CCR5 deficiency decreases susceptibility to experimental cerebral malaria

Elodie Belnoue, Michèle Kayibanda, Jean-Christophe Deschemin, Mireille Viguier, Matthias Mack, William A. Kuziel, and Laurent Rénia

Infection of susceptible mouse strains with *Plasmodium berghei* ANKA (PbA) is a valuable experimental model of cerebral malaria (CM). Two major pathologic features of CM are the intravascular sequestration of infected erythrocytes and leukocytes inside brain microvessels. We have recently shown that only the CD8+ T-cell subset of these brain-sequestered leukocytes is critical for progression to CM. Chemokine receptor–5 (CCR5) is an important regulator of leukocyte trafficking in the brain in response to fungal and viral infection. Therefore, we investigated whether CCR5 plays a role in the pathogenesis of experimental CM. Approximately 70% to 85% of wild-type and CCR5+/− mice infected with PbA developed CM, whereas only about 20% of PbA-infected CCR5-deficient mice exhibited the characteristic neurologic signs of CM. The brains of wild-type mice with CM showed significant increases in CCR5+ leukocytes, particularly CCR5+ CD8+ T cells, as well as increases in T-helper 1 (Th1) cytokine production. The few PbA-infected CCR5-deficient mice that developed CM exhibited a similar increase in CD8+ T cells. Significant leukocyte accumulation in the brain and Th1 cytokine production did not occur in PbA-infected CCR5-deficient mice that did not develop CM. Moreover, experiments using bone marrow (BM)–chimeric mice showed that a reduced but significant proportion of deficient mice grafted with CCR5+ BM develop CM, indicating that CCR5 expression on a radiation-resistant brain cell population is necessary for CM to occur. Taken together, these results suggest that CCR5 is an important factor in the development of experimental CM. (Blood. 2003; 101:4253-4259)

© 2003 by The American Society of Hematology

Introduction

With more than 300 million new cases and approximately 2 million deaths annually, malaria represents the most important worldwide parasitic disease. The most severe complication of this disease is cerebral malaria (CM), which involves the cytoadherence of parasitized erythrocytes (PEs) to the cerebral microvasculature.[1] However, the sequestration of infected erythrocytes, though necessary for CM pathogenesis, does not alone explain the progression to severe disease,[2] as some investigators have observed cerebral PE sequestration in non-CM cases.[3] Histopathologic studies have revealed the presence of mononuclear cells in brain capillaries of CM patients[4-7] (Terrie Taylor, personal communication). However, the role of these cells in CM pathogenesis remains unclear.

Infection of susceptible mouse strains with *Plasmodium berghei* ANKA (PbA) provides an experimental model of CM that shares some characteristics with the human disease. PEs together with leukocytes are sequestered in brain capillaries in PbA-infected mice developing CM.[8-12] We have recently demonstrated that leukocyte accumulation in the brain strongly correlates with CM development in susceptible mice and that CD8+ T cells are critical for both the neurologic defects and the ensuing death of animals with CM.[13]

Chemokines and chemokine receptors are well-known regulators of leukocyte trafficking, and it is possible that some of these molecules are involved in the pathogenesis of experimental CM. A good candidate for a role in experimental CM is chemokine receptor–5 (CCR5). Like other chemokine receptors, CCR5 is a member of the 7-transmembrane, G protein–coupled receptor superfamily. CCR5 binds to multiple chemokines, including CCR ligand–3 (CCL3) (macrophage inflammatory protein–1α [MIP-1α], CCL4 (MIP-1β) and CCL5 (RANTES; regulated on activation, normal T-cell expressed and secreted),[14] and it is expressed on a variety of leukocytes, including monocytes, T-helper 1 (Th1) cells, cytotoxic T cells, and immature dendritic cells.[15,16] CCR5 is also expressed on certain nonhematopoietic cell types, most notably endothelial cells of the brain microvasculature.[17,18] CCR5-bearing cells are among the infiltrating leukocytes in a variety of inflammatory processes, including lymphocytes in hepatitis C–infected liver,[19] mononuclear cells in rheumatoid arthritis[20] or infiltrated in the graft during acute and chronic transplant rejection,[21] and mononuclear phagocytes accumulated in the central nervous system of patients with multiple sclerosis.[22] Infection models with CCR5-deficient mice demonstrated that CCR5 is a key regulator of neuroinflammatory responses. CCR5-deficient mice infected with *Cryptococcus neoformans* or with mouse hepatitis virus exhibit major defects in leukocyte recruitment to the brain.[23,24] However, it is not clear whether these defects in the neuromigratory behavior of leukocytes in CCR5-deficient mice reflect the absence of CCR5 from leukocytes or from the brain endothelial cells or from both cell types. Nevertheless, these results prompted us to investigate whether CCR5 might contribute to the pathogenesis of experimental CM. Our results clearly show that CCR5 is involved in murine CM.

© 2003 by The American Society of Hematology

Materials and methods

Mice

All experimental mice were on a mixed C57BL/6J × 129/Ola genetic background and included wild-type animals (WT), CCR5-deficient animals (B6129F2/J-Cmkbr5[tm1Kuz]) (CCR5 knock-out [CCR5 KO]), and CCR5^{+/−} (F$_1$) animals from a cross of WT × CCR5 KO mice. All mice were bred under specific pathogen-free conditions and were used between 8 and 12 weeks of age. In each experiment, mice were matched for age and sex. The production of the CCR5 KO mice has been previously described.[23]

Parasites and pathology

The blood stage of a cloned line of PbA[8] was maintained as a stabilate (parasitized erythrocytes [PEs] at 10[7]/mL in Alsever solution) in liquid nitrogen. Batches were prepared through in vivo passage in C57BL/6J mice, and they were free of any infectious agents. Mice were infected intraperitoneally with 10[6] PEs. Animals were monitored for the neurologic signs of CM, including paralysis, deviation of the head, ataxia, convulsions, and coma. CM incidence was calculated as the percentage of infected mice that developed CM.

