The American Journal of Pathology, Vol. 172, No. 1, January 2008

DOI: 10.2353/ajpath.2008.070567

Neurobiology

Both TLR2 and TLR4 Are Required for the Effective Immune Response in *Staphylococcus aureus*-Induced Experimental Murine Brain Abscess

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Toll-like receptors (TLRs) play central roles in the innate reaction to bacterial products and transmit specific immune responses against these pathogens. TLRs are expressed on numerous cell types, including innate immune cells, and on astrocytes, neurons, and microglial cells of the central nervous system (CNS). Lipoproteins and lipopolysaccharides are specifically recognized by TLR2 and TLR4, respectively. We examined the *in vivo* role of TLR2 and TLR4 in *Staphylococcus aureus*-induced brain abscess. Phenotypically, 87% of TLR2$^{-/-}$ mice and 43% of TLR4$^{-/-}$ mice died whereas all wild-type (WT) mice recovered. Clearance of bacteria from the CNS was significantly delayed in TLR2$^{-/-}$ mice compared with TLR4$^{-/-}$ and WT animals. Recruitment of granulocytes and macrophages to the CNS, as well as microglial activation and expansion, was up-regulated in TLR2$^{-/-}$ mice. Although inflammation persisted especially in the CNS of TLR2$^{-/-}$ mice, but also of TLR4$^{-/-}$ mice, WT mice terminated the infection more effectively. Collectively, these data show that the immune response to experimental *S. aureus*-induced brain abscess depends crucially on the recognition of *S. aureus* by TLR2 but that TLR4 is also required for an optimal intracerebral immune response in this disorder. *(Am J Pathol 2008, 172:132–145; DOI: 10.2353/ajpath.2008.070567)*

Toll-like receptors (TLRs) are germline-encoded receptors that recognize microbial pathogens.[1–3] As pattern recognition receptors, they mediate innate first-line defense mechanisms closely connected to the adaptive immune system.[4,5] TLR activation can lead directly to antimicrobial killing but may also regulate the ensuing immune response by inducing the release of cytokines, production of reactive oxygen intermediates, adhesion molecules, caspase recruitment, and further effector mechanisms,[6,7] all of which have been shown to play major roles in experimental brain abscess.[8,9] These reactions are destined to eliminate the pathogen and, thereafter, terminate the immune response but may also trigger possible collateral damage to the central nervous system (CNS) in case of deregulation.[8,9]

So far, 12 different TLRs have been identified in mice (10 in humans), each specifically mediating the immune response to a wide set of molecular motifs derived from microbial ligands.[5,10–12] Whereas the heterodimer TLR2 recognizes ligands such as lipopeptides, lipoproteins, zymosan, heat shock proteins HSP60 and HSP70, peptidoglycan, and others, the homodimer TLR4 recognizes mainly lipopolysaccaride of Gram-negative bacteria, in conjunction with CD14 and MD2, but also host ligands like fibronectin, fibrinogen, and β-defensin, which are nonspecifically released in situations of tissue damage.[13,14] Molecular mechanisms underlying the specific ligand-receptor binding and the subsequent host immune response have not been elucidated for many pathological conditions. TLRs are expressed on many cell types and in the CNS of mice and men, a broad spectrum of TLR expression on astrocytes, oligodendrocytes, microglial cells, and neurons was identified recently.[15–19] Astrocytes express both surface and intracellular TLR3 and

Supported by the Else Kröner-Fresenius Stiftung (grant no. P12/06// A82/05 to W.S.).

Accepted for publication September 13, 2007.

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TLR4, and oligodendrocytes primarily express TLR2 and TLR3 on their cell surface, whereas microglia express cell surface TLR 2,[18] whereas TLR3 and TLR4 expression is restricted to intracellular vesicles in the latter.[15–19]

Brain abscess is an infectious disease of the CNS associated with a high mortality despite modern therapeutics with *Staphylococcus aureus* being the most frequent and important pathogen of traumatic brain abscess.[20,21] In murine *S. aureus*-induced brain abscess, a plethora of cytokines and chemokines is produced, including interleukins (IL)-1α, IL-1β, IL-6, IL-10, and IL-12p40; tumor necrosis factor (TNF); interferon-γ (IFN-γ); monocyte chemoattractant protein-1α; macrophage inflammatory proteins-1, -1β, and -2; regulated on activation normal T cell expressed and secreted; and inducible nitric-oxide synthase (iNOS).[9,22–24] Functional analysis has shown that TNF and IL-10 are important to eliminate the bacterium and to resolve the inflammation. In addition to inflammatory leukocytes recruited to the CNS, brain resident cell populations, especially microglia and astrocytes, play central roles in the control of this infectious CNS disorder.[8,9,25]

The precise involvement of different TLRs in brain abscess has not yet been defined *in vivo*. However, some recent data from *in vitro* research illustrate that TLR2 plays a pivotal role in recognition of peptidoglycan from *S. aureus* in microglial cells[26] and that TLR2 was required for a maximal cytokine response of primary astrocytes stimulated with peptidoglycan and *S. aureus*.[27] Additionally, Kielian et al[28] have postulated that, because of similar mortality rates, bacterial titers, and blood-brain barrier permeability after *S. aureus*-induced brain abscess in TLR2$^{-/-}$ and wild-type (WT) mice, alternative pattern recognition receptors may play a role. However, early publications on the role of TLR2 in recognition of *S. aureus* after systemic infection underscore the importance of dose dependency in detecting a significant phenotypic reaction pattern.[29] The role of TLR4 in brain abscess has not yet been defined, and thus far, rare reports have pointed to a possible involvement of TLR4 in pneumococcal infection *in vitro*, but *in vivo* studies have not yet been published.[30,31]

In this study, we characterized the *in vivo* roles of TLR2 and TLR4 in experimental *S. aureus*-induced brain abscess. The analysis of TLR2$^{-/-}$ and TLR4$^{-/-}$ mice revealed that TLR2 and, importantly, TLR4 are important for survival and regulate an effective clearance of the bacterium from the brain and an appropriate cytokine and chemokine production leading to coordinated leukocyte recruitment. The data presented illustrate that in the CNS, specific factors may determine the functional importance of different TLRs in cerebral infections.

Materials and Methods

Animals and Induction of Brain Abscess

Age- and sex-matched TLR2$^{-/-}$ mice and TLR4$^{-/-}$ mice on a C57BL/6 $\times$ 129 SV background[13,32] and homozygous C57BL/6 $\times$ 129SV WT mice (n = 60 per group) were used for the experiments. All animals were housed in an isolation facility throughout the breeding period and the experiments. The local ethics committee approved the experiments.

Brain abscess was stereotaxically induced as described previously.[9,25] Mice of the three genotypes, which had received a stereotaxic injection of sterile agarose beads, and uninfected mice of either strain served as controls. The intracerebral bacterial load was evaluated by plating aliquots of whole brain tissue homogenates, which were obtained from animals that had received an i.c. injection of either *S. aureus*-laden or sterile beads, on tryptose soy agar and cultivated subsequently at 37°C for 24 hours.

Clinical Assessment

Throughout the experiment, animals were examined to determine the clinical disease activity on days 1, 3, 7, 14, and 21 after infection (p.i.), with regard to motility, uptake of food and water, weight, fur status, presence of neurological deficits, and manifestation of seizures according to an assessment scale described previously.[9,25] Scoring of animals for signs of clinical disease was performed in a blinded manner.

Experimental Tissue Processing and Immunohistological Staining

On the indicated days p.i., *S. aureus*-infected TLR2$^{-/-}$ mice, TLR4$^{-/-}$ mice, and WT mice, or mice injected with sterile beads and uninfected mice, were perfused intracardially with 0.9% saline under deep isoflurane (Isofluran-Baxter; Baxter, München, Germany) anesthesia. The brains of the animals were removed, mounted on thick filter paper with Tissue Tek O.T.C. compound (Miles Scientific, Naperville, IL), snap-frozen in isopentane (Fluka, Neu-Ulm, Germany), precooled on dry ice, and stored at −80°C.

For immunohistochemistry, 10-μm frozen sections were prepared. The following rat anti-mouse monoclonal antibodies were obtained as hybridomas from the American Type Culture Collection (Manassas, VA) were used for staining procedures: CD4 (clone G.K.1.5), CD8 (clone 2.43), CD45 (LCA, clone M1/9.3.4.HL.2), F4/80, major histocompatibility complex (MHC) class I (clone M 1/42.3.9.8 HLK), MHC class II (I-A^{b,d,q} haplotypes, clone M5.114.15.2), and Ly6-G (GR1) (clone RB6–8C5). In addition, iNOS rabbit anti-mouse serum (Alexis, Grünberg, Germany) and rabbit anti-active caspase-3 (BD Pharmingen, Heidelberg, Germany) were used. Double immunofluorescence stainings were performed by use of iNOS or activated caspase-3 antiserum followed by biotinylated goat-anti-rabbit antibody (Dianova, Hamburg, Germany) and extravidin-conjugated Cy3 (Sigma, München, Germany). Thereafter, sections were incubated with MHC class II and GR1, as primary monoclonal antibodies, followed by biotinylated mouse-anti-rat antibody (Dianova) and extravidin-conjugated fluorescein isothiocyanate (Dianova).

