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The Ly-6C^{high} Monocyte Subpopulation Transports *Listeria monocytogenes* into the Brain during Systemic Infection of Mice[1]

Douglas A. Drevets,[2]* Marilyn J. Dillon,* Jennifer S. Schawang,* Nico van Rooijen,† Jan Ehrchen,‡ Cord Sunderkötter,[3‡§] and Pieter J. M. Leenen[3¶]

Mononuclear phagocytes can be used by intracellular pathogens to disseminate throughout the host. In the bloodstream these cells are generically referred to as monocytes. However, blood monocytes are a heterogeneous population, and the exact identity of the leukocyte(s) relevant for microbial spreading is not known. Experiments reported in this study used *Listeria monocytogenes*-infected mice to establish the phenotype of parasitized blood leukocytes and to test their role in systemic dissemination of intracellular bacteria. More than 90% of the blood leukocytes that were associated with bacteria were CD11b$^+$ mononuclear cells. Analysis of newly described monocyte subsets showed that most infected cells belonged to the Ly-6C^{high} monocyte subset and that Ly-6C^{high} and Ly-6C$^{neg\text{-}low}$ monocytes harbored similar numbers of bacteria per cell. Interestingly, systemic infection with wild-type or Δ*actA* mutants of *L. monocytogenes*, both of which escape from phagosomes and replicate intracellularly, caused expansion of the Ly-6C^{high} subset. In contrast, this was not evident after infection with Δ*hly* mutants, which neither escape phagosomes nor replicate intracellularly. Importantly, when CD11b$^+$ leukocytes were isolated from the brains of lethally infected mice, 88% of these cells were identified as Ly-6C^{high} monocytes. Kinetic analysis showed a significant influx of Ly-6C^{high} monocytes into the brain 2 days after systemic infection. This coincided with both bacterial invasion and up-regulation of brain macrophage chemoattractant protein-1 gene expression. These data indicate that the Ly-6C^{high} monocyte subset transports *L. monocytogenes* into the brain and establish their role as Trojan horses in vivo. ***The Journal of Immunology*, 2004, 172: 4418–4424.**

Intracellular parasitism of leukocytes is a mechanism used by some pathogenic microorganisms to avoid host defenses and to disseminate away from the site of primary infection. For example, recent data show that dendritic cells transport intracellular *Listeria monocytogenes*, *Leishmania major*, *Mycobacterium tuberculosis*, and *Salmonella typhimurium* away from mucosal areas and toward draining lymph nodes (1–5). Phagocytes in the bloodstream also have a role in systemic dissemination of intracellular pathogens, although the cells and mechanisms used for this are less well defined (6). Understanding this process is critical because systemic dissemination is necessary for pathogenic microbes to establish distant foci of infection and, in particular, for invading protected spaces such as the CNS (7). Targeting these steps pharmaceutically could present a new avenue for limiting the spread of neurotropic pathogens.

L. monocytogenes is a facultative intracellular bacterium that invades humans via the gastrointestinal tract and causes bacteremia as well as a variety of CNS infections (8). In the mouse model of systemic listeriosis, bacteremia typically precedes CNS infection and is composed of both cell-free bacteria and infected leukocytes (9, 10). Because intracellular and extracellular *L. monocytogenes* are present together in the circulation, it has been unclear whether extracellular bacteria invade the CNS directly or whether parasitized leukocytes transport them into the CNS. Data supporting the latter mechanism come from histological studies of experimentally infected mice that identified infected phagocytes in the choroid plexus (11). In addition, studies from our laboratory showed that killing extracellular *L. monocytogenes* in blood with gentamicin did not prevent bacterial infection of the brain (12). This finding suggests that migration of parasitized leukocytes from the bloodstream into the CNS is instrumental in neuroinvasion. Taken together, these data support a central role for infected phagocytes in systemic dissemination and neuroinvasion by *L. monocytogenes*.

Most *L. monocytogenes*-infected leukocytes in the blood have been identified morphologically as mononuclear cells (10), but the exact phenotype of the leukocytes that transport these bacteria through the bloodstream and into the brain is not yet known. Moreover, precise identification of parasitized mononuclear phagocytes in the blood during experimental infection of mice is complicated by the fact that no single marker exclusively recognizes mouse monocytes and distinguishes them from granulocytes (13–18). Recent studies from our group and others indicate that mouse monocytes are a heterogeneous population composed of different subsets that function differentially in steady state and inflammation (18–20). These data offer an exciting new paradigm for the study of mouse blood monocytes and their roles during infection with

*Department of Medicine, Oklahoma University Health Sciences Center, and Veterans Affairs Medical Center, Oklahoma City, OK 73014; †Department of Molecular Cell Biology, Free University Medical Center, Amsterdam, The Netherlands; ‡Institute of Experimental Dermatology and Department of Dermatology, University of Münster, Münster, Germany; §Department of Dermatology and Allergy, University of Ulm, Ulm, Germany; and ¶Department of Immunology, Erasmus MC, Rotterdam, The Netherlands

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[2] Address correspondence and reprint requests to Dr. Douglas A. Drevets, Veterans Affairs Medical Center 111/c, 921 NE 13th Street, Oklahoma City, OK 73014. E-mail address: douglas-drevets@ouhsc.edu

[3] C.S. and P.J.M.L. contributed equally to this work.

