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Ly6C^{high} Monocytes Control Cerebral Toxoplasmosis

Aindrila Biswas,* Dunja Bruder,*⁺ Susanne A. Wolf,‡ Andreas Jeron,* Matthias Mack,§ Markus M. Heimesaat,‖ and Ildiko Rita Dunay*‡

Cerebral infection with the parasite *Toxoplasma gondii* is followed by activation of resident cells and recruitment of immune cells from the periphery to the CNS. In this study, we show that a subset of myeloid cells, namely Ly6C^{high}CCR2^{+} inflammatory monocytes that infiltrate the brain upon chronic *T. gondii* infection, plays a decisive role in host defense. Depletion of this monocyte subset resulted in elevated parasite load and decreased survival of infected mice, suggesting their crucial role. Notably, Ly6C^{high}CCR2^{+} monocytes governed parasite control due to production of proinflammatory mediators, such as IL-1β, IL-6, inducible NO synthase, TNF, and reactive oxygen intermediate. Interestingly, Ly6C^{high}CCR2^{+} monocytes were also able to produce the regulatory cytokine IL-10, revealing their dual feature. Moreover, we confirmed by adoptive transfer that the recruited monocytes further develop into two distinct subpopulations contributing to parasite control and profound host defense. The differentiated Ly6C^{int}CCR2^{+}F4/80^{int} subset upregulated MHC I and MHC II molecules, suggesting dendritic cell properties against this intracellular pathogen (10, 11). In our previous study, we detected that Ly6C^{high}Gr1^{+} monocytes were crucial during management of inflammatory Ly6C^{high} monocytes and their macrophage noninflamed state (34). Recent reports indicate that the recruitment of Ly6C^{high} monocytes to the CNS is regulated by P-selectin glycoprotein ligand-1. These results indicate the critical importance of recruited Ly6C^{high} monocytes upon cerebral toxoplasmosis and reveal the behavior of further differentiated myeloid-derived mononuclear cell subsets in parasite control and immune regulation of the CNS. The Journal of Immunology, 2015, 194: 3223–3235.

Toxoplasmosis is a common worldwide zoonotic infection caused by the opportunistic intracellular parasite *Toxoplasma gondii* (1). After oral uptake, the parasites cross the intestinal epithelium as well as the blood-brain barrier; while traveling within mononuclear cells, they reach immune-privileged sites such as the CNS (2–5).

The primary infection with *T. gondii* is followed by the recruitment of neutrophil granulocytes, monocytes, and dendritic cells (DC) to the site of infection (6–8). We have recently shown that, in the acute stage of infection, DCs secrete high levels of IL-12, triggering adaptive immunity (9). The proinflammatory cytokine IFN-γ produced mainly by activated innate lymphoid cells, NK, and T cells is the major driving factor for host protection against this intracellular pathogen (10, 11). In our previous studies, we detected that Ly6C^{high}Gr1^{+} monocytes were crucial during the acute stage of *T. gondii* infection, producing high amounts of TNF, inducible NO synthase (iNOS), and reactive oxygen intermediates (ROS), which directly contributed to the control of the parasite burden in the host (6, 7, 12, 13). In addition, we have shown that neutrophil granulocytes rather contributed to the ileal pathology in the acute stage (7).

After reaching the brain, the parasites persist lifelong within tissue cysts, strictly controlled by the host immune system. IFN-γ-dependent immune response during the chronic stage suggests ongoing basal inflammation associated with resident cell activation in the CNS (14, 15). Upon immunosuppression, the latent infection can reactivate and develop into life-threatening encephalomyelitis (1, 6, 7, 16–18). Within the inflamed brain, resident immune cells, such as microglia and astrocytes, become activated, displaying significant antiparasitic properties (19–21).

Despite limited access, the CNS is inundated by immune cells from the periphery during the chronic stage of *T. gondii* infection. The adaptive immune responses in the CNS have been extensively investigated, describing an important contribution to the local parasite control (22–25). Besides, several studies have highlighted the function and phenotype of certain mononuclear cells such as microglia, brain DCs, and macrophages upon cerebral toxoplasmosis; however, the role of recruited newly described myeloid cell subpopulations remains undefined (19, 26, 27).

Monocytes are generated from macrophage and DC precursors in the bone marrow and are released in the bloodstream in a CCR2-dependent manner (28–30). On the basis of chemokine receptor expression and specific surface molecules (31), monocytes are divided into two major populations: CXCR1^{low}CCR2^{+}Ly6C^{high} and CXCR1^{+}CCR2^{−}Ly6C^{low} (6, 30). A short-lived CXCR1^{low}CCR2^{+}Ly6C^{high} inflammatory monocyte subset that is actively recruited to sites of infection and inflammation (32) serves as an immediate precursor for Ag-presenting DCs and macrophages (32, 33). CXCR1^{−}CCR2^{+}Ly6C^{low} patrolling resident monocytes adhere and migrate to luminal surfaces of blood vessels in the noninflamed state (34). Recent reports indicate that the recruitment of inflammatory Ly6C^{high} monocytes and their macrophage or DC progeny reflects the changing needs of the affected tissue along the course of inflammation, which includes cytokine...
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production, Ag presentation, and phagocytosis of cellular debris (12, 13, 35, 36). In the CNS, functional macrophage heterogeneity has been demonstrated in several models of autoimmune pathology as well as in neurodegenerative and infectious diseases (19, 26, 27, 36, 37), but the precise role of the recruited mononuclear cell subsets in cerebral toxoplasmosis remains to be addressed.

Subsequently, the availability of new surface markers and fate mapping led to a paradigm shift describing the behavior of distinct monocyte-derived macrophages and their difference to tissue-resident mononuclear cells (26, 33, 38, 39).

