Toll-like receptor 2 (TLR2) deficiency leads to increased Th17 infiltrates in experimental brain abscesses[†]

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Abstract

TLR2 plays a pivotal role in recognizing *S. aureus*, a common etiologic agent of CNS parenchymal infections, such as brain abscess. We previously reported that brain abscesses of TLR2 knockout (KO) mice exhibited elevated IL-17 levels, suggesting the presence of an alternative pathway available to respond to *S. aureus* infection that may involve Th17 cells. Both CD4^{+} and CD8^{+} T cell infiltrates were elevated in brain abscesses of TLR2 KO mice at days 3, 7, and 14 post-infection compared to wild type animals. Intracellular cytokine staining revealed a significant increase in the frequency of IL-17-producing Th17 cells in TLR2 KO mice with relatively few IFN-γ-positive cells.γδ T cells were also a source of IL-17 in brain abscesses. Microglia, astrocytes, and macrophages were shown to express both IL-17RA and IL-17RC. Despite receptor expression, IL-17 was relatively ineffective at eliciting glial activation, whereas the cytokine augmented the ability of TNF-α to induce CXCL2 and CCL2 expression by macrophages. Based on the ability of IL-17 to elicit the release of chemokines and other pro-inflammatory mediators, we propose that the exaggerated IL-17 response that occurs in TLR2 KO mice functions in a compensatory manner to control brain abscess pathogenesis, with cells other than glia as targets for IL-17 action. This is supported by our findings where innate immune infiltrates were not significantly different between TLR2 KO and WT mice in conjunction with the lack of prolonged alterations in the synthesis of other pro-inflammatory molecules during the course of infection.

Keywords

Toll-like receptor 2; Th17; IL-17; *S. aureus*; brain abscess; central nervous system

INTRODUCTION

Brain abscesses are serious CNS infections that develop in response to parenchymal colonization with pyogenic bacteria such as *S. aureus* or *Streptococcus* strains (1,2). Abscesses begin as localized areas of cerebritis that evolve into suppurative lesions surrounded by a well-vascularized fibrotic capsule. While antibiotic and surgical treatment options are available,

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brain abscesses remain serious CNS infections with the potential for long-term complications including seizures, loss of mental acuity, and other neurologic defects (1,2).

Toll-like receptors (TLRs) are an innate immune receptor family that is responsible for recognizing highly conserved microbial motifs (3,4). In the CNS, TLRs are expressed in many cell types including microglia (5–10), astrocytes (10–13), and neurons (14,15). TLR2 has been implicated as a critical receptor for eliciting responses to Gram-positive bacteria such as *S. aureus* (16,17), which is mediated by its ability to recognize lipoproteins and peptidoglycan (PGN) associated with the bacterial cell wall. Earlier studies from our laboratory have demonstrated that TLR2 plays an important role in both microglial and astrocyte activation in response to *S. aureus* (7,11). Although we previously reported that the expression of several pro-inflammatory mediators was transiently attenuated in brain abscesses of TLR2 KO mice compared to wild type (WT) animals, unexpectedly there were no significant adverse effects on survival or bacterial burdens in the former (18). One possibility that could have accounted for these results was the finding that brain abscesses of TLR2 KO mice exhibited elevated IL-17 levels (18). IL-17 is a potent inflammatory cytokine involved in immune amplification and is produced primarily by CD4^{+} Th17 cells, although other cellular sources of IL-17 have also been reported (19–26). IL-17 can stimulate the expression of chemokines and cytokines, either alone or in synergy with other pro-inflammatory mediators such as TNF-α 20, 27–30). Th17 cells play an important role in the management of extracellular bacterial infections (31, 32) and more recently these cells have been implicated as a key player in autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) (33–36).

There is limited information currently available regarding the expression of IL-17 in models of CNS bacterial infection. An earlier report described low levels of IL-17 expression in the brain following a systemic infection with *Cryptococcus neoformans* (37) and IL-17 expression is augmented in the CNS of mice chronically infected with *Toxoplasma gondii* (38). The finding that IL-17 levels were significantly exaggerated in brain abscesses of TLR2 KO mice led us to envision that these animals may exhibit enhanced Th17 infiltrates that could functionally compensate for the loss of TLR2-dependent signals through the pro-inflammatory effects of IL-17. Indeed, Th17 influx was increased in TLR2 KO mice compared to WT animals, whereas the recruitment of other innate immune effector cells (i.e. PMNs, macrophages, microglia, or dendritic cells) was not affected. Microglia and astrocytes were found to express both IL-17RA and IL-17RC; however, these cells were relatively non-responsive to IL-17 in terms of cytokine/chemokine production. In contrast, IL-17 potentiated chemokine release from TNF-α stimulated macrophages, suggesting that these cells may be a target of IL-17 in the infected CNS. We propose that the exaggerated IL-17 response that occurs in TLR2 KO mice functions as a compensatory mechanism to facilitate the effective control of brain abscess progression.