Immunohistochemistry was performed by use of the Vectastain Elite ABC kit (Vector, Burlingame, CA) with

appropriate biotinylated secondary antibodies. The peroxidase reaction product was visualized using 3,3′-diaminobenzidine (Sigma) as chromogene and H_2O_2 as co-substrate.

Histopathological alterations were microscopically evaluated on H&E-stained and immunostained horizontal brain sections. To determine the abscess size, maximal diameters of the necrotic center were measured in serial sections of the lesions in three mice per group at the respective days p.i.

RNA Extraction

Total RNA was extracted from brain tissue samples of the respective time points using the TRIzol/chloroform method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and resuspended in 60 μl of diethyl pyrocarbonate-treated water. Concentration of total RNA was determined using the Qubit fluorometer according to the manufacturer's protocol (Invitrogen).

Quantitative Real-Time PCR

The RNA was reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol using 1 μg total RNA per sample as described previously.[8]

Briefly, the expression level of cytokines and chemokines and the endogenous control gene hypoxanthin-guanine-phosphoribosyl-transferase (*hprt*) in the specimens was analyzed by real-time quantitative RT-PCR using the 5′-nuclease technology on an ABI PRISM 7300HT Sequence Detection System and the Mouse TaqMan pre-developed assay reagents (both from Applied Biosystems). The assay identification numbers are as follows: TNF, Mm00443258_m1; IFN-γ, Mm00801778_m1; IL-1β, Mm00434228_m1; IL-6, Mm00446190_m1; IL-10, Mm00439616_m1; iNOS, Mm00440485_m1; CCL2, Mm00441242_m1; CCL3, Mm00441258_m1; CCL4, Mm00443111_m1; CCL5, Mm01302428_m1; CXCL5, Mm00436451_g1; and CXCL10, Mm00445235_m1 and for the internal control: hypoxanthine-guanine phosphoribosyl/transferase, Mm00446968_m1.

PCRs were prepared in a final volume of 20 μl, with final concentrations of 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and a cDNA equivalent of 5 ng RNA. All analyses were performed in triplicate, and the threshold cycle (C_t) was determined. Gene expression was concomitantly measured in normal murine brain as calibrator to allow comparison between the different samples of TLR2$^{-/-}$, TLR4$^{-/-}$, and WT mice using the $\Delta\Delta C_t$ method.[33]

Flow Cytometry

Cerebral leukocytes were isolated from brains after perfusion with 0.9% NaCl. Brain tissue was minced through a 70-μm mesh sieve (BD Pharmingen), leukocytes were separated by Percoll gradient centrifugation (Amersham-Pharmacia, Freiburg, Germany),[34,35] and brain-derived leukocytes were subjected to double or triple immunofluorescence staining followed by flow cytometry as described previously.[8,9]

Statistical Evaluation

Student's *t*-test was performed to determine the significance of differences in the i.c. bacterial load, abscess size, the number of i.c. leukocytes between TLR2$^{-/-}$, TLR4$^{-/-}$, and WT mice, and quantitative differences in mRNA transcripts. For body weight during the infection, the Wilcoxon rank sum test was applied. For clinical score, the two-tailed Mann-Whitney *U*-test was applied. Data are presented as means ± SD. The level of confidence for significance was $P < 0.05$. All experiments were performed in duplicate. A representative experiment is shown in each figure.

Results

Clinical Disease in TLR2$^{-/-}$, TLR4$^{-/-}$, and WT Mice

Whereas WT mice survived the infection, 87% of TLR2$^{-/-}$ mice and 43% of TLR4$^{-/-}$ mice died (Figure 1A). TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were more severely affected throughout the disease compared with WT mice. Clinical symptoms included a reduced motor activity, hemiparesis, epileptic fits, prominent weight loss, and ruffled fur, symptoms that persisted throughout the disease ($P < 0.01$ at all experimental days for TLR2$^{-/-}$ mice versus WT mice, and $P < 0.05$ at days 1 and 3 p.i. and $P < 0.01$ at later time points for TLR4$^{-/-}$ mice versus WT mice; Figure 1B). Furthermore, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice remained severely ill when WT mice had already recovered clinically (Figure 1B). Persistent reduction of body weight was pronounced in TLR2$^{-/-}$ mice compared with WT mice at days 1 ($P \leq 0.05$), 7, 14, and 21 p.i. ($P < 0.01$, Figure 1C). In TLR4$^{-/-}$ mice, loss of body weight was significantly pronounced at days 1 and 7 p.i. compared with WT animals ($P < 0.05$ and 0.01, respectively). Control mice, which had received sterile agarose beads, did not show any clinical symptoms.

These data show a significantly increased mortality and morbidity of TLR2$^{-/-}$ mice, which remained ill until day 21 p.i. Also, TLR4$^{-/-}$ mice became significantly more severely ill than WT mice and exhibited a high mortality rate during the 1st week, and morbidity was increased until day 21 p.i.

Abscess Size and Bacterial Load in TLR2$^{-/-}$, TLR4$^{-/-}$, and WT Mice

Differences in clinical disease activity and weight loss matched the marked divergence in kinetics of abscess development and resolution between the three groups. The abscess size in TLR2$^{-/-}$ mice was significantly increased compared with WT animals at all days analyzed, and large abscesses persisted throughout the experiment ($P \leq 0.01$, Figure 2A). In TLR4$^{-/-}$ mice, the ab-

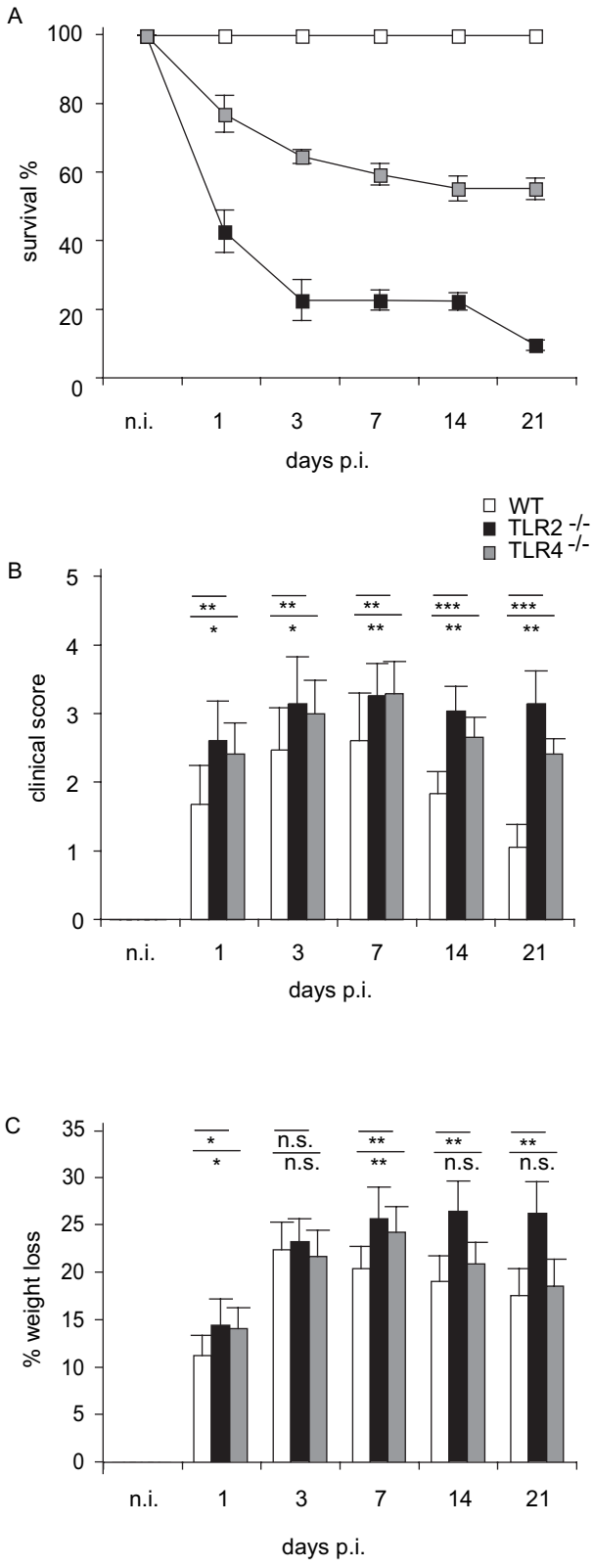


Figure 1. Survival (**A**), clinical score (**B**), and percentage of body weight loss (**C**) of WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice with stereotaxic *S. aureus*-induced brain abscess at days 0 to 21 p.i. Data represent the mean of initially 60 mice per group and time point in one experiment. Differences between TLR2$^{-/-}$, TLR4$^{-/-}$, and WT mice were analyzed by the Kaplan-Meyer survival analysis (**A**), the Mann-Whitney *U*-test (two-tailed) (**B**), and the Wilcoxon rank sum test (**C**), with $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$; and n.s., not significant.

