CXC Chemokine Receptor-2 Ligands Are Required for Neutrophil-Mediated Host Defense in Experimental Brain Abscesses

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CXC Chemokine Receptor-2 Ligands Are Required for Neutrophil-Mediated Host Defense in Experimental Brain Abscesses

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We have developed a mouse brain abscess model by using *Staphylococcus aureus*, one of the main etiologic agents of brain abscesses in humans. Direct damage to the blood-brain barrier was observed from 24 h to 7 days after *S. aureus* exposure as demonstrated by the accumulation of serum IgG in the brain parenchyma. Evaluation of brain abscesses by immunohistochemistry and flow cytometry revealed a prominent neutrophil infiltrate. To address the importance of neutrophils in the early containment of *S. aureus* infection in the brain, mice were transiently depleted of neutrophils before implantation of bacteria-laden beads. Neutrophil-depleted animals demonstrated more severe brain abscesses and higher CNS bacterial burdens compared with control animals. *S. aureus* led to the induction of numerous chemokines in the brain, including macrophage-inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL1, monocyte chemoattractant protein-1/CCL2, and TCA-3/CCL1, within 6 h after bacterial exposure. These chemokines were expressed by both primary cultures of neonatal mouse microglia and astrocytes exposed to heat-inactivated *S. aureus* in vitro. Because neutrophils constitute the majority of the cellular infiltrate in early brain abscess development, subsequent analysis focused on MIP-2 and KC/CXCL1, two neutrophil-attracting CXC chemokines. Both MIP-2 and KC protein levels were significantly elevated in the brain after *S. aureus* exposure. Neutrophil extravasation into the brain parenchyma was impaired in CXCR2 knockout mice and was associated with increased bacterial burdens. These studies demonstrate the importance of the CXCR2 ligands MIP-2 and KC and neutrophils in the acute host response to *S. aureus* in the brain. *The Journal of Immunology*, 2001, 166: 4634–4643.

Brain abscesses represent a significant medical problem, accounting for 1 in every 10,000 hospital admissions in the United States (1). The end result of a brain abscess can include the replacement of the abscessed area with a fibrotic scar, loss of brain tissue by surgical removal, and/or abscess rupture and death. The most common etiologic agents of brain abscesses in humans are the *Streptococcal* strains and *Staphylococcus aureus* (1, 2). We have developed an experimental brain abscess model in the mouse by using *S. aureus*, which allows for dissection of the basic cellular and immunological mechanisms involved in the progression and resolution of brain abscesses. Previous studies have established that the rat brain abscess model closely mimics human disease (3). For example, both progress through a series of well-defined inflammatory stages (2, 3).

*S. aureus* induces the temporal influx of leukocytes into the CNS during the course of an evolving brain abscess (3). However, the chemokines induced after infection and their functional significance in vivo remain largely undefined. Previous studies have examined a select subset of chemokines in the rat brain abscess model (4). The objective of this study was to perform a comprehensive analysis of chemokine expression during the acute phase of brain abscess formation in the mouse and to determine the functional importance of chemokines in neutrophil recruitment into the brain in vivo.

Chemokines represent a family of small-molecular weight chemotactic cytokines, classified into groups based on the presence and position of conserved cysteine residues (5–7). These mediators play an important role in recruiting leukocytes into areas of active inflammation. They are synthesized locally at sites of developing inflammation and establish a concentration gradient to which target cell populations migrate. The CXC chemokine family is subdivided based on the presence or absence of a 3-aa ELR (glutamic acid-leucine-arginine) motif. The mouse ELR-containing chemokines KC (rodent homologue of growth related oncogene-α/CXCL1), and macrophage inflammatory protein (MIP)1-2 (MIP-2/CXCL1) have potent neutrophil chemotactic activity and are also angiogenic (6, 8, 9). The sole receptor for MIP-2 and KC in the mouse is CXCR2, which is expressed on neutrophils, basophils, and epithelial cells (10). Both MIP-2 and KC have been shown to play a critical role in several infectious disease models including invasive pulmonary aspergillosis (11), *Norcardia asteroides* infections (12), and *Pseudomonas aeruginosa*-induced pneumonia (13). Neutrophils from CXCR2-deficient mice (14) fail to respond to MIP-2 or KC in vivo but are still sensitive to chemotaxis induced by CSa (15). Members of the CC chemokine family are chemotactic for monocytes and T cells and include such members as MIP-1α/CCL3, monocyte chemoattractant protein-1 (MCP-1/CCL2), and RANTES/CCL5.