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intracellular pathogens. To capitalize on this, the experiments reported in this study analyzed mouse monocytes and their subsets during systemic infection with *L. monocytogenes*. Our results show that a subset of monocytes distinguished by high level expression of Ly-6C (Ly-6C^{high}) harbors the majority of *L. monocytogenes* in the bloodstream. Importantly, we also demonstrate that systemic infection stimulates an influx of these cells, some of which contain bacteria, into the brain coincident with bacterial invasion. The monocyte influx coincides with up-regulation of macrophage chemoattractant protein-1 (MCP-1;[4] CCL2) gene expression.

Materials and Methods

Antibodies

CD3-PE, CD11b (M1/70)-PE-Cy5 and -Cy5.5, CD19-PE, CD62L-PE (MEL-14), GR-1-PE (RB6-8C5), Ly-6G-PE (1A8), NK1.1-biotin, and isotype control mAb were purchased from BD PharMingen (San Diego, CA) as direct conjugates. Rat anti-mouse Ly-6C (ER-MP20) was used as hybridoma culture supernatant and as direct FITC conjugate (21).

Bacteria

Bacteria were stored in brain heart infusion broth (Difco, Detroit, MI) at 10^9 CFU/ml at −70°C. Wild-type *L. monocytogenes* strains included EGD and 10403s. Gene deletion mutants of *L. monocytogenes* strain 10403s were obtained from D. Portnoy (University of California, Berkeley, CA) and included the listeriolysin O-deficient (Δ*hly*) DP-L2161 and *actA*-deficient (Δ*actA*) DP-L1942 (22, 23). *L. monocytogenes* strain NF-L512 containing a chromosomal *actA-gfpuv-plcB* transcriptional fusion was obtained from N. Freitag (Seattle Biomedical Research Institute, Seattle, WA) (12). For experiments, 0.5 ml of stock culture was diluted in 4 ml of broth and then cultured for 4.5 h at 37°C. Bacteria were diluted in sterile PBS before injection into mice.

Mouse infection

Female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), 8–16 wk old, were used in all experiments. They were infected by i.p. or i.v. injection of 1–2 LD_{50} of wild-type *L. monocytogenes* or 10^6–10^7 CFU of other bacteria and then were euthanized at the indicated time with ketamine/xylazine (Vedco, St. Joseph, MO). Blood was collected into PBS containing 10 mM EDTA, and blood leukocytes were isolated as previously described (10, 12). In experiments that required harvesting of bacteria, leukocytes, or RNA from the brain, the animals were perfused with 30 ml of PBS via the left ventricle to remove blood from the brain. Leukocytes were isolated from whole brains by enzymatic digestion with 0.1% collagenase D (Roche, Indianapolis, IN) and 10 μg/ml DNase I (Sigma-Aldrich, St. Louis, MO), followed by immunomagnetic collection of $CD45^+$ or $CD11b^+$ cells on a miniMACS column (Miltenyi Biotech, Auburn, CA) (24). In other experiments the brain was divided lengthwise along the main sagittal fissure, with half the specimen used for quantifying CFU of bacteria in the brain and the other half being processed for real time-PCR (described below).

In some experiments mice were infected with *L. monocytogenes* strain NF-L512. Eighteen hours later they underwent surgical implantation of Alzet osmotic pumps (Durect, Cupertino, CA) filled with gentamicin (Sigma-Aldrich; 100 mg/ml in PBS) as previously described (12) and were injected i.v. with 0.2 ml of clodronate liposome into the lateral tail vein to eliminate monocytes in vivo (20). Dichloromethylene-bisphosphonate (clodronate) was a gift from Roche (Mannheim, Germany) and was incorporated into liposomes as previously described (25). Repopulating monocytes were labeled 24 h after depletion by i.v. injection of 0.2 ml of PBS-containing liposomes labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) (Molecular Probes, Eugene, OR).

Flow cytometry and cell sorting

Samples of 10^5 blood leukocytes, or the entire cell pellet from individual brains, were incubated in 96-well microtiter plates with 3% normal mouse serum and anti-CD16/32 mAb (BD PharMingen) for 30 min on ice before addition of isotype-matched control or test mAb. Cells were incubated with mAb for 30 min and then were washed three times with PBS/BSA/azide and postfixed with 1% paraformaldehyde. Flow cytometry was performed on a FACSCalibur (BD PharMingen), whereas cell sorting was performed on a MoStar (DakoCytomation Colorado, Ft. Collins, CO).

Microscopy

Leukocytes were cytocentrifuged onto coverslips, then fixed with 2% paraformaldehyde for 10 min at room temperature and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich). Nonspecific Ab binding was blocked using preincubation with PBS plus 5% donkey serum and 2% mouse serum. Then the cells were immunolabeled with CD11b or Ly-6C mAb, followed by fluorochrome-conjugated $F(ab')_2$ of donkey anti-rat secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and nuclear counterstaining with 4′,6-diamido-2-phenylindole hydrochloride (DAPI; Molecular Probes, Eugene, OR). Bacteria were labeled with *L. monocytogenes* antiserum (Difco), followed by fluorochrome-conjugated $F(ab')_2$ of donkey anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories). Fluorescence microscopy under oil immersion (×1000) was used to quantify Ab-labeled cells and bacteria. Confocal microscopy was performed on a TNS NT microscope (Leica, Deerfield, IL) with four-laser stimulation and four-channel image collection.

