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Ly6C^high Monocytes Control Cerebral Toxoplasmosis

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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^highCCR2^+ 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^highCCR2^+ monocytes governed parasite control due to production of proinflammatory mediators, such as IL-1α, IL-1β, IL-6, inducible NO synthase, TNF, and reactive oxygen intermediate. Interestingly, Ly6C^highCCR2^+ 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^lowCCR2^+F4/80^int^ subset upregulated MHC I and MHC II molecules, suggesting dendritic cell properties recruited monocytes further develop into two subpopulations contributing to parasite control and profound host defense. The differentiated Ly6C^lowCCR2^+F4/80^int^ subset upregulated MHC I and MHC II molecules, suggesting dendritic cell properties such as interaction with T cells, whereas the Ly6C^negF4/80^high^ cell subset displayed elevated phagocytic capacity while upregulating triggering receptor expressed on myeloid cells-2. Finally, we have shown 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: 000–000.

T oxoplasmosis 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^highGr1^+ 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^lowCCR2^+Ly6C^high^ and CXCR1^highCCR2^+Ly6C^low^ (6, 30). A short-lived CXCR1^lowCCR2^+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^highCCR2^+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 infection, which includes cytokine

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Abbreviations used in this article: DC, dendritic cell; iNOS, inducible NO synthase; PSGL-1, P-selectin glycoprotein ligand-1; ROS, reactive oxygen intermediate; TREM2, triggering receptor expressed on myeloid cells-2.

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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>high</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>high</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 performance with cytoclasin D (Sigma-Aldrich), and cells were incubated 2 h in untreated medium with 5% CO<sub>2</sub> for further experiments. Peripheral blood obtained by cardiac puncture was washed and used immediately.

**Monitoring of reactive oxygen species**

Single-cell suspensions from the brains (5 × 10<sup>5</sup> cells/well) were stimulated in 24-well flat-bottom plates in the presence of Toxoplasma lysate Ag (5 μg/ml), brefeldin A (10 μg/ml, GolgiPlug; BD Biosciences), and monensin (10 μg/ml; BioLegend). After 6 h, cells were incubated with FcR-blocking Ab (clone 93) for 15 min on ice and surface stained for CD45 (30-F11), CD11b (M1/70), CD11c (N418), Ly6C (HK1.4), F4/80 (BM8), MHC II (Ly6C<sup>int</sup>CCR2<sup>int</sup>CD11c<sup>+</sup>), adopting DC properties. The two distinct mononuclear cell subpopulations. One subset downregulated Ly6C, CCR2, and upregulated CD11c, MHC II (Ly6C<sup>high</sup>CCR2<sup>high</sup>CD11c<sup>+</sup>), adopting DC properties. The other myeloid cell subpopulation did not exhibit Ly6C expression, but upregulated F4/80 and TREM2, presenting potent phagocytic capacity (Ly6C<sup>high</sup>CCR2<sup>high</sup>F4/80<sup>high</sup>), respectively. Finally, we show that P-selectin glycoprotein ligand-1 (PSGL-1) plays a role in the recruitment of Ly6C<sup>high</sup> monocytes to the CNS. Our results indicate the critical importance of the recruited Ly6C<sup>high</sup> monocytes in the CNS upon chronic T. gondii infection, thus further exposing the dynamics and behavior of antiparasitic and inflammatory properties of the mononuclear cell subsets in a murine model of cerebral toxoplasmosis.

**Materials and Methods**

**Animals**

All animal experiments were approved by the local authorities according to German and European legislation. Experiments were conducted with adult C57BL/6 female mice (8 wk old; purchased from Janvier, Saint Berthevin, France). Four to five animals per group in up to four independent experiments were investigated.

**Infection**

T. gondii cysts of type II ME49 strain were harvested from the brains of female NMRI mice strain infected i.p. with T. gondii cysts 5–6 mo earlier, as described previously (15). Brains obtained from infected mice were mechanically homogenized in 1 ml sterile PBS, and the cyst numbers were determined using a light microscope. Three cysts were administered i.p. into mice in a total volume of 200 μL. Control mice were mock infected with sterile PBS.

