INFECTION AND IMMUNITY, June 2003, p. 3648–3651 Vol. 71, No. 6
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.6.3648–3651.2003

Chemokine Receptor CCR2 Is Not Essential for the Development of Experimental Cerebral Malaria

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Received 13 January 2003/Returned for modification 14 February 2003/Accepted 6 March 2003

Infection with *Plasmodium berghei* ANKA induces cerebral malaria in susceptible mice. Brain-sequestered $CD8^+$ T cells are responsible for this pathology. We have evaluated the role of CCR2, a chemokine receptor expressed on $CD8^+$ T cells. Infected CCR2-deficient mice were as susceptible to cerebral malaria as wild-type mice were, and $CD8^+$ T-cell migration to the brain was not abolished.

Cerebral malaria (CM) contributes to around 2 million deaths annually, mainly in African children. Brain sequestration of parasitized erythrocytes (PE) is thought to be responsible for this pathology (4, 18). However, though necessary, PE sequestration cannot account alone for CM, since this phenomenon has been observed in non-CM cases (25). Leukocyte sequestration has often been described within brain postcapillary venules from patients who died of CM (9, 21); however, ethical considerations limit investigation of the role of these cells in pathogenesis. In a mouse model of CM with *Plasmodium berghei* ANKA, characterized by paralysis, deviation of the head, ataxia, convulsions, and coma, histological studies have shown that PE and leukocytes are sequestered in brain capillaries (10, 12, 20, 22). We have recently demonstrated that recruitment of macrophages, neutrophils, and T lymphocytes to the brain is associated with the onset of the disease and that the recruited $CD8^+$ T-cell subset is responsible for the neurological symptoms and the ensuing death (2). We postulated that a chemokine receptor(s) must be necessary for the migration of these pathogenic $CD8^+$ T cells to the brain. We focused on one of these chemokine receptors, CCR2, since it has been shown previously to be expressed on $CD8^+$ T cells migrating to the brain after a viral infection (19). CCR2 is a member of the seven-transmembrane G protein-coupled receptor superfamily and binds ligands such as CCL2 (MCP-1), CCL7 (MCP-3), and CCL12 (MCP-5) (29). In the mouse, CCR2 is expressed on monocytes; T cells, in particular $CD8^+$ T cells (17); endothelial cells; and brain cells like astrocytes and microglial cells (5, 11). CCR2 has been shown elsewhere to be implicated in leukocyte adhesion, monocyte recruitment (13, 26), and dendritic cell trafficking (23).

With the use of a recently described monoclonal antibody (MAb) to mouse CCR2 (17), expression of this molecule was investigated by cytofluorometry on total brain-sequestered leukocytes (BSL) and on the cell populations (macrophages and T lymphocytes) which are known to express CCR2 (17), isolated from 129/Ola × C57BL/6J F_2 wild-type (WT) naive mice or *P. berghei* ANKA-infected WT mice with or without CM. BSL were isolated as previously described (2), and leukocyte subsets were identified with the following antibodies: biotinylated rat immunoglobulin G2b (IgG2b) MAb anti-mouse F4/80 (Tebu, Le Perray-en-Yvelines, France), hamster IgG MAb anti-mouse CD3 conjugated to phycoerythrin (clone 17A2; PharMingen), rat IgG2a antibody anti-mouse CD8α conjugated to quantum red (clone 53-6.7; Sigma), rat IgG2a MAb anti-mouse CD4 conjugated to quantum red (clone H129-19; Sigma), and purified rat antibody anti-mouse CCR2 (17). Ultravidin conjugated to phycoerythrin (Leinco Technologies Inc., St. Louis, Mo.) and goat IgG anti-rat IgG conjugated to fluorescein isothiocyanate (Polysciences, Inc., Warrington, Pa.) were used as secondary reagents. For each sample, 5,000 cells were analyzed. $CCR2^+$ BSL were more numerous in WT mice with CM than in those without CM (NCM) or in naive mice (Fig. 1). BSL from WT mice with CM also expressed more CCR2 on their surface (mean fluorescence intensity [MFI], 57.1 ± 10.4) than did BSL from mice without CM (MFI, 30.1 ± 2.9; one-factor analysis of variance and Tukey test, $P < 0.05$; five mice per group) or BSL from naive mice (MFI, 25.15 ± 2; $P < 0.01$). Moreover, a strong and significant accumulation of $CD8^+$ T cells expressing CCR2 was observed in the brains of CM mice but not in those from NCM or naive mice (Fig. 1B).