In this study, we have characterized the importance of neutrophils and the CXCR2 ligands MIP-2 and KC in the host response...
to *S. aureus* in the brain. Depletion studies revealed that neutrophils are required for containing bacterial burdens and minimizing tissue damage to surrounding normal brain parenchyma. *S. aureus* was found to induce the rapid and sustained expression of numerous chemokines in the brain, including MIP-2 and KC. The importance of MIP-2 and KC in neutrophil recruitment into brain abscesses in vivo was demonstrated in CXCR2 knockout mice. Neutrophil extravasation into the brain parenchyma was impaired in CXCR2 knockout mice and was associated with increased bacterial burdens. The data presented here demonstrate the importance of MIP-2, KC, and infiltrating neutrophils during the acute phase of experimental brain abscess development.

**Materials and Methods**

**Mouse strains**

AKR/J, CXCR2 knockout, and homozygous wild-type littermates 6–8 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Pregnant AKR/J females also were obtained from The Jackson Laboratory for microglia and astrocyte isolation from neonatal mice. The animal use protocol has been approved by the Dartmouth College Institutional Animal Care and Use Committee and is in accord with National Institutes of Health guidelines for the use of rodents.

**Preparation of *S. aureus*-laden agarose beads**

Live *S. aureus* were encapsulated in agarose beads before implantation in the brain as described previously (3, 4). The use of agarose beads prevents bacterial dissemination or rapid wound sterilization by the host. Briefly, *S. aureus* strain RN6390 (a gift of Ambrose Cheung, Dartmouth Medical School, Lebanon, NH) was grown to postexponential phase at 37°C in brain heart infusion medium (Difco, Detroit, MI). A total of 1 × 10^7* S. aureus* bacteria were added to a solution of 1.4% low-melt agarose (type XII; Sigma, St. Louis, MO) at 40°C. The mixture then was added to rapidly swirling heavy mineral oil (Sigma) prewarmed to 37°C, and quickly cooled to 0°C on crushed ice. Beads were washed four times in 1× Dulbecco’s PBS (DPBS) (Mediatech Cellgro, Herndon, VA) to remove mineral oil. Beads with dimensions between 50 and 100 μm, as determined by phase contrast microscopy, were used for implantation into the brain. Control animals were implanted with sterile agarose beads. The bacterial viability or stability of bead preparations was confirmed by overnight culture in Luria-Bertani medium and quantitative culture onto blood agar plates (Becton Dickinson, Franklin Lakes, NJ).

**Generation of experimental brain abscesses**

Mice were anesthetized with avertin, and a 1-cm longitudinal incision was made along the vertex of the skull extending from the ear to the eye, exposing the frontal bone. A burr hole was drilled 1 mm anterior and 1 mm lateral to the frontal suture of the calvarium. A Hamilton syringe fitted with a 23-gauge needle was inserted 1 mm anteriorly until the bone was penetrated. The needle was rapidly withdrawn, and agarose beads were slowly infused 3 mm deep from the frontal bone. A mixture of 10^7 S. aureus or *E. coli* bacteria was added to the agarose beads and rapidly mixed with heavy mineral oil. The mixture then was added to swirling brain heart infusion medium (Difco, Detroit, MI). A total of 1 × 10^7 bacteria in 20 μl was injected into the brain as described previously (3, 4). The use of agarose beads prevents bacterial dissemination or rapid wound sterilization by the host. Briefly, S. aureus strain RN6390 (a gift of Ambrose Cheung, Dartmouth Medical School, Lebanon, NH) was grown to postexponential phase at 37°C in brain heart infusion medium (Difco, Detroit, MI). A total of 1 × 10^7* S. aureus* bacteria were added to a solution of 1.4% low-melt agarose (type XII; Sigma, St. Louis, MO) at 40°C. The mixture then was added to rapidly swirling heavy mineral oil (Sigma) prewarmed to 37°C, and quickly cooled to 0°C on crushed ice. Beads were washed four times in 1× Dulbecco’s PBS (DPBS) (Mediatech Cellgro, Herndon, VA) to remove mineral oil. Beads with dimensions between 50 and 100 μm, as determined by phase contrast microscopy, were used for implantation into the brain. Control animals were implanted with sterile agarose beads. The bacterial viability or stability of bead preparations was confirmed by overnight culture in Luria-Bertani medium and quantitative culture onto blood agar plates (Becton Dickinson, Franklin Lakes, NJ).

**Quantitation of viable bacteria from brain abscesses**

To quantitate the numbers of viable bacteria associated with brain abscesses in vivo, serial 10-fold dilutions of brain abscess homogenates were plated onto blood agar plates (Becton Dickinson). Titer values were calculated by enumerating colony growth and were expressed as CFU per milliliter of homogenate.