In this study, we set out to investigate the phenotype and particular function of the recruited Ly6<sup>ch</sup>CCR2<sup>+</sup> monocytes along the course of a low-cyst dose-induced chronic T. gondii infection. We demonstrate that, during cerebral T. gondii infection, Ly6<sup>ch</sup>CCR2<sup>+</sup> inflammatory monocytes are recruited to the brain and perform crucial antiparasitic functions. Their specific ablation resulted in decreased survival, suggesting pivotal defense function performed crucial antiparasitic functions. Their specific ablation resulted in decreased survival, suggesting pivotal defense function.

Ablation of monocytes was performed by administration of anti-CCR2 mAb (clone MC-21; provided by M. Mack, University of Regensburg). Mice were injected with 75 μg (40) Ab i.p. on days 20, 22, 24, 26, and 28 postinfection. Twenty-four hours after the last treatment on day 29, the mice were sacrificed. Rat IgG (ebioscience, San Diego, CA) was used as a control mAb.

Cell isolation

Brains were homogenized in a buffer containing 1 M HEPES (pH 7.3) and 45% glucose and then sieved through a 70 μm strainer. The cell suspension was washed and fractionated on 25–75% Percoll gradient (GE Healthcare) for 25 min at 800 × g without brake. The cells in the interphase comprised of mononuclear cells, which were washed with PBS and used immediately for further experiments. Peripheral blood obtained by cardiac puncture was treated with erythrocyte (RBC) lysis buffer (ebioscience) and then washed twice with ice-cold PBS at 300 × g for 10 min, followed by staining with the desired fluorophore-conjugated Abs.

Flow cytometry

Mononuclear cells isolated from different organs were incubated with an anti-FcγRIIII/II Ab (clone 93) to block unspecific staining. Thereafter, cells were stained with fluorochrome-conjugated Abs against cell surface markers, as follows: CD45 (50-F11), CD11b (M1/70), CD11c (N418), Ly6C (HK.14), F4/80 (BM8), MHC II-2D<sup>b</sup> (28-14-8, MHCII-I-A/E (M5/114.15.2), VLA-4 (R1-2), and LFA-1 (M17/4), all purchased from ebioscience; Ly6G (1A8), PSGL-1 (2PH1), and CD62L (M-EL-14) from BD Biosciences; and CXCR1 (polycyonal goat), Trem2 (237520), and CCR2 (475301) from R&D Systems for 30 min on ice, and then washed twice with 4% paraformaldehyde. Cells were then permeabilized using BD Perm/Wash Buffer. To measure cytokine expression, cells were stained with fluorochrome-conjugated Abs against cell surface markers, as follows: CD45 (50-F11), CD11b (M1/70), CD11c (N418), Ly6C (HK.14), and CD11c (N418) in FACS buffer for 30 min on ice; washed twice in FACS buffer; and fixed in 4% paraformaldehyde. Cells were then permeabilized using BD Perm/Wash Buffer. To measure cytokine expression, cells were stained with the following Abs: TNF (MP5-20F3), IL-6 (NJTEN3), IL-1α (ALF-161), IL-1β (NJTEN3), IL-6 (MP5-20F3), and IL-10 (JES5-16E3) from ebioscience and TNF (M-19) from Santa Cruz for 45 min in BD Perm/Wash Buffer.

A total of 100,000 cells was acquired using a flow cytometer (BD FACS Canto II), and flow cytometric data were analyzed using FlowJo software (Version 9.6.4; Tree Star). Matched isotype controls were used to subtract the nonspecific background staining.

Detection of reactive oxygen species

Single-cell suspensions from brain after blocking their FcγRs were surface stained for CD45 (30-F11), CD11b (M1/70), Ly6G (1A8), Ly6C (HK.14), and CD11c (N418) in FACS buffer for 30 min on ice. ROS production was measured by Total ROS Detection Kit (ENZO, 51011), according to the manufacturer’s instructions.

In vitro phagocytosis assay

The isolated mononuclear cells were cultured in 12-well chambers (Nalge Nunc International) at a density of 4 × 10<sup>5</sup> cells/ml. A total of 50 μl Fluospheres latex beads were added (Molecular Probes) after 1-h pre-treatment with cytochalasin D (Sigma-Aldrich), and cells were incubated under standard culture conditions for 8 h. Samples were acquired on flow cytometer (BD FACS Canto II) and analyzed with FlowJo software (Version 9.6.4; Tree Star).

Immunofluorescence

Frozen sections were acetone fixed for 10 min and then rehydrated and blocked by incubation for 10 min in dilute (PBS containing 2.0% normal goat serum, 1% BSA, 0.1% gelatin, 0.05% Tween 20, and 0.05% sodium
azide). Sections were incubated in primary Abs (Ly6C clone HK1-1, eBioscience; CCR2, GenWay Biotech) in diluent for 60 min, rinsed in wash buffer (PBS containing 0.5% FBS), and incubated for 60 min with secondary Abs conjugated to Alexa 594 and Alexa 488 (Life Technologies), and 1 x 10^5 cells were injected i.v. into 4-wk T. gondii (ME49)-infected C57BL/6 mice. Forty-eight hours later, the recipient mice were sacrificed and the brain mononuclear cells were isolated and analyzed by flow cytometry.

**Anti–PSGL-1 treatment**

To assess the transmigration capacity of the cells, 2 mg/kg rat anti-mouse PSGL-1 (4RA10; BioXCell) was administered i.p. every alternate day from day 16 to day 26 postinfection. Twenty-four hours after the last treatment on day 27, the mice were sacrificed. Isotype-matched rat IgG (eBioscience) was administered to the controls.

**Statistical analysis**

Data were analyzed by Student t test for two groups or one-way ANOVA for several groups, followed by Tukey's posttest with GraphPad Prism 6 (San Diego, CA). In all cases, results were presented as mean ± SD and were considered significant with p < 0.05.