Real-time PCR for MCP-1

Perfused brains from infected and uninfected control mice were flash-frozen in liquid nitrogen and stored at −80°C until RNA extraction was performed with the BD Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA). Total RNA was reverse transcribed in 10-μl reactions using TaqMan reverse transcription reagents (PE Applied Biosystems, Foster City, CA) in 96-well optical reaction plates with optical caps (PE Applied Biosystems). Conditions for the reaction consisted of hold steps of 10 min at 25°C and 30 min at 48°C, followed by denaturation at 95°C for 5 min in an ABI PRISM SDS 7700 thermocycler (PE Applied Biosystems). Reverse-transcribed cDNA and RT-negative controls were diluted to 1 ng/μl. Then real-time PCR reactions were run with SYBR Green PCR Master Mix (PE Applied Biosystems), custom-made primers from IDT Technologies (Coralville, IA) for MCP-1 (forward, 5′-CCCAAAGAAGCTG TAGTTTTTGTCA-3′; reverse, 5′-CAGCACAGACCTCTCTCTTGAGC 3′), and the housekeeping gene, hypoxanthine phosphoribosyl transferase (forward, 5′-GTTGAAGATATAATTGACACTGGTAAAACA-3′; reverse, 5′-AGCTTGCAACCTTAACCATTTTG-3′), with a forward:reverse primer concentration ratio of 6:1. In other experiments commercial primers for mouse MCP-1 (BioSource, Camarillo, CA) were used to confirm the results. Real-time PCR reactions were run at 50-μl volumes in 96-well optical reaction plates using the ABI PRISM SDS 7700 system. Thermocycling conditions were as follows: hold at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, hold at 95°C for 15 s, hold at 60°C for 20 s, ramp to 95°C in 19 min 59 s (for a dissociation curve), hold at 95°C for 15 s.

Statistical analysis

Tests performed included one-way ANOVA with Tukey's multiple comparison test and two-tailed Student's *t* test with equal variance (PRISM, GraphPad, San Diego, CA). Both tests used a level of significance set at $p < 0.05$.

Results

Mice were infected with wild-type *L. monocytogenes*, and blood leukocytes were harvested 3–4 days later. Immunostaining and fluorescence microscopy were used to characterize *L. monocytogenes*-infected cells as CD11b-positive or -negative, and mononuclear or polymorphonuclear using criteria established by Biermann et al. (16). The results showed that 98.5% of all blood leukocytes associated with bacteria were $CD11b^+$ ($n = 15$ mice). Of these, 95.3 ± 1.9% (mean ± SEM) were mononuclear, whereas only 4.7 ± 1.2% were neutrophils, consistent with previous findings (10). These data indicate that $CD11b^+$ monocytes are the leukocytes that transport bacteria in the blood.

Because subpopulations of mouse monocytes have been identified recently (18, 20), further analysis of monocyte subsets was performed using recently established immunophenotypic criteria (20). Identification of monocytes on the basis of low orthogonal light scatter, as performed in steady state (20), was not possible, because this property was dramatically altered by *L. monocytogenes* infection (Fig. 1). Therefore, monocytes were distinguished

[4] Abbreviations used in this paper: MCP-1, macrophage chemoattractant protein-1; clodronate, dichloromethylene-bisphosphonate; DAPI, 4′,6-diamido-2-phenylindole hydrochloride; F-actin, filamentous actin; GFP, green fluorescence protein; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate.

