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Pivotal Role for CD16+ Monocytes in Immune Surveillance of the Central Nervous System

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Monocytes represent a heterogeneous population of primary immune effector cells. At least three different subsets can be distinguished based on expression of the low-affinity FcγRIII: CD14++CD16 classical monocytes, CD14++CD16 intermediate monocytes, and CD14++CD16++ non-classical monocytes. Whereas CD16 classical monocytes are considered key players in multiple sclerosis (MS), little is known on CD16 monocytes and how they contribute to the disease. In this study, we examined the frequency and phenotype of monocyte subpopulations in peripheral blood, cerebrospinal fluid (CSF), and brain biopsy material derived from MS patients and controls. Furthermore, we addressed a possible monocyte dysfunction in MS and analyzed migratory properties of monocyte subsets using human brain microvascular endothelial cells. Our ex vivo studies demonstrated that CD16 monocyte subpopulations are functional but numerically reduced in the peripheral blood of MS patients. CD16 monocytes with an intermediate-like phenotype were found to be enriched in CSF and dominated the CSF monocyte population under noninflammatory conditions. In contrast, an increased CD16 to CD16 CSF monocyte ratio was observed in MS patients with relapsing-remitting disease. Newly infiltrating, hematogenous CD16 monocytes were detected in a perivascular location within active MS lesions, and CD16 monocytes facilitated CD4 T cell trafficking in a blood–brain barrier model. Our findings support an important role of CD16 monocytes in the steady-state immune surveillance of the CNS and suggest that CD16 monocytes shift to sites of inflammation and contribute to the breakdown of the blood–brain barrier in CNS autoimmune diseases. The Journal of Immunology, 2016, 196: 1558–1567.

Antigen-presenting cells are considered key players in the immune surveillance of the CNS. At the same time they are critically involved in the pathogenesis of CNS autoimmune diseases. Sequestration of immune cells in the periphery and/or inhibition of their migration into the CNS are promising treatment strategies of CNS autoimmune diseases. On the downside, these therapies interfere with CNS immune surveillance and bear the risk of opportunistic CNS infections (1). Studying CNS immune surveillance with a focus on the different cellular players may therefore not only be key to a better understanding of the pathogenesis of CNS autoimmune diseases but also a prerequisite for tailoring future immunomodulatory therapies.

Among the cells of the innate immune system, monocytes have long been recognized to play a pivotal role in multiple sclerosis (MS) (2). Recent data from the animal model confirms that monocyte infiltration triggers disease progression (3) and monocyte-derived macrophages initiate demyelination at disease onset (4). However, monocytes represent a heterogeneous population of primary immune effector cells with distinct phenotypical and functional characteristics, and their differential roles in steady-state immune surveillance and the pathogenesis of human CNS disease are poorly understood.

Three different monocyte subsets can be distinguished based on the expression of CD14 and the low-affinity FcγRIII CD16. CD14++CD16 classical monocytes are the most prevalent subset in the peripheral blood, whereas CD14++CD16 intermediate and CD14++CD16++ non-classical monocytes together make up approximately only 10% of circulating monocytes (5). Animal studies have demonstrated their functional diversities in the peripheral immune compartment. CCR2LY6Chi monocytes, which are the murine counterpart of human CD16 classical monocytes, are rapidly recruited to sites of inflammation, where they mediate the inflammatory process by cytokine secretion and phagocytosis. C3CR1LY6Clo monocytes, which resemble CD16 monocyte subsets in humans, have been attributed a patrolling behavior with the primary function to survey endothelial integrity in the steady-state (6).

Increased frequencies of circulating CD16 monocytes have been reported in various autoimmune diseases such as sarcoidosis (7, 8), rheumatoid arthritis (9–11), inflammatory bowel diseases (12), Sjögren syndrome (13), asthma (14), and immune thrombocytopenia (15).
The most detailed work has been performed in rheumatoid arthritis. In these studies, it was demonstrated that the expansion of CD16+ monocytes is driven by the intermediate monocyte subset (9–11) and that these CD16+ monocytes actively shape T cell responses by favoring Th17 differentiation (9). Accordingly, it has been suggested that CD16+ monocytes play a pivotal role in the pathophysiology of autoimmune diseases.