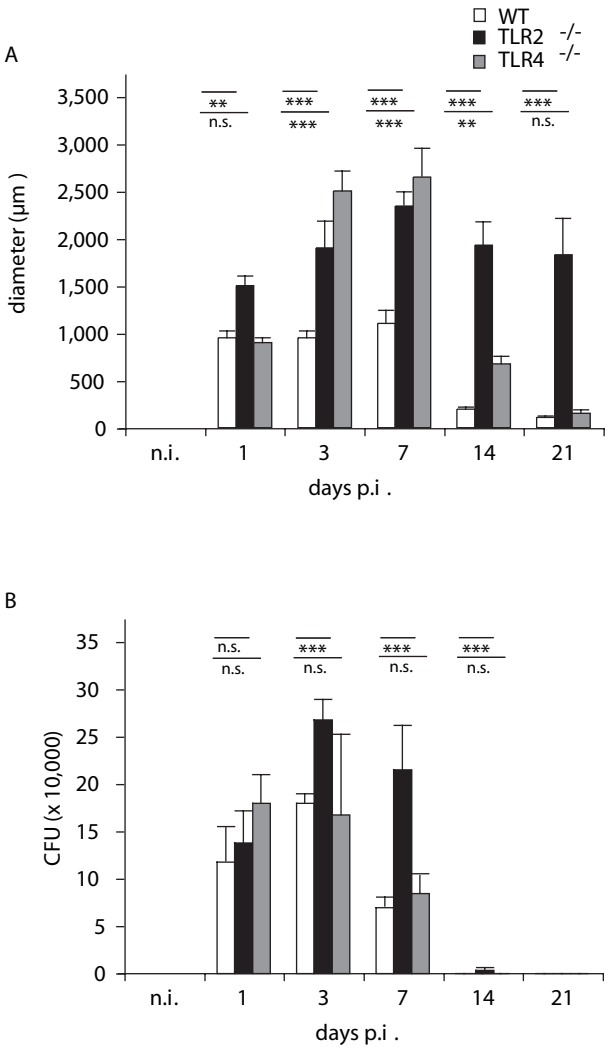
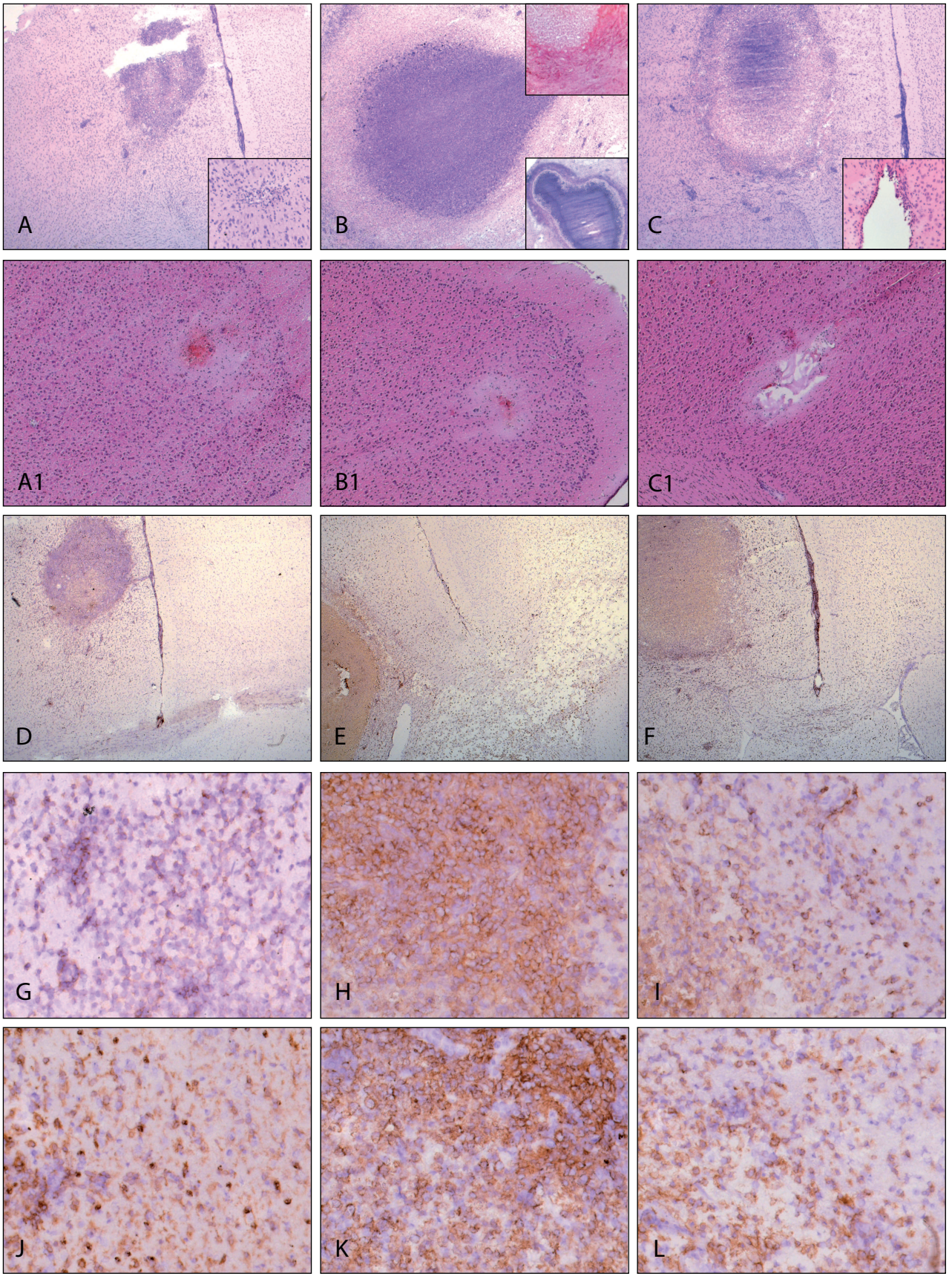


Figure 2. Lesion size and bacterial load of WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice with stereotaxic *S. aureus*-induced brain abscess at days 0 to 21 p.i. The lesion size (**A**) was determined by measuring the diameter of the necrosis in three mice per group and time point over a 21-day observation period in WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice. Data represent the mean ± SD Statistical analysis between WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice was performed by use of the Student's *t*-test, with $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$; and n.s., not significant. The intracerebral bacterial load (CFU) (**B**) of WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice was counted after 24 hours/37°C incubation of whole brains of mice, plated on agarose. Data represent the mean ± SD of three animals per time point and experimental group. Statistical differences between TLR2$^{-/-}$ and WT mice were found from day 3 up to day 14, whereas CFUs in TLR4$^{-/-}$ mice and WT mice did not reach statistical difference. Statistical analysis between WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice was performed by use of the Student's *t*-test, with $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$; and n.s., not significant.

scess size peaked at day 7 p.i. and steadily declined thereafter. Importantly, the abscess diameters were significantly increased in TLR4$^{-/-}$ mice compared with WT mice at days 3, 7, and 14 p.i. ($P \leq 0.01$).

Bacterial titers of *S. aureus* in mice of either genotype were not significantly different at day 1 p.i. However, colony-forming units (CFUs) of TLR2$^{-/-}$ mice were significantly increased compared with WT mice at days 3, 7, and 14 p.i. ($P < 0.001$). Differences between bacterial titers of TLR4$^{-/-}$ and WT mice did not reach statistical significance at any day of the experiment (Figure 2B).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These data demonstrate a superior role of TLR2 in restriction of the abscess size and elimination of *S. aureus* from the brain. Although TLR4 was not involved in clearance of *S. aureus*, this TLR participated in the restriction of the abscess size, pointing to an important role of TLR4 in the pathophysiology of *S. aureus*-induced brain abscess.

Both TLR2 and TLR4 Are Important for Abscess Containment

To analyze whether the clinically increased disease severity in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice correlated with a more pronounced tissue damage of the CNS, a detailed histopathological study was performed. In both TLR2$^{-/-}$ and TLR4$^{-/-}$ mice, abscesses with a necrotic center accompanied by an outer rim of leukocytes developed at the peak of disease activity (day 7 p.i., Figure 3, A–C). In contrast, abscesses of WT mice harbored smaller central tissue necrosis and lacked the prominent outer rim of leukocytes (Figure 3, A–C). In single surviving TLR2$^{-/-}$ mice, abscesses persisted and were surrounded by a capsule (day 21 p.i., Figure 3B, top inset). In contrast, TLR4$^{-/-}$ mice and WT mice were able to resolve the abscess with only minor residues like small scars with a central cystic defect at day 21 p.i. (Figure 3, A–C, bottom insets). In mice of either genotype, which were inoculated with sterile beads, only limited histopathological alterations are demonstrated (day 1 p.i., Figure 3, A1–C1).