**Results**

**Myeloid cells are recruited to the brain upon chronic T. gondii infection**

Cerebral toxoplasmosis is associated with activation of resident immune cells and peripheral cell recruitment to the CNS; however, the heterogeneity of infiltrating immune cell subsets has not been fully characterized. Accordingly, we investigated the characteristic features and fate of the newly described Ly6C<sup>high</sup> monocytes following extravasation in the brains of infected mice. We observed an ingress of CD45<sup>high</sup> populations in the brains of C57BL/6 mice after 4 wk with a low-dose T. gondii infection (Fig. 1B), whereas, in uninfected controls, the major leukocyte population in the brain was resting resident microglia, (CD45<sup>low</sup>CD11b<sup>+</sup>; Fig. 1A), corroborating previous reports (19, 27, 38). Further phenotyping of the cell subsets (Fig. 1B) subdivided the CD45<sup>high</sup> population into a CD45<sup>high</sup>CD11b<sup>+</sup> subset (ungated) comprised mainly of recruited lymphocytes and a CD45<sup>high</sup>CD11b<sup>+</sup> population (upper gate: 8.0 ± 1.06% of the parent population) encompassing myeloid-derived cells, namely monocytes, neutrophils, macrophages, and DCs. Brain resident–activated microglia expressed elevated levels of CD45 upon the infection (CD45<sup>low</sup>CD11b<sup>+</sup> gated, lower: 4.1 ± 0.31% of the parent population).

The Ly6G (1A8) Ab (7, 40) was used to distinguish between Ly6G<sup>+</sup> monocytes (Fig. 1C; 96.0 ± 2.11% of the CD45<sup>high</sup>CD11b<sup>+</sup>) and Ly6G<sup>+</sup> neutrophils (4.0 ± 2.02% of the CD45<sup>high</sup>CD11b<sup>+</sup>). Relative expression of Ly6C and F4/80 was used to further differentiate between the myeloid cell subsets (Fig. 1D). Based on these surface markers, we identified three distinct myeloid cell subpopulations, as follows: Ly6C<sup>neg</sup>F4/80<sup>+</sup>, Ly6C<sup>int</sup>F4/80<sup>+</sup>, and Ly6C<sup>high</sup>F4/80<sup>+</sup>. These data suggest that, during chronic T. gondii infection, alongside with the activation of resident microglia, a heterogeneous population of myeloid cells infiltrates the CNS that can be further divided into three distinct subsets based on their Ly6C and F4/80 expression.

**Ly6C<sup>high</sup>CCR2<sup>+</sup> cells localize in the parenchyma during cerebral toxoplasmosis**

Immunofluorescence analysis of brain sections of T. gondii–infected mice revealed accumulation of Iba1-positive cells (Fig. 2E) in the meninges as well as in the cortex compared with noninfected controls (Fig. 2A). Closer examination suggested robust cell activation of Iba-positive microglia with less ramification, bigger soma, marked increase of average surface area, and rather amoeboid morphology (Fig. 2I). Importantly, inflammatory foci of infected brains contained Ly6C (Fig. 2F, 2J) and CCR2 (Fig. 2G, 2K)-positive cells, suggesting Ly6C<sup>high</sup>CCR2<sup>+</sup> monocytes (Fig. 2H, 2L). These amoeboid-shaped inflammatory cells were mainly localized adjacent to the lesions in the cortex close to the vessels. These observations further indicate that Ly6C<sup>high</sup> CCR2<sup>+</sup> monocytes are recruited to the CNS upon T. gondii infection, which is in accordance with previous studies describing rapid influx of monocytes in the CNS upon inflammatory processes (16, 38, 41).

**Ablation of CCR2<sup>+</sup>Ly6C<sup>high</sup> monocytes upon cerebral toxoplasmosis is detrimental**

To evaluate the contribution of CCR2<sup>+</sup>Ly6C<sup>high</sup> monocytes in the parasite control, we took advantage of the newly available depleting anti-CCR2 Ab (MC-21) (35, 42, 43). Thus, we applied either anti-CCR2 Ab or isotype control IgG mAb, on alternating days from 20 d postinfection to 28 d postinfection, to two groups of infected mice, respectively. Twenty-four hours after the last Ab treatment, mice were sacrificed and the successful depletion of CCR2<sup>+</sup>Ly6C<sup>high</sup> monocytes in the blood was confirmed by flow cytometry by staining for Ly6C and CD11b (2.0 ± 0.23–0% of peripheral blood leukocytes; Fig. 3A, 3B, 3D).

Importantly, we noticed a reduction of recruited myeloid cells (8.0 ± 1.5% to 2.0 ± 0.28%) in the brains of anti-CCR2–treated mice (Fig. 3C, upper panel). This observation was further confirmed with a compelling decrease of Ly6C<sup>high</sup> monocytes (lower panel; 66.5 ± 8.6% to 11.2 ± 6.2%) and a slight decrease in the Ly6C<sup>neg</sup> population (30.3 ± 2.7% to 24.2 ± 1.6%). In addition, a rise of the Ly6C<sup>neg</sup> population was detected (4.0 ± 0.7% to 65.0 ± 4.8%) that might have entered the brain before depletion occurred (before day 20). Alterations in the absolute cell numbers revealed a significant reduction of Ly6C<sup>high</sup> (p < 0.001) and Ly6C<sup>neg</sup> (p < 0.01) cell subsets. Moreover, there was an increase in the Ly6C<sup>neg</sup> (p < 0.001) cell subset, whereas the microglia compartment remained unaltered (Fig. 3E).

