-1890.*
32. Schreier, T., Degen, E., and Baschong, W. 1993. Fibroblast migration and proliferation during in vitro wound healing. *Res. Exp. Med. 193: 195-205.*
33. Baum, J. and Duffy, H.S. 2011. Fibroblasts and myofibroblasts: what are we talking about? *J. Cardiovasc. Pharmacol. 57: 376.*
34. Stary, H. 1992. Composition and classification of human atherosclerotic lesions. *Virchows Archiv A 421: 277-290.*
35. Shaposhnik, Z., Wang, X., and Lusis, A.J. 2010. Arterial colony stimulating factor-1 influences atherosclerotic lesions by regulating monocyte migration and apoptosis. *J. Lipid Res. 51: 1962-1970.*
36. Brochériou, I., Maouche, S., Durand, H., Braunersreuther, V., Le Naour, G., Gratchev, A., Koskas, F., Mach, F., Kzhyshkowska, J., and Ninio, E. 2011. Antagonistic regulation of macrophage phenotype by M-CSF and GM-CSF: implication in atherosclerosis. *Atherosclerosis 214: 316-324.*
37. Yıldırım-Buharalıoğlu, G., Bond, M., Sala-Newby, G.B., Hindmarch, C.C., and Newby, A.C. 2017. Regulation of epigenetic modifiers, including KDM6B, by interferon-γ and interleukin-4 in human macrophages. *Front. Immunol. 8: 92.*

38. Khallou-Laschet, J., Varthaman, A., Fornasa, G., Compain, C., Gaston, A., Clement, M., Dussiot, M., Levillain, O., Graff-Dubois, S., and Nicoletti, A. 2010. Macrophage plasticity in experimental atherosclerosis. *PloS one 5: e8852*.

39. Subramanian, M., Thorp, E., and Tabas, I. 2015. Identification of a non-growth factor role for GM-CSF in advanced atherosclerosis: promotion of macrophage apoptosis and plaque necrosis through IL-23 signaling. *Circ. Res. 116: e13-e24*.

40. Van Roon, J.A., Van Vuuren, A.J., Wijngaarden, S., Jacobs, K.M., Bijlsma, J.W., Lafeber, F.P., Thepen, T., and Van De Winkel, Jan GJ. 2003. Selective elimination of synovial inflammatory macrophages in rheumatoid arthritis by an Fcγ receptor I–directed immunotoxin. *Arthritis Rheumatol. 48: 1229-1238*.

41. Clifford, A.H., Murphy, E.M., Burrell, S.C., Bligh, M.P., MacDougall, R.F., Heathcote, J.G., Castonguay, M.C., Lee, M.S., Matheson, K., and Hanly, J.G. 2017. Positron emission tomography/computerized tomography in newly diagnosed patients with giant cell arteritis who are taking glucocorticoids. *J. Rheumatol. 44: 1859-1866*.

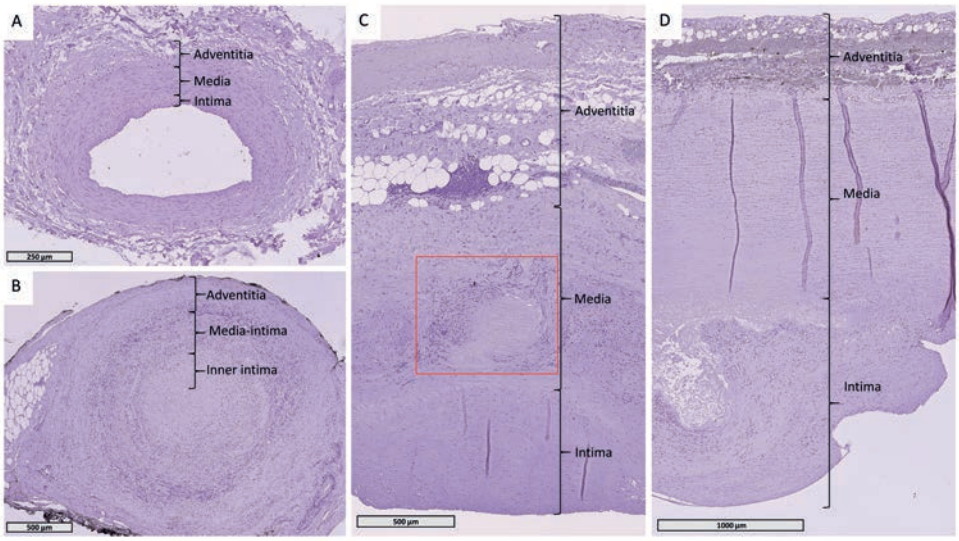
42. Maleszewski, J.J., Younge, B.R., Fritzlen, J.T., Hunder, G.G., Goronzy, J.J., Warrington, K.J., and Weyand, C.M. 2017. Clinical and pathological evolution of giant cell arteritis: a prospective study of follow-up temporal artery biopsies in 40 treated patients. *Mod. Pathol.*

43. Gloor A.D., Yerly D., Adler S., Reichenbach S., Kuchen S., Seitz M., Villiger P.M. 2018. Immuno-monitoring reveals an extended subclinical disease activity in tocilizumab-treated giant cell arteritis. *Rheumatology (Oxford) 57:1795-1801*.

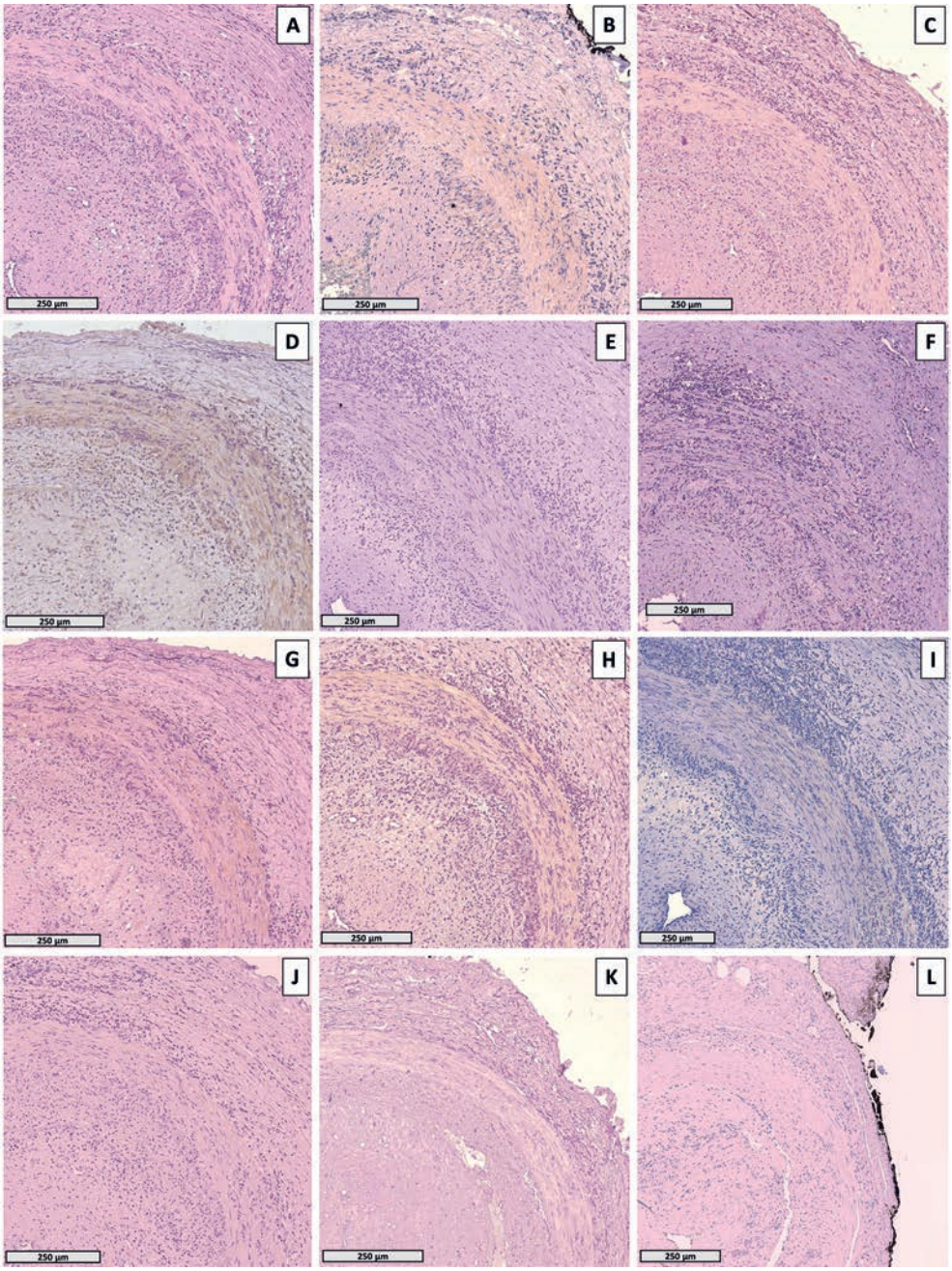
44. Reichenbach, S., Adler, S., Bonel, H., Cullmann, J.L., Kuchen, S., Bütikofer, L., Seitz, M., and Villiger, P.M. 2018. Magnetic resonance angiography in giant cell arteritis: results of a randomized controlled trial of tocilizumab in giant cell arteritis. *Rheumatology (Oxford) 57: 982-986*.

45. Jiemy, W.F., Heeringa, P., Kamps, J.A., van der Laken, Conny J, Slart, R.H., and Brouwer, E. 2018. Positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging of macrophages in large vessel vasculitis: current status and future prospects. *Autoimm. Rev. 17: 715-726*.

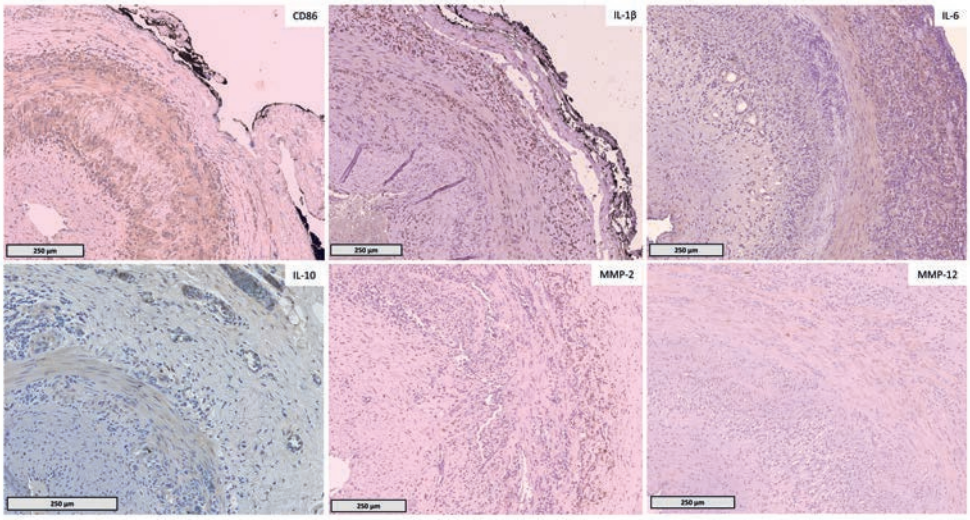
SUPPLEMENTARY DATA



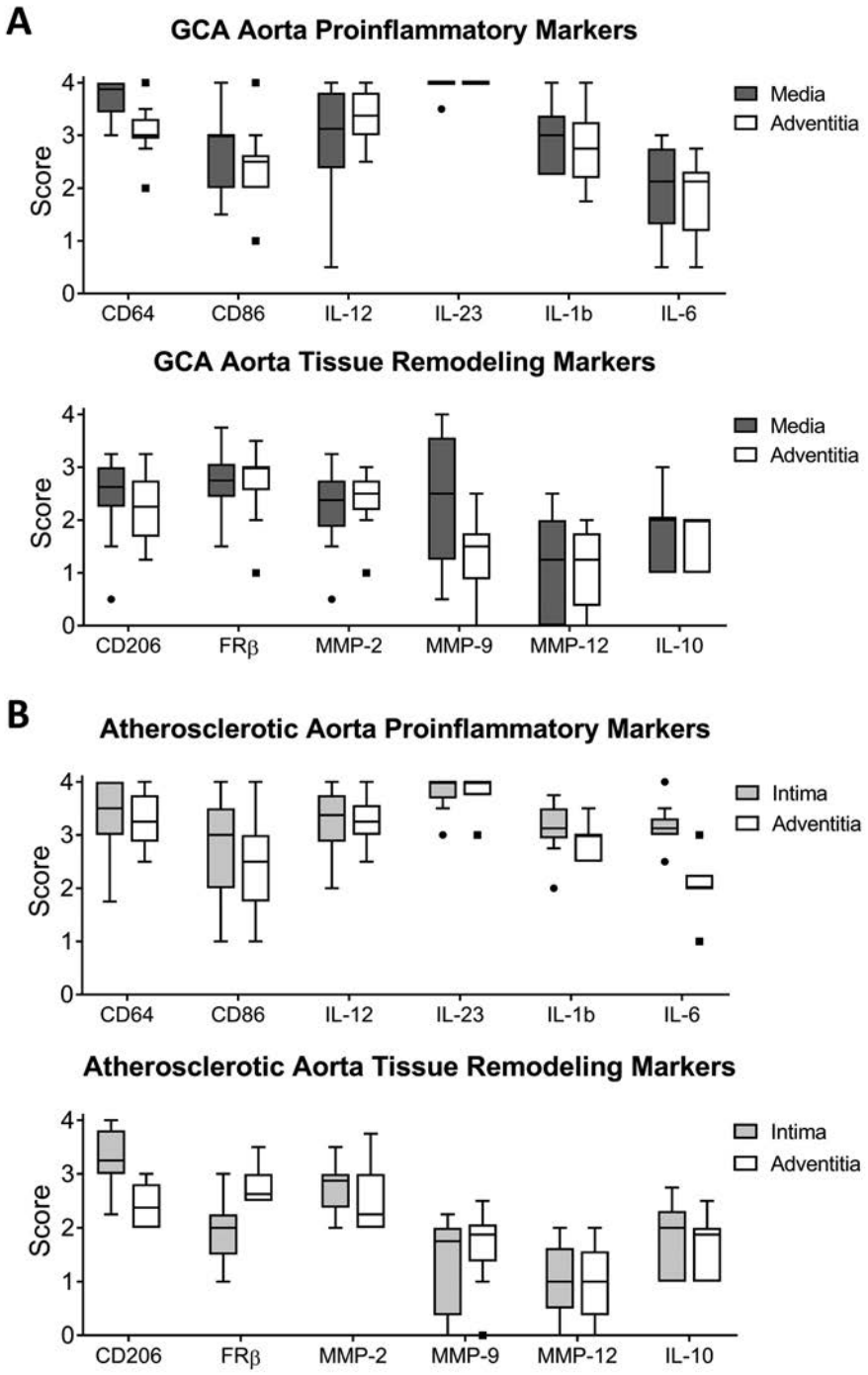
Supplementary Figure 1. Tissue topology of the healthy and unhealthy vessel wall. Representative hematoxylin staining of a GCA-negative temporal artery biopsy (**A**), GCA-positive temporal artery biopsy (**B**), GCA-positive aorta (**C**) and atherosclerotic aorta (**D**). Infiltrating leukocytes can be found in all three layers of GCA-positive TABs, whereas no infiltrates were found in GCA-negative TABs. In GCA-positive aortas, infiltrating leukocytes localized mainly in the adventitia and media. In contrast, atherosclerotic aortas showed massive intimal infiltration with minimal infiltrates in the media. The red box shows a necrotizing granuloma with a leukocyte rim present in GCA-affected aortas but not in atherosclerotic aortas. GCA: giant cell arteritis, TAB: temporal artery biopsy.



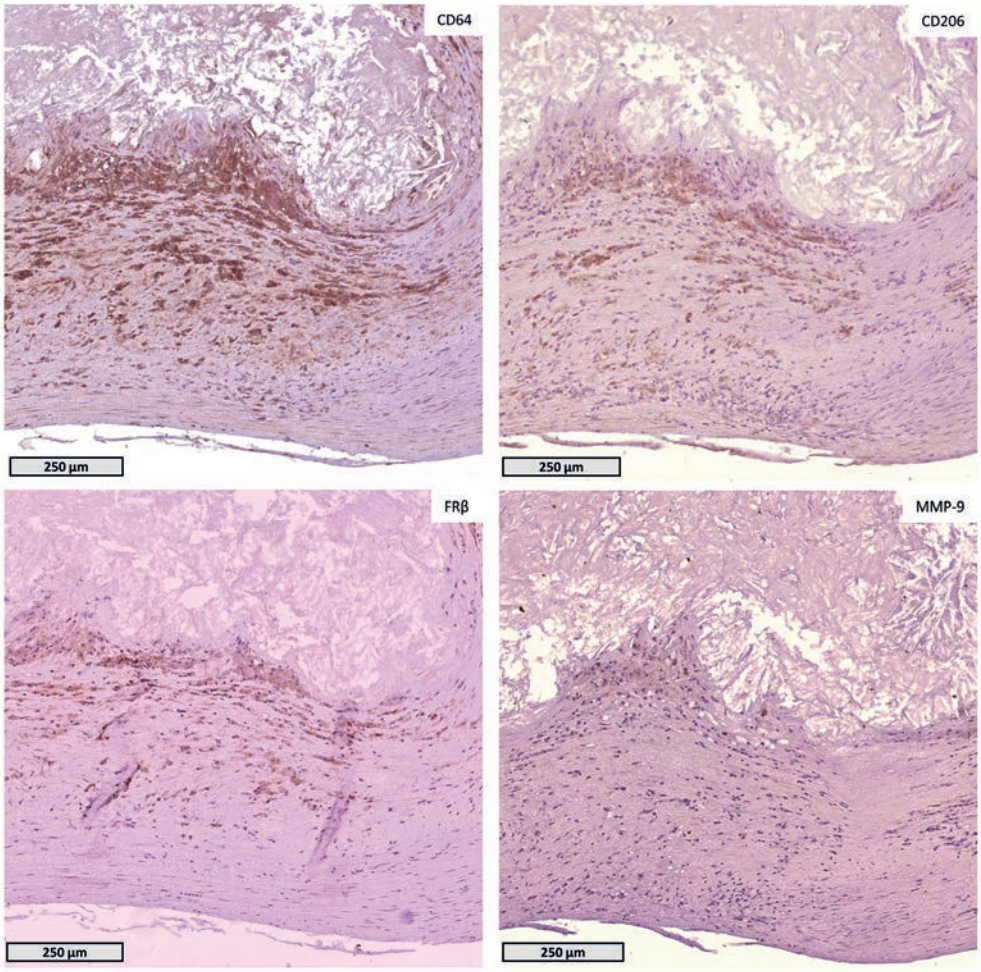
Supplementary Figure 2. Isotype control for CD64 & MMP-9 (**A**), CD86 (**B**), CD206 (**C**), FR-β (**D**), IL-1β (**E**), IL-6 (**F**), IL-12 & GM-CSF (**G**), IL-23 (**H**), M-CSF (**I**), MMP-2 (**J**), MMP-12 (**K**), and IL-10 (**L**).



Supplementary Figure 3. Single-staining immunohistochemistry of CD86, IL-1β, IL-6, IL-10, MMP-2 and MMP-2.



Supplementary Figure 4. Localization of proinflammatory and tissue remodeling macrophage markers in the aorta. GCA-affected aortas (n=10, **A**) and atherosclerotic aortas (n=10, **B**) were semiquantitatively scored. Data are expressed as Tukey boxplots. The intimal layer of GCA aortas and the medial layer of atherosclerotic aortas were not scored to a lack of infiltrating cells. GCA: giant cell arteritis, MMP: matrix-metalloproteinase



Supplementary Figure 5. No distinct distribution pattern in atherosclerotic aortas. Shown are consecutive tissue staining experiments for CD64, FRβ, CD206 and MMP-9 in the intimal layer.

Supplementary Table 1. List of antibodies.

Single Staining				
Target	**Antigen retrieval**	**Manufacturer & code**	**Host**	**Isotype**
IL-6	pH 6	Santa Cruz, SC-130326	Mouse	IgG2b
IL-1β	pH 9	Abcam, Ab156791	Mouse	IgG1
IL-12p35	pH 6	Atlas Antibodies, HPA001886	Rabbit	Polyclonal (IgG)
IL-23p19	pH 6	Abcam, Ab45420	Rabbit	Polyclonal (IgG)
CD206	pH 9	Abcam, Ab201927	Mouse	IgG2b
CD64	pH 9	Abcam, Ab140779	Mouse	IgG1
MMP-2*	pH 6	Abcam, Ab86607	Mouse	IgG2a
MMP-9*	pH 9	Abcam, Ab58803	Mouse	IgG1
MMP-12*	N/A	Abcam, Ab56305	Mouse	IgG2b
FR-β	pH 9	Origene, TA808017	Mouse	IgG1
IL-10	pH 6	Abcam, Ab34843	Rabbit	Poly antisera
M-CSF	pH 9	Abcam, Ab52864	rabbit	IgG
CD86	pH 9	Abcam, Ab234000	mouse	IgG1
GM-CSF	pH 6	Abcam, Ab9741	rabbit	Polyclonal (IgG)
Double Staining				
Target	**Antigen retrieval**	**Manufacturer & code**	**Host & Isotype**	**Dilution**
CD64	pH 9	Abcam, Ab140779	Mouse, IgG1	1:150
PU.1		Abcam, Ab76543	Rabbit monoclonal IgG	1:150
CD206	pH 9	Abcam, Ab201927	Mouse, IgG2b	1:25
PU.1		Abcam, Ab76543	Rabbit monoclonal IgG	1:150
FR-β	pH 9	Origene, TA808017	Mouse, IgG1	1:200
PU.1		Abcam, Ab76543	Rabbit monoclonal IgG	1:150
MMP-9	pH 9	Abcam, Ab58803	Mouse, IgG1	1:200
PU.1		Abcam, Ab76543	Rabbit monoclonal IgG	1:150
IL-12	pH 9	Atlas Antibodies, HPA001886	Rabbit polyclonal IgG	1:50
PU.1		Biolegend, 658012	Mouse, IgG1	1:20
IL-23	pH 9	Abcam, Ab45420	Rabbit polyclonal IgG	1:150
PU.1		Biolegend, 658012	Mouse, IgG1	1:20

***: Antibodies against MMPs detect both pro-MMPs and active MMPs**

Dilution	Secondary antibody	Manufacturer & code	Dilution
1:100	Rabbit α-mouse Ig-HRP	DAKO, P260	1:50
1:100	Envision α-mouse polymer-HRP	DAKO, K4006	Undiluted
1:25	Envision α-rabbit polymer-HRP	DAKO, K4003	undiluted
1:100	Goat α-rabbit Ig-HRP	DAKO, P448	1:50
1:25	Envision α-mouse polymer-HRP	DAKO, K4006	Undiluted
1:150	Rabbit α-mouse Ig-HRP	DAKO, P260	1:50
1:500	Rabbit α-mouse Ig-HRP	DAKO, P260	1:50
1:200	Rabbit α-mouse Ig-HRP	DAKO, P260	1:50
1:100	Rabbit α-mouse Ig-HRP	DAKO, P260	1:50
1:150	Rabbit α-mouse Ig-HRP	DAKO, P260	1:50
1:350	Envision α-rabbit polymer-HRP	DAKO, K4003	undiluted
1:100	Goat α-rabbit Ig-HRP	DAKO, P448	1:50
1: 50	Rabbit α-mouse Ig-HRP	DAKO, P260	1:50
1:250	Envision α-rabbit polymer-HRP	DAKO, K4003	undiluted

Secondary antibody	Manufacturer & code	Dilution
MultiVision polymer cocktail	ThermoFisher, TL-012-MARH	undiluted
Envision α-mouse polymer-HRP	DAKO, K4006	Undiluted
Donkey rabbit-AP	SouthernBiotech, 6441-04	1:50
MultiVision polymer cocktail	ThermoFisher, TL-012-MARH	undiluted
MultiVision polymer cocktail	ThermoFisher, TL-012-MARH	undiluted
MultiVision polymer cocktail	ThermoFisher, TL-012-MARH	undiluted
MultiVision polymer cocktail	ThermoFisher, TL-012-MARH	undiluted

van Sleen Y,

Jiemy WF,

Pringle S,

Abdulahad WH,

van der Geest KSM,

Sandovici M,

Heeringa P,

Brouwer E,

Boots AMH

SIX

Macrophages Skewed by GM-CSF Produce YKL-40, Instigating Angiogenesis in Giant Cell Arteritis

Work in progress

INTRODUCTION

Giant cell arteritis (GCA) is an inflammatory disease affecting the medium- and large-sized arteries [1] with potential serious acute complications such as blindness and stroke. Chronic complications can also occur, as long-term aortic inflammation is associated with aneurysms and dissection [2, 3]. GCA is commonly treated with glucocorticoids (GCs). More recently, tocilizumab (interleukin (IL)-6 receptor blockade) has become available as GC sparing therapy in GCA [4]. GCs and tocilizumab treatment generally manage to suppress disease symptoms. It is less clear, however, if GCs and tocilizumab suppress smoldering vascular inflammation, which is likely associated with patient relapses and chronic complications of GCA [5-9]. Reliable serum markers of vascular inflammation may aid the development of treatment regimens targeting persistent vascular inflammation.

An interesting candidate serum marker of ongoing vascular inflammation is YKL-40. We previously showed that serum levels of YKL-40 are elevated in GCA at diagnosis, but do not normalize after initiation of GC treatment. In contrast, acute-phase markers such as C-reactive protein (CRP) are strongly suppressed by treatment with GCs and tocilizumab, as their levels are highly dependent on IL-6 in GCA and PMR [7, 10]. YKL-40 is a chitinase-like protein, which means it is able to bind to chitin but is not able to cleave it [11]. YKL-40 production by innate immune cells, including macrophages, is induced by various cytokines, not only IL-6 but also IL-1β and interferon γ (IFNγ) [12]. Cancer studies have implicated YKL-40 production by tumor-associated macrophages in various inflammatory and tissue remodeling processes, including angiogenesis. These tumor-associated macrophages thereby promote tumor growth and are associated with poor survival [11]. Less is known about the role of YKL-40 in inflammatory diseases such as GCA. In this study, we investigated the cellular source and the pro-angiogenic function of YKL-40 in GCA patients.

MATERIALS AND METHODS

YKL-40 production by macrophages in vivo and *in vitro*

For this study we performed immunohistochemistry (IHC) and cell culture experiments. IHC was performed on GCA positive temporal artery biopsies (TABs; n=12) of treatment-naive patients. In addition, we stained aortas (n=10) obtained from GCA patients who were undergoing surgery for an aortic aneurysm, not using any GCs. Staining for YKL-40 (R&D System, Minneapolis, MN, USA) was compared to staining for CD206 and matrix-metalloproteinase-9 (MMP-9), stainings that were performed in the context of our previous study [13]. Expression of YKL-40 by macrophages was confirmed by double staining with macrophage transcription factor PU.1 (Abcam, Cambridge, UK; upper right panel). Additionally, the TABs were stained for IL-13Rα2, the receptor for YKL-40 (Proteintech, Rosemont, IL, USA).

To assess the effect of skewing signals on YKL-40 production by macrophages, we cultured monocytes of 8 treatment-naive GCA patients for seven days in the presence of either 100 ng/mL GM-CSF (Peprotech, Rocky Hill, NJ, USA) or 100 ng/mL M-CSF (Peprotech) to generate macrophages (GM-MØ and M-MØ, respectively). 100 ng/mL of lipopolysaccharide (LPS; O55:B5 from E.coli,

Sigma-Aldrich, St. Louis, Missouri, USA) was added to activate the cells on the fifth day of culture. On day 7, culture supernatants were assayed for YKL-40 by Luminex.

Angiogenic potential of YKL-40 *in vitro*

The angiogenic potential of YKL-40 was studied in vitro. Human microvascular endothelial cells (HMVECs, Lonza, Basel, Switzerland) were starved in endothelial cell basal medium containing 0.5% FBS for 24 hours before the experiment. To visualize the cells, the PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich, Saint Louis, MO, USA) was used, prior to adding the cells to the culture. HMVECs in plain medium, with or without added factors, were seeded in 96-well plates containing a growth factor-reduced Matrigel (Corning, Corning, NY, USA) at 30,000 cells per well. Each condition was measured by three technical replicates for the following conditions: control, 150 ng/mL YKL-40 (Org 39141, MSD, Oss, The Netherlands), 1500 ng/mL YKL-40 and 20 ng/mL of the well-known angiogenic instigator VEGF (Peprotech). HMVECs were cultured for 16 hours, after which the wells were scanned by TissueFAXS (TissueGnostics, Vienna, Austria). Tube formation was assessed by counting the number of enclosed fields visible.