Distribution of CD45^{+} leukocytes also differed markedly between the different strains of mice. Whereas the leukocytic infiltrate was confined to the center of the lesion and its immediate border in WT mice, CD45^{+} leukocytes were found widespread around the lesion in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (Figure 3, D and E). Additional analysis by flow cytometry revealed that numbers of intracerebral leukocytes were increased in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice compared with WT animals. Maximal numbers of leukocytes were observed at day 7 p.i. in all strains. Beyond day 14 p.i., there were no significant differences between TLR4$^{-/-}$ and WT mice, whereas TLR2$^{-/-}$ mice still contained significantly more leukocytes compared with WT animals (Figure 4).

TLR2 but Not TLR4 Signaling Regulates Recruitment and Bactericidal Function of Granulocytes and Macrophages

Because macrophages and granulocytes are important for the control of *S. aureus* infections, the infiltration of these cells in *S. aureus*-induced abscess was studied. A dense infiltration of the abscess and the adjacent CNS tissue by GR1^{+} granulocytes and CD11b^{+} or F4/80^{+} macrophages/microglia was detectable in TLR2$^{-/-}$ mice (Figure 3, H and K). Although distribution of granulocytes was more widespread in TLR4$^{-/-}$ mice (Figure 3, G versus I), no differences in the distribution of macrophages between TLR4$^{-/-}$ and WT mice were detected (Figure 3, J versus L). A detailed quantitative analysis of the intracerebral leukocyte populations showed that the kinetics of GR1^{+} granulocytes paralleled that of CD11b^{+}CD45^{high} macrophages throughout the disease in all three genotypes with significantly higher numbers of granulocytes and macrophages in TLR2$^{-/-}$ mice from days 1 through 21 p.i. ($P < 0.01$ TLR2$^{-/-}$ versus WT mice), whereas significant differences between TLR4$^{-/-}$ mice and WT mice were not detectable (Figure 4). Macrophages and granulocytes showed an early and prominent increase, peaked at day 7 p.i., and gradually declined thereafter in all three genotypes, albeit with a less eminent peak in TLR4$^{-/-}$ and WT mice. Granulocytes and macrophages persisted at significantly elevated levels in TLR2$^{-/-}$ mice up to day 21 p.i. ($P < 0.01$), whereas these populations had declined to low levels in TLR4$^{-/-}$ mice and WT mice at day 21 p.i., showing that recruitment and kinetics of both cell types was unaffected by TLR4 deficiency (Figure 4).

In a next step, we compared cytokine and chemokine mRNA transcription in the CNS of the three genotypes after brain abscess induction. In TLR2$^{-/-}$ mice, transcription of most chemokines and cytokines was not different or even slightly reduced at day 1 p.i. compared with the WT, with the exception of IFN-γ, which was not detected at day 1 p.i. in the WT. At late stages of the disease (≥day 7 p.i., ie, the clinical and morphological maximum of the disease), chemokines implicated in leukocyte entry like CCL2, CCL3, CCL4, CCL5, and CXCL10

Figure 3. Neuroimmunological characteristics of *S. aureus*-induced brain abscess in WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice. **A:** In a WT mouse, a centrally necrotic lesion is present within the CNS parenchyma at day 7 p.i. (H&E staining; original magnification, ×50), whereas at day 21 p.i., only a residual scar was found (**bottom inset**) by H&E staining (original magnification, ×200). **B:** In an TLR2$^{-/-}$ mouse, abscess size is increased, a rim of leukocytes surrounds the necrotic center, and edema is more pronounced at day 7 p.i. (H&E staining; original magnification, ×50). A large abscess exhibits prominent capsule formation at day 21 p.i. (**top inset**). Elastica van Gieson staining; original magnification, ×200 (**bottom inset**). **C:** In a TLR4$^{-/-}$ mouse, also a huge abscess with a leukocytic rim and massive edema had formed at day 7 p.i. (H&E staining; original magnification, ×50). At day 21 p.i., only a residual scar was found (H&E staining; original magnification, ×200). **A1–C1:** In a WT mouse (**A1**), in a TLR2$^{-/-}$ mouse (**B1**), and in a TLR4$^{-/-}$ mouse (**C1**), a tiny traumatic lesion is present within the right frontal white matter at day 1 after injection with sterile beads (H&E staining; original magnification, ×50). **D:** In a WT mouse, CD45^{+} leukocytes are found in the center and at the border of the abscess at day 7 p.i. (anti-CD45 immunostaining; original magnification, ×50). **E:** In an TLR2$^{-/-}$ mouse, CD45^{+} leukocytes additionally spread extensively throughout the brain, even infiltrating the contralateral hemisphere. Additionally, important edema adjacent to the abscess and also within the contralateral hemisphere is visualized at day 7 p.i. (anti-CD45 immunostaining; original magnification, ×50). **F:** In an TLR4$^{-/-}$ mouse, CD45^{+} leukocytes spread less extensively throughout the brain at day 7 p.i. (anti-CD45 immunostaining; original magnification, ×50). **G:** GR1^{+} granulocytes in the vicinity of an abscess in a WT mouse at day 7 p.i. (anti-GR1 immunostaining; original magnification, ×200). **H:** In contrast to G, a dense infiltrate of GR1^{+} granulocytes is demonstrated in a TLR2$^{-/-}$ mouse at day 7 p.i. (anti-GR1 immunostaining; original magnification, ×200). **I:** In contrast to G, in a TLR4$^{-/-}$ mouse, prominent and widespread infiltration of GR1^{+} granulocytes was noted at day 7 p.i. (anti-GR1 immunostaining; original magnification, ×200). **J:** F4/80^{+} macrophages/microglia in the surroundings of an abscess in a WT mouse at day 7 p.i. (anti-F4/80 immunostaining; original magnification, ×50). **K:** In a TLR2$^{-/-}$ mouse, the abscess is associated with more F4/80^{+} macrophages/microglia compared with **J** at day 7 p.i. (anti-F4/80 immunostaining; original magnification, ×200). **I:** Macrophages/microglia were similarly distributed in a TLR4$^{-/-}$ mouse compared with the WT (**J**) at day 7 p.i. (anti-F4/80 immunostaining; original magnification, ×200).

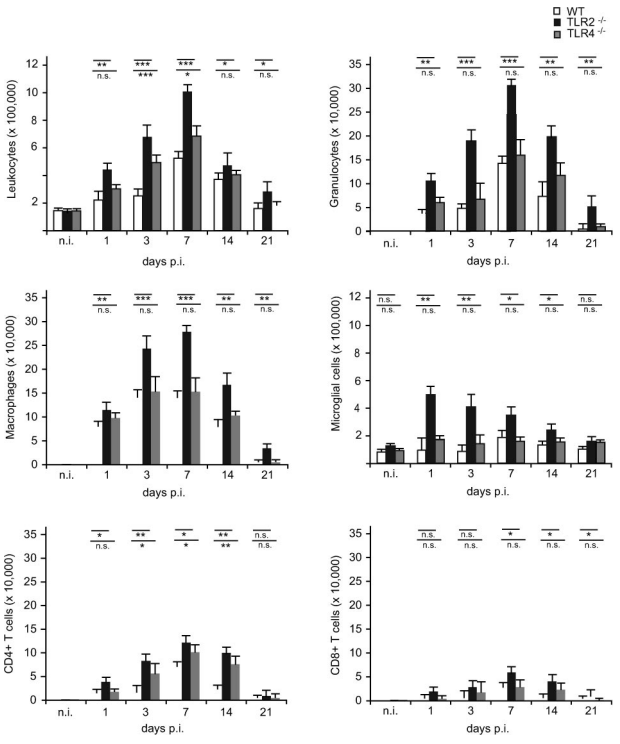


Figure 4. Phenotypic composition of intracerebral leukocyte infiltrates in WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice. For fluorescence-activated cell sorting analysis of intracerebral leukocytes, brains of three mice were pooled at each day as indicated, following i.c. infection with *S. aureus*-laden agarose beads, in WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice. Cells were isolated, stained for GR1, CD11b, CD4, CD8, and CD45, and analyzed by flow cytometry. Data are expressed as numbers of cells of the respective cell type per brain. Statistical analysis between WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice was performed by use of the Student's *t*-test, with $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$; and n.s., not significant.