Data from MS patients were not consistent concerning the frequency of CD16+ monocytes within the peripheral blood (16, 17). In the present study, we analyzed the frequency of CD16+ and CD16– monocyte subsets in the peripheral blood of patients with relapsing-remitting MS (RRMS) and CD16+ monocyte subsets in the peripheral blood of patients with relapsing-remitting MS (RRMS) and inflammatory and noninflammatory neurologic diseases (NIND). We further show that CD16+ monocytes are functional in MS and may contribute to the breakdown of the blood–brain barrier in MS by facilitating T cell trafficking into the CNS.

Materials and Methods

Patients and sample collection

This study was approved by the Ethics Committee of the Friedrich-Alexander University Erlangen (no. 257_13B). Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. To be eligible for peripheral blood analysis, patients had to be diagnosed with MS or clinically isolated syndrome (CIS) according to the revised McDonald criteria (18). Patients treated with glucocorticoids within the last 6 wk before study inclusion were excluded. Patients had to be either treatment naïve (group 1) or treated for >2 mo with immunomodulatory drugs (group 2) as specified below. Additionally, age- and sex-matched healthy donors (HD) were recruited for blood donation. Patients’ characteristics are summarized in Table I. Peripheral blood was obtained by venipuncture and processed immediately as described below.

For CD16+ monocyte subset frequency analysis, 100 μl EDTA containing whole blood were stained in Trucount tubes (BD Biosciences, San Jose, CA) with anti-CD45 (2D1), anti-HLA-DR (G46-6), anti-CD14 (M60), and anti-CD16 (3G8) Ab or the respective isotype control Abs in a fluorescence minus one control staining for 30 min at 4˚C (all Abs were from BD Biosciences). Following erythrocyte lysis using an ammonium-potassium-chloride buffer, cells were washed twice and analyzed on a BD FACSCanto II using FACSDiva software. The gating strategy, which is detailed in Supplemental Fig. 1 (peripheral blood) and Supplemental Fig. 2 (CSF), was adapted from Heimbeck et al. (19). Instead of an oblique line to the right of non-classical events, a straight vertical line was placed to the left of non-classical monocytes. This gating strategy, also used by other authors (discussed in Ref. 5), was chosen after staining for additional markers differentially expressed in non-classical versus intermediate monocyte subsets following the manufacturer’s instructions. CD4+ T cells were purified from freshly isolated PBMC using the CD4+ T cell isolation kit for negative selection. The purity of isolated cell populations was controlled by flow cytometry (typically > 90%).

Monocyte function assays

For the phagocytosis assay, following opsonization with human AB serum, Fluoresbrite YG carboxylate microspheres (0.75 μm; Polysciences Europe, Warrington, PA, Germany) were added to 100 μl whole blood (citrate) and coincubated for 60 min either at 37˚C or on ice (control). Afterward, all samples were placed on ice and stained with fluorescence-conjugated anti-CD14, anti-CD14, and anti-CD16 Abs. After erythrocyte lysis and extensive washing, the percentage of cells with incorporated beads was assessed by flow cytometry.

For reactive oxygen species (ROS) production, 1 × 10^6 PBMC were incubated with 10 μM carboxy-H2DFFDA (Life Technologies, Darmstadt, Germany) for 15 min at 37˚C or PBS only (control). Following extensive washing, cells were coincubated with anti-CD14 and anti-CD16 Abs. The intracellular ROS levels within the specific monocyte subsets were determined by flow cytometry.

For cytokine secretion, 5 × 10^6 MACS-purified CD16+ monocytes per well derived from RRMS patients of HD were cultured for 24 h in 96-well plates with the presence of 1 μg/ml LPS (100 μg/ml). A human inflammatory cytokine BD cyometric bead array was used for the simultaneous detection of IL-1β, IL-6, IL-8, IL-10, TNF-α, and IL-12p70 according to the manufacturer’s instructions (BD Biosciences).

