Increased Subpopulations of CD16<sup>+</sup> and CD56<sup>+</sup> Blood Monocytes in Patients with Active Crohn’s Disease

Olof Grip, MD, PhD*; Anders Bredberg, MD, PhD†; Stefan Lindgren, MD, PhD*; and Gunnel Henriksson MD, PhD†

Background: Circulating monocytes may be subdivided according to the presence or absence of the Fcγ receptor CD16 and the neural cell adhesion molecule CD56. Monocytes classified into these subpopulations are characterized by distinct phenotypic and functional features. We hypothesized that patients with active Crohn’s disease differ in their peripheral monocyte subpopulations.

Methods: Using flow cytometry we investigated the expression of CD16 and CD56 on circulating monocytes in 11 patients with active Crohn’s disease and 11 controls. These monocyte subpopulations were then analyzed for expression of the chemokine receptor fractalkine, CX<sub>3</sub>CR1, and the monocyte chemoattractant protein-1, CCR2.

Results: We found a median 3.7-fold increase in the number of CD16<sup>+</sup> monocytes related to the population with high expression of the pattern recognition receptor CD14 compared to that in the controls (\(P < 0.001\)). By studying the percentage of monocytes expressing CX<sub>3</sub>CR1, and their relative fluorescence intensity (RFI), we found significant differences, with both the highest percentage and the highest RFI in the CD14<sup>high</sup>CD16<sup>+</sup> subgroup, whereas the CD14<sup>high</sup>CD16<sup>+</sup> subgroup represented an intermediate population. Inversely, CCR2 expression was highest in the populations with high expression of CD14, whereas the CD14<sup>low</sup>CD16<sup>+</sup> subpopulation showed the lowest percentage and the lowest RFI for CCR2. We found the percentage of CD14<sup>+</sup>CD56<sup>+</sup> monocytes in patients with active Crohn’s disease to be increased 2.7 times compared to the controls (\(P = 0.011\)).

Conclusions: These results show that subsets of peripheral monocytes with a more mature phenotype are expanded in patients with active Crohn’s disease.

Key Words: Crohn’s disease, monocytes, cellular differentiation, chemokine receptors

Monocytes play a pivotal role in various stages of self-defense, including phagocytosis of opsonized pathogens, digestion and processing of foreign antigens, antigen presentation in association with class I or class II molecules, and, finally, release of several inflammatory effector molecules. They also determine the direction and intensity of inflammatory reactions elicited in response to given pathogens by production of selected cytokines with immunomodulating activity. Grip et al provide a more detailed description of the role of monocytes in inflammatory bowel diseases in a previous review.

It is now better understood that monocytes are heterogeneous, and subpopulations have been identified according to physical, functional, and surface marker criteria. Circulating monocytes can be distinguished by the presence of the Fcγ receptor CD16. Monocytes identified as belonging to 1 of these 2 subpopulations are characterized by distinct spectra of cytokine production and chemokine receptor expression. The major classical subpopulation expresses a high level of the pattern recognition receptor CD14, often used as a monocyte/macrophage marker, without coexpressing CD16 (CD14<sup>high</sup>CD16<sup>+</sup>), whereas the minor subpopulation, which make up 5%–10% of the monocytes, concurrently expresses CD16 and a low level of CD14 receptors (CD14<sup>low</sup>CD16<sup>+</sup>) on the cell surface. The classical subpopulation is known to produce anti-inflammatory cytokines, including IL-10, whereas the minor subpopulation is increased during acute inflammatory illness as well as during chronic inflammatory illnesses such as rheumatoid arthritis and HIV infections.

A minor subset of peripheral monocytes recently identified and defined by the presence of the neural cell adhesion molecule CD56 is speculated to have a regulatory function in inflammatory cells. They make up 1.3% of mononuclear cells and were described as more mature than classical CD14<sup>+</sup> monocytes.
We hypothesized that in patients with active Crohn’s disease the peripheral monocyte subpopulations are different, possibly because the disease elicits differentiation of blood monocytes. By classifying peripheral monocytes according to expression of cell-surface markers for differentiated cells using flow cytometry, we defined subpopulations whose phenotypic properties we then examined for expression of 2 major monocyte chemokine receptors, CCR2 and CX3CR1.