Parasitemia and hematologic parameters

Parasitemia was determined daily by means of blood smears stained with Giemsa and a count of the number of PEs in at least 1000 erythrocytes. Values from parasitemia were then transformed by means of the formula $x' = \log(x + 1)$ as described.[25] Hemoglobin (Hb) concentration was determined every 2 days as previously described.[26] Briefly, 2 μL tail-vein blood was diluted in 500 μL Drabkin solution (Sigma, St Quentin L'Arbresle, France), and Hb was assayed in 96-well microtiter plates (Costar, Cambridge, MA) in a volume of 100 μL by measuring the absorption at 405 nm (OD$_{405nm}$) in an enzyme-linked immunosorbent assay reader (Victor 1420, Wallac, Turku, Finland). Values were converted to milligrams per millimeter by means of a standard curve of human Hb (Sigma) dissolved in Drabkin solution.

Histology and immunostaining

One of the 2 hemispheres of brain from mice with or without CM was fixed in Carnoy solution (ethanol–chloroform–acetic acid, 6:3:1) for 24 hours and stored in butanol. Paraffin sections (5 μm) were cut from the midbrain region of 2 animals per group and stained with hematoxylin and eosin. For immunohistochemistry, brains from mice with or without CM were frozen in isopentane, cooled in liquid nitrogen, and stored at −80°C. Cryosections (6 μm) were cut and fixed in acetone (Prolabo, Fontenay sous Bois, France) for 10 minutes. Sections were incubated overnight at room temperature with biotinylated hamster antimouse intercellular adhesion molecule–1 (ICAM-1) antibody (clone 3E2; Pharmingen, San Diego, CA) diluted in phoshate-buffered saline (PBS). After washing, the sections were treated for 30 minutes with extravidin–alkaline phosphatase (Sigma). Phosphatase activity was detected by addition of the alkaline phosphatase substrate chromogen Fuchsin (DAKO, Carpinteria, CA). The sections were then counterstained with hematoxylin.

Purification of whole brain–sequestered leukocytes

Mice were killed and perfused intracardially with PBS to remove circulating red blood cells and leukocytes from the brain. The brains were then removed, and leukocytes were isolated by means of a previously described protocol.[13] Briefly, brains were removed and crushed in HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer: 100 mM NaCl, 2 mM KCl, 0.3 mM Na$_2$HPO4.12H$_2$O, 0.01 M HEPES (Sigma), and 100 000 IU penicillin/streptomycin (Life Technologies, Paisley, Scotland), and containing 0.05% collagenase (Boehringer Mannheim, Meylan, France) and 2 U/mL DNAse (Sigma). The tissue extract was then centrifuged at 400g for 5 minutes. The pelleted cells were further purified on a 30% Percoll gradient (Pharmacia Biotech, Uppsala, Sweden). Residual red blood cells were removed by hypotonic shock by means of ammonium chloride–potassium (ACK) lysis buffer. Brain-sequestered leukocytes (BSLs) were resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS containing 1% fetal calf serum [FCS] and 0.01% NaN$_3$) and counted.

Immunolabeling and flow cytometry analysis

BSLs were identified by their size (forward scatter [FSC]) and granulosity (side scatter [SSC]), as previously described.[13] BSL subsets were further phenotyped by cytofluorimetry. Macrophages were identified as F4/80[+] by means of biotinylated rat antimouse F4/80 (clone C1:A3-1; Tebu, Le Perray-en-Yvelines, France). Neutrophils were identified as F4/80[−] and Gr-1^{+} by means of rat antimouse Gr-1 conjugated to fluorescein isothiocyanate (FITC) (clone RB6-8C5; Pharmingen). T cells were identified by their small size and with the use of the following antibodies: hamster antimouse CD3 conjugated to phycoerythrin (clone 17A2; Pharmingen), rat antimouse CD8α conjugated to FITC (clone 53-6.7; Pharmingen), rat antimouse CD8α conjugated to quantum red (QR) (clone 53-6.7; Sigma), rat antimouse CD4 conjugated to QR (clone H129-19; Sigma), rat antimouse CD4 conjugated to phycoerythrin (clone H129-19; Pharmingen), and rat antimouse CCR5.[27] Antibodies were diluted at the appropriate concentration in FACS buffer. Ultravidin conjugated to phycoerythrin (Leinco Technologies, St Louis, MO) and goat antirat immunoglobulin G (IgG) conjugated to FITC (Polysciences,Warrington, PA) were used as secondary reagents. For each sample, 5000 cells were screened. Data were collected by means of a FACScalibur flow cytometer and analyzed by means of Cell Quest software (Becton Dickinson, Le Pont de Claix, France).

In vivo leukocyte subset depletion

Rat antimouse CD8 (clone 2.43; ATCC [Manassas, VA] TIB 210), rat antimouse CD4 (clone GK1.5; ATCC TIB 207), or anti–polymorphonuclear cells (anti-PMNs)[28] were purified from ascites or supernatant after ammonium sulfate precipitation. We injected 1 mg anti-CD8, anti-CD4, or anti-PMN monoclonal antibodies (mAbs) intraperitoneally at day 6 after parasite injection, and just before the onset of CM. More than 98% of blood CD8^{+} or CD4^{+} T cells were depleted by this procedure as verified by FACS analysis with the use of anti-CD4 (clone H129-19; Sigma) and anti-CD8 (clone 53-6.7; Pharmingen) mAbs that recognized epitopes different from those recognized by the depleting mAbs. Depletion of blood neutrophils was more than 80% as verified by FACS analysis with the use of anti–Gr-1 mAb. Purified rat IgG (Sigma) was used as a negative control. Macrophages were depleted at day 5 after PbA injection by intravenous injection of 0.2 mL PBS containing approximately 1 mg dichloromethylenediphosphonate (Cl$_2$-MDP) encapsulated in liposomes.[29] More than 90% of blood F4/80^{+} cells were depleted as verified by FACS analysis 2 days later.