Migration assays

Cells from the human cerebral microvascular endothelial cell line hCMEC/D3 were cultured in endothelial basal medium-2 (Lonza, Walkersville, MD) supplemented with EGM-2 (Quincke Pharmaceuticals, Washington, GA) and endothelial basal medium-2 (Lonza, Walkersville, MD)-coated flasks under standard conditions. Only cell passages between 25 and 35 were used. For migration assays, 7.0 × 10^5 hCMEC/D3 cells per insert were seeded on 12-well polycarbonate inserts (pore diameter, 3.0 μm; Corning, New York, NY) coated with type I rat collagen. In some experiments human primary brain microvascular endothelial cells (HBMEC; Sciellencel Research Laboratories) were used instead of the hCMEC/D3 cell line. The inserts were grown in fibronectin-coated flasks using endothelial cell medium (Sciencel Research Laboratories) under standard conditions. HBMEC (1.5 × 10^5) were seeded on fibronectin-coated Transwell inserts (see above). Cells were grown until confluence of the endothelial monolayer. The integrity of the endothelial barrier was assessed using dextran-FITC (40 kDa, Sigma-Aldrich) and a fluorescence plate reader (VICTOR). Prior to each experiment Transwell inserts were transferred to a new 12-well cell culture plate and the medium was changed to RPMI 1640 0.5% FCS. PBMC (2.5 × 10^6) derived from HD were added to the upper chamber and PBS (control), 10% FBS, CCL2 (50 ng/ml), or CX3CL1 (50 ng/ml) was added to the lower chamber to build a serum or chemokine gradient. Additionally, a tube of untouched PBMC (resting) was stored to assess individual differences in the percentage of CD16+ monocytes in PBMC samples of our donors. Following a 5-h incubation at 37˚C, 90% CO2, Transwell inserts were transferred to a cell culture dish and rinsed three times with PBS. Adherent cells were detached from the insert by using Accutase solution (Sigma-Aldrich) and transferred to a FACS tube. Additionally, cells were recovered from the lower well after adding 50 μl CountBright absolute counting beads (Life Technologies). The content of the lower well (transmigrated cells) was transferred to a FACS tube. All samples were stained with anti-HLA-DR, anti-CD16, and anti-CD14 Abs in addition to fluorescence minus one controls and analyzed by flow cytometry. To assess possible effects of purified monocytes on CD4+ T cell migration in the hCMEC/D3 cell-based model, CD4+ T cells were isolated as described above, stained with CFDA-SE, and added to the upper well either alone or together with purified, autologous PAN monocytes, CD16+ or CD16– monocytes in a 4:1 T cell/monocyte ratio. Following a 5-h incubation, transmigrated T cells were recovered from the lower well after addition of CountBright absolute counting beads (Life Technologies) and were analyzed by flow cytometry.

Immunohistochemistry

To demonstrate the presence of CD14+CD16+ mononuclear phagocytes in acute MS lesions, we performed double immunofluorescence staining on PBMC isolation and magnetic cell purification

PBMC were isolated by density gradient centrifugation (Lymphoprep, Axis Shield, Oslo, Norway). The PAN monocyte isolation kit (Miltenyi Biotec, Bielefeld, Germany) was used for depletion of non-monocytes (negative selection) to obtain highly pure unlabelled monocytes (PAN monocytes) according to the manufacturer’s protocol. In a second step, CD16+ monocytes (Miltenyi Biotec) were used for purification of CD16+ and CD16– monocyte subsets following the manufacturer’s instructions. CD4+ T cells were purified from freshly isolated PBMC using the CD4+ T cell isolation kit for negative selection. The purity of isolated cell populations was controlled by flow cytometry (typically > 90%).
formalin-fixed, paraffin-embedded archival brain biopsy samples (n = 2). Additionally, we investigated brain specimens obtained from patients diagnosed with CNS vasculitis (n = 1) or stroke (n = 1). We used the tyramide-enhanced avidin-biotin immunostaining method (TSATM detection kit, Invitrogen, Darmstadt, Germany) and the mouse monoclonal anti-human Abs anti-CD14 (Novocastra, clone 7, 1:500; incubated with HRP and visualized with tyramide-coupled Alexa Fluor 555) and anti-CD16 (Novocastra, clone 2H7, 1:25; incubated with anti-rabbit IgG conjugated with Alexa Fluor 488). Slides were counterstained with DAPI dihydrochloride (1:10,000, Molecular Probes/Invitrogen, Carlsbad, CA) and analyzed with an Olympus BX 51 light microscope (Olympus, Hamburg, Germany). Images were taken with an Olympus XM10 camera and Cell F software.