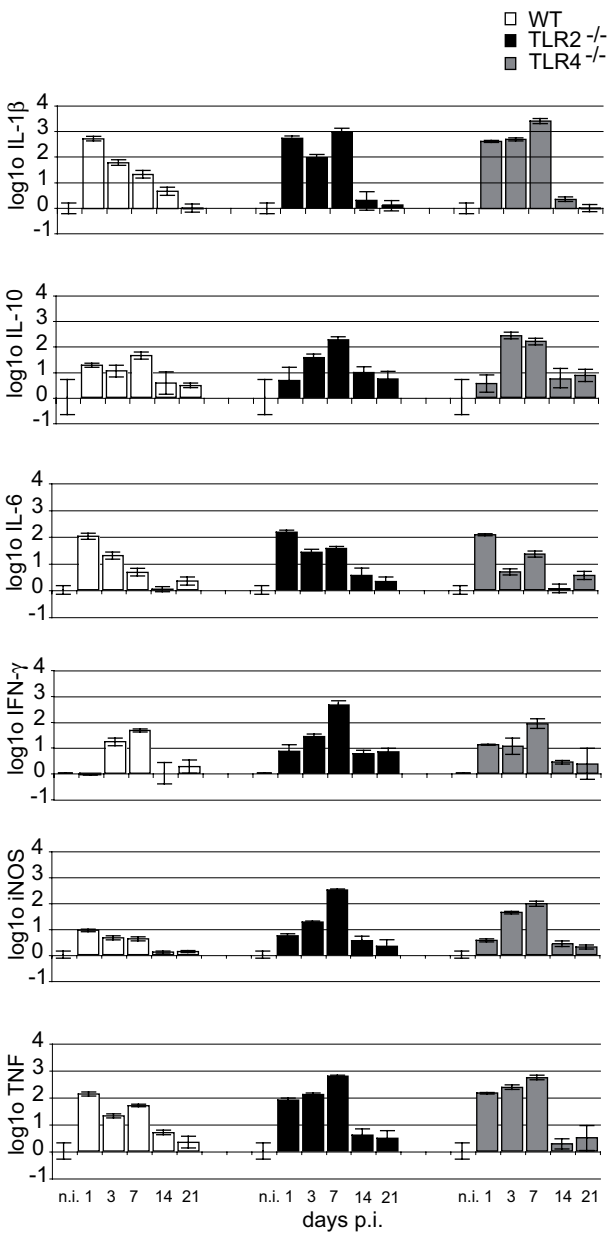


Figure 5. Quantitative analysis of mRNA expression of mediators of the inflammatory response in brain tissue homogenates of WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice and uninfected mice by real-time PCR. IL-1β, IL-6, iNOS, and TNF mRNA is maximally transcribed at day 1 p.i., INF-γ and IL-10 at day 7 p.i. in WT mice, whereas TLR2$^{-/-}$, and TLR4$^{-/-}$ mice reached maximal values of mRNA transcripts mostly at day 7 p.i. No or only low levels were transcribed in the brains of uninfected controls, which served as calibrators. Expression levels were calculated from a group of three replicates. Error bars display the SEM expression level, with a relative quantification confidence level of 97%.

and most proinflammatory cytokines were prominently transcribed. Remarkably, the important granulocyte chemoattractant CXCL5 was reduced at day 3 p.i. compared with the WT. In TLR4$^{-/-}$ mice, no differences in the induction of any chemokine and proinflammatory cytokine mRNA were noted at day 1 p.i., with the exception of IL-10 and iNOS, which were produced at reduced levels compared with the WT, and again IFN-γ, which was not detected at day 1 p.i. in the WT. Conversely, levels of IL-1β, IL-6, IL-10, IFN-γ, iNOS, TNF, CCL2, CCL3, CCL4, CCL5, and CXCL10 mRNA were increased compared with WT mice at day 7 p.i. and beyond (Figures 5 and 6). These results show that the early cytokine induction during brain abscess may not be TLR dependent, whereas the extent of chemokine and cytokine transcription in the late phase, which is characterized by induced leukocyte recruitment and/or leukocyte persistence, is regulated by TLR2 and TLR4.

In TLR2$^{-/-}$ mice at day 7 p.i. and even at day 21 p.i. (data not shown), iNOS-producing GR1^{+} granulocytes [Figure 7H, yellow overlay of granulocytes (green) and iNOS (red)] formed an inner rim (Figure 7H, arrows) within the centrally necrotic lesion (Figure 7H, asterisk). iNOS production in GR1^{+} granulocytes was less conspicuous at day 7 p.i. in TLR4$^{-/-}$ mice (Figure 7I) and had subsided at day 21 p.i. (data not shown). iNOS-producing GR1^{+} granulocytes in WT mice were rarely detected at days 3 and 7 p.i. (Figure 7G). iNOS production was mostly confined to GR1^{+} granulocytes in TLR2$^{-/-}$ mice, but some iNOS-producing MHC class II^{+} macrophages were also detected within the inner rim of the abscess (Figure 7K, asterisk), whereas iNOS^{-} macrophages were localized mostly outside the lesion (Figure 7K, arrows). No iNOS-producing MHC class II^{+} macrophages were found in either WT or TLR4$^{-/-}$ mice (Figure 7, J and L). In accordance, iNOS mRNA was transcribed at low levels in WT mice at days 1 and 3 p.i., whereas in TLR2$^{-/-}$ and

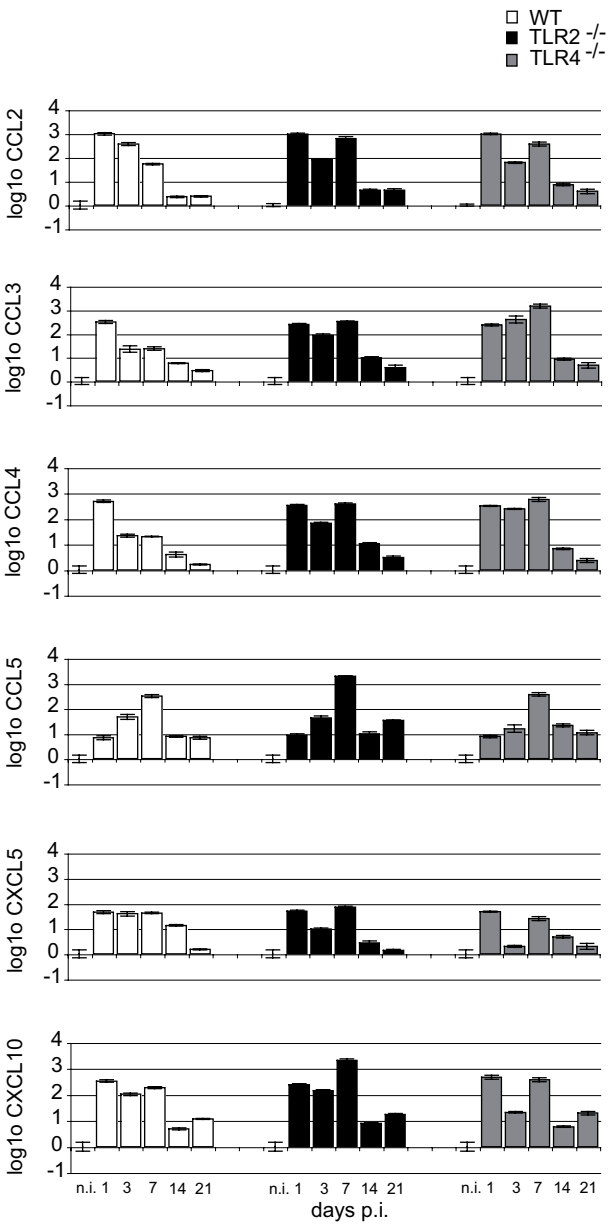


Figure 6. Quantitative analysis of mRNA expression of chemokines in brain tissue homogenates of WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice and uninfected mice by real-time PCR. CCL2, CCL3, CCL4, CXCL5, and CXCL10 mRNA are maximally transcribed at day 1 p.i., and CCL5 mRNA are maximally transcribed at day 7 p.i. in WT mice, whereas TLR2$^{-/-}$ and TLR4$^{-/-}$ mice reached highest values of mRNA transcripts mostly at day 7 p.i. No or only low levels were transcribed in the brains of uninfected controls, which served as calibrators. Expression levels were calculated from a group of three replicates. Error bars display the SEM expression level, with a relative quantification confidence level of 97%.

TLR4$^{-/-}$ mice, iNOS was transcribed at high levels until day 7 p.i.

These data demonstrate that granulocyte and macrophage recruitment are regulated by TLR2, leading to hyperinflammation in TLR2 deficiency. Death in TLR2$^{-/-}$ mice and in TLR4$^{-/-}$ mice during the 1st week occurred due to severely inflamed CNS tissue as a consequence of uncontrolled space occupying abscesses. In addition, prominent differences between the mouse strains with respect to the topographical distribution of granulocytes and their production of iNOS as an attempt to control the infection are demonstrated.

