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NK Cells Stimulate Recruitment of CXCR3⁺ T Cells to the Brain during Plasmodium berghei-Mediated Cerebral Malaria

Diana S. Hansen, Nicholas J. Bernard, Catherine Q. Nie, and Louis Schofield

NK cells are cytotoxic lymphocytes that also secrete regulatory cytokines and can therefore influence adaptive immune responses. NK cell function is largely controlled by genes present in a genomic region named the NK complex. It has been shown that the NK complex is a genetic determinant of murine cerebral malaria pathogenesis mediated by Plasmodium berghei ANKA. In this study, we show that NK cells are required for cerebral malaria disease induction and the control of parasitemia. NK cells were found infiltrating brains of cerebral malaria-afflicted mice. NK cell depletion resulted in inhibition of T cell recruitment to the brain of P. berghei-infected animals. NK cell-depleted mice displayed down-regulation of CXCR3 expression and a significant reduction of T cells migrating in response to IFN-γ-inducible protein 10, indicating that this chemokine pathway plays an essential role in leukocyte trafficking leading to cerebral disease and fatalities. The Journal of Immunology, 2007, 178: 5779 – 5788.

Natural killer cells are large granular lymphocytes that constitute the major lymphoid cell population involved in innate immunity. Cytotoxicity is perhaps the best characterized effector function of NK cells and main targets include “stressed” cells such as tumor and viral-infected cells (1). Apart from their killing capacity, activated NK cells also produce several regulatory cytokines including IL-3, GM-CSF, TNF-α, TGF-β, and IFN-γ (2). Immune responses mediated by NK cells are essential for immune surveillance and to provide a first line of defense against infection. Furthermore, evidence from both human and mouse studies suggests that NK cells can also influence adaptive immune responses by modulating dendritic cell (DC) function (3–5) and by regulating Th1 polarization through the production of IFN-γ (6, 7).

NK cell function is largely controlled by genes encoded within a conserved genomic region named the NK complex (NKC) (8). The murine NKC, located on chromosome 6, comprises several genes such as Cdx6 and Cdx4 as well as multigene families including Nkrl, Nkg2, and Lyp49. Many of these genes encode type II integral membrane proteins with C-lectin domains, having inhibitory or activation function depending on the presence or absence of ITIMs in their intracellular domains (9, 10). Interaction of inhibitory receptors with their MHC class I (MHC I) or MHC I-like ligands results in inhibition of cytotoxic activity, which protects normal cells from the attack of autologous NK cells (11). Conversely, ligation of activation receptors induces NK cell activation, IFN-γ secretion, and killing of target cells (12, 13).

The NKC appears to be a highly polymorphic region and allelic variability of various NKC loci has been demonstrated in inbred mice providing evidence for NKC haplotypes (14, 15). Apart from genes encoding known protein products, the NKC has been shown to encode several phenotypically defined loci involved in the control of susceptibility and resistance to various infections. For example, resistance to mousepox and murine CMV infection has been shown to be under the control of loci named Rmp1 and Cmv1, respectively, which map to the NKC (16, 17). Further work indicated that Cmv1 maps to the activation receptor Ly49H (12). More recently, it was found that the NKC is also a genetic determinant of murine cerebral malaria pathogenesis mediated by Plasmodium berghei ANKA, since BALB.B6-Cmv1 congenic mice, in which the NKC from disease-susceptible C57BL/6 mice has been introduced in the resistant BALB/c background (18), were found to be significantly more susceptible to disease than wild-type control animals (19, 20).

Plasmodium falciparum-mediated cerebral malaria is responsible for ~2.5 million deaths each year (21) and the most susceptible population are children under the age of five. This neurological syndrome is characterized by the occurrence of convulsions, seizures, and coma (22). P. berghei ANKA murine malaria has many features in common with human disease and is thus the best available model for certain important aspects of clinical malaria (23–25). Despite extensive research, the precise mechanisms leading to pathogenesis during cerebral malaria are not fully understood. A large body of work indicates that proinflammatory responses are involved in disease induction. Systemic and local inflammation appear to be orchestrated by the action of cytokines produced in response to infection such as lymphotixin-α (LT-α) (26), TNF-α (27, 28), and IFN-γ (29, 30), which were shown to play a role in pathogenesis. Thus, cerebral malaria seems to arise from a strong Th1 response and causal roles for CD4⁺ (31, 32) and CD8⁺ T cells (33, 34) in disease promotion have been determined experimentally. Although the mechanism by which host effector cells mediate cerebral malaria has not been fully characterized, it has been suggested that sequestration of leukocytes together with
parasitized RBC within brain blood vessels contribute to disease induction (33–37). Recent studies indicate that αβ T cells accumulate in brains of malaria-infected mice and play a pathogenic role during disease (34). Particularly, brain-sequestered cytotoxic CD8⁺ lymphocytes were shown to mediate cerebral malaria pathogenesis in a perforin-dependent fashion (33).