Results

**CD16**+ circulating monocytes are reduced in treatment-naive RRMS patients

The frequency of CD16** and CD16**+ monocyte subpopulations in the peripheral blood of treatment-naive RRMS patients (n = 40) and HD (n = 40) was assessed by flow cytometry (Table I). As compared with HD, the percentage of circulating CD16**+ monocytes in untreated RRMS patients was reduced by 35% (Fig. 1A). This effect was largely driven by a reduction of circulating CD14**CD16**+ non-classical monocytes (Fig. 1B, 1C) and was also observed when absolute cell numbers were taken into account (subgroup analysis, 40.47 CD16** monocytes/μL in RRMS [n = 29] versus 60.13 CD16** monocytes/μL in HD [n = 38], p = 0.033, two-tailed, unpaired t test).

We addressed possible treatment effects on the CD16**+ monocyte population by analyzing the frequency of non-classical and intermediate monocytes in the peripheral blood of RRMS patients treated with either natalizumab (n = 32), fingolimod (n = 12, FTY) or IFNs (n = 23). The percentage of circulating CD16**+ monocytes within the whole monocyte population was found to be within or above normal levels in all MS treatment groups (Fig. 1D). The highest percentages of CD14**CD16** intermediate and CD14**CD16**+ non-classical monocytes were found in IFN-β–treated RRMS patients (6.87 ± 0.8 intermediate, 8.87 ± 0.9 non-classical), exceeding the levels of these subpopulations in both untreated RRMS patients (2.83 ± 0.3 intermediate, 4.39 ± 0.5 non-classical; p < 0.0001) and HD (4.49 ± 0.6 non-classical; p = 0.028).

**CD16**+ circulating monocytes are functional in MS

Functional characteristics of CD16** monocytes were assessed in RRMS patients and controls to exclude a possible dysfunction of

![FIGURE 1](http://www.jimmunol.org/)

**Table I. Clinical characteristics of HD, treatment-naive patients with RRMS, as well as natalizumab-, fingolimod, and β IFN–treated RRMS patients included in the peripheral blood monocyte analysis**

<table>
<thead>
<tr>
<th>Disease Category</th>
<th>No. of Subjects</th>
<th>Mean Age (y)</th>
<th>Female/Male</th>
<th>Median EDSS</th>
<th>Disease Duration (y): Median (Mean)</th>
<th>Clinical Disease Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>40</td>
<td>37</td>
<td>1.4:1</td>
<td>n/a</td>
<td>n/a</td>
<td>&lt;5 mo</td>
</tr>
<tr>
<td>RRMS</td>
<td>40</td>
<td>36</td>
<td>1.6:1</td>
<td>1.5</td>
<td>0.5 (2.7)</td>
<td>n/a</td>
</tr>
<tr>
<td>NAT</td>
<td>32</td>
<td>34</td>
<td>1.9:1</td>
<td>2.5</td>
<td>6 (6.7)</td>
<td>50</td>
</tr>
<tr>
<td>FTY</td>
<td>12</td>
<td>37</td>
<td>5.0:1</td>
<td>2</td>
<td>6.25 (6.25)</td>
<td>12.5</td>
</tr>
<tr>
<td>IFN</td>
<td>23</td>
<td>35</td>
<td>5.0:1</td>
<td>2.5</td>
<td>3 (6)</td>
<td>16.7</td>
</tr>
</tbody>
</table>

EDSS, expanded disability status scale; FTY, fingolimod; IFN, β IFN; NAT, natalizumab.
this low frequent cell population in MS. Bead-based phagocytosis assays were performed to detect a possible CD16+ monocyte dysfunction in MS. The phagocytic activity of monocyte subpopulations in HD (n = 15, dark gray) versus RRMS patients (n = 15, light gray) was assessed in bead-based phagocytosis assays. The mean percentage of incorporated beads (±SEM) is shown (Mann–Whitney U test). Basal production of ROS was assessed by flow cytometry in HD (n = 19, dark gray) versus RRMS patients (n = 19, light gray). The specific fluorescence index (SFI) of the indicator dye (dichlorofluorescein [DCF], mean ± SEM) is shown (Mann–Whitney U test). Cytokine secretion by purified CD16+ monocytes was assessed by flow cytometric bead array. The basal cytokine secretion of CD16+ monocytes derived from HD (n = 8) and RRMS patients (n = 8) is shown (mean ± SEM, two-tailed paired t test).

CD14+CD16+ cells are enriched in the CSF and share characteristics with peripheral blood intermediate monocytes

Paired CSF and peripheral blood samples derived from RRMS/CIS patients and from patients with NIND and OIND (Table II) were analyzed for the expression of CD16 and CD14 on mononuclear phagocytes. CD16+CD14+ cells were found to be enriched in the CSF compared with peripheral blood in all groups as exemplary shown for NIND patients (n = 26; Fig. 3A).