MATERIALS AND METHODS

Mouse strains

TLR2 KO mice (generously provided by Dr. Shizuo Akira, Osaka University, Japan) were backcrossed with C57BL/6 mice for a total of eight generations prior to use in these studies. Age- and sex-matched C57BL/6 mice (Harlan Labs, Indianapolis, IN) were used as WT controls. For all brain abscess studies, TLR2 KO and WT mice were used between 6 and 8 weeks of age.

Generation of experimental brain abscesses

Brain abscesses were induced by the stereotactic intracerebral injection of *S. aureus* strain RN6390 encapsulated in agarose beads as previously described (39). Briefly, mice were anesthetized with 2.5% avertin i.p. and a 1 cm longitudinal incision was made in the scalp to

expose the underlying skull sutures to facilitate the identification of bregma. A rodent stereotaxic apparatus equipped with a Cunningham mouse adaptor (Stoelting, Kiel, WI) was used to implant *S. aureus*-encapsulated beads into the caudate-putamen region of the brain using the following coordinates relative to bregma: + 1.0 mm rostral, + 2.0 mm lateral, and −3.0 mm deep from the surface of the brain. A burr hole was made and a 5 μl Hamilton syringe fitted with a 26-gauge needle was used to slowly deliver 2 μl of *S. aureus*-laden beads (10^4 colony forming units [CFU]) into the brain parenchyma. The needle remained in place for 2.5 min following injection to minimize bead efflux and potential leakage into the meninges. The superficial skin incision was closed using surgical glue. Animals were closely monitored over the course of each study for clinical indices of infection. The animal use protocol, approved by the University of Nebraska Medical Center Animal Care and Use Committee, is in accord with the National Institutes of Health guidelines for the use of rodents.

Quantitation of T cells, microglia, macrophages, PMNs, and dendritic cells (DC) in brain abscesses

To determine whether the loss of TLR2-dependent signals influenced the influx of T cells, PMNs, macrophages, DCs, and/or microglia into lesions, abscess-associated cells were quantitated by FACS analysis as previously described (39–42). Briefly, mice were perfused to eliminate leukocytes from the vasculature, whereupon the entire infected hemisphere was collected to recover abscess-associated cells. This approach ensured that equivalent tissue regions were procured from both TLR2 KO and WT mice for downstream comparisons of leukocyte infiltrates. Following vascular perfusion, tissues were minced in HBSS (Mediatech, Herndon, VA) supplemented with 10% FBS (HyClone, Logan, UT) and filtered through a 70 μm nylon mesh cell strainer using a rubber policeman. The resulting slurry was digested for 30 min at 37° C in HBSS supplemented with 2 mg/ml collagenase type I (Sigma, St. Louis, MO) and 5000 U/ml DNAse I (Invitrogen, Carlsbad, CA) to obtain a single-cell suspension. Following enzyme neutralization, cells were layered onto a discontinuous Percoll gradient (1.03 to 1.088 g/ml) and centrifuged at 2,400 rpm for 20 min at room-temperature in a swinging bucket rotor. After centrifugation, myelin debris was carefully aspirated and the cell interface collected. Following extensive washes and incubation in Fc Block™ (BD Biosciences, San Diego, CA) to minimize non-specific antibody binding to Fc receptors, cells were stained with directly conjugated antibodies for 4-color FACS to detect PMNs (Gr-1$^+$, CD11b$^+$, CD45^{hi}), macrophages (Gr-1$^-$, CD11b$^+$, CD45^{hi}), DCs (Gr-1$^-$, CD11c$^+$, CD45^{hi}) and microglia (Gr-1$^-$, CD11b$^+$, CD45$^{lo-intermediate}$). To enumerate abscess-associated T cell infiltrates, cells were analyzed by 3-color FACS using CD3, CD4, and CD8. All antibodies were purchased from BD Biosciences. Cells were analyzed using a BD FACSAria with compensation set based on the staining of each individual fluorochrome alone (CD11b-AlexaFluor488, CD11c-PECy7, Gr1-APC, CD45-FITC, CD3-FITC, CD4-PE, or CD8-APC) and correction for autofluorescence with unstained cells. Microglial, astrocyte, and macrophage IL-17 receptor A (IL-17RA) and IL-17RC expression was examined using goat anti-mouse primary antibodies directed against each receptor isoform (R & D Systems, Minneapolis, MN) followed by a donkey anti-goat IgG-FITC conjugate (Jackson Immunoresearch, West Grove, PA). Controls included cells stained with isotype control antibodies to assess the degree of non-specific staining.

