Subpopulations of Mouse Blood Monocytes Differ in Maturation Stage and Inflammatory Response

Cord Sunderkötter, Tatjana Nikolic, Marilyn J. Dillon, Nico van Rooijen, Martin Stehling, Douglas A. Drevets and Pieter J. M. Leenen

*J Immunol* 2004; 172:4410-4417; ; doi: 10.4049/jimmunol.172.7.4410
http://www.jimmunol.org/content/172/7/4410

**References**
This article cites 32 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/172/7/4410.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Subpopulations of Mouse Blood Monocytes Differ in Maturation Stage and Inflammatory Response

Cord Sunderkötter, Tatjana Nikolic, Marilyn J. Dillon, Nico van Rooijen, Martin Stehling, Douglas A. Drevets, and Pieter J. M. Leenen

Blood monocytes are well-characterized precursors for macrophages and dendritic cells. Subsets of human monocytes with differential representation in various disease states are well known. In contrast, mouse monocyte subsets have been characterized minimally. In this study we identify three subpopulations of mouse monocytes that can be distinguished by differential expression of Ly-6C, CD43, CD11c, MBR, and CD62L. The subsets share the characteristics of extensive phagocytosis, similar expression of M-CSF receptor (CD115), and development into macrophages upon M-CSF stimulation. By eliminating blood monocytes with dichloromethylene-bisphosphonate-loaded liposomes and monitoring their repopulation, we showed a developmental relationship between the subsets. Monocytes were maximally depleted 18 h after liposome application and subsequently reappeared in the circulation. These cells were exclusively of the Ly-6C<sup>hi</sup> subset, resembling bone marrow monocytes. Serial flow cytometric analyses of newly released Ly-6C<sup>hi</sup> monocytes showed that Ly-6C expression on these cells was down-regulated while in circulation. Under inflammatory conditions elicited either by acute infection with <i>Listeria monocytogenes</i> or chronic infection with <i>Leishmania major</i>, there was a significant increase in immature Ly-6C<sup>hi</sup> monocytes, resembling the inflammatory left shift of granulocytes. In addition, acute peritoneal inflammation recruited preferentially Ly-6C<sup>med</sup>-high monocytes. Taken together, these data identify distinct subpopulations of mouse blood monocytes that differ in maturation stage and capacity to become recruited to inflammatory sites. The Journal of Immunology, 2004, 172: 4410–4417.

Compared with what is known about the human system, knowledge about mouse monocytes is much more limited. Bone marrow (BM) monocytes have been shown to develop into mature macrophages in vitro as well as in vivo (4, 5). In addition, at least a fraction has the potential to develop into DC (6–8). Monocytes can be identified in the BM on the basis of high level expression of Ly-6C (ER-MP20) and the absence of CD31 (ER-MP12), but an exclusive marker for mouse monocytes in the BM or the bloodstream is not yet available (4, 9, 10). Characterizing peripheral blood monocytes, Lagasse and Weissman (11) and, more recently, Henderson et al. (12) showed peripheral blood monocytes to be a homogeneous population. Nevertheless, experiments conducted by Palframan et al. (13) and Geissman et al. (14) using <i>CX<sub>C</sub>CRI<sub>GFP/F<sub>R</sub></sub></i> mice indicated that peripheral blood monocytes in these mice also encompass subtypes, differing in <i>CX<sub>C</sub></i>, <i>CX<sub>R1</sub></i>, <i>CCR2</i>, and CD62L expression. Interestingly, the monocytes expressing CCR2, CD62L, and low levels of <i>CX<sub>C</sub></i> appeared to be preferentially recruited to inflamed peripheral sites by virtue of their recognition of CCL2/macrophage chemoattractant protein-1 and the CD62L-mediated interaction with high endothelial venules. Conversely, the <i>CX<sub>C</sub>CRI<sub>hi</sub></i> monocytes migrated into noninflamed sites (14).