RESULTS AND DISCUSSION

YKL-40, produced by a specialized macrophage subset, serves as a marker of ongoing vascular inflammation in GCA

YKL-40 is produced by a distinct subset of macrophages in GCA temporal artery biopsies (TABs) and aortas. Previously, we showed strong expression of YKL-40 in the media and the media borders, which is in congruence with an earlier report [7, 14]. Our recent characterization of macrophage phenotypes revealed the presence of a CD206+ macrophage subset in GCA lesions, which is thought to be involved in tissue destruction by the production of MMP-9 [13]. We here show YKL-40 to be expressed by CD206+/MMP-9+ macrophages in all GCA TABs (Figure 1A). A similar pattern was observed in aortas of GCA patients with an aortic aneurysm (Figure 1B), a late-stage disease complication which may develop when the vascular inflammation is not sufficiently suppressed (9). Bonneh-Barkay et al. showed that YKL-40 production by macrophages may be insensitive to anti-inflammatory drugs [15]. These findings suggest that the high serum levels of YKL-40 in treated patients may originate from the CD206+/ MMP-9+ macrophages in vascular tissue. Moreover, IFNγ, vastly produced by T-cells in the GCA TAB, appears to be largely unaffected by GC treatment [16, 17]. Tissue production of IFNγ, however, is not reflected by elevated levels in the blood [18]. We therefore suggest that YKL-40 qualifies as a candidate biomarker of smoldering vessel inflammation caused by persistent IFNγ signaling in GCA tissues. It remains to be established if IFNγ is indeed the driver of YKL-40 production in GCA, and if this ongoing process is accurately reflected by serum levels of YKL-40.

Previously, we provided evidence that this CD206+ macrophage phenotype is dependent on local GM-CSF production in GCA tissues [13]. To validate if YKL-40 production is elevated in GM-CSF skewed macrophages, monocytes of GCA patients were differentiated into macrophages in the presence of GM-CSF or M-CSF. Our results show that GM-CSF skewed macrophages, highly

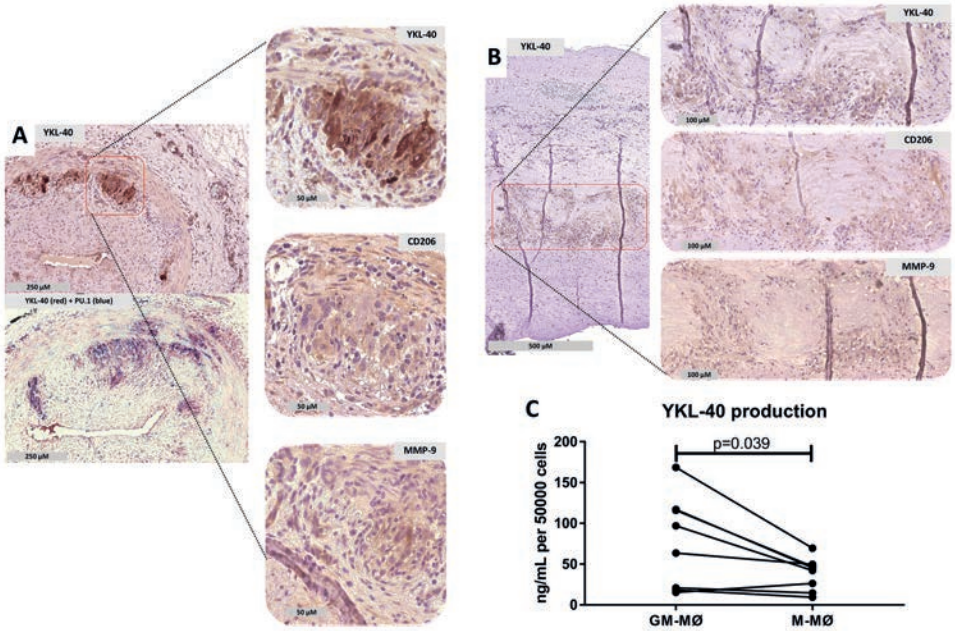


Figure 1. YKL-40 is likely produced by CD206+, MMP-9+, GM-CSF skewed macrophages in GCA affected medium- and large-sized arteries. TABs (N=12) of treatment-naive GCA patients were stained for YKL-40 by IHC (**A**, upper left panel). Double staining with macrophage transcription factor PU.1 shows YKL-40 production by macrophages (lower left panel). Higher magnification pictures show expression of YLK-40 and CD206 within the same region of the TAB (right panels). In **B**, we document a similar pattern of YKL-40 production within the region of CD206 expressing cells, located at the site of the granuloma in the media of GCA affected aortas. In vitro, we show that GM-CSF differentiated macrophages are specialized in producing YKL-40 (**C**). The Wilcoxon signed rank test showed significantly higher concentrations of YKL-40 in the supernatant of GM-MØ compared to M-MØ.

expressing CD206, produced higher levels of YKL-40 than M-CSF macrophages (Figure 1C). This finding corresponds to our data on expression of CD206 and YKL-40 in the same regions of the GCA TABs. Indeed, others have reported that CD206 expression distinguishes YKL-40 positive macrophages from YKL-40 negative macrophages [15]. Taken together, the CD206+/MMP9+ macrophage subset skewed by local GM-CSF signals is likely the main producer of YKL-40 in GCA TABs and aortas.

YKL-40 as instigator of angiogenesis in GCA

We next wondered what function YKL-40 serves at the inflammatory site in GCA. The high angiogenic potential of YKL-40 was previously reported showing that YKL-40 performs equally well as VEGF in stimulating tube formation of HMVECs [19]. Indeed, our preliminary data confirm that YKL-40 stimulation induces more tube formation compared to unstimulated HMVECs (Figure 2AB) and that YKL-40 has a potential equal to VEGF to stimulate tube formation [19]. Angiogenic effects of YKL-40 are thought to be governed by its receptor IL-13 receptor α2 (IL-13Rα2),

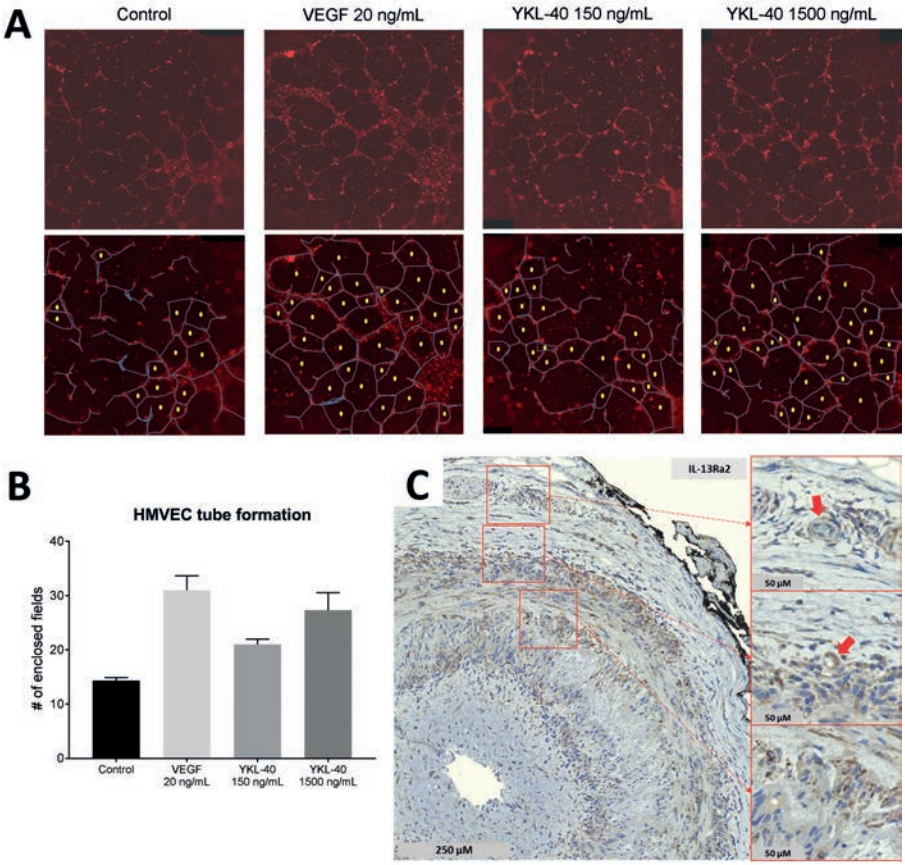


Figure 2. The angiogenic potential of YKL-40, as assessed by tube formation of HMVECs, is relevant for GCA. Shown are representative photos and the analysis of HMVEC tube formation with or without stimulation with YKL-40 or VEGF (**A**). The visible HMVEC membranes are colored blue, the enclosed fields are marked with a yellow dot. The number of enclosed fields was higher for YKL-40 and VEGF stimulated HMVECs than for HMVECs in plain medium (**B**). Data are expressed as mean and standard deviation. To identify whether YKL-40 can signal in GCA lesions, we stained GCA TABs for expression of IL-13Rα2 by IHC. IL-13Rα2 expression was observed in the inflammatory infiltrate in all three layers, as well as by endothelial cells of the vasa vasorum (**C**). Two zoomed in regions identifying the vasa vasorum are shown in the upper right corner (red arrows indicate IL-13Rα2-positive capillaries), and a close-up of the inflammatory infiltrate in the intima is shown in the lower right corner.

a decoy receptor of IL-13 [20]. We here show that IL-13Rα2 is also abundantly expressed in GCA TABs (Figure 2C). YKL-40 induced angiogenesis, as seen in tumor growth and metastasis, may thus also fuel vascular inflammation in diseases such as GCA and PMR [21, 22]. Moreover, abundant expression of IL-13Rα2 is observed in macrophage-rich areas. Indeed, YKL-40 can signal macrophages, inducing the production of inflammatory, migratory and tissue remodeling mediators such as CCL-2, CXCL2, IL-8 and MMP-9 [11]. In line with this, YKL-40 has been shown to significantly enhance the migration of macrophages [23].

CONCLUSION

Taken together, we show here that a distinct subset of macrophages, skewed by GM-CSF, is responsible for the production of YKL-40 in GCA. This YKL-40 production may be involved in angiogenesis in GCA tissues, a process important for the continuation of the inflammatory process. The high serum levels of YKL-40 in patients during treatment suggest that this pathogenic macrophage subset is not properly targeted by GCs. Currently, a trial is ongoing targeting GM-CSF signaling in GCA (NCT03827018), which possibly also targets this YKL-40 producing macrophage subset.

REFERENCES

1. Samson, M., Corbera-Bellalta, M., Audia, S., Planas-Rigol, E., Martin, L., Cid, M.C., and Bonnotte, B. 2017. Recent advances in our understanding of giant cell arteritis pathogenesis. *Autoimmun. Rev. 16: 833-844.*
2. Gonzalez-Gay, M.A., Garcia-Porrua, C., Piñeiro, A., Pego-Reigosa, R., Llorca, J., and Hunder, G.G. 2004. Aortic aneurysm and dissection in patients with biopsy-proven giant cell arteritis from northwestern Spain: a population-based study. *Medicine 83: 335-341.*
3. Nuenninghoff, D.M., Hunder, G.G., Christianson, T.J., McClelland, R.L., and Matteson, E.L. 2003. Incidence and predictors of large-artery complication (aortic aneurysm, aortic dissection, and/or large-artery stenosis) in patients with giant cell arteritis: a population-based study over 50 years. *Arthritis Rheumatol. 48: 3522-3531.*
4. Stone, J.H., Tuckwell, K., Dimonaco, S., Klearman, M., Aringer, M., Blockmans, D., Brouwer, E., Cid, M.C., Dasgupta, B., Rech, J., Salvarani, C., Schett, G., Schulze-Koops, H., Spiera, R., Unizony, S.H., and Collinson, N. 2017. Trial of Tocilizumab in Giant-Cell Arteritis. *N. Engl. J. Med. 377: 317-328.*
5. Schmidt, W.A. 2018. Ultrasound in the diagnosis and management of giant cell arteritis. *Rheumatology (Oxford) 57: ii22-ii31.*
6. Maleszewski, J.J., Younge, B.R., Fritzlen, J.T., Hunder, G.G., Goronzy, J.J., Warrington, K.J., and Weyand, C.M. 2017. Clinical and pathological evolution of giant cell arteritis: a prospective study of follow-up temporal artery biopsies in 40 treated patients. *Mod. Pathol. 30(6):788-796.*
7. van Sleen, Y., Sandovici, M., Abdulahad, W., Bijzet, J., van der Geest, K., Boots, A.M., and van der Geest, Kornelis SM. 2019. Markers of Angiogenesis and Macrophage Products for Predicting Disease Course and Monitoring Vascular Inflammation in Giant Cell Arteritis. *Rheumatology (Oxford) 58: 1383–1392.*
8. Gloor A.D., Yerly D., Adler S., Reichenbach S., Kuchen S., Seitz M., Villiger P.M. 2018. Immuno-monitoring reveals an extended subclinical disease activity in tocilizumab-treated giant cell arteritis. *Rheumatology (Oxford) 57:1795-1801*
9. Reichenbach, S., Adler, S., Bonel, H., Cullmann, J.L., Kuchen, S., Bütikofer, L., Seitz, M., and Villiger, P.M. 2018. Magnetic resonance angiography in giant cell arteritis: results of a randomized controlled trial of tocilizumab in giant cell arteritis. *Rheumatology (Oxford) 57: 982-986.*
10. Stone J.H., Tuckwell K., Dimonaco S., Klearman M., Aringer M., Blockmans D., Brouwer E., Cid M.C., Dasgupta B., Rech J., Salvarani C., Schulze-Koops H., Schett G., Spiera R., Unizony S.H., Collinson N. Glucocorticoid Doses and Acute-Phase Reactants at Giant Cell Arteritis Flare in a Randomized Trial of Tocilizumab. *Arthritis Rheumatol. 71:1329-1338.*
11. Libreros, S. and Iragavarapu-Charyulu, V. 2015. YKL-40/CHI3L1 drives inflammation on the road of tumor progression. *J. Leukoc. Biol. 98: 931-936.*
12. Kzhyshkowska, J., Mamidi, S., Gratchev, A., Kremmer, E., Schmuttermaier, C., Krusell, L., Haus, G., Utikal, J., Schledzewski, K., Scholtze, J., and Goerdt, S. 2006. Novel stabilin-1 interacting chitinase-like protein (SI-CLP) is up-regulated in alternatively activated macrophages and secreted via lysosomal pathway. *Blood 107: 3221-3228.*
13. Jiemy, W.F., van Sleen, Y., van der Geest, K.S.M., ten Berge, H.A., Abdulahad, W.H., Sandovici, M., Boots, A.M.H., Heeringa, P., and Brouwer, E. 2019. Distinct Macrophage Subsets Dictated by Local GM-CSF and M-CSF expression are Associated with Tissue Destruction and Intimal Hyperplasia in Giant Cell Arteritis. *Submitted*
14. Johansen, J.S., Baslund, B., Garbarsch, C., Hansen, M., Stoltenberg, M., Lorenzen, I., and Price, P.A. 1999. YKL-40 in giant cells and macrophages from patients with giant cell arteritis. *Arthritis Rheumatol. 42: 2624-2630.*
15. Bonneh-Barkay, D., Bissel, S.J., Kofler, J., Starkey, A., Wang, G., and Wiley, C.A. 2012. Astrocyte and macrophage regulation of YKL-40 expression and cellular response in neuroinflammation. *Brain Path. 22: 530-546.*

16. Weyand, C.M., Hicok, K.C., Hunder, G.G., and Goronzy, J.J. 1994. Tissue cytokine patterns in patients with polymyalgia rheumatica and giant cell arteritis. *Ann. Intern. Med. 121: 484-491.*

17. Deng, J., Younge, B.R., Olshen, R.A., Goronzy, J.J., and Weyand, C.M. 2010. Th17 and Th1 T-cell responses in giant cell arteritis. *Circulation 121: 906-915.*

18. van der Geest, K.S., Abdulahad, W.H., Rutgers, A., Horst, G., Bijzet, J., Arends, S., Roffel, M.P., Boots, A.M., and Brouwer, E. 2015. Serum markers associated with disease activity in giant cell arteritis and polymyalgia rheumatica. *Rheumatology (Oxford) 54: 1397-1402.*

19. Shao, R., Hamel, K., Petersen, L., Cao, Q., Arenas, R.B., Bigelow, C., Bentley, B., and Yan, W. 2009. YKL-40, a secreted glycoprotein, promotes tumor angiogenesis. *Oncogene 28: 4456.*

20. Ma, B., Herzog, E.L., Lee, C.G., Peng, X., Lee, C.M., Chen, X., Rockwell, S., Koo, J.S., Kluger, H., Herbst, R.S., Sznol, M., and Elias, J.A. 2015. Role of chitinase 3-like-1 and semaphorin 7a in pulmonary melanoma metastasis. *Cancer Res. 75: 487-496.*

21. Faibish, M., Francescone, R., Bentley, B., Yan, W., and Shao, R. 2011. A YKL-40-neutralizing antibody blocks tumor angiogenesis and progression: a potential therapeutic agent in cancers. *Mol. Cancer. Ther. 10: 742-751.*

22. Kaiser, M., Younge, B., Bjornsson, J., Goronzy, J.J., and Weyand, C.M. 1999. Formation of new vasa vasorum in vasculitis. Production of angiogenic cytokines by multinucleated giant cells. *Am. J. Pathol. 155: 765-774.*

23. Kawada, M., Seno, H., Kanda, K., Nakanishi, Y., Akitake, R., Komekado, H., Kawada, K., Sakai, Y., Mizoguchi, E., and Chiba, T. 2012. Chitinase 3-like 1 promotes macrophage recruitment and angiogenesis in colorectal cancer. *Oncogene 31: 3111.*

van Sleen Y,

Sandovici M,

Abdulahad WH,

Bijzet J,

van der Geest KSM,

Boots AMH,

Brouwer E

SEVEN

Markers of Angiogenesis and Macrophage Products for Predicting Disease Course and Monitoring Vascular Inflammation in Giant Cell Arteritis

Rheumatology (Oxford) 2019 58;8: 1383-1392

ABSTRACT

Giant Cell Arteritis (GCA), a systemic vasculitis, is characterised by an interleukin (IL)-6-dependent acute-phase response. This response is typically suppressed by treatment rendering CRP/ESR unreliable for monitoring vascular inflammation. Also, there are no accurate biomarkers predicting a non-favourable disease course. Here, we investigated macrophage products and markers of angiogenesis as biomarkers for prognosis and monitoring of vascular inflammation.

Forty-one newly diagnosed, glucocorticoid-naive GCA patients were prospectively followed for relapses and glucocorticoid requirement for 30 months (median; range 0-71). Serum markers at baseline and during follow-up were compared with 33 age-matched healthy controls and 13 infection controls. Concentrations of IL-6, serum amyloid A (SAA), soluble CD163 (sCD163), calprotectin, YKL-40, vascular endothelial growth factor (VEGF), angiopoietin-1 and -2, and sTie2 were determined by ELISA/Luminex assay.

Serum concentrations of all markers, but not angiopoietin-1, were elevated in GCA patients at baseline if compared to healthy controls. High VEGF ($p=0.0025$) and angiopoietin-1 ($p=0.0174$), and low YKL-40 ($p=0.0369$) levels at baseline were predictive of a short time to glucocorticoid-free remission. Raised angiopoietin-2 levels were associated with an imminent relapse during treatment ($p<0.05$). IL-6 correlated strongly with acute-phase markers and sCD163, but not with markers of angiogenesis, YKL-40 or calprotectin. Glucocorticoid treatment down-modulated all markers, except for calprotectin and YKL-40. Tissue expression of markers in temporal arteries

was confirmed.

Markers of angiogenesis at baseline and during treatment predict GCA disease course, suggesting utility in patient stratification for glucocorticoid-sparing therapy. Calprotectin and YKL-40 are candidate markers for monitoring vessel wall inflammation.

INTRODUCTION

Giant cell arteritis (GCA) is the most frequent inflammatory disease of medium and large arteries [1]. Involvement of cranial arteries in GCA (Cranial (C)-GCA) can lead to symptoms like headache, jaw claudication and vision loss [2]. Signs and symptoms of inflammation of the aorta and its branches (Large Vessel (LV)-GCA) are less specific and include weight loss and low-grade fever. Ultimately, LV-GCA can lead to the formation of aneurysms and aortic dissection [3, 4].

The most common treatment for GCA remains high-dose and long-term glucocorticoid (GC) monotherapy. However, many patients relapse, and the burden of GC treatment adds onto that of the disease itself, with a great impact on the patients' quality of life [5-7]. Still, a subset of patients experience a more favourable, non-relapsing disease course requiring short-term GC treatment. To prevent GC toxicity and the risk of relapses, there is an urgent need for biomarkers that, either at baseline or during treatment, can predict disease course in GCA. Recently, interleukin-6 receptor (IL-6R) blocking therapy (tocilizumab) has become available as GC-sparing treatment [8].

Classically, IL-6-dependent acute-phase markers C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are used in diagnosis and monitoring of GCA [6, 7]. However, in 5-16% of newly diagnosed GCA patients CRP and ESR levels are within the normal ranges [9, 10]. In addition, although both GC and tocilizumab treatment strongly suppress the synthesis of these markers [7, 8], disease activity may persist [7, 11, 12]. In line with this notion, recent studies showed ongoing vessel inflammation, despite normalization of CRP and ESR, both under GC treatment [13] as well as under tocilizumab treatment [11, 12]. Recent meta-analyses on serum markers in GCA concluded that there are no reliable serological markers for monitoring or prognosis [14, 15]. Thus, there is an unmet need for IL-6-independent biomarkers that accurately reflect disease activity and vessel wall inflammation during treatment with GCs or tocilizumab.

In an effort to identify prognostic biomarkers and biomarkers for monitoring disease activity, we took clues from GCA characteristic pathogenic processes at the tissue level; these include vessel wall granulomatous infiltrates and neoangiogenesis [16-18]. Consequently, we hypothesized a role for macrophage products and markers of angiogenesis as novel candidate biomarkers. Monocytes and macrophages are capable producers of IL-6 [19], a cytokine known to stimulate hepatocytes to produce acute-phase response markers including SAA [20, 21]. During inflammation, monocytes/macrophages also release calprotectin, sCD163, and YKL-40 [22-24]. Inflamed GCA vessels are characterized by new vessel formation involving VEGF, angiopoietin-1/2 and sTie2 as key regulators in this process [25-27].

We thus compared the performance of these nine soluble markers at baseline with that of CRP and ESR using serum samples prospectively collected over seven years in our GCA cohort. We established their association with the IL-6-driven acute-phase response. Next, we investigated these markers for prediction of disease course and analysed the effects of GC treatment on these markers to identify candidates for monitoring of ongoing vascular inflammation.

PATIENTS AND METHODS

Baseline

Forty-one newly diagnosed, treatment-naive GCA patients participated in the study (Table 1). Patients were diagnosed based on clinical signs and symptoms in combination with either a positive temporal artery biopsy (TAB) and/or a positive 18F-fluorodeoxyglucose-positron emission tomography-computed tomography (PET-CT). In this study, 27 of the 41 GCA patients fulfilled the 1990 ACR criteria. The ACR criteria are useful in diagnosis of C-GCA rather than LV-GCA. Blood samples were obtained before noon and all donors were non-fasted. Thirty-three age- and sex-matched healthy controls (HCs) and 13 age-matched infection controls (INFs) were included as well. HCs were screened for past and present morbidities. Hospitalised INFs were included only if diagnosed with pneumonia or a urinary tract infection. They were excluded in case of comorbid diseases, like cancer or diabetes, and/or treatment with immunosuppressive drugs. Written informed consent was obtained from all study participants. All procedures were in compliance with the declaration of Helsinki. The study was approved by the institutional review board of the UMCG (METc2010/222 for GCA and INF, and METc2012/375 for HC).

GCA clinic

At baseline, patients were scored as having cranial symptoms if one of the following symptoms was noted: new headache, temporal artery abnormality, scalp tenderness, jaw/tongue claudication, vision loss, amaurosis fugax, transient ischemic attack (TIA) or cerebrovascular accident (CVA). Systemic symptoms were scored if patients presented with arm/leg claudication or polymyalgia rheumatica. Moreover, systemic symptoms were noted as well if two of the following symptoms occurred: fever, weight loss, malaise or night sweats.

Table 1. Baseline characteristics of newly diagnosed, treatment-naive GCA patients, aged healthy controls and aged infection controls. [a] The three groups did not significantly differ in age, but significantly less infectious controls were female compared to the other groups (Chi-square $p<0.05$).

	HC	GCA	INF
N	33	41	13
Age in years; median (range)	67 (50-83)	71 (52-89)	74 (47-97)
Females (%)	22 (67)	28 (68)	4 (31)[a]
GCA diagnosis: TAB/ PET-CT/ Both	NA	13 / 19 / 9	NA
GCA symptoms: Cranial/ Systemic/ Combined	NA	11 / 8 / 22	NA
Fulfilled ACR criteria (%)	NA	27 (66)	NA
PMR clinic (%)	NA	10 (24)	NA
Ischemic ocular involvement (%)	NA	11 (27)	NA
Claudication (%)	NA	22 (54)	NA
Follow-up in months; median (range)	NA	30 (0-71)	NA

GCA: giant cell arteritis, HC: healthy control, INF: infection control, PET-CT: positron emission tomography-computed tomography, TAB: temporal artery biopsy, ACR: American college of rheumatology, PMR: polymyalgia rheumatica, NA: not applicable.

Ischemic ocular involvement was scored if a patient suffered from either vision loss or amaurosis fugax. Other ischemic symptoms were scored under claudication: jaw/tongue claudication, TIA, CVA, arm/leg claudication.

Symptoms were scored only if they could not be explained by other causes such as infection.

Follow-up

GCA patients were prospectively followed during which they visited the outpatient clinic according to a fixed study protocol. In case of re-appearance of clinical signs and symptoms a relapse visit was planned. Remission or relapse was defined based on clinical signs and symptoms of GCA. CRP or ESR levels were not taken into account in line with the analysis of the GiACTA trial [8]. At 3 months (± 4 weeks; N=30), 6 or 9 (± 10 weeks; N=5) and 12 months (± 10 weeks; N=29) follow-up samples were collected as per protocol (supplementary Figure 1 and supplementary Table 1).

To investigate differences in biomarker levels in remission patients who would or would not relapse within a time frame of 4 months, samples were identified, grouped and compared (supplementary Figure 1, supplementary Table 1).

Treatment

All patients were treated with GCs, which were tapered in agreement with the BSR guidelines [28]. In short, starting dose of 40-60 mg per day and tapering by 10 mg per 2-3 weeks to 20 mg per day, followed by more gradual tapering. Tapering was done when clinical signs and symptoms of disease activity were absent, preferably with normalisation of the CRP and ESR. In case of a relapse, the GC dose was increased and/or a csDMARD was added (methotrexate or leflunomide). GC-free remission was defined as an absence of signs and symptoms, no GC use, and no return of active disease within at least 6 months of follow-up. Treatment-free remission was defined as no signs and symptoms, no GCs or other DMARDs and no return of active disease for a period of at least 6 months follow-up. Serum marker levels were assessed in samples of 8 patients having achieved treatment-free remission.

Serum marker measurements

Blood samples were drawn at the rheumatology and clinical immunology outpatient clinic of the University Medical Center Groningen. Blood serum was stored at -20°C until use. CRP and ESR were assessed in the context of standard medical care. Levels of serum IL-6 (standard curve range 4.8 - 1154; sensitivity 1.7 pg/ml), sCD163 (5196 - 1262520; 530 pg/ml), VEGF (0.55 - 2250; 2.1 pg/ml), YKL40 (352 - 85610; 3.3 pg/ml), angiopoietin-1 (114 - 27610; 9.43 pg/ml), angiopoietin-2 (90.5 - 22000; 17.1 pg/ml) and sTie2 (614 - 149166; 211 pg/ml) were measured with Human premix Magnetic Luminex screening assay kits (R&DSystems, Abingdon, UK) according to the manufacturer's instructions and read on a Luminex Magpix instrument (Luminex, Austin, TX, USA). Data were analysed with xPONENT 4.2 software (Luminex). Levels of SAA (standard curve range 1.7 – 219; detection level 1.6 ng/ ml) and calprotectin (1.6 – 100; 1.6 ng/ ml) were measured by ELISA (SAA by in house ELISA and calprotectin by Hycult Biotech, Uden, the Netherlands).

Immunohistochemistry (IHC)

Five positive TABs were used for immunohistochemical detection of markers as described previously [17]. In brief, formalin-fixed, paraffin-embedded tissue sections (3 μm) were deparaffinised and rehydrated. After antigen retrieval and endogenous peroxidase blocking, the tissues were incubated with antibodies detecting IL-6 (Santa Cruz Biotechnology, Dallas, TX, USA), SAA (Reu86.2; Sanbio, Uden, The Netherlands), calprotectin (Hycult), YKL40 (R&D), VEGF (Santa Cruz), or angiopoietin-2 (Thermofisher Scientific, Waltham, MA, USA). To identify macrophage-rich areas, neutrophil-rich areas and areas of angiogenesis we employed anti-CD68 (2), CD15 (Abcam) and anti-CD34 (Roche, Basel, Switzerland). After incubation with secondary antibody and peroxidase, counterstaining with hematoxilin was performed. Stained sections were scanned using a Nanozoomer Digital Pathology Scanner (NDP Scan U10074–01, Hamamatsu Photonics K.K., Hamamatsu, Japan).

Statistical analysis

Non-parametric tests (2-tailed) were used to analyse the data (differences between groups). Comparisons between baseline patients and control groups were done by Kruskal Wallis and Mann Whitney U tests. Also, the Mann Whitney U test was used for comparison of follow-up samples with HCs, comparison of samples from active patients and patients in remission during treatment and comparison of remission patients who would or would not relapse within 4 months. Paired testing was performed to compare follow-up samples and baseline samples using the Wilcoxon signed rank test. Correlations between biomarkers were assessed using Spearman's rank correlation coefficient. To compare the time to GC-free remission of patients with high levels of serum markers at baseline to patients with low levels, the log rank test was used. The log rank test was used as well to calculate hazard ratios for long-term GC requirement. Analyses were performed with IBM SPSS 23 and GraphPad Prism 7.0 software.