Since CCR2 is expressed on pathogenic $CD8^+$ T cells, we next investigated susceptibility in CCR2-knockout (KO) mice (14). These mice display severe deficits in macrophage (7, 14), neutrophil (6), and T-cell (8) migration in response to either antigenic or nonantigenic challenge and an impaired type 1 cytokine response (7). CCR2-KO and WT mice were infected with 10^6 PE, and their parasitemia and anemia (hemoglobin levels) were determined every other day as previously described (28). All the KO mice but only 60 to 80% of WT mice developed CM and died between days 6 and 10 after infection (Fig. 2A and B). Though parasite levels were not significantly different between the two groups during the first week, the remaining WT mice died 2 weeks later (Fig. 2B) of hyperparasitemia (Fig. 2C) and anemia (Fig. 2D).

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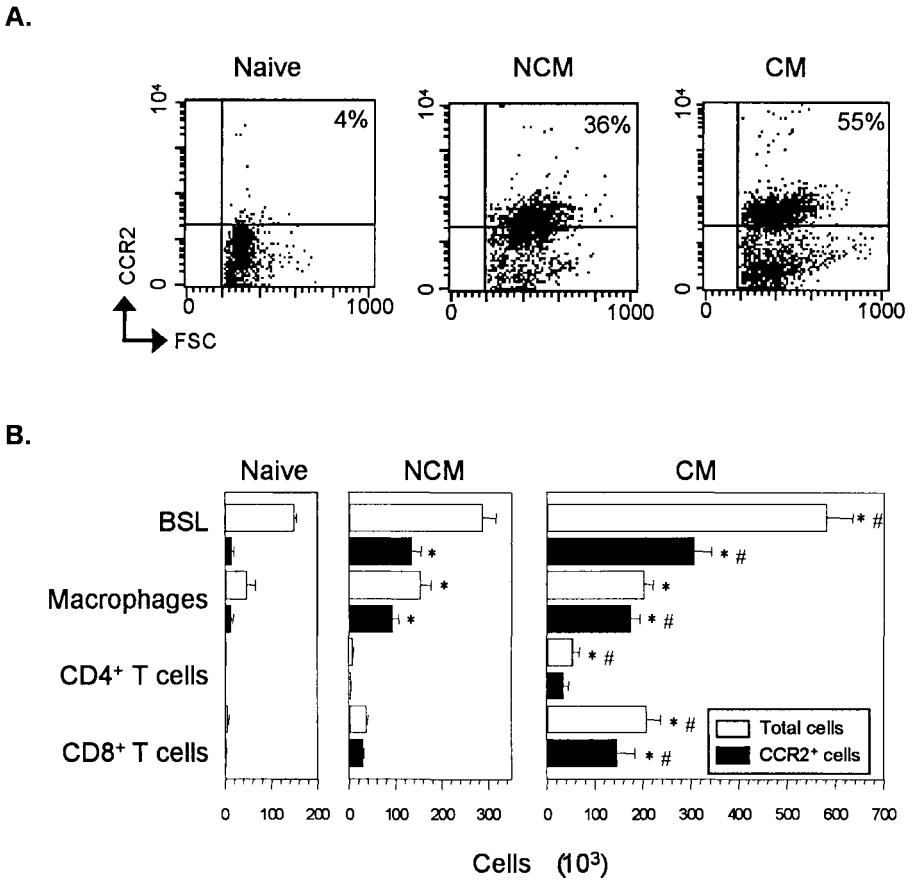