The goals of the present study were to identify and characterize the putative subpopulations of monocytes in normal mouse peripheral blood with respect to phenotype and function and to establish their hitherto unknown developmental relationship. The initial criteria used to distinguish monocytes from other leukocyte types were their mononuclearity, which is read as low orthogonal (side) scatter in the flow cytometer, and their myeloid nature, as indicated by high level expression of CD11b/Mac-1. Additional required characteristics were high phagocytic capacity and the ability to develop into macrophages upon stimulation with M-CSF. Our results show that distinct subpopulations of monocytes can be identified in steady state peripheral blood of normal mice by differential expression of various surface markers, in particular Ly-6C.
(ER-MP20), Ly-6C<sup>high</sup> monocytes are recent immigrants from the BM and have the capacity to migrate into sites of peripheral inflammation. In contrast, Ly-6C<sup>low</sup> monocytes have lost this potential. Moreover, we show that Ly-6C<sup>high</sup> monocytes mature in the circulation and are the precursors for Ly-6C<sup>low</sup> monocytes.

Materials and Methods
As the reported experiments comprise a collaborative effort among three different laboratories, materials and methods differed slightly between experiments performed at different locations, as indicated. However, results were validated by repetitions of experiments and extensive comparisons between our laboratories. All data shown are representative of at least three independently performed experiments.

Mice
Specific pathogen-free C57BL/6J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME), Charles River (Sulzfeld, Germany), or Harlan (Horst, The Netherlands) and were housed in microisolator cages and given mouse chow and water ad libitum. Mice were 8–16 wk of age when used in experiments. All experiments with mice were performed with the approval of the animal care and use committees of Veterans Affairs Medical Center (Oklahoma City, OK), Erasmus Medical Center (Rotterdam, The Netherlands), or the State Review Board of Munster (Germany).

Antibodies
Specifications of mAbs and fluorescent conjugates against surface markers used in this study are listed in Table I. Directly conjugated isotype control mAb were purchased from BD PharMingen (San Diego, CA). Anti-rat IgG conjugated with FITC was obtained from DianoVA (Hamburg, Germany).

Mouse models of inflammation
*L. monocytogenes* strain EGD was stored in brain heart infusion broth (Difco, Detroit, MI) at 10<sup>7</sup> CFU/ml at −70°C. For experiments, 10 µl of stock culture were inoculated into 4 ml of broth and incubated overnight at 37°C with shaking. An aliquot of the overnight culture was diluted 1/10 into fresh broth and cultured an additional 4.5 h. Bacteria were diluted to the desired concentration with sterile PBS before injection. *Leishmania major* (World Health Organization nomenclature: MHOM/IL/81/SE/BNI) were maintained by monthly passages in BALB/c mice. Promastigotes were grown in a 5% CO<sub>2</sub> atmosphere at 25°C in Schneider’s Drosophila medium (Promocell, Heidelberg, Germany) supplemented with 10% FCS, 2% human urine, glutamine, and HEPES buffer, with penicillin and streptomycin as antibiotics. Mice were infected i.p. with 5.1 log<sub>10</sub> CFU (1 medium (Promocell, Heidelberg, Germany) supplemented with 10% FCS, or by s.c. application of 2 × 10<sup>7</sup> promastigotes (World Health Organization nomenclature: MHOM/IL/81/SE/BNI) in 2 ml of HBSS. Suspensions of freshly sorted cells were cytocentrifuged onto slides, air-dried, and then fixed with equal parts ethanol/methanol. Differential counts, according to the morphological criteria established previously (10), were performed on May–Gru¨newald-Giemsa-stained cytospin preparations of peripheral blood leukocytes and blood smears by counting at least 200 cells/cytospin or blood smear at ×400 magnification.