Adoptive bone marrow reconstitution

Chimeric mice were prepared by irradiating animals twice (3 hours apart) with 5.5 Gy and then engrafting them with 10[6] fresh bone marrow (BM) cells injected intravenously. Mice were maintained on antibiotics for 4 weeks after BM cell injection. CCR5-deficient mice received either KO or WT BM cells, and WT mice received either KO or WT BM cells. To test for successful adoptive bone marrow cell transfer, CCR5 expression on peripheral blood leukocytes (PBLs) from reconstituted mice was determined 10 weeks after reconstitution by FACS analysis. Mice were infected with 10[6] PEs 10 weeks after receiving BM cells.

Splenocyte culture

Naive mice were infected with PbA, and spleens were harvested from animals with CM (at the day of the neurologic symptoms) or without CM (at day 10 after infection). Splenocytes were dissociated in RPMI glutamax medium (Life Technologies) supplemented with 10% FCS, 50 U/mL penicillin-streptomycin (Life Technologies), 50 μM β-2-mercaptoethanol (Sigma), and 10 mM HEPES (complete RPMI medium). Red blood cells were lyzed by osmotic shock, and leukocytes were cultured at 10[7] cells per

well in complete RPMI medium in a 24-well plate (Costar). The supernatants were removed 48 hours after the beginning of culture and stored at $-20°C$.

TNF bioassay

Sera from naive and from infected mice with or without CM were harvested and kept at $-20°C$ until analyzed. Filtered sera or splenocyte culture supernatants were assayed as previously described.[30] Murine WEHI 164.13 cells (3×10^4 cells per well) were placed in 96-well microtiter plates in 50 µL complete RPMI medium supplemented with 40 mM LiCl and 2 µg/mL actinomycin D (Sigma). We added 50 µL serum samples, supernatants, or dilutions of recombinant murine tumor necrosis factor–α (TNF-α) (Genzyme, Paris, France) in duplicate into the wells. The plates were incubated overnight at 37°C in 5% CO_2 atmosphere. Cell viability was determined by methylthiozolyl tetrazolium (MTT) reduction[31] by dissolving crystals in ethanol/dimethyl sulfoxide (DMSO) (vol/vol) and measuring absorbance at 560 nm with a reference wavelength of 630 nm. The TNF-α detection limit was 0.3 U/mL.

Enzyme-linked immunosorbent assay (ELISA) detection of IFN-γ and IL-10

The wells of 96-well plates (Nunc, Napperville, IL) were coated with anti–interferon-γ (anti–IFN-γ) (clone R4-6A2; Pharmingen) or anti–interleukin 10 (anti–IL-10) (clone JES5-2A5; Pharmingen) mAbs, and the plates were incubated overnight at 4°C. Supernatants or sera were added and incubated overnight at 4°C. A second biotinylated antibody against IFN-γ (clone XMG1.2) or IL-10 (clone SXC-1) (both clones from Pharmingen) was added to the appropriate wells, and the plates were incubated for 1 hour at room temperature (RT). This was followed by addition of extravidin–alkaline phosphatase for 1 hour at RT. Phosphatase activity was measured by means of 4-methylumbelliferyl phosphate (Sigma) as substrate and reading fluorescence at 360/460 nm with the use of a spectrophotometer (Victor 1420; Wallac). Dilutions of known quantities of IFN-γ or IL-10 were used for the standard curve. Detection limits were 50 pg/mL for IFN-γ and 80 pg/mL for IL-10.

Statistical analysis

Differences in CM incidence were analyzed by means of the Fisher exact test, and differences between multiple groups were analyzed for statistical significance by means of the one-way ANOVA followed by the Tukey multiple comparison test and with $P < .05$ as the level of significance.

Results

CCR5-deficient mice infected with PbA have increased resistance to CM

As shown in Figure 1, approximately 70% of WT mice and 85% of F_1 mice infected with PbA exhibited CM-specific neurologic signs within 7 to 10 days after infection and died within the next 24 hours. In sharp contrast, only about 20% of PbA-infected CCR5-deficient mice exhibited these neurologic signs and died. In the animals from all 3 groups (WT, F_1, and CCR5 KO) that did not develop CM (NCM), the infection progressed and the mice died within the third or fourth week after infection as a result of hyperparasitemia (Figure 1C) and anemia (Figure 1D). Histologic analysis of brain sections from infected WT and CCR5-deficient mice with and without CM revealed the presence of infected red blood cells in both sample sets, but characteristic petechial hemorrhages were observed only in CM brains (data not shown). In contrast, the capillaries of infected NCM WT and CCR5-deficient mice were normal with no evidence of hemorrhage (data not shown). ICAM-1, which is involved in leukocyte sequestration in
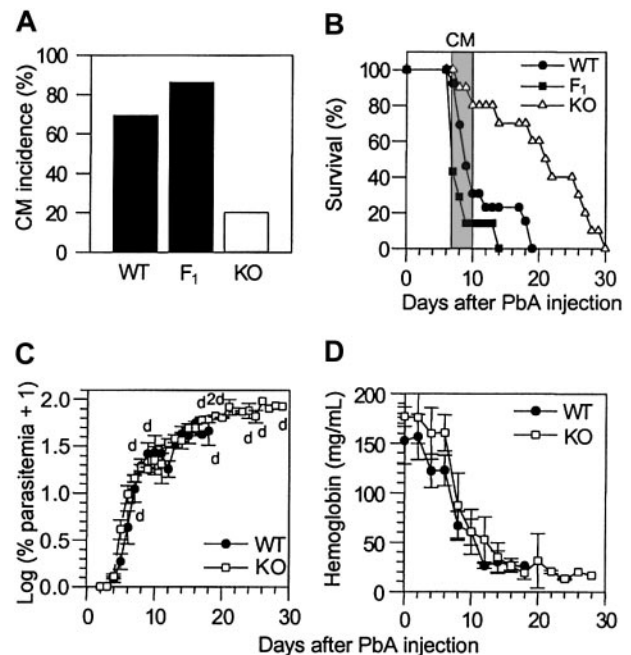

Figure 1. Increased resistance to CM in CCR5-deficient mice infected with PbA. (A-B) CM incidence occurring between day 7 and day 10 (A) and survival time (B) of WT (n = 13), F_1 (n = 7), and KO (n = 10) mice infected with PbA (10^6 PEs). Neurologic signs first appear late on days 7 through 10 (B, shaded area), with death occurring in less than 24 hours after their onset. On day 10, as calculated by Fisher exact test, $P < .05$ between WT (or F_1) and KO mice. This experiment is representative of 3 experiments. (C-D) Parasitemia (C) and hemoglobin levels (D) of PbA-infected WT (n = 5) and KO (n = 5) mice. Mortality is indicated on the first graph at the top (WT mice) and at the bottom (KO mice) as the number of dead mice (d) on that day. F_1 mice have parasitemia and hemoglobin level profiles similar to these observed for the WT mice. Error bars represent mean (SE) of 5 mice.

the brain during CM,[31] was expressed equally on large and small vessels of the brains of WT and CCR5-deficient mice with CM, whereas in NCM WT and CCR5-deficient mice, ICAM expression was restricted to large vessels in the brain (data not shown).