FIG. 1. CCR2 is expressed on BSL. (A) Representative dot plot of BSL from CCR2 WT mice (naive, NCM, and CM) stained with an anti-CCR2 MAb versus size (forward size scatter [FSC]). Data are representative of five animals per group. (B) Number of total sequestered leukocyte subsets (white bars) and $CCR2^+$ leukocyte subsets (black bars) from the whole brain of WT mice (naive, NCM, and CM). Samples of brain leukocyte suspension from mice infected with 10^6 PE and healthy mice were stained with MAbs specific for neutrophils, macrophages, and $CD4^+$ and $CD8^+$ T cells and for CCR2 and analyzed by flow cytometry. Absolute numbers of a given subset were calculated by multiplying the percentage of positive cells for this subset by the total number of BSL. $CCR2^+$ cell numbers were determined by using the percentage of CCR2 positive cells within each subset multiplied by the total number of this subset. *, $P < 0.05$ versus CM mice (one-factor analysis of variance followed by Tukey test). #, $P < 0.05$ versus NCM mice. This experiment is representative of three.

Histopathological analysis of the midbrain region of infected mice was performed as described previously (1) and revealed petechial hemorrhages and leukocyte accumulation in the capillaries of WT mice with CM, whereas these changes were not observed in infected WT mice without CM (data not shown). Brains of infected KO mice with CM showed ring hemorrhages with apparently fewer leukocytes in the capillaries than in those of WT mice with CM (data not shown). We thus quantified the total number of BSL from WT and KO mice. As shown in Fig. 3, BSL from KO mice with CM were less numerous than BSL from WT mice with CM. Nevertheless, there was a significant threefold increase in BSL number in infected KO mice compared to naive KO mice. There were eight times more BSL from WT mice with CM than from naive WT mice. NCM WT mice contained the same number of BSL as did naive WT mice (Fig. 3). BSL from the different mouse groups were further phenotyped by cytofluorometry. Macrophages were identified as $F4/80^+$, neutrophils were identified as $F4/80^-$ and $Gr\text{-}1^+$ (rat IgG2b MAb anti-mouse Gr-1 conjugated to fluorescein isothiocyanate, clone RB6-8C5; PharMingen), and T cells were identified as described above. We observed a significant increase in the numbers of macrophages, neutrophils, and $CD4^+$ and $CD8^+$ T lymphocytes (but not of other cell types) in CM WT mice compared with naive or NCM WT mice. Macrophages and $CD8^+$ T cells, but no other cell types, increased in infected KO mice with CM compared with naive KO mice. However, the number of macrophages in KO mice with CM was significantly lower than in CM WT mice (Fig. 3). In contrast, similar numbers of $CD8^+$ T cells, the subset responsible for CM in WT mice, were found in CM WT and CM KO mice. Depletion experiments were carried out to investigate the role of brain-sequestered $CD8^+$ T cells in CCR2-KO mice with CM. Depletion of BSL subsets was performed at day 6, just before the onset of CM, by injecting intraperitoneally 1 mg of the following MAbs: rat IgG anti-mouse CD8 (clone 2.43; ATCC TIB 210), rat IgG anti-mouse CD4 (clone GK1.5; ATCC TIB 207), or antipolymorphonuclear cells (15). More than 98% of blood $CD8^+$ or $CD4^+$ T cells were depleted as verified by fluorescence-activated cell sorting (FACS) analysis. Depletion of blood neutrophils was more than 80% as verified