Whereas CD16+ monocytes in the peripheral blood can be subdivided based on a high or low expression of CD14 into a CD14++CD16+ intermediate and a CD14+CD16++ non-classical monocyte population, all CSF monocytes expressed high levels of CD14. A mean of 57.6 ± 3.13% (NIND) of these cells coexpressed the low affinity Fcγ receptor CD16. Based on the expression level of CD14 and CD16 on these cells, they most closely resembled the peripheral blood intermediate monocyte subset (compare Fig. 4C and Supplemental Fig. 2). A detailed phenotypic characterization revealed that in addition these CSF CD14+ cells expressed intermediate levels of the chemokine receptor CX3CR1 and high levels of CCR5, CD64, and the costimulatory molecule CD86 (Fig. 3B). In contrast, ILT-4 and CD163 were only expressed at low levels (Fig. 3B). The expression level of CD14 on CSF monocytes, which was assessed in a post hoc analysis of CSF cohort 1 data, did not differ significantly between RRMS (n = 13) and NIND patients (n = 26; 2.45 versus 2.89; NS, Mann–Whitney U test).

CD16+ monocytes dominate the CSF under noninflammatory conditions

The percentage of CD16+ monocytes within the CSF was assessed in patients with RRMS/CIS (n = 13) and NIND (n = 26). Flow

Table II. Clinical characteristics of patients with NIND, OIND, RRMS, and SPMS or PPMS included in the CSF analysis

<table>
<thead>
<tr>
<th>CSF Analysis</th>
<th>Disease Category</th>
<th>No. of Subjects</th>
<th>Mean Age (y) Female/Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort 1</td>
<td>NIND</td>
<td>26</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>RRMS</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>OIND</td>
<td>19</td>
<td>55</td>
</tr>
<tr>
<td>Cohort 2</td>
<td>NIND</td>
<td>15</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>RRMS</td>
<td>10</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>SPMS/PPMS</td>
<td>7</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>OIND</td>
<td>19</td>
<td>55</td>
</tr>
</tbody>
</table>
cytometric analysis revealed that under noninflammatory conditions CD14⁺CD16⁺ mononuclear phagocytes dominate the CSF, whereas the percentage of CD16⁺ CSF monocytes is reduced in RRMS patients (Fig. 4A, 4C). This finding was confirmed in a second, independent cohort of 10 RRMS patients and controls (Fig. 4B). In contrast to patients with relapsing-remitting disease, the CD16⁺ monocyte fraction was not reduced in progressive MS patients (PPMS/SPMS, n = 7), providing further evidence for the hypothesis that distinct pathological processes are involved in the progressive stage of the disease (Fig. 4B). A combined analysis demonstrated a CD16⁺ to CD16⁻ CSF monocyte ratio of 2.09 ± 0.3 SEM in NIND versus 0.72 ± 0.089 in RRMS (p = 0.0084, ANOVA), and 1.232 ± 0.35 in PPMS/SPMS patients (NS). However, a low percentage of CD16⁺ cells was not specific for RRMS patients, as confirmed by analyzing an additional group of patients with OIND (Fig. 4B, 4C).

**FIGURE 3.** CD16⁺ monocytes are enriched in CSF. Paired peripheral blood and CSF samples were analyzed for the expression of CD14 and CD16 on monocytes. (A) The line plot shows the percentage of CD14⁺CD16⁺ monocytes within the CD14⁺ monocyte population in corresponding peripheral blood and CSF samples derived from patients with NIND (n = 26, paired two-tailed t test). (B) Surface molecules known to be differentially expressed on classical, non-classical, and intermediate monocytes were analyzed on peripheral blood and CSF monocytes (CX3CR1, CCR5, CD64, CD86, ILT4, CD163, n = 6, Friedmann test with Dunn posttest, *p < 0.05, **p < 0.005). The specific fluorescence index (SFI) is shown.