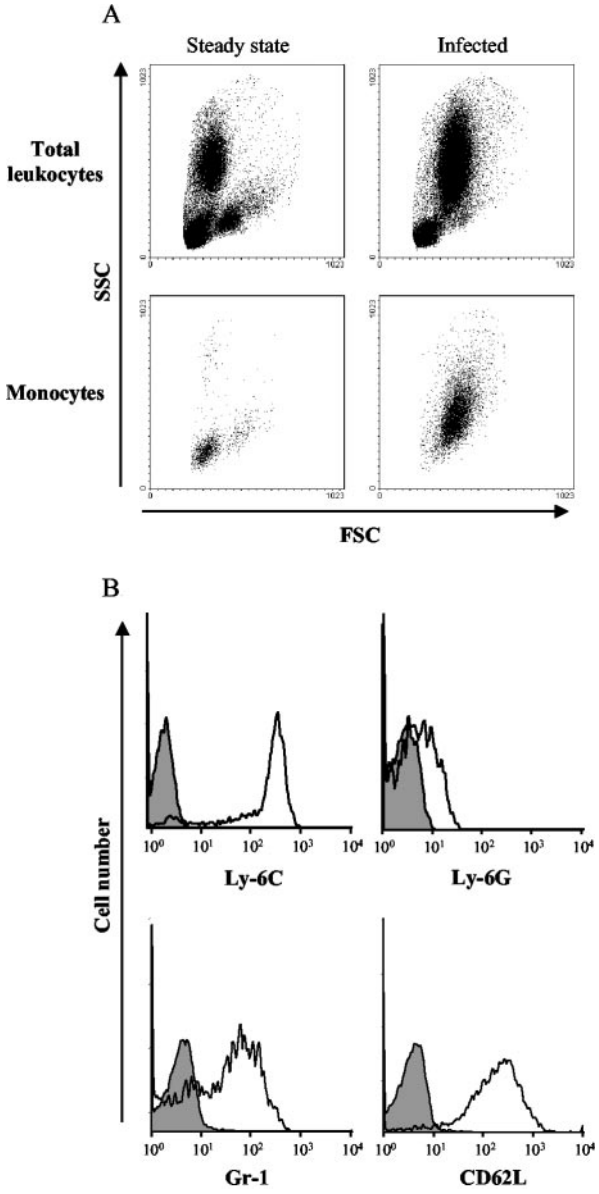

FIGURE 1. *L. monocytogenes* infection alters the forward and orthogonal light scatter properties of blood monocytes. Blood leukocytes were collected from uninfected mice and from mice infected 3 days previously with *L. monocytogenes*. The cells were immunolabeled and analyzed by flow cytometry. Monocytes were identified by first selecting the CD11b^{high} population, and then excluding neutrophils (Ly-6G^{high} or GR-1^{high}) on the Ly-6C vs Ly-6G plot. *A*, Forward and orthogonal light scatter properties of total blood leukocytes and monocytes from steady state and infected mice. *B*, Open histograms show the expression of Ly-6C and Ly-6G on total monocytes, and GR-1 and CD62L on gated Ly-6C^{high} monocytes only, from infected mice. Shaded histograms represent control mAb.

from neutrophils on the basis of their differential expressions of GR-1 or the more neutrophil specific Ly-6G when plotted against the Ly-6C expression of gated CD11b^{high} cells. These studies confirmed that monocytes from infected mice were CD11b^{high}/Ly-6G$^{neg\text{-}low}$, expressed a variable amount of Ly-6C, did not express other lineage markers, including NK1.1, CD3, and CD19, and displayed typical monocytic morphology (20) (data not shown). Further analysis of Ly-6C^{high} monocytes from infected mice showed that they were GR-1^{+} and CD62L^{+} (Fig. 1) and thus correspond to the CX3CR1^{low}/GR-1^{+} monocytes identified by Geissmann et al. (18).

Recent data show that infection of mice with *L. monocytogenes* or *L. major* skews the ratio of Ly-6C^{high} to Ly-6C$^{neg\text{-}low}$ monocytes in favor of the less mature Ly-6C^{high} cells, reflecting a left shift in the monocyte compartment (20). Interestingly, the ability to alter the steady state ratio between Ly-6C^{high} and Ly-6C$^{neg\text{-}low}$ subsets was not a common feature of infection with any bacteria. Although both Δ*actA* and Δ*hly* mutants of *L. monocytogenes* are avirulent in mice, infection with Δ*actA* bacteria elicited a percentage of Ly-6C^{high} monocytes similar to wild-type bacteria (Fig. 2). In contrast, infection with Δ*hly* mutants did not do so on a consistent basis, and if present, the shift was always minimal.

To test for differential uptake of bacteria by monocyte subsets in vivo, bacteria-containing mononuclear cells from mice infected with wild-type *L. monocytogenes* were identified by fluorescence microscopy and then categorized as Ly-6C^{high} or Ly-6C$^{neg\text{-}low}$. Monocytes were designated Ly-6C^{high} if their fluorescence intensity was greater than or equal to that of neutrophils on the same slide, or as Ly-6C$^{neg\text{-}low}$ if their intensity was less (Fig. 3). The number of bacteria per infected cell was similar in either group with 4.5 ± 0.7 bacteria/Ly-6C^{high} monocyte (mean ± SEM; n = 10 mice) compared with 5.4 ± 1.3 bacteria/Ly-6C$^{neg\text{-}low}$ monocyte ($p > 0.05$). By comparison, the majority (75.3 ± 4.1%) of monocytes with bacteria were Ly-6C^{high}, most likely reflecting expansion of this subset during infection, rather than preferential bacterial uptake (20). This finding establishes that the Ly-6C^{high} blood monocyte is the cell that harbors most of the cell-associated *L. monocytogenes* in vivo.

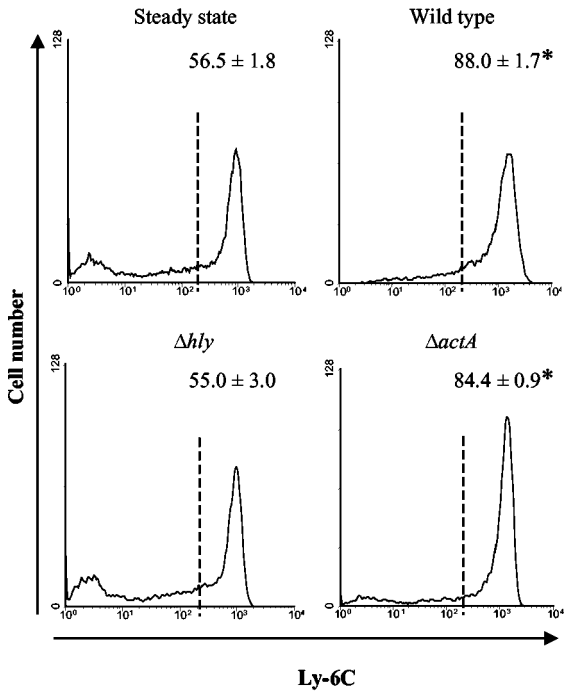

FIGURE 2. Avirulent *L. monocytogenes* mutants differ in their abilities to increase the ratio of Ly-6C^{high} to Ly-6C$^{neg\text{-}low}$ monocytes. Blood leukocytes were collected from uninfected mice and from mice infected 3 days previously with the indicated bacteria. The cells were immunolabeled and analyzed by flow cytometry. The histograms show the expression of Ly-6C on monocytes from representative uninfected mice (steady state) and from mice infected with 10^5 CFU of wild-type *L. monocytogenes* strain 10403s or with 10^6 CFU of Δ*hly* or Δ*actA L. monocytogenes* mutants. The mean ± SEM percentages of Ly-6C^{high} monocytes for four to eight mice in each group are shown. *, $p < 0.001$, by Student's t test comparing infected mice to steady state mice.

