The absolute number of circulating nonclassical (CD14⁺CD16⁺⁺) monocytes negatively correlates with DAS28 and swollen joint count in patients with peripheral spondyloarthritis

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KEY WORDS
Disease Activity Score 28, monocytes, spondyloarthritis

ABSTRACT

INTRODUCTION A different clinical course and pattern of skeletal involvement in peripheral and axial spondyloarthritis (SpA) suggests a distinct pathophysiology of these 2 phenotypic manifestations of SpA. Monocytes, as part of the innate immune system, seem to play an important role in the pathogenesis of SpA, but the exact inflammatory pathways remain to be elucidated. Regulatory T lymphocytes (T_{reg}) and Th17 lymphocytes are also known to influence proinflammatory and anti-inflammatory reactions.

OBJECTIVES The aim of our study was to compare the absolute numbers of monocyte subpopulations, T_{reg} and Th17 lymphocytes with clinical measures of disease activity in patients with peripheral and axial SpA.

PATIENTS AND METHODS We enrolled 21 patients with peripheral SpA and 27 patients with axial SpA diagnosed according to the Assessment of SpondyloArthritis International Society classification criteria, as well as 23 healthy controls. Patients were under 45 years, naïve to synthetic and biological disease-modifying antirheumatic drugs and without the administration of systemic glucocorticoids. The absolute numbers of classical, intermediate, nonclassical monocytes, T_{reg}, and Th17 in peripheral blood were analyzed. Disease activity was assessed using the Ankylosing Spondylitis Disease Activity Score (ASDAS-CRP), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), and Disease Activity Score 28 (DAS28).

RESULTS In patients with SpA, the number of circulating nonclassical monocytes was decreased in comparison with controls. Only in the peripheral SpA group, a significant negative correlation was found between the concentration of nonclassical monocytes and DAS28 and the number of swollen joints. The 3 groups did not differ in terms of the concentrations of classical or intermediate monocytes and T_{reg} or Th17 lymphocytes.

CONCLUSIONS Nonclassical monocytes may play a role in induction and perpetuation of peripheral joint inflammation, at least in peripheral SpA, as cells infiltrating the synovium.

INTRODUCTION Spondyloarthritis (SpA) is a heterogeneous group of diseases that can be classified as axial or peripheral SpA, according to the Assessment of SpondyloArthritis International Society classification criteria.¹ ¹ The classification depends on the predominance of axial (sacroiliitis, spondylitis) or peripheral (arthritis, enthesitis, dactylitis) symptoms. While joint involvement in peripheral SpA is related to more expressed pain and stiffness of peripheral tissues, axial disease...
leads to more pronounced new bone formation and ankylosis of the spine and sacroiliac joints. Differences in the clinical course and pattern of skeleton and joint involvement suggest a distinct pathomechanism of these 2 subsets of SpA, but the exact inflammatory and bone remodeling mechanisms, especially in early disease, remain unknown.

Monocytes, as part of the innate immune system, seem to play an important role in the pathogenesis of SpA. Under physiological and inflammatory conditions, circulating monocytes, upon leaving blood vessels, may transform into tissue macrophages, antigen-presenting cells, and osteoclasts. Monocytes are a heterogeneous cell population, divided at least into 3 subsets: classical (CD14++CD16–), intermediate (CD14–CD16+), and nonclassical (CD14–CD16–) cells. The latter 2 subsets, collectively called CD16+ monocytes, are considered to possess proinflammatory activities. Moreover, several studies revealed the role of CD16+ monocytes as precursors of osteoclasts in psoriatic arthritis (PsA) or promoters of the expansion of the Th17 lymphocytes in rheumatoid arthritis (RA). However, each monocyte subset may express distinct, sometimes contradictory functions in inflammation.

Nonclassical monocytes form 5% to 15% of circulating monocyte pool and, in comparison with the classical subtype, are considered as more mature. They also have a higher expression of human leukocyte antigens (HLA) class II (mostly HLA-DR). After stimulation with bacterial products (ie, lipopolysaccharide), they express and secrete large amounts of proinflammatory cytokines, for example, tumor necrosis factor (TNF) and interleukins (IL) IL-12 and IL-1, and almost no anti-inflammatory IL-10. They also exhibit a lower capacity to phagocytosis but higher ability to present antigens, that is why they seem to functionally resemble dendritic cells. Due to the high expression of the integrins of the β family (CD11a, CD11c, and CD18), very late antigen 4 (VLA-4), fractalkine receptor CX3CR1, and chemokine receptor CCR5, they tend to strongly adhere to the endothelium, which may lead to enhanced transmigration to inflammatory lesions.