Differential migratory behavior of CD16⁺ and CD16⁻ monocytes

The migration of CD16⁺ or CD16⁻ monocytes via an endothelial monolayer of human brain-derived microvascular cells...
FIGURE 4. The percentage of CD16+ CSF monocytes differs between patients with NIND and RRMS. The percentage of CD14+CD16+ cells within the CSF monocyte population of patients with NIND, patients with RRMS or CIS and OIND was assessed by flow cytometry. (A) Scatter plot of the percentage of CD14+CD16+ CSF monocytes in NIND (n = 26) and RRMS patients (n = 13, two-tailed unpaired t test). (B) Scatter plot of the percentage of CD14+CD16+ CSF monocytes in a second cohort of NIND (n = 15), RRMS patients (n = 10), MS patients with primary or secondary progressive MS (PPMS/SPMS, n = 7), and OIND patients (n = 19; ANOVA with Bonferroni correction). (C) CD14 and CD16 dot plots of representative multicolor flow cytometry stainings in paired CSF (right) and peripheral blood samples (left) of patients with NIND (I, II), OIND (III), RRMS (IV, V), and PPMS (VI). For gating strategy, see Supplemental Fig. 2.
(hCMEC/D3) was studied in modified Boyden chamber experiments. Whereas classical monocytes migrated readily toward a serum or chemokine gradient (Fig. 5A), CD16+ monocytes were rarely detectable in the transmigrated fraction (Fig. 5B). Instead, CD16+ monocytes were found to enrich in the adhering fraction as exemplified for the migration toward a 10% serum gradient (Fig. 5C, left). Comparable results were obtained when HBMEC were used instead of hCMEC/D3 cells (Fig. 5C, right).

**CD16+ monocytes facilitate CD4+ T cell migration**

The transendothelial migration of T cells toward a serum gradient was assessed in the presence or absence of CD14+ monocytes (PAN monocytes) and CD16+ or CD16− purified monocyte subsets. The numbers of migrated CD4+ T cells were almost doubled in the presence of CD16+ monocytes, whereas CD16− or PAN monocytes had no significant effects on the numbers of migrated T cells (Fig. 6).

**Perivascular CD14+CD16+ mononuclear phagocytes are present in active MS lesions**

Double immunofluorescence staining was performed to determine the presence of CD14+CD16+ mononuclear phagocytes in acute MS lesions. In acute MS lesions, newly infiltrating CD14+CD16+ hematogenous monocytes were mostly detectable within the perivascular infiltrates and, occasionally, in the parenchyma (Fig. 7A). CD14+CD16+ mononuclear phagocytes were also present in lesions caused by ischemic stroke and CNS vasculitis (Fig. 7B). Please note that CD14 immunoreactivity is not restricted to infiltrating monocytes/macrophages, as activated microglia may also express CD14 in MS lesions (21).

**Discussion**

In this study, we report perturbations within the circulating monocyte population in RRMS patients and highlight the role of CD16+ monocytes in CNS immune surveillance by providing novel data on the phenotype of CSF monocytes and the impact of CD16+ monocytes on T cell trafficking. Our data suggest a compartmentalization of hematogenous CD16+ monocytes to CSF-filled spaces and adjacent tissues, putting these cells in the prime position to actively contribute to the immune surveillance of the CNS and orchestrate inflammatory processes in CNS autoimmune diseases.

The reduction of circulating CD16+ monocytes in the peripheral blood of treatment-naive RRMS patients was mainly driven by...
ongoing debate. Therefore, direct consequences of a reduction of the fate and plasticity of monocyte subpopulations are subjects of associated with disease activity. The developmental sequence and pool may therefore be characteristic of early MS pathology and/or cohort whereas patients on treatment had considerably longer disease duration, 0.5 y) and represented a rather active, early MS patients in our treatment-naive group were newly diagnosed (median drug. Additionally, it has to be taken into account that most pa-

flect the overall disease-stabilizing effect of immunomodulatory 

differentiation process, or a compartmentalization of these cells to recruitment from the bone marrow, an imbalanced monocyte differentiation process, or a compartmentalization of these cells to the CNS as suggested by our findings on CSF and brain tissue. In contrast to treatment-naive RRMS patients, those treated with disease-modifying drugs had normal (natalizumab, fingolimod) to high (IFN-β) percentages of circulating CD16+ monocytes. Although this could be possibly attributed to direct effects of immunomodulatory drugs on the composition of the blood monocyte pool as previously suggested for IFN-β (22), it might as well reflect the overall disease-stabilizing effect of immunomodulatory drugs. Additionally, it has to be taken into account that most patients in our treatment-naive group were newly diagnosed (median disease duration, 0.5 y) and represented a rather active, early MS cohort whereas patients on treatment had considerably longer disease durations (median, 6–7 y). Perturbations in the blood monocyte pool may therefore be characteristic of early MS pathology and/or associated with disease activity. The developmental sequence and the fate and plasticity of monocyte subpopulations are subjects of ongoing debate. Therefore, direct consequences of a reduction of this heterogeneous population of circulating CD16+ monocytes for monocyte descendents and their differentiation (e.g., M1 and M2 macrophages) cannot be foreseen.