Elements of the innate immune system have also been implicated in cerebral malaria disease induction. Previous studies showed that CD1d-restricted NKT cells mediate susceptibility or resistance to P. berghei-induced cerebral malaria depending on the genetic background of the host (20). Our previous data has also indicated that the differential expression of NKC loci in CD1d-restricted NKT cells determines their immunological properties during malaria infection (20). Although NKC receptors are mainly expressed in NK cells, a contribution of this cell population to the development of cerebral malaria pathogenesis has not been established. Therefore, in this study, we sought to investigate the role that NK cells play in malarial disease induction. We found that NK cells are required for cerebral malaria and are among the early recruits to brains of P. berghei-infected animals. Moreover, cell depletion experiments indicated that NK cells stimulate T cell recruitment to the brain of cerebral malaria-affected animals, providing evidence that immune responses mediated by NK cells can also regulate adaptive immunity by modulating the ability of T lymphocytes to migrate to the site of inflammation in response to chemotactic stimuli.

Materials and Methods

Mice and infections

Eight- to 12-wk-old C57BL/6 mice were used throughout the study. Groups of 10–15 mice were injected i.p. with 1 × 10⁸ P. berghei ANKA-infected RBC. In some experiments, mice were injected i.v with 15 μl of anti-asialo GM1 Ab (Wako) before challenge to deplete NK cells. Control mice received normal rabbit serum. NK cell depletion was confirmed by flow cytometry. Parasitemia was assessed from Giemsa-stained smears of infected mice were taken into 10% neutral-buffered formalin, sectioned (5 μm), and stained with H&E. Slides were coded and scored “blind” for parasite burden within brain blood vessels.

Brain histology

For histological analysis of cerebral pathology, brains from P. berghei-infected mice were taken into 10% neutral-buffered formalin, sectioned (5 μm), and stained with H&E. Slides were coded and scored “blind” for histological evidence of cerebral syndrome.

Flow cytometry

Splenic cells from C57BL/6 mice were incubated with anti-CD16 Ab (Fc-block), washed, and then stained with FITC- or PE-conjugated anti-NK1.1 (PK136) and FITC-conjugated or allophycocyanin-conjugated anti-TCR (H57-597) Abs. Some samples were simultaneously stained with PE-conjugated anti-CXCR3 (220803) (R&D Systems) or with biotinylated anti-CCR5 (C343-3448) followed by incubation for 1 h on ice with a streptavidin-PerCP-Cy5.5 conjugate. For analysis of T cell activation, splenocytes were stained with CyChrome-conjugated anti-CD4 (L3T4) or PerCP-Cy5.5-conjugated anti-CD8 (53-6.7) together with PE-conjugated anti-NK1.1 and either FITC-conjugated anti-CD69 (H1.2F3), anti-CD25 (7D4), or unlabeled anti-asialo GM1 (Wako). Alternatively, samples were stained with allophycocyanin-conjugated anti-TCR, PE-conjugated anti-CXCR3, PerCP-Cy5.5-conjugated anti-NK1.1 (all Abs and conjugates are from BD Pharmingen), except otherwise indicated) and anti-asialo GM1. Samples stained with appropriate isotype controls or normal rabbit serum were included as background controls. After washing, samples stained with unlabeled Abs were incubated for 1 h on ice with a FITC-anti-rabbit IgG conjugate. The cells were then washed two times with PBS containing 1% FCS and suspended in 200 μl of PBS. The cells were then analyzed in a FACS Calibur cytometer (BD Biosciences) using CellQuest software. Viable lymphocytes were gated by forward and side scatter.

BrdU incorporation assay

P. berghei-infected C57BL/6 mice were maintained ad libitum for 1 wk on light-shielded, BrdU-supplemented drinking water (0.8 mg/ml) replaced every 2–3 days. Spleenocyte suspensions from individual mice collected at different time points p.i. with P. berghei were incubated with anti-CD16 Ab, washed, and stained with anti-NK1.1-PE-conjugated and anti-TCR-allophycocyanin-conjugated Abs as described above. For staining of incorporated BrdU, cells were then fixed, permeabilized, treated with DNase I, and stained with FITC-anti-BrdU Ab using the BrdU Flow kit (BD Pharmingen), according to the manufacturer’s instructions. Cells were then resuspended in PBS and analyzed by flow cytometry.