For reporting differences in cellular influx between TLR2 KO and WT mice, one of two approaches was utilized. In the first, we normalized (i.e. divided) the numbers of FACS-purified T cells recovered from TLR2 WT mice by those collected from TLR2 KO animals to express the latter as a percentage of WT (set to 100%). This approach was required since it is difficult to achieve identical bacterial burdens in mice between independent brain abscess experiments. As a result, the absolute numbers of infiltrating cells within brain abscesses of TLR2 WT and KO mice differed between individual studies, requiring us to normalize our

data within each independent replicate. Therefore, this method enabled us to pool the results from three independent experiments since the relative ratio of each cell type between TLR2 KO and WT mice remained consistent throughout our independent replicates. As an alternative approach, in some experiments to report immune cell influx into brain abscesses we used the percentage of positive cells for each innate immune cell population (i.e. PMN, macrophages, DCs, and microglia) and pooled data from a minimum of three independent experiments.

Intracellular cytokine staining

To characterize the cytokine expression profiles of infiltrating T cells into brain abscesses of TLR2 KO and WT mice, abscess-associated T cells were collected and evaluated by intracellular cytokine staining and FACS analysis. As described above, TLR2 KO and WT mice harboring brain abscesses were perfused to remove leukocytes from the vasculature and the entire infected hemispheres were collected. Brain tissues were manually disassociated as described above, followed by enzyme digestion and purification using a Percoll gradient. The abscess-associated cells were then incubated with CD90-conjugated magnetic beads and purified using MACS magnetic cell separation columns (Miltenyi Biotec, Auburn CA). Cells recovered from magnetic cell separation columns were stimulated with a cocktail of PMA + ionomycin (Leukocyte Activation Cocktail) in the presence of GolgiPlug™ for 12 h, whereupon cells were incubated with Fc Block™ (all from BD Biosciences) to minimize non-specific antibody binding. Subsequently, T cells were stained with directly-conjugated antibodies against CD4$^+$, CD8$^+$, or γδ TcR, permeabilized with a CytoFix/CytoPerm kit, and stained for intracellular IL-17 or IFN-γ (all from BD Biosciences). Cells were analyzed using a BD FACSAria with compensation set based on the staining of each individual fluorochrome alone (CD4-FITC, CD8-APC, γδ TcR-PE-Cy5, IL17-PE, IFNγ-PE-Cy7) and correction for autofluorescence with unstained cells. Controls included cells stained with directly-conjugated isotype control antibodies to assess the degree of non-specific staining.

Primary glial cell culture, macrophage isolation, and reagents

Primary microglia and astrocytes were isolated from the cerebral cortex of TLR2 KO and WT mice (2–4 days of age) as previously described (43,44). Briefly, when mixed glial cultures reached confluency (7 to 10 days), flasks were shaken at 200 rpm overnight at 37° C to recover microglia. This was repeated for a minimum of three times, whereupon flasks were trypsinized and cells resuspended in culture medium supplemented with 0.1 mM of the microglial cytotoxic agent L-leucine methyl ester (L-LME; Sigma). Astrocytes were cultured in L-LME-containing medium for at least 2 weeks prior to use in experiments and were not utilized until the third passage in culture (approximately day 30 *in vitro*). Microglia and astrocyte cultures were evaluated by immunohistochemical staining using antibodies against CD11b (BD Biosciences) and glial fibrillary acidic protein (GFAP; Invitrogen), with purities approximating 98% and 95%, respectively. We acknowledge the fact that findings obtained with primary astrocyte cultures can often be confounded by the presence of contaminating microglia (45); however, several lines of evidence argue against this possibility using our culture paradigm as previously addressed (46).

Primary macrophages were elicited from the abdominal cavity of C57BL/6 mice four days following an i.p. injection of 4% Brewer's thioglycollate broth. Cells were collected by peritoneal lavage with ice-cold PBS and maintained in RPMI medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT).

To examine whether microglia, astrocytes, and/or macrophages were responsive to IL-17, cells were treated with varying doses of recombinant mouse IL-17 (R & D Systems) in the presence or absence of recombinant mouse IL-1β or TNF-α (both from Biosource, Camarillo, CA). After

a 24 h stimulation period, cell-conditioned supernatants were evaluated for inflammatory mediator release by ELISA.