Leukocyte trafficking to the brain is reduced in CM-resistant CCR5-deficient mice

The total number of brain-sequestered leukocytes (BSLs) in PbA-infected WT and CCR5-deficient animals that did not develop CM were only slightly increased relative to the number of these cells in naive WT and CCR5-deficient mice, respectively. There were no significant differences in the accumulation of leukocyte subsets, including macrophages, neutrophils, and CD4+ and CD8+ T cells (Table 1). In contrast, PbA-infected WT and CCR5-deficient mice with CM exhibited significant accumulations of BSLs relative to naive and PbA-infected NCM mice. Neutrophils and CD4+ and CD8+ T cells were increased in number in WT mice compared with NCM WT mice. Only CD8+ T cells were significantly increased in number in CCR5-deficient mice with CM compared with NCM CCR5-deficient mice.

Distribution of CCR5+ leukocytes in naive and PbA-infected WT mice with and without CM

Using a recently described rat antimouse CCR5 monoclonal antibody,[27] we characterized the CCR5+ cells in total BSLs from naive WT mice and PbA-infected WT mice with and without CM. This antibody recognizes CCR5 on peripheral blood mononuclear cells from WT mice but does not react on such cells obtained from CCR5-deficient animals

Table 1. Quantification and characterization of brain-sequestered leukocytes of naive, CM, NCM, WT, and KO mice

				T lymphocytes	
Mice*	Leukocytes†	Macrophages	Neutrophils	CD3⁺ CD4⁺	CD3⁺ CD8⁺
WT					
Naive	138.1 ± 17.8	66.4 ± 11.6	14.7 ± 5.1	6.9 ± 3.05	6.3 ± 2.5
NCM	180.5 ± 36.9	136.1 ± 30.5	28.5 ± 5.2	4.5 ± 0.9	12.4 ± 4.9
CM	697 ± 73‡§	278.8 ± 27.8‡	240 ± 38.2‡§	41.1 ± 10.2‡§	190.3 ± 64.1‡§
KO					
Naive	231.3 ± 37.5	99.6 ± 22.9	37.5 ± 10.5	4.7 ± 1	3.5 ± 0.5
NCM	310.8 ± 48.6	190.1 ± 42.5	82.7 ± 17.5	10 ± 5.3	4.5 ± 1.9
CM	619.1 ± 87.8‖¶	239.5 ± 66.6‖	183.2 ± 43.7‖	33.6 ± 5‖	181.1 ± 31.3‖¶

*PbA-infected mice were killed between day 7 and day 10 after infection, when mice displayed neurologic symptoms. Mice without CM were killed at day 10.
†Results are mean ± SEM (×10³) of 3 to 9 mice per group.
‡$P < .05$ versus naive WT mice.
§$P < .05$ versus NCM WT mice.
‖$P < .05$ versus naive KO mice.
¶$P < .05$ versus NCM KO mice.

(data not shown). As shown in Figure 2, populations of BSLs isolated from naive WT, WT CM, and WT NCM mice all contain a substantial proportion of CCR5⁺ cells, and these cells are significantly more abundant in the WT CM animals (Figure 2A). In naive WT mice, approximately 50% of the BSLs are CCR5⁺ (Figure 2B). Many of these cells are also F4/80⁺, and a small fraction are CD8⁺. The majority of CCR5⁺ cells in the BSLs isolated from PbA-infected WT mice without CM are also F4/80⁺, and most of the remaining cells are CD8⁺. As with the BSLs from WT NCM mice, most of the BSLs in WT CM mice are CCR5⁺, but in striking contrast, the predominant subset of CCR5⁺ cells in the BSLs from WT CM mice are also CD8⁺ (Figure 2B). Most of the remaining cells are F4/80⁺, but a significant fraction are also CD4⁺. We and others have previously demonstrated that CD8⁺ cells are critical for the development of CM in PbA-infected WT mice.[13,32,33] Depleting CD8⁺ cells by treatment of WT or CCR5-deficient mice with anti-CD8 antibodies completely abrogated development of CM in both strains, confirming our earlier results (data not shown).

Development of CM in CCR5 KO or WT mice reconstituted with bone marrow from KO or WT mice

To evaluate whether the presence of CCR5 on leukocytes, on brain cells, or on both was essential for CM development, we generated

Figure 2. Expression of CCR5 on BSLs. (A) Dot plots representing size and CCR5 expression on whole BSLs from WT mice (naive, NCM, and CM). (B) Number of sequestered leukocyte subsets expressing CCR5 from the whole brain of WT mice (naive, NCM, and CM). *$P < .05$ versus naive mice. #$P < .05$ versus NCM mice. Error bars represent mean (SE) of 5 to 10 mice per group.