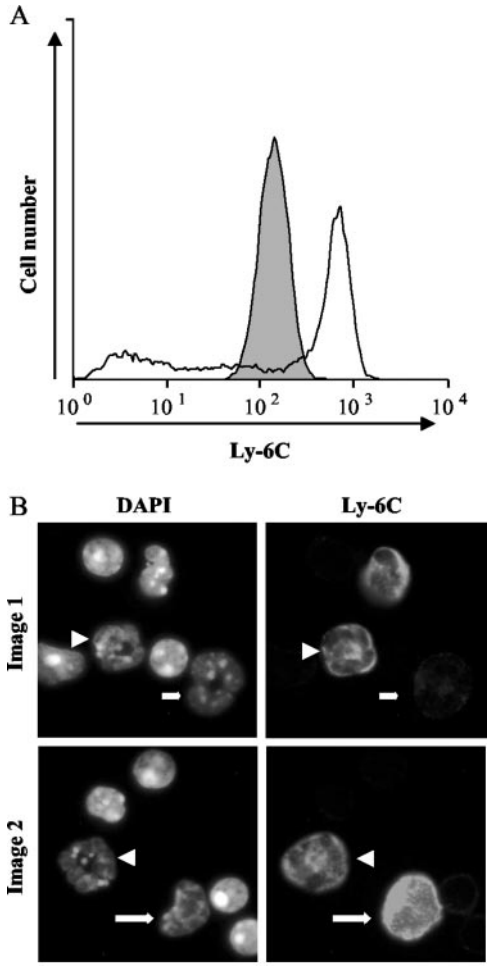

FIGURE 3. Use of fluorescence microscopy to identify Ly-6C$^{neg\text{-}low}$ and Ly-6C^{high} monocytes. Blood leukocytes were harvested from control mice, immunolabeled, and analyzed by FACS (*A*) or were harvested from *L. monocytogenes*-infected animals and then cytocentrifuged onto coverslips and immunolabeled with Ly-6C and counterstained with DAPI (*B*). The histogram represents overlays of the expression of Ly-6C on blood monocytes (open histogram) and neutrophils (shaded histogram; *A*). *B*, Images 1 and 2 depict gray scale representations using DAPI to demonstrate nuclear morphology and Ly-6C immunostaining of the same cells for Ly-6C expression to identify neutrophils (arrowheads), Ly-6C$^{neg\text{-}low}$ monocytes (short arrows, image 1), or Ly-6C^{high} monocytes (long arrows, image 2).

Previous studies using gentamicin-treated mice infected with green fluorescence protein (GFP)-expressing *L. monocytogenes* demonstrated intracellular parasitism of blood leukocytes in vivo (12). To establish whether Ly-6C^{high} monocytes were parasitized in vivo, we modified this system by also eliminating existing blood monocytes with clodronate-loaded liposomes and then labeling newly produced monocytes with liposomes containing DiD as previously described (20). Flow cytometric analysis showed that there was near-complete depletion of monocytes within 18 h of clodronate injection, with significant repopulation in another 24 h (data not shown). Moreover, 98% of repopulating monocytes labeled by DiD were Ly-6C^{high} (data not shown). DiD$^+$ cells containing GFP$^+$ bacteria were identified easily by fluorescence microscopy; some bacteria also had filamentous actin (F-actin) tails, confirming intracellular parasitism (see Fig. 4). Quantification of DiD$^+$ monocytes with and without GFP$^+$ bacteria from 11 different mice showed that 14.9 $\pm$ 5.1% (mean $\pm$ SEM) of DiD$^+$ monocytes were infected (range, 2.4–48.5%) and contained, on the average, 1.68 $\pm$ 0.12 GFP$^+$ bacteria/cell (mean $\pm$ SEM). These data clearly demonstrate intracellular parasitism of Ly-6C^{high} monocytes in vivo.

The next series of experiments tested whether infected Ly-6C^{high} blood monocytes also had a role in establishing CNS infection. For this, CD11b$^+$ cells from the brains of infected mice were isolated by immunomagnetic sorting and were analyzed by fluorescence microscopy for the presence of bacteria, Ly-6C expression, as well as nuclear morphology (Fig. 5). There were five to >60 infected cells identified/brain ($n = 6$), with a mean $\pm$ SEM of 26.2 $\pm$ 11.4 infected cells/brain that contained, on the average ($\pm$SEM), a total of 173 $\pm$ 97 bacteria/brain. Ly-6C^{high} mononuclear cells accounted for 88% of all infected cells recovered, whereas Ly-6C$^{neg\text{-}low}$ mononuclear cells and neutrophils comprised only 6.3 and 5.7% of infected cells, respectively. These data suggest that infected Ly-6C^{high} monocytes are candidate cells for trafficking intracellular bacteria into the CNS. Nevertheless, it was still imperative to determine whether Ly-6C^{high} monocytes initiated infection via phagocyte-facilitated trafficking or were recruited into the CNS in response to existing bacterial infection.

