Ageing of innate immunity in health and vasculitic diseases
Wang, Qi

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CHAPTER 5

Altered distribution of monocyte subsets in giant cell arteritis and polymyalgia rheumatica

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¹Department of Rheumatology and Clinical Immunology, ²Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

* Contributed equally

MANUSCRIPT IN PREPARATION
ABSTRACT

Background
Macroangiitis are critical tissue destructive cells in the immunopathology of patients with giant cell arteritis (GCA) and polymyalgia rheumatica (PMR). As tissue macrophages in the vascular wall are derived from blood monocytes, we aimed to study the distribution of three different monocyte subsets in GCA and PMR patients and to investigate their traits in temporal artery biopsies (TAB) of GCA patients.

Methods
Total monocytes were enumerated in peripheral blood samples obtained from newly-diagnosed GCA and PMR patients and in follow-up samples after corticosteroid-induced remission. The distribution of circulating classical (CD14brightCD16-) and the more pro-inflammatory intermediate (CD14brightCD16+) and non-classical (CD14dimCD16+) monocyte subsets was analyzed by flow cytometry. The phenotype of macrophages in the different layers of the TAB was studied by immunohistochemistry and by double staining using immunofluorescence-labeled antibodies. Expression of chemokines in serum and in TAB were determined by multiplex assays and immunohistochemistry.

Results
Total monocyte numbers were found to be elevated in newly diagnosed GCA and PMR patients compared to HC and this was largely explained by elevated numbers of classical monocytes. The increase in classical monocytes led to significant proportional decreases of non-classical monocytes. Corticosteroid treatment suppressed numbers of non-classical monocytes in both GCA and PMR patients. Most macrophages in the TAB of GCA patients were found to express non-classical monocyte markers CD16 and CX3CR1. CX3CL1 was highly expressed in the TAB and systemic levels increased after corticosteroid treatment of GCA patients.

Conclusion
We report an altered distribution of monocyte subsets with a proportional decrease of non-classical monocytes in newly diagnosed GCA and PMR patients. Macrophages in the TAB of GCA patients resembled the phenotype of non-classical monocytes. Moreover, tissue migration of CD16+ non-classical monocytes is likely driven by the CX3CR1-CX3CL1 axis.
INTRODUCTION

Giant cell arteritis (GCA) and polymyalgia rheumatica (PMR) are two closely related syndromes affecting older people. GCA is a granulomatous vasculitis which mainly affects the medium and large arteries causing vascular occlusion leading to blindness or stroke [1]. GCA and PMR are known to frequently co-occur as 50% of GCA patients also have PMR [1]. PMR is a rheumatic disease characterized by pain and stiffness of both shoulders and hips and by systemic inflammation. GCA and PMR develop in people over 50 years of age with a median age of onset of 70. Therefore, it has been suggested that ageing-associated changes of the immune system and the vascular bed contribute in tandem to GCA development [2]. Corticosteroid therapy is currently the first choice treatment option for GCA and PMR, but long-term corticosteroid therapy is associated with severe side effects [3]. 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 GCA and PMR is complex and not yet well understood. There is consensus, however, that GCA pathology is characterized by local dendritic cell activation followed by infiltration of CD4+ T-cells and monocytes/macrophages into the vessel wall niche through the vaso vasorum [4]. Within the vessel wall, migrated monocytes/macrophages produce pro-inflammatory cytokines and matrix metalloproteases thereby 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 [5]. Three monocyte subsets can be distinguished by phenotypic and functional characteristics: classical monocytes (CD14brightCD16-), intermediate monocytes (CD14brightCD16+) and non-classical monocytes (CD14dimCD16+) [6]. CD14brightCD16- 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 [7]. CD16+ monocytes are the more mature cells compared to the classical monocytes; a developmental relationship has been established, and their numbers increase with age [8]. Importantly, increased proportions of CD16+ monocytes have been associated with numerous vascular and inflammatory diseases like rheumatoid arthritis [9], sarcoidosis [10, 11], SLE [7] and ANCA-associated vasculitis [12, 13].