RESULTS

Follow-up patient characteristics

The median follow-up duration of GCA patients was 30 months (range 0-71). Out of 41 patients in the cohort, fifteen reached GC-free remission in a median of 21 months (range 8-47, supplementary Table 1).

Elevated levels of inflammatory and angiogenesis serum markers in newly diagnosed GCA patients

Macrophage products (calprotectin, YKL-40, sCD163) and markers of angiogenesis (VEGF, angiopoietin-2, sTie2) were significantly higher in newly diagnosed, GC treatment-naive GCA patients (N=41) compared to age- and sex-matched healthy controls (N=33, Figure 1, supplementary Figure 2
and supplementary Table 2). In contrast, angiopoietin-1 levels were not elevated. As expected, ESR and acute-phase markers (CRP, IL-6 and SAA) were also elevated. Most markers were also found elevated in INFs (N=13), indicating that these markers are not disease specific. Interestingly, ESR and

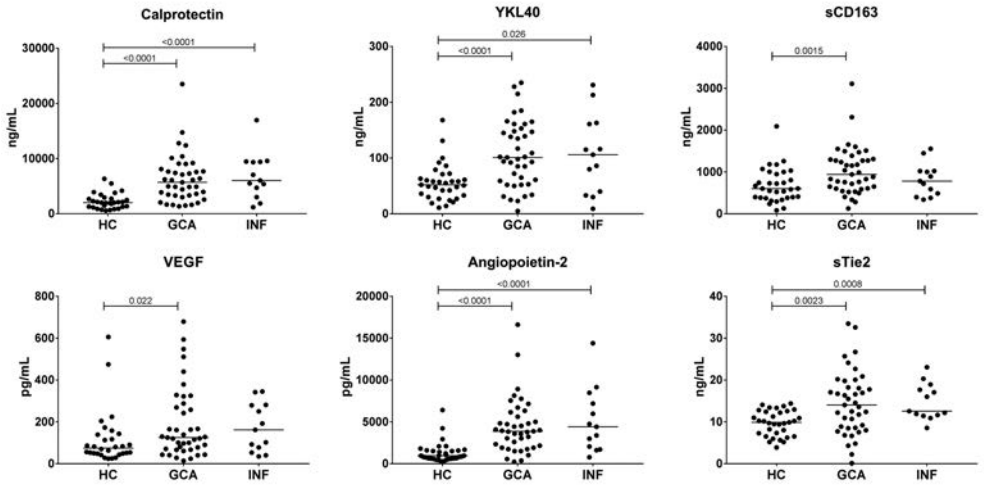


Figure 1. Serum marker levels in newly diagnosed GCA patients compared to infection and healthy controls. Serum levels of calprotectin, YKL-40, sCD163, VEGF, angiopoietin-2 and sTie2 in newly diagnosed GCA patients compared to infection and healthy controls. All markers were significantly higher in newly diagnosed, treatment-naive GCA (N=41) and in infection controls (INF, N=13) as compared to age-matched healthy controls (HC, N=33). As the Kruskal Wallis test showed a significant difference between groups ($p<0.05$), differences between individual groups were tested with the Mann-Whitney U test. The horizontal line represents the median. Statistical significance is indicated by p-values in the graphs.

angiopoietin-2 levels were clearly elevated in 4 out of 5 patients with low CRP levels, suggesting that these markers could add to diagnosis (supplementary Table 3).

Acute-phase response and GCA clinic

Patients with combined cranial and systemic symptoms had a significantly higher acute-phase response compared to patients with isolated cranial or systemic symptoms (Table 1, GCA clinic in supplementary Patients and Methods). CRP and ESR were significantly higher in the combined group (N=22) when compared to the isolated cranial group (N=11, $p<0.05$) or the isolated systemic group (N=8, $p<0.05$). IL-6, SAA and sCD163 were also significantly higher in the combined group compared to the isolated cranial group ($p<0.05$). No differences were found for the other macrophage and angiogenesis markers.

Levels of acute-phase markers were lower in patients with ischemic ocular involvement (N=11, $p<0.05$ for IL-6 and SAA). Interestingly, this was not typical for patients with other ischemic symptoms in both C- and LV-GCA (e.g. jaw claudication and limb claudication, N=22). No differences were found for the other markers.

Baseline inflammatory and angiogenesis serum marker correlations

Next, we investigated serum marker correlations. As expected, levels of CRP, ESR, and SAA were strongly correlated with IL-6 in newly diagnosed GCA patients (N=41, Figure 2A). Also, we found a strong correlation of sCD163 with IL-6 and the acute-phase markers. In contrast, YKL-40

A)

	CRP	ESR	IL-6	SAA	sCD163	Calp	YKL40	VEGF	Angpt1	Angpt2
ESR	0.795**									
IL-6	0.778**	0.746**								
SAA	0.699**	0.668**	0.811**							
sCD163	0.538**	0.518**	0.506**	0.442**						
Calp	0,311	0,114	0,134	0,247	0,168					
YKL40	0,292	0,248	0.476**	0.352*	0,261	0,147				
VEGF	0,216	0.390*	0.410**	0.335*	0.496**	0,112	0,155			
Angpt1	0,007	0,232	0,095	0,250	0,251	0,257	-0,125	0.403**		
Angpt2	0.334*	0.415**	0,266	0,104	0,257	-0,011	0,124	0,061	0,119	
sTie2	0,042	0.339*	0,195	0,095	0,156	-0,198	-0,098	0,113	0,184	0.448**

Legend:

Spearman R = >0,75 / >0,5 / >0,25 / < -0,25 / < -0,5

B)

	CRP	ESR	IL-6	SAA	sCD163	Calp	YKL40	VEGF	Angpt1	Angpt2
ESR	0,886*									
IL-6	0,835**	0,657								
SAA	0,610*	0,600	0,291							
sCD163	0,313	0,143	0,192	0,297						
Calp	0,659*	0,543	0,775**	0,434	0,302					
YKL40	0,676*	0,600	0,736**	0,434	0,203	0,632*				
VEGF	0,253	0,200	0,341	0,192	0,005	0,275	0,016			
Angpt1	-0,143	-0,086	-0,069	0,151	0,047	0,344	-0,278	0,558*		
Angpt2	0,758**	0,829*	0,742**	0,374	0,374	0,731**	0,874**	-0,016	-0,256	
sTie2	0,022	-0,486	0,209	-0,049	-0,121	0,121	0,088	0,132	0,264	-0,137

Figure 2. Baseline inflammatory and angiogenesis marker correlations. Depicted are Spearman's correlation coefficients for all markers in newly diagnosed, treatment-naive GCA patients (**A**) and infection controls (**B**). Strength of correlation is indicated by cell colours. Statistical significance is indicated as * ($p < 0.05$) and ** ($p<0.01$).

correlated only weakly with IL-6 and SAA. No correlations were found for IL-6 and macrophage marker calprotectin. No or weak correlations were seen for markers of angiogenesis with IL-6 or the acute-phase response.

Interestingly, in INF, IL-6 correlated strongly with calprotectin, YKL-40 and angiopoietin-2 whereas correlations of IL-6 with SAA and sCD163 were absent (N=13, Figure 2B).

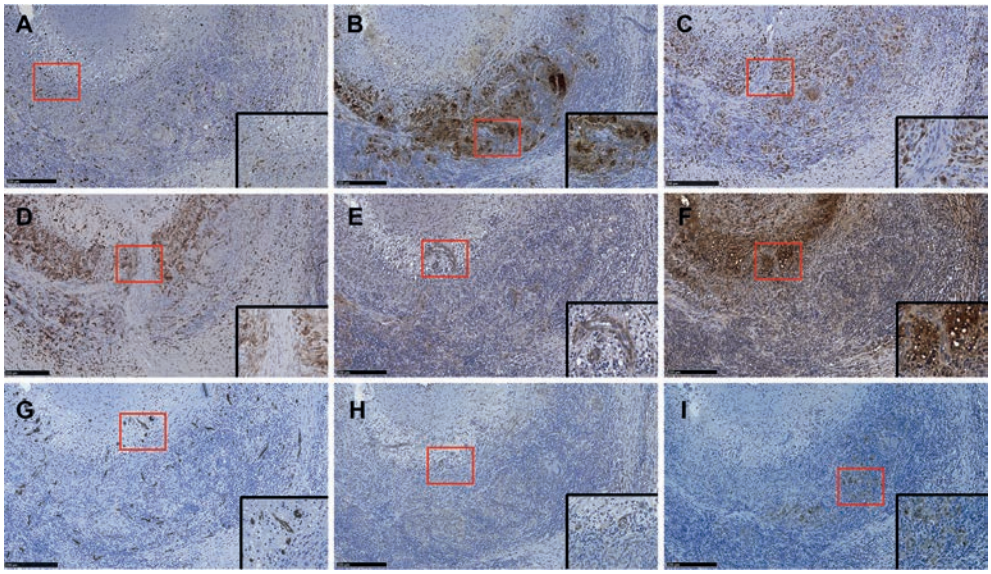


Figure 3. Representative IHC stainings of consecutive sections in a positive TAB of a treatment-naive GCA patient. Paraffin-embedded tissues were stained with antibodies against calprotectin (**A**), YKL-40 (**B**), CD68 (**C**), CD163 (**D**), VEGF (**E**), angiopoietin-2 (**F**), CD34 (**G**), IL-6 (**H**) and SAA (**I**). Regions of interest (red) are magnified and are shown in the lower right corner.

Expression of macrophage and angiogenesis markers in TAB at diagnosis

To confirm that markers of macrophages are expressed at the site of GCA pathology, consecutive TAB sections (N=5) were stained for calprotectin and YKL-40 by IHC (Figure 3). To identify macrophage-rich areas, sections were stained with CD68 and CD163. In addition, we stained for VEGF and angiopoietin-2 as markers of angiogenesis. Newly formed vessels were identified by staining of CD34+ endothelial cells. Expression of IL-6 and SAA was assessed as markers of the acute-phase response. We did not investigate expression of angiopoietin-1, as serum levels were not modulated in GCA, nor sTie-2, as there are no IHC reagents available.

All markers were found to be expressed in the tissue. Massive staining was observed for YKL-40 and angiopoietin-2. As expected, expression of all markers was found mostly in macrophage-rich areas, but endothelial cells also appeared to express IL-6, VEGF and angiopoietin-2. As calprotectin may also be expressed by neutrophils, we checked their presence by CD15 staining. Few CD15+ cells were found (data not shown).

Angiogenic markers at baseline predict time to glucocorticoid-free remission

Next, we determined if baseline serum marker levels could predict disease outcome. To that end, we compared the time to GC-free remission in patients with serum levels below the median (low) and above the median (high). High relative levels of VEGF and angiopoietin-1 and low relative levels

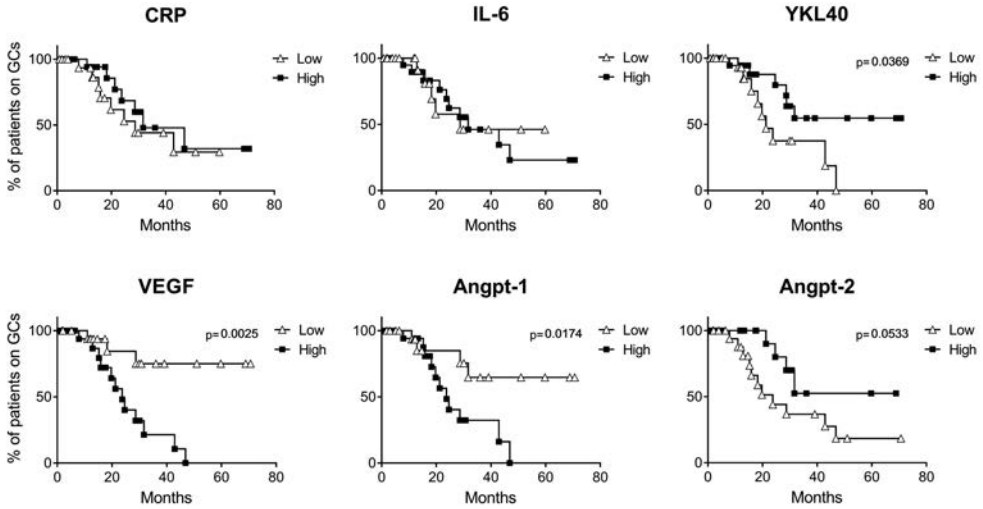


Figure 4. Angiogenesis markers and YKL-40 at baseline predicted a short-term glucocorticoid treatment in GCA. Baseline serum marker levels were split in GCA patients by low or high levels (based on the median) and were plotted in a Kaplan-Meier curve against time to GC-free remission. Strong trends and significant differences of the log-rank test are indicated as p-values in the graphs. Like CRP and IL-6, baseline levels of ESR, SAA, sCD163, calprotectin and sTie2 were not predictive of time to GC-free remission (data not shown).

of YKL-40 at baseline were found to predict a short time to GC-free remission (Figure 4). In addition, a strong trend was seen for low levels of angiopoietin-2 predicting a short time to GC-free remission. The hazard ratio for long-term GC requirement per biomarker was calculated: 5.5 for lower than median VEGF (95% confidence interval: 2.0-15.3), 3.5 for lower angiopoietin-1 (1.3-9.8), 2.8 for higher YKL-40 (1.0-8.2), and 2.9 for higher angiopoietin-2 (1.1-8.0).

Calprotectin and YKL-40 remain elevated during glucocorticoid treatment

To identify markers associated with ongoing vascular inflammation in spite of GC treatment, we investigated the effects of GC treatment on all candidate markers. After 3 and 12 months (N=30 and N=29, respectively, Figure 5A, B) of GC treatment, levels of most markers were found to be decreased compared to baseline, even though many remained significantly elevated compared to HCs. Importantly, calprotectin and YKL-40 levels remained mostly unaffected by GCs and could thus reflect asymptomatic smouldering vessel wall inflammation. Angiopoietin-1 levels were significantly higher in active patients compared to patients in remission at 12 months (Figure 5B, $p<0.05$). YKL-40 correlated with the treatment-reduced ESR, CRP and IL-6, suggesting that YKL-40 may identify ongoing subclinical inflammation in spite of treatment (supplementary Figure 3).

Angiopoetin-2 elevated in remission patients with an imminent relapse

Next, we assessed marker levels associated with future relapses in remission patients (all within 12 months of GC treatment, see supplementary Figure 1, supplementary Table 1). To this end we compared remission patients that would relapse within a period of 4 months (N=14, future relapse)

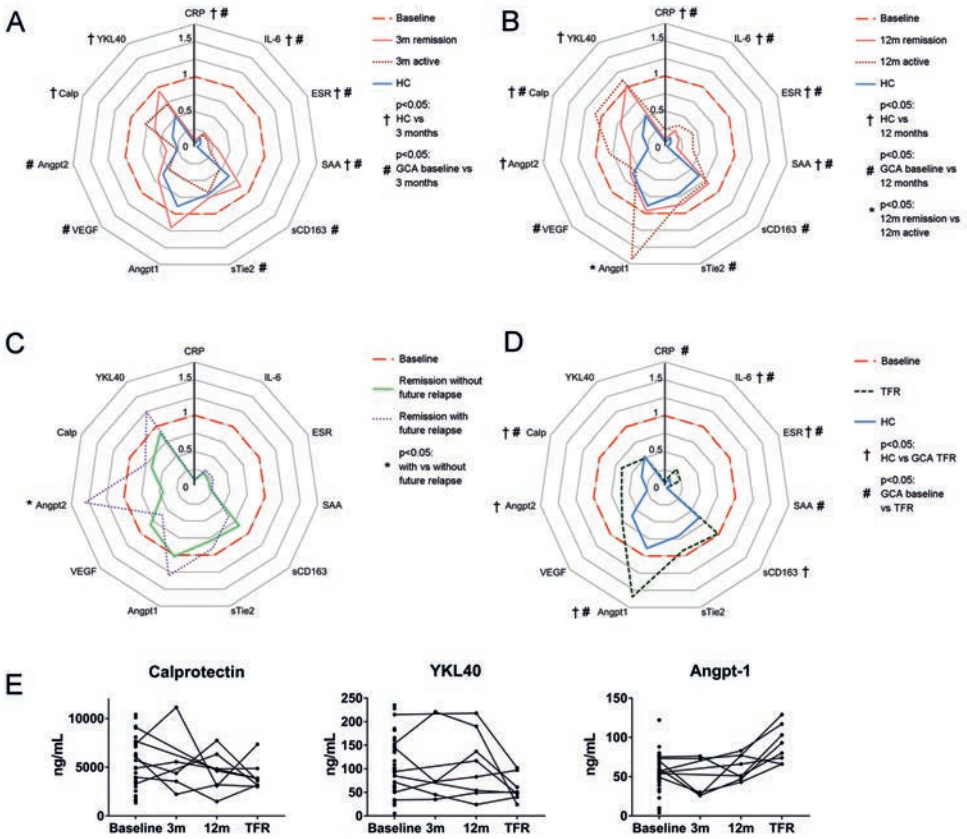


Figure 5. Changes in serum biomarker concentrations during and after treatment. In A-D, radar plots present biomarker levels expressed as fold changes compared to GCA baseline values. **A**, Patients in remission (N=24) or active disease (N=6) at 3 months after start of treatment. **B**, Patients in remission (N=20) or active disease (N=9) at 12 months. **C**, Samples from remission patients who would (N=14) or would not relapse (N=35) within 4 months. **D**, Patients in treatment-free remission (TFR, N=8). **E**, Serum markers over time in GCA patients in treatment-free remission (N=8). HC= healthy control (N=33). Statistical significance by Mann-Whitney U test.

with those that would not relapse in 4 months (N=35). Angiopoietin-2 levels were significantly higher in the future-relapsing group (Figure 5C). The data are in line with increased hazard ratio's for long-term GC requirement associated with high angiopoietin-2 levels (see above, Figure 4).

Extended elevation of markers in treatment-free remission

There is a paucity of data on serum markers in treatment-free remission as these samples are rarely available. To answer the question if treatment leads to normalisation of serum markers in treatment-free remission, we assessed serum marker levels in a small group of patients (N=8; supplementary Figure 1). Levels of IL-6, ESR, sCD163, angiopoietin-1, angiopoietin-2 and calprotectin remained significantly elevated compared to HC levels (Figure 5D). Calprotectin levels were persistently high throughout the whole disease course, while angiopoietin-1 levels increased only after at least 12

months of treatment (Figure 5E). Interestingly, YKL-40 levels remained elevated during 12 months of treatment but eventually normalised in treatment-free remission. Fluctuations of the other markers over time are shown in supplementary Figure 4.

DISCUSSION

Giant Cell Arteritis remains difficult to treat, as the current treatment strategy with GCs is a trade-off between their variable efficacy and their side effects. The goal of treatment in GCA is to reach stable GC-free remission as quickly as possible. Currently, only scarce evidence suggests that serum markers may predict disease course in GCA [14]. In this study, we found that serum markers of angiogenesis at baseline predicted not only time to GC-free remission (VEGF, angiopoietin-1 and YKL-40) but were also associated with an imminent relapse while on treatment (angiopoietin-2). Thus, these markers may aid the stratification of patients eligible for a quick or slow GC tapering scheme**.** In addition, we have identified macrophage products as markers of vessel wall inflammation that may be used for monitoring vascular disease during and after treatment.

We found several markers of angiogenesis (VEGF, angiopoietin-2 and sTie2) to be upregulated in GCA. Neoangiogenesis is instigated by disruption of homeostatic angiopoietin-1 – Tie2 signalling by angiopoietin-2 (competing for binding the Tie-2 receptor) and sTie2 (as decoy receptor), in the presence of VEGF [26, 27]. In GCA TAB, we indeed found VEGF and angiopoietin-2 expressed in neoangiogenic areas, likely triggered by hypoxia [30]. Our findings highlight the importance of new vessel formation at the site of inflammation in GCA to fuel the ongoing inflammatory process and are in line with previous studies reporting on elevated levels of VEGF in GCA. These studies, however, did not investigate this marker in longitudinal follow-up studies [25, 31, 32].

We found that high levels of serum VEGF and angiopoietin-1 at baseline were predictive of short time to GC-free remission. In contrast, high levels of angiopoietin-2, tended to be predictive of a non-favourable disease course. Moreover, elevation of angiopoietin-2 preceded relapses during treatment. Thus, markers of angiogenesis impact the disease course in GCA. The protective effect of VEGF may be explained by its potential to repress CD4+ T cell proliferation and activation. CD4+ T-cells, key players in GCA pathogenesis, express VEGFR2, but not the angiopoietin receptor Tie2 [33]. The notion of protective features of VEGF, however, was not substantiated in another study in which VEGF was reported to amplify T cell pathogenic effector functions in GCA [34].

Worldwide, CRP is increasingly being used for GCA diagnosis instead of ESR; however, diagnosis is typically difficult in patients with low CRP [9, 10]. We propose that elevated angiopoietin-2 may have utility in diagnosis of a small subset of GCA patients with low CRP. Angiopoietin-1 levels were not altered at baseline and during treatment. In treatment-free remission patients, however, increased levels of angiopoietin-1 were found, which may suggest a role in microvessel stabilization. So far, serum levels of angiopoietins and their decoy receptor sTie2 [27] have not been documented in GCA. Clearly, more fundamental studies are needed to elucidate the role of angiopoietins in GCA.

In this study, we provide evidence for the notion of IL-6-independent biomarkers of vessel inflammation at baseline and under the cover of treatment. The monocyte/macrophage products calprotectin, and to a lesser extent YKL-40, are IL-6-independent. Moreover, both markers remain

elevated in spite of treatment and thus qualify as candidate biomarkers of smouldering vessel inflammation. Our findings are in line with the notion that GCs do not sufficiently suppress vascular inflammation [13]. Calprotectin (MRP8/14 or S100A8/9) is a calcium binding protein that acts as a DAMP signal on the TLR4 and RAGE receptors [35]. It is released by monocytes and neutrophils after interaction with endothelial cells during migration [36]. Importantly, calprotectin levels did not correlate with IL-6 and the acute-phase response. Calprotectin levels remained high in treated patients, suggesting ongoing monocyte/neutrophil tissue migration and innate immune activation. Surprisingly, calprotectin levels remained elevated in treatment-free remission. YKL-40 is a marker expressed by mature macrophages, thought to be involved in tissue remodelling and angiogenesis [37, 38]. It is expressed by non-classical monocytes in the blood and by macrophages and giant cells in GCA TABs [24, 39]. In vitro, YKL-40 production by macrophages is sensitive to GCs [40]. In our study, however, long-term high dose GC treatment did not lead to a direct decrease in serum YKL-40 levels, suggesting that YKL-40 producing cells are GC resistant. High YKL-40 levels at baseline predicted a long time to GC-free remission. Our observation of strong YKL-40 expression in TABs in the intima-media border region suggests that this protein is mainly released in fully developed GCA with transmural inflammation, which may be more GC-resistant. Interestingly, YKL-40 levels were clearly decreased in treatment-free remission, which may point towards resolution of inflammation.

We found a strong correlation between IL-6, CRP/ESR and SAA at baseline in GCA patients. This was not the case in infection controls where levels of SAA were not correlated with IL-6, implying that other cytokines stimulate hepatocytes to produce SAA, such as IL-1-or TNF-, cytokines that are reportedly not increased in GCA [20, 41]. We found SAA also expressed at the tissue level. SAA may amplify the local inflammatory response as O'Neill et al showed that stimulation with SAA induced the production of IL-6, VEGF and angiopoietin-2 in TAB explants [21].

We observed a stronger acute-phase response in patients with overlapping cranial and large vessel GCA compared to patients with C-GCA or LV-GCA alone. Recent reviews addressed the similarities and differences between C-GCA and LV-GCA patients [2, 3, 14]. It is currently still debated which patient group expresses the strongest acute-phase response: C-GCA, LV-GCA or patients with overlapping symptoms [3, 42]. High levels of acute-phase proteins in patients with overlapping symptoms may be due to a higher inflammatory load (more inflamed vessels) and consequently a higher net IL-6 production and ensuing acute-phase response.

Patients with ischemic ocular involvement presented with a weaker acute-phase response in line with previous reports [43-45]. In contrast, we did not observe a weak acute-phase response in patients presenting with other ischemic symptoms such as claudication. It has been suggested that high levels of IL-6 are protective against ischemic events by promoting neo-angiogenesis [45]. Thus, it could be expected that IL-6, via a similar mechanism, is protective against claudication as well, which was not the case in our cohort. Therefore, it is more likely that patients with visual symptoms present earlier in the disease course not yet having developed a more extensive vessel wall inflammation.

This study has several strengths. The selection of biomarkers was based on a strong rationale; their potential involvement in GCA immunopathogenesis. Also, we included newly diagnosed GCA patients before start of GC treatment. This is an important strength as we observed a strong effect

of GCs on most serum markers. Furthermore, newly diagnosed, treatment-naive patients were prospectively followed for up to 7 years and samples were taken at fixed time points. Patients were intensively monitored by frequent follow-up visits according to protocol and extra visits in between in case of suspicion of a relapse. This allowed us to calculate the exact time to GC-free remission. Due to the already longstanding follow-up, we were also able to include treatment-free remission samples. This revealed that many serum markers are still elevated for an extended period. Another advantage of our study design is the inclusion of two control populations: age- and sex-matched healthy controls and age-matched infection controls allowing to discriminate between disease-specific and non-specific events.

Our study has the following limitations: low numbers of patients with active disease during treatment and low numbers of patients in treatment-free remission (both N<10). The latter limitation is obviously due to the length of the disease course. This implies that the data in active disease and in treatment-free remission should be taken with caution. Data from this study cannot yet be extended to GCA patients treated with tocilizumab.

The serum markers in this study may aid in designing personalized medicine for easy (short-term GC requiring) and difficult to treat (long-term GC requiring) GCA patients. Patients at baseline may be stratified based on VEGF, angiopoietins and possibly YKL-40 levels for a quick or a slow GC tapering scheme. It is yet unclear whether these markers have a similar predictive value in patients on IL-6R blockade treatment. The predictive values of angiogenesis-related serum markers require further confirmation. Future studies on tissue inflammation markers may focus on calprotectin or YKL-40, especially to prevent aneurysms and aortic dissection. PET-CT or follow-up biopsies would allow to determine whether these markers correlate with silently ongoing tissue inflammation. If calprotectin and/or YKL-40 are confirmed as markers of tissue inflammation, monitoring their levels would be implied to prevent recurrence of disease in GCA patients in remission.

To conclude, this prospective study identified a profile of angiogenic and macrophage serum markers that predict disease course in GCA. This profile outperformed the classical GCA biomarkers CRP and ESR. In addition, calprotectin and/or YKL-40 may prove useful as IL-6-independent biomarkers monitoring vessel inflammation during treatment.