ELISAs

Brain abscess homogenates or cell culture supernatants from primary glia and macrophages stimulated with IL-17 alone or in combination with IL-1β or TNF-α were analyzed by ELISA for IL-17, IL-17F, CXCL1, and CXCL2 (all from R & D Systems) and IL-1β, IL-12 p40, and CCL2 (all from BD Biosciences) according to the manufacturer's instructions. For quantitating IL-17 and IL-17F expression in brain abscess homogenates, cytokine levels were normalized to the amount of total protein extracted from tissues to correct for differences in sampling size using a BioRad protein assay kit (Hercules, CA).

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA from TLR2 WT and KO glia was isolated using the TriZol reagent and treated with DNAse1 (both from Invitrogen, Carlsbad, CA) prior to use in qRT-PCR studies. The experimental procedure was performed as previously described (7). GAPDH primers and TAMRA Taqman probe were designed as previously described (7,11) and synthesized by Applied Biosystems (ABI, Foster City, CA). ABI Assays-on-Demand™ Taqman kits were utilized to examine IL-17RA, IL-17RC, and β-actin expression. Comparisons in gene expression between TLR2 WT and KO primary glia were calculated after normalizing cycle thresholds against the housekeeping genes GAPDH or β-actin and are presented as the difference in cycle threshold (ΔCt).

Statistics

Significant differences between macrophages treated with IL-17 or TNF-α alone versus a combination of the two cytokines were determined using one-way ANOVA followed by the Holm-Sidak method for multiple pair-wise comparisons with Sigma Stat (SPSS Science, Chicago, IL). Significant differences between all other experimental groups were determined using the unpaired Student's t test with Sigma Stat.

RESULTS

CD4^{+} and CD8^{+} T cell infiltrates are enhanced in TLR2 KO mice

We have previously demonstrated that IL-17 levels were significantly elevated in TLR2 KO mice compared to WT animals from days 3 to 7 post-infection (18). In the current study this interval was extended out to 3 weeks following *S. aureus* infection, the latest time point examined (Figure 1). IL-17 is produced by Th17 cells and is known to play a role in granulopoiesis, PMN recruitment, and pro-inflammatory cytokine production in response to extracellular infections (20,32). Given the elevated levels of a T cell-derived cytokine in brain abscesses of TLR2 knockout mice, it was expected that T cell infiltrates would be elevated in these mice compared to WT animals. To investigate this possibility, we quantitated T cell influx in the brains of infected TLR2 WT and KO mice by collecting abscess-associated cells at days 3, 7, and 14 following infection. Both CD4^{+} and CD8^{+} T cell influx into brain abscesses was greater in TLR2 KO mice compared to WT animals at all time points examined, although only CD8^{+} T cell infiltrates reached statistical significance at day 3 post-infection (Figure 2). The inevitable variability between individual experiments resulted in these differences not reaching statistical significance, although there was a strong trend towards elevated CD4^{+} and CD8^{+} infiltrates in TLR2 KO mice. The frequency of CD4^{+} T cells, in particular, increased from days 3 to 14 post-infection, corresponding with the elevated IL-17 expression detected throughout the course of brain abscess development in TLR2 KO mice (Figure 1). The increase in T cell

infiltrates observed in mice deficient in TLR2 signaling may explain the enhanced IL-17 levels noted in these animals.

IL-17 producing cells are more frequent in brain abscesses of TLR2 KO mice

IL-17 is produced by Th17 cells, recently described as a distinct T cell subset involved in extracellular infections and autoimmune diseases (19,47). Given the high levels of IL-17 and increased T cell infiltrates noted in infected TLR2 KO mice, we next examined the frequency of Th17 cells by intracellular cytokine staining to determine whether a shift in T cell subtypes was observed in brain abscesses of TLR2 KO animals. Abscess-associated T cells were recovered from TLR2 KO and WT mice using CD90-conjugated magnetic beads, whereupon Th17 (IL-17) and Th1 (IFN-γ) cells were quantitated by FACS. At day 14 post-infection, brain abscesses of TLR2 KO mice displayed an increase in the frequency of IL-17-producing Th17 cells (Figures 3 and 4), with relatively few IFN-γ-positive cells present (Figure 4). Although differences were observed between the percentages of IL-17$^+$ and IFN-γ$^+$ populations in TLR2 KO and WT mice at day 7 post-infection, the fold-differences at this earlier time point were not consistently reproducible, whereas changes in the frequency of IL-17$^+$ and/or IFN-γ$^+$ cells were consistent across several independent experiments after two weeks post-infection and typically reached a magnitude of several-fold higher in TLR2 KO mice (Figure 4). A small proportion of dual IL-17- and IFN-γ-producing T cells were also associated with brain abscesses of both TLR2 WT and KO animals (Figure 4) in agreement with earlier reports (48,49). The majority of Th17 cells were observed in the brain, with relatively few Th17 cells detected in the draining cervical lymph nodes (CLNs) or spleens of infected TLR2 KO or WT animals (Figure 4). We also observed a population of IL-17 producing cells that were CD4$^-$ and CD8$^-$ and were more frequent in brain abscesses of TLR2 KO mice (Figure 3). Based on the recent literature, γδ T cells and NK cells have also been reported to express IL-17 (21); therefore, intracellular cytokine staining was repeated utilizing antibodies reactive with each subset (γδ TcR and NK1.1, respectively). From this analysis, we confirmed that CD4$^+$ Th17 cells represent the majority of IL-17-producing cells in brain abscesses (Figure 5). However, γδ T cells were also found to express IL-17, albeit at a reduced frequency compared to CD4$^+$ cells (i.e. ~ 8% versus 28%, respectively; Figure 5). We did not find any evidence for NK cell (NK1.1$^+$) production of IL-17 at the time points examined in this study (data not shown); however, we cannot discount a potential contribution by NKT cells since recent studies have demonstrated IL-17 production by CD4$^-$NK1.1$^-$ cells (25,50,51). The increased proportion of IL-17 producing cells detected in brain abscesses of TLR2 KO mice explains the elevated cytokine levels noted in previous experiments with these animals.