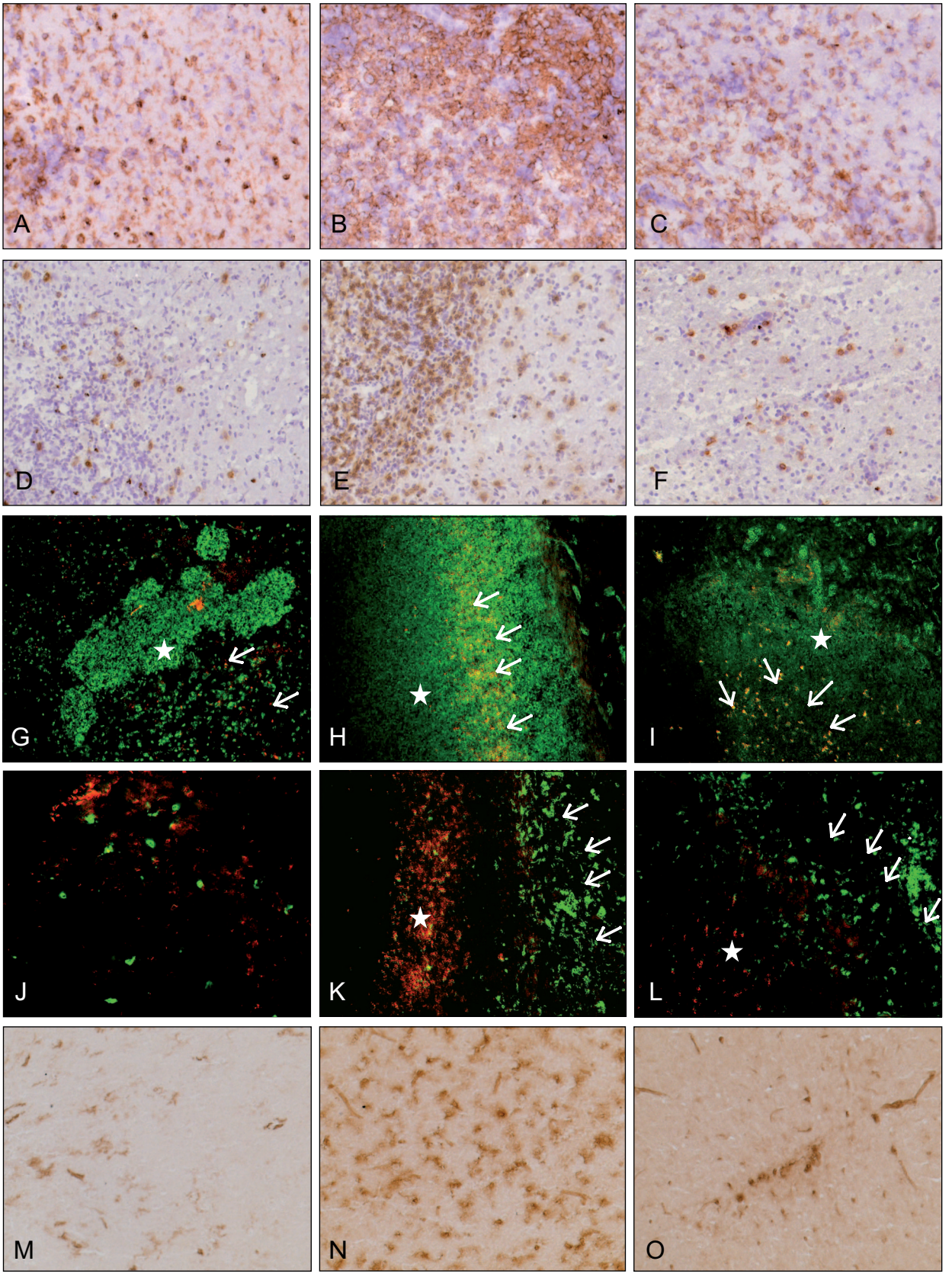
TLR2- and TLR4-Dependent Regulation of Microglial Activation

TLRs have been implicated in the regulation of microglial cell activation in bacterial CNS infections, and because this cell population may play an important role in the control of *S. aureus* and in the regulation of the intracerebral immune response, we next studied the response of microglial cells in our model. There were overt differences in the reaction pattern of microglial cells, with a more pronounced activation—as measured by expression of MHC class II, CD11b, and F4/80 protein by immunohistochemistry—in TLR2$^{-/-}$ mice, followed by TLR4$^{-/-}$ mice compared with WT littermates at all days of the experiment. In addition to their focal accumulation, ubiquitous prominent activation within the brain parenchyma was detected in TLR2$^{-/-}$ mice from days 1 to 21 p.i., whereas microglial activation had largely subsided in TLR4$^{-/-}$ and WT mice beyond day 14 p.i. (Figure 7, M–O). Microglial expansion, as measured by the number of CD11b^{+}CD45^{low} microglial cells, was significantly elevated compared with the WT exclusively in TLR2$^{-/-}$ mice from days 1 through 14 p.i. In WT mice and TLR4$^{-/-}$ mice, microglial numbers increased slightly with a maximum at day 7 p.i., returning to baseline levels of noninfected animals at day 21 p.i. without significant differences at any time point (Figure 4). These data show an early and sustained TLR2-dependent increase of microglial cell numbers, whereas TLR4 is not involved in microglial cell expansion.

Differential Regulation of T-Cell Responses by TLR2 and TLR4

Because T cells have been previously implicated in the pathogenesis of brain abscess, we further analyzed infiltration of T-cell subsets after brain abscess induction by *S. aureus* in the setting of TLR2 and TLR4 deficiency. Both CD4^{+} and CD8^{+} T cells were detected within the abscess and diffusely scattered throughout the affected hemisphere in TLR2$^{-/-}$ mice, a finding that was absent in WT mice (Figure 7, B and E). TLR4$^{-/-}$ mice also showed a more widespread distribution of CD4^{+} T cells in the brain tissue compared with the WT (Figure 7, A and C), whereas CD8^{+} T cells were detected in comparable localization in both WT and TLR4$^{-/-}$ mice (Figure 7, D and F).

In addition to its anatomical distribution, we analyzed how TLR2 and TLR4 deficiency affected T-cell recruitment to the CNS of *S. aureus*-infected mice. Quantification by flow cytometry showed that CD4^{+} and CD8^{+} T cells infiltrated the CNS of all genotypes, reached a maximum at day 7 p.i., and declined thereafter (Figure 4). In comparison with WT mice, a significantly increased recruitment of CD4^{+} T cells was observed in TLR2$^{-/-}$ mice from days 1 to 14 p.i. ($P < 0.05$). CD4^{+} T-cell recruitment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was also increased in TLR4$^{-/-}$ mice from days 3 to 14 p.i. compared with WT mice ($P < 0.05$). Whereas TLR4$^{-/-}$ mice recruited CD8$^+$ T cells in numbers comparable with WT mice, TLR2$^{-/-}$ mice showed a significantly enhanced recruitment at late time points, ie, from days 7 to 14 p.i. ($P < 0.05$, Figure 4). Corresponding to the increased numbers of T cells in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice, the important T-cell chemoattractant CCL4 was sustained at high levels from days 7 to 21 p.i. in both TLR2$^{-/-}$ and TLR4$^{-/-}$ mice compared with the WT (Figure 5). Furthermore, transcription of CCL5 mRNA in TLR2$^{-/-}$ mice compared with the WT (day 7 p.i.) and transcription of CCL5 mRNA in TLR4$^{-/-}$ mice compared with the WT (day 14 p.i.) were elevated. CXCL10 mRNA transcripts were elevated at day 7 p.i. in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice compared with the WT and less prominent at day 14 p.i. in TLR2$^{-/-}$ mice compared with the WT.

In conclusion, these findings point to a role of TLR2 and, to a lesser extent, also of TLR4 in the CD4$^+$ T-cell response after *S. aureus* infection of the CNS, whereas the recruitment of CD8$^+$ T cells was only regulated by TLR2 in our model.

TLR2 and TLR4 Signaling Regulates Leukocyte Apoptosis

We have previously shown that apoptosis of leukocytes during the disease is an important immunoregulatory mechanism that is differentially regulated by different cytokines in *S. aureus*-induced brain abscess. Very recently, TLR signaling via TLR3, TLR4, or TLR5 has been implicated in cell proliferation and survival *in vitro* via the adaptor protein Toll/IL-1R-containing adaptor-inducing IFN-β.[36] We therefore sought to analyze the impact of TLR2 and TLR4 for leukocyte apoptosis in brain abscess *in vivo*.

The kinetics of apoptotic leukocytes differed substantially in the three genotypes. In TLR2$^{-/-}$ mice, the maximum of apoptotic cells within the abscess was observed at day 21 p.i. In TLR4$^{-/-}$ mice, most leukocytes underwent apoptosis around day 7, whereas in WT mice, apoptosis was maximally induced at day 1 p.i. (data not shown). Double immunofluorescence staining of activated caspase-3 (Figure 8, A, D, and G) and GR1 identified granulocytes (Figure 8, B, E, and H) as the main population undergoing apoptosis. In TLR2$^{-/-}$ mice, granulocytes prominently underwent apoptosis at day 7 p.i. (Figure 8F), whereas in TLR4$^{-/-}$ mice, colocalization of activated caspase-3-positive granulocytes was less pronounced (Figure 8, G–I) and was even minor in WT mice (Figure 8, A–C). Thus, histology revealed that defective TLR2 and TLR4 signaling critically impacts effector mechanisms such as the extent of leukocyte apoptosis.