To study the contribution of monocytes/macrophages to the immunopathogenesis of GCA, it is crucial to understand the precursors of the tissue macrophages and the factors involved in tissue migration. Tissue migration of different monocyte subsets is determined by differential expression of chemokine receptors [14]. Classical monocytes show a marked CCR2brightCX3CR1dim expression whereas non-classical
Monocytes show CCR2\textsuperscript{neg}CX3CR1\textsuperscript{bright} expression [15]. Also, CD16+ monocytes show an increased capacity to adhere to endothelial cells and thereby more readily migrate across the endothelium when compared to CD16-negative monocytes [16, 17]. Migration of CD16+ monocytes is guided by fractalkine (CX3CL1) – CX3CR1 interaction and inhibition of this interaction reduces transmigration [18, 19].

GCA and PMR are ageing-related inflammatory syndromes and 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 and PMR pathogenesis. We therefore studied monocyte subset distribution in newly diagnosed GCA and PMR patients and effects of steroid treatment on these subsets. Next, we assessed if CD16 was expressed by macrophages in temporal arteries 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 population
In this prospective study, 43 newly-diagnosed patients with GCA (n=22) and PMR (n=21) were consecutively enrolled (Table 1). None of the patients received corticosteroids or disease-modifying anti-rheumatic drugs (DMARDs) at the time of blood withdrawal. GCA patients either fulfilled the ACR 1990 classification criteria or had cranial symptoms of GCA (new or different headache, scalp or temporal artery tenderness, ischemia-related vision loss, or jaw claudication or had systemic (fever, weight loss, malaise, night sweats) or PMR symptoms, defined as proximal morning stiffness and pain longer than 60 minutes in neck shoulder or pelvic region (at least 2 regions) together with a positive biopsy or positive 18FDG PET/CT scan performed before start of steroids using “vascular uptake > liver” as cutoff point as most appropriate [20]. PMR patients needed to have a morning stiffness longer than 1 hour, to fulfill the Chuang/Hunder criteria bilateral pain (for at least one month) involving 2 of the following areas neck/torso, shoulders and pelvic girdle/upper legs, age older than 50 years, ESR above 40 mm/hr, and exclusion of other diagnoses.

We collected 30 follow-up samples of GCA (n=15) and PMR (n=15) patients, who were in remission after 3 months of corticosteroid treatment. Remission was defined as absence of symptoms and a normal ESR (<30 mm/hr). Eleven of the 22 newly-diagnosed GCA patients were included in the TAB study (Table 2). As controls, we obtained blood samples from 24 age-matched, healthy controls (HC) who were screened for past or actual morbidities. Written informed consent was
Table 1. Baseline characteristics of patients and controls in the peripheral blood analyses

<table>
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<tr>
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<th>GCA patients (N = 22)</th>
<th>PMR (N=21)</th>
<th>HC (N = 24)</th>
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<td>Age (years); Median (range)</td>
<td>71 (52-81)</td>
<td>70 (54-84)</td>
<td>72 (53-83)</td>
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<tr>
<td>Females (%)</td>
<td>77</td>
<td>71</td>
<td>75</td>
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<tr>
<td>BMI (kg/m2); Median (range)</td>
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<td>25.7 (19.2-35.8)</td>
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<td>1633 (1033-3300)</td>
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<tr>
<td>Fullfilling ACR/Chuang criteria</td>
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<td>21</td>
<td>NA</td>
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<tr>
<td>Positive biopsies / Total number of biopsies</td>
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<td>NA</td>
</tr>
<tr>
<td>Positive PET scan/Total number of PET scans</td>
<td>15/18</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Leukocytes (10^9/L); Median (Range)</td>
<td>9.2 (5.0-18.4) ***</td>
<td>8.7 (4.5-14.4) ***</td>
<td>6.0 (4.2-9.6) ***</td>
</tr>
<tr>
<td>Hb (mmol/l); Median (Range)</td>
<td>7.0 (5.5-8.5) ***</td>
<td>7.4 (5.6-9.3) ***</td>
<td>8.7 (7.2-9.9) ***</td>
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<tr>
<td>ESR (mm/h); Median (Range)</td>
<td>65 (31-118) ***</td>
<td>52 (30-124) ***</td>
<td>12 (2-30)</td>
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<tr>
<td>CRP (mg/l); Median (Range)</td>
<td>47 (11-138) ***</td>
<td>46 (7-186) ***</td>
<td>2 (2-5)</td>
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</tbody>
</table>