**Histopathology**

Brains were removed and immersed in 4% paraformaldehyde for several days and sectioned coronally at 4 μm. Paraffin-embedded, 4-μm-thick sections were deparaffinized and convenionally stained with H&E. The isolated mononuclear cells were cultured in 12-well chambers (Nalge Nunc International) at a density of 4 × 10<sup>3</sup> cells/ml. A total of 50 μl Fluosphere latex beads were added (Molecular Probes) after 1-h pretreatment 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; 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 (50-F11), CD11b (M1/70), Ly6G (IA8), Ly6C (HK1.4), 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 cytotoxic expression, cells were stained with the following Abs: TNF (MP6-XT22), IL-12p60 (C17.8), IL-1α (ALF-161), IL-1β (JETN3), IL-6 (MP5-20F3), and IL-10 (JES5-16E3) from eBioscience and iNOS (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.

**Immunofluorescence**

Frozen sections were acetone fixed for 10 min and then rehydrated and blocked by incubation for 10 min in diluent (PBS containing 2.0% normal goat serum, 1% BSA, 0.1% gelatin, 0.05% Tween 20, and 0.05% sodium
Bone marrow cells were harvested from femora and tibiae of wild-type noninfected mice, lysed with erythrocyte (RBC) lysis buffer (eBioscience), and washed twice with ice-cold PBS at 300 × g for 10 min, before staining with the desired fluorescent conjugated Abs. CD11b+Ly6Chigh monocytes were sorted using BD FACS Diva with Digital Vantage Option from wild-type C57BL/6 bone marrow cells and labeled with 5μM CFSE (Life Technologies), and 1 × 10^6 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 ± 5D 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^high monocytes following extravasation in the brains of infected mice. We observed an ingress of CD45^high 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 were resting resident microglia, (CD45^lowCD11b^; Fig. 1A), corroborating previous reports (19, 27, 38). Further phenotyping of the cell subsets (Fig. 1B) subdivided the CD45^high population into a CD45^highCD11b^ subset (ungated) comprised mainly of recruited lymphocytes and a CD45^highCD11b^ 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^highCD11b^-gated, lower; 4.1 ± 0.31% of the parent population).

The Ly6G (1A8) Ab (7, 40) was used to distinguish between Ly6G^- monocytes (Fig. 1C; 96.0 ± 2.11% of the CD45^high CD11b^-) and Ly6G^- neutrophils (4.0 ± 2.02% of the CD45^high CD11b^-). 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^highF4/80^, Ly6C^highF4/80^- and Ly6C^negF4/80^high. 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^highCCR2^- 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^highCCR2^- monocytes (Fig. 2H, 2L). These amoeboid-shaped inflammatory cells were mainly located adjacent to the lesions in the cortex close to the vessels. These observations further indicate that Ly6C^high CCR2^- 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^-Ly6C^high monocytes upon cerebral toxoplasmosis is detrimental

To evaluate the contribution of CCR2^-Ly6C^high 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^-Ly6C^high 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 observed 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^high monocytes (lower panel; 66.5 ± 8.6% to 11.2 ± 6.2%) and a slight decrease in the Ly6C^neg population (30.3 ± 2.7% to 24.2 ± 1.6%). In addition, a rise of the Ly6C^neg 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^high (p < 0.001) and Ly6C^neg (p < 0.01) cell subsets. Moreover, there was an increase in the Ly6C^neg (p < 0.001) cell subset, whereas the microglia compartment remained unaltered (Fig. 3E).

Next, we investigated whether the selective ablation of CCR2^-Ly6C^high 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^ 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 non-treated infected control brains. Most importantly, depletion of Ly6C^high monocytes was followed by decreased survival rates, as by day 60 all anti-CCR2–treated mice succumbed due to the infection, whereas all non-treated infected control mice survived (Fig. 4J). Together these results demonstrate that Ly6C^highCCR2^- 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 Ly6Clow subpopulations expressed low levels of TREM2, implying little phagocytic activity. Parallel to their ele-
activated CD11c expression, Ly6Cint 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 Ly6Cneg cells also expressed MHC I and II, but with less intensity (Fig. 5D, 5H, 5L, 5P). Collectively, the extensive phenotypic characterization of the myeloid cell subsets revealed that these cells are distinct from microglia, and they express different levels of specific surface markers, suggesting definite functions.