Table I. Monoclonal Abs

<table>
<thead>
<tr>
<th>Marker</th>
<th>mAb</th>
<th>Used Form</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>145-2C11</td>
<td>FITC, phycoerythrin conjugate</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>CD11b/Mac-1</td>
<td>M1/70</td>
<td>FITC, PE, PerCP-Cy5, PE-CY5,</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>allophycocyanin conjugate</td>
<td></td>
</tr>
<tr>
<td>CD11c</td>
<td>HL3</td>
<td>FITC, PE conjugate</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>CD16/32/FcγRII/III</td>
<td>2.4G2</td>
<td>Hybridoma supernatant</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CD19</td>
<td>ID3</td>
<td>PE conjugate</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>CD43</td>
<td>S7</td>
<td>FITC, PE conjugate</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>CD54/ICAM-1</td>
<td>YN1/1.7</td>
<td>Hybridoma supernatant</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CD62L/L-selectin</td>
<td>MEL-14</td>
<td>PE conjugate</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>CD115/M-CSF R</td>
<td>AF598</td>
<td>Hybridoma supernatant</td>
<td>Dr. Nishikawa (Kyoto, Japan)</td>
</tr>
<tr>
<td>ER-MP58</td>
<td>ER-MP58</td>
<td>Hybridoma supernatant</td>
<td>Own laboratory</td>
</tr>
<tr>
<td>Ly-6C</td>
<td>F4/80</td>
<td>FITC conjugate</td>
<td>Callag Laboratories</td>
</tr>
<tr>
<td></td>
<td>F4/80</td>
<td>FITC conjugate</td>
<td></td>
</tr>
<tr>
<td>MBR</td>
<td>MIV 38</td>
<td>Hybridoma supernatant</td>
<td>Dr. Falkenberg (Bochum, Germany)</td>
</tr>
<tr>
<td>MHC class II</td>
<td>M5/114.15.2</td>
<td>PE conjugate</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PK136</td>
<td>PE conjugate</td>
<td>BD PharMingen</td>
</tr>
</tbody>
</table>

Preparation of leukocytes
Mice were euthanized with an overdose of ketamine (2 mg; Parke-Davis, Berlin, Germany; or Morris Plains, NJ) and xylazine (0.2 mg; CEVA Tiergesundheit, Dusseldorf, Germany; or Vedco, St. Joseph, MO) or by CO<sub>2</sub> exposure. Blood was obtained by axillary or femoral artery puncture or by heart puncture after exposing the organ and was collected in heparin-coated tubes or in syringes containing 1.0 ml of PBS with 8 mM EDTA. Control experiments showed that similar results were obtained by either method of exsanguination and anticoagulation. Erythrocytes were lysed using ACK lysis solution, and then leukocytes were washed twice by centrifugation at 250 × g in DMEM (Life Technologies, Gaithersburg, MD) containing 5 mM EDTA and 0.5% BSA. Peritoneal cells were collected 18–24 h after induction of inflammation by lavage with 8 ml of sterile iced PBS and were similarly prepared for further analysis.

Flow cytometry and cell sorting
Aliquots of ~10<sup>6</sup> cells in 1% BSA in HBSS (Biochrom, Berlin, Germany; or Life Technologies) were put into 96-well microtiter plates and then incubated with unlabeled mAb or directly conjugated primary mAb for 30 min on ice. In experiments using PE-Cy5-conjugated mAbs, the cells were first incubated with 3% normal mouse serum and anti-CD16/32 mAb (BD PharMingen) for 30 min on ice before addition of directly conjugated mAb to block nonspecific binding. After the first incubation, the cells were washed three times and then incubated with fluorochrome-conjugated secondary Abs as needed. Controls included cells incubated with or without fluorochrome-conjugated control Abs and with unspecific isotype Ab, followed by fluorochrome-conjugated secondary Abs as needed. The cells were analyzed using a FACSCalibur equipped for four-color flow cytometry. Each measurement contained a defined number of 2 × 10<sup>6</sup> cells. Data were analyzed using CellQuest (BD Biosciences, Mountain View, CA) or WinMDI 2.8 software. Cell sorting was performed on a MoFlo High Speed Cell Sorter and Analyzer (Cytometry, Ft. Collins, CO) with cell acquisition using CyCLOPS version 2.1 software (Cytometry).