Characteristics of newly diagnosed corticosteroid/DMARD-free GCA (nGCA) and PMR patients (nPMR) and of healthy controls (HC). As data are not normally distributed, the Kruskal-Wallis test was performed to compare data among study groups (HC, GCA and PMR). The Mann-Whitney U test was used to compare each patient group with healthy controls. LVV = large vessel vasculitis. TAB = temporal artery biopsy. ESR = erythrocyte sedimentation rate. CRP = C-reactive protein. NA = not applicable. ***p value <0.001 compared with HC.

Table 2. Patient characteristics of the TAB study

<table>
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<tr>
<td>Number of Subjects</td>
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<tr>
<td>Sex (female/male)</td>
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</tr>
<tr>
<td>Age (years)</td>
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</tr>
<tr>
<td>Positive TAB/positive PET scan/both /neither</td>
<td>10/4/6/2</td>
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<tr>
<td>PMR co-occurrence</td>
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<tr>
<td>Number of subjects starting glucocorticoid treatment at the time of biopsy</td>
<td>5</td>
</tr>
</tbody>
</table>

Characteristics of the GCA patients participating in the TAB study. Eleven of these GCA patients also participated in the peripheral blood analysis study.
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 prednisolone, respectively. Tapering of corticosteroid treatment was started after 3-4 weeks and was further continued based on clinical and laboratory findings during follow-up. After 3 months, the median prednisolone dose was 25 mg/day (range 15-50) in GCA patients and 15mg/day (range 5-17.5) in PMR patients.

Flowcytometry
Absolute numbers of monocytes in freshly drawn blood samples were determined by BD MultiTest TruCount, as described by the manufacturer. Data were acquired on a FACS Canto-II (BD Biosciences) and analyzed with FACSCanto Clinical Software (BD). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood with Lymphoprep (Axis-Shield) and 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 monoclonal antibodies to quantify monocyte subsets and their chemokine receptor expression: CD3, CD14, CD16, CD19, CD56, CD66b, CCR2, CX3CR1 (see product information in supplementary Table 1). Proper isotype controls were included. Cells were fixed and analyzed using a LSR-II (BD) flowcytometer. Flowcytometry data was analyzed using Kaluza software (BD). Classical (CD14brightCD16-), intermediate (CD14brightCD16+) and non-classical (CD14dimCD16+) monocytes subsets were gated as previously described [21].

Immunohistochemistry
The temporal artery biopsies studied were obtained from 16 biopsy positive GCA patients (12 patients were without any treatment at the time of biopsy, 4 patients started corticosteroid treatment 2-14 days before the biopsy was taken) and from 6 biopsy negative GCA patients (5 patients were without any treatment at the time of biopsy, 1 patient started corticosteroid treatment 1 week before the biopsy was taken, Table 2). The tissue was fixed in 10% neutral buffered formalin and paraffin embedded. Sections of 3 μm thickness were deparaffinized in xylene and rehydrated in graded ethanol. After antigen retrieval by EDTA buffer (1mM, pH 9.0) 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 (see product information
in supplementary Table 2). Proper isotype controls were also included. Next, slides were incubated with secondary antibody rabbit anti-mouse HRP (DAKO P0260) or Goat anti-rabbit HPR (DAKO P0448). Following washing, slides were incubated with peroxidase-DAKO Envision polymer (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% estimated positive), 3 = moderate numbers of positive cells (20-50% estimated positive), 4 = high numbers of positive cells (more than 50% estimated positive). 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>1x10^4 µm^2) within infiltrated areas using software of Aperio ImageScope (V11.2.0.780 Aperio Technologies, CA, USA. Setting: Hue value-0.03, Hue width-0.5, Color saturation threshold-0.04).