Discussion

In this study, we have analyzed the *in vivo* role of TLR2 and TLR4 in experimental *S. aureus*-induced brain abscess. The absence of TLR2 dramatically increased the severity of the clinical disease, the chemokine and cytokine response, the recruitment of inflammatory leukocytes, and the immune response of microglia. Interestingly, the absence of TLR4 led to reduced survival during the 1st week and an increased morbidity throughout the disease. TLR4 also regulated chemokine and cytokine production and controlled the T-cell response, whereas macrophage and granulocyte recruitment and microglial reactivity were not significantly affected.

Worsening of the course of disease, with highly increased mortality in TLR2$^{-/-}$ mice and less pronounced mortality in TLR4$^{-/-}$ mice, occurred because of important mass effects of persisting large abscesses accompanied by resulting edema of the CNS tissue. In murine *S. pneumoniae*-induced meningitis, mortality and morbidity were also enhanced in TLR2$^{-/-}$ mice compared with WT mice, which was attributed to a higher bacterial load in the brain parenchyma and increased meningeal leukocytic inflammation.[31,37] Accordingly, in *S. aureus*-induced brain abscess, the severe adverse course of the disease in TLR2$^{-/-}$ mice was paralleled by significantly increased CFU and a delayed clearance of staphylococci compared with WT animals. TLR4 deficiency, however, did not affect clearance of *S. aureus* from the CNS. Correspondingly, in systemic *S. aureus*-induced infections, TLR2$^{-/-}$ mice were also highly susceptible and displayed elevated numbers of bacteria in peripheral organs (eg, kidney) compared with WT mice.[29]

The function of TLRs is not limited to pathogen control, and TLR2 and TLR4 have been implicated in wound healing in a noninfectious lung injury model.[38] In addition, TLR2 and TLR4 have not only been reported to recognize

Figure 7. Neuroimmunological characteristics of *S. aureus*-induced brain abscess in WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice at day 7 p.i. **A:** Few CD4$^+$ T cells are found in a WT mouse in the vicinity of the abscess at day 7 p.i. (anti-CD4 immunostaining; original magnification, ×200). **B:** A dense infiltrate of CD4$^+$ T cells is found in a TLR2$^{-/-}$ mouse in the vicinity of the abscess at day 7 p.i. (anti-CD4 immunostaining; original magnification, ×200). **C:** In contrast to WT mice (**A**), more CD4$^+$ T cells are found in a TLR4$^{-/-}$ mouse in the center and the vicinity of the abscess at day 7 p.i. (anti-CD4 immunostaining; original magnification, ×200). **D:** Few CD8$^+$ T cells are found in a WT mouse in the vicinity of the abscess at day 7 p.i. (anti-CD8 immunostaining; original magnification, ×200). **E:** A dense infiltrate of CD8$^+$ T cells is found in a TLR2$^{-/-}$ mouse in the vicinity of the abscess at day 7 p.i. (anti-CD8 immunostaining; original magnification, ×200). **F:** Similarly to WT mice (**D**), few CD8$^+$ T cells are found in a TLR4$^{-/-}$ mouse in the center and the vicinity of the abscess at day 7 p.i. (anti-CD8 immunostaining; original magnification, ×200). **G–I:** In a WT mouse (**G**), a few iNOS$^+$ cells (red) colocalize with GR1$^+$ granulocytes (green) in the abscess vicinity (**arrows**) at day 7 p.i. (anti-iNOS and anti-GR1 double-immunofluorescence staining; original magnification, ×200). Numerous iNOS$^+$ granulocytes form an inner rim at the necrotic border of the abscess (**arrows**) in a TLR2$^{-/-}$ mouse (**H**), whereas only few iNOS$^+$ granulocytes are found in this localization a TLR4$^{-/-}$ mouse (**I**) at day 7 p.i. (anti-iNOS and anti-GR1 double-immunofluorescence staining; original magnification, ×200). **J–L:** In a WT mouse (**J**) and a TLR4$^{-/-}$ mouse (**L**), no colocalization of iNOS (red) with MHC class II$^+$ cells (green) in the abscess vicinity (**arrows**) was found at day 7 p.i. In a TLR2$^{-/-}$ mouse (**K**), some iNOS$^+$ MHC class II$^+$ cells were detected within the inner rim of the necrotic center, whereas most MHC class II$^+$ cells were localized in the abscess vicinity at day 7 p.i. (anti-iNOS and anti- MHC class II double-immunofluorescence staining; original magnification, ×200). **M–O:** Few I-A$^+$ microglia with ramifying processes are detectable in a WT mouse (**M**) at day 21 p.i., whereas in a TLR2$^{-/-}$ mouse (**N**), activation of microglia was much more pronounced. Also, in a TLR4$^{-/-}$ mouse (**O**), microglial activation had largely subsided at day 21 p.i. (anti-I-A immunostaining; original magnification, ×200).

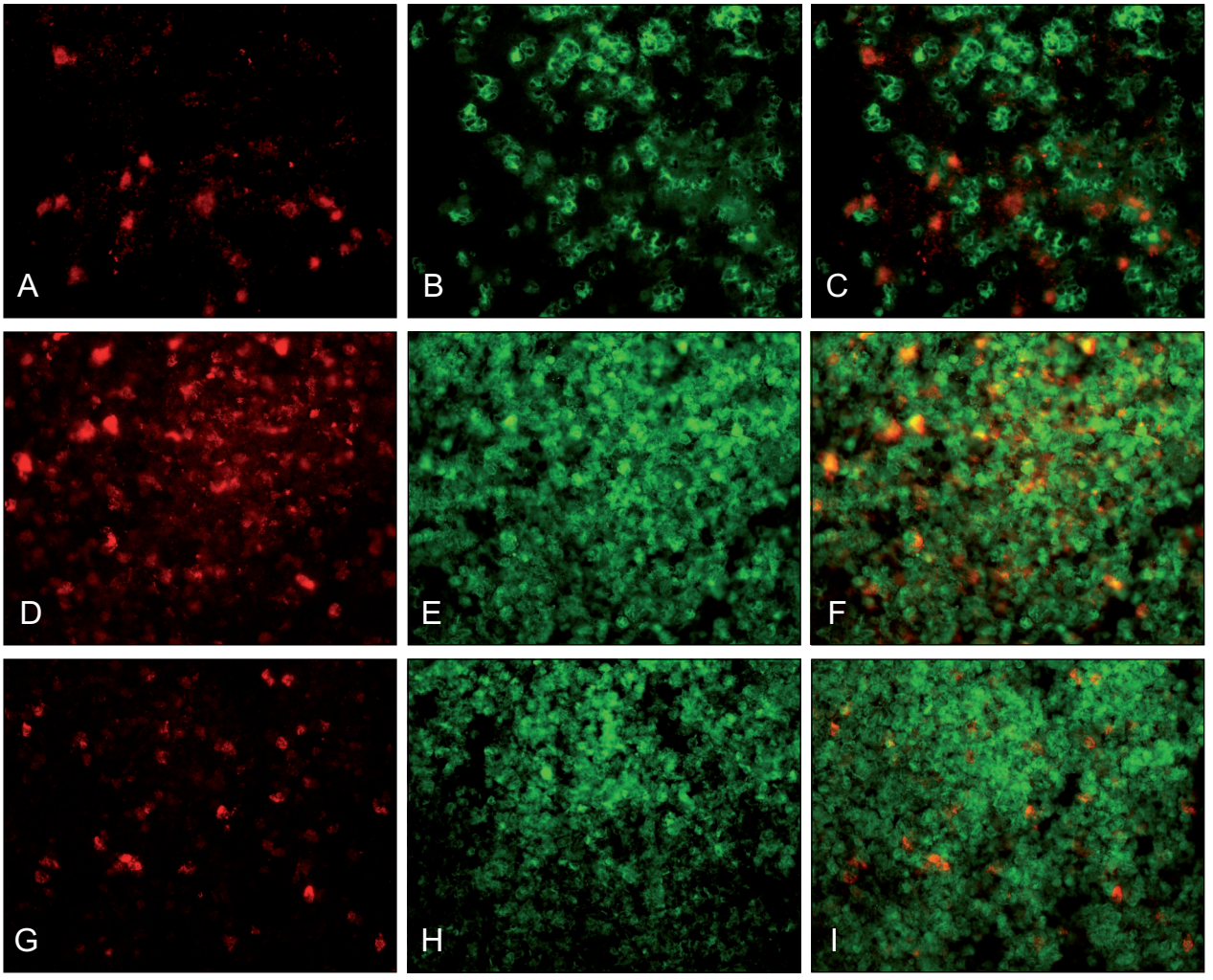

Figure 8. Immunohistochemical detection of apoptosis in WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice at day 7 p.i. **A–C:** In a WT mouse, very few activated caspase 3^{+} cells (**A**) colocalize (**C**) with GR1^{+} granulocytes (**B**) in the abscess center at day 7 p.i. (anti-activated caspase 3 and anti-GR1 immunostaining; original magnification, ×200). **D–I:** Numerous activated caspase 3^{+} cells (**D**) show a conspicuous colocalization (**F**) with GR1^{+} granulocytes (**E**) in a TLR2$^{-/-}$ mouse at day 7 p.i. Colocalization (**I**) of these antibodies is less pronounced in a TLR4$^{-/-}$ mouse at day 7 p.i. (**G–I**) compared with a TLR2$^{-/-}$ mouse (**D–F**). Anti-activated caspase 3 and anti-GR1 immunostaining; original magnification, ×200.