PMN, macrophage and dendritic cell (DC) infiltrates into brain abscesses remain unchanged in TLR2 KO mice compared to WT animals

One function of IL-17 is to recruit PMNs and other innate immune cells to sites of infection by eliciting chemokine production by many parenchymal and immune cell types (20,31,52, 53). In the *S. aureus* brain abscess model, augmented IL-17 levels observed in TLR2 KO mice may serve to facilitate the influx and activation of innate immune cells to control bacterial infection despite the lack of TLR2 signaling. To examine this possibility, we analyzed the brains of infected TLR2 KO and WT mice using 4-color FACS to determine the relative proportions of innate immune cells present. There were no significant differences in the numbers of PMNs, macrophages, DCs, or microglia associated with brain abscesses of TLR2 KO or WT mice at either 7 or 14 days post-infection (Figure 6A and B, respectively). To our knowledge, this study is the first to report the presence of myeloid DCs (CD11b$^+$, CD11c$^+$) in brain abscesses. DCs represented a small percentage of abscess-associated infiltrates; however, these cells did increase in number from day 7 to 14 post-infection (2.75% to 5% respectively, Figure 6). The fact that immune cell influx in TLR2 KO mice is comparable to WT animals in

the face of relatively similar pro-inflammatory mediator levels likely explains the similarity in brain abscess severity between the two strains as demonstrated in our previous study (18).

IL-17 receptor expression patterns in primary glia and macrophages

Unlike the more limited array of cells capable of producing IL-17, IL-17 receptors are present on multiple cell types, including myeloid lineages as well as parenchymal cells in a variety of mouse and human tissues (20). The main isoform of IL-17 (i.e. IL-17A) interacts with IL-17 receptor A (IL-17RA) to transduce activation signals (47,54). Despite two studies demonstrating that IL-17 triggers pro-inflammatory mediator release from microglia and astrocytes (55,56), to our knowledge no reports have described whether astrocytes express IL-17RA, whereas one study has demonstrated IL-17RA in microglia (55). Both primary microglia and astrocytes were found to express IL-17RA mRNA (Figures 7 and 8, respectively and Supplementary Figure 1), suggesting that these cells may be primed to respond to IL-17 in evolving brain abscesses. IL-17RA expression was confirmed at the protein level by FACS where equivalent levels were observed in both primary microglia and macrophages (Figure 9). In contrast, IL-17RA expression on astrocytes was low. Although human IL-17 is capable of binding to both IL-17RA and IL-17RC, recent studies have demonstrated that mouse IL-17 is only capable of engaging IL-17RA (54). Since we were interested in examining other IL-17R family members in glia, we evaluated IL-17RC expression in primary microglia and astrocytes, which to our knowledge, has not yet been reported in the literature. Similar to IL-17RA, IL-17RC expression was roughly equivalent in microglia and macrophages, whereas receptor levels on astrocytes were virtually undetectable (Figure 9). Neither IL-17RA nor IL-17RC expression was altered in response to *S. aureus* stimulation in any of the cell types examined (Figures 7–9). Expression of the ligand for IL-17RC, IL-17F, was transiently elevated in brain abscesses of TLR2 KO mice, albeit with different kinetics compared to IL-17 (Figure 10). Therefore, ligands for both IL-17RA and IL-17RC are upregulated during the course of brain abscess development in TLR2 KO mice.