Next, we investigated whether the selective ablation of CCR2<sup>+</sup> Ly6C<sup>high</sup> monocytes had an impact on brain pathology and survival of chronically infected mice. Histological examination of infected anti-CCR2 Ab-depleted mice revealed a higher frequency of inflammatory foci (Fig. 4B, 4D), macrophage 1 Ag<sup>+</sup> microglia, and mononuclear cells, and infiltration of immune cells into the cortex (Fig. 4D, 4F). Notably, we observed increased numbers of T. gondii cyst numbers (Fig. 4H, 4I) when compared with noninfected control brains. Most importantly, depletion of Ly6C<sup>high</sup> monocytes was followed by decreased survival rates, as by day 60 all anti-CCR2–treated mice succumbed due to the infection, whereas all nontreated infected control mice survived (Fig. 4J). Together these results demonstrate that Ly6C<sup>high</sup>CCR2<sup>+</sup> monocytes carry out fundamental functions in parasite control.
during cerebral toxoplasmosis, and their depletion exacerbates the outcome of the infection.

Characterization of mononuclear cell subsets upon cerebral T. gondii infection

The heterogeneity of monocyte–macrophage populations and their multifunctionality in the CNS have been intensively studied recently (33, 35, 38, 44). The different mononuclear cell subsets can be distinguished by their pattern of characteristic surface markers, making their discrimination between the subpopulations a complex assignment (45, 46). Therefore, to perceive the distinct myeloid cell subsets in the brain upon T. gondii infection, we first compared the expression of specific surface markers. Brain mononuclear cells from infected and control mice were isolated, and comprehensive flow cytometry analysis was performed.

Microglia from noninfected and infected mice did not express Ly6C, in contrast to monocytes (Fig. 5A, 5E). CCR2, which is a receptor for monocyte chemokine protein-1 (or CCL2), has been established to play a determining role for inflammatory monocyte egress from bone marrow (6, 32). As expected, Ly6Chigh monocytes notoriously expressed high levels of CCR2, and its expression on Ly6Cint and Ly6Cneg population was lower, whereas CCR2 was absent on microglia. This observation initiated our hypothesis that the Ly6Cint and Ly6Cneg subpopulations might have differentiated from the infiltrating Ly6Chigh monocyte subset (Fig. 5I, 5M).

Next, we measured the expression of the chemokine receptor CX3CR1. Fractalkine, the ligand of the CX3CR1 receptor, is expressed by neurons along with other cells and plays a role in maintaining certain microglial function (47–49). Microglia expressed high amounts of CX3CR1, as described previously (30, 34, 35, 38, 50, 51), whereas the receptor on recruited myeloid cells was present in low levels (Fig. 5B, 5F). The F4/80 Ag, which is exhibited by mature macrophages, was expressed predominantly by activated microglia and by myeloid-derived Ly6Cneg cells. The expression intensity of F4/80+ within the Ly6Cint and Ly6Cneg population was weaker (Fig. 5J, 5N). We observed a significant upregulation of a common DC marker CD11c on activated microglia, in line with previous studies highlighting their activation status (27). Furthermore, expression of CD11c was high on Ly6Cint and Ly6Cneg myeloid-derived cells (Fig. 5C, 5G). The surface marker TREM2 was strongly upregulated on activated microglia and myeloid-derived Ly6Cneg cells (Fig. 5K, 5O), pointing toward a potentially elevated phagocytic capacity. The Ly6Chigh and Ly6Cint subpopulations expressed low levels of TREM2, implying little phagocytic activity. Parallel to their ele-

FIGURE 1. Myeloid cell recruitment and activation of microglia cells. Leukocytes were isolated from brains of noninfected (A) and T. gondii–infected (B–D) C57BL/6 mice and analyzed by flow cytometry. Following the basic forward light scatter–side light scatter gating, the singlet cells were selected for further characterization. (A) Shows resident microglia (CD11b+CD45low, lower gate), and (B) shows the percentage of activated microglia (CD11b+CD45int, lower gate) and the myeloid population (CD11b+CD45high, upper gate). (C) The myeloid population consists of neutrophils (CD11bLy6G+, upper gate) and monocytes (CD11bLy6G-, lower gate). (D) The monocytes can be further divided according to their expression of Ly6C and F4/80: Ly6CintF4/80int, Ly6CnegF4/80int, and Ly6CnegF4/80high. Numbers represent percentage of parent population. Data shown are representative of four individual experiments (n = 4); results are shown as mean ± SD.

FIGURE 2. Immunofluorescence staining of microglia and monocytes in brain slides. We show the cortex and the cortical meninges in noninfected control (A–D) and infected (E–L) brains. In the noninfected brain (A), Iba+ microglia have a ramified morphology, whereas in the infected brain (E) they display a rather amoeboid morphology with bigger soma. The Ly6C and CCR2 staining are negative in the control brains, but positive in the parenchyma of the infected brains (F and G), displaying the recruitment of inflammatory monocytes (H) during T. gondii infection. Five to six coronal slides per mouse were analyzed; n = 4 mice per group. This experiment was repeated three times. Scale bars, 100 μm in (A)–(H) and 25 μm in (I)–(L).
vated CD11c expression, Ly6C<sup>int</sup> cells upregulated primarily the activation markers MHC I and II, indicating their efficacy to initiate adaptive immune responses by Ag presentation. Activated microglia, Ly6Chigh, and Ly6C<sup>neg</sup> resident monocytes (lower gate) in the blood. The expression of Ly6C and CCR2 of the inflammatory monocytes (Ly6C<sup>high</sup>) is shown further (A, right). (D) The bar graph represents the percentage of Ly6C<sup>high</sup> in the blood. (C) After the basic forward light scatter–side light scatter and singlet gating, upper plots show the gating of activated microglia (CD11b<sup>+</sup>CD45<sup>int</sup>) and the myeloid population (CD11b<sup>+</sup>CD45<sup>high</sup>) in the brain. Lower plots display the monocyte subsets (from the myeloid gate): Ly6C<sup>high</sup>, Ly6C<sup>int</sup>, and Ly6C<sup>neg</sup>. (E) The bar graphs represent the total cell number of the respective subset in the brain. Numbers in the plots represent percentage of parent population. Data shown are representative of five individual experiments (with n = 4 individual mice); results shown as mean ± SD. Significant differences (**p < 0.01, ***p < 0.001) were determined using the Student t test. Act. microglia, activated microglia.