**Processing of tissues for immunohistochemistry and protein extraction from brain abscesses**

To prepare tissues for immunohistochemistry, animals were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was removed, postfixed in paraformaldehyde for 30 min, and washed in 0.2 M phosphate buffer, pH 7.4, overnight. Tissues were cryoprotected in 30% sucrose for 24 h and then snap-frozen in OCT for immunohistochemistry.

For ELISA, brain abscess extracts were prepared by disrupting tissues in DPBS supplemented with a complete protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Samples were sonicated, centrifuged at 14,000 rpm for 15 min at 4°C, and stored at −70°C until use.

**Immunohistochemistry**

Frozen sections of fixed tissue were processed for immunohistochemistry by using the avidin-peroxidase method as described previously (16). The following Abs were used for analysis: F4/80 and the anti-neutrophil Ab 7/4 (both from Serotec, Raleigh, NC), anti-GR-1, anti-CD11b, anti-CD45, and anti-class II MHC (BD Pharmingen, San Diego, CA), and the isotype control Abs rat IgG2b (BD Pharmingen) and rabbit IgG (Vector Laboratories, Burlingame, CA). Biotinylated secondary Abs included anti-rat or anti-rabbit IgG (both from Vector Laboratories). Slides were developed by using the substrate 3,3′-diaminobenzidine.

**ELISA**

Murine MIP-2, KC, and IgG levels were quantitated in brain abscess homogenates by using the murine Quantikine M MIP-2 and KC ELISA kits (R&D Systems, Minneapolis, MN), and a standard sandwich ELISA for mouse IgG. MIP-2 and KC ELISAs were performed according to the manufacturer’s instructions (with slight modifications of sensitivity 7.8 and 15.6 ng/ml, respectively). For IgG quantitation, horseradish peroxidase-conjugated goat-anti-mouse IgG was used to assess the effect of neutrophils on abscess development. Peripheral blood smears were performed on each animal before and at days 1, 3, and 5 after Ab injection to monitor neutrophil levels. Animals were euthanized and perfusion-fixed 48 h after *S. aureus* exposure to compare brain abscess pathology between groups. In addition, immunohistochemical staining of abscesses from neutrophil-depleted and control animals was performed to confirm a reduction in neutrophil infiltration in the former.

**Neutrophil depletion studies**

Mice were injected i.p. with either 100 μg of RB6-C85 (anti-GR-1; rat IgG2b) or rat IgG2b isotype control Abs on day 0. Both Abs were obtained in a no azide/low endotoxin form from BD Pharmingen. The following day, animals received an intracerebral inoculation of *S. aureus* to assess the effect of neutrophils on abscess development. Peripheral blood smears were performed on each animal before and at days 1, 3, and 5 after Ab injection to monitor neutrophil levels. Animals were euthanized and perfusion-fixed 48 h after *S. aureus* exposure to compare brain abscess pathology between groups. In addition, immunohistochemical staining of abscesses from neutrophil-depleted and control animals was performed to confirm a reduction in neutrophil infiltration in the former.

**Recovery of cells from brain abscesses and identification by flow cytometric staining**

Brain abscesses were collected from animals at days 3 and 5 after bacterial exposure to recover infiltrating and resident cells. Briefly, mice were perfused intracardially with DPBS to eliminate contamination from peripheral blood leukocytes. Brain abscess tissues were pooled, minced into fine pieces with forceps, and incubated with collagenase type II (1 mg/ml final concentration; Sigma) for 20 min at 37°C. The resulting cell suspension was layered onto a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Piscataway, NJ) to separate myelin debris from cells. The following fluorescently labeled Abs were used for two-color flow cytometric analysis; anti-CD45-PE, anti-CD11b-FITC, and anti-GR-1-PE (all from BD Pharmingen), F4/80-FITC (Serotec), and rat IgG2b isotype control Abs directly conjugated to PE. Normal rat serum was added at 2% to the amount of total protein extracted from brain abscesses to correct for differences in tissue sampling size.