IL-17 potentiates TNF-α-induced inflammatory mediator release in macrophages but not microglia

As noted above, IL-17 is known to induce pro-inflammatory mediator production both independently and in synergy with TNF-α and IL-1β in diverse parenchymal cell types (28–30,47). In addition to demonstrating the presence of IL-17 receptors in glia, we attempted to establish the *in vitro* effects of IL-17 on astrocyte and microglial activation. Primary glial cultures were stimulated with various concentrations of IL-17 for 24 h, whereupon pro-inflammatory mediator expression was evaluated by ELISA. Of all the molecules examined (CXCL1, CXCL2, CCL2, IL-12 p40, and IL-1β), IL-17 was only capable of inducing the PMN chemokine CXCL1 (KC) in a dose-dependent manner by astrocytes (Figure 11). Interestingly, we did not observe any reproducible effects of IL-17 on the induction of inflammatory products by microglia or macrophages (i.e. CXCL2, CCL2, IL-1β, and TNF-α) despite the fact that they express IL-17RA.

Since IL-17 has been reported to synergize with other pro-inflammatory cytokines such as TNF-α and IL-1β in other cell types (28–30,47), we next examined whether this relationship would hold true for glia. Primary astrocytes and microglia were treated with IL-17 in combination with varying doses of either TNF-α or IL-1β, whereupon conditioned supernatants were evaluated by ELISA. Although TNF-α and IL-1β alone were capable of eliciting the production of a few inflammatory mediators by glia including CXCL2 and CCL2, the addition of IL-17 did not significantly alter the release of these factors (Figure 12 and data not shown). In contrast, IL-17 potentiated TNF-α-induced CXCL2 and CCL2 production in macrophages (Figure 12), which are also relevant to brain abscess pathogenesis since these cells represent a significant constituent of the inflammatory infiltrate in response to infection (39,57,58).

Importantly, only a select number of mediators were augmented by IL-17 in TNF-α-stimulated macrophages (i.e. CXCL2 and CCL2), whereas others were not affected (i.e. IL-12, IL-1β; Figure 12 and data not shown). Interestingly, we failed to detect any synergy with IL-1β and IL-17 in macrophages, regardless of the concentrations examined, revealing specificity in the responses obtained (data not shown). Collectively, the limited response of glial cells to IL-17 stimulation suggests that the primary target of increased IL-17 levels in brain abscesses of TLR2 KO mice may be peripheral immune cells infiltrating the CNS such as macrophages and/or other parenchymal cell types.

DISCUSSION

Toll-like receptors initiate the production of pro-inflammatory mediators that influence the genesis of adaptive immunity against microbes, effectively bridging the innate and adaptive immune responses (4,59). Despite an important role for TLR2 in mediating host defense during systemic Gram-positive infections (17,60), our earlier study using TLR2 KO mice in the experimental brain abscess model did not reveal striking differences compared to WT animals, with one notable exception (18). Namely, IL-17 levels were consistently and markedly elevated in brain abscesses of TLR2 KO mice, a relationship that we extend out to three weeks following infection in the current study. Another novel finding was that a second IL-17 isoform, IL-17F, was also elevated during brain abscess development. These observations served as the basis for the hypothesis that elevated IL-17 expression in the absence of TLR2 could represent a compensatory signal to ensure the genesis of an adequate host anti-bacterial immune response based upon the numerous pro-inflammatory effects ascribed to IL-17 (20).

The major finding of this study was that Th17 influx was augmented in brain abscesses of TLR2 KO mice, which was demonstrated by enhanced CD4$^+$ IL-17 producing cells by intracellular cytokine staining. Our findings demonstrating elevated CD4$^+$ and CD8$^+$ T cell infiltrates in brain abscesses of TLR2 KO mice is in agreement with a recent study by Stenzel et al. (61), providing further evidence that T cell dynamics are indeed altered in the infected CNS of mice lacking TLR2. However, these authors did not investigate IL-17 expression in their study, which remains a novel aspect of the current work. In addition to Th17 cells, γδ T cells were also found to produce IL-17 in brain abscesses, albeit at a much lower frequency than the former. It is worth noting that elevated Th17 influx in TLR2 KO animals appears to represent a non-traditional pathway since although Th17 cells are present in the CNS of infected WT mice, they do not predominate the adaptive response since Th1 cells producing IFN-γ are also a significant component of the T cell infiltrate. A few studies have reported that astrocytes (26,62) and oligodendrocytes (26) produce IL-17 in the context of neuroinflammation. However, in our hands we were not able to detect any production of IL-17 in either microglia or astrocytes in response to *S. aureus* or PGN *in vitro* (data not shown). Possibilities to explain these discrepancies could relate to the context of the inciting stimulus, variations between *in vitro* and *in vivo* studies, and/or species differences since the reports describing IL-17 production by glia were all performed with human cells/tissues (26,62).