Immunofluorescence
To identify if CD68 and CD16 are co-expressed by cells in situ, and to exclude CCR2 expression by CD16 positive cells, a double-labeling immunofluorescence protocol was used. Formalin-fixed paraffin-embedded temporal artery biopsy tissue was deparaffinized and antigen retrieval was performed as described above for IHC. Anti-CD68 (dilution 1:50), anti-CD16 (dilution 1:50) or anti-CCR2 antibodies (dilution 1:50) were added and incubated overnight. Following washing, FITC-labeled goat anti-rabbit IgG (A11008, Lifetechnologies, Carlsbad, CA, USA) and AF555-labeled goat 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 measurement
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 (mean fluorescence intensity, MFI) were analyzed 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 flowcytometry 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 non paired data of patient groups with healthy controls. 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 of the IHC data (e.g. CD16 and CD68 scores in the different layers of the vascular wall) 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
Absolute numbers of circulating monocytes were enumerated in peripheral blood of patients who were newly diagnosed as having GCA or PMR and compared to age- and sex- matched healthy controls (Table 1). Numbers of circulating monocytes were clearly higher in patients with newly diagnosed GCA (nGCA) or PMR (nPMR) compared to healthy controls (Figure 1A). In addition, we assessed the effects of prednisolone after 3 months of treatment. Three months of treatment led to normalization of monocyte numbers in remission PMR patients (rPMR) but this was not the case for monocyte numbers in remission GCA patients (rGCA) patients (Figure 1B and C).

Altered distribution of circulating monocyte subsets in GCA and PMR
As a clear monocytosis was observed in newly diagnosed GCA/PMR, we next analyzed the distribution of the three different monocyte populations defined by CD14 and CD16 expression (Figure 2A). Our further analysis revealed numerical increases of classical monocytes in nGCA and nPMR patients when compared to healthy controls, whereas numbers of intermediate and non-classical monocytes were largely comparable (Figure 2B and Suppl Figure 1A). The increase in classical
monocytes led to significant proportional decreases of non-classical monocytes in both nGCA and nPMR patients, but did not significantly alter intermediate type monocyte proportions in nGCA and nPMR patients (Suppl Figure 1B). Following prednisolone treatment, all three monocyte subsets were significantly reduced in PMR patients (Figure 2B). Interestingly, prednisolone treatment reduced numbers of non-classical monocytes in rGCA patients 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.

Figure 1. Total monocyte counts are elevated in newly diagnosed GCA and PMR patients. (A) Absolute numbers of monocytes in freshly drawn whole blood obtained from healthy controls (HCs, n=20), newly-diagnosed patients with GCA (nGCA; n=21) and PMR (nPMR; n=20). (B) Absolute numbers of monocytes from nGCA patients samples (n=21) and in follow-up samples from GCA patients in remission after 3 months of treatment (rGCA; n=14). (C) Absolute numbers of monocytes in freshly drawn whole blood obtained from nPMR patients samples (n=20) and in follow-up samples from PMR patients in remission after 3 month of treatment (rPMR; n=15). Data are expressed as box and whisker plots. The Mann-Whitney U test was used to compare each patient group with HC. Statistical significance is indicated as *** p value <0.001. Paired samples (e.g. nGCA vs rGCA and nPMR vs rPMR) were compared with the Wilcoxon signed rank test. Statistical significance is indicated as + p value <0.05.
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 (CD14brightCD16−), intermediate (CD14brightCD16+) and non-classical (CD14dimCD16+) monocytes subsets (left panel). A representative flow cytometry dot plot showing samples from one HC, one newly diagnosed GCA patient (nGCA) and one newly diagnosed PMR patient (nPMR) is shown in the right panel. (B) 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=20) 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 box and whisker plots. 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. Statistical significance is indicated as *** p value <0.001. Paired samples were compared with the Wilcoxon signed rank test. Statistical significance is indicated as ++ p value <0.01, +++ p value <0.001.
CD16+ macrophages are readily detected in GCA TAB lesions