REFERENCES

1. Crowson Cynthia S CS. 2011-3. The lifetime risk of adult-onset rheumatoid arthritis and other inflammatory autoimmune rheumatic diseases. *Arthritis Care Res. 63: 633-9.*
2. Dejaco, C., Duftner, C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2016. The spectrum of giant cell arteritis and polymyalgia rheumatica: revisiting the concept of the disease. *Rheumatology (Oxford) 56(4):506-515.*
3. Koster, M.J., Matteson, E.L., and Warrington, K.J. 2018. Large-vessel giant cell arteritis: diagnosis, monitoring and management. *Rheumatology (Oxford) 57: ii32-ii42.*
4. Dejaco, C., Brouwer, E., Mason, J.C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2017. Giant cell arteritis and polymyalgia rheumatica: current challenges and opportunities. *Nat. Rev. Rheumatol. 13(10):578-592.*
5. Broder, M.S., Sarsour, K., Chang, E., Collinson, N., Tuckwell, K., Napalkov, P., and Klearman, M. 2016. Corticosteroid-related adverse events in patients with giant cell arteritis: A claims-based analysis. *Semin. Arthritis Rheum. 46: 246-252.*
6. Restuccia, G., Boiardi, L., Cavazza, A., Catanoso, M., Macchioni, P., Muratore, F., Cimino, L., Aldigeri, R., Crescentini, F., Pipitone, N., and Salvarani, C. 2016. Flares in Biopsy-Proven Giant Cell Arteritis in Northern Italy: Characteristics and Predictors in a Long-Term Follow-Up Study. *Medicine (Baltimore) 95: e3524.*
7. Alba, M.A., Garcia-Martinez, A., Prieto-Gonzalez, S., Tavera-Bahillo, I., Corbera-Bellalta, M., Planas-Rigol, E., Espigol-Frigole, G., Butjosa, M., Hernandez-Rodriguez, J., and Cid, M.C. 2014. Relapses in patients with giant cell arteritis: prevalence, characteristics, and associated clinical findings in a longitudinally followed cohort of 106 patients. *Medicine (Baltimore) 93: 194-201.*
8. Stone, J.H., Tuckwell, K., Dimonaco, S., Klearman, M., Aringer, M., Blockmans, D., Brouwer, E., Cid, M.C., Dasgupta, B., Rech, J., Salvarani, C., Schett, G., Schulze-Koops, H., Spiera, R., Unizony, S.H., and Collinson, N. 2017. Trial of Tocilizumab in Giant-Cell Arteritis. *N. Engl. J. Med. 377: 317-328.*
9. Salvarani, C. and Hunder, G.G. 2001. Giant cell arteritis with low erythrocyte sedimentation rate: frequency of occurence in a population-based study. *Arthritis Rheum. 45: 140-145.*
10. Kermani, T.A., Schmidt, J., Crowson, C.S., Ytterberg, S.R., Hunder, G.G., Matteson, E.L., and Warrington, K.J. 2012. Utility of erythrocyte sedimentation rate and C-reactive protein for the diagnosis of giant cell arteritis. *Semin. Arthritis Rheum. 41: 866-871.*
11. Gloor A.D., Yerly D., Adler S., Reichenbach S., Kuchen S., Seitz M., Villiger P.M. 2018. Immuno-monitoring reveals an extended subclinical disease activity in tocilizumab-treated giant cell arteritis. *Rheumatology (Oxford) 57:1795-1801*
12. Reichenbach, S., Adler, S., Bonel, H., Cullmann, J.L., Kuchen, S., Bütikofer, L., Seitz, M., and Villiger, P.M. 2018. Magnetic resonance angiography in giant cell arteritis: results of a randomized controlled trial of tocilizumab in giant cell arteritis. *Rheumatology (Oxford) 57: 982-986.*
13. Maleszewski, J.J., Younge, B.R., Fritzlen, J.T., Hunder, G.G., Goronzy, J.J., Warrington, K.J., and Weyand, C.M. 2017. Clinical and pathological evolution of giant cell arteritis: a prospective study of follow-up temporal artery biopsies in 40 treated patients. *Mod. Pathol. 30:788-796..*
14. van der Geest, K.S.M., Sandovici, M., van Sleen, Y., Sanders, J.S., Bos, N.A., Abdulahad, W.H., Stegeman, C.A., Heeringa, P., Rutgers, A., Kallenberg, C.G.M., Boots, A.M.H., and Brouwer, E. 2018. What is the Current Evidence for Disease Subsets in Giant Cell Arteritis? *Arthritis Rheumatol. 70: 1366-1376*
15. Burja, B., Kuret, T., Sodin-Semrl, S., Lakota, K., Rotar, Z., Jese, R., Mrak-Poljsak, K., Zigon, P., Thallinger, G.G., Feichtinger, J., Cucnik, S., Tomsic, M., Praprotnik, S., and Hocevar, A. 2017. A concise review of significantly modified serological biomarkers in Giant cell arteritis, as detected by different methods. *Autoimmun. Rev. 17:188-194*
16. Weyand, C.M. and Goronzy, J.J. 2013. Immune mechanisms in medium and large-vessel vasculitis. *Nat. Rev. Rheumatol. 9: 731-740.*

17. van Sleen, Y., Wang, Q., van der Geest, K.S.M., Westra, J., Abdulahad, W.H., Heeringa, P., Boots, A.M.H., and Brouwer, E. 2017. Involvement of Monocyte Subsets in the Immunopathology of Giant Cell Arteritis. *Sci. Rep. 7: 6553-017-06826-4.*

18. Samson, M., Corbera-Bellalta, M., Audia, S., Planas-Rigol, E., Martin, L., Cid, M.C., and Bonnotte, B. 2017. Recent advances in our understanding of giant cell arteritis pathogenesis. *Autoimmun. Rev. 16: 833-844.*

19. Thaler, B., Hohensinner, P.J., Krychtiuk, K.A., Matzneller, P., Koller, L., Brekalo, M., Maurer, G., Huber, K., Zeitlinger, M., Jilma, B., Wojta, J., and Speidl, W.S. 2016. Differential in vivo activation of monocyte subsets during low-grade inflammation through experimental endotoxemia in humans. *Sci. Rep. 6: 30162.*

20. Westra J J. 2006-7. Differential influence of p38 mitogen activated protein kinase (MAPK) inhibition on acute phase protein synthesis in human hepatoma cell lines. *Ann. Rheum. Dis. 65: 929-35.*

21. O'Neill, L., Rooney, P., Molloy, D., Connolly, M., McCormick, J., McCarthy, G., Veale, D.J., Murphy, C.C., Fearon, U., and Molloy, E. 2015. Regulation of Inflammation and Angiogenesis in Giant Cell Arteritis by Acute-Phase Serum Amyloid A. *Arthritis Rheumatol. 67: 2447-2456.*

22. Foell, D., Hernandez-Rodriguez, J., Sanchez, M., Vogl, T., Cid, M.C., and Roth, J. 2004. Early recruitment of phagocytes contributes to the vascular inflammation of giant cell arteritis. *J. Pathol. 204: 311-316.*

23. Weaver, L.K., Hintz-Goldstein, K.A., Pioli, P.A., Wardwell, K., Qureshi, N., Vogel, S.N., and Guyre, P.M. 2006. Pivotal advance: activation of cell surface Toll-like receptors causes shedding of the hemoglobin scavenger receptor CD163. *J. Leukoc. Biol. 80: 26-35.*

24. Baeten, D., Boots, A.M., Steenbakkers, P.G., Elewaut, D., Bos, E., Verheijden, G.F., Berheijden, G., Miltenburg, A.M., Rijnders, A.W., Veys, E.M., and De Keyser, F. 2000. Human cartilage gp-39+,CD16+ monocytes in peripheral blood and synovium: correlation with joint destruction in rheumatoid arthritis. *Arthritis Rheumatol. 43: 1233-1243.*

25. Goodfellow, N., Morlet, J., Singh, S., Sabokbar, A., Hutchings, A., Sharma, V., Vaskova, J., Masters, S., Zarei, A., and Luqmani, R. 2017. Is vascular endothelial growth factor a useful biomarker in giant cell arteritis? *RMD Open 3: e000353.*

26. Moritz, F., Schniering, J., Distler, J.H.W., Gay, R.E., Gay, S., Distler, O., and Maurer, B. 2017. Tie2 as a novel key factor of microangiopathy in systemic sclerosis. *Arthritis Res. Ther. 19: 105-017-1304-2.*

27. Milam, K.E. and Parikh, S.M. 2015. The angiopoietin-Tie2 signaling axis in the vascular leakage of systemic inflammation. *Tissue Barriers 3: e957508.*

28. Dasgupta, B., Borg, F.A., Hassan, N., Alexander, L., Barraclough, K., Bourke, B., Fulcher, J., Hollywood, J., Hutchings, A., and James, P. 2010. BSR and BHPR guidelines for the management of giant cell arteritis. *Rheumatology (Oxford) 49: 1594-1597.*

29. Kaiser, M., Younge, B., Bjornsson, J., Goronzy, J.J., and Weyand, C.M. 1999. Formation of new vasa vasorum in vasculitis. Production of angiogenic cytokines by multinucleated giant cells. *Am. J. Pathol. 155: 765-774.*

30. Baldini, M., Maugeri, N., Ramirez, G.A., Giacomassi, C., Castiglioni, A., Prieto-Gonzalez, S., Corbera-Bellalta, M., Di Comite, G., Papa, I., Dell'antonio, G., Ammirati, E., Cuccovillo, I., Vecchio, V., Mantovani, A., Rovere-Querini, P., Sabbadini, M.G., Cid, M.C., and Manfredi, A.A. 2012. Selective up-regulation of the soluble pattern-recognition receptor pentraxin 3 and of vascular endothelial growth factor in giant cell arteritis: relevance for recent optic nerve ischemia. *Arthritis Rheumatol. 64: 854-865.*

31. Smets, P., Devauchelle-Pensec, V., Rouzaire, P.O., Pereira, B., Andre, M., and Soubrier, M. 2016. Vascular endothelial growth factor levels and rheumatic diseases of the elderly. *Arthritis Res. Ther. 18: 283-016-1184-x.*

32. Ziogas Apostolos C AC. 2012-2-15. VEGF directly suppresses activation of T cells from ovarian cancer patients and healthy individuals via VEGF receptor Type 2. *Int. J. Canc. 130: 857-64.*

33. Wen Zhenke Z. 2017-7-19. The microvascular niche instructs T cells in large vessel vasculitis via the VEGF-Jagged1-Notch pathway. *Sci. Transl. Med 9:*

34. Wang Liqun L. 2014. Functional characterization of S100A8 and S100A9 in altering monolayer

permeability of human umbilical endothelial cells. *PLoS ONE 9:*

35. Soulas, C., Conerly, C., Kim, W.K., Burdo, T.H., Alvarez, X., Lackner, A.A., and Williams, K.C. 2011. Recently infiltrating MAC387(+) monocytes/macrophages a third macrophage population involved in SIV and HIV encephalitic lesion formation. *Am. J. Pathol. 178: 2121-2135.*
36. Chupp Geoffrey L GL. 2007-11-15. A chitinase-like protein in the lung and circulation of patients with severe asthma. *N. Engl. J. Med. 357: 2016-27.*
37. Johansen, J.S., Jensen, B.V., Roslind, A., Nielsen, D., and Price, P.A. 2006. Serum YKL-40, a new prognostic biomarker in cancer patients? *Cancer Epidemiol. Biomarkers Prev. 15: 194-202.*
38. Johansen, J.S., Baslund, B., Garbarsch, C., Hansen, M., Stoltenberg, M., Lorenzen, I., and Price, P.A. 1999. YKL-40 in giant cells and macrophages from patients with giant cell arteritis. *Arthritis Rheumatol. 42: 2624-2630.*
39. Kunz, L.I., van't Wout, E.F., van Schadewijk, A., Postma, D.S., Kerstjens, H.A., Sterk, P.J., and Hiemstra, P.S. 2015. Regulation of YKL-40 expression by corticosteroids: effect on pro-inflammatory macrophages in vitro and its modulation in COPD in vivo. *Respir. Res. 16: 154-015-0314-3.*
40. van der Geest, K.S., Abdulahad, W.H., Rutgers, A., Horst, G., Bijzet, J., Arends, S., Roffel, M.P., Boots, A.M., and Brouwer, E. 2015. Serum markers associated with disease activity in giant cell arteritis and polymyalgia rheumatica. *Rheumatology (Oxford) 54: 1397-1402.*
41. Prieto-González Sergio S. 2012-7. Large vessel involvement in biopsy-proven giant cell arteritis: prospective study in 40 newly diagnosed patients using CT angiography. *Ann. Rheum. Dis. 71: 1170-6.*
42. Cid M C MC. 1998-1. Association between strong inflammatory response and low risk of developing visual loss and other cranial ischemic complications in giant cell (temporal) arteritis. *Arthritis Rheumatol. 41: 26-32.*
43. Hernandez-Rodriguez, J., Garcia-Martinez, A., Casademont, J., Filella, X., Esteban, M.J., Lopez-Soto, A., Fernandez-Sola, J., Urbano-Marquez, A., Grau, J.M., and Cid, M.C. 2002. A strong initial systemic inflammatory response is associated with higher corticosteroid requirements and longer duration of therapy in patients with giant-cell arteritis. *Arthritis Rheumatol. 47: 29-35.*
44. Hernández-Rodríguez José J. 2003-5-20. Elevated production of interleukin-6 is associated with a lower incidence of disease-related ischemic events in patients with giant-cell arteritis: angiogenic activity of interleukin-6 as a potential protective mechanism. *Circulation 107: 2428-34.*

Supplementary Table 1. Overview of patient samples, follow-up time, time to GC-free remission, and additional csDMARD use (at any moment throughout the disease course). At baseline, 3 months (± 4 weeks; N=30), 6 or 9 (± 10 weeks; N=5),12 months (± 10 weeks; N=29) and in treatment-free remission (N=8) follow-up samples were collected and are indicated with X. At 3, 6 or 9, and 12 months, disease status of patients is indicated by colours: green for patient in remission who would not relapse within 4 months; yellow for patient in remission who would relapse within 4 months; and lila for patient with active disease. Reasons for missing samples are indicated with a-e. **a**: patient visit delayed; **b**: patient not yet reached time point in follow-up; **c**: serum sample lost in work-up; **d**: patient was lost to follow-up due to death (unrelated to GCA); **e**: patient was lost to follow-up due to inability to visit the outpatient clinic.

Patient ID	Baseline Fig 1 Suppl. tab 3 Suppl. fig 2	3 months Fig 5A,C Suppl. fig 3A	6 or 9 months (Fig.5C) Fig 5C	12 months Fig 5B,C Suppl. fig 3B	Treatment-free remission Fig 5D Suppl. fig 4	Follow-up time (days)	Time to GC-free remission (days) Fig 4	DMARD added
GCA1	X	X		d		195	NA	
GCA2	X	X		a		2170	483	MTX
GCA4	X	X		X		2093	NA	
GCA5	X	X	X	X		2148	NA	MTX
GCA6	X	X	X	X	X	2053	1304	MTX
GCA7	X	X		X		1840	963	MTX
GCA8	X	X		X	X	1961	1425	
GCA9	X	X		X		431	NA	
GCA10	X	X		a		1784	874	MTX
GCA11	X	X		X		1816	NA	
GCA12	X	a		X	X	1830	392	
GCA13	X	X		X		1550	NA	
GCA14	X	X		e		92	NA	
GCA15	X	X	X	X	X	1582	601	MTX
GCA16	X	a		X		1449	871	MTX
GCA17	X	a		X	X	1158	464	
GCA18	X	X		X		1190	NA	MTX&LEF
GCA19	X	c		X		1067	749	
GCA20	X	X		X	X	1085	241	

Supplementary Table 1. (continued)

Patient ID	Baseline Fig 1 Suppl. tab 3 Suppl. fig 2	3 months Fig 5A,C Suppl. fig 3A	6 or 9 months (Fig.5C) Fig 5C	12 months Fig 5B,C Suppl. fig 3B	Treatment-free remission Fig 5D Suppl. fig 4	Follow-up time (days)	Time to GC-free remission (days) Fig 4	DMARD added
GCA21	X	X		X	X	1200	334	
GCA22	X	X		X	X	1176	647	
GCA23	X	X		X		1097	NA	
GCA24	X	X		X		934	NA	MTX
GCA26	X	X		X		914	NA	MTX
GCA27	X	X	X	X		909	NA	
GCA28	X	a		X		914	722	
GCA30	X	a		X		382	NA	
GCA31	X	X	X	X		594	554	MTX
GCA32	X	X		X		446	NA	MTX
GCA33	X	X		X		530	NA	MTX
GCA34	X	X		X		537	NA	MTX
GCA35	X	X		X		367	NA	
GCA36	X	X		b		403	NA	MTX&LEF
GCA37	X	X		b		374	NA	
GCA38	X	X		X		360	NA	MTX
GCA39	X	e		e		0	NA	
GCA40	X	X		b		157	NA	MTX
GCA41	X	b		b		63	NA	
GCA42	X	b		b		56	NA	
GCA43	X	b		b		25	NA	
GCA1031	X	b		b		8	NA	
TOTAL	**41**	**30**	**5**	**29**	**8**			**17**

ID = identification number; GC = glucocorticoid; DMARD = disease-modifying anti- rheumatic drug; NA = not applicable; MTX = methotrexate; LEF = leflunomide

Supplementary Table 2. Absolute values of serum markers in GCA, before and during treatment (baseline, and at three and twelve months after start of GC treatment) and in control groups. Values at each time point are expressed as median, 25th percentile, and 75th percentile.

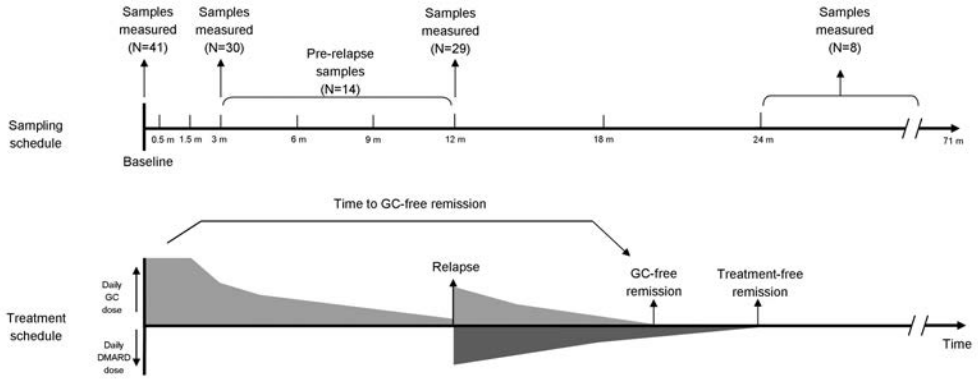
	Disease status/ Control group	Baseline	3 months	12 months
N	Remission	NA	24	20
	Active disease	41	6	9
	Healthy controls	33	NA	NA
	Infection controls	13	NA	NA
Calp ng/mL	Remission	NA	**3583** (2681-7166)	**3961** (2270-4899)
	Active disease	**5711** (3336-7680)	**4444** (4283-5557)	**6251** (2958-6974)
	Healthy controls	**2011** (1270-2718)	NA	NA
	Infection controls	**6030** (4715-9411)	NA	NA
YKL40 ng/ml	Remission	NA	**95** (65-171)	**103** (55-152)
	Active disease	**101** (59-148)	**72** (44-84)	**112** (81-123)
	Healthy controls	**52** (33-63)	NA	NA
	Infection controls	**106** (36-162)	NA	NA
sCD163 ng/mL	Remission	NA	**812** (579-1033)	**767** (481-1066)
	Active disease	**941** (607-1296)	**436** (305-786)	**733** (494-1286)
	Healthy controls	**603** (385-949)	NA	NA
	Infection controls	**782** (488-1021)	NA	NA
VEGF pg/mL	Remission	NA	**86** (60-161)	**81** (53-129)
	Active disease	**125** (71-269)	**61** (49-87)	**68** (58-115)
	Healthy controls	**75** (52-143)	NA	NA
	Infection controls	**162** (78-279)	NA	NA
Angpt-2 pg/ml	Remission	NA	**1583** (478-4437)	**1958** (1151-4372)
	Active disease	**3877** (2158-5610)	**1021** (693-1594)	**3086** (2390-6431)
	Healthy controls	**952** (616-1570)	NA	NA
	Infection controls	**4417** (2054-7190)	NA	NA
sTie2 ng/mL	Remission	NA	**10.5** (7.7-15.6)	**12.2** (8.0-14.8)
	Active disease	**14** (8.6-18.2)	**9.6** (8.4-13.7)	**11.4** (9.5-17.9)
	Healthy controls	**9.9** (7.3-12.4)	NA	NA
	Infection controls	**12.6** (11.6-17.7)	NA	NA
Angpt-1 ng/ml	Remission	NA	**65** (39-78)	**52** (45-64)
	Active disease	**54** (47-67)	**27** (24-46)	**91** (70-112)
	Healthy controls	**48** (41-60)	NA	NA
	Infection controls	**64** (52-80)	NA	NA
CRP mg/L	Remission	NA	**5** (3-7)	**5** (3-12)
	Active disease	**50** (21-90)	**2** (1-4)	**12** (6-20)
	Healthy controls	**3** (1-3)	NA	NA
	Infection controls	**71** (35-111)	NA	NA

Supplementary Table 2. (continued)

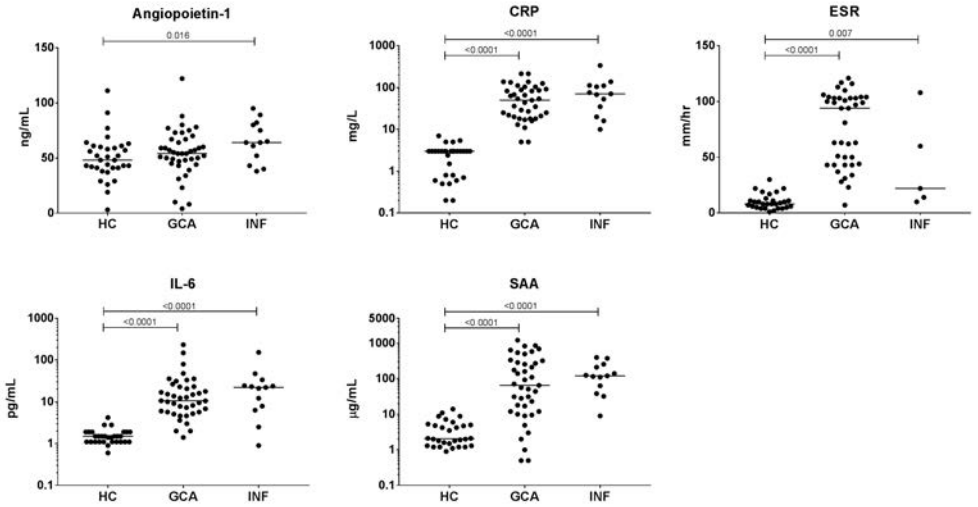
	Disease status/ Control group	Baseline	3 months	12 months
ESR mm/hr	Remission	NA	**21** (11-25)	**20** (13-31)
	Active disease	**94** (44-103)	**19** (12-22)	**41** (17-63)
	Healthy controls	**8** (5-11)	NA	NA
	Infection controls	**41** (10-110)	NA	NA
IL-6 pg/mL	Remission	NA	**2.4** (1.5-5.0)	**2.8** (1.7-3.6)
	Active disease	**10.6** (5.7-22.9)	**2.2** (1.9-2.9)	**3.7** (1.5-10.9)
	Healthy controls	**1.5** (1.1-1.9)	NA	NA
	Infection controls	**22.1** (7.9-24.3)	NA	NA
SAA µg/mL	Remission	NA	**22** (9.8-61)	**11** (5.5-40)
	Active disease	**65** (12-285)	**13** (8.8-145)	**26** (10-125)
	Healthy controls	**2.1** (1.4-5.0)	NA	NA
	Infection controls	**120** (63-210)	NA	NA

Supplementary Table 3. Biomarker levels in five CRP-low GCA patients at baseline compared to 90th percentile levels in HCs. Biomarker levels in GCA patients higher than 90th percentile levels in HCs are indicated in green, lower levels in red. In four out of five patients that presented with the lowest CRP levels, ESR and angiopoietin-2 levels were higher than in 90% of HCs.

	CRP (mg/L)	ESR (mm/hr)	IL-6 (pg/mL)	SAA (µg/mL)	sCD163 (ng/mL)	Calp (ng/mL)	YKL40 (ng/mL)	VEGF (pg/mL)	Angpt2 (pg/mL)	sTie2 (ng/mL)
HC 90%	5	20	2.0	8.8	1156	3944	91	212	2093	13.4
GCA42	5	7	1.9	3	335	1351	25	97	1740	6.8
GCA43	5	28	1.4	0.5	603	1621	31	163	4084	16.7
GCA39	11	23	5.7	2	282	1495	59	41	4437	16.9
GCA9	13	43	6.0	12	987	7405	153	139	2158	6.7
GCA27	16	50	5.2	31	882	7111	61	40	3941	10.7



Supplementary Figure 1. Schematic presentation of the prospective biomarker study in GCA. The upper timeline shows the time points of patients scheduled visits at the outpatient clinic. The lower timeline shows the treatment schedule. GC dose taper and increase after a relapse are indicated in blue. Start of DMARD after a relapse and taper indicated in red.



Supplementary Figure 2. The ESR and serum levels of angiopoietin-1, CRP, IL-6, and SAA in newly diagnosed GCA patients (N=39 for CRP and ESR and N=41 for the other markers) compared to infection (INF; N=13) and healthy controls (HC; N=33). CRP, ESR, IL-6 and SAA were significantly increased in newly diagnosed GCA patients compared to HCs. N=5 for ESR in INF. The horizontal line represents the median. Significance by Mann-Whitney U test is indicated in the graphs, as the Kruskal Wallis test was significant for all markers.

	CRP	ESR	IL-6	SAA	sCD163	Calp	YKL40	VEGF	Angpt1	Angpt2
ESR	0.272									
IL-6	0.573**	0.306								
SAA	0.619**	-0.044	0.405*							
sCD163	-0.221	0.031	-0.145	-0.155						
Calp	0.239	0.115	0.128	0.203	0.095					
YKL40	0.572**	0.614**	0.622**	0.357	-0.017	0.305				
VEGF	-0.002	0.006	-0.060	-0.050	0.130	0.290	0.124			
Angpt1	0.080	-0.191	0.173	0.055	-0.027	-0.215	-0.069	0.029		
Angpt2	-0.084	0.124	0.108	-0.074	-0.159	-0.423	0.041	-0.177	0.530**	
sTie2	-0.041	-0.001	0.041	-0.159	0.104	-0.325	-0.174	0.326	0.427*	0.374*

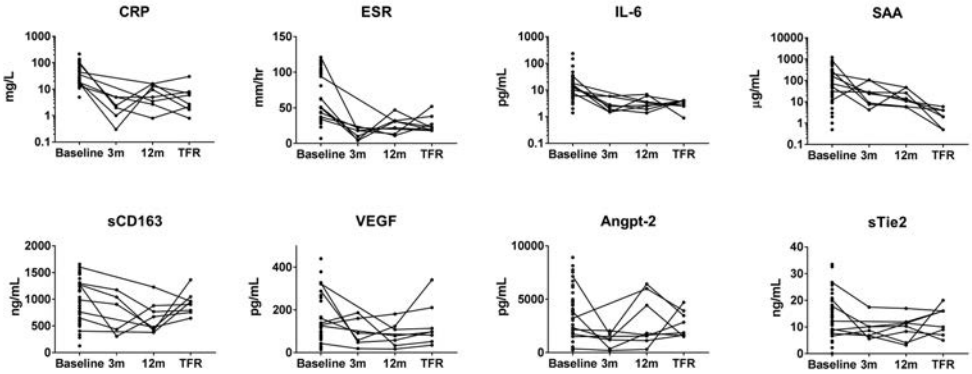
Legend:

Spearman R =

- >0.75
- >0.5
- >0.25
- < -0.25
- < -0.5

	CRP	ESR	IL-6	SAA	sCD163	Calp	YKL40	VEGF	Angpt1	Angpt2
ESR	0.721**									
IL-6	0.478*	0.492*								
SAA	0.703**	0.801**	0.495**							
sCD163	0.105	0.005	0.120	0.100						
Calp	0.434*	0.366	0.051	0.329	-0.300					
YKL40	0.481*	0.505*	0.439*	0.342	0.140	0.272				
VEGF	-0.128	-0.403*	-0.034	-0.276	0.108	-0.073	-0.147			
Angpt1	-0.152	-0.204	-0.140	-0.140	0.180	0.046	-0.085	0.253		
Angpt2	0.110	0.166	0.073	0.065	0.136	-0.032	0.325	0.063	0.318	
sTie2	0.028	-0.054	0.050	-0.081	0.275	-0.200	-0.024	0.469*	0.235	0.486**

Supplementary Figure 3. Correlations between markers in GCA patients during treatment. Depicted are Spearman's correlation coefficients of all markers at three (A) and twelve (B) months after start of GC treatment. Colours indicate strength of correlation. Statistical significance is indicated as * ($p<0.05$) and ** ($p<0.01$).



Supplementary Figure 4. ESR and levels of CRP, IL-6, SAA, sCD163, VEGF, angiopoietin-2 and sTie2 over time in GCA patients in treatment-free remission (TFR), which is defined as no signs and symptoms, no GCs or other DMARDs and no return of active disease for a period of at least 6 months follow-up (N=8).

van Sleen Y,

Boots AMH,

Abdulahad WH,

Bijzet J,

Sandovici M,

van der Geest KSM #,

Brouwer E #

#: Shared last author

EIGHT

High Angiopoietin-2 Levels Associate with Arterial Inflammation and Long-Term Glucocorticoid Requirement in Polymyalgia Rheumatica

Rheumatology (Oxford) 2020 59;1: 176-184

ABSTRACT

PMR frequently co-occurs with GCA. So far, a simple biomarker for detecting concomitant arterial inflammation in PMR patients is lacking. Furthermore, biomarkers predicting disease course in PMR are awaited. We here investigated the diagnostic and prognostic value of acute-phase markers (ESR, CRP, IL-6, serum amyloid A) and angiogenesis markers (VEGF, soluble Tie2, angiopoietin-1, angiopoietin-2) in isolated PMR and PMR/GCA overlap patients.

We prospectively included 39 treatment-naive PMR patients, of whom 10 patients also showed evidence of large vessel GCA by PET-CT. Age-matched healthy controls (n=32) and infection controls (n=13) were included for comparison. Serum marker levels were measured by ELISA or Luminex. Receiver operating characteristic (ROC) and Kaplan Meier analyses were used to asses diagnostic and prognostic accuracy, respectively.

All acute-phase and angiogenesis markers, except angiopoietin-1, were higher in isolated PMR patients than in healthy controls. Angiopoietin-2, ESR and soluble Tie-2 were significantly higher in patients with PMR/GCA overlap than in isolated PMR patients. Angiopoietin-2, but not soluble Tie2, outperformed ESR and CRP in discriminating patients with and without overlapping GCA (area under the curve: 0.90, sensitivity 100%, specificity 76%). Moreover, high angiopoietin-2 levels were associated with long-term glucocorticoid requirement.

Assessment of angiopoietin-2 at baseline may assist diagnosis of concomitant vasculitis in PMR. Moreover, high levels of angiopoietin-2 were associated with an unfavorable disease course in isolated PMR patients. These findings imply that angiopoietin-2 is an interesting diagnostic and prognostic biomarker in PMR.

INTRODUCTION

PMR is the most common inflammatory rheumatic disease in the elderly (1). PMR is characterized by (peri-)articular inflammation which is typically accompanied by a strong acute-phase response(1). Symptoms of PMR include pain and morning stiffness of shoulders, proximal limbs, neck and hip girdle. The main treatment strategy of PMR is long-term glucocorticoids (GCs), which are associated with severe side-effects such as diabetes and infections (2,3).