chimeric mice by adoptive bone marrow transplantation after total body irradiation. After total reconstitution (10 weeks), as verified by CCR5 expression in PBLs (Figure 3A), mice were infected by PbA. As shown in Figure 3, all WT mice reconstituted with WT bone marrow cells (WT plus WT BM) developed CM (Figure 3B) and died between day 9 and day 13 (Figure 3C). These mice took longer to die from CM than normal infected WT mice (Figure 1). This was associated with a longer delay between the time of the appearance of neurologic symptoms (day 7 to 8) and death by CM. Only 50% of WT mice reconstituted with KO bone marrow cells (WT plus KO BM) developed CM. Forty-three percent of CCR5-deficient mice reconstituted with KO and 50% of CCR5-deficient mice reconstituted with WT mice bone marrow cells developed CM. It should be noted that in the KO mice group reconstituted with KO BM, a slightly higher proportion of mice died of CM than in the normal KO mice. However, when the WT plus WT BM group is compared with the KO plus KO BM group, there is a significant difference in CM incidence, confirming a role for CCR5 in CM. No differences in parasitemia among the different experimental groups were observed (data not shown), confirming data in Figure 1 on the absence of influence of CCR5 on parasite development. Taken together, these results demonstrate that the presence of CCR5 on both hematopoietic and nonhematopoietic cells is necessary for CM development.
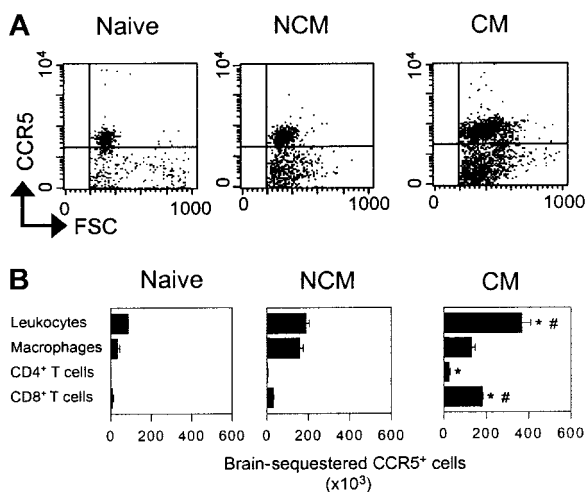
Cytokine production

IFN-γ and TNF-α are thought to be involved in the pathogenesis of CM.[34,35] Therefore, we investigated whether the absence of CCR5 affected production of these 2 Th1 cytokines during infection with PbA. Levels of IFN-γ, TNF-α, and IL-10 were measured in cultured splenocytes and in the serum from naive WT and CCR5-deficient mice and from PbA-infected WT and CCR5-deficient mice with and without CM.

Cultured splenocytes from WT mice with CM spontaneously produced more IFN-γ than splenocytes from WT NCM ($P < .05$) or naive WT mice ($P < .01$). Cultured splenocytes from PbA-infected CCR5-deficient mice with or without CM spontaneously produced higher levels of IFN-γ than splenocytes from naive CCR5-deficient animals, but these IFN-γ levels were lower than the amount of IFN-γ detected in splenocytes from WT CM mice (Figure 4A). A higher level of IFN-γ was detected in the sera of WT CM mice compared with WT NCM animals ($P < .001$) or naive WT mice ($P < .001$) (Figure 4B). In contrast, IFN-γ was not
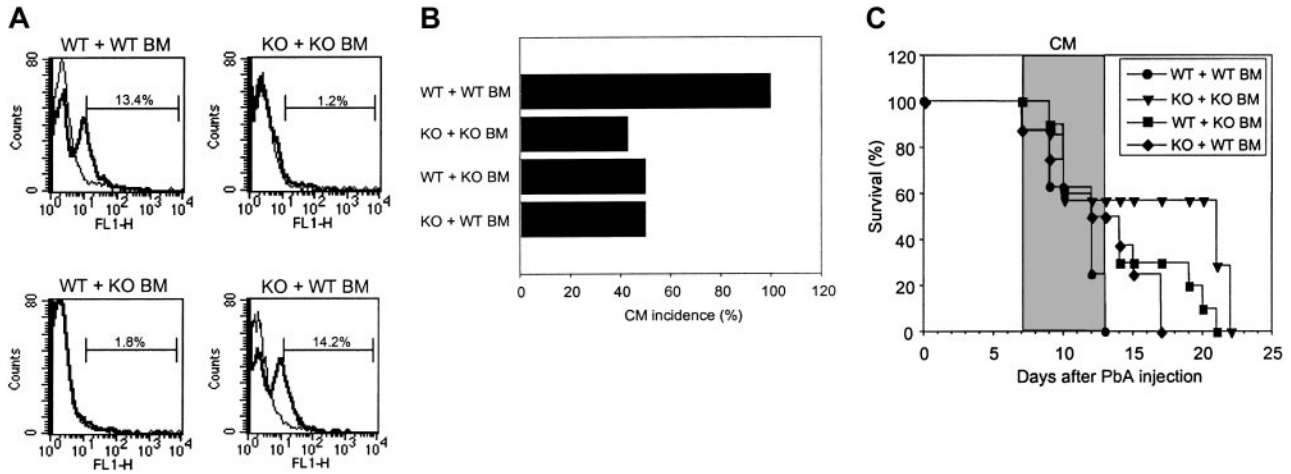
Figure 3. Relationship of CM to CCR5 expression on leukocytes and a radio-resistant brain cell. CCR5 expression on leukocytes and a radio-resistant cell in the brain is essential for CM. (A) Histograms of CCR5 expression on peripheral blood mononuclear cells of WT mice engrafted with WT BM, KO mice engrafted with KO BM, WT mice engrafted with KO BM, and KO mice engrafted with WT BM. Expression of CCR5 was assessed on peripheral blood leukocytes 10 weeks after BM engraftment for all reconstituted mice (7 to 10 per group). These histograms are representative of one mouse per group. Control is shown as a thin line. (B-C) CM incidence occurring between day 7 and day 13 (B) and survival time (C) of WT mice engrafted with WT BM (n = 8), KO mice engrafted with KO BM (n = 8), WT mice engrafted with KO BM (n = 10), and KO mice engrafted with WT BM (n = 8) and all infected with PbA (10^6 PE) 10 weeks after reconstitution. Neurologic signs first appear late on days 7 to 10, with death occurring 2 to 3 days after their onset (shaded area). On day 13, as calculated by Fisher exact test, $P < .05$ between WT mice engrafted with WT BM and KO mice engrafted with KO BM.

detected in the sera obtained from any of the 3 experimental groups of CCR5-deficient animals: naive, CM, or NCM.