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 positive and negative (skip lesions) GCA biopsies and evaluated expression of CD16 and of macrophage marker CD68 (Figure 3A). All positive GCA biopsies studied showed evidence of transmural inflammation. CD68 and CD16 were both abundantly expressed within the infiltrates of the adventitia, media and intima layer of the vessel wall (Figure 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 (Figure 3C).

To rule out an involvement of NK cells which can also express CD16, we stained for CD56 and found this marker to be virtually absent in the vessel wall (data not shown).

To further confirm the co-localization of CD68 and CD16, double staining using immunofluorescence was performed. Indeed, within infiltrated regions, substantial co-localization of CD16 and CD68 was found, showing that the majority of macrophages in the vascular wall express CD16 (Figure 3D).

Monocyte CCR2 and CX3CR1 expression in GCA and PMR patients

Recruitment of monocytes to peripheral sites of inflammation is driven by specific chemokine and chemokine receptor interactions. Interestingly, the three different monocyte subsets can also be distinguished by differences in their chemokine receptor expression profiles, in particular by CCR2 and CX3CR1 (fractalkine receptor). Therefore, we assessed expression of CCR2 and CX3CR1 on classical, intermediate and non-classical monocytes. In accordance with previous studies [22], classical monocytes demonstrated a high per cell expression (MFI) of CCR2 with low expression of CX3CR1, while non-classical monocytes demonstrated high per cell expression of CX3CR1 and a lack of CCR2 expression (Figure 4A and B).

Following corticosteroid treatment, CX3CR1 expression was down-modulated by all monocyte subsets in rGCA and rPMR patients (Suppl Figure 2). In contrast, CCR2 expression by monocyte subsets was not sensitive to steroid treatment.

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 (Table 3). Systemic levels of CCL2 and CCL11, the CCR2 receptor ligands, were significantly lower in nGCA patients but normalized after corticosteroid 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 and CCL26 were not altered in GCA and PMR patients when compared to healthy controls. Steroid treatment upregulated serum levels of CX3CL1 in rGCA patients.
Figure 3. CD16+ cells co-localize with CD68+ macrophages in GCA temporal artery biopsy lesions. (A) Immunohistochemistry staining for CD68 and CD16 in a representative inflamed temporal artery biopsy (TAB) positive GCA patient. (B) Semi-quantitative mean scores of CD68+ cells and CD16+ cells in inflammatory areas of 16 TAB positive GCA patients. 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 16 TAB positive GCA patients, as determined by Spearman’s rank correlation coefficient. (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 positive GCA patient. 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.
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 (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 temporal arteries

Next, we determined expression of CCR2, CX3CR1 and their ligands in the inflamed arteries of GCA patients. In non-inflamed temporal artery biopsies, only slight CCR2 and CX3CR1 expression by vascular smooth muscle cells (VSMCs) was detected (data not shown). In the inflamed vascular wall, high numbers (median score 3-4) of CX3CR1-positive cells were detected (Figure 4C and D; Suppl Figure 3 for isotype control stainings). CX3CR1-positive cells were clearly detected in macrophage-rich areas of adventitia, media and intima. In contrast, only few to moderate numbers of CCR2-positive cells (median scores 2-3) were found in these same areas (Figure 4C and D). Double staining for CD16 and CCR2 confirmed that CD16+ cells rarely co-localize with CCR2 (Suppl Figure 4).