A key question for every physician dealing with a PMR patient is whether or not the patient also has inflammation of medium and large arteries (i.e. GCA)(4,5). The frequency of GCA among PMR patients has been reported to range from 16 to 21% (1). Arterial inflammation in PMR is likely underdiagnosed since symptoms of GCA can be non-specific (4,6). As GCA patients require higher daily GC dosages than PMR patients in order to prevent ischemic complications such as vision loss, timely diagnosis of GCA is essential(6). Diagnostic workup for GCA includes imaging and/or a temporal artery biopsy (TAB). These techniques are costly and/or not readily available to every physician. To identify GCA in patients presenting with PMR, sensitive biomarkers reflecting arterial inflammation are highly needed.

In addition, better prognostic biomarkers at baseline are awaited in PMR. The best-studied biomarker in this regard is ESR, which is associated with a worse disease course (i.e. longer GC requirement)(7). However, multiple studies have failed to confirm this finding (8,9). Another report indicated that an elevated plasma viscosity at baseline is associated with a lower probability of stopping GCs within five years (10).

Little is known about the pathogenesis of PMR. In the blood, PMR shows overlap with GCA, as both diseases are characterized by a strong IL-6-dependent acute-phase response as well as altered leukocyte subset counts and functionality (11-13). Our prior work has shown that markers of angiogenesis, including VEGF, are elevated in serum of GCA patients (14). Angiogenesis is considered an important process in amplifying arterial inflammation. Interestingly, PMR patients may also show elevated levels of VEGF (15,16), whereas little is known about other angiogenesis markers in PMR.

We hypothesized that markers of angiogenesis may mirror arterial inflammation in PMR patients with concomitant GCA, and that their diagnostic accuracy for concomitant GCA outperforms the acute-phase response markers. In addition, we investigated the prognostic value of acute-phase markers and angiogenic markers in PMR. To that end we performed a comprehensive analysis of acute-phase markers (CRP, ESR, serum amyloid A (SAA)), IL-6, and angiogenic markers (VEGF, soluble Tie2 (sTie2), angiopoietin-1, angiopoietin-2) in our prospective cohort of treatment-naive isolated PMR patients and PMR/GCA overlap patients.

PATIENTS AND METHODS

Patient characteristics

Twenty-nine newly-diagnosed and treatment-naive (GCs or DMARDs) PMR patients participated in this study. Diagnosis of PMR was based on clinical signs and symptoms, acute-phase markers and imaging by 18F-fluorodeoxyglucose PET-CT. Five isolated PMR patients did not fulfil the Chuang

criteria (17) due to a low ESR. In these five cases, patients had elevated CRP (>10 mg/L) and/or were diagnosed based on imaging. In 26 isolated PMR patients no evidence of GCA was found, i.e. by TAB (n=6), vascular ultrasound (n=8) and/or PET-CT scan (n=24, see Table 1). In the other three isolated PMR patients, no additional testing for GCA was performed due to lack of symptoms. In addition, we included ten newly-diagnosed, treatment-naive PMR patients who all had a positive PET-CT for GCA. Thirty-three age-and sex-matched healthy controls and 13 age-matched infection controls were included. Volunteers in both control groups were excluded in case of past and current morbidities and immunomodulatory drug use. Hospitalized infection controls all had either pneumonia or urinary tract infection. Patients and controls started participation (as a consecutive series) in our cohort between 2010 and 2018 and were all seen by a rheumatologist at the University Medical Center Groningen and METc2012/375).

Treatment

PMR patients were initially treated with 15 mg prednisolone daily (median, range 15-30), whereas PMR/GCA overlap patients started with 40-60 mg prednisolone daily. When remission was achieved, GCs were tapered in accordance with the British Society for Rheumatology guidelines (18,19).In case of relapse, GC dose was increased and/or a conventional synthetic DMARD was added. Relapse was defined as return of disease-specific clinical signs and symptoms. Upon remission, GCs were tapered until GC-free remission was achieved. GC-free remission was defined as: an absence of signs and symptoms, no GC use, and no return of active disease within at least 6 months of follow-up.

Serum marker measurements

Blood samples were drawn at the Rheumatology and Clinical Immunology outpatient clinic of the University Medical Center Groningen, and were stored at -20°C. CRP and ESR were measured in the context of standard medical care. Levels of serum IL-6 (standard curve range 4.8 - 1154; sensitivity 1.7 pg/ml), VEGF (0.55 - 2250; 2.1 pg/ml), sTie2 (614 - 149166; 211 pg/ml), angiopoietin-1 (114 - 27610; 9.43 pg/ml) and angiopoietin-2 (90.5 - 22000; 17.1 pg/ml) were determined with Human premix Magnetic Luminex screening assay kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions and read on a Luminex Magpix instrument (Luminex, Austin, TX, USA). Data analysis was performed with xPONENT 4.2 software (Luminex). Levels of SAA (standard curve range 1.7 - 219; detection level 1.6 ng/ ml) were measured by in house ELISA (20). Clinical information was blinded to the performers of the measurements.

Statistics

Data were analyzed by non-parametric testing. Differences in serum marker levels between study populations were tested by Kruskal Wallis and Mann Whitney U tests. Spearman's rank correlation coefficient was used to assess the strength of correlations between markers. To assess which marker independently associated with vasculitis in PMR patients, multiple regression analysis was performed. ROC analysis with area under the curve (AUC) was used to evaluate the markers' discriminatory performance. To identify optimal cut-off points, the maximum of the sum of

sensitivity and specificity was assessed, according to the Youden index. To compare time to GC-free remission, Kaplan Meier analysis and log rank tests were used. Analyses were performed with IBM SPSS 23 and GraphPad Prism 7.02 software.

RESULTS

Baseline characteristics of patient groups

Baseline characteristics of patients with isolated PMR, PMR/GCA overlap patients, healthy controls and infection controls are displayed in Table 1. Age and sex were not significantly different between isolated PMR patients and the other groups. At baseline, significantly more amaurosis fugax (p=0.013) and weight loss (p=0.007) was found in PMR/GCA overlap patients compared to isolated PMR patients. Isolated PMR and PMR/GCA overlap patients were followed for a median of 46 months (range 0-76) and 34 months (3-69), respectively. Two patients with isolated PMR developed GCA later in the disease course.

Elevated serum markers in newly-diagnosed, treatment-naive PMR patients

The ESR and levels of CRP, SAA, IL-6, VEGF, sTie2 and angiopoietin-2 were significantly higher in isolated PMR patients than in healthy controls (Table 1). Similar levels of these markers were found in infection controls, except for angiopoietin-2 which was significantly lower in isolated PMR (p=0.018). Angiopoietin-2 correlated moderately with the ESR in PMR patients (rho= 0.49, p<0.01), but negatively with VEGF levels (rho= -0.37, p<0.05; supplementary Figure 1). Serum IL-6 levels correlated strongly with CRP and SAA, but not with the ESR.

Angiopoietin-2 outperforms CRP and ESR in identifying patients with PMR/GCA overlap

The ESR and serum levels of angiopoietin-2 and sTie2 were lower in isolated PMR patients than in patients with PMR/GCA overlap (Table 1). Multiple logistic regression confirmed that angiopoietin-2, but not ESR and sTie2, was an independent predictor for presence of overlapping GCA in PMR patients (supplementary Table 1).

Next, we further assessed the diagnostic accuracy of these markers for concomitant vasculitis in PMR. ROC analyses (Figure 1) showed poor discrimination (AUC<0.80) between isolated PMR and PMR/GCA overlap patients for the acute-phase markers and for VEGF, angiopoietin-1 and sTie2. In contrast, angiopoietin-2 discriminated well between these patient groups, as evidenced by an AUC of 0.90, sensitivity of 100% and specificity of 76%. The angiopoietin-2/angiopoietin-1 ratio also discriminated well but did not further improve accuracy (AUC 0.88).

High baseline angiopoietin-2 predicts an unfavorable disease course in PMR patients

We determined time to GC-free remission in isolated PMR patients as a reflection of a favorable or unfavorable disease course. First, we determined optimal prognostic cut-off values for each marker based on the number of patients in GC-free remission at 24 months after start of treatment

Table 1. Baseline characteristics of newly diagnosed, treatment-naive patients with isolated PMR and PMR/GCA overlap. Differences in sex and symptoms at baseline between the groups were tested by the Fisher's exact test. Differences in age and biomarker levels were tested with the Mann Whitney U test. [a] : all isolated PMR patients with cranial symptoms suggestive of GCA were PET-CT negative for GCA and had either a negative ultrasound or negative biopsy.

	Healthy controls	Isolated PMR	PMR/GCA overlap	Infection controls	p-value isolated PMR vs healthy controls	p-value isolated PMR vs PMR/GCA overlap	p-value isolated PMR vs infection controls
N	33	29	10	13	-	-	-
Age in years; mean (SEM)	68 (66-70)	72 (70-74)	70 (67-73)	73 (69-77)	NS	NS	NS
Females (%)	22 (67)	18 (62)	8 (80)	4 (31)	NS	NS	NS
PET-CT positive for PMR (%):	NA	24 (83)	10 (100)	NA	-	-	-
PET-CT for GCA: Positive / Negative / Not done	NA	0 / 24 / 5	10 / 0 / 0	NA	-	-	-
TAB: Positive / Negative / Not done	NA	0 / 6 / 23	1 / 6 / 3	NA	-	-	-
Ultrasound for GCA: Positive / Negative / Not done	NA	0 / 8 / 21	2 / 4 / 4	NA	-	-	-
Follow-up time in months; median (range)	NA	46 (0-76)	34 (3-69)	NA	-	-	-
Symptoms at baseline (%)							
New headache	NA	5 (17) [a]	4 (40)	NA	-	NS	-
Jaw claudication	NA	3 (10) [a]	2 (20)	NA	-	NS	-
Temporal artery abnormal	NA	1 (3) [a]	0 (0)	NA	-	NS	-
Amaurosis fugax	NA	0 (0)	3 (30)	NA	-	0.013	-
Vision loss	NA	0 (0)	1 (10)	NA	-	NS	-
Fever (>38°C)	NA	6 (21)	3 (30)	NA	-	NS	-
Weight loss (>2 kg)	NA	15 (51)	10 (100)	NA	-	0.007	-
Malaise	NA	26 (90)	7 (70)	NA	-	NS	-
Night sweats	NA	11 (38)	5 (50)	NA	-	NS	-

Table 1. (continued)

	Healthy controls	Isolated PMR	PMR/GCA overlap	Infection controls	p-value isolated PMR vs healthy controls	p-value isolated PMR vs PMR/GCA overlap	p-value isolated PMR vs infection controls
Biomarker levels; median (range)							
CRP mg/L	**3** (0.2-7)	**42** (3.2-186)	**50** (25-215)	**71** (10-339)	<0.0001	NS	NS
ESR mm/hr	**8** (1-30)	**57** (8-109)	**94** (43-117)	NA	<0.0001	0.014	-
SAA µg/mL	**2.1** (0.9-14)	**74** (3.1-515)	**102** (1.0-540)	**120** (9.0-395)	<0.0001	NS	NS
IL-6 pg/mL	**1.5** (0.6-4.1)	**19.8** (2.0-117)	**15.1** (2.0-233)	**22.1** (0.9-152)	<0.0001	NS	NS
VEGF pg/mL	**75** (24-606)	**190** (47-536)	**100** (14-548)	**161** (35-345)	0.0001	NS	NS
sTie-2 ng/mL	**9.88** (3.87-14.4)	**12.1** (2.94-30.8)	**18.4** (4.31-32.6)	**12.6** (8.50-23.1)	0.016	0.026	NS
Angiopoietin-1 ng/mL	**48** (3.5-111)	**48** (29-87)	**52** (4-88)	**64** (39-96)	NS	NS	NS
Angiopoietin-2 pg/mL	**952** (288-6411)	**1848** (535-8350)	**5222** (3220-16622)	**4417** (775-14404)	<0.0001	<0.0001	0.018
Angpt-2/angpt-1 ratio	**0.017** (0.005-0.52)	**0.038** (0.010-0.29)	**0.13** (0.054-0.89)	**0.055** (0.015-0.24)	0.0025	0.0002	NS

NS: not significant, SAA: serum amyloid A, sTie2: soluble Tie2.

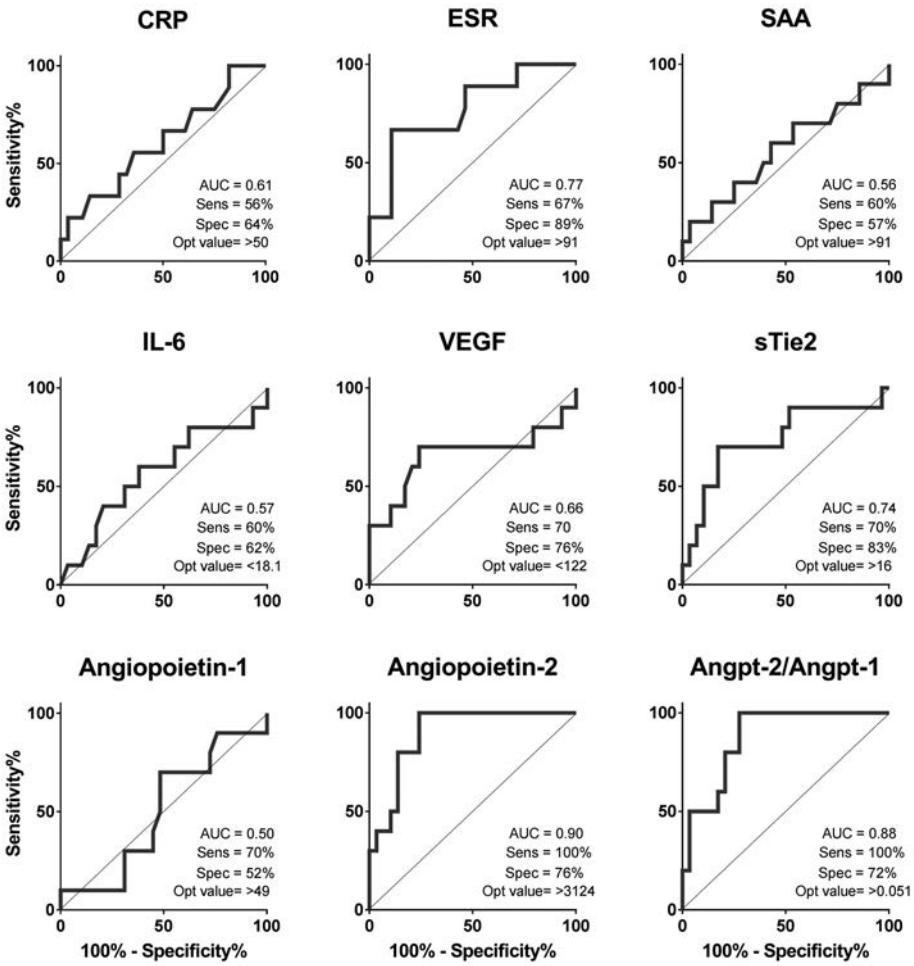


Figure 1. High angiopoietin-2 levels discriminate between PMR/GCA overlap patients and isolated PMR patients. ROC curves reflect the ability of each acute-phase marker and angiogenesis marker to detect arterial inflammation in PMR patients. The optimal sensitivity, specificity and cut-off value are identified according to the Youden index. AUC, sensitivity, specificity and optimal cut-off value for each marker are depicted in the graph. ROC: receiver operating characteristic, AUC: area under the curve, sens: sensitivity, spec: specificity, opt: optimal, SAA: serum-amyloid A, sTie2: soluble Tie2.

(Table 2). Baseline angiopoietin-2 levels (p=0.0045) and angiopoietin-2/angiopoietin-1 ratio (p=0.013) were higher in patients who were still on GC treatment at 24 months than in patients in GC-free remission at that time point. Next, we used the optimal cut-off values to assess differences in a Kaplan-Meier graph throughout the whole disease course (Figure 2). High baseline levels of angiopoietin-2 (p=0.0010), ESR (p=0.041) and SAA (p=0.041), or low levels of VEGF (p=0.031), significantly predicted a long-term GC requirement. The angiopoietin-2/angiopoietin-1 ratio performed even better than angiopoietin-2 levels alone: p< 0.0001.

Table 2. Baseline biomarker levels of patients in GC-free remission or on GC treatment at 24 months. At 24 months after start of treatment (n=19), ten isolated PMR patients had achieved GC-free remission and nine patients with PMR-only patients were still on GC treatment. Displayed are the median biomarker values at baseline (before start of treatment) in patients that were in GC-free remission (n=10) and in patients that were still on GC-treatment at 24 months after start of treatment (n=9). Optimal cut-off values of the ROC curves are calculated according to the Youden index. AUC values > 0.8 and p-values < 0.05 are indicated in bold.

Baseline biomarker	GC-free remission	On GC treatment	Cut-off value	AUC	p-value
CRP (mg/L)	48	49	>49	0.54	0.81
ESR (mm/hr)	50	63	>74	0.63	0.35
SAA (µg/mL)	97	120	>108	0.60	0.50
IL-6 (pg/mL)	21	31	>29	0.62	0.40
VEGF (pg/mL)	231	141	<149	0.69	0.18
sTie2 (ng/mL)	12	14	>19	0.69	0.18
Angiopoietin-1 (ng/mL)	55	48	>71	0.53	0.84
Angiopoietin-2 (pg/mL)	1177	2637	>2134	**0.87**	**0.0045**
Angpt-2/angpt-1 ratio	0.029	0.048	>0.038	**0.83**	**0.013**

8

We then compared time to GC-free remission in patients with isolated PMR and patients with PMR/GCA overlap (supplementary Figure 2). Patients with isolated PMR that had high angiopoietin-2 levels at baseline and PMR/GCA overlap patients had a comparable disease course as assessed by the time to GC-free remission. In contrast, angiopoietin-2^{low} patients with isolated PMR had a shorter time to GC-free remission than patients with PMR/GCA overlap (p=0.017).

DISCUSSION

Estimating the probability of concomitant vasculitis in PMR patients is challenging (4,6). In addition, good prognostic markers are lacking (7). Here we show that angiopoietin-2, a marker of angiogenesis relevant to vascular inflammation, helps to identify PMR patients with concomitant GCA. Furthermore, high levels of angiopoietin-2 at diagnosis identified PMR patients with an unfavourable long-term disease course. In Figure 3 we propose the possible utility of angiopoietin-2 as a diagnostic and prognostic biomarker in a flow chart. In both instances, angiopoietin-2 clearly outperformed classical biomarkers CRP and ESR. To the best of our knowledge, this is the first study investigating angiogenesis markers angiopoietin-1, angiopoietin-2 and sTie2 in PMR patients.

This study has identified angiopoietin-2 as the most robust marker of arterial inflammation in PMR patients. In the majority of our PMR patients, concomitant vasculitis could be excluded based on low levels of angiopoietin-2. The pro-angiogenic sTie2 also distinguished isolated PMR patients from PMR/GCA overlap patients, albeit with lesser accuracy. Previously, the ESR was found to be higher in patients with PMR/GCA overlap compared to isolated PMR (21). This was confirmed in our study, whereas CRP is not different between the two disease populations. One clinical study indicated that new headache, followed by age and abnormal TAB were the best predictors of arterial inflammation in PMR patients (22). In accordance with this study, we observed that only overlapping

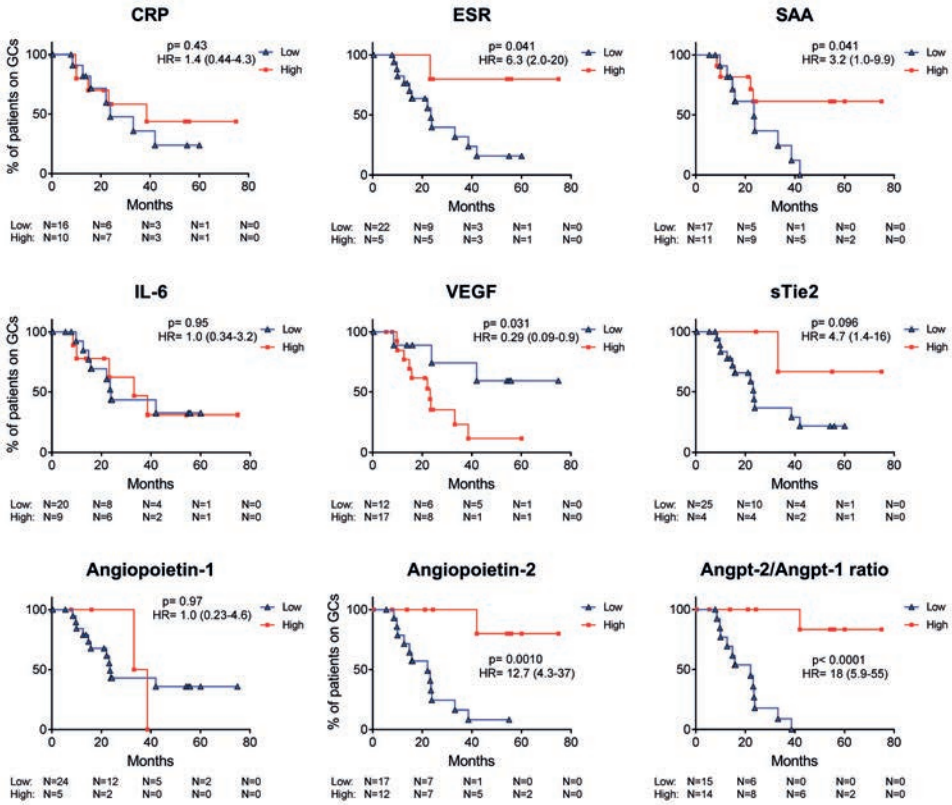


Figure 2. Long-term GC requirement is best predicted by baseline angiopoietin-2/angiopoietin-1 ratio. Baseline biomarker levels (or ratio) of PMR patients were split into low or high levels (based on the optimal cut-off value at 24 months after start of treatment) and were plotted in a Kaplan-Meier curve against time to GC-free remission. p-value and hazard ratio (HR; including 95% confidence interval) of the log- rank test are depicted in the graphs. GC: glucocorticoid, SAA: serum-amyloid A, sTie2: soluble Tie2.

patients have amaurosis fugax. Moreover, we observed that all PMR/GCA overlap patients suffered from weight loss, while this symptom was noted in only half of the isolated PMR patients.

Besides aiding detection of overlapping GCA, baseline angiopoietin-2 levels may also have prognostic utility. The time to GC-free remission was significantly longer in patients with high baseline levels of angiopoietin-2. The angiopoietin-2/angiopoietin-1 ratio, commonly used to indicate a pro-angiogenic shift (23), performed even better than angiopoietin-2 levels alone. Difficult-to-treat patients require long-term GC treatment as tapering of GCs leads to return of signs and symptoms in these patients. Long-term GC requirement is detrimental for these patients, as this is associated with serious side-effects such as diabetes and infections (2). Therefore, patients with high baseline angiopoietin-2 levels could possibly benefit from starting with a GC-sparing DMARD upon diagnosis. In a prior study, PMR patients with a typical 'extracapsular' pattern of inflammation on a MRI scan, were more likely to require GC treatment for >1 year (24). Possibly, this subset of patients overlaps with our angpiopoietin-2^{high} subset of isolated PMR patients.

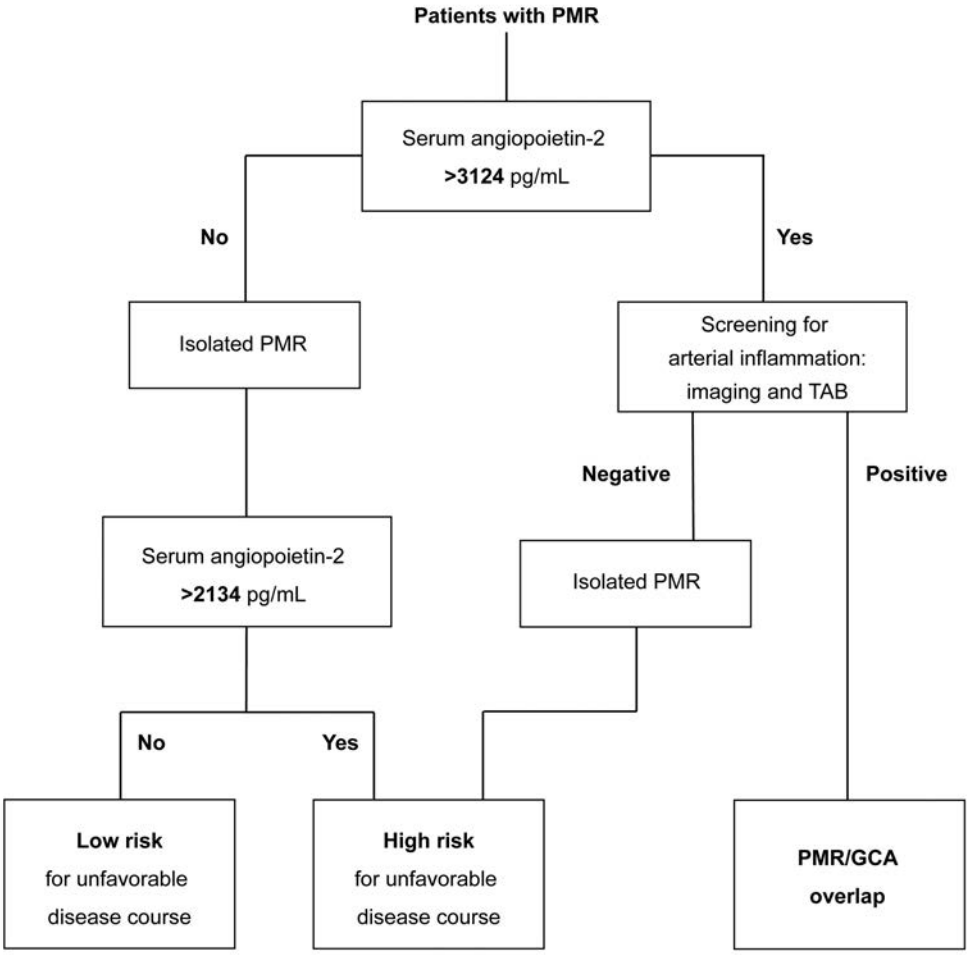


Figure 3. Proposed flow chart to assess the risk for concomitant GCA or unfavorable disease course in PMR patients. This flow chart represents a proposed algorithm based on observations within our cohort. In our cohort, treatment-naive patients presenting with PMR are at risk for overlapping GCA if serum angiopoietin-2 levels are higher than 3124 pg/mL. In absence of vasculitis, patients with serum angiopoietin levels higher than 2134 pg/mL have a high risk for an unfavorable disease course (i.e. long-term GC requirement).

The high levels of angiopoietin-2 in both patients with PMR/GCA overlap and patients with isolated PMR requiring long-term GCs could suggest the presence of vasculitis in these angiopoietin-2^{high} PMR patients. Presence of inflammation of large systemic arteries was precluded by FDG-PET/CT in all patients with isolated PMR and high angiopoietin-2 levels. In case of cranial symptoms, concomitant inflammation of cranial arteries was further excluded by ultrasound and/or TAB. Moreover, we observed no changes in the clinical diagnosis during the first 6 months after diagnosis. Hence, we are confident that occult vasculitis did not substantially affect our findings regarding patients with isolated PMR and high angiopoietin-2 levels. Indeed, three isolated PMR patients with low angiopoietin-2 levels had no further examination by imaging or TAB. However,

the presence of concomitant vasculitis in these patients would have ameliorated rather than augmented the prognostic differences that we observed between patients with low and high angiopoietin-2 levels. Thus, although we cannot fully exclude the possibility of undetected vasculitis in some of our isolated PMR patients, it is unlikely that such misclassification heavily influenced our findings.

Interestingly, we observed that high VEGF levels at baseline were protective against long-term GC requirement. This was also observed in GCA patients, and may thus suggest a similar protective mechanism in PMR (14). More studies are needed to elucidate why one pro-angiogenic marker (VEGF) appears to be protective against long-term GC requirement whilst another pro-angiogenic marker (angiopoietin-2) shows the opposite effect. Moreover, we observed a moderate negative correlation between angiopoietin-2 and VEGF levels at baseline. Also high SAA and ESR levels at baseline predicted a long-term GC requirement, although statistical significance levels and hazard ratios were less convincing for these markers.

Overall, serum levels of angiopoietin-2, sTie-2 and VEGF were elevated in PMR patients when compared to healthy controls. Indeed, earlier studies also reported higher VEGF serum levels in PMR patients (15,16). Angiopoietin-2 instigates angiogenesis by competing with the homeostatic angiopoietin-1 for signalling by Tie2 (25). During hypoxia and inflammation, angiopoietin-2 is released from Weibel-Palade bodies, aiding the loss of vessel integrity that leads to small vessel sprouting if VEGF is present. VEGF has been documented in synovia of PMR patients (16) but angiopoietin-2 expression has not been assessed in PMR tissues so far. Importantly, elevated angiogenic signaling is not specific for GCA and PMR, as our infection controls show higher levels of angiogenesis markers as well. Thus, to properly interpret the diagnostic and prognostic value of these markers, the presence of infections needs to be excluded in PMR patients.