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 T. gondii infection. As anticipated, the Ly6C high monocytes expressed high amount of CCR2, whereas the resident monocytes were CCR2neg (Supplemental Fig. 1E, 1F). CX3CR1 and F4/80 were predominantly expressed on resident monocytes (Supplemental Fig. 1G–J). The surface Ag CD11c was weakly expressed on all investigated cell populations in the blood; however, the highest appearance was observed on Ly6Chigh monocytes (Supplemental Fig. 1K, 1L). Similarly, MHC I was upregulated on Ly6Chigh inflammatory monocytes upon T. gondii 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 high monocytes were able to produce high amounts of proinflammatory mediators such as IL-1α, IL-1β, IL-6, TNF, and iNOS (Fig. 6A–F). The surface Ag CD11c was weakly expressed on all investigated cell populations in the blood; however, the highest appearance was observed on Ly6Chigh monocytes (Supplemental Fig. 1G–J). The surface Ag CD11c was weakly expressed on all investigated cell populations in the blood; however, the highest appearance was observed on Ly6Chigh monocytes (Supplemental Fig. 1G–J). Similarly, MHC I was upregulated on Ly6Chigh inflammatory monocytes upon T. gondii infection, and MHC II was mainly expressed on resident monocytes during infection (Supplemental Fig. 1M–P).

FIGURE 3. Depletion of CCR2+Ly6Chigh monocytes. C57BL/6 mice were infected with T. gondii and from day 20 to day 28 postinfection alternatively treated with either IgG mAb or anti-CCR2 to deplete inflammatory monocytes. After the standard forward light scatter–side light scatter gating, single cells were selected for further characterization. Panel (A left, B) displays the representative plots to define Ly6Chigh inflammatory monocytes (upper gate), Ly6Cint neutrophils (middle gate), and Ly6Cneg resident monocytes (lower gate) in the blood. The coexpression of Ly6C and CCR2 of the inflammatory monocytes (Ly6Chigh) is shown further (A, right). (D) The bar graph represents the percentage of Ly6Chigh 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+CD45+++) and the myeloid population (CD11b+CD45++) in the brain. Lower plots display the monocyte subsets (from the myeloid gate): Ly6Chigh, Ly6Cint, and Ly6Cneg. (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.

FIGURE 4. Increased parasite load and decreased survival in anti-CCR2–treated T. gondii–infected mice (A–H). Immunohistological representation of control (left panel) and anti-CCR2 (right panel) brains shows more inflammation by H&E staining (D, arrows), increased habitation of mononuclear cells (F), and more parasites (H) in the anti-CCR2 brain. (I) The bar graph is the quantification of the total cyst burden in the control and anti-CCR2 brains. (J) Survival curve of the T. gondii–infected control and anti-CCR2–treated mice was monitored from day 0 until day 60. Five to six coronal slides per mouse were analyzed; n = 4 mice per group. Total cyst load was determined from brain lysates; n = 4 mice per group. The survival experiment was repeated twice for a total of n = 8–10 mice per experiment. Significant differences (*p < 0.05) were determined using Student t test.
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 Ly6C high and Ly6C int cells exhibited low phagocytic capacity (31.1 ± 3.6% and 25.0 ± 2.2%, respectively), Ly6C neg cells demonstrated prominent phagocytic ability (80.2 ± 5.6%), alongside with those of activated resident microglia (60.2 ± 1.2%; Fig. 6E).
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+CD45highLy6G+Ly6Cint), Ly6Cint monocytes (CD11b+CD45highLy6G2Ly6Cint), Ly6Cneg monocytes (CD11b+CD45highLy6G2Ly6Cneg), 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.
Ly6C<sup>high</sup> cells infiltrate and differentiate in the brain upon adoptive transfer

To confirm that recruited Ly6C<sup>high</sup> monocytes further differentiate to the previously described Ly6C<sup>int</sup> and Ly6C<sup>neg</sup> cells following extravasation in the CNS, we conducted adoptive transfer experiments. To address this, we injected 1 x 10<sup>5</sup> sorted, CFSE-labeled Ly6C<sup>high</sup>CD11b<sup>+</sup> 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 Ly6C<sup>high</sup> cells were found in the CNS as early as 24 h after the transfer (data not shown). Forty-eight hours after the transfer, Ly6C<sup>high</sup> cells (27.2 ± 3.2% of CD11b<sup>+</sup>CFSE<sup>+</sup> gate) downregulated Ly6C and apparently differentiated into Ly6C<sup>int</sup> (32.0 ± 1.0% of CD11b<sup>+</sup>CFSE<sup>+</sup> gate) and Ly6C<sup>neg</sup> cells (41.0 ± 2.0% of CD11b<sup>+</sup>CFSE<sup>+</sup> gate; Fig. 7B). These data suggest that Ly6C<sup>high</sup> monocytes are recruited to the brain upon T. gondii infection, in which a subset of them loses their Ly6C expression and further generates the Ly6C<sup>int</sup> and Ly6C<sup>neg</sup> subsets.