Morphological analysis
For morphological characterization of sorted cells, at least 2 × 10<sup>4</sup> cells from each population were sorted into FCS-coated plastic tubes filled with 2 ml of HBSS. Suspensions of freshly sorted cells were cytocentrifuged onto slides, air-dried, and then fixed with equal parts ethanol/methanol. Differential counts, according to the morphological criteria established previously (10), were performed on May–Grünewald-Giemsa-stained cytosin preparations of peripheral blood leukocytes and blood smears by counting at least 200 cells/cytospin or blood smear at ×400 magnification.
Culture of sorted cells

For analysis of in vitro development of sorted populations, 6–9 × 10^6 cells from each population were collected into 24-well glass tissue culture chambers (Nunc, Roskilde, Denmark). To test for macrophage development, cells were seeded at a density of 1 × 10^4 or 2 × 10^5 cells/ml in DMEM supplemented with 10% heat-inactivated FCS, 10% L cell-conditioned medium (as a source of M-CSF), l-glutamine, kanamycin, and nonessential amino acids. The cells were incubated at 37°C with 7% CO_2 for 1–8 days, after which time they were fixed and stained for differential counting as described above.

Adherence and proliferation of cultured cells were evaluated daily with an inverted microscope using an ocular with eyepiece graticule. When several cells were found in close contact with each other and distinctly separated from neighboring cells, they were referred to as groups of 2–10 cells, clusters of 10–50 cells, or colonies when counting >50 cells.

Phagocytosis of latex particles and L. major promastigotes

Phagocytic activity was quantified by adding 2.5 μl of a suspension of 5.4 × 10^6 latex particles/ml with an average diameter of 0.81 μm (Difco) to adherent cells on 24-well plates for a final ratio of cells to latex particles of 1:14. Cells and latex particles were incubated for 4 h at 37°C and then fixed with ethanol/aceto (50/50, v/v). In some experiments, phagocytosis was studied using L. major promastigotes. For this, freshly sorted cells were coincubated with promastigotes for 4 h at a ratio of 1:5. Cells were washed and fixed in ethanol/aceto (50/50, v/v), and phagocytosis was quantified after staining with May-Grünewald-Giemsa. Cells were examined by light microscopy at ×200 and ×400 for the presence of intracellular parasites or ingested latex particles, respectively.

Results

Mouse blood monocytes are side scatterlow (SSC^low)CD11b^high cells heterogeneous for Ly-6C

To establish the flow cytometric identification of monocytes in normal mouse peripheral blood, we hypothesized that monocytes should be distinguishable from granulocytes and lymphocytes by their lower granular content, as reflected in low SSC, and their high level expression of Mac-1/CD11b (4, 10, 11). To test this, we sorted different peripheral blood subsets based on SSC and Mac-1 expression (Fig. 1A) and determined which population contained monocytes. Morphological characterization by May-Grünewald-Giemsa staining showed that 95% of SSC^lowCD11b^high cells were monocytes (see below). In addition, we observed that almost 95% of SSC^lowM-CSF receptor^+ (M-CSF R)^+ cells could be found within the CD11b^high window (Fig. 1A), suggesting that virtually all monocytes were contained in this population. When sorted, these SSC^lowCD11b^high cells became adherent when cultured in M-CSF-containing medium and proliferated to some degree (Fig. 1B). In contrast, almost all cells from the SSC^highCD11b^high population were granulocytes, whereas lymphocytes constituted the majority of SSC^lowCD11b^low cells. Neither of these populations survived in the culture conditions for macrophages (Fig. 1B). More than 90% of the SSC^lowCD11b^high cells were able to phagocytose L. major, whereas only SSC^high granulocytes, but not cells from other sorted populations, showed relevant phagocytic activity (Fig. 1C).

Further analysis of the SSC^lowCD11b^high population with markers for NK cells (NK1.1), T cells (CD3), or B cells (CD19) showed that the SSC^lowCD11b^high population contained no NK cells or lymphocytes (Fig. 2A). In contrast, SSC^lowCD11b^high cells uniformly expressed significant levels of M-CSF R. Together, these findings indicate that the SSC^lowCD11b^high population in peripheral blood encompasses almost exclusively monocytes on the basis of phenotype, morphology, and function.