Next, we elucidated the surface markers of the mononuclear cell subsets in the blood before they entered the infected CNS at 4 wk after the <i>T. gondii</i> infection. As anticipated, the Ly6C<sup>high</sup> monocytes expressed high amount of CCR2, whereas the resident monocytes were CCR2<sup>neg</sup> (Supplemental Fig. 1E, 1F). CX<sub>3</sub>CR1 and F4/80 were predominantly expressed on resident monocytes and Ly6C<sup>high</sup> inflammatory monocytes upon <i>T. gondii</i> infection, and MHC II was mainly expressed on resident monocytes during infection (Supplemental Fig. 1M–P).

**Unique cytokine profile of myeloid cell subsets in cerebral toxoplasmosis**

Consequently, to find out the particular cytokine production of these three distinct myeloid-derived cell subsets, we performed intracellular flow cytometry analysis. We detected that Ly6C<sup>high</sup> monocytes were able to produce high amounts of proinflammatory mediators such as IL-1α, IL-1β, IL-6, TNF, and iNOS.
ROS secretion was measured explicitly by Ly6C high monocytes, suggesting their strong potential to eliminate the parasites (Fig. 6L, 6P). The Ly6C int and Ly6C neg population produced lesser amounts of cytokines; however, IL-12p40 was mainly produced by Ly6C int cells, suggesting their role to shape the adaptive immune system (Fig. 6D, 6I). Activated microglia contributed with low levels of cytokine production to the host defense in this model with low-dose *T. gondii*–induced chronic infection in the brain. Interestingly, alongside their proinflammatory and antiparasitic functions, Ly6Chigh cells also expressed the immunoregulatory cytokine IL-10 upon in vitro stimulation, implicating their possible dual nature to maintain tissue homeostasis and counterbalance the ongoing CNS inflammation (Fig. 6M, 6Q).

**Ly6C neg myeloid cells perform strong phagocytic capacity**

To examine the phagocytic properties of microglia and recruited myeloid cell subsets, we performed ex vivo phagocytosis assay. To this end, the respective cell subsets were isolated, sorted, and then incubated with fluorescent latex beads. Whereas recruited Ly6Chigh and Ly6Cint cells exhibited low phagocytic capacity (31.1 ± 3.6% and 25.0 ± 2.2%, respectively), Ly6Cneg cells demonstrated prominent phagocytic ability (80.2 ± 5.6%), alongside with those of activated resident microglia (60.2 ± 1.2%; Fig. 6E).

6C, 6F–6H, 6J, 6K, 6N, 6O). ROS secretion was measured explicitly by Ly6C high monocytes, suggesting their strong potential to eliminate the parasites (Fig. 6L, 6P). The Ly6C int and Ly6C neg population produced lesser amounts of cytokines; however, IL-12p40 was mainly produced by Ly6C int cells, suggesting their role to shape the adaptive immune system (Fig. 6D, 6I). Activated microglia contributed with low levels of cytokine production to the host defense in this model with low-dose *T. gondii*–induced chronic infection in the brain. Interestingly, alongside their proinflammatory and antiparasitic functions, Ly6C high cells also expressed the immunoregulatory cytokine IL-10 upon in vitro stimulation, implicating their possible dual nature to maintain tissue homeostasis and counterbalance the ongoing CNS inflammation (Fig. 6M, 6Q).

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FIGURE 6. Cytokine production and phagocytic capacity of the cell subsets in the brain. (A–D and F–Q) Intracellular cytokine production was analyzed in cells isolated from brains of noninfected and infected mice after in vitro stimulation with *Toxoplasma* lysate Ag. The cells were gated as shown in the representative plots of Fig. 1A–D. (A–D and J–M) Representative histograms showing cytokine expression by cell population. Bars mark the cells positive for the particular cytokine. Numbers above bars display the percentage of cells positive for the cytokine of the respective population: resident microglia (CD11b+/CD45low), activated microglia (CD11b+/CD45int), inflammatory monocytes (CD11b+/CD45high/Ly6G−Ly6Chigh), Ly6Cint monocytes (CD11b+/CD45high/Ly6G−Ly6Cint), Ly6Cneg monocytes (CD11b+/CD45high/Ly6G−Ly6Cneg), isotype control, tinted. (F–I and N–Q) Bar graphs represent the median fluorescence intensity (MFI) of the respective fluorochrome for a particular cytokine, MFI ± SD (n = 4). Data are representative of four independent experiments. (E) The uptake of fluorescent latex beads by brain mononuclear cells ex vivo was measured by flow cytometry. After the standard forward light scatter–side light scatter gating and singlet gating, CD11b+ cells were further gated, as shown in Fig. 1. Bar graph shows the percentage of phagocytosis of the latex beads by the respective population. Data are representative of three experiments with n = 4; results are shown as mean ± SD. One-way ANOVA analysis followed by Tukey’s post hoc test was performed for multiple comparisons (*p < 0.05, **p < 0.01, ***p < 0.001). Act. microglia, activated microglia; ns, not significant; Res. microglia, resident microglia.
Ly6Chigh cells infiltrate and differentiate in the brain upon adoptive transfer