To test this, mice were infected i.v., and the kinetics of bacterial invasion of the brain and the numbers of CD11b^{high}Ly-6C$^{med\text{-}hi}$ leukocytes in the brain were quantified. Typical of this system, bacteremia developed 48 h after infection, and bacteria entered the brain over the ensuing 24 h (Fig. 6). Only a few CD11b^{high}Ly-6C$^{med\text{-}hi}$ leukocytes were present in the brain at steady state; however, a substantial influx of cells was evident 48 h after infection. Further analysis using differential GR-1 staining showed that this was due to a significant increase in the numbers of Ly-6C^{high} monocytes, whereas the numbers of neutrophils did not change. Moreover, the initial monocytic influx proceeded or at least was contemporaneous with bacterial invasion.

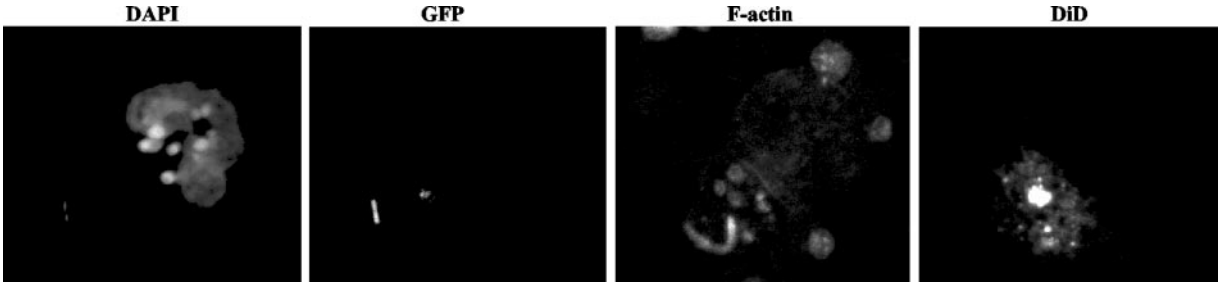

FIGURE 4. *L. monocytogenes* parasitize inflammatory mononuclear phagocytes in the blood. Mice were infected with *L. monocytogenes* strain NF-512, then were sequentially injected with clodronate liposome (Clo-lip) and with PBS-lip labeled with DiD. Leukocytes isolated from blood were cytocentrifuged onto coverslips and stained with Alexa 568-phalloidin to identify F-actin and DAPI to reveal nuclear morphology. Images of the same cell were collected with a confocal microscope.

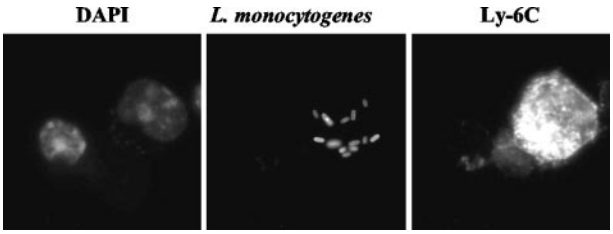

FIGURE 5. Infected Ly-6C^{high} mononuclear leukocytes are present in the brain. Perfused brains were harvested from mice infected 4–5 days previously with 1–2 LD_{50} of *L. monocytogenes*. Leukocytes isolated by immunomagnetic selection of CD11b^{+} cells were cytocentrifuged onto coverslips and then immunostained with anti-Ly-6C and anti-*L. monocytogenes* Ab, followed by incubation in DAPI. The figures shown are gray scale images of the same cell using different filters.

Finally, we tested whether recruitment of Ly-6C^{high} monocytes into the brain was associated with expression of a corresponding chemokine. Because Ly-6C^{high} monocytes correspond to the CX3CR1^{low}/GR-1^{+} monocytes, these cells are CCR2^{+} and thus can be recruited by MCP-1 (18, 19). Therefore, we tested whether there was evidence for up-regulation of this chemokine in the brain (Fig. 7). Real-time PCR showed a significant increase in the expression of MCP-1 mRNA compared with PBS-injected control animals. An increase was noted at 24 h, although this did not reach statistical significance. At 48 h postinfection, i.e., at the same time as the influx of Ly-6C^{high} monocytes, significantly increased MCP-1 mRNA levels were found in the brain and continued to increase throughout the experiment. Thus, Ly-6C^{high} monocytes, some of which contain bacteria, are recruited into the brain coincident with bacterial invasion of that organ. These findings support the hypothesis that CNS infection is established by transportation of intracellular bacteria by Ly-6C^{high} monocytes and suggest that MCP-1 is an important chemoattractant in this process (Fig. 7).

Discussion

Recent data showing that mouse monocytes are comprised of phenotypically distinct subpopulations have emphasized the beneficial functions that they could have in host defense (18–20, 26, 27). In marked contrast, the studies presented here show the other side of the host:pathogen encounter, in which blood monocytes help disseminate intracellular bacteria throughout the host and facilitate brain invasion. Previous studies from our laboratory indicated that parasitized mononuclear phagocytes were present in the blood of *L. monocytogenes*-infected mice and suggested that these cells had a role in CNS invasion (10, 12). In the present study we found that nearly all cell-associated bacteria colocalized with CD11b^{+} monocytes. Subset analysis of the monocytes according to Ly-6C expression revealed that most of the infected monocytes belonged to the Ly-6C^{high} subset. These cells are comparable to $CX_3CR1^{low}CCR2^{+}Gr\text{-}1^{+}$ blood monocytes in mice and to CD14^{+}CD16^{-} blood monocytes in humans (18).