**Isolation of primary astrocytes and microglia**

Astrocytes and microglia were isolated from neonatal AKR/J mouse brains by using a technique exploiting the differential adherence characteristics of...
astrocytes and microglia, as described previously (17). Mice were euthanized with halothane, and brains were removed aseptically and immediately placed in ice-cold DPBS. Meninges were removed and brains homogenized through a vacuum filtration apparatus fitted with a 100-μm nylon mesh. Cells were resuspended in defined medium (DMEM 4, 500 mg/L glucose supplemented with 10% FBS, 200 mM l-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and fungizone) and cultured in 75-cm² flasks for 5–7 days until monolayers reached confluence. During this initial culture period, medium was supplemented with 250 U/ml of GM-CSF (R&D Systems) to allow for microglial expansion. After 7 days, confluent cultures were vigorously agitated on a rotary shaker for 15 h (37°C, 180 rpm) to dislodge microglia and oligodendrocytes. The resulting cell suspension, rich in microglia, was transferred to 75-cm² culture flasks and allowed to adhere at 37°C. After a 3-h adherence interval, loosely adherent cells and cells in suspension (most of which were oligodendrocytes) were removed by gently shaking flasks at room temperature. Astrocytes remained attached to the culture flask after the initial microglia disassociation step. To ensure the elimination of contaminating microglial cells, astrocyte cultures were treated with 1 mM t-leucine methyl ester (Sigma), a lysosomotrophic agent that selectively destroys mononuclear cells, including microglia. The purity of both astrocyte and microglia cell preparations were verified by immunohistochemical staining with anti-glial fibrillary acidic protein (GFAP) and anti-CD11b Abs, respectively. This isolation procedure routinely yielded cell populations with a purity of >95%.

RNA interference assay (RPA)
Chemokine mRNA expression in brain abscess tissues and primary glial cells was examined by RPA with the RiboQuant RPA kit and mCK-5 multiprobe template set (BD PharMingen). The mCK-5 template set contains probes for the housekeeping genes L32 and GAPDH, which serve as internal controls for the assay. Probes were synthesized by using [a-33P]UTP, resulting in an average specific activity of 4 × 10⁶ cpm/μl. RPA was performed according to the manufacturer’s instructions by using 5–10 μg of total RNA per sample. Products were resolved on a 6% acrylamide gel, dried, and exposed to film (Kodak BioMax MR, Rochester, NY).

Statistics
Significant differences between experimental groups were determined by using the Student’s paired t test at the 95% confidence interval.

Results
S. aureus leads to the chronic disruption of the blood-brain barrier (BBB)
The BBB normally functions as a safeguard restricting the entry of most macromolecules and cells into the CNS. However, during inflammation, vasoactive mediators and cytokines compromise the integrity of the BBB. To determine whether S. aureus infection results in a transient or chronic disruption of the BBB, the leakage of serum IgG into the brain parenchyma was quantified in brain abscesses by ELISA. As shown in Fig. 1, BBB permeability was significantly elevated in those animals receiving S. aureus. Evidence of BBB dysfunction persisted to 7 days after bacterial exposure, the last time point assessed, and most probably continues for a more extended period. However, the amount of IgG in brain abscesses at later periods may also reflect local Ab production by infiltrating plasma cells which are detected at day 10 after bacterial exposure (3). IgG levels in brain tissue of animals implanted with sterile beads were negligible, indicating that the stab wound created by injections does not significantly alter BBB permeability (Fig. 1). The chronic disruption of the BBB correlates with the continued influx of inflammatory cells observed in evolving brain abscesses.

S. aureus induces the influx and accumulation of neutrophils in brain abscesses
Pyogenic bacteria, such as S. aureus, induce the migration and accumulation of neutrophils in peripheral tissues. Previous studies in the rat brain abscess model have demonstrated the rapid influx of neutrophils in response to S. aureus (Ref. 3 and T.K., unpublished observations). To demonstrate whether S. aureus elicits a similar response in the mouse brain abscess model, we examined neutrophil influx into abscesses by using immunohistochemistry. As shown in Fig. 2, brain abscesses were associated with a prominent neutrophil infiltrate as demonstrated by staining with the neutrophil-specific Abs 7/4 (Fig. 2) and anti-GR-1 (data not shown). Infiltrating neutrophils were localized within the necrotic abscess and in the surrounding normal brain parenchyma. The kinetics of neutrophil influx into the brain was rapid, with cells easily detected by 24 h after S. aureus exposure (data not shown). Evidence of microglial and astrocyte activation within abscesses was demonstrated on the basis of immunohistochemical staining for CD11b and F4/80, and ICAM-1 and GFAP, respectively (Fig. 2).

To quantify the percentage of neutrophils associated with brain abscesses in vivo, cells were isolated from brain abscesses and evaluated by two-color flow cytometry. As shown in Fig. 3, neutrophils (CD45<sup>high</sup>, CD11b<sup>+</sup>, GR-1<sup>+</sup>) were the predominant cell type, representing >90% of the cells recovered from brain abscesses at day 5 after bacterial exposure. The relative numbers of neutrophils compared with resident microglia and infiltrating macrophages precluded the detection of the latter populations in our analysis. These findings, in combination with those obtained by using immunohistochemistry, demonstrate that neutrophils are the predominant cell type associated with brain abscesses in vivo.