Purification and analysis of brain-sequestered leukocytes

C57BL/6 mice were sacrificed at different time points p.i. with P. berghei ANKA. In some experiments, mice were perfused intracardially with PBS to remove circulating leukocytes from the brain. Brains were then removed, crushed in RPMI 1640 medium, and passed through a cell mesh. The tissue extract was then centrifuged at 200 × g for 10 min and the pellet was dissolved in 3 ml of RPMI 1640 containing 0.05% collagenase D (Worthington) and 2 U/ml DNase I (Sigma-Aldrich). The mixture was rotated for 60 min at 22°C and filtered through a 70-μm cell strainer. The supernatant was then seeded on a 35% Percoll (Amersham Bioscience) cushion and centrifugated at 400 × g for 20 min at 22°C. The pellet was collected and erythrocytes were lysed with Tris-NH₄Cl Buffer. Leukocytes were then washed, counted, and resuspended in 1% FCS, PBS. The cells were then incubated with anti-CD16 Ab, washed, and stained with PE-anti-NK1.1, allophycocyanin-anti-TCR, FITC-anti-CD4, and biotinylated-anti-CD8. Alternatively, cells were stained with FITC-anti-NK1.1, allophycocyanin-anti-TCR, PE-anti-CXCR3, and biotinylated anti-CCR5. The cells were then washed and incubated with a streptavidin-PerCP-Cy5.5 conjugate. After washing twice with PBS 1% FCS, the cells were suspended in 200 μl of PBS and analyzed by flow cytometry.

Chemotaxis assays

T lymphocytes were purified from naıve or malaria-infected C57BL/6 mice splenocyte suspensions using a negative isolation kit (Dynal Biotech) following the manufacturer’s instructions. Migration studies were done by placing 100 μl of cells (5 × 10⁵ cells/ml) in the upper wells of 24-well Transwell inserts (Corning Costar). Inserts were then placed in 24-well plate wells containing optimal concentration of mouse recombinant CXCL-9 (monokine induced by IFN-γ (Mig-9)), CXCL-10 (IFN-γ-inducible protein 10 (IP-10)), or CXCL-11 (IFN-inducible α-chemoattractant (I-TAC)) (all chemokines from PeproTech). The cells were incubated for 4 h at 37°C with 5% CO₂ and then at 4°C for 20 min to loosen leukocytes bound to the undersides of the membranes. The cells in the lower chambers were then collected and counted in a Neubauer hemocytometer. A chemotaxis index was calculated by dividing the number of cells migrating in response to a chemokine by the number of cells migrating in wells containing medium alone.

Adaptive transfer experiments

C57BL/6 recipient mice were depleted of NK cells by injection with anti-asialo GM1 Ab. Two days later, mice were infected with P. berghei ANKA (1 × 10⁸ pRBC) and were then injected i.v with 4 × 10⁷ wild-type or IFN-γ−/− NK cells, isolated from C57BL/6 or IFN-γ−/− splenocytes (The Jackson Laboratory) by negative selection using an NK cell isolation kit (Miltenyi Biotec). Purity of purified NK cells was 93% as assessed by flow cytometry. Adoptively transferred and control mice were sacrificed at day 6 p.i. and spleens and brains were collected. Single-cell suspensions were then prepared for flow cytometry and functional assays as indicated in the text.

Statistic analysis

A two-tailed paired-sample Student’s t test was used for data evaluation. Differences in mortality rates of P. berghei-infected mice during the period of susceptibility were assessed by Cox-Mantel log-rank analysis.

Results

NK cell depletion protects C57BL/6 mice against P. berghei-mediated cerebral malaria

To investigate the contribution of NK cells to the development of cerebral malaria pathogenesis, resistant BALB/c and susceptible C57BL/6 mice were infected with anti-asialo GM1 Ab. Unlike
depletion with anti-NK1.1 Ab, this procedure depletes NK cells without affecting CD1d-restricted NKT cells. Specificity and efficiency of NK cell depletion was confirmed in splenocytes by flow cytometry (Fig. 1A). Groups of 10 anti-asialo GM1-treated and control mice were infected with *P. berghei* ANKA (1 × 10⁶). The percentage survival (B) was monitored daily, *p* < 0.05. Parasitemia (C) was assessed from Giemsa-stained blood films. Each point represents the mean parasitemia ± SEM of the surviving animals; *, *p* < 0.005. This infection is representative of four separate experiments. D, Histological examination of brains from control (left panel) and anti-asialo GM1-treated mice (right panel) infected with *P. berghei*. Magnification, ×200.

CS7BL/6 anti-asialo GM1-treated mice developed cerebral malaria (Fig. 1B). Parasite burdens were significantly higher in anti-asialo GM1-treated animals compared with control mice at day 7 p.i. (Fig. 1C). Parasitemia levels remained elevated in cerebral malaria-protected anti-asialo GM1-treated animals, resembling parasite growth rates observed in disease-resistant mouse strains such as BALB/c (20, 38) and explaining the death of some of these animals during the second week of infection due to hemolytic anemia. The diagnoses of...
cerebral malaria were confirmed by histological examination of brains taken at day 6 p.i. Control mice dying of cerebral syndrome displayed typical pathology including high levels of vascular occlusion with both parasitized erythrocytes and leukocytes (Fig. 1D). In contrast, anti-asialo GM1-treated animals showed very reduced vascular occlusion despite higher parasite burdens (Fig. 1D). Taken together, these results indicated that NK cells contribute to the induction of cerebral malarial pathogenesis and that they are also involved in the control of parasitemia.