"stranger signals " from bacteria but also to transduce endogenous "danger signals " like fibronectin,[39] hyaluron acid,[40] and HMGB1,[41] all of which may be found at abundance within large areas of tissue necrosis in the setting of brain abscess. Thus, in our model, TLR2 and TLR4 might be required to limit the size and to resolve the abscess.

Although both TLR2 and TLR4 played a protective role in the *S. aureus*-brain abscess model, TLR2 was clearly much more important than TLR4 concerning survival and pathogen control. In addition, only TLR2$^{-/-}$ mice developed a capsule, which surrounded the abscess at late stages of the disease. Previously, we have identified that the severe course of *S. aureus*-induced brain abscess in TNF$^{-/-}$ mice also results in capsule formation at late stages of the disease and correlated with persistence of large abscesses as well.[9] We hypothesize that capsule formation in the CNS with deposition of collagen and other extracellular matrix substances occurs in response to a large abscesses with increased CFU and prolonged persistence.

Recently, the relevance of a prolonged inadequate cytokine and chemokine production in the context of brain abscess as a major factor contributing to high morbidity was emphasized in IL-10 deficiency.[8] In the present study, TLR2 additionally regulated the production of proinflammatory mediators and chemokines during the course of disease. Thus, it is attractive to ascribe these chemokine-mediated effects to recruitment of granulocytes, macrophages, and T cells to the CNS, because leukocyte entry into the CNS was shown to be dependent on CCL2/CCR2 signaling.[42–44] CXCL5 is an important chemoattractant for granulocytes in the periphery but has not yet been studied in the CNS. Because its expression was not increased but was reduced and low even at late stages of the disease, when neutrophils were still present, its induction by *S. aureus* might be TLR2 and TLR4 independent, and other chemokines may play a

more important role in neutrophil recruitment to the CNS.[45]

The proinflammatory cytokines TNF and IFN-γ were highly elevated at the clinical and morphological maximum of abscess formation in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice, illustrating that these two TLRs are not essential for the induction of these cytokines. However, the role of TLRs concerning the regulation of TNF production in cerebral infections with Gram-positive pathogens is controversial. Whereas Koedel et al[31] reported a reduced expression of TNF in murine *S. pneumoniae* meningitis, Echchannaoui et al[37] described an increased *in vivo* induction of TNF in TLR2$^{-/-}$ mice compared with WT animals in the same disorder. However, Echchannaoui and colleagues[46] stressed that *Listeria monocytogenes*-infected TLR2$^{-/-}$ and WT mice had similar TNF activity in the CSF, arguing for different roles of this receptor in Gram-positive CNS infections.

Neutrophil activation by purified lipoteichoic acid from *S. aureus in vitro* has been found to directly stimulate cytokine production via CD14 in a manner independent of TLR2 or TLR4.[47] CD14 is required not only for TLR4 signaling but also for TLR2/TLR6 signaling,[48,49] and it has therefore been hypothesized that TLR2 and TLR4 are anatomically linked as part of a single large sensing complex on the cell surface.[50] Cooperation between TLR2 and TLR4 on iNOS and TNF production was described just recently.[51] The authors suggest that shared intracellular adaptor protein activation cascades may be an attractive explanation for these phenomena. TLR synergy was also suggested by the observation that recognition of *Mycobacterium avium* was only partially affected in TLR2- and TLR4-deficient mice, whereas MyD88-deficient mice, which lack the central adaptor protein used by both receptors, displayed a severely reduced capacity to control the infection.[52] The involvement of TLR4 in Gram-positive infections of the CNS was first suggested by Koedel et al,[31] who have demonstrated responsiveness to pneumococci by ectopic TLR2 and TLR4 expression in HEK293 cells. In addition, TNF release from macrophage isolates from TLR2- and/or TLR4-deficient mice did not differ, arguing for synergistic effects of these receptors or involvement of further pattern recognition receptors in mediating the immune response.[31] Peptidoglycan of *S. aureus* has been shown to cause a priming effect on lipopolysaccaride signaling in an *ex vivo* whole human blood model, causing an up-regulation not only of TLR2 but also of TLR4.[53] Synergy was also described between the ligand for TLR4, lipopolysaccharide, and a ligand for TLR2, muramyl dipeptide, in the release of TNF in an *in vitro* system.[54]

In contrast to results obtained in murine *S. pneumoniae* meningitis,[31,37] we observed a remarkable impact of TLR2 and TLR4 on leukocyte recruitment to the CNS and a specific effect of these TLRs on chemokine and cytokine expression. Increased numbers of CD4^{+} T cells in TLR4$^{-/-}$ mice may be explained at least in part by high levels of CCL2, CCL3, CCL4, CCL5, and CXCL10, chemokines that can mediate T-cell entry to the CNS.[42,43,55,56] These data together with the clinical and immunohistochemical results illustrate the role of TLR4, especially in the early phase of the disease. However, it has to be stressed that the increased numbers of bacteria in TLR2$^{-/-}$ mice may play a role for leukocyte recruitment, because bacterial products also exert a chemotactic effect on leukocytes.

TLR2 signaling, but not TLR4 signaling, regulated microglial activation and expansion in *S. aureus*-induced brain abscess. Already early after the i.c. infection, microglial cell numbers were strongly increased in TLR2$^{-/-}$ mice compared with WT animals, indicating that TLR2 expression either on microglia or on surrounding astrocytes exerts a suppressive effect inhibiting microglial proliferation. Expansion of microglia has also been shown after cuprizone treatment and in response to transsection of axons in the entorhinal cortex by several investigators.[43,57–59] In contrast to these "noninfectious " models, in which lesion-induced microglial proliferation was reduced in TLR2$^{-/-}$ mice in the early phase,[43] a prominent activation of microglial cells was remarkable in TLR2$^{-/-}$ mice until late stages of brain abscess, which may also be fostered by high levels of chemokines like CCL2 during the later course of the disease.

Apoptosis of inflammatory leukocytes is a tightly controlled mechanism within the CNS, because on one hand, cells of the innate and adaptive immune system execute effector functions for an adequate time span in the case of bacterial challenge. On the other hand, prolonged presence of these cells may cause secondary damage to the CNS. We show here that in TLR2$^{-/-}$ mice, apoptosis of granulocytes was highly elevated, even after 3 weeks p.i., which reflects the high cellular turnover, a situation also characteristic for IL-10$^{-/-}$ mice.[8] This is in line with observations of TLR2-mediated delay or inhibition of apoptosis in granulocytes.[60–62]

In contrast to our results obtained in TLR2$^{-/-}$ mice, Kielian et al[28] have recently described lack of a significant impact for TLR2 on survival and bacterial load in a model of the early phase of *S. aureus*-induced murine brain abscess. Furthermore, proinflammatory mediators like TNF, MIP-2, and iNOS were reduced at day 1 or 3 p.i. in TLR2$^{-/-}$ mice, which is in marked contrast to our findings. Two explanations for these discrepancies may be discussed. First, the *S. aureus* strain used for the i.c. inoculation was different (RN 6390 versus ATCC 25923). The ATCC 25923 strain is Panton-Valentine leukocidin positive, which was found to be associated with increased virulence.[63,64] The RN6390 strain is sigma B deficient and has been shown to be *agr* negative, which may lead to a completely avirulent state *in vitro*. RN6390 is also deficient in biofilm formation, a process that is important for both pathogenesis and antimicrobial resistance.[65] Furthermore, the strains show differences in solvent tolerance[66] and antibiotic susceptibility.[67] The number of bacteria used for the infection is also of outmost importance for the ensuing immune response, which has been shown clearly by use of two different doses in systemic *S. aureus* infection[29] and in systemic group B streptococcal disease.[68]

In conclusion, the present study underscores that innate immune recognition of *S. aureus* within the CNS by TLR2 is absolutely mandatory and that TLR4 also plays

an important role in this disease. Both receptors have specific roles in leukocyte and microglial activation and migration and in regulating the rate of apoptosis.

Acknowledgments

Meike Brenkmann, Sonja Klemm, Nina Plewka, and Laura Wilden are very kindly acknowledged for expert technical assistance. We further thank Oleg Krut (M.D.) for helpful discussion.

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