By contrast, classical monocytes have been suggested to have a potential to produce anti-inflammatory IL-10 and exhibit a relatively high phagocytic activity. Intermediate monocytes are suggested to be the primary producers of soluble CD18 integrins, which inhibit leukocyte migration by antagonistic binding to intercellular adhesion molecule 1 (ICAM-1) in the endothelium and synovium. They form a transient pool of circulating monocytes that functionally resemble nonclassical cells.

Several studies suggested an important role of CD16+ monocytes (intermediate and nonclassical) in the pathogenesis of different inflammatory rheumatic diseases. In patients with RA, an increased monocyte count (especially the CD14+CD16++ subpopulation) correlates with clinical manifestations and elevated inflammatory parameters localized in periarticular tissue. Migration of this subpopulation to synovial tissue is mediated by a fractalkine interaction with CX3CR1. Moreover, dendritic cells originating from migrating monocytes seem to participate in osteogenesis and inflammation-mediated destruction of bone tissue in inflammatory arthritis. Patients with RA present a significantly higher percentage of CD16+ monocytes, as compared with healthy subjects, with a positive correlation between the Disease Activity Score 28 (DAS28) and ultrasound composite score US7. Patients with PsA and psoriasis have a higher percentage of circulating nonclassical CD14–CD16+ cells than healthy controls. On the contrary, in patients with axial SpA and ankylosing spondylitis (AS), the percentage of intermediate and nonclassical subsets was reduced, as compared with healthy controls. In a recent study by Perpétuo et al, a lower frequency of nonclassical monocytes was reported in AS, but there were no differences in classical and intermediate monocytes. It was also suggested that monocytes favor maintenance of inflammation in periaricular tissues in patients with AS.

Previous studies suggested the involvement of particular monocyte subsets in the pathogenesis of inflammation in SpA (Table 1), although the results were contradictory. To our best knowledge, so far there have been no data linking the absolute numbers of monocyte subpopulations in the context of $T_{reg}$ and Th17 lymphocytes with clinical measures of joint inflammation in peripheral and axial SpA.

**PATIENTS AND METHODS**

**Patients and controls** We enrolled 27 patients with axial SpA and 21 patients with peripheral SpA, diagnosed according to the Assessment of SpondyloArthritis International Society classification criteria, as well as 23 healthy controls. Eligible patients were younger than 45 years of age, were naive to treatment with synthetic or biological disease-modifying antirheumatic drugs, and were not using systemic glucocorticoids. Patients provided a signed informed consent and the study protocol was approved by a local bioethics committee.

**Cytometric analysis** Whole peripheral blood samples from patients with SpA and from healthy controls were drawn to EDTA-containing tubes (Vacutainer System; Becton Dickinson Biosciences, San Jose, California, United States). The monocyte subsets were analyzed as previously described by our group and others. Briefly, blood samples were washed with 0.9% sodium chloride in polypropylene round-bottom tubes (BD Biosciences) and centrifuged (1000 × g). Then, cell suspension was placed in the TruCOUNT™ tubes (BD Biosciences) along with monoclonal antibodies (mAbs): anti–CD45-APC, anti–HLA-DR-PerCP, anti–CD14-FITC, and anti–CD16-PE (BD Biosciences), and incubated for 30 minutes at 4°C.
The samples were then treated with FACS Lysing Solution (BD Biosciences) until erythrocyte lysis and immediately processed in the FACS Canto flow cytometer (Becton Dickinson Immunocytometry Systems, BD Biosciences). The absolute numbers of monocytes were calculated with reference to the bead count.

Peripheral blood samples were also incubated in the TruCOUNT™ tubes with anti–CD3–FITC and anti–CD4–PE (BD Simultest, BD Biosciences) mAbs, followed by erythrocyte lysis and analysis on a FACS Canto flow cytometer, in order to count the absolute numbers of CD3⁻/CD4⁺ T lymphocytes.

For Treg and Th17 lymphocyte analysis, peripheral blood mononuclear cells (PBMC) were isolated from EDTA-treated peripheral blood samples by standard Ficoll-Paque density gradient centrifugation (Pharmacia, Uppsala, Sweden). PBMC samples were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and streptomycin (50 μg/ml, Gibco BRL, Karlsruhe, Germany). Then, PBMC samples were stimulated with PMA (phorbol-12-myristate-13-acetate) and ionomycin (at 50 ng/ml and 1 μg/ml, respectively) for 5 hours at 37°C. To inhibit cytokine secretion, monensin (2 μM, Golgistop Protein Transport Inhibitor, BD Biosciences) was added at the beginning of the culture. Next, the cells were harvested and stained with the following mAbs: anti–CD4–PerCP-Cy5.5, anti–IL-17A–PE, and anti–FoxP3–Alexa Fluor 647, using the human Th17/Treg Phenotyping Kit (BD Pharmingen, BD Biosciences) according to the manufacturer’s instructions. The samples were analyzed in the FACS Canto flow cytometer using the FACSDiva software. The absolute numbers of Treg and Th17 cells were calculated on the basis of their percentage of CD4⁺ T cells and the absolute number of CD3⁻/CD4⁺ T cells.