To confirm that recruited Ly6Chigh monocytes further differentiate to the previously described Ly6Cint and Ly6Cneg cells following extravasation in the CNS, we conducted adoptive transfer experiments. To address this, we injected 1 x 10^6 sorted, CFSE-labeled Ly6ChighCD11b+ cells isolated from the bone marrow of wild-type mice i.v. into T. gondii-infected recipient mice at day 28 postinfection. The cells were injected when the ongoing inflammation had already affected the blood-brain barrier permeability and the recruitment of the inflammatory cells to the CNS reached its peak (data not shown). Notably, CFSE-labeled Ly6Chigh cells were found in the CNS as early as 24 h after the transfer (data not shown). Forty-eight hours after the transfer, Ly6Chigh cells (27.2 ± 3.2% of CD11b+CFSE+ gate) downregulated Ly6C and apparently differentiated into Ly6Cint (32.0 ± 1.6% of CD11b+CFSE+ gate) and Ly6Cneg cells (41.0 ± 2.0% of CD11b+CFSE+ gate; Fig. 7B).

These data suggest that Ly6Chigh monocytes are recruited to the brain upon T. gondii infection, in which a subset of them loses their Ly6C expression and further generates the Ly6Cint and Ly6Cneg subsets.

Migration of CCR2+Ly6Chigh monocytes to the brain is PSGL-1 dependent

Selective leukocyte homing to the site of inflammation has been shown to be dependent on chemokines and distinct adhesion molecules (52–55). To evaluate whether any particular adhesion molecule plays a role in CCR2+Ly6Chigh monocyte recruitment to the CNS in toxoplasmosis, we measured the expression of L selectin (CD62L), LFA-1, and PSGL-1 on Ly6Chigh monocytes.

CD62L, which is known to be important for leukocyte rolling on the inflamed endothelium (56, 57), was highly expressed on Ly6Chigh inflammatory monocytes in the periphery, but was downregulated upon entry to the brain. Resident monocytes in the blood expressed only low levels of CD62L (Fig. 8A, 8D, 8G, 8J). LFA-1 was expressed both in the periphery and in the brain by the recruited monocytes as well as on Ly6Cint and Ly6Cneg cells in the CNS. Resident monocytes in the blood upregulated LFA-1 upon T. gondii infection, similarly to resident microglia cells in the brain (Fig. 8B, 8E, 8H, 8K).

PSGL-1 expression on the Ly6C high inflammatory monocytes was significantly higher compared with the percentage (40.6 ± 1.67%) to 25.2 ± 2.1% of the CD45+Ly6G+ population) and in the total cell numbers within the Ly6Chigh compartment (Fig. 9C [lower panel], 9D, respectively). No significant differences were detected in the frequencies and total numbers of other cell subsets (Fig. 9C, lower panel). The inhibition of Ly6Chigh cell recruitment to the brain was markedly diminished (p < 0.01; Fig. 9E), confirming that PSGL-1 is an important mediator for monocyte homing to the CNS.

Discussion

Due to the recent discovery of several novel characteristic markers and transcription factors, our knowledge of the myeloid cell heterogeneity has advanced extensively (26, 30, 33, 36, 38, 41, 45, 58, 59). Previous studies have suggested that, alongside the resident microglia, recruited mononuclear cells are important to control CNS inflammation and (different) CNS infections (16, 27, 36, 37, 39). However, differences in the experimental setups have made it difficult to precisely correlate results and determine the relative importance of the definitive cell subpopulations during the inflammatory processes and infections.

The role of bone marrow–derived CCR2+Ly6Chigh monocytes was investigated under various infectious conditions in the periphery, as well as in the CNS. We have previously shown that inflammatory monocytes control acute T. gondii infection in the ileum, by producing antimicrobial mediators (6, 7). Also, during Listeria monocytogenes infection, the absence of Ly6Chigh monocytes leads to rapid death of mice, demonstrating their important role in host defense (32, 60, 61). Furthermore, we recently observed that Ly6Chigh monocytes are substantially involved in brain inflammation and immune cell recruitment to the CNS, leading to experimental cerebral malaria upon Plasmodium ANKA infection (our unpublished observations). In cases of viral encephalitis, Ly6Chigh monocytes infiltrate the CNS and, although contributing to viral clearance, they also induce significant immunopathology (16, 62). The opposing beneficial and detrimental nature of Ly6Chigh monocytes, which seemingly depends on the type of infection, warrants further characterization to understand their intricate behavior.

In the chronic phase of T. gondii infection, parasites persist in cysts within immune-privileged sites (15). The latent stage is associated with marginal inflammation and cell recruitment to the CNS, which is necessary to provide adequate IFN-γ levels, the major driving force for parasite control. In the murine models of chronic infection, the characteristics of T cell subsets (24, 63–65), specific mononuclear cells, for example, resident microglia and APCs, have been extensively studied. In contrast, the role of the newly described myeloid cell subsets is controversial and thus requires further investigation (19–21). Microglia cells have been shown to eliminate parasites in an IFN-γ-dependent manner, in addition to their efficient phagocytic capacity and cytokine production (19, 20, 66, 67). Some early studies suggest that brain DCs differentiate from local resident cells, whereas others have proposed a peripheral hematopoietic cell origin upon cerebral toxoplasmosis (27, 39, 68).