One main issue that remains unresolved relates to the mechanism(s) by which TLR2 loss impacts exaggerated Th17 entry into the infected CNS. One possibility is that the absence of TLR2 may alter the cellular response to infection at the level of antigen presenting cells (APCs). For example, the inability to engage TLR2 may re-program APCs towards a phenotype that favors Th17 development by enhancing the production of positive regulators of Th17 cells such as TGF-β, IL-6, and/or IL-23 (33,47,63) or the overexpression of cytokines known to negatively impact Th17 development such as IL-27 (38,64). Alternatively, a reduction in Th1 cytokines that normally antagonize Th17 development (i.e. IFN-γ) would also have a similar net effect in augmenting Th17 levels (33,63). This possibility is supported by our data where IFN-γ-producing T cells were reduced in brain abscesses of TLR2 KO mice concomitant with

an increase in Th17 infiltrates, a reciprocal regulatory relationship that has been described by other laboratories (33,63). Another explanation to account for the skewing towards Th17 infiltrates during brain abscess development in TLR2 KO mice could be directly dictated by effects on the T cells themselves. Several studies have reported TLR2 expression on various T cell populations in the mouse and TLR2 engagement by microbial ligands is capable of T cell activation (65–67). By extension, it is possible that the lack of TLR2 signaling may lead to defects in the autocrine/paracrine regulation of Th17 cells, such as the failure to initiate a negative signal(s) to quell Th17 expansion. An alternative mechanism could be at the transcriptional level, where the absence of TLR2 signals may lead to the inability to trigger/ negatively regulate certain transcription factors (TFs) that promote/repress Th17 development (49,68). However, these possibilities have one important caveat; namely that although it seems clear that mouse T cells express TLR2, the evidence for TLR expression on human T cells is more controversial (65). Because of this apparent lack of TLR2 on human T cells coupled with the observation that IL-17 is a major constituent of the pro-inflammatory response following injury/infection in humans (69,70), it is tempting to speculate that with the loss of TLR2 on T cells from KO mice we have in effect made these cells more like their human counterparts. A schematic summarizing the potential mechanisms of exaggerated IL-17 expression in brain abscesses of TLR2 KO mice is presented in Figure 13. The potential identity of a non-TLR2 receptor(s) for PGN in our experimental model is currently speculative. One candidate is the intracellular PRR nucleotide-binding oligomerization domain 2 (NOD2), which has been implicated in the recognition of PGN breakdown products (i.e. muramyl dipeptide; MDP) from Gram-positive bacteria such as *S. aureus* (71–73). Although NOD2 is a cytoplasmic PRR, studies have demonstrated that the receptor is capable of being engaged following the application of its ligand extracellularly (72,73). Currently, the mechanism(s) by which NOD ligands enter the cell for direct engagement of intracellular PRRs such as NOD2 is unknown; however, possibilities including cytoplasmic translocation via a MDP-specific transporter on the plasma membrane (i.e. PepT1) or cytoplasmic release from phagolysosomes have been proposed (74–76). Since PGN is an extensively cross-linked insoluble polymer with a heterogeneous structure, it is conceivable that it is engulfed via a phagocytic pathway, whereupon degradation products could be liberated to engage intracellular PRRs such as NOD2. It is not known whether MDP is present in the extracellular milieu during brain abscess infection; however, this is plausible since *S. aureus* expresses a wide array of PGN hydrolases that serve to regulate bacterial cell death and lysis (77–79).

An interesting relationship between Th17 and CD8+ T cells has emerged based on the production of IL-21 by the former, which serves as a potent regulator of CD8+ T cell proliferation (20,80,81). Since Th17 infiltrates are augmented in brain abscesses, IL-21 release by these cells may represent a positive feedback loop to augment CD8+ T cell expansion. This possibility would explain the finding that CD8+ T cell infiltrates were also increased in brain abscesses of TLR2 KO mice.