The Ly-6C^{high} subset is expanded by infection with *L. monocytogenes* or with the protozoan pathogen *Leishmania major*, resulting in a monocyte left shift toward a predominance of less mature cells that are recently released from the bone marrow (20). Interestingly, avirulent *L. monocytogenes* Δ*hly* mutants, which do not produce listeriolysin O and typically neither escape phagosomes nor replicate intracellularly, did not stimulate this shift. By comparison, listeriolysin O-producing Δ*actA* mutants, which do escape phagosomes and replicate intracellularly, but are avirulent because they lack F-actin-based motility, did elicit a subpopulation shift similar to that of wild-type bacteria. *L. major* is an obligate

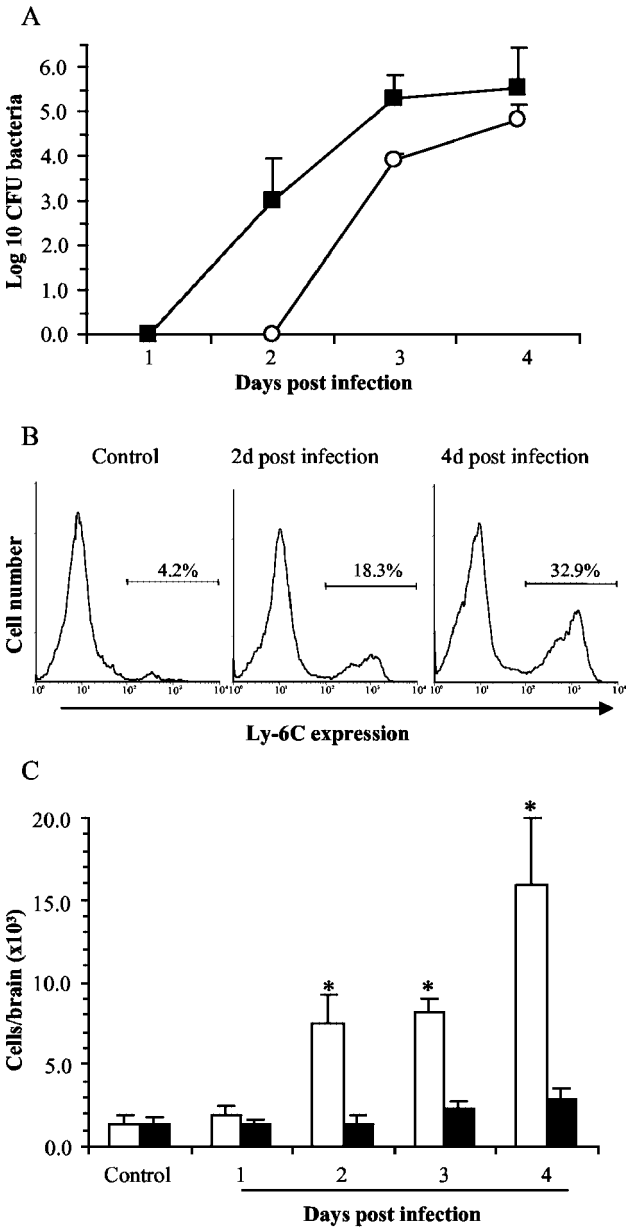

FIGURE 6. Ly-6C^{high} monocytes enter the brain during systemic *L. monocytogenes* infection. Mice were infected i.v. with 1–2 LD_{50} of *L. monocytogenes* and then euthanized at the indicated time, or were not infected (control). Different groups of mice were used for microbiological studies and for flow cytometry. Leukocytes were isolated from the brains of perfused animals by enzymatic digestion and immunomagnetic selection of CD45^{+} cells. *A*, Mean + SEM $\log_{10}$ CFU of bacteria in blood (■) and brain (○) were determined by serial dilution and plating. *B*, Histograms show the Ly-6C expression of CD11b^{high} cells from representative control and infected animals. *C*, The CD11b^{high}Ly-6C$^{med\text{-}high}$ cell population was selected, and numbers of monocytes and neutrophils were determined based on differential staining of GR-1. Data shown are the mean ± SEM Ly-6C$^{med\text{-}high}$ monocytes (□) and neutrophils (■) per brain from four to six mice per group. *, $p < 0.002$, infected mice compared with uninfected control animals.

intracellular protozoan that resides in modified phagosomes and does not escape from them until the parasitized cell ruptures (28). However, *L. major* does produce a pore-forming cytolysin at 37°C that is maximally active at pH 5.0–5.5, similar to listeriolysin O (29, 30). Although its role in leishmaniasis is not clear, it is probably produced during intracellular growth within mammalian macrophages. Taken together, this suggests that the hemopoietic

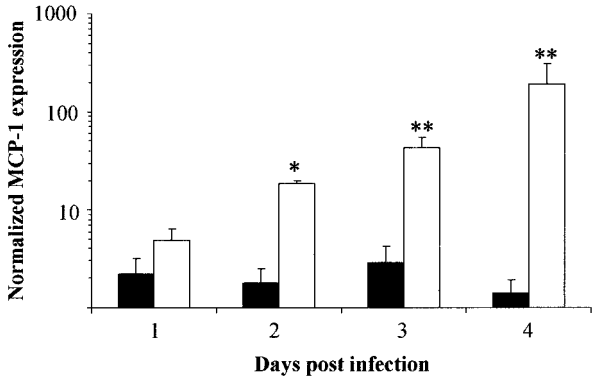

FIGURE 7. MCP-1 expression in then brain is up-regulated during systemic *L. monocytogenes* infection. Mice were infected i.v. with 1–2 LD_{50} of *L. monocytogenes*, then euthanized at the indicated time, or were injected i.v. with PBS (control). The brains were harvested and frozen until total RNA was extracted. Expressions of MCP-1 and the housekeeping gene hypoxanthine phosphoribosyl transferase were determined by real-time PCR. Data shown are the expressions of MCP-1 in control (■) and infected (□) mice normalized to the expression of hypoxanthine phosphoribosyl transferase in the same brain ($n = 5$ mice/group). *, $p < 0.01$; **, $p < 0.001$, normalized MCP-1 expression in infected mice compared with same day control animals.