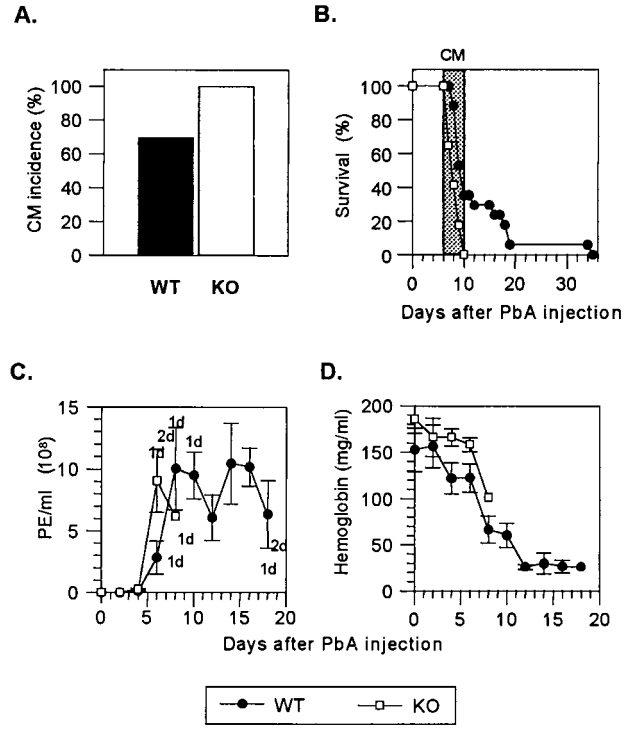

FIG. 2. CM incidence, survival, parasite load, and hemoglobin levels after *P. berghei* ANKA (PbA) infection of CCR2 WT and KO mice. (A) CM incidence occurring between day 6 and day 10 in WT ($n = 32$) and KO ($n = 27$) mice infected with 10^6 PE. On day 10, as calculated by Fisher's exact test, P was 0.0049 between WT and KO mice. (B) Survival of WT ($n = 17$) and KO ($n = 17$) mice infected with 10^6 PE. Neurological signs first appear late on days 6 to 10 (shaded area), with death occurring in <24 h after their onset. (C) PE per milliliter of blood ± standard errors of the means. WT ($n = 5$) and KO ($n = 5$) mice were infected with 10^6 PE. Mortality is indicated at the top (KO mice) and at the bottom (WT mice) as the number of dead mice (d) on that day. The difference between WT and KO mice on day 6 was not significant. (D) Hemoglobin levels (means ± standard errors of the means) in WT ($n = 5$) and KO ($n = 5$) mice infected with 10^6 PE.

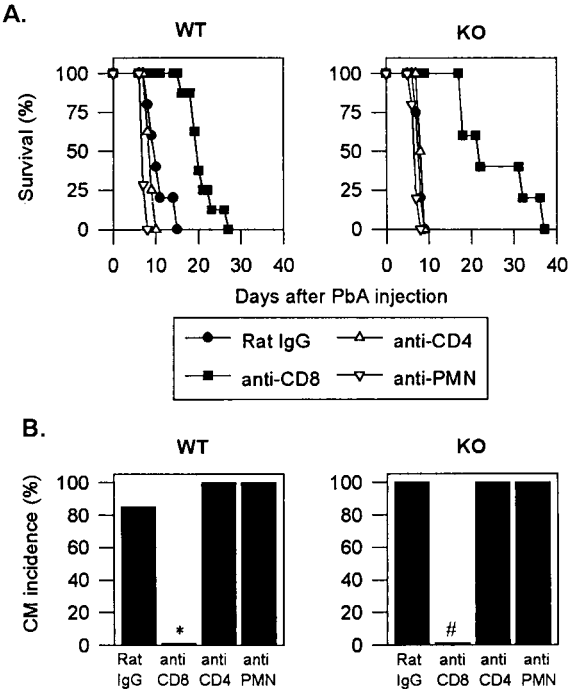

FIG. 4. Role of $CD8^+$ T cells in CM in CCR2 KO mice. The effector role of $CD8^+$ T cells was demonstrated through a series of depletion experiments with infected CCR2-KO and WT mice. The figure shows survival (A) and CM incidence (B) in infected WT or KO mice injected with the following rat antibodies: control IgG ($n = 5$), anti-CD8 ($n = 5$), anti-CD4 ($n = 5$), and anti-polymorphonuclear cell (PMN) ($n = 5$) on day 6. *, $P < 0.05$ (Fisher test) versus *P. berghei* ANKA-infected WT mice treated with control rat IgG; #, $P < 0.0001$ (Fisher test) versus rat IgG-treated KO mice. This experiment is representative of two.

by FACS analysis with anti-Gr-1 MAb. Purified rat IgG (Sigma) was used as a negative control. Macrophages were depleted at day 5 after *P. berghei* ANKA injection by intravenous injection of 0.2 ml of phosphate-buffered saline containing approximately 1 mg of dichloromethylenediphosphonate (Cl_2-MDP) encapsulated in liposomes (27). More than 90% of blood $F4/80^+$ cells were depleted as verified by FACS analysis 2 days later. All CCR2-KO mice depleted of $CD4^+$ T cells, neutrophils, or macrophages died of CM (Fig. 4 and data not shown), whereas none of the anti-CD8-treated KO mice de-