After establishing parameters to discriminate monocytes from other leukocytes by flow cytometry, we tested whether this population displayed phenotypic heterogeneity. Initial analysis indicated that Ly-6C (ER-MP20) was variably expressed among SSC^lowCD11b^high monocytes and allowed the distinction between subpopulations: Ly-6C^low, Ly-6C^med, and Ly-6C^high cells (Fig. 2A). The level of Ly-6C expression found on Ly-6C^high cells resembles that on BM monocytes (4). Sorting and analysis of SSC^lowCD11b^high cells according to the Ly-6C (ER-MP20) expression level showed that virtually all cells presented monocytic morphology (Fig. 2B) and extensive phagocytosis of latex beads and L. major.

Analysis of their proliferative activity in M-CSF-containing medium showed that cells from each subpopulation formed several groups of 2–10 cells in culture after a few days. Larger clusters (11–50 cells) and colonies (>50 cells) were most frequently seen in cultures of the Ly-6C^high population. This indicates that monocytes in all three subpopulations have the capacity to proliferate, but that cells with the highest proliferative potential reside in the Ly-6C^high population. Cells developing in these cultures were typically adherent and showed macrophage morphology (our unpublished observations). When we applied culture conditions suitable...
In particular, CD62L was expressed by Ly-6Chigh monocytes, whereas CD11c and CD43 were expressed by Ly-6C low monocytes. Representative data are shown for at least four independent experiments.

For the development of DC, using GM-CSF and IL-4, cells with morphological characteristics of DC and CD11c and MHC class II expression developed in a few days from all three different subsets (our unpublished observations), in accordance with the previously characterized development of DC from mouse monocytes (8, 17).

Further phenotypic characterization of the Ly-6C-defined monocyte subsets showed a uniform high level expression of ER-MP58, characteristic for cells of the myeloid lineage (18) (Fig. 2D). The ability to detect F4/80 varied between experiments, with all subsets expressing the Ag in some experiments, whereas in others no positive subsets were present. In the currently shown profile, most, but not all, monocytes in the different subsets express F4/80. The various subpopulations expressed only marginal amounts of mature macrophage markers such as FcγRII/RII (CD16/CD32) or scavenger receptor type A (Fig. 2D). However, the subsets did show clear heterogeneity with regard to the expression of CD11c, CD43, and CD62L, with the latter being present primarily on Ly-6Chigh monocytes, whereas CD11c and CD43 were found in particular on Ly-6C–/low cells.

**Down-regulation of Ly-6C marks the maturation of mouse blood monocytes**

Previous studies have indicated that BM monocytes are Ly-6Chigh and that Ly-6C expression is rapidly lost during in vitro development into macrophages or DC (4, 8). Using the same gating as established for the peripheral blood monocytes, we confirmed that BM monocytes, identified as either SSClowCD11bhigh or SSChighM-CSF R+ cells, were almost exclusively Ly-6Chigh (Fig. 3A). Therefore, we investigated the possibility that Ly-6Chigh peripheral blood monocytes might correspond to BM monocytes, which developed into Ly-6C–/low cells subsequently. In vitro we found that sorted Ly-6Chigh peripheral blood monocytes lost expression of Ly-6C within 3 days (our unpublished observations).

To analyze the subset relationship in vivo we injected mice i.v. with liposomes loaded with clodronate to deplete all phagocytic cells in the circulation and followed the kinetics of monocyte repopulation in the blood. Fig. 3B shows that almost 90% of the SSClowCD11bhigh monocytes were eliminated by this procedure 18 h after liposome injection. This result seemingly contrasts with the reported finding that blood monocytes could be depleted only partially by this method (19). However, in that study monocyte depletion was evaluated only 48 h after depletion, whereas we found that after 24 h monocytes had started to repopulate and had reached normal numbers in 3–4 days (Fig. 3B). The monocytes reappearing at 2 and 4 days after depletion were almost exclusively of the Ly-6Chigh BM phenotype (Fig. 3C). Ly-6C–/low monocytes were found in the circulation in significant numbers only from 7 days after depletion. These findings are consistent with the interpretation that Ly-6Chigh monocytes give rise to Ly-6Cmed and Ly-6C– monocytes.