Migration of CCR<sup>2</sup>Ly6C<sup>high</sup> 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<sup>+</sup>Ly6C<sup>high</sup> monocyte recruitment to the CNS in toxoplasmosis, we measured the expression of L selectin (CD62L), LFA-1, and PSGL-1 on Ly6C<sup>high</sup> monocytes. CD62L, which is known to be important for leukocyte rolling on the inflamed endothelium (56, 57), was highly expressed on Ly6C<sup>high</sup> 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 Ly6C<sup>int</sup> and Ly6C<sup>neg</sup> 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<sup>high</sup> inflammatory monocytes was significantly higher compared with resident cells in the blood and in the CNS (Fig. 8C, 8F, 8I, 8L).

Hence, to test whether the high PSGL-1 levels detected on the surface of Ly6C<sup>high</sup> monocytes had a functional role in their migration to the CNS, mice were treated i.p. with anti–PSGL-1 Ab from day 16 to day 26 after T. gondii infection. After 24 h of the last Ab application, we observed an increased proportion of Ly6C<sup>high</sup> 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<sup>high</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> population and in the total cell numbers within the Ly6C<sup>high</sup> 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 Ly6C<sup>high</sup> 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<sup>+</sup>Ly6C<sup>high</sup> 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 Ly6C<sup>high</sup> monocytes leads to rapid death of mice, demonstrating their important role in host defense (32, 60, 61). Furthermore, we recently observed that Ly6C<sup>high</sup> 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, Ly6C<sup>high</sup> monocytes infiltrate the CNS and, although contributing to viral clearance, they also induce significant immunopathology (16, 62). The opposing beneficial and detrimental nature of Ly6C<sup>high</sup> 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
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>Ly6ChighF4/80<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>Ly6Chigh 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-

**FIGURE 8.** CCR2<sup>−/−</sup>Ly6C<sup>high</sup> monocytes express PSGL-1. Cells from the peripheral blood (A–F) (gating strategy: Supplemental Fig. 1) and brains (G–L) (gating strategy: Fig. 1) of noninfected and infected mice were measured for the expression of adhesion molecules. (A–C and G–I) Representative histograms showing adhesion molecule expression by cell population. Bars mark the cells positive for the particular adhesion molecule. Numbers above bars display the percentage of cells positive for the marker of the concerned population. Blood cell subsets (D–F) are indicated as follows: resident monocytes, resident monocytes from infected mice, inflammatory monocytes, inflammatory monocytes from infected mice, isotype control, tinted. Brain leukocyte subsets (J–L) are as follows: resident microglia (CD11b<sup>+</sup>CD45<sup>low</sup>), activated microglia (CD11b<sup>+</sup>CD45<sup>int</sup>), inflammatory monocytes (CD11b<sup>+</sup>CD45<sup>high</sup>Ly6G<sup>2</sup>Ly6C<sup>high</sup>), Ly6C<sup>int</sup> monocytes (CD11b<sup>+</sup>CD45<sup>high</sup>Ly6G<sup>−</sup>Ly6C<sup>int</sup>), Ly6C<sup>neg</sup> monocytes (CD11b<sup>+</sup>CD45<sup>high</sup>Ly6G<sup>−</sup>Ly6C<sup>neg</sup>), isotype control, tinted. (D–F and J–L) Bar graphs represent the median fluorescence intensity for the Ab, median fluorescence intensity (MFI) ± SD (n = 5). Data are representative of two independent experiments. 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; Inf. Ly6C<sup>high</sup>, infected Ly6C<sup>high</sup>; Inf. res. mono., infected resident monocytes; ns, not significant; Res. microglia, resident microglia; Res. mono., resident monocytes.
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 Ly6C<sup>high</sup>CCR2<sup>+</sup> 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<sup>+</sup>Ly6C<sup>high</sup> monocytes, Ly6C<sup>int</sup> and Ly6C<sup>neg</sup> 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 Ly6C<sup>high</sup> cells, and was lower on Ly6C<sup>int</sup> and Ly6C<sup>neg</sup> cells. The chemokine receptor CX<sub>C</sub>CR1 was predominantly expressed on microglia and was absent on Ly6C<sup>high</sup> 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 Ly6C<sup>neg</sup> cells, suggesting their macrophage-like nature. Microglia upregulated CD11c upon infection-induced activation, as previously reported (27). CD11c expression was also elevated in Ly6C<sup>int</sup> and Ly6C<sup>neg</sup> cells, pointing toward their DC phenotype. Interestingly, MHC I and II were most intensely expressed on the surface of Ly6C<sup>int</sup> 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 Ly6C<sup>high</sup> 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 Ly6C<sup>high</sup> monocytes, further suggesting their critical role in the host defense arsenal. This proinflammatory signature of Ly6C<sup>high</sup> cells was reaffirmed in cerebral viral infections (62), and in other parasitic models (72, 73). IL-12p40 secretion was the highest by Ly6C<sup>int</sup> cells, well in line with their CD11c and MHC expression, suggesting their capability to initiate adaptive immune responses. In comparison with CD11b<sup>+</sup> Ly6C<sup>high</sup> 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, Ly6C<sup>high</sup> 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 Ly6C<sup>high</sup> 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 Ly6C<sup>high</sup> 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 Ly6C<sup>neg</sup> myeloid-derived cells displayed phagocytic potential, whereas...
Ly6C<sup>high</sup> and Ly6C<sup>int</sup> 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 Ly6C<sup>high</sup> inflammatory monocytes. The diverse behavior of the monocyte-derived macrophages was demonstrated in experimental autoimmune uveitis, in which the kinetics of CX<sub>3</sub>CR<sub>1</sub><sup>low</sup>Ly6C<sup>+</sup> and CX<sub>3</sub>CR<sub>1</sub><sup>high</sup>Ly6C<sup>−</sup> changed along the course of the disease (36). Such heterogeneity was also shown in an acute model of colitis, in which the recruited CX<sub>3</sub>CR<sub>1</sub><sup>int</sup>Ly6C<sup>high</sup> monocytes promoted inflammation, but over time gave rise to a CX<sub>3</sub>CR<sub>1</sub><sup>int</sup> Ly6C<sup>low</sup> population that displayed all the trademarks of a DC (35).