Table 3. Serum levels of chemokines in healthy controls and in GCA and PMR patients before and after CS treatment.

<table>
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<th>HC</th>
<th>nGCA</th>
<th>rGCA</th>
<th>nPMR</th>
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<tr>
<td><strong>CCR2 ligands</strong></td>
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</table>

Systemic levels of CCL2 and CCL11 (CCR2 receptor ligands) and CX3CL1 and CCL26 (CX3CR1 ligands) were measured in healthy controls (HCs, 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. Statistical significance is indicated as * p value <0.05, ** p value <0.01. Paired samples were compared with the Wilcoxon signed rank test. Statistical significance is indicated as + p value <0.05, ++ p value <0.01.
Figure 4. Differential expression of CCR2 and CX3CR1 by monocyte subsets in peripheral blood of GCA and PMR and in GCA temporal artery biopsies. (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 non-classical, intermediate and classical monocytes in newly diagnosed GCA patients (nGCA; n =21). Data are expressed as box-and-whisker plots. (C) Immunohistochemistry staining for CCR2 and CX3CR1 in a representative temporal artery biopsy specimen from a GCA patient. (D) Semi-quantitative score of CCR2-, CX3CR1-positive cells in the inflammatory area of inflamed temporal artery biopsy specimens from 16 GCA patients. Intensity of staining was not taken into account. Scores are given for the adventitia (Adv), media (Med, only the infiltrating cells) and intima (Int). Scores of 2 investigators were averaged. Data are presented as box and whisker plots with the median value indicated. (E) Immunohistochemistry staining for CCL2, CX3CL1 in a representative inflamed temporal artery biopsy specimen from a GCA patient (F). Quantification of staining intensity (positive pixels/total number of pixels) of CCL2 and CX3CL1 in the inflammatory vessel wall was performed using software of ImageScope Viewer. Data are presented as box and whisker plots with the median value indicated.
Next, we analyzed tissue expression of the chemokines CCL2 and CX3CL1. Both in inflamed and non-inflamed temporal artery biopsies, we detected expression of CCL2 and CX3CL1 by vascular smooth muscle cells. Quantification of CCL2 and CX3CL1 in inflamed temporal arteries of GCA demonstrated expression of both chemokine receptor ligands in all three vessel layers with highest expression in the media (Figure 4E and F). Thus, the phenotype of tissue macrophages in GCA TAB lesions resembles the phenotype of the non-classical monocytes in blood. In addition, high expression of CX3CR1 by non-classical monocytes in the inflamed vessels suggests tissue migration of non-classical monocytes driven by the CX3CR1-CX3CL1 axis.

DISCUSSION

Monocytes/macrophages are critical contributors to vascular inflammation. Improved understanding of the role of monocyte/macrophages in GCA and PMR pathogenesis is expected to provide a rational for novel treatment options for these patients. Our studies revealed a clear monocytosis in newly diagnosed GCA/PMR patients which is consistent with the notion of inflammation-induced monocyte recruitment from the bone marrow as observed in other inflammatory diseases [23, 24]. When analysing the contribution of the different monocyte subsets to this rise in total blood monocytes, we found numbers of classical monocytes to be significantly 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, corticosteroid treatment demonstrated further reduction of 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 bone marrow [25, 26], monocyte recruitment from the bone marrow niche is largely driven by the CCL2/CCR2 pathway which would explain preferential recruitment of classical monocytes expressing high levels of CCR2 [27]. In line with this notion, we found systemic CCL2 levels decreased in newly diagnosed GCA patients, suggesting binding of this ligand to increased numbers of classical monocytes. Corticosteroid treatment normalised serum levels of CCL2. A similar pattern was observed in PMR patients. Classical monocytes may consequently give rise to intermediate and non-classical monocytes [8, 28]. However, in newly-diagnosed GCA and PMR patients we did not detect an increase of intermediate or non-classical monocytes.