**MATERIALS AND METHODS**

**Patients and Controls**

We obtained peripheral blood from 11 patients with active Crohn’s disease visiting our outpatient clinic because of increased clinical symptoms and from 11 healthy controls. Table 1 summarizes the demographic characteristics of the patients. The number of peripheral-blood monocytes in patients with active Crohn’s disease (median 0.5 × 10^9/L, range 0.3–1.6 × 10^9/L) was equal to that in the controls (0.5 × 10^9/L, 0.3–1.1 × 10^9/L), as analyzed with a Beckman Coulter LH 7450 (Bromma, Sweden). Blood samples for flow cytometry were collected in Vacutainer tubes (BD Diagnostics, Stockholm, Sweden) containing a 3.2% sodium-citrate solution as anticoagulant. Samples were preferably taken from patients not taking immunomodulators or before they had been prescribed to control flare-ups; nonetheless, some of our patients were already on IBD-related drugs. The Harvey Bradshaw Severity Index for Crohn’s disease was used to quantify disease activity, and patients who scored at least 7 in combination with having elevated levels of plasma C-reactive protein (> 3.0 mg/L) were included in the study. The controls were sex-matched and age-matched healthy volunteers who had normal levels of C-reactive protein (< 2.0 mg/L). Informed consent was obtained from all patients, and the study was approved by the local medical ethics committee.

**Flow Cytometry Analysis**

Blood samples were stained according to a whole-blood technique using a protocol described previously. Briefly, Falcon tubes containing 50 μL of whole blood were stained in the dark for 15 minutes at 4°C with the appropriate amount of fluorescein isothiocyanate–conjugated anti-CX3CR1 from Nordic Biosite (Täby, Sweden), allophycocyanin-conjugated anti-CD56 from MACS, Miltenyi Biotec (GT, Västra Frölunda, Sweden), phycoerythrin-conjugated anti-CD16, and Alexa Fluor 647 anti-CCR2 and peridin chlorophyll protein–conjugated anti-CD14 from BD Pharmingen (Stockholm, Sweden). Fluorochrome-conjugated isotype-matched control antibodies against rat IgG2b (Nordic Biosite) as well as mouse IgG1 and IgG2b (BD Pharmingen) were used to check for nonspecific reactivity. Erythrocytes were lysed and leukocytes fixated for 10 minutes with BD FACT Lysing Solution (BD Pharmingen) before being washed twice with phosphate buffered saline (PBS) and centrifuged for 3 minutes at 1500 g. Flow cytometry was performed on a FACS Calibur (BD Biosciences) 4-color flow cytometer, and data were acquired from at least 4000 monocytes, gated morphologically (bivariate plot of forward versus side scatter) with CellQuest (BD Biosciences) software, before analysis using WinList software (Verity Software House Inc., Topsham, Maine). Relative fluorescence intensity (RFI) was displayed as the ratio of the linearized positive median to the linearized isotype control median.

<table>
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<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Duration of disease (years)</th>
<th>Involvement</th>
<th>HBSI</th>
<th>P-CRP (mg/L)</th>
<th>B-Monocyte (× 10^9/L)</th>
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HBSI, Harvey Bradshaw Severity Index; CRP, C-reactive protein; Bud, budesonide; Metron, metronidazole; AzA, azathioprine; Cipro, ciprofloxacin; 5-ASA, 5-aminosalicylate; MTX, methotrexate.
Statistical Analysis

Results are expressed as medians with interquartile ranges (IQR). Differences were analyzed by the Mann-Whitney rank sum test and the Kruskal-Wallis one-way analysis of variance on ranks using SigmaStat 3.1 software (Systat Software GmbH, Germany). A \( P \) value of less than 0.05 was considered significant.

RESULTS

CD14\textsuperscript{high}CD16\textsuperscript{+} Monocytes Are Expanded in Patients with Active Crohn’s Disease

We found a 1.7-fold increase in the expression of CD14\textsuperscript{+} monocytes expressing CD16 (\( P = 0.003 \)) in the patients with active Crohn’s disease (median 13.9, IQR 10.6%–17.4%) compared to the controls (9.2, 6.8%–11.1%). By further dividing the monocyte subpopulations into CD14\textsuperscript{high}CD16\textsuperscript{+}, CD14\textsuperscript{high}CD16\textsuperscript{+}, and CD14\textsuperscript{low}CD16\textsuperscript{+} (Fig. 1), we found that the increased expression of CD16 monocytes was related to the population with high expression of CD14. In the subpopulation with high expression of CD14 the median increase was 3.7-fold compared to the controls (10.3, 7.3%–13.7%, versus 2.8, 2.0%–4.3%; \( P < 0.001 \); Fig. 2). No significant differences with the controls were detected in the 2 other subpopulations. These subpopulations were further analyzed for their expression of the chemokine receptors CX\textsubscript{3}CR1 and CCR2.