This serum marker study has strengths and limitations. It is performed in our cohort that prospectively enrolled treatment-naive GCA and PMR patients. Patients in this cohort have gone through an intense diagnostic work-up, which provided a confident diagnosis. Specifically, overlapping vasculitis was excluded in PMR patients by a combination of clinical signs and symptoms, imaging and biopsies. Importantly, this diagnosis did not change for at least six months during follow-up. Another strength is the longstanding protocolized follow-up, which allowed us to determine the time to GC-free remission in most patients. Limitations are the limited number of patients that are included in the PMR/GCA overlap group. This is because only a subset of PMR patients have concomitant GCA (1). Therefore, validation of our findings in a prognostic study is necessary before implementing these biomarkers in daily clinical practice.

In conclusion, this study provides evidence for the use of angiopoietin-2 as a diagnostic marker for concomitant vasculitis in PMR patients. When confirmed, PMR patients presenting with high angiopoietin-2 levels should be more intensively screened for the presence of arteritis. In addition, assessment of baseline angiopoietin-2 levels may help to identify a subset of PMR patients that would qualify for intensive treatment and disease-monitoring.

REFERENCES

1. Salvarani, C., Cantini, F., and Hunder, G.G. 2008. Polymyalgia rheumatica and giant-cell arteritis. Lancet 372: 234-245.
2. Broder, M.S., Sarsour, K., Chang, E., Collinson, N., Tuckwell, K., Napalkov, P., and Klearman, M. 2016. Corticosteroid-related adverse events in patients with giant cell arteritis: A claims-based analysis. Semin. Arthritis Rheum. 46: 246-252.
3. Buttgereit F., Matteson E.L., Dejaco C., Dasgupta B. 2018. Prevention of glucocorticoid morbidity in giant cell arteritis. Rheumatology (Oxford) 57(suppl_2): ii11-ii21.
4. Dejaco, C., Duftner, C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2016. The spectrum of giant cell arteritis and polymyalgia rheumatica: revisiting the concept of the disease. Rheumatology (Oxford) 56(4):506-515.
5. van der Geest, K.S.M., Sandovici, M., van Sleen, Y., Sanders, J.S., Bos, N.A., Abdulahad, W.H., Stegeman, C.A., Heeringa, P., Rutgers, A., Kallenberg, C.G.M., Boots, A.M.H., and Brouwer, E. 2018. What is the Current Evidence for Disease Subsets in Giant Cell Arteritis? Arthritis Rheumatol. 70: 1366-1376
6. J. Narváez, P. Estrada, L. López-Vives, M. Ricse, A. Zacarías, S. Heredia, C. Gómez-Vaquero, J.M. Nolla. Prevalence of ischemic complications in patients with giant cell arteritis presenting with apparently isolated polymyalgia rheumatica. Semin Arthritis Rheum. 45(3):328-33.
7. Dejaco, C., Singh, Y.P., Perel, P., Hutchings, A., Camellino, D., Mackie, S., Matteson, E.L., and Dasgupta, B. 2015. Current evidence for therapeutic interventions and prognostic factors in polymyalgia rheumatica: a systematic literature review informing the 2015 European League Against Rheumatism/American College of Rheumatology recommendations for the management of polymyalgia rheumatica. Ann. Rheum. Dis. 74: 1808-1817.
8. Kanik, K.S., Bridgefor, P.H., Germain, B.F., Espinosa, L.R., Lowenstein, M., Valeriano-Marcet, J., Seleznick, M., and Vases, F.B. 1997. Polymyalgia rheumatica with a low erythrocyte sedimentation rate: comparison of 10 cases with 10 cases with high erythrocyte sedimentation rate. J. Clin. Rheumatol. 3: 319-323.
9. Larrosa, M., Gratacos, J., and Sala, M. 2000. Polymyalgia rheumatica with low erythrocyte sedimentation rate at diagnosis. J. Rheumatol. 27: 1815-1816.
10. Mackie, S.L., Hensor, E.M., Haugeberg, G., Bhakta, B., and Pease, C.T. 2010. Can the prognosis of polymyalgia rheumatica be predicted at disease onset? Results from a 5-year prospective study. Rheumatology (Oxford) 49: 716-722.
11. van der Geest, K.S., Abdulahad, W.H., Rutgers, A., Horst, G., Bijzet, J., Arends, S., Roffel, M.P., Boots, A.M., and Brouwer, E. 2015. Serum markers associated with disease activity in giant cell arteritis and polymyalgia rheumatica. Rheumatology (Oxford) 54: 1397-1402.
12. van Sleen, Y., Wang, Q., van der Geest, K.S.M., Westra, J., Abdulahad, W.H., Heeringa, P., Boots, A.M.H., and Brouwer, E. 2017. Involvement of Monocyte Subsets in the Immunopathology of Giant Cell Arteritis. Sci. Rep. 7: 6553.
13. Macchioni, P., Boiardi, L., Salvarani, C., Rossi, F., Casadei-Maldini, M., Mancini, R., Beltrandi, E., and Portioli, I. 1993. Lymphocyte subpopulations analysis in peripheral blood in polymyalgia rheumatica/giant cell arteritis. Br. J. Rheumatol. 32: 666-670.
14. van Sleen, Y., Sandovici, M., Abdulahad, W., Bijzet, J., van der Geest, K., Boots, A.M., and van der Geest, Kornelis SM. 2019. Markers of Angiogenesis and Macrophage Products for Predicting Disease Course and Monitoring Vascular Inflammation in Giant Cell Arteritis. Rheumatology (Oxford) 58: 1383–1392.
15. Camellino D., Solano S., Cutolo M., Cimmino M.A. 2018. Dissecting the inflammatory response in polymyalgia rheumatica: the relative role of IL-6 and its inhibition. Rheumatol. Int. 38:1699-170 4
16. Meliconi, R., Pulsatelli, L., Dolzani, P., Boiardi, L., Macchioni, P., Salvarani, C., Silvestri, T., Frizziero, L., and Facchini, A. 2000. Vascular endothelial

growth factor production in polymyalgia rheumatica. Arthritis Rheumatol. 43: 2472-2480.

17. Chuang, T.Y., Hunder, G.G., Ilstrup, D.M., and Kurland, L.T. 1982. Polymyalgia rheumatica: a 10-year epidemiologic and clinical study. Ann. Intern. Med. 97: 672-680.

18. Dasgupta, B., Borg, F.A., Hassan, N., Barraclough, K., Bourke, B., Fulcher, J., Hollywood, J., Hutchings, A., Kyle, V., and Nott, J. 2009. BSR and BHPR guidelines for the management of polymyalgia rheumatica. Rheumatology (Oxford) 49: 186-190.

19. Dasgupta, B., Borg, F.A., Hassan, N., Alexander, L., Barraclough, K., Bourke, B., Fulcher, J., Hollywood, J., Hutchings, A., and James, P. 2010. BSR and BHPR guidelines for the management of giant cell arteritis. Rheumatology (Oxford) 49: 1594-1597.

20. Hazenberg, B.P., Limburg, P.C., Bijzet, J., and van Rijswijk, M.H. 1999. A quantitative method for detecting deposits of amyloid A protein in aspirated fat tissue of patients with arthritis. Ann. Rheum. Dis. 58: 96-102.

21. Gonzalez-Gay, M.A., Barros, S., Lopez-Diaz, M.J., Garcia-Porrua, C., Sanchez-Andrade, A., and Llorca, J. 2005. Giant cell arteritis: disease patterns of clinical presentation in a series of 240 patients. Medicine 84: 269-276.

22. Rodriguez-Valverde, V., Sarabia, J.M., González-Gay, M.A., Figueroa, M., Armona, J., Blanco, R., Fernández-Sueiro, J.L., and Martínez-Taboada, V.M. 1997. Risk factors and predictive models of giant cell arteritis in polymyalgia rheumatica. Am. J. Med. 102: 331-336.

23. Saharinen, P., Eklund, L., and Alitalo, K. 2017. Therapeutic targeting of the angiopoietin–TIE pathway. Nat. Rev. Drug Discov. 16: 635.

24. Mackie, S.L., Pease, C.T., Fukuba, E., Harris, E., Emery, P., Hodgson, R., Freeston, J., and McGonagle, D. 2015. Whole-body MRI of patients with polymyalgia rheumatica identifies a distinct subset with complete patient-reported response to glucocorticoids. Ann. Rheum. Dis. 74: 2188-2192.

25. Milam, K.E. and Parikh, S.M. 2015. The angiopoietin-Tie2 signaling axis in the vascular leakage of systemic inflammation. Tissue Barriers 3: e957508.

SUPPLEMENTARY DATA

Supplemental Table 1. Results of the logistic regression analysis (enter method) to predict overlapping vasculitis in PMR patients. Angiopoietin-2, but not ESR and sTie2, contributed significantly to the logistic regression model. Two patients (one isolated PMR and one PMR/GCA overlap) were excluded for this analysis, as no ESR values were available. Analysis was performed in SPSS.

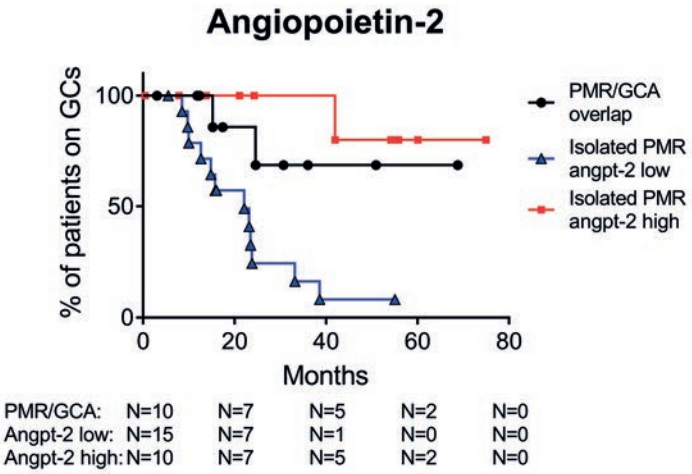
Predicting Variables	B	S.E.M.	p-value	Exp(B)	95% C.I. for Exp(B)	
					Lower	Upper
ESR	.02240	.02270	.32385	1.02265	.97814	1.06918
Angpt-2	.00045	.00023	.04502	1.00045	1.00001	1.00089
sTie2	.06808	.06989	.33004	1.07045	.93341	1.22761
Constant	-5.7017	2.0140	.00464	.00334		

B: logistic regression coefficient, Exp(B): odds ratio, C.I.: confidence interval.

	CRP	ESR	IL-6	SAA	VEGF	Angpt1	Angpt2
ESR	0.45*						
IL-6	0.66**	0,29					
SAA	0.84**	0.63**	0.58**				
VEGF	0,15	-0,29	0,09	-0,07			
Angpt1	-0,01	-0,28	-0,21	-0,07	0,31		
Angpt2	-0,04	0.49**	-0,03	0,08	-0.37*	-0,15	
sTie2	-0,11	-0,13	0,07	-0,03	0,20	0,08	0,11

Legend:
Spearman R = >0.75 / >0.5 / >0.25 / < -0.25 / < -0.5

Supplementary Figure 1. Correlations between biomarkers in baseline PMR patients. Strength of correlations in newly-diagnosed, treatment-naive patients with isolated PMR (N=29) are depicted as Spearman's correlation coefficients. Strengths of the correlations are indicated by cell colors and statistical significance is shown as * ($p<0.05$) and ** ($p<0.01$).



Supplementary Figure 2. Time to GC-free remission is comparable between angiopoietin-2-low isolated PMR patients and PMR/GCA overlap patients. Time to GC-free remission was compared between PMR/GCA overlap patients and isolated PMR patients with baseline angiopoietin-2 levels lower and higher than the cut-off value of 2134 pg/mL. Data was plotted in a Kaplan Meier curve and the log rank test was used to identify significant differences. Time to GC-free remission was similar between PMR/GCA overlap patients and angiopoietin-2^{high} isolated PMR patients (p=0.39). The difference between PMR/GCA overlap patients and angiopoietin-2-low isolated PMR patients, however, was statistically significant (p=0.017).

NINE

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 (IFNγ 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-naive 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-naive 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 (FR)β-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.

THESIS 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 wells 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 vasorum 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 CD4+ T-cells. Recently, a CD4+ 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-13Rα2, 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.

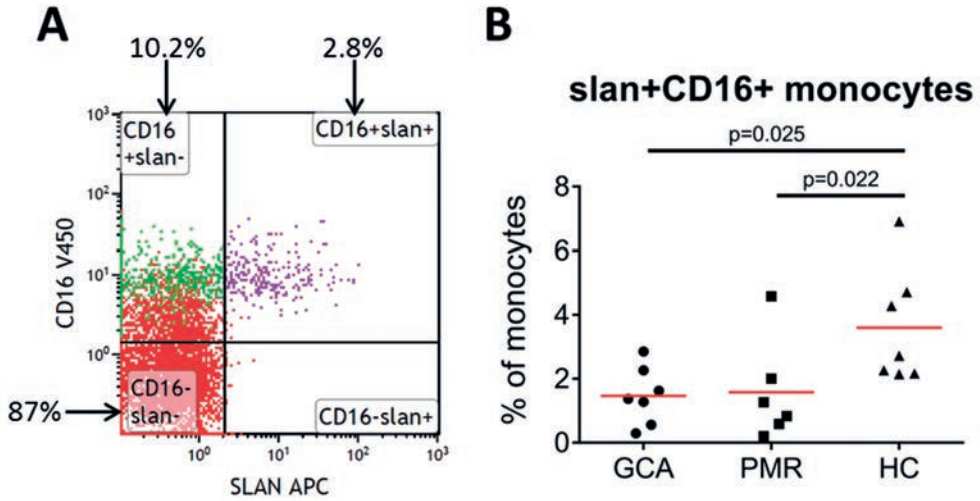


Figure 1. The proportions of non-classical monocytes, as defined by the slan approach, is lower in both GCA and PMR patients compared to healthy controls. Thawed peripheral blood mononuclear cells were stained for CD14 and HLA-DR to identify monocytes. Slan expression (identified by the M-DC8 antibody, 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-naive, newly-diagnosed GCA and PMR patients were compared to age- and sex-matched controls (**B**, N=7 for each group).

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 wrongfully 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- [24, 27]. 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

angiopoietin-2, serving as a chemoattractant [44]. This could be important in GCA and PMR, as angiopoietin-2 was discovered as a promising serum biomarker in **Chapter 7** and **8**. Moreover, we observed high expression of angiopoietin-2 in inflamed GCA lesions.

Thus, monocytes and macrophages are important cells in GCA (and PMR), with various functions. Figure 2 displays the migration of monocytes to the vessel wall, where these monocytes differentiate in macrophages with inflammatory, tissue destructive and pro-fibrotic functions. The phenotype of these macrophages is driven by environmental cues such as GM-CSF and M-CSF.

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

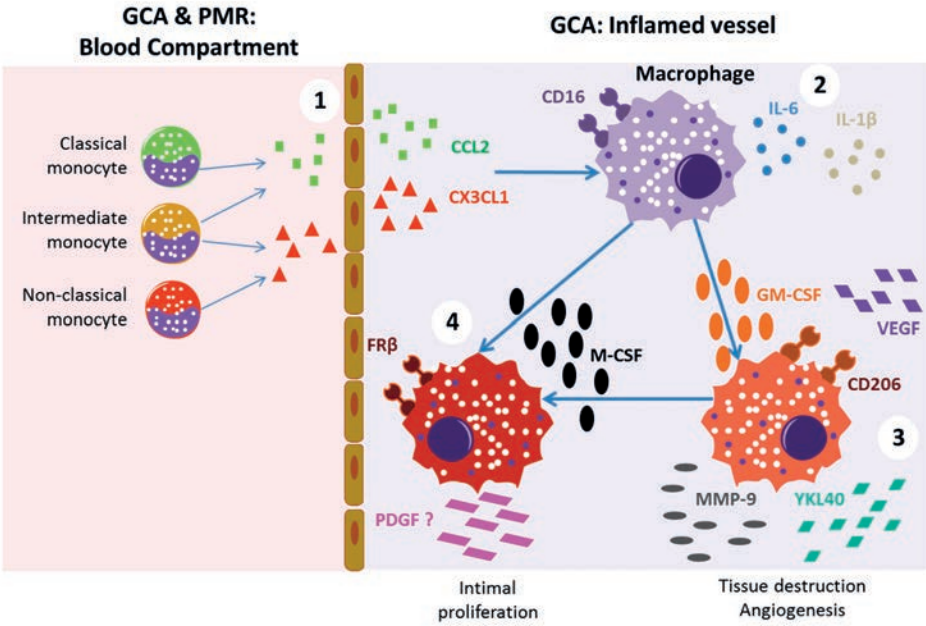


Figure 2. Monocytes and macrophages in GCA. Macrophages are derived from infiltrating monocytes. Monocytes can enter the vessel wall guided by CCL2, for classical and intermediate monocytes, and CX3CL1, for CD16+ intermediate and non-classical monocytes (1). Macrophages in the vessel wall of GCA patients 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).

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 [5, 45, 46]. It would therefore be interesting to investigate whether mDCs of GCA and PMR patients have a lower threshold for TLR2-initiated activation. TLR2 senses numerous bacterial (mainly gram+), viral (such as varicella zoster virus), and endogeneous (serum amyloid A (SAA)) ligands, many of which are implicated in GCA pathogenesis [47-50].

The remarkably reduced counts of circulating mDCs implicates them in the local inflammatory response in GCA/PMR. Myeloid DCs are derived from classical monocytes [22]. It is therefore counterintuitive that lower counts of myeloid DCs were noted whereas classical monocyte counts were elevated. In this chapter, we propose that mDCs are trapped at the inflammatory site, hence their relative absence in the blood [46]. This reasoning is similar in our explanation for the lower non-classical monocyte proportions in the blood, as non-classical monocytes are also likely derived from classical monocytes. Alternatively, some unknown signaling may be responsible in preventing classical monocytes to develop in either the mDC phenotype or the non-classical monocyte phenotype. Moreover, studies tracking circulating mDCs and non-classical monocytes could reveal if these cells indeed migrate to the inflammatory site in GCA and PMR.

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]. Many of these lineage differentiation 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, blocking IL-12 and IL-23 by a neutralizing antibody was found to reduce IFNγ and IL-17 production in cultured GCA arteries [53]. Indeed, 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).

Given the importance of Th1 and Th17 cells at the inflammatory site of GCA, we expected to see a representation of these skewing signals in the blood. Th17 skewing cytokine IL-6 is indeed elevated in the blood of GCA/PMR patients, whereas IL-12, IL-1β and IL-23 serum or plasma levels were similar to controls [53, 57]. This may be in line with previous reports showing increased frequencies of CD4+ T cells with the capacity to produce IL-17. In **Chapter 4**, however, we did not see expansion of Th1 and Th17 cells in GCA/PMR. This is in contrast to previous research observing higher proportions of cells capable of producing IFNγ (Th1) and IL-17 (Th17) [58-60]. It could be that circulating T-helper subsets do not accurately reflect the T-helper subsets in the tissue. Currently, the field awaits more sensitive/reliable methods to document true Th1 and Th17 cells in blood and tissue.

Interestingly, GM-CSF producing T-cells may also skew macrophage phenotypes at local sites and thereby contribute to tissue destruction as suggested by the findings in this thesis. Further investigation of GM-CSF producing T-cells (and other GM-CSF producing cells) and their interaction

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, prednis(ol)one. 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

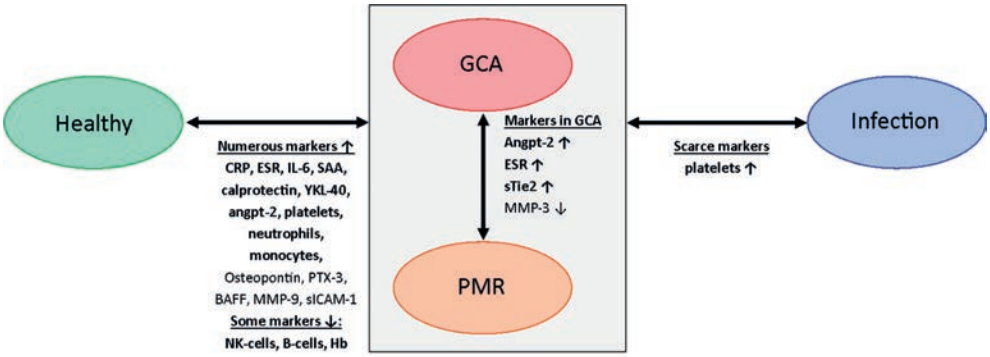


Figure 3. Diagnostic biomarkers in GCA and PMR. Numerous markers (serum markers, cell counts or other) are altered in GCA and/or PMR compared to healthy controls. Scarce studies have looked at markers that are altered in GCA/PMR versus patients with an infection, and so far only platelet counts were 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).

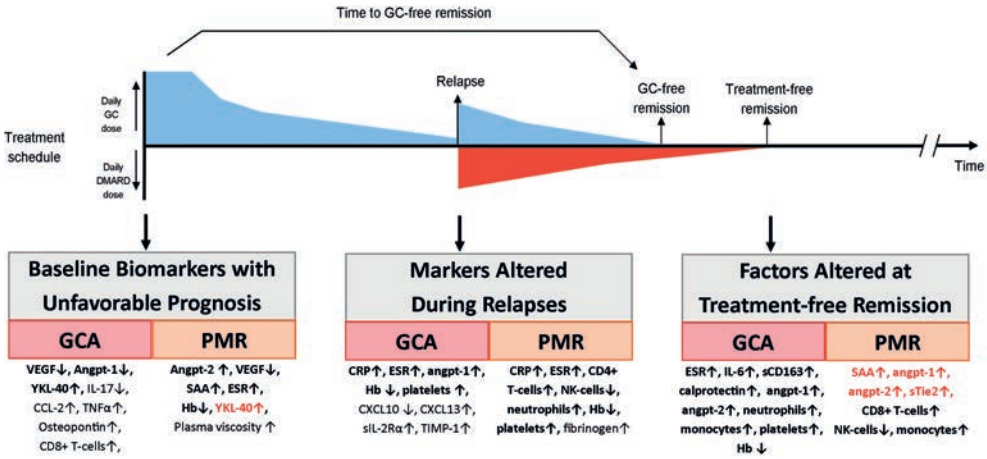


Figure 4. Markers during the disease course of GCA/PMR patients. Patients in our studies are all treated with GCs that are tapered over time, unless a patient experiences a relapse. If a patient relapses, the GC dose is increased, and a DMARD can be added (in our study methotrexate or leflunomide). In 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.

platelet 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 a higher starting GC dose. Arterial inflammation in patients with PMR manifests itself mostly in the aorta and its branches [65], and this type of GCA (LV-GCA) has mostly no specific symptoms. Moreover, both GCA and PMR have high levels of acute-phase markers, rendering them mostly useless in making a distinction between these two diseases. ESR and MMP-3 levels have been identified to discriminate GCA from PMR, albeit with a low sensitivity/specificity [66, 67].

In **Chapter 8**, we identified angiopoietin-2 as the best marker to identify arterial inflammation in PMR patients, with a high sensitivity/specificity (Figure 5). This finding needs to be validated in a second cohort, and preferably also in a dedicated prospective study. If confirmed, a ready-to-use test for measuring angiopoietin-2 needs to be implemented in daily clinical practice. Patients with angiopoietin-2 levels higher than the cut-off value will be at risk for GCA and will require additional, more costly/invasive diagnostic tools, such as FDG-PET-CT, ultrasound or TAB.

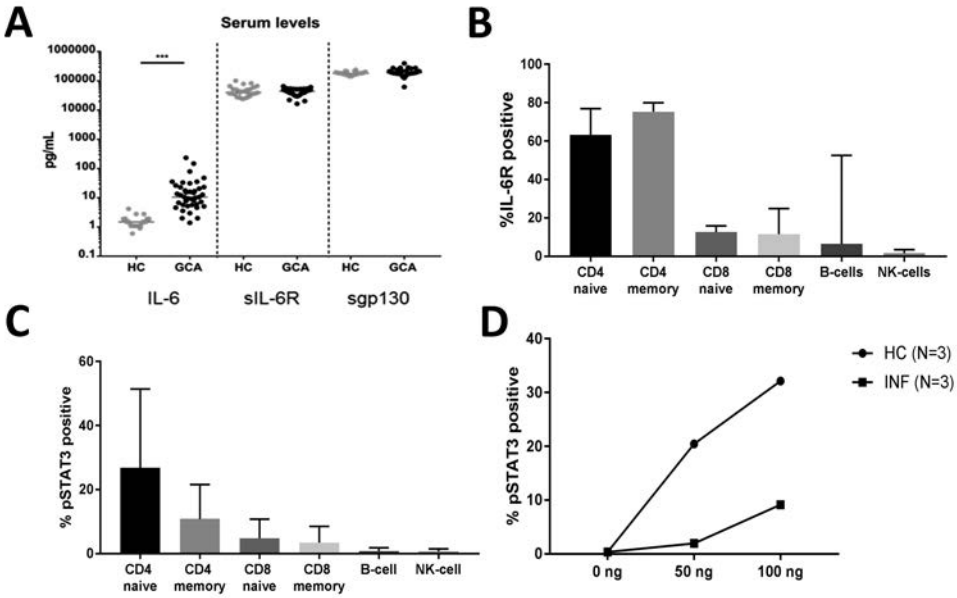


Figure 5. IL-6 signaling in CD4+ T-cells. In A, we show serum levels of IL-6 (Chapter 7), soluble IL-6 receptor (sIL-6R) and soluble gp130 (sgp130) in treatment naive GCA and healthy control (HC). Levels of sIL-6R and sgp130 were assessed by ELISA. Next, we show the percentage of membrane-bound IL-6R (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.

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.

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]. Maleszewski 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]). 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

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 naive CD4+ T-cells and memory CD4+ T cells, as opposed to CD8 T-cells, B-cell and NK-cells (Figure 5). Naive 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 naive CD4+ T cells from peripheral blood. Continuous exposure to IL-6 in GCA and PMR is likely associated with a T-cell STAT 3 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.