Although monocytosis is a characteristic of many inflammatory diseases, such as rheumatoid arthritis [23], Crohn’s disease [24] and sepsis [29], these studies all showed elevated numbers or proportions of CD16+ monocytes [30-32]. Yet,
our study in newly diagnosed GCA and PMR patients showed a remarkable, proportional decrease of non-classical monocytes particularly. This may be explained by the selective loss of non-classical monocytes through spontaneous apoptosis, to which the CD16+ monocytes are more susceptible [33]. This explanation is unlikely, however, as CX3CR1, highly expressed by non-classical monocytes enforces survival of CD16+ monocytes [34] and CX3CR1 expression by non-classical monocytes was found unaltered in the newly diagnosed GCA/PMR patients compared to healthy controls (data not shown). Alternatively, the proportional decrease of non-classical monocytes could be due to blunted (cause unknown) differentiation of intermediate monocytes to non-classical monocytes. However, this is also unlikely as we did not notice an accumulation of intermediate monocytes. Thus, we sought for an alternative explanation for the relative decrease of non-classical monocytes in GCA/PMR.

Previous studies have shed light on the role of non-classical monocytes in homeostasis and in inflammation. It has become more and more clear that non-classical monocytes actively patrol the vascular endothelium and are preferentially found in the marginal pool [35, 36]. 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 [36, 37]. In the resting state, they clear damaged cells and debris and in infection or inflammation they are thought to be important in wound healing and 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 [7, 30]. More recently, Mukherjee et al showed that non-classical monocytes, investigated following a robust gating approach of untouched cells, indeed become pro-inflammatory upon activation [7, 38]. 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 [35], thereby facilitating preferential migration of non-classical monocytes to sites of inflammation in GCA and PMR. This notion was studied here in GCA temporal artery tissue only.

We here describe for the first time, a massive accumulation of CD16+ macrophages in temporal arteries 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 injury in GCA [39], this transmural inflammation is likely achieved following entry via the vasa vasora in the adventitial layer, progressing to the medial and intimal layers of the vascular wall. Accumulation of CX3CR1+CD16+ macrophages, not co-localizing with CCR2, indicates that most of the macrophages in the GCA TAB resemble the phenotype of non-classical monocytes in blood. Although we found a predominance of macrophages resembling the phenotype
of non-classical monocytes in GCA TAB, we cannot exclude the possibility that both CD16 and CX3CR1 expression can be acquired after migration of classical monocytes to tissue. Indeed, a prior study showed that IL-10 can induce classical monocytes to become CD16+ macrophages [40]. Interestingly, systemic IL-10 levels are increased in newly-diagnosed GCA patients [41]. Yet, we are not aware of any data on IL-10 expression in GCA temporal artery tissue and therefore, local IL-10 expression should be studied. Also, classical monocytes may gain CX3CR1 and lose CCR2 upon migration to tissue [42]. Furthermore, it has been proposed that local proliferation of tissue-resident precursors may give rise to macrophage expansion at local sites rather than infiltration by monocytes [43]. The latter option, however, is not likely here as normal vessels do not show infiltrates. Moreover, our preliminary data employing the proliferation marker Ki-67 was largely negative in inflamed temporal artery tissues of GCA patients (data not shown). Taken together, it is highly unlikely that accumulation of macrophages in the TAB of GCA patients is caused by proliferation of resident cells. Thus, monocytes are likely the precursors of tissue macrophages in the inflamed vessel wall. Although the majority of tissue macrophages was found to express CD16 and were largely negative for CCR2, it remains to be established if non-classical monocytes are the only precursors of tissue macrophages in GCA.