CD14\textsuperscript{high}CD16\textsuperscript{+} Monocytes Have High Expression of CCR2 and Intermediate Expression of CX\textsubscript{3}CR1

By studying the percentage of monocytes expressing CX\textsubscript{3}CR1 and their relative fluorescence intensity (RFI), we found significant differences between all 3 subpopulations, with both the highest percentage and highest RFI in the CD14\textsuperscript{low}CD16\textsuperscript{+} subpopulation and intermediate expression of both in the CD14\textsuperscript{high}CD16\textsuperscript{+} subpopulation. The percentages of monocytes expressing CX\textsubscript{3}CR1 in the defined subpopulations (CD14\textsuperscript{high}CD16\textsuperscript{+}, CD14\textsuperscript{high}CD16\textsuperscript{+}, and CD14\textsuperscript{low}CD16\textsuperscript{+}) were 53.3%, 72.9%, and 86.8%, respectively, in the patients (\( P < 0.05 \)) and 70.2%, 89.3%, and 96.5%, respectively, in the controls (\( P < 0.05 \)). In the patient subpopulations positive for CD16, we found a significantly lower percentage of monocytes expressing CX\textsubscript{3}CR1 than in the controls (\( P = 0.049 \) in the CD14\textsuperscript{high}CD16\textsuperscript{+} subpopulation and \( P = 0.003 \) in the CD14\textsuperscript{low}CD16\textsuperscript{+} subpopulation). The RFI of CX\textsubscript{3}CR1 increased in a similar way, with the highest expression in the CD14\textsuperscript{low}CD16\textsuperscript{+} subpopulation (3.5%, 5.1%, and 11.7%, respectively, in the patient subpopulations [\( P < 0.05 \)] and 3.1%, 4.9%, and 10.9%, respectively, in the controls [\( P < 0.05 \)]).

We observed an inverse situation for CCR2 expression, detecting the lowest percentage and lowest RFI in the CD14\textsuperscript{low}CD16\textsuperscript{+} subpopulation. Significant differences were detected in the expression of the CD14\textsuperscript{high}CD16\textsuperscript{+}, CD14\textsuperscript{high}CD16\textsuperscript{+}, and CD14\textsuperscript{low}CD16\textsuperscript{+} monocyte subpopulations (88.9%, 77.1%, and 28.3%, respectively, in the patients, and 92.9%, 66.7%, and 19.6%, respectively, in the controls; \( P < 0.05 \)). Furthermore, we observed a trend that the patient subpopulations had a higher percentage of monocytes expressing CCR2 than did the control subpopulations. The RFI of CCR2 decreased in a similar way, with the lowest expression in the CD14\textsuperscript{low}CD16\textsuperscript{+} subpopulation (12.0%, 12.6%, and 6.3%, respectively, in the patient subpopulations and 10.8%, 7.6%, and 3.0%, respectively, in the controls; \( P < 0.05 \)). In the controls there were significant differences between all the subpopulations, with the CD14\textsuperscript{high}CD16\textsuperscript{+} cells representing an intermediate population, whereas in the patient subpopulations the expression of CCR2 in the intermediate CD14\textsuperscript{high}CD16\textsuperscript{+} population was as high as that in the CD16\textsuperscript{+} population. We found significantly higher RFIs of CCR2 in the patient subpopulations positive for CD16 than in the controls (\( P = 0.008 \) in the CD14\textsuperscript{high}CD16\textsuperscript{+} subpopulation and \( P = 0.010 \) in the CD14\textsuperscript{low}CD16\textsuperscript{+} subpopulation; Fig. 3). Thus, CCR2 was the dominant chemokine receptor in both the largest monocyte population, the classical CD14\textsuperscript{high}CD16\textsuperscript{+} cells, and in the population that was expanded in patients with active Crohn’s disease, the CD14\textsuperscript{high}CD16\textsuperscript{+} monocytes.