growth factor-driven monocyte left shift may be elicited in response to microbial proteins, perhaps hemolysins, that are expressed intracellularly during infection (31, 32).

The finding that Ly-6C^{high} monocytes were the main transporters of intracellular bacteria in the bloodstream prompted us to test whether these cells also entered the brain. Indeed, we found that systemic *L. monocytogenes* infection induced a significant influx of Ly-6C^{high} monocytes into the brain. Moreover, nearly 90% of infected CD11b^{+} leukocytes in the brain were Ly-6C^{high} mononuclear phagocytes, consistent with the hypothesis that bloodborne *L. monocytogenes* enter the brain via phagocyte-facilitated invasion, with Ly-6C^{high} monocytes acting as the Trojan horse (7). As Ly-6C^{high} monocytes are CCR2^{+} and migrate to MCP-1 (18, 19), this selective recruitment into the brain is probably mediated at least in part by MCP-1, as we observed a significantly up-regulated expression of this chemokine in the brains of infected mice. MCP-1 has an established role in mediating recruitment of CCR2^{+} monocytes into the CNS (33, 34). Several different cell types, such as astrocytes, brain endothelial cells, and the newly recruited monocytes themselves, could have produced the MCP-1 mRNA detected in these experiments (35). Nevertheless, it remains to be determined the extent to which MCP-1 provides the initial stimulus for monocyte recruitment or just amplifies an ongoing monocytic influx, and whether other monocyte-attracting chemokines are also involved.

Data from the mouse model of systemic *L. monocytogenes* infection suggests that peripheral infection initiates a cascade of events causing recruitment of Ly-6C^{high} monocytes, and the bacteria they contain, into the brain. Key components of this cascade probably include translocation of NF-κB in cerebral vessels (36) and up-regulation of adhesion molecules, including P-selectin, ICAM-1, and VCAM-1, on brain endothelial cells (7, 37). In vitro data suggest that once infected cells arrive in the CNS, bacteria from monocytes can invade a variety of cells, including neurons and endothelial cells, by cell-to-cell spread (10, 38–40). How these events relate to human infection is not completely clear. In this light, Hertzig et al. (41) recently reported that normal human serum contains IgG against the *L. monocytogenes* invasion protein InlB, thus inhibiting direct bacterial invasion of human brain microvascular endothelial cells. These data support a role for phagocyte-facilitated invasion of the CNS in humans as well.

A key finding of the present study is that a relevant leukocyte influx occurs before significant bacterial invasion. This is similar to recent data in systemic listeriosis in mice showing that *Listeria*-specific T cells also enter the brain in the absence of CNS infection (42). Our finding that monocytes enter the brain before neutrophils contrasts with the sequence reported by others. Lopez et al. (37) used immunohistochemistry to study leukocyte recruitment in the brains of s.c. infected mice and found that the first leukocytes to enter the CNS were neutrophils. These discrepant results may be attributable to differences between the infection models or mouse strains, as well as to the relative sensitivities of immunohistochemistry of brain sections vs flow cytometry of whole brain isolates for detecting and identifying small numbers of cells. In addition, recent data show that the mAb used to identify neutrophils by immunohistochemistry also reacts with GR-1^{+} monocytes (17). Thus, newly recruited cells identified as neutrophils could have been Ly-6C^{high} monocytes. After intracranial inoculation of *L. monocytogenes*, neutrophils are the first leukocytes recruited into the brain (43–45). There are obvious differences between systemic and intracranial routes of infection, in particular, the fact that leukocyte recruitment is triggered by the presence of bacteria and bacterial products in the CNS after intracranial inoculation.

An interesting point in the context of this study is that we observed in preliminary experiments that the same Ly-6C^{high} subpopulation of blood monocytes also can harbor the phylogenetically unrelated intracellular pathogen, *L. major* (our unpublished observation). *L. major* disseminates in susceptible mice to visceral organs, including liver, spleen, and bone marrow (46, 47). The fact that some, albeit few, Ly-6C^{high} monocytes contain *L. major* is remarkable for several reasons: firstly, because *L. major* is thought to disseminate mostly through lymphatics; secondly, because this Ly-6C^{high} monocyte subset is apparently permissive for different microbes; and thirdly, because this subset shows the strongest increase in size in the course of infection. Although the percentage of infected Ly-6C^{high} blood monocytes was very low (0.15%), they may add a novel aspect to dissemination of *L. major*. Now that it has become possible to correlate more accurately mouse and human monocyte subsets (18, 20), our data indicate that the Ly-6C^{high} monocyte subset in the mouse and its human counterpart, identified as CD14^{+}CD16^{-}, represent a pathway for microbes to disseminate within a mammalian host that is conserved among a wide variety of intracellular pathogens.

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