The strong local expression of CX3CL1 in the GCA temporal artery is consistent with massive expression of CX3CR1. It is therefore highly possible that the presence of CD16+ monocytes in the tissue of GCA is 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 [44]. It was also evidenced that in the absence of CCL2 action, i.e., in CCR2-/- mice, monocytes can still traffic into sites of infection [27]. Importantly, many studies described the importance for chemokine receptor CX3CR1 for tissue migration and survival [19, 45, 46].

Furthermore, our study illustrated that corticosteroid treatment led to clear reductions of non-classical monocyte counts in both GCA and PMR patients. Steroid treatment has been described to induce apoptosis of non-classical monocytes via a caspase-dependent mechanism [47, 48]. Selective GC-induced apoptosis of non-classical monocytes may be explained by high expression of the glucocorticoid receptor (GR) in this monocyte subset [47]. Whether GC treatment will also deplete the CD16+ macrophages in the GCA temporal artery, or in PMR synovial tissues, is not clear yet and awaits further studies. Corticosteroid treatment led to clear reduction of CX3CR1 per cell expression by peripheral blood
monocytes and an upregulation of serum CX3CL1. Thus, steroid 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 migration of CD16+ monocyte to 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. This is an unexpected finding as steroid dose, and also the accumulated steroid dose, is substantially lower in PMR patients. An unexplored possibility is that classical monocytes in PMR are more sensitive to GC treatment due to their altered characteristics as evidence exists that monocytes can be functionally different in PMR patients compared with GCA patients and HC [49], a notion to be further explored. Taken together, the presence of CD16+ macrophages in GCA temporal artery tissue as well as their sensitivity in response to effective steroid treatment point towards an immuno-pathogenic role of CD16+ monocytes in the development of GCA and possibly PMR.

In conclusion, we report an altered monocyte subset distribution in GCA/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. Tissue migration of CD16+ non-classical monocytes is likely driven by the CX3CR1-CX3CL1 axis. Further study is required to investigate whether CD16+ monocytes are indeed the main precursors of CD16+ tissue macrophages with pro-inflammatory and tissue destructive properties. New insights into the role of monocytes in the immunopathogenesis of large vessel vasculitis as seen in GCA and PMR may provide a rational for novel treatment options and may help the identification of highly awaited, disease-specific biomarkers.
REFERENCES


27. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. Nature immunology 2006;7(3):311-17


37. Rennert K, Heisig K, Groeger M, et al. 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 2016;83:41-52
## SUPPLEMENTARY MATERIAL

### Supplementary Table 1. Primary antibody information used in flow cytometry

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### Supplementary Table 2. Primary antibody information used in immunohistochemistry

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# 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=20) 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 based on HC (n=24), nGCA (n=22) and nPMR (n=21). The Kruskal-Wallis test was performed to compare data among study groups (HC, GCA and PMR). The Mann-Whitney U test was used to compare nGCA and nPMR with HC, respectively. Statistical significance is indicated as ** p value <0.01; *** p value <0.001. Paired samples were compared with the Wilcoxon signed rank test. Statistical significance is indicated as ++ p value <0.01, +++ p value <0.001.
Supplementary Figure 2. CX3CR1 expression but not CCR2 expression on monocyte subsets is modulated by steroid treatment in GCA and PMR. Mean fluorescence intensity (MFI) of CCR2 (A) and CX3CR1 (B) on classical, intermediate and non-classical monocytes in newly diagnosed GCA (nGCA; n=21) and PMR (nPMR; n=21) 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 data. The Wilcoxon signed rank test was used to compare paired samples. Statistical significance is indicated as +++ p value <0.001.
Supplementary Figure 3. Isotype controls for immunohistochemistry staining. Immunohistochemistry 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 4. CCR2 and CD16 do not co-localize in temporal artery biopsy specimens from GCA patients. Single staining for CD16, CCR2 and double staining for CD16 and CCR2 respectively from left to right in the a temporal artery positive from a GCA patient. Blue= DAPI staining of nuclei; Green(FITC)= CD16 expression, Red(AF555)= CCR2 expression.