In this study, we have elucidated the nature of the recruited myeloid cells within the brain upon cerebral toxoplasmosis. We from day 16 to day 26 after T. gondii infection. After 24 h of the last Ab application, we observed an increased proportion of Ly6Chigh monocytes in the peripheral blood (Fig. 9A, 9B; 3.1 ± 0.6% to 5.4 ± 0.92%) of the parent population of the treated mice, implying that, in the absence of PSGL-1, sufficient transmigration does not occur and cells accumulate in the blood. In the brains of infected anti-PSGL-1–treated mice, we observed a reduction in the percentage (40.6 ± 1.67% to 25.2 ± 2.1% of the CD45+Ly6G+ population) and in the total cell numbers within the Ly6Chigh compartment (Fig. 9C [lower panel], 9D, respectively). No significant differences were detected in the frequencies and total numbers of other cell subsets (Fig. 9C, lower panel). The inhibition of Ly6Chigh cell recruitment to the brain was markedly diminished (p < 0.01; Fig. 9E), confirming that PSGL-1 is an important mediator for monocyte homing to the CNS.
found that, upon low-dose infection with *T. gondii*, myeloid-derived Ly6C<sup>high</sup> cells infiltrated the brain and expressed specific surface markers CD45<sup>high</sup>CD11b<sup>+</sup>Ly6G<sup>2</sup>Ly6C<sup>high</sup>F4/80<sup>int</sup>. In addition, based on their Ly6C and F4/80 expression, there were two more CD45<sup>+</sup> myeloid populations present in the CNS, namely Ly6C<sup>int</sup> F4/80<sup>int</sup> and Ly6C<sup>neg</sup> F4/80<sup>high</sup>.

In our previous studies, we detected that CCR2<sup>−/−</sup> mice were extremely sensitive to *T. gondii* infection, with 90% of mice succumbing in the acute phase of infection. We therefore concluded that CCR2<sup>+</sup> Ly6C<sup>high</sup> monocytes were necessary to control the acute *T. gondii* infection and to limit the small intestinal pathology (6). The remaining 10% of infected mice displayed elevated parasite numbers in the CNS, suggesting that these cells might also be involved in the chronic phase of the infection (6). Thus, to study the role of CCR2<sup>+</sup> Ly6C<sup>high</sup> monocytes in the chronic stage, the depletion of this particular cell subset was necessary to perform after the infection overcame the acute phase and parasites were present in the CNS. Previously, we compared the effect of commonly used Abs to ablate inflammatory monocytes and addressed the concerns surrounding the expression of Gr1, Ly6C, and Ly6G, using the available depletion strategies of neutrophil granulocytes and inflammatory monocytes (7). In these earlier studies, we used the Gr-1–specific RB6-8C5 Ab; however, the application of this Ab led to a depletion of both Ly6C<sup>high</sup>Ly6G<sup>2</sup> Ly6C<sup>high</sup> monocytes, as well as Ly6G<sup>+</sup>Ly6C<sup>+</sup> neutrophils (40). As control, we used the 1A8 Ab that reacts specifically to Ly6G and depletes solely neutrophils (40). Importantly, our results confirmed that Ly6C<sup>high</sup> monocytes are crucial in the control of the acute *T. gondii* infection, and that Ly6G<sup>+</sup> neutrophils rather contributed to the immunopathology (7).

In the current study, taking advantage of a new depletion strategy of Ly6C<sup>high</sup>CCR2<sup>+</sup> monocytes, we applied anti-CCR2 Ab (MC-21), which selectively depletes CCR2<sup>+</sup> inflammatory monocytes (69, 70). We observed an increase in immunopathology and ele-
vated parasite numbers in the CNS during cerebral toxoplasmosis. Most importantly, mice were unable to control the parasites and succumbed to infection. This finding strongly suggests that Ly6ChighCCR2+ monocytes play a critical role in governing the chronic phase of Toxoplasma infection.

Due to the differential expression of Ly6C and F4/80 among the myeloid compartment, we characterized the phenotype of the recruited CCR2+Ly6Chigh monocytes, Ly6Cint and Ly6Cneg myeloid-derived cells, and resident microglia. Therefore, we conducted comprehensive surface and intracellular stainings using highly specific markers. CCR2 expression was the highest on the newly recruited Ly6Chigh cells, and was lower on Ly6Cint and Ly6Cneg cells. The chemokine receptor CX3CR1 was predominantly expressed on microglia and was absent on Ly6Chigh monocytes, in accordance with previous studies (31, 33, 34, 38, 71). The mature macrophage marker F4/80 was expressed mainly by activated microglia and by Ly6Cneg cells, suggesting their macrophage-like nature. Microglia upregulated CD11c upon infection-induced activation, as previously reported (27). CD11c expression was also elevated in Ly6Cint and Ly6Cneg cells, pointing toward their DC phenotype. Interestingly, MHC I and II were most intensely expressed on the surface of Ly6Cint cells, implying that these cells may represent the previously characterized brain DCs (27). These results proved that the recruited myeloid cell populations are different from the resident microglia.

Comparing the cytokine profile between the resident and recruited mononuclear cell subsets in the CNS revealed that Ly6Chigh monocytes produced the highest levels of proinflammatory molecules, such as IL-1α, IL-1β, IL-6, TNF, and iNOS. These results indicate that monocytes are essential to control T. gondii infection in the brain, similar to our previous findings in the periphery (6, 7). Additionally, the elevated ROS production was observed exclusively by the Ly6Chigh monocytes, further suggesting their critical role in the host defense arsenal. This proinflammatory signature of Ly6Chigh cells was reaffirmed in cerebral viral infections (62), and in other parasitic models (72, 73). IL-12p40 secretion was the highest by Ly6Cint cells, well in line with their CD11c and MHC expression, suggesting their capability to initiate adaptive immune responses. In comparison with CD11b+ CD11b+ myeloid-derived cells, the activated resident microglia contributed to lesser extent to the secretion of proinflammatory mediators in cerebral toxoplasmosis.