CD14\textsuperscript{+}CD56\textsuperscript{+} Monocytes Are Expanded in Patients with Active Crohn’s Disease

Because the myeloid CD56\textsuperscript{+} cell population may have a regulatory function in the immune response, we also wanted to define its presence in patients with Crohn’s disease. We found that the number of CD14\textsuperscript{+}CD56\textsuperscript{+} monocytes in patients with active Crohn’s disease was increased 2.7 times over that in the controls (6.2, 3.7%–13.4%, versus 2.3, 1.1%–3.8%; \( P = 0.011 \); Fig. 4). We also analyzed expression of CD16 and found that most monocytes expressing CD56 did not concurrently express CD16. In the patients with Crohn’s disease 12.6% (7.7%–20.2%) of the CD14\textsuperscript{+}CD56\textsuperscript{+} monocytes were CD16\textsuperscript{+} compared to 6.9% (4.7%–9.6%) in the controls. Thus, the expression of CD16 was similar to that in the general monocyte population. Finally, we analyzed the expression of the chemokine CX\textsubscript{3}CR1 in CD14\textsuperscript{+}CD56\textsuperscript{+} monocytes from the patients and found that 57.5% (39.2%–81.2%) of the cells were positive for CX\textsubscript{3}CR1 and that the RFI of CX\textsubscript{3}CR1 was 3.7 (2.7–4.6), with similar findings for the CD14\textsuperscript{high}CD16\textsuperscript{+} cells. The RFI was 2.9 (2.6–3.6). Accordingly, the expression of chemokine CX\textsubscript{3}CR1 in the CD56\textsuperscript{+} myeloid cell population was similar to that observed in classical CD14\textsuperscript{high}CD16\textsuperscript{+} monocytes.

Monocytes Are Expanded in Patients with Active Crohn’s Disease

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Inflamm Bowel Dis Monocyte Subpopulations in Crohn’s Disease

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Peripheral monocytes analyzed by flow cytometry. Leukocytes initially separated in bivariate plot of forward scatter (FSC) versus side scatter (SSC) to identify monocytes and ellipse gated cells in R1. Then gated monocytes were subdivided by expression of CD14, CD16, and CD56. (A and B) Patient with active Crohn’s disease; (C and D) control subject. Subpopulations defined as CD14^{high}CD16^{low}, region 2; CD14^{high}CD16^{high}, region 3; CD14^{low}CD16^{low}, region 5; and CD14^{low}CD56^{low}, region 9.
DISCUSSION

The existence of monocyte subsets in humans has been known and studied for many years, in most cases on the basis of CD16 expression. Because no studies have been performed on patients with Crohn’s disease, we wanted to evaluate the relevance of CD16 expression in this chronic inflammatory disease. Our results showed clear expansion of the CD16+ monocytes, indicating they may play an important role in the pathogenesis of Crohn’s disease. CD16+ monocytes have been described as “proinflammatory,” producing high levels on patients with Crohn’s disease, we wanted to evaluate the relevance of CD16 expression in this chronic inflammatory disease.
of TNF, with the anti-inflammatory cytokine IL-10 low or absent. CD16+ cells also demonstrate features of differentiated monocytes or tissue macrophages such as increased migration into tissue, and it has been suggested that they contribute significantly to precursors of dendritic cells. Studies of inflamed tissues analyzed from patients with inflammatory bowel disease have identified subpopulations of CD16-expressing macrophages near to lamina propria, which are rarely present in normal, noninflamed tissue. Engagement of CD16 has been shown to have an important role in signaling survival by preventing apoptosis of monocytes in response to IL-10. This mechanism operating in vivo might preserve the viability of phagocytes with the capacity to migrate to lymph nodes as antigen-presenting dendritic cells.

Previous studies indicated that the maturation of monocytes is reflected in increased surface expression of CD16 and diminished expression of CD14. It is possible that bacterial antigens can induce CD16 monocytes ex vivo, suggesting that CD14lowCD16+ monocytes may mature from CD14highCD16monocytes. The assumption of progressive differentiation of monocytes in circulation under inflammatory conditions in vivo is supported by experimental data from adoptively transferred cells in mice. However, maturation of circulating monocytes is likely to be only one contributing source. Data reported by other authors support the theory that CD16+ monocytes are more mature cells entering circulation from tissue in extreme inflammatory conditions.