Immune cells that enter CSF-filled spaces via the choroid plexus have been attributed a major role in the steady-state immune surveillance of the CNS, and it is within the subarachnoid spaces that autoreactive T cells encounter their cognate Ags presented to them by monocyte-derived meningeal or perivascular macrophages and dendritic cells. In this study, we demonstrate an enrichment of CD16+ monocytes within the CSF, which is in line with a previous publication (23). Moreover, our phenotypical analysis revealed that these CD16+ monocytes most closely resemble the peripheral blood intermediate and not the non-classical monocyte subset based on a high expression of CD14 and CD16 and intermediate levels of the chemokine receptor CX3CR1. However, there were also differences in the expression of defining markers between peripheral blood and CSF monocytes; that is, CSF monocytes expressed even higher levels of CCR5, CD86, and CD64 compared with intermediate monocytes and lacked relevant levels of CD163 or ILT4, which may be secondary to differences in the activation state or changes acquired en route to the CSF-filled spaces.

Interestingly, the highest proportion of CD16+ monocytes within the CSF was detected in patients with NIND, providing strong evidence for a role of CD16+ monocytes in the steady-state immune surveillance of CNS-drained spaces. Compared to NIND, RRMS patients displayed considerably lower proportions of CD14+CD16+ monocytes resulting in an inversion of the CD16+ to CSF monocyte ratio, which may reflect a stronger recruitment of classical monocytes to the CSF secondary to the inflammatory process. Although this may be the sole valid explanation for acute inflammatory CNS diseases (i.e., meningitis), alternative options may come into play in RRMS. It has been repeatedly demonstrated that in RRMS the whole monocyte frequency in the CSF is markedly reduced (24–26). This has been attributed to a shift of monocytes to the meninges and the inflamed parenchyma (24, 25). Our data suggest that the decrease in CSF monocytes is foremost driven by a reduction in the CD16+ monocyte subset, and we hypothesize that this is due to a higher propensity of CD16+ monocytes to adhere to adjacent tissue and turn into monocyte-derived subarachnoid-space macrophages compared with their classical counterparts. Given the high expression of CCR5 on CD16+ intermediate/CSF monocytes, previous reports demonstrating an accumulation of infiltrating CCR5+ monocytes within perivascular spaces of early MS lesions but not noninflamed control tissue provide further indirect support (27). This is further substantiated by our immunofluorescence stainings that, besides CD14+ activated microglia, show perivascular, newly infiltrating CD14+CD16+ hematogenous monocytes within active MS lesions.

FIGURE 6. CD16+ monocytes facilitate T cell migration via an endothelial barrier. To analyze possible effects of distinct monocyte subsets on transendothelial T cell migration, CFSE-stained T cells were added to the upper chamber of modified Boyden chambers either alone (control), in the presence of MACS-purified whole monocytes (PAN), or with CD16+ or CD16− monocyte subsets at a 4:1 ratio. Following a 5-h incubation period, the absolute numbers of migrated T cells within the lower chamber were assessed by flow cytometry using counting beads (**p = 0.0022 vs. control, n.s., n = 7, repeated measurement ANOVA with Bonferroni correction).

FIGURE 7. Perivascular CD14+CD16+ mononuclear phagocytes are present in active MS lesions. Perivascular CD14+CD16+ cells are found around blood vessels in active MS lesions (A) and within the inflamed vessels in CNS vasculitis (B). Double immunofluorescence staining with anti-CD14 Ab (Alexa Fluor 555) and anti-CD16 Ab (Alexa Fluor 488) was used. Scale bars, 50 μm.
In summary, alterations in the composition of the mobile CSF monocyte pool may reflect a shift of CD16+ monocytes from the periphery to sites of inflammation. Such a compartmentalization, which is typical for MS, may account for the difference in monocyte subgroup distributions in other autoimmune diseases (7–15).