Remarkably, in addition to their antiparasitic capacity, Ly6Chigh cells were also able to secrete the regulatory cytokine IL-10, suggesting a dual function of limiting pathogen expansion and regulating detrimental immunopathology in the CNS. Our observations are consistent with a recent study by Grainger et al. (72), reporting similar dual features of Ly6Chigh cells in the acute T. gondii infection model. Additionally, this study also described that monocytes regulate neutrophil function by secreting PGE2, hence contributing to our understanding of their complex functions. Such decisive roles played by the Ly6Chigh cells during the resolution of inflammation, tissue regeneration, and debris clearance were also seen in models of autoimmunity and neurodegeneration (26, 36, 37).

We also found that activated microglia alongside with Ly6Cneg myeloid-derived cells displayed phagocytic potential, whereas...
Ly6Chigh and Ly6Cint cells were less capable in this activity. The F4/80 and particularly the TREM2 expression of those cells followed the same pattern, as described recently for the latter molecule (49, 74–77), suggesting that the activated microglia has the capacity to engulf invading microorganisms and dead tissue remains during the ongoing inflammation (49, 76–79). The current paradigm suggests that monocytes do not substantially contribute to tissue-resident macrophages under steady state conditions; rather, resident macrophages and microglia in the CNS develop in the embryonic stage (38). However, during infection and inflammation, macrophage-like cells can differentiate from infiltrating Ly6Chigh inflammatory monocytes. The diverse behavior of the monocyte-derived macrophages was demonstrated in experimental autoimmune uveitis, in which the kinetics of CX3CR1lowLy6C+ and CX3CR1highLy6C− changed along the course of the disease (36). Such heterogeneity was also shown in an acute model of colitis, in which the recruited CX3CR1highLy6Chigh monocytes promoted inflammation, but over time gave rise to a CX3CR1int Ly6Clow population that displayed all the trademarks of a DC (35).

In cerebral toxoplasmosis, the conversion of monocytes to brain DCs upon recruitment was also proposed by a previous study (27). Confirming their plasticity, we also detected that recruited monocytes in the brain further differentiate into two distinct population CD11cLy6Chigh brain DCs and Ly6CnegF4/80+ macrophages and carry out particular functions in parasite clearance.

Blood monocytes are comprised of two distinct populations, the inflammatory CX3CR1lowCCR2Ly6Chigh and the patrolling CX3CR1highCCR2Ly6Clow cells. Correspondingly, in our current experiments, CX3CR1lowCCR2Ly6Chigh monocytes and CX3CR1highCCR2Ly6Clow were present in the blood, and, upon Toxoplasma infection, the common DC marker CD11c and the MHC I molecule were upregulated on the surface of Ly6Chigh monocytes, suggesting modified activation status even before reaching the site of infection.

Leukocyte transmigration in the blood through the blood-brain barrier upon cerebral inflammation is dependent on adhesion molecules and their receptors. Inflammatory monocytes use the CCR2–CCL2 axis to egress from the bone marrow, but the mechanism of crossing the blood-brain barrier is poorly understood (6, 78). Therefore, we investigated the involvement of certain key molecules in the recruitment of Ly6Chigh inflammatory monocytes to the CNS. CD62L (L-selectin), which assists immune cells to enter different tissues (56, 57), was highly expressed on Ly6Chigh monocytes in the blood, but was downregulated upon their entry into the CNS. Consistent with previous studies, the CD62L expression was low on resident monocytes and microglia (38). LFA-1, which can interact with P- and E-selectin on the activated endothelial cells (promoting recruitment), was present both in the periphery and in the CNS on Ly6Chigh monocytes, as well as on differentiated Ly6Cint and Ly6Cneg monocytes. Previous studies have described elevated LFA-1 expression on resident microglia in cerebral toxoplasmosis and their functional role on recruited. Additionally, the functional role of LFA-1 on recruited DCs is well characterized (27). We measured highest PSGL-1 expression on the surface of Ly6Chigh monocytes, which led us to the hypothesis that this molecule might play a role in monocyte entry.

The dependence on PSGL-1 for lymphocyte migration is well established (56, 57, 79–82). However, to date, the function of PSGL-1 in monocyte recruitment has not been sufficiently addressed. One previous study described the role of PSGL-1 in Ly6Chigh monocyte homing to the site of atherosclerosis in blood vessels of mice (54). This study revealed that Ly6Chigh monocytes, which are PSGL-1high and CD62L*, preferentially interacted with P- and E-selectin on activated endothelium or with CD62L on a rolling/adherent leukocyte under flow by secondary tethering. However, the authors speculated that other adhesion molecules such as LFA-1 and VCAM-1 (ligand VLA-4) may not be key factors in monocyte homing, as their ligands were expressed at lower levels on Ly6Chigh cells. Furthermore, other studies detected PSGL-1–dependent monocyte migration in the periphery during Leishmania major infection, tumor metastasis, and thrombus formation (48, 81, 82). Thus, to our knowledge, we investigated for the first time PSGL-1–dependent monocyte recruitment to the CNS. Treatment of mice with anti–PSGL-1 Ab revealed a significant inhibition of Ly6Chigh cell recruitment to the brain upon chronic T. gondii infection, confirming that PSGL-1 is critical for monocyte homing to the CNS.

Altogether, our findings combined with emerging evidence from other murine models further highlight the plasticity of recruited Ly6Chigh monocytes. Cerebral T. gondii infection leads to cytokine production by the inundated Ly6Chigh monocytes, which play an influential role in the protection of the inflamed brain. The Ly6Chigh monocytes further give rise to Ly6Cint and Ly6Cneg subsets and perform divergent functions such as Ag presentation and phagocytosis. Thus, monocytes and their descendants play multifaceted functions to control cerebral toxoplasmosis. In conclusion, our findings indicate that, during cerebral T. gondii infection, Ly6Chigh inflammatory monocytes infiltrate the CNS and differentiate into phenotypically and functionally distinct cell subsets and carry out pivotal functions to control the chronic stage.

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Disclosures
The authors have no financial conflicts of interest.

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