**Statistical analysis** Database management and analysis were performed using SAS 9.2 (SAS Institute Inc., Cary, North Carolina, United States) and GraphPad PRISM (GraphPad Software Inc., San Diego, California, United States) software packages. The variables following a nonnormal distribution were presented as medians (IQR), and those that were normally distributed, as means (SD). The nonnormally distributed data were analyzed using nonparametric tests (Wilcoxon rank sum test for comparison of unpaired continuous data, and Spearman rank correlation analysis for correlation analysis). The means of normally distributed variables were compared using the t test. The proportions were compared using the χ² test. All P values were 2-tailed, and 5% was considered as the threshold for significance. For the analysis of the cell subsets, we used
The principal findings of our study were as follows: 1) Axial SpA had a significantly higher percentage of CD16+ monocytes compared with peripheral SpA, healthy controls, and 2 and 3 in the intermediate and classical monocyte counts (FIGURE 1B and 1C, respectively). The groups did not differ in the absolute number of circulating T<sub>reg</sub> and Th17 lymphocytes (FIGURE 2A and 2B, respectively).

Monocyte subpopulations and disease activity
In the peripheral SpA group, there were no correlations between the absolute numbers of the 3 monocyte subpopulations and the ASDAS-CRP score, BASDAI score, CRP level, and ESR value (all P > 0.27). Similar findings were observed for the axial SpA group (all P > 0.29). In patients with peripheral SpA, we found a significant negative correlation between the concentration of nonclassical monocytes and the DAS28 (r = −0.59, P = 0.02) and the number of swollen joints (r = −0.64, P = 0.004). We found no correlations between the indicators of the clinical disease activity and T<sub>reg</sub> or Th17 levels in either group of patients. Moreover, in the entire study group and the subgroups, we did not observe correlations between the number of monocyte subpopulations, T<sub>reg</sub> or Th17 and the duration of SpA (P > 0.51 for the entire group; P > 0.11 for the subgroup analyses).

DISCUSSION
The principal findings of our study based on the measurement of the absolute numbers of circulating cell subsets are as follows: 1) the numbers of nonclassical monocytes are significantly lower in both peripheral and axial SpA as compared with healthy controls; 2) there was no disease-type (peripheral and axial SpA) specific association between the ASDAS-CRP score, BASDAI score, CRP levels, and ESR and monocyte subpopulation levels; 3) there is a strong negative correlation between the number of nonclassical monocytes and the clinical indicators of peripheral joint involvement (DAS28 and the number of swollen joints) in peripheral SpA; and finally 4) there is no significant association between T<sub>reg</sub> or Th17 concentrations and clinical measures of disease activity either in peripheral or axial SpA. Taking these data together, we assume that nonclassical monocytes may be involved in the pathogenesis of peripheral SpA.

Our findings in patients with SpA are in contrast to the results of Amoruso et al who reported that patients with RA presented a significantly higher percentage of CD16+ monocytes compared with healthy controls. However, Amoruso et al analyzed monocyte proportions rather than the absolute numbers. The incompatibility of methodology makes it impossible to draw conclusions on the similarities or differences of the involvement of monocyte subsets in the pathology of RA and peripheral SpA in the context of the DAS28 score. Additionally, in our patients with axial SpA, we found no association between the ASDAS-CRP or BASDAI...
Concentrations of nonclassical (CD14+CD16++; A), intermediate (CD14++CD16++; B), and classical monocytes (CD14++CD16--; C) in patients with axial and peripheral spondyloarthritis (SpA), and healthy controls. Individual data points, medians, and interquartile ranges are shown.
clinical activity indicators and the numbers of monocyte subsets. However, this finding does not preclude the possibility that the nonclassical monocytes are involved in the pathogenesis of axial SpA and may indicate that these clinical indicators are not sensitive enough with respect to monocyte-induced peripheral inflammatory changes, or inflammation might not be the leading pathology in axial SpA, dependent on nonclassical monocytes.