CD16+ non-classical so-called patrolling monocytes exert important functions in the peripheral immune compartment. Their primary function is sensing and scanning the endothelial surface for damage or the presence of pathogens and to coordinate inflammatory processes/repair (6). Recent studies on LY6C<sup>lo</sup> monocytes, the murine counterpart of non-classical CD16<sup>+</sup> monocytes, suggest that the number of cells adhering to the vasculature at any time makes up a third of total circulating LY6C<sup>lo</sup> monocytes, which brought them the name accessory cells of the endothelium (28). In the present study, we transfer this concept to humans and studied the migratory behavior of monocyte subsets in a basic model of the blood–brain barrier employing a well-characterized human cerebral microvascular endothelial cell line (hCMEC/D3) (29) or primary HBMEC.

In this static model and under noninflammatory conditions, CD16<sup>+</sup> monocytes were found to enrich in the fraction adhering to the brain microvascular endothelium and were only rarely observed in the transmigrated fraction. This finding is in line with other reports that observed a higher spontaneous migration of human CD16<sup>-</sup> monocytes via endothelial monolayers in similar models employing human umbilical cells (30) or HBMEC (31) and that demonstrated that adhesion of CD16<sup>+</sup> but not CD16<sup>-</sup> monocytes to the endothelial barrier may even be amplified under inflammatory conditions (31). Please note that in our proof-of-principle experiments the high concentration of CX3CL1 may not efficiently trigger CD16<sup>+</sup> monocyte migration via the barrier. It has previously been shown that concentrations of 1–10 ng/ml but not 50–100 ng/ml induce CD16<sup>+</sup> monocyte migration via brain microvascular endothelial cells (31).

Monocyte–endothelial interactions may impact lymphocyte migration via endothelial barriers (32). In this study, we demonstrate that it is the CD16<sup>+</sup> and not the CD16<sup>-</sup> classical monocyte subset that promotes CD4<sup>+</sup> T cell trafficking via the endothelial barrier. Our experiments support the hypothesis that in RRMS, during early lesion formation, CD16<sup>+</sup> monocytes adhere to the brain microvasculature and contribute to the breakdown of the blood–brain barrier by promoting T cell entry into the CNS and may then gain access themselves as suggested by our biopsy findings in active MS lesions.

Recently, differences in the locomotor behavior of specific monocyte subsets were confirmed in a dynamic endothelial barrier model that also revealed divergent behavior between non-classical and intermediate monocytes, providing further evidence for the heterogeneity of CD16 monocytes and their specific function at endothelial barriers (33). Therefore, it is necessary to emphasize that in assessing the impact of CD16<sup>+</sup> monocytes on T cell migration, we did not distinguish between intermediate and non-classical monocytes and thus cannot draw conclusions on a specific CD16<sup>+</sup> monocyte subset.

In conclusion, in this study we demonstrate a perturbed balance in circulating monocytes in untreated RRMS patients. The patrolling behavior of these accessory cells of the endothelium suggests a shift from the circulation to sites of inflammation where CD16<sup>+</sup> monocytes contribute to the pathological cascade in MS by promoting T cell trafficking to the CNS. Accordingly, CD16<sup>+</sup> monocytes may represent an interesting target for future therapeutic strategies in MS (6). Furthermore, the abundance of CD16<sup>+</sup> monocytes within the CSF under steady-state conditions suggests an engagement in the immune surveillance of the healthy as well as inflamed CNS. Manipulating these cells for therapeutic benefit may therefore also imply a significant risk for opportunistic CNS infections, which needs to be taken into account when designing novel drugs that target CD16<sup>+</sup> monocytes or their receptors (34).

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References


lance of the central nervous system in multiple sclerosis—relevance for therapeu-

tic strategies in MS (6). Furthermore, the abundance of CD16<sup>+</sup> monocytes may represent an interesting target for future thera-

pic strategies in MS (6). Furthermore, the abundance of CD16<sup>+</sup> monocytes may represent an interesting target for future thera-

2. Brück, W., P. Porada, S. Porer, P. Rieckmann, F. Hanefeld, H. A. Kretzschmar,


doi:10.1371/journal.pone.0109775.

12. Koch, S.-T., Kucharzik, J. Heidemann, A. Nusrat, and A. Luegering. 2010. In-

vestigating the role of proinflammatory CD16<sup>+</sup> monocytes in the pathogenesis of inflammatoty bowel disease. Gastroenterology 139: 332–341.


immunol. 112: 197–205.