Apart from strongly binding to NK cells, it has been shown that anti-asialo GM1 might bind to activated but not naive T cells (39) and thioglycolate-elicited macrophages (40). Therefore, to examine whether traces of anti-asialo GM1 injected before parasitic challenge could target T cells and macrophages as they become activated at later times during infection, the absolute number and percentage of activated macrophages, CD4+ and CD8+ T cells were examined in splenocytes of mice injected with anti-asialo GM1 Ab before infection and control animals. Preliminary experiments indicated that P. berghei infection does not result in significant up-regulation of asialo GM1 in macrophages (data not shown). Therefore, this cell population was not further analyzed. No significant differences were found in the absolute number of CD4+ and CD8+ T cells in splenocytes from anti-asialo GM1-injected and control mice (Fig. 2, A and B). The percentage of activated CD4+ and CD8+ T cells, expressing CD69, CD25, and asialo GM1 itself was also determined. The expression of CD25 and CD69 in CD4+ and CD8+ T cells gradually increased as the infection developed, reaching a peak on days 4–5 p.i. (Fig. 2C). No differences were found in the percentage of activated T cells from anti-asialo GM1-treated and control mice (Fig. 2C). Around 5–8% of CD4+ and 10–15% CD8+ naive T cells were found to express asialo GM1. However, injection of anti-asialo GM1 Ab did not result in significant depletion of these populations (Fig. 2C), presumably due to their intermediate expression levels as compared with NK cells, which are asialo GM1bright and therefore become more easily targeted by the depleting Ab (data not shown). The expression of asialo GM1 increased in CD4+ and CD8+ T cells, reaching a peak at day 5 p.i. (Fig. 2C). Similar percentages of asialo GM1+ T cells were found in both injected and control groups. Taken together, these results indicate that in this experimental model anti-asialo GM1 injection of mice performed before parasitic challenge does not result in significant depletion of activated T cells and it constitutes a valuable tool to assess NK cell-mediated immune responses.

The absolute number of splenic NK cells decreases in response to malaria infection

The spleen is an important organ in the initiation of immune responses to malaria. Therefore, we sought to characterize the dynamics of the splenic NK cell pool. To that end, mice were challenged with P. berghei and splenocytes were stained with anti-NK1.1 and anti-TCR Abs at different time points p.i. Even though the total number of splenocytes increased in response to malaria (Fig. 3D), both the percentage (Fig. 3, A and B) and as the absolute number (Fig. 3C) of NK cells markedly decreased as the infection developed. To determine whether this reduction in NK cell numbers reflected a low turnover or a poor proliferation rate, mice were fed the thymidine analog BrdU and NK cell proliferation in response to P. berghei was analyzed in vivo. As shown in Fig. 3, E and F, a very high percentage of NK cells proliferated in response to malaria, with nearly 80% of the splenic NK cell pool dividing at days 5–7 p.i. This high proliferation rate was not sufficient to restore normal NK cell numbers, because the absolute number of cells continued to decrease (Fig. 3G), suggesting that during malaria, NK cells either have a very high turnover or migrate out of the spleen.

NK cells are recruited to the brain of malaria-infected mice

To determine whether NK cells were recruited to the brain of malaria-infected animals, C57BL/6 mice were infected with P. berghei and brains were collected at different time points p.i. after extensive perfusion of the sacrificed animals. The brain-sequestered leukocytes (BSL) were purified as described in Materials and Methods and analyzed by flow cytometry. A high percentage of NK cells was found in brains of malaria-infected animals as early as day 4 p.i., accounting for 15–18% of the total BSL pool (Fig. 4A). At day 5 p.i., a significant amount of NK1.1+ αβ TCR+ and conventional αβ T cells could also be found. Interestingly, at day 6 p.i., correlating with the onset of clear cerebral disease signs, there was a significant increase in the absolute number of BSL recovered from brains of infected animals (Fig. 4B). Fifteen percent of those cells were NK cells and ~60% consisted of αβ T cells (Fig. 4A). Further analysis revealed that 70% of the sequestered αβ T cells were CD4+ and ~30% were CD8+ (data not shown). Thus, NK cells are recruited to the brain of malaria-infected mice and are abundant at early times p.i.

NK cells stimulate T cell recruitment to the brain of cerebral malaria affected mice

To investigate the mechanism by which NK cells mediate cerebral malaria, mice were injected with anti-asialo GM1 Ab to deplete...
NK cells and then challenged with *P. berghei*. Brains of anti-asialo GM1-injected animals and non-NK cell-depleted controls were harvested at day 6 p.i. and the composition of the BSL was analyzed by flow cytometry. As expected, brains from anti-asialo GM1-treated animals lacked NK cells (Fig. 5, A and B). Brains from NK cell-depleted mice also displayed a significant decrease in the percentage of αβ T cells (Fig. 5, A). In fact, anti-asialo GM1 treatment resulted in a 95% reduction of the absolute number of αβ T cells recruited to the brains of malaria-infected animals (Fig. 5, C). NK cell depletion affected recruitment of both CD4+ as well as CD8+ T cells (Fig. 5, D–F). However, the inhibition of migration was more pronounced in CD8+ compared with CD4+ lymphocytes. Thus, during cerebral malaria T cell recruitment to the brain of affected mice appears to be stimulated by NK cells.