Attempting to approach this maturation sequence more directly, we sorted Ly-6Chigh monocytes from green fluorescent protein (GFP)-transgenic mice and injected these into normal, unconditioned recipients. However, at no time point after transfer could GFP+ cells be retrieved from the circulation of recipient mice, suggesting that manipulation of these Ly-6Chigh monocytes might induce aberrant behavior, leading to rapid margination in vivo. As an alternative approach, we synchronized monocyte development by clodronate liposome-mediated depletion and then labeled reappearing monocytes in vivo to follow their kinetic response. At 48 h after depletion, virtually all circulating monocytes were Ly-6Chigh (Fig. 3C). When we then injected fluorochrome (DiI)-labeled liposomes, we found that the vast majority of circulating monocytes became labeled and retained the Ly-6Chigh phenotype for 2 days (Fig. 3D). From 3 days after fluorochrome application, DiI-labeled Ly-6C–/low monocytes were observed in the circulation, indicating that these cells had indeed developed from Ly-6Chigh monocytes. In contrast, the vast majority of Ly-6Chigh monocytes were not labeled at this point, demonstrating that they had been released into circulation after the fluorochrome pulse labeling. These experiments strongly support the concept that monocytes enter the circulation from the BM as Ly-6Chigh cells and then develop into Ly-6C–/low monocytes before emigrating into peripheral tissues.
To follow the kinetics of the distinct monocyte populations in steady state, we attempted to label all monocytes by i.v. injection of fluorochrome (DiD)-labeled liposomes. Unexpectedly, we found that all monocytes, identified as SSC low CD11b high cells, disappeared from the circulation within 30 min after liposome injection (Fig. 3E) suggesting that phagocytosis of liposomes triggered monocyte margination. The first cells reappeared 2 days after clodronate liposome injection and were BM-like Ly-6C<sup>high</sup> monocytes, whereas Ly-6C<sup>low</sup> monocytes were detected in the circulation from 7 days after depletion. Monocytes that reappeared on day 2 after depletion were pulse-labeled in vivo by injecting Dil-loaded liposomes. By 3 days after the Dil labeling, Ly-6C<sup>high</sup> monocytes had developed into Ly-6C<sup>low</sup> monocytes, whereas the vast majority of unlabeled monocytes at that time were of the Ly-6C<sup>high</sup> BM type. Injection of DiD-loaded liposomes into steady state mice caused all monocytes to disappear temporarily from the circulation within 30 min after injection. Measurable numbers of monocytes were found in circulation 2–4 h later, but these were unlabeled Ly-6C<sup>high</sup> BM-type cells. DiD-labeled monocytes were again present 16 h after application of DiD-labeled liposomes and expressed only low levels, if any, of Ly-6C reflecting their relatively mature status.

The immature Ly-6C<sup>med-high</sup> monocyte subset is selectively involved in inflammatory conditions

Previous studies have shown that monocytes recruited to peripheral inflammatory sites typically show a high level expression of Ly-6C (20, 21). In view of our current findings, we asked whether this was due to a selective recruitment of immature BM-type monocytes. Therefore, sterile inflammation was elicited by i.p. injection of FCS. In the steady state peritoneal cavity, we observed that monocyte-like SSC<sup>low</sup>CD11b<sup>high</sup> cells were present, characterized by an intermediate expression of the macrophage marker BM8, well below that of resident peritoneal macrophages (Fig. 4A). These resident peritoneal monocytes were uniformly lacking Ly-6C (Fig. 4B). In contrast, the first inflammatory monocytes examined 18 h after FCS injection clearly showed significant Ly-6C expression (Fig. 4B). By comparison, the ratio between...
monocyte subsets in peripheral blood was unchanged. This suggested that Ly-6C high immature monocytes were selectively recruited to the site of inflammation, in accordance with recent findings (14). In contrast, immigration of Ly-6C high monocytes was blocked completely when the inflammatory trigger was given to mice that were simultaneously depleted of circulating monocytes by i.v. injection of clodronate liposomes (Fig. 4B). The latter finding also indicates that FCS injection does not induce Ly-6C expression in resident peritoneal monocytes.