Splenocytes from WT CM mice spontaneously produced more TNF-α than splenocytes from naive WT mice or WT NCM animals (Figure 5A). No significant differences in TNF-α concentration were observed between the different groups of CCR5-deficient mice (Figure 5A). TNF-α was detected in the sera from both WT and CCR5-deficient mice that developed CM, but the levels were significantly lower in the CCR5-deficient CM animals ($P < .01$). Serum TNF-α was not detected in naive WT, WT NCM, and CCR5-deficient mice (Figure 5B)

There was detectable production of IL-10 by splenocytes from PbA-infected WT and CCR5-deficient mice regardless of whether or not they had CM (Figure 6A). Serum IL-10 levels were significantly higher in WT and CCR5-deficient NCM mice than in naive WT and CCR5-deficient animals ($P < .01$) (Figure 6B).

Discussion

The results presented here strongly support the idea that CCR5 is important for progression to CM in mice after infection with *P berghei* ANKA. We show here that expression on both brain-sequestered leukocytes and nonhematopoietic cells, possibly endothelial cells, astrocytes, and microglial cells in the brain, is required for CM to develop in the majority of the infected animals.

We have previously demonstrated that leukocyte sequestration in the brain of PbA-infected mice correlates with the development of CM and that CD8+ T cells in the BSLs are essential for the neurologic signs and ensuing death of mice with CM,[13] confirming previous indirect evidence that these cells are a key effector in the severity of malaria.[32,33,36] We have now shown that a substantial number of CD8+ T cells in the BSLs of WT CM mice are CCR5+ (Figure 2B) and that in the majority of KO mice (those that do not develop CM), there was a defective trafficking of leukocytes, and in particular of CD8+ T cells, to the brain. A similar defect in leukocyte trafficking to the brain has been shown to occur in
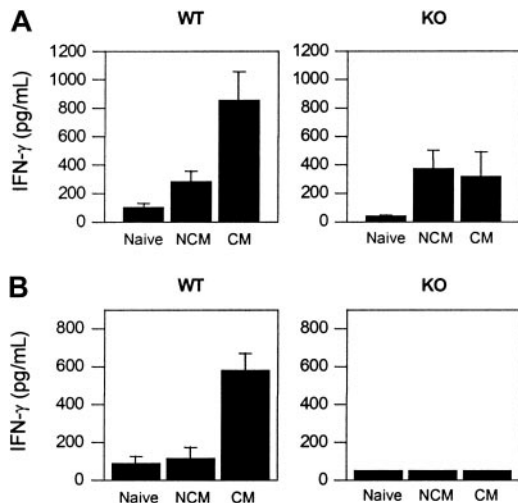

Figure 4. IFN-γ production in naive and PbA-infected WT and CCR5-deficient mice. (A) Spontaneous cytokine release from splenocytes. Mean (SE) of 3 to 5 mice per group. (B) Cytokine in the serum. Mean (SE) of 4 to 12 mice per group.

Figure 5. TNF-α production in naive and PbA-infected WT and CCR5-deficient mice. (A) Spontaneous cytokine release from splenocytes. Mean (SE) of 3 to 5 mice per group. (B) Cytokine in the serum. Mean (SE) of 4 to 12 mice per group.
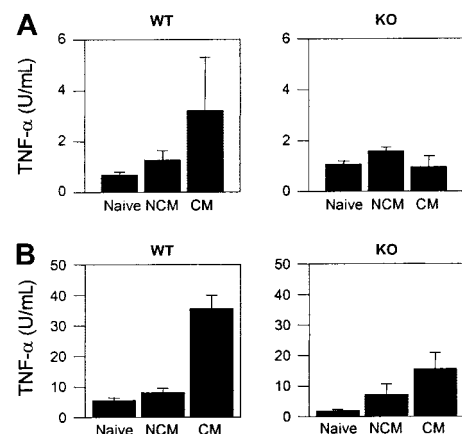
Figure 6. IL-10 production in naive and PbA-infected WT and CCR5-deficient mice. (A) Spontaneous cytokine release from splenocytes. Mean (SE) of 3 to 5 mice per group. (B) Cytokine in the serum. Mean (SE) of 4 to 12 mice per group.
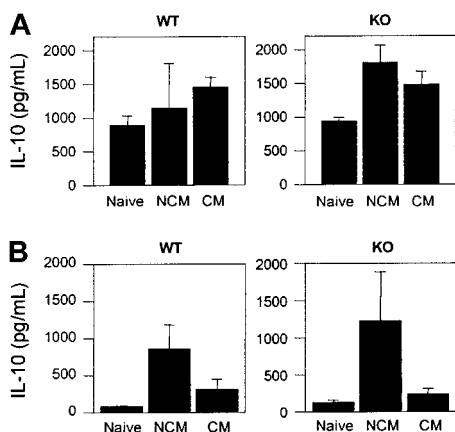
CCR5-deficient mice after disseminated *Cryptococcus neoformans* infection or intracranial infection with mouse hepatitis virus.[23,24] In CCR5-deficient mice infected with mouse hepatitis virus, there was a reduction in acute CD8^{+} T-cell accumulation in the brain that correlated with reduced early virus clearance.[24] In our model of experimental CM, infection of mice with PbA may lead to the activation of leukocytes and consequent CCR5 expression on their surface. Endothelial cells interacting with PEs through membrane contacts or after ingesting parasite antigens[37] may be induced to produce and present specific chemokines, including ligands for CCR5, such as CCL3, CCL4, and CCL5, (MIP-1α, MIP-1β, and RANTES, respectively),[38-40] which would serve to lure the CCR5-bearing leukocytes to the brain.[41] Then, adhesion molecules expressed on activated brain endothelial cells (ECs) could interact with their ligands expressed on the migrating leukocytes. We have proposed that brain-recruited effector CD8^{+} T cells destroy ECs, thus leading to the breakdown of the blood-brain barrier and the subsequent hemorrhages and perturbation of neuronal communication.[13]

Reconstitution experiments of irradiated WT or KO mice with bone marrow from WT or KO mice indicated that CCR5 expression on radio-resistant cells of nonhematopietic origin was involved in CM. Brain endothelial cells, microglia, and astrocytes are likely to be involved since they express CCR5 on their surface.[18,42,43] The binding of chemokines to CCR5 on these cells can serve to further activate them and enhance breakdown of the blood-brain barrier.