**CXCR3 expression increases in NK cells and T cells during malaria infection**

As discussed above, cerebral malaria pathogenesis results from a strong proinflammatory response mediated by Th1 cytokines such as IFN-γ. Several studies indicate that two chemokine receptors, CCR5 and CXCR3, are preferentially expressed in Th1 lymphocytes and define a subset of T cells associated with certain inflammatory conditions (41, 42). Interestingly, microarray analysis from our group indicated that the expression of CCR5, CXCR3, and several of their chemokines significantly increases during malaria infection (19, 43). To explore the mechanism of leukocyte migration during malaria, the expression of CXCR3 and CCR5 was analyzed in gated splenic αβ T cells and NK1.1+ TCR- cells at different times p.i. with *P. berghei* ANKA. CCR5 expression gradually increased in both NK cells and T lymphocytes as the infection developed, although in general this response was modest (Fig. 6A). Around half of the splenic NK cell pool expressed CXCR3 before malaria infection. At days 5–6 p.i., when animals show disease signs, the expression of CXCR3 remained high in NK and was highly up-regulated in αβ T cells, with 50% of the cells expressing this receptor (Fig. 6A). The percentage of CXCR3+ and CCR5+ NK and αβ T cells was also determined in BSL isolated at day 6 p.i. with *P. berghei*. Only 20% of T lymphocytes and ~10% of NK cells infiltrating brains of infected animals expressed CCR5. In contrast, >80% of NK cells and 90% of αβ T cells recovered from brains of cerebral malaria-affected mice were found to express CXCR3 (Fig. 6B). Taken together, these results

![FIGURE 4](http://www.jimmunol.org) Both NK cells and αβ T cells accumulate in brains of malaria-infected mice. C57BL/6 mice were infected with *P. berghei*. Brains were collected at different times p.i. after extensive perfusion of the sacrificed animals. The BSL were isolated, stained with anti-NK1.1 and anti-TCR Abs, and analyzed by flow cytometry. The percentage (A) and absolute number (B) of NK cells, T cells, and NK1.1+ TCR- cells were calculated. Each experiment is representative of four separate infections. Representative dot plots are shown. Each bar represents the mean of three samples ± SEM.

![FIGURE 5](http://www.jimmunol.org) NK cells stimulate T cell recruitment to the brain of cerebral malaria-affected mice. C57BL/6 mice were injected twice with anti-asialo GM1 Ab or rabbit antiserum, 2 days apart. Control and anti-asialo GM1-injected mice were infected with *P. berghei* ANKA. Brains were collected on day 6 p.i. and the BSL were stained with anti-NK1.1, anti-TCR, anti-CD4, and anti-CD8 Abs. The percentage (A and D) and absolute number of NK cells (B), total T cells (C), CD4+ (E), and CD8+ (F) cells were calculated. Each experiment is representative of four separate infections. Representative dot plots are shown. Each bar represents the mean of three samples ± SEM; *, p < 0.0001.
indicate that CXCR3 expression is associated with lymphocyte trafficking during malaria infection.

**NK cells regulate the ability of T cells to migrate in response to CXCR3 chemokines**

To determine whether T cells from malaria-infected animals could in fact migrate in response to CXCR3 chemokines, T cell-enriched splenocytes from *P. berghei*-infected mice were placed in the upper wells of Transwell inserts and the chemotactic response to IP-10 was determined. Despite low CXCR3 expression levels, naive cells were unable to migrate in response to this chemokine (Fig. 7A). To the contrary, cells from malaria-infected mice showed an increasing chemotactic response to IP-10, which peaked at day 6 p.i. Similar results were obtained when cells from splenocytes from *P. berghei*-infected mice were placed in the upper wells of Transwell inserts and the chemotactic response to IP-10 was determined. Despite low CXCR3 expression levels, naive cells were unable to migrate in response to this chemokine (Fig. 7A). To the contrary, cells from malaria-infected mice showed an increasing chemotactic response to IP-10, which peaked at day 6 p.i. Similar results were obtained when cells from

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**FIGURE 6.** CXCR3 is up-regulated in T cells and NK cells in response to malaria infection. A, C57BL/6 mice (*n* = 4) were infected with *P. berghei*. Spleen cells were prepared at different times p.i. and the expression of CCR5 and CXCR3 was analyzed by flow cytometry on gated NK1.1^− TCR^−^ and TCR^NK1.1^+^ cells. B, Brains were collected on day 6 p.i. and the BSL were stained with anti-NK1.1, anti-TCR, anti-CCR5, and anti-CXCR3 Abs. The percentage of CCR5^+^ and CXCR3^+^ cells was calculated on gated NK cells and T lymphocytes. Each experiment is representative of two separate infections. Representative histograms and contours are shown.