Neutrophils play a pivotal role in the host immune response to S. aureus in the brain
Neutrophils constitute the majority of infiltrating leukocytes during the acute phase of brain abscess development. In addition to bacterial neutralization, neutrophils have the ability to mediate tissue injury through the release of proteolytic enzymes and production of reactive oxygen intermediates. To assess the importance of neutrophils in the host immune response to S. aureus in the brain, mice were treated with the mAb RB6-C85, which has been used extensively for in vivo neutrophil depletion studies (18–21). As shown in Fig. 4B, RB6-C85-treated mice had significantly fewer circulating neutrophils one day after Ab administration (p < 0.001), whereas neutrophil levels in animals receiving an isotype-matched control Ab remained unaffected. Intracerebral inoculation of S. aureus resulted in a prominent neutrophilia in control animals, whereas neutrophil numbers remained significantly depressed in RB6-C85-treated animals (p < 0.05; Fig. 4B). These
findings confirmed the reduction in circulating neutrophils in RB6-C85-treated mice throughout the duration of the experiment. Brain abscesses were collected from isotype control- or RB6-C85-treated animals 48 h after S. aureus exposure and compared for differences in pathology. As shown in Fig. 4A, abscesses were more severe in neutrophil-depleted animals as typified by more extensive tissue necrosis and loss of normal brain architecture. Numerous extracellular bacterial foci were detected in abscesses from RB6-C85-treated animals, a phenomena that is never observed in immune-competent animals (Fig. 4A, arrows). The importance of neutrophils in controlling bacterial replication in the brain was evaluated by comparing bacterial burdens in RB6-C85-treated and control mice. As shown in Fig. 5, neutrophil-depleted animals had significantly higher bacterial burdens at day 5 after infection compared with control mice with titers >1 log in the former. Immunohistochemistry confirmed that abscesses in RB6-C85-treated mice had fewer infiltrating neutrophils (data not shown). During the acute phase of infection, neutrophils appear critical for containing bacterial levels and preventing widespread necrosis of the surrounding normal brain parenchyma.

S. aureus induces rapid chemokine expression in the brain

Leukocytes infiltrate brain abscesses in a temporal fashion, with neutrophils being the first to appear followed by monocytes/macrophages (3). To investigate the potential signal(s) involved in leukocyte recruitment into brain abscesses in vivo, we examined the kinetics of chemokine induction immediately after S. aureus exposure in the brain by RPA. As shown in Fig. 6, numerous chemokines were induced within 6 h after S. aureus exposure, including MIP-2, IP-10/CXCL10, MIP-1α and β, MCP-1, and TCA-3. The induction of MIP-2, a potent CXC neutrophil chemoattractant, preceded the influx of neutrophils into brain abscesses that occurs within 24 h after bacterial exposure. Likewise, the expression of MIP-1α and β, MCP-1, and TCA-3 preceded the accumulation of monocytes into brain abscesses that occurs at 4 days after S. aureus exposure. Importantly, none of these mediators were detected in normal mouse brain (Fig. 6). Previous studies have established that the implantation of sterile beads induces negligible and transient chemokine expression in the brain (4). These findings demonstrate that S. aureus stimulates chemokine expression in the brain, which likely serves as a signal to recruit leukocytes into brain abscesses in vivo.

S. aureus induces chemokine expression by primary microglia and astrocytes in vitro

The rapidity at which chemokine production was induced in response to S. aureus in the brain suggested that these chemotactic moieties may be synthesized early in infection by resident glial cells as opposed to newly recruited hematogenous cells. This was supported by the finding that chemokine expression is detected in early brain abscesses before the accumulation of infiltrating leukocytes. As shown in Fig. 7, both primary microglia and astrocytes rapidly expressed numerous chemokines in response to S. aureus.
in vitro, reflecting their ability to recognize and respond to bacterial components. Microglia were the major source of MIP-1α, whereas astrocytes were the major producers of RANTES (Fig. 7). Both cell types expressed equivalent levels of MIP-1β, MCP-1, and TCA-3. These products were not detected in unstimulated microglia or astrocytes (Fig. 7). These results indicate that microglia and astrocytes express numerous chemokines in response to *S. aureus* and may serve as the source for mediator production during

**FIGURE 3.** Neutrophils constitute the majority of the cellular infiltrate in experimental brain abscesses. Mice were implanted with *S. aureus*-encapsulated agarose beads as described in Materials and Methods. Animals were euthanized at day 5 after injection and perfused intracardially with DPBS. Cells were recovered from brain abscess tissues as described in Materials and Methods and stained with directly conjugated Abs against CD45, CD11b, or GR-1 to identify neutrophils. Cells were incubated with isotype control Abs (IgG2b-FITC and IgG2b-PE) to account for nonspecific staining. Percentages of cells are denoted in each dot plot quadrant. Results are representative of three independent experiments.