There is a role for cytokines in this process as well. The absence of CCR5 from PbA-infected animals resulted in a reduced Th1 cytokine production. This production was reported to up-regulate ICAM-1 on the surface of ECs.[44] Low ICAM-1 expression may contribute to the absence of BSLs in the brains of CCR5-deficient mice that do not develop CM. A defect in production of inflammatory cytokines (both IFN-γ and TNF-α) was also observed in CCR5-deficient mice with CM where leukocyte recruitment was normal. However, IL-10 production in these animals was reduced, and we have previously shown that administration of IL-10 was able to protect susceptible mice from CM.[45] KO mice with CM had levels of TNF-α similar to those of KO mice without CM. This suggests that TNF-α is not involved CM pathogenesis. This is an agreement with recent results showing that TNF-α deficient mice are fully susceptible to CM.[46] Reduced cytokine production (mainly of IFN-γ) has also been observed in *Leishmania donovani*–infected CCR5-deficient mice, which led to a delay in controlling the infection in these animals.[47]

In the few cases of PbA-infected CCR5-deficient mice that developed CM, the absence of CCR5 function may have been compensated for by one or more additional chemokine receptors, such as CCR1, CCR2, and CCR3. CCR1 and CCR3 bind some of the same ligands that bind to CCR5 (CCR1 binds to CCL3 and CCL5; CCR3 binds to CCL5),[14] and CCR2 regulates the trafficking of several of the same leukocyte subsets as CCR5.[48] In addition, CCR1 and CCR2, like CCR5, are expressed on brain endothelial cells.[49,50] If chemokine receptors on brain endothelial cells are involved in CM development, CCR1, CCR2, or other molecules might provide sufficient functional redundancy to promote CM in the absence of CCR5. Consistent with hypothesis, we have observed in a preliminary experiment that more than 90% of brain-sequestered leukocytes express CCR2^{+} (E.B. and L.R., unpublished results).

Recently, a number of polymorphisms and mutations in human CCR5 genes that alter, sometimes drastically, CCR5 functional activity or expression levels, have been identified.[51,52] For example, individuals homozygous for the delta32 mutation are naturally deficient in CCR5. Because there is growing evidence that leukocytes may be involved in human CM[4-7] (Terrie Taylor, personal communication), it will be of interest to determine whether natural CCR5 deficiency correlates with protection against CM. The results could suggest new therapeutic strategies to decrease the mortality associated with this disease.

Acknowledgments

References

1. Berendt AR, Turner GDH, Newbold CI. Cerebral malaria: the sequestration hypothesis. Parasitol Today. 1994;10:412-414.

2. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature. 2002;415:673-679.

3. Silamut K, Phu NH, Whitty C, et al. A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain. Am J Pathol. 1999;155:395-410.

4. Patnaik JK, Das BS, Mishra SK, Mohanty S, Satpathy SK, Mohanty D. Vascular clogging, mononuclear cell margination, and enhanced vascular permeability in the pathogenesis of human cerebral malaria. Am J Trop Med Hyg. 1994;51:642-647.

5. Porta J, Carota A, Pizzolato GP, et al. Immunopathological changes in human cerebral malaria. Clin Neuropathol. 1993;12:142-146.

6. Brown HC, Chau TT, Mai NT, et al. Blood-brain barrier function in cerebral malaria and CNS infections in Vietnam. Neurology. 2000;55:104-111.

7. Brown H, Hien TT, Day N, et al. Evidence of blood-brain barrier dysfunction in human cerebral malaria. Neuropathol Appl Neurobiol. 1999;25:331-340.

8. Hearn J, Rayment N, Landon DN, Katz DR, De Souza JB. Immunopathology of cerebral malaria: morphological evidence of parasite sequestration in murine brain microvasculature. Infect Immun. 2000;68:5364-5376.

9. Jennings VM, Lal AA, Hunter RL. Evidence for multiple pathologic and protective mechanisms of murine cerebral malaria. Infect Immun. 1998;66:5972-5979.

10. Rest JR. Cerebral malaria in inbred mice, I: a new model and its pathology. Trans R Soc Trop Med Hyg. 1982;76:410-415.

11. Neill AL, Hunt NH. Pathology of fatal and resolving *Plasmodium berghei* cerebral malaria in mice. Parasitology. 1992;105:165-175.

12. Polder TW, Eling WMC, Curfs JHAJ, Jerusalem CR, Wijers-Rouw M. Ultrastructural changes in the blood-brain barrier of mice infected with *Plasmodium berghei*. Acta Leiden. 1992;60:31-46.

13. Belnoue E, Kayibanda M, Vigário AM, et al. On the pathogenic role of brain-sequestered αβ

CD8⁺ T cells in experimental cerebral malaria. J Immunol. 2002;169:6369-6375.

14. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity. 2000;12:121-127.

15. Mantovani A. The chemokine system: redundancy for robust outputs. Immunol Today. 1999; 20:254-257.

16. Bonecchi R, Bianchi G, Bordignon PP, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med. 1998;187:129-134.

17. Edinger, A, Mankowski J, Doranz BJ, et al. CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain. Proc Natl Acad Sci U S A. 1997;94:14742-14747.

18. Berger O, Gan X, Gujuluva C, Burns AR, et al. CXC and CC chemokine receptors on coronary and brain endothelia. Mol Med. 1999;5:795-805.

19. Shields PL, Morland CM, Salmon M, Qin S, Hubscher SG, Adams DH. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. J Immunol. 1999;163:6236-6243.

20. Mack M, Bruhl H, Gruber R, et al. Predominance of mononuclear cells expressing the chemokine receptor CCR5 in synovial effusions of patients with different forms of arthritis. Arthritis Rheum. 1999;42:981-988.