Much of the extensive research into leukocyte trafficking has focused on neutrophils and T cells. Intrinsic monocytic diversity has made specific studies on monocyte trafficking more difficult. However, in recent years our knowledge of the mechanisms underlying monocyte recruitment has increased. An initial step in monocyte migration is the activation of leukocyte integrins, which results in the firm adhesion of leukocytes to the endothelium before they emigrate through the vessel wall. Chemokines may initiate signals that elicit β1 and β2 integrins, and the chemokines that control the positioning of monocyte-derived cells in tissues are those that activate the leukocyte integrins α4β2 (LFA1), αMβ2 (MAC1), α5β2, and α4β1 (VLA4). CD16+ monocytes have been described as exhibiting preferential migration across endothelial layers in response to the chemokine fractalkine (CX3CL1). Studies of fractalkine have shown that it may enhance adhesion of macrophage-like cell lines through both β1 integrin- and β2 integrin-dependent and -independent pathways. By elegant experiments performed in mice, 2 monocyte subsets have been identified in detail, in part through knock-in of a green fluorescent protein (GFP) reporter into the fractalkine receptor (CX3CR1). Studies using this marker indicated that cells expressing low levels of CX3CR1 give rise to tissue macrophages through fractalkine-dependent recruitment. Unlike the recruitment of monocytes to tissue that is not inflamed, recruitment to inflamed tissue mainly depends on the monocyte chemoattractant protein-1 (MCP-1), the ligand of chemokine receptor CCR2. MCP-1 has been shown to contribute to the adhesion of monocytes to inflamed human endothelium via β2-integrin pathways. Thus, 2 subsets have been identified: CCR2-CX3CR1high monocytes, which constitutively home in on tissue, and CCR2+CX3CR1low monocytes, which home in on tissue only if it is inflamed. In our study, we found that the CD14highCD16monocyte population closely resembles the CCR2'CX3CR1low subset and that the CD14lowCD16+ population corresponds to the CCR2−CX3CR1high subpopulation. However, the monocytes that were expanded in the patients with active Crohn’s disease in our study were CD14highCD16+ cells that had high expression of CCR2 and intermediate expression of CX3CR1. This subpopulation, which is suggested to be more mature than the classical inflammatory CD14highCD16+ population, may thus home in on inflamed tissues. Immunohistochemical staining of colonic specimens taken from patients with Crohn’s disease have shown increased expression of chemokines including MCP-1 that correlated with disease activity. In inflamed mucosa, multiple cells are able to produce MCP-1, including lamina propria macrophages, endothelial cells, spindle cells, and intestinal epithelial cells. In previous studies we also detected elevated MCP-1 in plasma taken from patients with active Crohn’s disease, indicating that the CCR2 pathway is central to monocyte recruitment in these patients.

The neural cell adhesion molecule (NCAM) CD56, which has an extracellular domain containing 5 immunoglobulin and 2 fibronectin type III domains, has been used by several studies as a marker to define populations of natural cytotoxic cells, including NK and NK-T cells. CD56 has also been used as a diagnostic and prognostic marker for myeloid malignancies. Furthermore, CD56 in combination with markers for dendritic cells (DCs) has facilitated the identification of a subset of plasmacytoid DCs. Little is known about the hematopoietic functions of CD56 other than its association with cells exhibiting natural immunity. It has been proposed that CD56+ cells cooperate with integrins LFA-1 and LFA-3 to enhance interactions between NK and other CD56+ cells, leading to increased cytotoxicity. However, other investigators failed to demonstrate direct involvement of CD56 in NK cell-mediated cytotoxicity. More recently, CD56 in combination with myeloid markers has been used to characterize a myeloid CD56 cell population. The authors found that this population had antigen-presenting functions that were superior to those of classical CD14+ monocytes, inducing greater T-cell proliferation when lymphocyte responders were HLA-mismatched. We can only speculate why we observed a clear expansion of CD56+...
monocytes in patients with Crohn’s disease. However, because Crohn’s disease is thought to be a consequence of a dysregulated T-cell response that leads to proliferation of the type 1 helper T-cell (Th1) phenotype, CD56+ monocytes may be an important mediator in this process.

In summary, we have characterized subsets of peripheral monocytes that have a more mature phenotype than that of the classic monocyte population and that are expanded in patients with active Crohn’s disease. These findings are probably not specific to Crohn’s disease and most likely can be found in other acute or chronic inflammatory conditions. However, studies of the CD14highCD16+ and CD56+ monocyte populations and their functions may enhance our understanding of this disease and influence our therapeutic strategies. Moreover, it would be interesting to see if the subpopulations determined can be used as biomarkers of inflammatory status in patients with Crohn’s disease, for instance, whether they are affected by medical treatment before the clinical effect is seen in patients.

REFERENCES