**FIGURE 7.** NK cells control the ability of T cells to migrate in response to CXCR3 chemokines. A, T cells from naive and *P. berghei*-infected mice were placed in the upper wells of Transwell inserts and the chemotactic response to IP-10 (100 ng/ml) was determined. B, C57BL/6 mice were injected with anti-asialo GM1 Ab and then infected with *P. berghei* ANKA. The chemotactic response to IP-10 was then analyzed in T cells from NK cell-depleted and control mice at different times p.i. The bars represent the mean of three samples ± SEM; *, *p < 0.05; **, *p < 0.01. C, Spleen cells from NK cell-depleted and control mice were prepared at different times p.i. The expression of CXCR3 was analyzed on gated TCR^NK1.1^− cells (C) and the expression of asialo GM1 was analyzed on gated TCR^NK1.1^− CXCR3^+^ cells (D) by flow cytometry. Each experiment is representative of two to three separate infections. Representative histograms and contour plots are shown.
infected animals were tested for their ability to migrate in response to MIG and I-TAC (data not shown), indicating that during malaria, splenic T lymphocytes acquire the capacity to migrate in response to CXCR3 chemokines.

To investigate whether NK cells could affect the ability of T lymphocytes to migrate in response to IP-10, T cells were isolated from control and anti-asialo GM1-treated malaria-infected mice and chemotaxis was analyzed in vitro. Anti-asialo GM1 treatment significantly reduced the chemotactic response of T lymphocytes to IP-10 (Fig. 7B). Consistent with this observation, splenic αβ T cells from anti-asialo GM1-treated mice displayed a significant reduction in the up-regulation of CXCR3 expression in response to infection (Fig. 7C). To exclude the possibility that anti-asialo GM1 injection resulted in in vivo depletion of CXCR3⁺/⁺-activated αβ T cells, explaining the reduced capacity of the splenic T cell pool from injected animals to migrate in response to IP-10 during infection, the expression levels of asialo GM1 were examined in gated CXCR3⁺/⁺ αβ T cells at different times p.i. Only 10–15% of CXCR3⁺ T cells expressed asialo GM1 throughout the infection period (Fig. 7D). Although the overall expression of CXCR3 was reduced in anti-asialo GM1-treated mice, no differences were found in the percentage of asialo GM1⁺CXCR3⁺ T cells from injected and control T cells (Fig. 7D). Taken together, these results suggest that during cerebral malaria NK cells stimulate T lymphocyte migration through the CXCR3 pathway.

**Control of T cell migration by NK cells requires IFN-γ**

To further understand the mechanism by which NK cells mediate up-regulation of the CXCR3 pathway in T lymphocytes during malaria infection, C57BL/6 mice were depleted of NK cells by injection with anti-asialo GM1 Ab and 2 days later the animals were challenged with *P. berghei*-ANKA. Infected mice were then adoptively transferred with either wild-type (A–C) or IFN-γ⁻/⁻ (D–F) purified NK cells and the splenocytes and BSL were isolated at day 6 p.i. The chemotactic response to IP-10 (100 ng/ml) (A and D) and the expression of CXCR3 (B and E) were determined in T cells from adoptively transferred, NK cell-depleted, and malaria-infected control mice. BSL (C and F) from adoptively transferred, NK cell-depleted, and malaria-infected control mice were stained with anti-NK1.1 and anti-TCR Abs and analyzed by flow cytometry. Each experiment is representative of two to three separate infections. Representative histograms and dot plots are shown. Each bar represents the mean of three samples ± SEM, *p* < 0.05 between adoptively transferred and NK cell-depleted mice.

**FIGURE 8.** NK cell-mediated T cell recruitment requires IFN-γ. C57BL/6 mice were depleted of NK cells by injection with anti-asialo GM1 Ab and 2 days later, the animals were challenged with *P. berghei*-ANKA. Infected mice were then adoptively transferred with either wild-type (A–C) or IFN-γ⁻/⁻ (D–F) purified NK cells and the splenocytes and BSL were isolated at day 6 p.i. The chemotactic response to IP-10 (100 ng/ml) (A and D) and the expression of CXCR3 (B and E) were determined in T cells from adoptively transferred, NK cell-depleted, and malaria-infected control mice. BSL (C and F) from adoptively transferred, NK cell-depleted, and malaria-infected control mice were stained with anti-NK1.1 and anti-TCR Abs and analyzed by flow cytometry. Each experiment is representative of two to three separate infections. Representative histograms and dot plots are shown. Each bar represents the mean of three samples ± SEM, *p* < 0.05 between adoptively transferred and NK cell-depleted mice.