In cerebral toxoplasmosis, the conversion of macrophages 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 CD11c<sup>+</sup>Ly6C<sup>int</sup> brain DCs and Ly6C<sup>−</sup>F4/80<sup>+</sup> macrophages and carry out particular functions in parasite clearance.

Blood monocytes are comprised of two distinct populations, the inflammatory CX<sub>3</sub>CR<sub>1</sub><sup>low</sup>CCR2<sup>Ly6C<sup>high</sup></sup> and the patrolling CX<sub>3</sub>CR<sub>1</sub><sup>high</sup>CCR2<sup>Ly6C<sup>low</sup></sup> cells. Correspondingly, in our current experiments, CX<sub>3</sub>CR<sub>1</sub><sup>low</sup>CCR2<sup>Ly6C<sup>high</sup></sup> monocytes and CX<sub>3</sub>CR<sub>1</sub><sup>high</sup>CCR2<sup>Ly6C<sup>low</sup></sup> 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 Ly6C<sup>high</sup> 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 Ly6C<sup>high</sup> inflammatory monocytes to the CNS. CD62L (L-selectin), which assists immune cells to enter different tissues (56, 57), was highly expressed on Ly6C<sup>high</sup> 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 vessel wall, is involved in lymphocyte migration in the periphery and in the CNS on Ly6C<sup>high</sup> monocytes, as well as on differentiated Ly6C<sup>int</sup> and Ly6C<sup>neg</sup> 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 Ly6C<sup>high</sup> 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 Ly6C<sup>high</sup> monocyte homing to the site of atherosclerosis in blood vessels of mice (54). This study revealed that Ly6C<sup>high</sup> monocytes, which are PSGL-1<sup>high</sup> and CD62L<sup>+</sup>, 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 Ly6C<sup>high</sup> 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 Ly6C<sup>high</sup> 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 highlighted the plasticity of recruited Ly6C<sup>high</sup> monocytes. Cerebral T. gondii infection leads to cytokine production by the inundated Ly6C<sup>high</sup> monocytes, which play an influential role in the protection of the inflamed brain. The Ly6C<sup>high</sup> monocytes further give rise to Ly6C<sup>int</sup> and Ly6C<sup>neg</sup> 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, Ly6C<sup>high</sup> 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

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References