To investigate the monocyte subset kinetics in acute bacterial inflammation, we infected mice with L. monocytogenes. Fig. 4C shows that the ratio between Ly-6C high and Ly-6C med/low monocytes did not change in the first 48 h after infection, but the absolute number of circulating monocytes decreased during this time. In contrast, there was a significant influx of Ly-6C high monocytes 72 h after infection, causing a notable monocytosis in the blood with a shift toward the immature Ly-6C high subset. To test whether chronic infection caused a similar shift in the monocyte balance, C57BL/6 mice were infected s.c. with L. major. After 4 wk, chronic infection was established as indicated by marked swelling of footpads and by dissemination of parasites into draining lymph nodes. At this stage also a significant shift was found toward the Ly-6C high immature monocyte subset (Fig. 4D). Taken together, these findings confirm the idea that inflammatory monocytes are exclusively Ly-6C med/high cells. Furthermore, both acute and chronic infection are reflected in the blood by a shift in the balance between monocyte subsets toward the immature Ly-6C high BM type.

**Discussion**

The circulating monocyte compartment in the mouse has been studied to only a limited extent due in part to the limited number of available cells and the lack of solid criteria to identify this cell type. In this study we characterized mouse peripheral blood monocytes in genetically nonmanipulated animals using an approach that is generally applicable in both steady state and experimental conditions. To identify monocytes among peripheral blood leukocytes, we used criteria that are universally recognized as characteristic of mononuclear phagocytes (22). These are 1) mononuclearity, seen as low orthogonal light scatter in the flow
cytometer; 2) myeloid nature, shown by high level expression of CD11b; 3) uniform phagocytic potential; and 4) the ability to develop into macrophages upon M-CSF stimulation. Application of these criteria indicated that monocytes in mouse peripheral blood were restricted to the SSClowCD11bhigh population and that this population included no other cells. These cells were uniformly positive for the M-CSF receptor (CD115), a proposed universal marker for mononuclear phagocytes (1). This way we identified ~6% of circulating leukocytes in the steady state as monocytes. With total leukocyte counts of ~10^9/ml, this amounts to 6 x 10^7 circulating monocytes/ml of mouse blood, which is consistent with earlier data (23).

In previous studies, Lagasse and Weissman used high level expression of CD11b, the absence or low level expression of Gr-1 and low SSC as discriminating criteria for monocytes and identified these cells as a single population (11). Similarly, Henderson and colleagues (12) found mouse monocytes to be a homogeneous population using high level expression of the myeloid marker 7/4 and intermediate expression of Gr-1 as discriminating criteria. In contrast, Geissmann and colleagues (14) recently recognized distinct subsets among circulating monocytes in CX3CR1GFP/+ mice which differed in phenotypic and functional characteristics. In accordance with these results, we distinguished monocyte subsets in normal mice according to differential expression of Ly-6C (ER-MP20), CD62L, CD11c, and CD43. Cells of each subset showed extensive phagocytic activity, displayed typical monocyte morphology and developed into macrophages in vitro upon M-CSF stimulation. Thus, each population of SSClowCD11bhigh leukocytes, distinguished by variable Ly-6C expression, contains bona fide monocytes.

To date a possible relationship between these monocyte subsets had not been addressed (13, 14). Using various approaches, we found that the monocyte subsets differing in Ly-6C expression represent different stages in a continuous maturation pathway. As such, recent immigrants from the BM enter circulation as Ly-6Chigh monocytes, which were shown to be monocytes, distinguished by variable Ly-6C expression, displayed typical monocyte morphology and developed into macrophages in vitro upon M-CSF stimulation. Thus, each population of SSClowCD11bhigh leukocytes, distinguished by variable Ly-6C expression, contains bona fide monocytes.