(data not shown). Therefore, this experimental protocol does not fully recapitulate the immunological scenario or the splenic architecture found in normal mice before infection. Despite this, adoptive transfer of wild-type NK cells resulted in a partial but significant reconstitution of both the chemotactic response to IP-10 as well as the up-regulation of CXCR3 expression in αβ T cells in response to malaria (Fig. 8, A and B). Wild-type NK cell transfer could also partially restore T cell recruitment to the brain of infected animals (Fig. 8C). These mice showed more severe symptoms than NK cell-depleted controls (data not shown). In marked contrast, animals transferred with IFN-γ⁻/⁻ NK cells failed to reinstate these responses (Fig. 8, D–F). Moreover, unlike adoptively transferred wild-type NK cells, IFN-γ⁻/⁻ NK cells were unable to migrate to the brain of infected animals. Taken together, these results suggest that IFN-γ secretion by activated NK cells is required for up-regulation of the CXCR3 chemotaxis pathway and to promote T cell recruitment to the brain of cerebral malaria-affected animals.

**Discussion**

This study shows that NK cells are important players in the induction of murine cerebral malaria pathogenesis. NK cells were found to migrate to the brains of malaria-infected animals, comprising a significant proportion of the total sequestered leukocyte pool. Our data has also provided evidence that during malaria infection, NK cells stimulate recruitment of CXCR3⁺ T cells to the brain of cerebral malaria-affected animals.

Despite high proliferation rates, the splenic NK cell pool was found to substantially decrease during infection with *P. berghei* blood-stage parasites, suggesting that NK cells migrate out of the spleen or undergo death by apoptosis. Interestingly, a similar phenomenon was observed in spleens of mice infected with *Plasmodium yoelii* sporozoites (44). In that infection model, the reduced cellularity of the splenic NK cell pool correlated with enhanced sensitivity of these cells to in vitro-induced apoptosis. Although our results here support the notion that splenic NK cells migrate to target organs such as the brain, the possibility of increased activation-induced cell death cannot be excluded.
Depletion of NK cells resulted in significantly increased parasitemia, demonstrating a role for this cell population in the control of *P. berghei* infection. These results are in agreement with previous observations showing that cytokine production by NK cells contributes to resistance against *Plasmodium chabaudi* blood stages (45). Despite higher parasitic burdens, NK cell depletion resulted in significant protection against cerebral malaria, indicating that control of disease induction by NK cells is independent of parasite growth rates. Moreover, this result and others (46) suggest that innate responses produced during malaria are in fact a “double-edged sword” because they can be responsible for parasitic clearance in the early stages of infection as well as severe disease induction.

The role of NK cells in the induction of cerebral malaria pathogenesis has been previously evaluated (32). Unlike the protective effect reported here after NK cell depletion with anti-asialo GM1 Ab, injection with anti-NK1.1 Ab failed to protect mice from *P. berghei*-mediated cerebral malaria. NK1.1 is an NK cell activation marker and it has been shown that ligation through this receptor triggers IFN-γ production (47) by NK cells and results in activation-induced cell death. It is therefore possible that this depletion protocol increases systemic IFN-γ levels, provoking a disease-enhancing effect and explaining the lack of protection against cerebral malaria in the absence of NK cells. In contrast, it has been shown that anti-asialo GM1 Ab may bind activated but not naive T lymphocytes (39, 48) and thioglycolate-elicited macrophages (40). In this study, T cell activation analysis in animals injected with anti-asialo GM1 before parasitic challenge indicated that treatment with the rabbit antiserum did not result in significant depletion of splenic T lymphocytes as they become activated in response to infection. Thus, when administered to naive animals before activation of the immune system anti-asialo GM1 injection constitutes a valuable tool for in vivo evaluation of NK cell-mediated responses and in the present study has allowed us to determine that lack of susceptibility to cerebral malaria can be attributed to the absence of NK cells.

It is becoming increasingly accepted that cerebral malaria pathogenesis results from the sequestration of parasitized RBC as well as intravascular infiltration of host monocytes and lymphocytes within blood vessels in the brain. Clearly, the analysis of cellular trafficking results from the sequestration of parasitized RBC as well as cytokine production by NK cells constitutes a valuable tool for in vivo evaluation of NK cell-mediated responses and in the present study has allowed us to determine that lack of susceptibility to cerebral malaria can be attributed to the absence of NK cells. Presumably, NK cells also constitute a significant proportion of the brain infiltrate, 2) confirming the role of infiltrating T lymphocytes in cerebral disease induction, 3) indicating that NK cells contribute to the series of immunological events resulting in T cell recruitment to the brain, and 4) providing evidence for a chemokine pathway governing T cell migration during malaria infection.