REFERENCES

1. van Beek, A.A., Van den Bossche, J., Mastroberardino, P.G., de Winther, M.P., and Leenen, P.J. 2019. Metabolic Alterations in Aging Macrophages: Ingredients for Inflammaging? *Trends Immunol. 40:113-127.*
2. Kovtonyuk, L.V., Fritsch, K., Feng, X., Manz, M.G., and Takizawa, H. 2016. Inflamm-aging of hematopoiesis, hematopoietic stem cells, and the bone marrow microenvironment. *Front. Immunol. 7: 502.*
3. Terrades-Garcia, N. and Cid, M.C. 2018. Pathogenesis of giant-cell arteritis: how targeted therapies are influencing our understanding of the mechanisms involved. *Rheumatology (Oxford) 57: ii51-ii62.*
4. Watanabe R., Maeda T., zhang H., Berry G.J., Zeisbrich M., Brockett R., Greenstein A.E., Tian L., Goronzy J.J., Weyand C.M. 2018. Matrix Metalloprotease-9 (MMP-9)-Producing Monocytes Enable T Cells to Invade the Vessel Wall and Cause Vasculitis. *Circ. Res. 123(6):700-715.*
5. Ma-Krupa, W., Jeon, M.S., Spoerl, S., Tedder, T.F., Goronzy, J.J., and Weyand, C.M. 2004. Activation of arterial wall dendritic cells and breakdown of self-tolerance in giant cell arteritis. *J. Exp. Med. 199: 173-183.*
6. Goldstein, B.L., Gedmintas, L., and Todd, D.J. 2014. Drug-associated polymyalgia rheumatica/giant cell arteritis occurring in two patients after treatment with ipilimumab, an antagonist of ctla-4. *Arthritis Rheumatol. 66: 768-769.*
7. Hid Cadena, R., Abdulahad, W.H., Hospers, G., Wind, T., Boots, A.M., Heeringa, P., and Brouwer, E. 2018. Checks and balances in autoimmune vasculitis. *Front. Immunol. 9: 315.*
8. Scholtens, S., Smidt, N., Swertz, M.A., Bakker, S.J., Dotinga, A., Vonk, J.M., van Dijk, F., van Zon, S.K., Wijmenga, C., and Wolffenbuttel, B.H. 2014. Cohort Profile: LifeLines, a three-generation cohort study and biobank. *Int. J. Epidemiol. 44: 1172-1180.*
9. Rodriguez-Pla, A., Bosch-Gil, J.A., Rossello-Urgell, J., Huguet-Redecilla, P., Stone, J.H., and Vilardell-Tarres, M. 2005. Metalloproteinase-2 and -9 in giant cell arteritis: involvement in vascular remodeling. *Circulation 112: 264-269.*
10. Goodfellow, N., Morlet, J., Singh, S., Sabokbar, A., Hutchings, A., Sharma, V., Vaskova, J., Masters, S., Zarei, A., and Luqmani, R. 2017. Is vascular endothelial growth factor a useful biomarker in giant cell arteritis? *RMD Open 3: e000353.*
11. Watanabe, S., Alexander, M., Misharin, A.V., and Budinger, G.S. 2019. The role of macrophages in the resolution of inflammation. *J. Clin. Invest. 129: 2619-2628*
12. Weyand, C.M., Wagner, A.D., Bjornsson, J., and Goronzy, J.J. 1996. Correlation of the topographical arrangement and the functional pattern of tissue-infiltrating macrophages in giant cell arteritis. *J. Clin. Invest. 98: 1642-1649.*
13. Martinez, F.O. and Gordon, S. 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep. 6: 13.*
14. Lacey, D.C., Achuthan, A., Fleetwood, A.J., Dinh, H., Roiniotis, J., Scholz, G.M., Chang, M.W., Beckman, S.K., Cook, A.D., and Hamilton, J.A. 2012. Defining GM-CSF–and macrophage-CSF–dependent macrophage responses by in vitro models. *J. Immunol. 188: 5752-5765.*
15. Dabritz, J., Weinhage, T., Varga, G., Wirth, T., Walscheid, K., Brockhausen, A., Schwarzmaier, D., Bruckner, M., Ross, M., Bettenworth, D., Roth, J., Ehrchen, J.M., and Foell, D. 2015. Reprogramming of monocytes by GM-CSF contributes to regulatory immune functions during intestinal inflammation. *J. Immunol. 194: 2424-2438.*
16. Noster, R., Riedel, R., Mashreghi, M.F., Radbruch, H., Harms, L., Haftmann, C., Chang, H.D., Radbruch, A., and Zielinski, C.E. 2014. IL-17 and GM-CSF expression are antagonistically regulated by human T helper cells. *Sci. Transl. Med. 6: 241ra80.*
17. Li, R., Rezk, A., Miyazaki, Y., Hilgenberg, E., Touil, H., Shen, P., Moore, C.S., Michel, L., Althekair, F., Rajasekharan, S., Gommerman, J.L., Prat, A., Fillatreau, S., Bar-Or, A., and Canadian B cells in MS Team. 2015. Proinflammatory GM-CSF-

producing B cells in multiple sclerosis and B cell depletion therapy. *Sci. Transl. Med. 7: 310ra166.*

18. van der Geest, K.S., Abdulahad, W.H., Chalan, P., Rutgers, A., Horst, G., Huitema, M.G., Roffel, M.P., Roozendaal, C., Kluin, P.M., Bos, N.A., Boots, A.M., and Brouwer, E. 2014. Disturbed B cell homeostasis in newly diagnosed giant cell arteritis and polymyalgia rheumatica. *Arthritis Rheumatol. 66: 1927-1938.*

19. Graver, J.C., Boots, A.M., Haacke, E.A., Diepstra, A., Brouwer, E., and Sandovici, M. 2019. Massive B-cell infiltration and Organization into Artery Tertiary Lymphoid Organs in the Aorta of Large Vessel Giant Cell Arteritis. *Front. Immunol. 10: 83.*

20. Jabłońska-Trypuć, A., Matejczyk, M., and Rosochacki, S. 2016. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. *J Enzyme Inhib Med Chem 31: 177-183.*

21. Rundhaug, J.E. 2005. Matrix metalloproteinases and angiogenesis. *J. Cell. Mol. Med. 9: 267-285.*

22. Boyette, L.B., Macedo, C., Hadi, K., Elinoff, B.D., Walters, J.T., Ramaswami, B., Chalasani, G., Taboas, J.M., Lakkis, F.G., and Metes, D.M. 2017. Phenotype, function, and differentiation potential of human monocyte subsets. *PLoS One 12: e0176460.*

23. Ziegler-Heitbrock, L. and Hofer, T.P. 2013. Toward a refined definition of monocyte subsets. *Front. Immunol. 4: 23.*

24. Hofer, T.P., Zawada, A.M., Frankenberger, M., Skokann, K., Satzl, A.A., Gesierich, W., Schuberth, M., Levin, J., Danek, A., Rotter, B., Heine, G.H., and Ziegler-Heitbrock, L. 2015. slan-defined subsets of CD16-positive monocytes: impact of granulomatous inflammation and M-CSF receptor mutation. *Blood 126: 2601-2610.*

25. Schäkel, K., Kannagi, R., Kniep, B., Goto, Y., Mitsuoka, C., Zwirner, J., Soruri, A., von Kietzell, M., and Rieber, E.P. 2002. 6-Sulfo LacNAc, a novel carbohydrate modification of PSGL-1, defines an inflammatory type of human dendritic cells. *Immunity 17: 289-301.*

26. van Leeuwen-Kerkhoff, N., Lundberg, K., Westers, T.M., Kordasti, S., Bontkes, H.J., de Gruijl, T.D., Lindstedt, M., and van de Loosdrecht, A.A. 2017. Transcriptional profiling reveals functional dichotomy between human slan+ non-classical monocytes and myeloid dendritic cells. *J. Leukoc. Biol. 102: 1055-1068*

27. Wong, K.L., Yeap, W.H., Tai, J.J., Ong, S.M., Dang, T.M., and Wong, S.C. 2012. The three human monocyte subsets: implications for health and disease. *Immunol. Res. 53: 41-57.*

28. Vermi, W., Micheletti, A., Finotti, G., Tecchio, C., Calzetti, F., Costa, S., Bugatti, M., Calza, S., Agostinelli, C., Pileri, S., Balzarini, P., Tucci, A., Rossi, G., Furlani, L., Todeschini, G., Zamo, A., Facchetti, F., Lorenzi, L., Lonardi, S., and Cassatella, M.A. 2018. slan(+) Monocytes and Macrophages Mediate CD20-Dependent B-cell Lymphoma Elimination via ADCC and ADCP. *Cancer Res. 78: 3544-3559.*

29. Schäkel, K., von Kietzell, M., Hänsel, A., Ebling, A., Schulze, L., Haase, M., Semmler, C., Sarfati, M., Barclay, A.N., and Randolph, G.J. 2006. Human 6-sulfo LacNAc-expressing dendritic cells are principal producers of early interleukin-12 and are controlled by erythrocytes. *Immunity 24: 767-777.*

30. Dutertre, C.A., Amraoui, S., DeRosa, A., Jourdain, J.P., Vimeux, L., Goguet, M., Degrelle, S., Feuillet, V., Liovat, A.S., Muller-Trutwin, M., Decroix, N., Deveau, C., Meyer, L., Goujard, C., Loulergue, P., Launay, O., Richard, Y., and Hosmalin, A. 2012. Pivotal role of M-DC8(+) monocytes from viremic HIV-infected patients in TNFalpha overproduction in response to microbial products. *Blood 120: 2259-2268.*

31. Vermi, W., Micheletti, A., Lonardi, S., Costantini, C., Calzetti, F., Nascimbeni, R., Bugatti, M., Codazzi, M., Pinter, P.C., and Schäkel, K. 2014. slanDCs selectively accumulate in carcinoma-draining lymph nodes and marginate metastatic cells. *Nature communications 5: 3029.*

32. Patel, A.A., Zhang, Y., Fullerton, J.N., Boelen, L., Rongvaux, A., Maini, A.A., Bigley, V., Flavell, R.A., Gilroy, D.W., Asquith, B., Macallan, D., and Yona, S. 2017. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J. Exp. Med. 214: 1913-1923.*

33. Rogacev, K.S., Zawada, A.M., Hundsdorfer, J., Achenbach, M., Held, G., Fliser, D., and Heine,

G.H. 2015. Immunosuppression and monocyte subsets. *Nephrol. Dial. Transplant. 30: 143-153.*

34. Dutertre, C.A., Amraoui, S., DeRosa, A., Jourdain, J.P., Vimeux, L., Goguet, M., Degrelle, S., Feuillet, V., Liovat, A.S., Muller-Trutwin, M., Decroix, N., Deveau, C., Meyer, L., Goujard, C., Loulergue, P., Launay, O., Richard, Y., and Hosmalin, A. 2012. Pivotal role of M-DC8(+) monocytes from viremic HIV-infected patients in TNFalpha overproduction in response to microbial products. *Blood 120: 2259-2268.*

35. Campos, T.M., Passos, S.T., Novais, F.O., Beiting, D.P., Costa, R.S., Queiroz, A., Mosser, D., Scott, P., Carvalho, E.M., and Carvalho, L.P. 2014. Matrix metalloproteinase 9 production by monocytes is enhanced by TNF and participates in the pathology of human cutaneous leishmaniasis. *PLoS Negl Trop Dis 8: e3282.*

36. Baeten, D., Boots, A.M., Steenbakkers, P.G., Elewaut, D., Bos, E., Verheijden, G.F., Berheijden, G., Miltenburg, A.M., Rijnders, A.W., Veys, E.M., and De Keyser, F. 2000. Human cartilage gp-39+,CD16+ monocytes in peripheral blood and synovium: correlation with joint destruction in rheumatoid arthritis. *Arthritis Rheumatol. 43: 1233-1243.*

37. Puchner, A., Saferding, V., Bonelli, M., Mikami, Y., Hofmann, M., Brunner, J.S., Caldera, M., Goncalves-Alves, E., Binder, N.B., Fischer, A., Simader, E., Steiner, C.W., Leiss, H., Hayer, S., Niederreiter, B., Karonitsch, T., Koenders, M.I., Podesser, B.K., O'Shea, J.J., Menche, J., Smolen, J.S., Redlich, K., and Bluml, S. 2018. Non-classical monocytes as mediators of tissue destruction in arthritis. *Ann. Rheum. Dis. 77: 1490-1497.*

38. Rossol, M., Kraus, S., Pierer, M., Baerwald, C., and Wagner, U. 2012. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheumatol. 64: 671-677.*

39. Tarzi, R.M., Liu, J., Schneiter, S., Hill, N.R., Page, T.H., Cook, H.T., Pusey, C.D., and Woollard, K.J. 2015. CD14 expression is increased on monocytes in patients with anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitis and correlates with the expression of ANCA autoantigens. *Clin. Exp. Immunol. 181: 65-75.*

40. Mandl, M., Schmitz, S., Weber, C., and Hristov, M. 2014. Characterization of the CD14++CD16+ monocyte population in human bone marrow. *PLoS One 9: e112140.*

41. Rossol, M., Kraus, S., Pierer, M., Baerwald, C., and Wagner, U. 2012. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheumatol. 64: 671-677.*

42. Parikh, S.M. 2017. The Angiopoietin-Tie2 Signaling Axis in Systemic Inflammation. *J. Am. Soc. Nephrol. 28: 1973-1982.*

43. Fujiyama, S., Amano, K., Uehira, K., Yoshida, M., Nishiwaki, Y., Nozawa, Y., Jin, D., Takai, S., Miyazaki, M., and Egashira, K. 2003. Bone marrow monocyte lineage cells adhere on injured endothelium in a monocyte chemoattractant protein-1–dependent manner and accelerate reendothelialization as endothelial progenitor cells. *Circ. Res. 93: 980-989.*

44. Murdoch, C., Tazzyman, S., Webster, S., and Lewis, C.E. 2007. Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. *J. Immunol. 178: 7405-7411.*

45. Han, J.W., Shimada, K., Ma-Krupa, W., Johnson, T.L., Nerem, R.M., Goronzy, J.J., and Weyand, C.M. 2008. Vessel wall–embedded dendritic cells induce T-cell autoreactivity and initiate vascular inflammation. *Circ. Res. 102: 546-553.*

46. Krupa, W.M., Dewan, M., Jeon, M., Kurtin, P.J., Younge, B.R., Goronzy, J.J., and Weyand, C.M. 2002. Trapping of misdirected dendritic cells in the granulomatous lesions of giant cell arteritis. *Am. J. Pathology 161: 1815-1823.*

47. O'Neill, L., Rooney, P., Molloy, D., Connolly, M., McCormick, J., McCarthy, G., Veale, D.J., Murphy, C.C., Fearon, U., and Molloy, E. 2015. Regulation of Inflammation and Angiogenesis in Giant Cell Arteritis by Acute-Phase Serum Amyloid A. *Arthritis Rheumatol. 67: 2447-2456.*

48. O'neill, L. and Molloy, E.S. 2016. The role of toll like receptors in giant cell arteritis. *Rheumatology (Oxford) 55: 1921-1931.*

49. Hoffman, G.S., Getz, T.M., Padmanabhan, R., Villa-Forte, A., Clifford, A.H., Funchain, P., Sankunny, M., Perry, J.D., Blandford, A.,

and Kosmorsky, G. 2019. The Microbiome of Temporal Arteries. *Pathogens & immunity 4: 21.*

50. Wang, J.P., Kurt-Jones, E.A., Shin, O.S., Manchak, M.D., Levin, M.J., and Finberg, R.W. 2005. Varicella-zoster virus activates inflammatory cytokines in human monocytes and macrophages via Toll-like receptor 2. *J. Virol. 79: 12658-12666.*

51. Samson, M., Corbera-Bellalta, M., Audia, S., Planas-Rigol, E., Martin, L., Cid, M.C., and Bonnotte, B. 2017. Recent advances in our understanding of giant cell arteritis pathogenesis. *Autoimmun. Rev. 16: 833-844.*

52. Geginat, J., Paroni, M., Maglie, S., Alfen, J.S., Kastirr, I., Gruarin, P., De Simone, M., Pagani, M., and Abrignani, S. 2014. Plasticity of human CD4 T cell subsets. *Front. Immunol. 5: 630.*

53. Espígol-Frigolé, G., Planas-Rigol, E., Lozano, E., Corbera-Bellalta, M., Terrades-García, N., Prieto-González, S., García-Martínez, A., Hernández-Rodríguez, J., Grau, J.M., and Cid, M.C. 2018. Expression and function of IL12/23 related cytokine subunits (p35, p40, and p19) in giant-cell arteritis lesions: contribution of p40 to Th1-and Th17-mediated inflammatory Pathways. *Front. Immunol. 9: 809.*

54. Weyand, C.M., Hicok, K.C., Hunder, G.G., and Goronzy, J.J. 1994. Tissue cytokine patterns in patients with polymyalgia rheumatica and giant cell arteritis. *Ann. Intern. Med. 121: 484-491.*

55. Conway, R., O'Neill, L., O'Flynn, E., Gallagher, P., McCarthy, G.M., Murphy, C.C., Veale, D.J., Fearon, U., and Molloy, E.S. 2016. Ustekinumab for the treatment of refractory giant cell arteritis. *Ann. Rheum. Dis. 75: 1578-1579.*

56. Samson, M., Ghesquiere, T., Berthier, S., and Bonnotte, B. 2018. Ustekinumab inhibits Th1 and Th17 polarisation in a patient with giant cell arteritis. *Ann. Rheum. Dis. 77: e6.*

57. van der Geest, K.S., Abdulahad, W.H., Rutgers, A., Horst, G., Bijzet, J., Arends, S., Roffel, M.P., Boots, A.M., and Brouwer, E. 2015. Serum markers associated with disease activity in giant cell arteritis and polymyalgia rheumatica. *Rheumatology (Oxford) 54: 1397-1402.*

58. Deng, J., Younge, B.R., Olshen, R.A., Goronzy, J.J., and Weyand, C.M. 2010. Th17 and Th1 T-cell responses in giant cell arteritis. *Circulation 121: 906-915.*

59. Saadoun, D., Garrido, M., Comarmond, C., Desbois, A., Domont, F., Savey, L., Terrier, B., Geri, G., Rosenzwajg, M., and Klatzmann, D. 2015. Th1 and Th17 cytokines drive inflammation in Takayasu arteritis. *Arthritis Rheumatol. 67: 1353-1360.*

60. Terrier, B., Geri, G., Chaara, W., Allenbach, Y., Rosenzwajg, M., Costedoat-Chalumeau, N., Fouret, P., Musset, L., Benveniste, O., and Six, A. 2012. Interleukin-21 modulates Th1 and Th17 responses in giant cell arteritis. *Arthritis Rheumatol. 64: 2001-2011.*

61. Burja, B., Kuret, T., Sodin-Semrl, S., Lakota, K., Rotar, Z., Jese, R., Mrak-Poljsak, K., Zigon, P., Thallinger, G.G., Feichtinger, J., Cucnik, S., Tomsic, M., Praprotnik, S., and Hocevar, A. 2017. A concise review of significantly modified serological biomarkers in Giant cell arteritis, as detected by different methods. *Autoimmun. Rev. 17:188-194*

62. Prieto-Gonzalez, S., Terrades-Garcia, N., Corbera-Bellalta, M., Planas-Rigol, E., Miyabe, C., Alba, M.A., Ponce, A., Tavera-Bahillo, I., Murgia, G., Espigol-Frigole, G., Marco-Hernandez, J., Hernandez-Rodriguez, J., Garcia-Martinez, A., Unizony, S.H., and Cid, M.C. 2017. Serum osteopontin: a biomarker of disease activity and predictor of relapsing course in patients with giant cell arteritis. Potential clinical usefulness in tocilizumab-treated patients. *RMD Open 3: e000570.*

63. Stone J.H., Tuckwell K., Dimonaco S., Klearman M., Aringer M., Blockmans D., Brouwer E., Cid M.C., Dasgupta B., Rech J., Salvarani C., Schulze-Koops H., Schett G., Spiera R., Unizony S.H., Collinson N. Glucocorticoid Doses and Acute-Phase Reactants at Giant Cell Arteritis Flare in a Randomized Trial of Tocilizumab. *Arthritis Rheumatol. 71:1329-1338.*

64. Caporali, R., Montecucco, C., Epis, O., Bobbio-Pallavicini, F., Maio, T., and Cimmino, M.A. 2001. Presenting features of polymyalgia rheumatica (PMR) and rheumatoid arthritis with PMR-like onset: a prospective study. *Ann. Rheum. Dis. 60: 1021-1024.*

65. Koster, M.J., Matteson, E.L., and Warrington, K.J. 2018. Large-vessel giant cell arteritis:

diagnosis, monitoring and management. *Rheumatology (Oxford) 57: ii32-ii42*.

66. Fukui, S., Nunokawa, T., Kobayashi, S., Kamei, S., Yokogawa, N., Takizawa, Y., Shimada, K., Sugii, S., and Setoguchi, K. 2016. MMP-3 can distinguish isolated PMR from PMR with GCA: A retrospective study regarding PMR and GCA in Japan. *Mod. Rheumatol. 26: 259-264*.

67. Gonzalez-Gay, M.A., Barros, S., Lopez-Diaz, M.J., Garcia-Porrua, C., Sanchez-Andrade, A., and Llorca, J. 2005. Giant cell arteritis: disease patterns of clinical presentation in a series of 240 patients. *Medicine 84: 269-276*.

68. Zen, M., Canova, M., Campana, C., Bettio, S., Nalotto, L., Rampudda, M., Ramonda, R., Iaccarino, L., and Doria, A. 2011. The kaleidoscope of glucorticoid effects on immune system. *Autoimmunity reviews 10: 305-310*.

69. Kermani, T.A., Schmidt, J., Crowson, C.S., Ytterberg, S.R., Hunder, G.G., Matteson, E.L., and Warrington, K.J. 2012. Utility of erythrocyte sedimentation rate and C-reactive protein for the diagnosis of giant cell arteritis. *Semin. Arthritis Rheum. 41: 866-871*.

70. McCarthy, E.M., MacMullan, P.A., Al-Mudhaffer, S., Madigan, A., Donnelly, S., McCarthy, C.J., Molloy, E.S., Kenny, D., and McCarthy, G.M. 2012. Plasma fibrinogen is an accurate marker of disease activity in patients with polymyalgia rheumatica. *Rheumatology (Oxford) 52: 465-471*.

71. Stone, J.H., Tuckwell, K., Dimonaco, S., Klearman, M., Aringer, M., Blockmans, D., Brouwer, E., Cid, M.C., Dasgupta, B., Rech, J., Salvarani, C., Schett, G., Schulze-Koops, H., Spiera, R., Unizony, S.H., and Collinson, N. 2017. Trial of Tocilizumab in Giant-Cell Arteritis. *N. Engl. J. Med. 377: 317-328*.

72. Dejaco, C., Brouwer, E., Mason, J.C., Buttgereit, F., Matteson, E.L., and Dasgupta, B. 2017. Giant cell arteritis and polymyalgia rheumatica: current challenges and opportunities. *Nat. Rev. Rheumatol. 13(10):578-592*.

73. Broder, M.S., Sarsour, K., Chang, E., Collinson, N., Tuckwell, K., Napalkov, P., and Klearman, M. 2016. Corticosteroid-related adverse events in patients with giant cell arteritis: A claims-based analysis. *Semin. Arthritis Rheum. 46: 246-252*.

74. Buttgereit F., Matteson E.L., Dejaco C., Dasgupta B. 2018. Prevention of glucocorticoid morbidity in giant cell arteritis. *Rheumatology (Oxford) 57(suppl_2): ii11-ii21*.

75. van der Geest, K.S.M., Sandovici, M., van Sleen, Y., Sanders, J.S., Bos, N.A., Abdulahad, W.H., Stegeman, C.A., Heeringa, P., Rutgers, A., Kallenberg, C.G.M., Boots, A.M.H., and Brouwer, E. 2018. What is the Current Evidence for Disease Subsets in Giant Cell Arteritis? *Arthritis Rheumatol. 70: 1366-1376*

76. Mackie, S.L., Hensor, E.M., Haugeberg, G., Bhakta, B., and Pease, C.T. 2010. Can the prognosis of polymyalgia rheumatica be predicted at disease onset? Results from a 5-year prospective study. *Rheumatology (Oxford) 49: 716-722*.

77. Schmidt, W.A. 2018. Ultrasound in the diagnosis and management of giant cell arteritis. *Rheumatology (Oxford) 57: ii22-ii31*.

78. Maleszewski, J.J., Younge, B.R., Fritzlen, J.T., Hunder, G.G., Goronzy, J.J., Warrington, K.J., and Weyand, C.M. 2017. Clinical and pathological evolution of giant cell arteritis: a prospective study of follow-up temporal artery biopsies in 40 treated patients. *Mod. Pathol. 30(6):788-796*.

79. Reichenbach, S., Adler, S., Bonel, H., Cullmann, J.L., Kuchen, S., Bütikofer, L., Seitz, M., and Villiger, P.M. 2018. Magnetic resonance angiography in giant cell arteritis: results of a randomized controlled trial of tocilizumab in giant cell arteritis. *Rheumatology (Oxford) 57: 982-986*.

80. Gloor A.D., Yerly D., Adler S., Reichenbach S., Kuchen S., Seitz M., Villiger P.M. 2018. Immuno-monitoring reveals an extended subclinical disease activity in tocilizumab-treated giant cell arteritis. *Rheumatology (Oxford) 57:1795-1801*.

81. Mohan, S.V., Liao, Y.J., Kim, J.W., Goronzy, J.J., and Weyand, C.M. 2011. Giant cell arteritis: immune and vascular aging as disease risk factors. *Arthritis Res. Ther. 13: 231*.

82. Van Deursen, J.M. 2014. The role of senescent cells in ageing. *Nature 509: 439*.

83. Yin, H. and Pickering, J.G. 2016. Cellular senescence and vascular disease: novel routes to better understanding and therapy. *Can. J. Cardiol. 32: 612-623*.

84. Victorelli, S. and Passos, J.F. 2017. Telomeres and cell senescence-size matters not. *EBioMedicine 21: 14-20*.

85. Dou, Z., Ghosh, K., Vizioli, M.G., Zhu, J., Sen, P., Wangensteen, K.J., Simithy, J., Lan, Y., Lin, Y., and Zhou, Z. 2017. Cytoplasmic chromatin triggers inflammation in senescence and cancer. *Nature 550: 402*.

86. Croci, S., Zerbini, A., Boiardi, L., Muratore, F., Bisagni, A., Nicoli, D., Farnetti, E., Pazzola, G., Cimino, L., Moramarco, A., Cavazza, A., Casali, B., Parmeggiani, M., and Salvarani, C. 2015. MicroRNA markers of inflammation and remodelling in temporal arteries from patients with giant cell arteritis. *Ann. Rheum. Dis. 75: 1527-1533*

87. Merino, A., Buendia, P., Martin-Malo, A., Aljama, P., Ramirez, R., and Carracedo, J. 2011. Senescent CD14+CD16+ monocytes exhibit proinflammatory and proatherosclerotic activity. *J. Immunol. 186: 1809-1815*.

88. Carracedo, J., Ramirez, R., Soriano, S., de Lara, Maria Antonia Alvarez, Rodriguez, M., Martin-Malo, A., and Aljama, P. 2005. Monocytes from dialysis patients exhibit characteristics of senescent cells: does it really mean inflammation? *Contrib Nephrol. 149:208-218*.

89. Coit, P., De Lott, L.B., Nan, B., Elner, V.M., and Sawalha, A.H. 2015. DNA methylation analysis of the temporal artery microenvironment in giant cell arteritis. *Ann. Rheum. Dis. 75:1196-202*.

90. Hakimi, M., Peters, A., Becker, A., Böckler, D., and Dihlmann, S. 2014. Inflammation-related induction of absent in melanoma 2 (AIM2) in vascular cells and atherosclerotic lesions suggests a role in vascular pathogenesis. *J Vasc Surg. 59: 794-803*.

91. Kirkland, J.L., Tchkonia, T., Zhu, Y., Niedernhofer, L.J., and Robbins, P.D. 2017. The clinical potential of senolytic drugs. *J. Am. Geriatr. Soc. 65: 2297-2301*.

92. Rose-John, S., Winthrop, K., and Calabrese, L. 2017. The role of IL-6 in host defence against infections: immunobiology and clinical implications. *Nat. Rev. Rheumatol. 13: 399*.

93. Anderson, A.E., Pratt, A.G., Sedhom, M.A., Doran, J.P., Routledge, C., Hargreaves, B., Brown, P.M., Le Cao, K.A., Isaacs, J.D., and Thomas, R. 2016. IL-6-driven STAT signalling in circulating CD4+ lymphocytes is a marker for early anticitrullinated peptide antibody-negative rheumatoid arthritis. *Ann. Rheum. Dis. 75: 466-473*.

94. Shen-Orr, S.S., Furman, D., Kidd, B.A., Hadad, F., Lovelace, P., Huang, Y., Rosenberg-Hasson, Y., Mackey, S., Grisar, F.A.G., and Pickman, Y. 2016. Defective signaling in the JAK-STAT pathway tracks with chronic inflammation and cardiovascular risk in aging humans. *Cell systems 3: 374-384*.

APPENDIX

Nederlandse samenvatting

Publications

Dankwoord

About the author

NEDERLANDSE SAMENVATTING

Reuscel arteriitis (RCA) en spierreuma (polymyalgia rheumatica, PMR) zijn ontstekingsziekten die exclusief voorkomen bij oudere volwassen. RCA patiënten hebben ontstoken middelgrote en grote slagaderen, met als gevolg symptomen zoals hoofdpijn, pijn in de kaken tijdens het kauwen, koorts en gewichtsverlies. Ernstige complicaties zoals blindheid en een verwijding/scheuring van de slagader (aneurysma) kunnen ook voorkomen. RCA overlapt geregeld met PMR. Deze ziekte wordt gekarakteriseerd door ontstoken gewrichten in de schouders en heupen, wat leidt tot ochtendstijfheid en pijn.

Beide ziekten worden getypeerd door een ontstekingsreactie meetbaar in het bloed en migratie van immuuncellen naar respectievelijk de vaatwand dan wel de gewrichten. De migrerende witte bloedcellen zijn voornamelijk T-helper cellen en monocyten.

RCA en PMR patiënten worden meestal behandeld met prednisolon. Vanwege het frequent opvlammen van de ziekte (reactivatie) is de behandeling vaak langdurig. Dit leidt onvermijdelijk tot ernstige bijwerkingen. Het ontstaan van RCA en PMR wordt nog niet volledig begrepen. Het doel van de studies beschreven in dit proefschrift is om meer kennis te krijgen over de ontstekingsprocessen die leiden tot het ontstaan van deze ziekten. Hierbij ligt de focus op twee aan elkaar verwante celtypen: de macrofagen, aangezien deze cellen de ontsteking in het weefsel aanjagen en zorgen voor schade, en de monocyten, de voorlopers van macrofagen die in het bloed circuleren (**Hoofdstuk 2-6**).

Als hulpmiddel bij de zorg voor RCA en PMR patiënten kunnen biomarkers een rol spelen. Biomarkers zijn meetbare indicatoren van een bepaalde biologische toestand. Een bekend voorbeeld van een (acute fase) biomarker is bijvoorbeeld de bezinking als maat voor ontsteking. Op dit moment zijn er helaas geen biomarkers die specifiek zijn voor RCA en PMR, en daarbij zijn er ook geen biomarkers die voorspellend zijn voor het ziekteverloop. Het doel van de huidige studies was dan ook om aan de hand van de huidige kennis van de ziektespecifieke processen nieuwe tools te ontwikkelen die kunnen helpen bij de diagnose, controle en voorspelling van ziekteverloop van RCA en PMR patiënten (**Hoofdstuk 7 & 8)**.

Hoofdstuk 2 betreft een omvangrijke lange termijn studie, waarbij we hebben gekeken naar de samenstelling van de witte bloedcellen in het bloed van RCA en PMR patiënten. We hebben de aantallen van verschillende typen witte bloedcellen in patiënten gemeten vóór, tijdens en ná de behandeling met prednisolon. Voor de start van de behandeling hadden RCA en PMR patiënten meer monocyten en neutrofielen, maar minder B-cellen en NK-cellen in het bloed, vergeleken met gezonde controles. Deze veranderingen zijn typisch voor een 'myeloïde verschuiving', een bloedprofiel dat wordt geassocieerd met een verouderd immuunsysteem, wat mogelijk vooraf gaat aan het ontstaan van RCA en PMR. Dit myeloïde profiel was blijvend, omdat ook tijdens de behandeling met prednisolon en zelfs lang na het staken van de behandeling de aantallen neutrofielen en de monocyten verhoogd bleven. Het behoud van dit myeloïde profiel wijst mogelijk op een sluimerend ontstekingsproces in deze patienten.