21. Segerer S, Mack M, Regele H, Kerjaschki D, Schlondorff D. Expression of the C-C chemokine receptor 5 in human kidney diseases. Kidney Int. 1999;56:52-64.

22. Trebst C, Sorensen TL, Kivisakk P, et al. CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis. Am J Pathol. 2001;159:1701-1710.

23. Huffnagle GB, McNeil LK, McDonald RA, et al. Role of C-C chemokine receptor 5 in organ-specific and innate immunity to *Cryptococcus neoformans.* J Immunol. 1999;163:4642-4646.

24. Glass WG, Liu MT, Kuziel WA, Lane TE. Reduced macrophage infiltration and demyelination in mice lacking the chemokine receptor CCR5 following infection with a neurotropic coronavirus. Virology. 2001;288:8-17.

25. Vigario AM, Belnoue E, Cumano A, et al. Inhibition of Plasmodium yoelii blood-stage malaria by interferon alpha through the inhibition of the production of its target cell, the reticulocyte. Blood. 2001;97:3966-3971.

26. Taverne J, Sheikh N, De Souza JB, Playfair JHL, Probert L, Kollias G. Anaemia and resistance to malaria in transgenic mice expressing human tumour necrosis factor. Immunology. 1994;82:397-403.

27. Mack M, Cihak J, Simonis C, et al. Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. J Immunol. 2000;166: 4697-4704.

28. Lopez AF, Strath M, Sanderson CJ. Differentiation antigens on mouse eosinophils and neutrophils identified by monoclonal antibodies. Br J Haematol. 1984;57:489-494.

29. Van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. J Immunol Methods. 1996;193:93-99.

30. Espevik T, Nissen-Meyer J. A highly sensitive cell line, WEHI clone 13, for measuring cytotoxic factor/tumor factor from human monocytes. J Immunol. 1986;95:99-107.

31. Mosmann TR. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55-63.

32. Yanez DM, Manning DD, Cooley AJ, Weidanz WP, van der Heyde HC. Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. J Immunol. 1996;157:1620-1624.

33. Boubou MI, Collette A, Voegtle D, Mazier D, Cazenave PA, Pied S. T cell response in malaria pathogenesis: selective increase in T cells carrying the TCR V(beta)8 during experimental cerebral malaria. Int Immunol. 1999;11:1553-1562.

34. Lou J, Lucas R, Grau GE. Pathogenesis of cerebral malaria: recent experimental data and possible applications for humans. Clin Microbiol Rev. 2001;14:810-820.

35. Amani V, Vigario AM, Belnoue E, et al. Involvement of IFN-γ receptor-mediated signaling in pathology and anti-malarial immunity induced by *Plasmodium berghei* infection. Eur J Immunol. 2000;30:1646-1655.

36. Chang WL, Jones SP, Lefer DJ, et al. CD8(+)-T-cell depletion ameliorates circulatory shock in Plasmodium berghei-infected mice. Infect Immun. 2001;69:7341-7348.

37. Robert C, Peyrol S, Pouvelle B, Gay-Andrieu F, Gysin J. Ultrastructural aspects of *Plasmodium falciparum*-infected erythrocyte adherence to endothelial cells of *Saimiri* brain microvasculature. Am J Trop Med Hyg. 1996;54:169-177.

38. Cinamon G, Grabovsky V, Winter E, et al. Novel chemokine functions in lymphocyte migration through vascular endothelium under shear flow. J Leukoc Biol. 2001;69:860-866.

39. Colotta F, Sciacca FL, Sironi M, Luini W, Rabiet MJ, Mantovani A. Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. Am J Pathol. 1994; 144:975-985.

40. Devergne O, Marfaing-Koka A, Schall TJ, et al. Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells. J Exp Med. 1994;179:1689-1694.

41. Pober JS, Cotran RS. Immunologic interactions of T lymphocytes with vascular endothelium. Adv Immunol. 1991;50:261-302.

42. Luo Y, Berman MA, Zhai Q, et al. RANTES stimulates inflammatory cascades and receptor modulation in murine astrocytes. Glia. 2002;39:19-30.

43. Hesselgesser J, Horuk R. Chemokine and chemokine receptor expression in the central nervous system. J Neurovirol. 1999;5:13-26.

44. Grau GE, Pointaire P, Piguet PF, et al. Late administration of monoclonal antibody to leukocyte function-antigen 1 abrogates incipient murine cerebral malaria. Eur J Immunol. 1991;21:2265-2267.

45. Eckwalanga M, Marussig M, Tavares MD, et al. Murine AIDS protects mice against experimental cerebral malaria: down-regulation by interleukin 10 of a T-helper type 1 CD4+ cell-mediated pathology. Proc Natl Acad Sci U S A. 1994;91:8097-8101.

46. Engwerda CR, Mynott TL, Sawhney S, De Souza JB, Bickle QD, Kaye PM. Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. J Exp Med. 2002;195:1371-1377.

47. Sato N, Kuziel WA, Melby PC, et al. Defects in the generation of IFN-gamma are overcome to control infection with *Leishmania donovani* in CC chemokine receptor (CCR) 5-, macrophage inflammatory protein-1alpha-, or CCR2-deficient mice. J Immunol. 1999;163:5519-5525.

48. Baggiolini M. Chemokines and leukocyte traffic. Nature. 1998;392:565-568.

49. Dzenko KA, Andjelkovic AV, Kuziel WA, Packter JS. The chemokine receptor CCR2 mediates binding and internalization of monocyte chemoattractant protein 1 along brain microvessels. J Neurosci. 2001;21:9214-9223.

50. Andjelkovic AV, Pachter JS. Characterization of binding sites for chemokines MCP-1 and MIP-1alpha on human brain microvessels. J Neurochem. 2000;75:1898-1906.

51. Blanpain C, Lee B, Tuckoen M, et al. Multiple nonfunctional alleles of CCR5 are frequent in various human populations. Blood. 2000;96: 1638-1645.

52. Mummidi S, Bamshad M, Ahuja SS, et al. Evolution of human and non-human primate CC chemokine receptor 5 gene and mRNA. J Biol Chem. 2000;275:18946-18961.