The most probable hypotheses to explain the low level of nonclassical monocytes in our patients are: 1) inhibition of their development, or 2) migration of monocytes from blood to peripheral tissues, that is, synovium of the joints. It was suggested that the essential role in the promotion of differentiation of classical monocytes to intermediate and nonclassical ones is mediated by macrophage colony-stimulating factor. Our preliminary observation that the macrophage colony-stimulating factor concentration in the sera of patients with SpA was significantly higher than in the control group, with no difference between axial and peripheral SpA (unpublished data), lends support to the latter hypothesis. Moreover, in our study, we found no differences in the number of intermediate monocytes, suggesting pronounced transmigration of nonclassical but not of intermediate cells. Additionally, the nonclassical monocytes appear to be accumulated in the marginal pool inside the vessels, where they are preferentially located because of a higher expression of adhesion molecules such as CD11d and VLA-4. The third hypothesis explaining the diminished number of nonclassical monocytes is their rapid destruction and elimination from the circulation, that is, in the spleen. However, it seems less probable in the context of a significant negative correlation between the low number of nonclassical monocytes and clinical manifestations of
Several years ago, Kragstrup et al,28 in a study performed on synovial tissue and peripheral blood samples from patients with peripheral SpA, demonstrated that the expression of the CCR5 receptor on peripheral blood mononuclear cells was higher in patients with peripheral SpA than in patients with psoriasis and PsA after depletion of CD14++ monocytes, supporting the hypothesis of transmigration of CCR5-expressing monocytes. This is consistent with the hypothesis proposed by Mack et al.,29 supporting the hypothesis of their transmigration or upregulation of CCR5 expression on the cells already present in the effusion.29 In fact, among monocyte subpopulations in peripheral blood, mainly nonclassical monocytes express CCR5, and as such they are predisposed to migration from blood to the sites of peripheral inflammation.39

All these observations may support the hypotheses of a more pronounced migration of monocytes into the synovium in peripheral SpA. It is also consistent with the hypothesis proposed by Kragstrup et al.28 that the insufficient upregulation of CD16 shedding from intermediate monocytes in patients with HLAB27+ SpA facilitates leukocyte migration to the entheses and joints and results in aggravating disease activity. Also, in a recent study on lupus nephritis, Garcia et al30 reported that patients with more severe forms of the disease had a higher grade of CD14+CD16++ cell infiltration in the kidneys and a lower peripheral blood level of nonclassical monocytes. Their observation may reflect the monocyte migration into renal tissue, which is in line with our hypothesis that in peripheral SpA nonclassical monocytes migrate to the synovium of peripheral joints, inducing and maintaining local inflammation. However, independent ex vivo experiments and animal model studies are needed to confirm these findings.

Another important aspect of our study is that contrary to the results for synovitis, there was no difference in the concentration of monocyte subsets according to the presence or absence of enthesitis, dactylitis, or inflammatory spinal and lower back pain. A possible explanation of this finding is that the process of migration of monocytes is more pronounced in active synovitis than in other peripheral or axial symptoms. However, this requires further confirmation, possibly based on a histological assessment of synovial biopsies.

Our results are not consistent with the finding of the elevated percentage of CD16+ monocytes in patients with PsA,9 as certain percentage of peripheral SpA may fit in the PsA CASPAR classification criteria.22 However, in contrast to the above study, our analysis was based on the absolute numbers of the respective cell types rather than on the percentages, which to some extent may explain the discrepancy. Additionally, there was no difference in the numbers of respective cells between patients with peripheral SpA with and without psoriasis (data not shown).

A clinical implication of our study is that blocking transmigration of nonclassical monocytes into peripheral joints could possibly be used in the treatment of synovitis, that is, with anti-CCR5 monoclonal antibodies. The clinical efficacy of glucocorticoids in peripheral arthritis could be explained by the depletion of nonclassical monocytes.33,34 This is consistent with the improvement of clinical symptoms in patients with psoriasis and PsA after depletion of CD14+16++ proinflammatory monocytes by adsorptive granulocyte and monocyte apheresis.35,36

In our study, we did not find any significant changes in the numbers of Treg and Th17 between any studied group and no correlations of the cell numbers with clinical disease activity measurements. However, some animal models and in vitro studies suggested a dysregulation between anti-inflammatory Treg lymphocytes and proinflammatory Th17 lymphocytes,37-39 as well as bilateral interactions between Treg and Th17 lymphocytes in these patients.40 However, these data must be confirmed by further investigations.

Our study has some limitations. First, we analyzed a relatively small number of patients, and the studied populations were heterogeneous in terms of the percentage of extra-articular signs and symptom duration. Second, our hypothesis of the enhanced migration of monocytes from blood to the site of inflammation is based on the number of monocytes in the peripheral blood and must be confirmed in an animal model setting. However, the strength of our study is the fact that patients were under 45 years of age and were treatment-naïve as any therapy may have influenced the levels of circulating immune cells.

In conclusion, our results suggest the important role of nonclassical monocytes in the early form of peripheral SpA, as these cells may infiltrate the synovium and play a role in the induction and maintenance of peripheral joint inflammation.

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Contribution statement ZG recruited patients and wrote the manuscript. MSt analyzed monocyte subsets and coordinated the study. MR-Z performed Treg lymphocytes analysis. ML performed Th17 lymphocytes analysis. MK recruited patients, analyzed results, and edited the manuscript. JG performed statistical analysis. JB and MB-K discussed and edited the manuscript. RS and JC assisted with the analysis of the results.
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