**FIGURE 4.** Neutrophils play an important role in the host response to *S. aureus* in the brain. Mice were treated with either 100 μg of RB6-C85 or isotype control Abs at day 0. The following day, animals were implanted with *S. aureus*-encapsulated agarose beads and euthanized 48 h later to compare brain abscess severity. A, Hematoxylin and eosin-stained sections of brain abscesses from animals treated with isotype control (I.C.) or RB6-C85 Abs. Abscesses from isotype control-treated animals had a significant neutrophil infiltrate, whereas those from RB6-C85-treated mice exhibited severe necrosis of the affected brain parenchyma, typified by few infiltrating neutrophils. Arrows denote extracellular *S. aureus* aggregates. B, Quantitation of peripheral blood neutrophils in isotype control- and RB6-C85-treated mice. Results are representative of two independent experiments.
the early phase of experimental brain abscess pathogenesis before inflammatory cell recruitment.

The CXCR2 ligands MIP-2 and KC are strongly induced after *S. aureus* exposure in the brain

Because neutrophils play a pivotal role in the host response to *S. aureus* in the brain, we narrowed our analysis to examine two neutrophil-attracting CXC chemokines, MIP-2 and KC. As shown in Fig. 8, *S. aureus* induced the rapid and sustained expression of both MIP-2 and KC in the brain. In contrast, the implantation of sterile beads had no effect, demonstrating that neither the stab wound itself nor the deposition of foreign material (agarose beads) into the brain stimulates MIP-2 or KC expression (Fig. 8). MIP-2 production remained elevated through day 7 after *S. aureus* exposure, whereas KC displayed different kinetics, with peak levels detected at 2 days. These findings demonstrate that a Gram-positive organism, such as *S. aureus*, induces the rapid and sustained expression of MIP-2 and KC in the brain, which probably participates in neutrophil recruitment into areas of acute cerebritis destined to become an abscess.

CXCR2 knockout mice exhibit an impairment in neutrophil extravasation and enhanced bacterial burdens in the brain

To demonstrate the importance of MIP-2 and KC in neutrophil recruitment in vivo, we examined the pathogenesis of *S. aureus*-induced abscesses in CXCR2 knockout mice. As shown in Fig. 9, neutrophil extravasation into the brain parenchyma was significantly impaired in CXCR2 knockout animals. The majority of neutrophils in CXCR2 knockout mice were sequestered within small blood vessels in infected brain tissue; this is most likely attributable to their inability to respond to MIP-2 and KC (Fig. 9B, arrows). Both MIP-2 and KC protein were detected in brain abscesses from CXCR2 knockout mice (Fig. 10), indicating that the defect in neutrophil recruitment was not a result of impaired chemokine production. The observed defect in neutrophil recruitment also had an effect on *S. aureus* survival in the brain. As shown in Fig. 11, bacterial burdens were higher in brain abscesses from...
CXCR2 knockout compared with wild-type littermates at day 5 after infection, suggesting that the ability to control bacterial replication is compromised in the former. Therefore, there exists an important role for the CXCR2 ligands MIP-2 and KC in neutrophil recruitment into experimental brain abscesses.

Discussion
Bacterial infection of the brain parenchyma confronts the host with a rapidly evolving, life-threatening challenge. The significance of understanding the immune response to pyogenic bacteria such as *S. aureus* in the brain is underscored by the fact that these CNS infections continue to occur despite advances made in detection and therapy. However, the pathogenesis of this potentially lethal malady and the details of the brain’s response to it, remain largely unknown. This report demonstrates the critical role of neutrophils and the CXC chemokines MIP-2 and KC in the containment and neutralization of bacteria in brain abscesses.

In addition to bacterial neutralization, neutrophils have the potential to induce normal tissue damage through the release of proteolytic enzymes and production of reactive oxygen intermediates. Indeed, previous studies have demonstrated that limiting neutrophil influx into the CNS provides some benefit in situations where inflammation accompanies CNS disease (22–24). This study examined the importance of neutrophils in brain abscess development. Experiments that transiently depleted mice of this leukocyte population demonstrated convincingly that neutrophils are critical for restricting bacterial numbers in the brain parenchyma. Neutrophil-depleted mice exhibited numerous extracellular bacterial foci and higher bacterial burdens compared with immunocompetent animals. Similar results demonstrating the vital role of neutrophils

FIGURE 8. *S. aureus* induces the rapid and sustained expression of MIP-2 and KC in the brain. Mice were implanted with *S. aureus*-encapsulated or sterile beads as described in Materials and Methods. Brain abscess homogenates were prepared at the indicated time points and analyzed for MIP-2 (A) and KC (B) protein expression by ELISA. Chemokine concentrations are normalized to the amount of total protein recovered and are reported as the amount of MIP-2 or KC (nanograms) per milligram of total protein. Significant differences are denoted with asterisks (*, *p* < 0.05). Results are representative of two independent experiments.