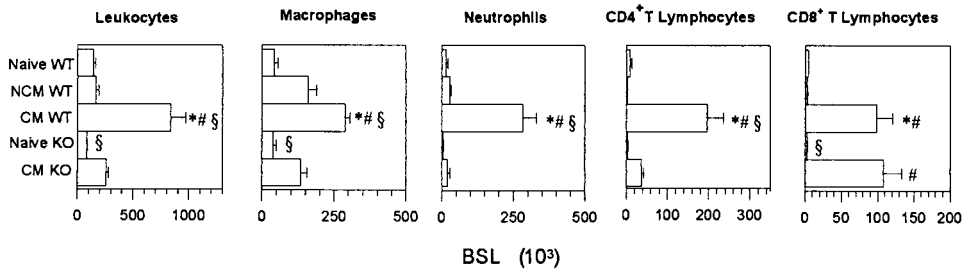

FIG. 3. Levels of whole-brain-sequestered leukocytes in CCR2-KO and WT mice after *P. berghei* ANKA infection. Enumeration of BSL was performed on perfused brains from KO ($n = 15$) and WT ($n = 8$) mice at the time when CM is diagnosable (days 6 to 10), NCM WT mice (days 9 to 10) ($n = 6$), and naive KO ($n = 10$) or WT ($n = 11$) mice. Cell numbers were determined as described for Fig. 1B. Values are expressed as means ± standard errors of the means. *, $P < 0.05$ (one-factor analysis of variance followed by Tukey test), significantly different from naive WT mice; #, $P < 0.05$, Tukey test, significantly different from NCM WT mice; and , $P < 0.05$, Tukey test, significantly different from CM KO mice. This experiment is representative of three.

veloped CM. Identical results were observed in infected and similarly depleted WT mice (Fig. 4 and data not shown).

Finally the role of cytokines was investigated, since a type 1 response, which is altered in CCR2-KO mice (7, 23, 24), has been associated elsewhere with CM development (1, 16). Both CM WT and KO mice, however, developed similar serological and cellular type 1 responses overall (data not shown).

Our results clearly show that CCR2 is not necessary for CM to occur. CCR2 deficiency was associated with a reduction in numbers of macrophages, neutrophils, and $CD4^+$ T cells but not of $CD8^+$ T cells. Our results further confirm that $CD8^+$ T cells are responsible for CM death (2). It is remarkable that the pathology in WT and CCR2-KO mice was due to the sequestration of less than 10^5 $CD8^+$ T cells in the vasculature of a whole brain. CCR2 has also been shown previously to be expressed on brain cells like endothelial cells, astrocytes, and microglial cells (5, 11), and signaling through this receptor may activate these cell types for chemokine and cytokine production. However, our results indicate that CCR2 signaling in these cells is not required for the development of CM. Since migration of $CD8^+$ T cells to the brain occurred normally in CCR2 KO mice, this implies that another chemokine receptor(s) is involved in this process. We have shown recently that CCR5 deficiency results in the decrease in CM susceptibility in mice of the same genetic background (3). Preliminary results indicate that more than 80% of brain-sequestered $CD8^+$ T cells from infected WT or CCR2 KO mice express CCR5 (data not shown). More studies are needed to determine if other chemokine receptors are involved in rodent and eventually in human CM.

We thank Georges Snounou for critical reading of the manuscript.

This work was supported in part by a grant from Junta Nacional de Investigação Cientifica e Tecnologica (JNICT) and Fondation de La Recherche Médicale to Laurent Rénia. Elodie Belnoue held a fellowship from MENRT. Fabio T. M Costa was supported by a fellowship from the CAPES foundation, Brazil. Ana Margarida Vigário held a fellowship from Junta Nacional de Investigação Cientifica e Tecnologica (JNICT), Portugal.

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Editor: W. A. Petri, Jr.