De bevindingen beschreven in hoofdstuk 2 toonden aan dat het aantal monocyten voortdurend verhoogd was in RCA en PMR patiënten. Monocyten zijn onder te verdelen in drie subsets,

gebaseerd op de expressie van de eiwitten CD14 en CD16: klassiek ($CD14^+CD16^-$, het meest voorkomend), intermediar ($CD14^+CD16^+$, het sterkst geassocieerd met ontsteking) en niet-klassiek($CD14^-CD16^+$, de meest 'volwassen' subset). In **Hoofdstuk 3** hebben wij ontdekt dat monocyt aantallen in het bloed zijn toegenomen vanwege een expansie van de klassieke monocyten. Prednisolon behandeling zorgde voor een sterke daling in de aantallen van de niet-klassieke monocyten, maar niet de klassieke monocyten. Als monocyten migreren naar de vaatwand veranderen ze in macrofagen. In het ontstoken bloedvat van RCA patiënten waren alle macrofagen positief voor CD16. Wij hebben ook de migratie van monocyten naar het ontstoken weefsel onderzocht. Dit gebeurt door middel van chemokines: klassieke monocyten, met hoge CCR2 expressie, migreren naar hoge concentraties CCL2, en niet-klassieke monocyten migreren naar hoge concentraties CX3CL1 vanwege hun hoge CX3CR1 expressie. In het weefsel werden er maar weinig CCR2-positieve macrofagen geobserveerd, wat leidde tot de conclusie dat macrofagen in het ontstoken RCA bloedvat waarschijnlijk ontstaan zijn uit non-classical monocyten.

In de ontstoken weefsels van RCA patiënten treffen we infiltraten aan met daarin macrofagen, dendritische cellen (DCs, gespecialiseerde antigeen-presenterende cellen) en ontstekingsbevorderende T-helper cellen (Th1 en Th17 cellen). Het wordt aangenomen dat met name de macrofagen en T-helper cellen in het weefsel afkomstig zijn uit het bloed. Onze hypothese luidde dan ook dat monocytsubsetaantallen, als voorlopers van macrofagen, gelinkt zouden kunnen zijn aan Th1 en Th17 cellen in het bloed. Daarom hebben wij in **Hoofdstuk 4** de aantallen en onderverdeling van de monocyten bepaald en vervolgens het aantal T-helper cellen die de cytokines IFNγ (Th1) en IL-17 (Th17) maken. Deze bepaling in het bloed werd gedaan in twee kleine cohorten van RCA/PMR patiënten. Echter, in tegenstelling tot eerdere studies, vonden wij geen veranderde aantallen Th1 en Th17 cellen in onbehandelde patiënten. Er was dan ook geen correlatie tussen monocyt subsets en Th1/Th17 aantallen. Waarschijnlijk vinden de interacties tussen deze cellen vooral plaats in het ontstoken weefsel, waardoor ze minder goed te karakteriseren zijn in het bloed. Naast bovengenoemde cellen, hebben wij ook het aantal DCs in het bloed bepaald voor twee subsets: myeloïde DCs (mDCs) en plasmacytoïde DCs (pDCs). Verlaagde aantallen mDCs werden gevonden in onbehandelde RCA en PMR patiënten. Vervolgens hebben wij gekeken naar de expressie van 'pattern recognition receptors' op monocyten en DCs. Deze receptoren zijn belangrijke initiators van ontstekingsprocessen. Een van deze pattern recognition receptors, TLR2, kwam hoger tot expressie op mDCs van RCA/PMR patiënten. Deze hogere TLR2 expressie op mDCs, en hun lagere aantallen in het bloed, suggereert dat deze cellen naar het ontstoken weefsel migreren, waar ze geactiveerd kunnen worden door TLR2 liganden (patterns).

Het is algemeen bekend dat macrofagen aanzienlijk heterogene fenotypes en functies kunnen hebben. Het fenotype van macrofagen wordt grotendeels bepaald door signalen uit de omgeving. Tot nu toe is er echter weinig kennis over de heterogeniteit van macrofagen in RCA. Onze hypothese was dat macrofagen locatie-afhankelijke kenmerken hebben die overeen komen met de activiteiten die op deze locatie plaatsvinden. Voorbeelden hiervan zijn de weefselschade bevorderende macrofagen en macrofagen die verantwoordelijk zijn voor obstructie van het lumen van het bloedvat. Deze verschillende macrofaag fenotypes zouden geïnduceerd kunnen worden door lokaal geproduceerde groeifactoren. In **Hoofdstuk 5** identificeren wij verschillende macrofaag fenotypes,

afhankelijk van de locatie in het ontstoken bloedvat: een subset met hoge CD206 expressie die verantwoordelijk is voor weefselschade (door middel van MMP-9 productie) en een subset met hoge FRβ expressie betrokken bij obstructie van het bloedvat. Opvallend is dat deze verschillende fenotypes niet alleen werden gevonden in ontstoken middelgrote arteriën in het hoofd, maar ook in RCA aorta's. Daarentegen hadden macrofagen in ontstoken atherosclerotische aorta's niet zulke heterogene fenotypes als in RCA. In experimenten met gekweekte macrofagen zagen wij dat CD206 expressie hoog was als cellen gestimuleerd werden met de groeifactor GM-CSF, terwijl de expressie van FRβ hoger was voor cellen gestimuleerd met M-CSF. Deze twee groeifactoren worden -binnen verschillende regio's- ook geproduceerd in RCA bloedvaten. De macrofaag heterogeniteit die wij beschrijven in RCA zou dus verklaard kunnen worden door de lokale aan- of afwezigheid van deze groeifactoren. De nieuwe inzichten die wij hiermee verkregen hebben kunnen mogelijk gebruikt worden voor de ontwikkeling van nieuwe therapieën zoals gerichte uitschakeling van de weefseldestructieve macrofagen. Ook wordt het misschien mogelijk om met behulp van deze markers macrofagen specifiek te volgen met PET-CT scans.

Naast fenotypering, hebben wij ook gekeken naar een door macrofagen geproduceerd product, YKL-40, dat regelmatig in het bloed gemeten wordt als biomarker voor ontsteking en weefseltransformatie. In **Hoofdstuk 6** gaan wij dieper in op de productie en functie van dit eiwit. Wij laten een hoge expressie van YKL-40 zien in ontstoken middelgrote bloedvaten en aorta's van RCA patiënten. Deze aorta's zijn afkomstig van patiënten met een aneurysma, wat wordt gezien als een laat stadium van de ziekte. Onze studies laten zien dat YKL-40, geproduceerd door macrofagen in de onstoken bloedvaten, ook gemeten kan worden in het bloed als marker van een nog sluimerende bloedvatontsteking. Wij laten zien dat YKL-40 wordt geproduceerd door macrofagen met hoge CD206 expressie; deze macrofaag subset ontstaat na stimulatie met GM-CSF. De rol van YKL-40 in RCA is nog niet duidelijk. Hier laten wij zien dat YKL-40 de groei van kleine bloedvaten stimuleert. Dit proces, ook wel angiogenese genoemd, is belangrijk om de ontsteking op gang te houden. YKL-40 kan angiogenese veroorzaken als het in contact komt met de receptor IL-13Rα2. Onze weefselstudies wijzen uit dat IL-13Rα2 inderdaad tot expressie komt op verschillende cellen in RCA bloedvaten.

Hoofdstuk 7 & 8 zijn het resultaat van onze vertaling van de gegenereerde kennis naar de kliniek. Deze hoofdstukken laten de potentie zien van macrofaag- en angiogeneproducten als klinische biomarkers. We proberen hiermee een oplossing te vinden voor het gebrek aan een aantal klinische tools: diagnostische biomarkers die specifiek zijn voor RCA en PMR, betrouwbare biomarkers die de opvlamming van actieve ziekte en weefselontsteking aantonen, en biomarkers die het ziekteverloop kunnen voorspellen voor RCA en PMR patiënten. Met behulp van nieuwe biomarkers kunnen complicaties, zoals blindheid en aneuysma's, voorkomen worden en de klachten veroorzaakt door bijwerkingen van langdurige prednisolon behandeling verminderd worden. Onze biomarker studies zijn wereldwijd uniek omdat we gebruik konden maken van het uitgebreide GCA PMR SENEX (GPS) cohort onderzoek in het UMCG, mogelijk gemaakt dankzij deelname van patiënten en vrijwilligers aan dit langlopend onderzoek. Ten tijde van het onderzoek geven deelnemende patiënten regelmatig bloed af in een langdurige studie (>7 jaar) naar hun ziekte. Dit gebeurt op het

moment van diagnose (voor de start van behandeling), tijdens de behandeling, maar ook als de patiënten al een lange tijd uitbehandeld zijn.

In **Hoofdstuk 7** keken wij naar potentiële klinische toepassingen van een aantal macrofaag- en angiogenesemarkers, geselecteerd aan de hand van pathogene processen die karakteristiek zijn voor RCA. Verhoogde waarden van biomarkers van systemische ontsteking, macrofagen en angiogenese werden gevonden in het bloed van RCA patiënten (en patiënten met een infectie). De behandeling met prednisolon had een sterk onderdrukkend effect op veel van de biomarkers, maar de waarden van calprotectine en YKL-40 bleven verhoogd, wat mogelijk een teken is van sluimerende bloedvatontsteking. We hebben ook gekeken naar het bloed van patiënten die geen behandeling meer kregen. Verschillende biomarkers waren zelfs in deze patiënten niet genormaliseerd. De belangrijkste bevinding van dit hoofdstuk was dat een profiel van angiogenese biomarkers –gemeten voor de start van de behandeling– voorspellend is voor het ziekte verloop. Deze biomarkers, VEGF, YKL-40, en angiopoietine-1 en -2, voorspelden een lange behandelduur met prednisolon, waarschijnlijk omdat deze patiënten vaker een opvlamming van ziekte activiteit hebben.

Hoofdstuk 8 was gericht op het identificeren van biomarkers die onderliggende vaatwandontsteking in PMR patiënten kunnen aantonen. Wederom werd hier gekeken naar het nut van biomarkers gebaseerd op ontsteking en angiogenese. In deze studie werden recentelijk gediagnosticeerde patiënten met geïsoleerde PMR, PMR met overlappend RCA, en controlegroepen onderzocht. Net als in RCA, waren de waarden van de meeste biomarkers verhoogd in patiënten met geïsoleerde PMR. De bezinking en sTie2 en angiopoietine-2 waren echter lager in patiënten met geïsoleerde PMR vergeleken met patiënten met overlappende PMR/RCA. Uit vervolgonderzoek bleek dat angiopoietine-2 een superieur vermogen had om onderscheid tussen de twee patiëntenpopulaties te maken. Dit onderscheid maken is van groot belang, omdat RCA geassocieerd is met ernstige complicaties en hoger prednisolon gebruik. Daarbovenop bleek dat de waarde van angiopoietine-2 –gemeten in recent gediagnosticeerde patiënten– ook goed de behandelduur van de geïsoleerde PMR patiënten konden voorspellen. De biomarkers beschreven in Hoofdstuk 7 en 8 zijn in het najaar van 2019 ook onderzocht in een onafhankelijk Deens cohort van GCA en PMR patienten. Deze validatie studie is van belang om deze biomarkers op termijn ook daadwerkelijk toe te passen in verbeteren van de zorg voor de patient met RCA en/of PMR.

PUBLICATIONS

van Sleen Y, Wang Q, van der Geest KSM, Westra J, Abdulahad WH, Heeringa P, Boots AMH, Brouwer E. Involvement of Monocyte Subsets in the Immunopathology of Giant Cell Arteritis. **Scientific Reports.** 2017 26;7(1):6553.

van Sleen Y, Sandovici M, Abdulahad WH, Bijzet J, van der Geest KSM, Boots AMH, Brouwer E. Markers of Angiogenesis and Macrophage Products for Predicting Disease Course and Monitoring Vascular Inflammation in Giant Cell Arteritis. **Rheumatology (Oxford)** 2019; 58;8: 1383-1392.

van Sleen Y, Boots AMH, Abdulahad WH, Bijzet J, Sandovici M, van der Geest KSM, Brouwer E. High Angiopoietin-2 Levels Associate with Arterial Inflammation and Long-Term Glucocorticoid Requirement in Polymyalgia Rheumatica. **Rheumatology (Oxford)** 2020 59;1: 176-184

van Sleen Y, Graver JC, Abdulahad WH, van der Geest KSM, Boots AMH, Sandovici M, Brouwer E. Leukocyte Dynamics Reveal a Persistent Myeloid Dominance in Giant Cell Arteritis and Polymyalgia Rheumatica. **Frontiers in Immunology.** 2019; 10:1981.

van der Geest KSM, Sandovici M, van Sleen Y, Sanders JS, Bos NA, Abdulahad WH, Stegeman, C.A.; Heeringa, P.; Rutgers, A.; Kallenberg, C.G.M.; Boots, A.M.H.; Brouwer, E. What is the Current Evidence for Disease Subsets in Giant Cell Arteritis? **Arthritis & Rheumatology** 2018 70(9): 1366-1376.

Hid Cadena R, Reitsema RD, Huitema MG, van Sleen Y, van der Geest KSM, Heeringa P, Boots AMH, Abdulahad WH, Brouwer E. Decreased expression of negative immune checkpoint VISTA by CD4+ T cells facilitates T helper 1, T helper 17 and T follicular helper lineage differentiation in GCA. **Frontiers in Immunology**, 2019; 10:1638.

Jiemy WF, van Sleen Y, ten Berge HA, van der Geest KSM, Abdulahad WH, Sandovici M, Boots AMH, Heeringa P, Brouwer E. Distinct Macrophage Subsets Dictated by Local GM-CSF and M-CSF Expression are Associated with Tissue Destruction and Intimal Hyperplasia in Giant Cell Arteritis. **Under review.**

DANKWOORD

Ik heb tijdens mijn studie en ook tijdens mijn PhD vaak spookverhalen gehoord over PhD studenten die hun (co)promotoren hooguit bij de nieuwjaarsborrel zien en verder op zichzelf zijn aangewezen. Manuscripten zouden maanden op bureaus liggen om ze vervolgens met twee extra komma's in de auteurslijst weer terug te krijgen. Deze duistere verwachtingen weerhielden mij er gelukkig niet van om een PhD te beginnen bij de afdeling Reumatologie en Klinische Immunologie in het UMCG en gelukkig zijn die verwachtingen ook totaal niet uitgekomen. Ik ben dankbaar voor alle hulp en steun die ik heb gekregen tijdens het tot stand komen van dit boekje.

Als eerste wil ik mijn promotor bedanken. Mieke, ik waardeer ontzettend hoe betrokken jij bent geweest bij mijn promotietraject. Als ik een versie van een manuscript opstuurde, kreeg ik al snel een volledig rood gekleurd document terug, vol met verbeteringen en opmerkingen. Soms was dat misschien even slikken, maar ik ben er van overtuigd dat het de kwaliteit van dit boekje naar een hoger plan getrokken heeft. Ik vond het erg fijn dat ik altijd langs kon komen om samen te brainstormen over nieuwe experimenten, beursaanvragen of de kansen van Tom Dumoulin in de Tour de France.

Translationeel onderzoek valt of staat bij goede klinische input. Liesbeth, ik vind het daarom fijn dat ik dit project met jou heb kunnen doen. Ik denk dat er maar weinig mensen op de wereld zijn die patiënten met GCA en PMR zo goed kennen als jij. Omdat er bij het organiseren en bijhouden van het patiëntencohort een hele boel zaken afgestemd moeten worden, betekende het dat ik erg vaak bij jou voor de deur heb gestaan. Ondanks alle hectiek met alle patiënten en alle administratieve rompslomp was je altijd zo aardig om tijd vrij te maken om mijn kleine en grotere vragen te beantwoorden.

Beste Wayel 'Nieuwe Subset!' Abdulahad, bedankt voor al jouw enthousiasme en bijdrages aan mijn project. Flow cytometry is een essentieel onderdeel geweest van mijn PhD en vraagt vaak een kritische blik op de data. Gelukkig heb jij mij het op zo'n manier aangeleerd dat ik het nu wel mag beschouwen als mijn favoriete labtechniek! Ik reken er op dat jij jouw belofte na gaat komen en op de dag van mijn promotie toch echt op de fiets naar Groningen zal komen.

Dit project had niet afgerond kunnen worden zonder jou, Niels. Sterker nog, het was waarschijnlijk nooit van start gegaan. Jouw betrokkenheid, vooral tijdens de laatste periode, hielp mij om goede keuzes te maken tussen dingen die wel en niet belangrijk waren. Jouw kennis over de literatuur en handige trucjes om figuren sterker te maken waren een unieke toevoeging de afgelopen tijd.

To the reading committee, Prof Daemen, Prof van Laar and Prof Ellen Hauge, thank you for all your time. In particular, I want to thank Ellen for all the help in writing the EFIS-IL grant proposal and for my amazing stay in Aarhus last year. Of course, I want to extend my gratitude to Philip, Berit and

Magdalena as part of the team in Aarhus, I am very happy with what we achieved. Tak! (I'm sorry but that is practically all the Danish I managed to learn unfortunately!)

Ik wil graag Reuma Nederland bedanken voor het financieren van dit project. Verder wil ik ook mijn waardering uitspreken voor de steun die ik gehad heb van de Vasculitis Stichting om de congressen in Tokyo en Philadelphia te bezoeken, en de Cock – Hadders stichting voor de financiering van de vele antilichamen die ik heb verbruikt. Daarnaast heb ik een deel van het werk uit dit proefschrift kunnen valideren in Aarhus met ondersteuning van een beurs van EFIS-Immunology Letters. Maar bovenal bedank ik alle patiënten en gezonde controles bedanken voor hun deelname aan het GPS cohort.

Beste Maria, jij was officieel geen onderdeel van mijn promotieteam, maar soms leek dat wel zo! Zeker bij hoofdstuk 2, 7 en 8 was jouw bijdrage essentieel om dit werk gepubliceerd te krijgen. Ik denk dat het voor de afdeling een enorme verbetering is dat jij sinds kort volledig bij het UMCG in dienst bent.

Beste Peter, sinds William hier in Groningen begon, hebben wij vaker samengewerkt. Ik waardeer alle tips, vaak omdat ze weer net uit een andere invalshoek kwamen. Bedankt ook voor het bestellen van die héérlijke biertjes in Philadelphia die niemand uiteindelijk op kon drinken.

Beste Sarah, jij verdient zeker een bedankje en aangezien jij een Brexit vluchteling bent doe ik dat in het Nederlands. Heel fijn dat jij mij hebt geholpen met de tube formation experimenten, met alle input voor mijn beursaanvraag en als voorbeeld dat een post-doc prima mee kan op borrels.

Beste Elisabeth, Berber, Henk (ik heb binnenkort weer een buisje HMVECs nodig trouwens), Geert en FACS-Johan, bedankt voor alle hulp als ik weer eens iets moest vragen, maar ook voor de goede sfeer op het lab. Beste Theo, bedankt voor alle goede inbreng tijdens de meetings, filmpjes en pubquizen!

Beste Minke, ik heb de laatste jaren veel tijd bij jou op lab doorgebracht, en helaas veel te veel uren hiervan werden besteed aan PBMCs isoleren. Bedankt voor de hulp hier mee en met de experimenten van hoofdstuk 4.

Beste Johan, jouw aanwezigheid was onmisbaar bij het maken van dit proefschrift. Soms zijn het maar kleine dingen, zoals antilichamen bestellen, maar soms ook grote dingen zoals samen de Luminex uitvoeren. Fijn dat je mij zelfs in Denemarken nog van advies en zelfs data analyses kon voorzien!

Dear editors of predatory journals, Greetings from Thesis of Yannick 2020! I hope you are doing great. Thank you for wishing me joy and prosperity in my life and that all my hard work will pay off in the end. Warm salutations to you too!

Ik heb veel geleerd van alle discussies en presentaties elke week bij de Vasculitis meeting en de Reumatologie meeting. Veel dank daarom aan Bram, Jan-Stephan, Nico, Hendrika, Hannie (hopelijk kan mijn kledingkeuze je ook bekoren tijdens mijn promotie!), Frans en iedereen die ik nog niet genoemd heb. Kiki, Janny en Marjolein, bedankt voor de hulp met alles wat maar geregeld moest worden, van zaaltjes tot adressenlijsten.

Ik waardeer de inzet van alle studenten met wie ik samen heb gewerkt (en die af en toe mijn PBMC isolatie konden overnemen). Janneke, Marjolein, Carolien, Marit, Hilde en Jannik: bedankt!

Ik vind het nog steeds bijzonder om terug te kijken op alle PhDs die ik heb leren kennen en nu over de hele wereld zijn uitgevlogen. Fleur, Judith, Christien, Lucas, Fiona, Koen, Qi, Gerjan, Anouk, Gerjan, Jolien, Gwenny, Jolien, Arno ('The Stud') en Marieke: bedankt voor het warme welkom, de goede samenwerking en de onvergetelijke borrels en PhD-weekenden.

Dear Qi, it was amazing to work with you on the monocyte subsets article, and I learned a lot from you. But moreover you apparently knew me so well you immediately expected it was Christien I was dating, when I hinted at something. It was so great staying with you and your family in New York, and I hope I can, at some point, pay you back the favor when you come back to Groningen!

Vooral aan het begin van je PhD loop je tegen allerlei zaken aan, zoals het vergoeden van congressen of het kiezen van de juiste flow antibodies. Het scheelde daarom veel dat ik Lucas tegenover mij had op de kamer. Lucas, of ook wel 'het Orakel van de 3e verdieping', jij hebt mij geweldig op weg geholpen in de eerste jaren van mijn PhD. Daarnaast was het super om jouw paranimf te zijn, ondanks mijn griep waardoor ik er uit zag als een lijk.

Er wordt natuurlijk hard gewerkt op onze kamer, maar er waren bepaalde zomerse dagen dat er opeens een aantal collega's, zoals Koen, op het zelfde moment opveerden. Bleek er opeens een massale valpartij te zijn in de Tour de France; het geeft wel een mooi soort van saamhorigheid! Gerjan, er is nog altijd geen definitieve winnaar uit onze strijd over welke cel cooler is, maar hopelijk kunnen we dat in de toekomst nog eens uitvechten! Beste Anouk, ik denk dat jouw idee van het PhD-weekend een traditie is die we nog lang zullen houden op onze afdeling. Veel succes in Australië! Over Australië gesproken, tof dat je weer terug bent Gwenny! Ik voel me vereerd dat de winnaar van zo veel prijzen toch nog zo normaal is gebleven om met ons op borrels te gaan.

In de loop van de afgelopen jaren kwamen er steeds meer nieuwe gezichten bij. Het was erg raar om opeens te beseffen dat ik op een gegeven moment opeens de langstzittende promovendus was. Ik weet nog goed dat ik eens een vraag van Rosanne beantwoorde en ik opeens dacht "wauw ik klink als Lucas". Raar. Met de nieuwe groep zijn weer nieuwe tradities begonnen zoals de epische kerstborrels waarbij mensen opeens ontzettend enthousiast kunnen zijn over kerstmutsen en lichtgevende whiskyglaasjes. Met de mensen die er bij zijn gekomen denk ik denk de afdeling er

een talentvolle en gezellige groep wetenschappers bij heeft gekregen! Lei, Jacolien, Rebeca, Idil, Rosanne, Xiaoyan, Mark, Carlo, Robin en Wietske: het was geweldig om jullie te leren kennen en veel succes met jullie onderzoeken!

During my PhD I have done a lot of immunohistochemistry experiments, but there can only be one expert: IHC King William the 1st. I really think you are a very talented researcher and I loved collaborating with you on the most awesome cells in the human body. Writing this, I am still laughing at your enthusiastic texts such as "Dude the YKL-40 luminex is awesome, you are gonna be very happy" or "This hospital is a maze of death".

Beste Rosanne, ik vond het erg gezellig dat iemand op de kamer een beetje mijn circadiane ritme had. Bedankt voor alle tips qua congresabstracts en foute e-mails van twijfelachtige journals. Veel succes/sterkte met het bijhouden van de biobank, volgens mij gaat dat helemaal goedkomen! Dear Rebeca, congratulations on your excellent PhD defense! At the time I wrote this you actually still had to defend, but I am sure it went great. Op de een of andere manier heb ik vooral herinneringen van Wietske buiten werk als we samen aan het zingen zijn. Dit was vaak in de karaoke bar maar kon ook prima de Jeugd van Tegenwoordig in de auto zijn. Voor herhaling vatbaar lijkt me!

Onderzoek doen is natuurlijk altijd een bevredigende bezigheid en nooit frustrerend. Maar alsnog heb ik de afgelopen jaren mijn best gedaan om ook buiten werk nog een sociaal leven te leiden. Vaak was dit sociale leven op zo'n 20 meter van het UMCG, bij café de Buurvrouw. Nu al weer 11,5 jaar geleden (ja toen was Hyves nog hip), hebben we elkaar leren kennen aan het begin van de studietijd, toch steeds zien we elkaar regelmatig: Mart, René, Randy, Roeland, Cynthia, Hessel, Tim en Ronald. Ik ben verbaasd dat we na zo'n lange tijd nog altijd zo goed kunnen lachen en bier drinken samen, ik waardeer het heel erg! De Fireman waardeer ik alleen nog steeds niet en waag het niet om dat ooit nog eens als cadeau te geven!

Sporten is een mooie uitlaatklep na een lange week typen. Alle vrienden die ik ken dankzij alle verregende tenniscompetitiedagen, kansloze zaalvoetbalavonden en helse wielrenweekenden (bijvoorbeeld Roosmarijn, Leoni, Joachim, Jelmer en Ed), ook jullie bedankt voor jullie steun en interesse!

Familie Rondaan, bedankt voor het warme welkom en de steun de afgelopen jaren!

Als mijn paranimfen heb ik Jacolien en Ronald gekozen, wat natuurlijk een enorme gok is, maar ik reken er op dat ze het goed zullen doen. Beste Jacolien, wij hebben al jaren lang gezamenlijk een wekelijkse meeting waarbij jij mij regelmatig goede adviezen hebt gegeven. Bovendien hebben we gezamenlijk heel mooi werk afgeleverd (al zeg ik het zelf) in onze publicatie over leukocyten subsets. Maar naast al die werk zaken vond ik het ook geweldig om zo'n toffe collega te hebben met wie ik altijd van alles kon bespreken. Sterker nog, het is beschamend genoeg al meerdere

keren gebeurd dat ik jou heb getroffen in ons lokale restaurant (dankzij jouw vurige aanprijzing). Ik weet zeker dat jij volgend jaar een super goed proefschrift gaat afleveren (en niet alleen omdat ons gezamenlijke hoofdstuk er in staat)!

Het is nu al weer een hele tijd terug dat ik uit de trein stapte in Haren en met een mede-eerstejaarsstudent, Ronald, naar college liep. We waren natuurlijk allebei te laat. Sindsdien hebben gelijke interesses en een gedeeld (vrij matig) gevoel voor humor er voor gezorgd dat we elkaar nog altijd wekelijks zien voor koffie of bier. Hoewel jij nu een wat andere richting bent opgeslagen, als Mister E-Learning/ Graaf TEL (ik kon niet kiezen), kunnen we nog regelmatig wat successen en frustraties over werk en andere dingen delen. Bedankt voor alle ondersteuning de afgelopen jaren, en super dat jij en Jacolien mijn paranimf willen zijn!

Ontzettend belangrijk voor mij was de steun van mijn hechte familie, in de bewogen afgelopen jaren. Papa en mama, jullie vertrouwen heeft mij altijd het doorzettingsvermogen gegeven om iets te maken waar ik zelf ook trots op ben. Rutger, Suzanne, Sebastian en Iris, bedankt dat jullie altijd je best deden om te luisteren als ik weer eens te lang aan het oreren was over cellen en Excellen. Sebas, jij hebt het jaren volgehouden om samen met mij in een huis te leven en te wachten tot ik eindelijk het beloofde eten zou meenemen, dat is niet te onderschatten.

Lieve Christien, het was wel even slikken toen ik de hele afdeling moest vertellen dat ik aan het daten was met een collega. Zo cliché natuurlijk. Toch is zelfs een jaar samen op het kantoor nooit saai geweest met jou en heeft het me juist altijd gesteund dat jij zo goed weet wat ik mee maak en waar ik mee bezig ben. We hebben de afgelopen jaren zoveel bijzondere dingen samen gedaan, veel van de wereld gezien en zelfs een (geweldige) kat in huis gehaald. Ik ben heel gelukkig dat ik jou ken en dat jij mij altijd hebt gesteund de afgelopen jaren!

ABOUT THE AUTHOR

Yannick van Sleen was born August 9th 1990 in Smilde, the Netherlands. In 2008 he graduated from VWO at the CS Vincent van Gogh in Assen. Hereafter he started the Bachelor Biology, with the major Behavioral and Neurosciences, at the University of Groningen. After completion of the Bachelor, he continued with the Master Biology. The two main research projects of the master were focused on the effects of overfeeding during pregnancy (under the supervision of Prof. Gertjan van Dijk) and the involvement of the formyl peptide receptor in glioblastoma (under the supervision of Prof. dr. Wilfred den Dunnen). His master thesis was about the immunological link between overfeeding and the development of neurodegenerative disease. In 2015 he started as a PhD candidate at the UMCG under the supervision of Prof. Annemieke Boots, Dr. Elisabeth Brouwer, Dr. Wayel Abdulahad and Dr. Kornelis van der Geest. This PhD project has resulted in this thesis, with the title 'Monocyte and Macrophage Heterogeneity in Giant Cell Arteritis and Polymyalgia Rheumatica: Central in Pathology and a Source of Clinically Relevant Biomarkers'. In 2019, he has worked in Aarhus, Denmark, as part of a follow-up project to validate work in this thesis and to continue his scientific career.