FIGURE 9. Impairment in neutrophil extravasation into the brain parenchyma in CXCR2 knockout mice. CXCR2 knockout and homozygous wild-type littermates (control) were implanted with *S. aureus*-encapsulated agarose beads as described in Materials and Methods. Animals were euthanized at 4 days after bacterial exposure and perfusion-fixed to collect brain abscesses for immunohistochemical analysis. Brain abscesses from control (CXCR2 wild-type) and CXCR2 knockout mice were stained with the neutrophil-specific Ab GR-1. Arrows denote small vessels congested with neutrophils in CXCR2 knockout mice. Note the paucity of neutrophils infiltrating the brain parenchyma of CXCR2 knockout mice. Magnifications, ×22.5 (A) and ×100 (B). Results are representative of two independent experiments.
restricting bacterial replication in the brain were obtained by using CXCR2 knockout mice. Neutrophil infiltration into brain abscesses was compromised in CXCR2 knockout mice, which correlated with higher bacterial burdens compared with wild-type littermates. Increased bacterial burdens in abscesses from neutrophil-depleted and CXCR2 knockout mice were evident only at later periods after bacterial exposure. In the first few days after infection, there were no significant differences in the number of bacteria associated with neutrophil-depleted or CXCR2 knockout and control animals. This finding suggests that *S. aureus* replication in the brain is initially controlled but with time, organisms overwhelm the meager immune response elicited in neutrophil-depleted and CXCR2 knockout animals. In this brain abscess model, bacteria are contained within agarose beads during the initial stages of infection. Over time, bacteria replicate and escape from the beads, allowing them to become accessible for phagocytosis (T.K., unpublished observations). It is probable that activated microglia contain bacterial levels initially; however, without assistance from infiltrating neutrophils, *S. aureus* replication soon overwhelms this response. This explanation would account for the inability to detect differences in bacterial burdens between neutrophil-depleted/CXCR2 knockout and control animals at early time points after bacterial exposure.

In addition to containing bacterial burdens, neutrophils were found to be important in minimizing tissue damage during infection. For example, the severity of brain abscesses was markedly enhanced in neutrophil-depleted animals compared with control animals. This finding implies that bacteria and their products directly contribute to tissue damage in the brain. We are currently examining the roles of virulence factors produced by *S. aureus* in brain abscess pathogenesis. Enhanced abscess severity in neutrophil-depleted animals agrees with a previous report by Lo et al. (25) examining the host response during acute cerebritis. However, Lo et al. found no evidence of extracellular bacteria in lesions from neutrophil-depleted animals, as was found in this study. This discrepancy may result from differences in the duration of neutrophil depletion. In the study by Lo et al., neutrophil levels returned to baseline within 30 h, whereas we achieved depletion for an extended period of time (>3 days), similar to results obtained from other groups that used the RB6-C85 Ab (11, 21). Importantly, the present work extends these findings to evaluate chemokine expression and the functional role of neutrophils in bacterial containment in the brain.

This study demonstrates a pivotal role for the CXCR2 ligands MIP-2 and KC in neutrophil recruitment into experimental brain abscesses. Strikingly, the majority of neutrophils in CXCR2 knockout mice were sequestered within small blood vessels surrounding the lesion, most probably because of their inability to respond to MIP-2 and KC. This finding indicates that there is either a defect in tight adhesion or extravasation but not in the localization of neutrophils to vascular endothelium in brain abscesses from CXCR2-deficient mice. Leukocyte extravasation requires a series of three or more steps (26). First, leukocyte rolling occurs along the vascular endothelium, a loosely adhesive event that is selectin-mediated. The next step requires an activation signal to stimulate tight adhesion to the endothelium that is integrin-mediated. This activation signal is often provided by the binding of chemokines decorated on the endothelial cell surface to chemokine receptors expressed on the leukocyte. If so, this cannot occur in CXCR2 knockout mice because of their lack of a functional receptor. Therefore, although the CXCR2 ligands MIP-2 and KC are present in the brain parenchyma, neutrophils are unable to respond, and...
hence the subsequent activation-dependent step of leukocyte emigration cannot occur. The result is that neutrophils can marginate but not extravasate. Interestingly, the expression of MIP-2 and KC in brain abscesses were increased 10-fold in CXCR2 knockout mice compared with wild-type littermates. However, the levels of MIP-2 detected in abscesses from CXCR2 knockout mice were only slightly elevated compared with those detected in AKR/J mice (Fig. 8), suggesting that this increase may represent normal variation. Elevated KC expression in brain abscesses from CXCR2 knockout mice was unexpected, and currently there is no reasonable explanation to account for this finding. One possibility is that CXCR2 engagement stimulates an autocrine negative feedback loop to attenuate further chemokine expression in normal animals, and that this mechanism is defective in CXCR2 knockout mice because of the lack of a functional receptor. However, to date there is no experimental evidence to support this possibility. The relative paucity of neutrophils in the brains of CXCR2 knockout mice indicates that CXCR2 ligands are the major chemotactic signals required for neutrophil influx into brain abscesses and that their activity cannot be substituted by alternative chemotactic factors such as complement split products (C3a, C5a), PGs, leukotrienes, or other chemokines.