The immature, Ly-6C^high subset of monocytes is characterized by a higher level of L-selectin (CD62L^hi) expression compared with Ly-6C^low monocytes. Given that CD62L mediates recruitment of leukocytes (29), we tested whether differential expression of this molecule was also manifested in differences in recruitment of the different subsets during an elicited peritoneal inflammation. We observed that the inflammatory Ly-6C^high monocytes (20, 21), but not the Ly-6C^low monocytes, were recruited into acute inflammation. Recently, Pfaffmann et al. (13) identified two populations of F4/80-positive mononuclear cells in peripheral blood of heterozygous CX3CR1GFP/+ mice, of which one subset expressed CD62L and was recruited to lymph nodes draining inflamed skin. In accordance, Geissmann et al. (14) showed preferential migration of the CD62L^Gr-1^CCR2^+ monocyte subset, which corresponds to our Ly-6C^high population, into the peritoneal cavity during thioglycolate-induced inflammation in CX3CR1GFP/+ mice. We found in preliminary studies that Ly-6C^high monocytes express elevated levels of CCR5 and CXCR4 compared with the mature Ly-6C^low monocytes. Together, these findings clearly show that particularly the immature Ly-6C^high monocytes, but not the mature Ly-6C^low monocytes have a high potential to migrate to inflammatory sites.

Peripheral inflammation stimulates the production of monocytes in the BM and their subsequent release into circulation. When this is substantial, there is a skewing of the monocyte population toward a higher frequency of immature Ly-6C^high cells. This can be considered a so-called left shift in the monocyte compartment comparable to that seen in the neutrophil compartment, when more immature bandforms are found during acute inflammation. Such a monocyte left shift is typically observed after 72 h during primary infection with L. monocytogenes. It results in greatly increased numbers of monocytes in circulation, whereas the BM is fully occupied with myelopoiesis (9). We observed a significant skewing toward immature cells also in chronic infection caused by L. major. This probably reflects the increased myelopoiesis that is long recognized in chronic infection with L. major (30).

The identification of distinct monocyte subsets in the mouse raises the question of how these relate to monocyte subsets present in humans. A recent comparison between human and mouse monocytes indicated that CX3CR1low (our Ly-6C^high) monocytes correspond to the classical CD14^highCD16^- monocytes, whereas CX3CR1high (our Ly-6C^-flow) monocytes resemble CD14lowCD16^high cells (14). Interestingly, in human patients with sepsis an increase has been observed of CD64^- monocytes, which primarily correspond to the major subset of CD14^16^- monocytes (31). In analogy to our findings in infected mice, this might represent a so-called left shift toward immature monocytes also in severe inflammations in humans.
Taken together, in this study we identify different subsets of mouse monocytes in peripheral blood, which behave differently depending on host conditions. Our data indicate that the cells enter the circulation from the BM as Ly-6Chigh cells. Under steady state conditions, monocytes mature in circulation, which is reflected in strong down-regulation of Ly-6C and up-regulation of CD43 expression. In peripheral inflammation, however, only Ly-6Cmed/high monocytes are recruited to the affected sites to become inflammatory exudate macrophages. Increased monocytopenosis under these circumstances is apparent from the increased frequency of immature Ly-6C<sub>high</sub> monocytes, which may be considered a left shift in the monocye compartment. The destiny of the Ly-6C<sub>++/low</sub> monocytes remains more speculative at present. Their phenotypic change suggests maturation toward DC with increased CD43 expression and suggests maturation toward DC with increased CD43 expression. In peripheral inflammation, however, only Ly-6C<sub>med/</sub>high monocytes are recruited to the affected sites to become inflammatory exudate macrophages. Increased monocytopenosis under these circumstances is apparent from the increased frequency of immature Ly-6C<sub>high</sub> monocytes, which may be considered a left shift in the monocye compartment. The destiny of the Ly-6C<sub>++/low</sub> monocytes remains more speculative at present. Their phenotypic change suggests maturation toward DC with increased CD43 expression.

Acknowledgments

Many people were important in the genesis of this study. We thank Tar van Os for preparation of the figures; Eva Nattkemper, Ruth Goez, Berlinda den Broeder, Karin Fischer, and Meike Steinert for technical support; and Jeremy Gill for his enthusiastic input. We are grateful to Ellen van Lochem for advice on human monocyte development, and to Jim Henthorn (William K. Warren Medical Research Institute) for expert assistance with flow cytometry and cell sorting. Dr. Nishikawa is thankfully acknowledged for making available the anti-M-CSF R Ab.

References