Stimulated T lymphocytes migrate to the site of inflammation through both cognate and systemic mechanisms and this study provides evidence for one of them. Adoptive transfer strategies revealed that IFN-γ secretion by NK cells is required to reconstitute the capacity of T cells to migrate in response to IP-10 and to be recruited to the brain of malaria-infected animals. This response probably reflects systemic effects of IFN-γ resulting in up-regulation of the CXCR3/IP-10 pathway, because most of the adoptively transferred NK cells did not return to the spleen and remained in circulation (data not shown). Elicitation of IFN-γ production in response to malaria has been previously reported. Human NK cells have been shown to be potent and rapid producers of IFN-γ in response to *P. falciparum*-infected RBC (50–52). Moreover, it has been suggested that NK cells may represent an important early source of IFN-γ during infection (51). The present data, however, does not rule out the possibility of cognate mechanisms by which NK cells might enhance the ability of T lymphocytes to migrate in response to chemokines. Emerging evidence demonstrated several NK cell-DC interactions, which are essential for the development of effective immunity (3–5). In fact, NK cells have been shown to promote DC maturation via Nkp30 engagement and the release of TNF-α and IFN-γ (4). Therefore, the possibility of NK cells enhancing DC function and subsequent T cell priming during malaria infection should not be excluded.

This study has mostly focused on the effect of NK cells in cerebral malaria pathogenesis through the control of T cell recruitment to the brain. NK cells are versatile lymphocytes exerting several different functions and with the present data, a direct role for NK cells in cytotoxic-mediated pathogenesis cannot be excluded. Perforin+ mice have been shown to be resistant to *P. berghei*-induced cerebral malaria (33) and NK cells are known to exert cytotoxic activity using this pathway (1). Moreover, brain microvascular endothelial cells from cerebral malaria susceptible mouse strains have been shown to express low levels of MHC I (53). Down-regulation of MHC I expression levels represents the main signal to induce cytolysis by activated NK cells (11).

In previous work, we showed that BALB.B6-Cmv1™ congenic mice (18), in which the NK cell region from C57BL/6 mice has been introduced in the resistant BALB/c background, are significantly more susceptible than BALB/c animals to *P. berghei*-mediated cerebral malaria (19, 20). Anti-asialo GM1 depletion experiments conducted in that genetic background did not reverse the susceptibility of those mice to cerebral malaria (20). In that mouse strain, NK cells are the main NK-cell expressing cell population contributing to malarial pathogenesis. However, NK cells appeared to be crucial determinants of malarial pathogenesis in wild-type C57BL/6 animals. It has been proposed that the repertoire of Ly49 receptors expressed by NK cells is influenced by the MHC I expression in the host (54, 55), suggesting that NK cells need to “adapt” to the MHC I environment in which they develop. It is therefore possible that the expression levels of the activity of some NK receptors in NK cells from H-2Db-expressing BALB.B6-Cmv1™ mice, differs from that normally found in malaria-susceptible C57BL/6 animals (H-2Dd), explaining the differential immunological behavior of NK cells in wild-type and congenic mouse strains during malaria. It is then reasonable to postulate the testable hypothesis that during malaria, NK cell activity is influenced by the expression of MHC I. We are currently addressing this proposition using BALB.b.B6-Cmv1™ mice, which is a strain on a BALB/c background congenic for both the NKC and MHC I receptors from C57BL/6 mice. Preliminary experiments suggest that NK cell-depletion has a protective effect against disease in the double congenic mouse strain.

CXCR3 is a G protein-coupled chemokine receptor expressed mainly in activated Th1 lymphocytes and NK cells. CXCR3 and IP-10 have been shown to play essential roles in leukocyte recruitment in many proinflammatory conditions including multiple sclerosis (56, 57), myasthenia gravis (58), allograft rejection (59), rheumatoid arthritis (60), experimental autoimmune encephalomyelitis (61), and pulmonary alveolitis in immune-compromised patients (62, 63). Moreover, experimental evidence indicates that interactions between CXCR3 and IP-10 are largely responsible for trafficking of activated T cells to the site of inflammation inside the
CNS (64, 65). Consistent with those observations, lack of T cell recruitment to the brain of malaria-infected animals after NK cell depletion correlated with down-regulation of CXCR3 expression and a dramatic reduction of T lymphocytes migrating in response to IP-10, suggesting that this trafficking pathway also plays an important role in lymphocyte migration leading to cerebral malaria pathogenesis and fatalities. Moreover, CXCR3−/− mice were found to be significantly more resistant to P. berghei ANKA-mediated cerebral malaria than wild-type controls (J. Miu and N. Hunt, unpublished observation). The understanding of chemokine networks responsible for migration of pathogenic cells to target organs could be informative in developing new therapeutic approaches to alleviate proinflammatory diseases (66). In fact, some networks responsible for migration of pathogenic cells to target organs. Our results here open the possibility in developing anti-inflammatory trafficking strategies designed to prevent local inflammation in the brain and cerebral malaria disease.

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Disclosures
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References

The Journal of Immunology 5787

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