In vitro both primary microglia and astrocytes elaborated numerous chemokines in response to heat-inactivated *S. aureus*. However, the receptors these glial cells use to recognize and respond to *S. aureus* remain to be fully defined. Microglia express CD14 (27), a receptor for LPS, which has also been implicated in mediating responses to Gram-positive bacteria and cell wall products (28–30). However, a recent report demonstrated that microglial activation in response to *Streptococcus pneumoniae*, a Gram-positive organism, was CD14-independent (31). In addition, microglia express Toll-like receptor 2 (T.K., unpublished observations), a pattern recognition receptor implicated in the innate immune response to Gram-positive bacteria (32–36). Further investigation of CD14 and Toll-like receptor 2 in bacterial-dependent microglial activation is required. In contrast, the receptor(s) involved in the recognition and responses to *S. aureus* in astrocytes remain unknown because these cells are not classically considered part of the innate immune system. It is possible that other CNS resident cells such as cerebral endothelial cells and perivascular microglia also contribute to the initial synthesis of chemokines immediately after *S. aureus* exposure. Perivascular cells are known to respond rapidly to LPS as evidenced by their elaboration of cyclooxygenase II for eicosanoid synthesis within a few hours of LPS exposure (37). However, the ability of these cells to express chemokines in the context of early brain abscesses has not been examined.

Beyond its neutrophil-attracting activity, MIP-2 may exert additional effects on the surrounding brain parenchyma. MIP-2 has been shown to induce angiogenesis in a model of bleomycin-induced lung injury (38). It is interesting to note that as a brain abscess evolves, the developing wall is associated with a significant vascular response (Ref. 3 and T.K., unpublished observations). Certainly, the development of a collag enous, highly vascular envelope around a site of infection in an organ that responds to virtually every other type of insult with astrocytic reaction and gliosis, remains one of the enigmas in the pathogenesis of brain abscesses. It is possible that MIP-2 contributes to the angiogenic response associated with the developing abscess wall. Further evidence to suggest this was demonstrated by the continued expression of MIP-2. Significant levels of MIP-2 were still detected at 7 days after bacterial exposure that preceded the appearance of the vascular response observed at day 10. Further studies are needed to define the potential role of MIP-2 in vascularization of the brain abscess wall.

Recently, it was demonstrated that *S. aureus* can survive intracellularly in neutrophils (39), revealing another mechanism by which this organism can evade the host immune response and establish chronic infections. We have demonstrated the intracellular survival of *S. aureus* within cells recovered from brain abscesses; however, the identity of those cells harboring organisms remains to be determined (T.K., unpublished observations). Yet this finding raises an important issue concerning neutrophil recruitment in brain abscesses. It is evident that neutrophils are required to contain bacterial burdens. However, the ensuing chemokine response and subsequent influx of neutrophils needs to be tightly regulated to prevent the induction of an overactive immune response. Dysregulated neutrophil recruitment into brain abscesses could lead to more extensive tissue damage and provide an avenue to establish a chronic infection through the ability of *S. aureus* to survive intracellularly within these newly recruited neutrophils. Alternatively, another cell type in the brain could harbor *S. aureus*, leading to the establishment of chronic infection. The sustained expression of MIP-2 and KC observed in this study may contribute to the severity of brain abscesses through the aforementioned mechanisms.

The experiments reported here demonstrate the activation of a potent innate immune response in the brain after exposure to *S. aureus* and reveals the importance of the CXCR2 ligands MIP-2 and KC and neutrophils in the early immune response to bacteria in the CNS. It is likely that these findings may extend to other bacterial infections in the CNS induced by pyogenic organisms.

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References