IL-17RA and IL-17RC are two receptor isoforms that are responsible for mediating effects via IL-17 and IL-17F, respectively (20,47). To our knowledge, the current study represents the first report of IL-17RC expression in either microglia or astrocytes, despite a few previous reports describing glial responsiveness to IL-17 (55,56). Despite earlier studies demonstrating that IL-17 was capable of inducing pro-inflammatory mediator release from microglia and astrocytes (55,56), we observed very limited effects of IL-17 on glial activation, even in the context of other inflammatory molecules (i.e. TNF-α and IL-1β) where synergistic effects have been reported in other cell types (20,27–30). The low levels of IL-17 responsiveness in primary glia suggest that alternative cell types within the infected CNS may be responsive to IL-17 such as infiltrating macrophages. Indeed, we demonstrated that unlike glia, IL-17 potentiated TNF-α-induced chemokine production in macrophages. Interestingly, IL-17RA was expressed at equivalent levels in both microglia and macrophages, although the former was not capable

of producing inflammatory mediators in response to IL-17 in our hands. This suggests that receptor expression does not directly equate with cytokine responsiveness and the mechanism behind the relative inability of microglia to respond to IL-17 is unknown. Since IL-17 has been proposed to signal through a heterodimeric receptor composed of IL-17RA and IL-17RC (82), it is possible that there are differences in receptor combinations between glia and macrophages (83). Alternatively, signaling and/or adaptor proteins downstream of IL-17Rs may be differentially expressed within these cell types. However, we cannot discount a role for IL-17 in regulating other functions of glia that were not examined in this study including phagocytosis, chemotaxis, and/or increased responsiveness to other inflammatory signals.

The biological effects of IL-17 make it an ideal candidate for the amplification of CNS antibacterial immune responses in the absence of TLR2 signaling. For example, IL-17 is a potent inducer of PMN chemokines (20,29,84–86) and previous work from our laboratory has identified PMNs as an essential immune effector population during brain abscess development (87). Also IL-17 can act synergistically with other inflammatory mediators to induce the expression of a wide array of inflammatory mediators including cytokines, prostaglandin metabolites, and reactive oxygen/nitrogen intermediates (20). Alternative products produced by Th17 cells, such as IL-21 and IL-22 may provide further amplification of inflammatory networks in the brain. Collectively, these possibilities may contribute to the fact that in our hands, brain abscess severity is remarkably similar between TLR2 KO and WT mice (18), a finding that we attribute to the redundancy of infiltrating Th17 cells. Directly implicating the potential compensatory actions of IL-17 will require future studies where the cytokine is specifically neutralized in the CNS of TLR2 KO mice or by crossing TLR2 KO with IL-17R KO animals. This line of research is ongoing in our laboratory.

In summary, the current study demonstrates several novel aspects related to brain abscess pathogenesis in TLR2 KO mice. Namely, Th17 infiltrates are elevated in TLR2 KO animals as revealed by intracellular cytokine staining and the fact that primary microglia and astrocytes express both IL-17RA and IL-17RC, although not at equivalent levels.γδ T cells were also identified as another source of IL-17 in the infected brain. In addition, low levels of DC influx were observed in brain abscesses with numbers increasing progressively from day 7 to 14 post-infection. Although DC entry has been associated with other CNS infectious diseases (88, 89), to our knowledge, DC influx has not yet been described in brain abscesses. Collectively, we propose that the exaggerated Th17 influx into brain abscesses of TLR2 KO mice represents a compensatory mechanism to circumvent the loss of this important pattern recognition receptor to ensure the induction of an effective anti-bacterial immune response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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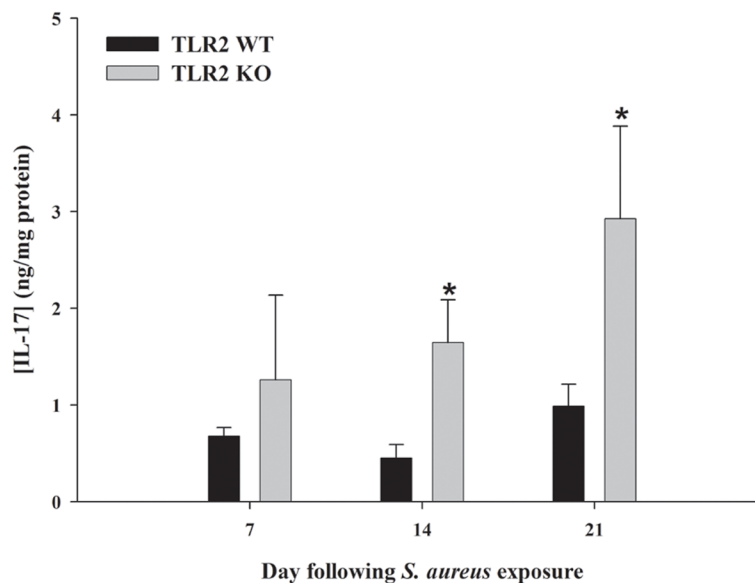
Figure 1. IL-17 production is augmented in brain abscesses of TLR2 KO mice out to 3 weeks post-infection

TLR2 KO and WT mice (n = 4 to 6 per group) were infected with *S. aureus* intracerebrally as described in the Materials and Methods. Animals were euthanized at 7, 14, or 21 days following bacterial exposure, whereupon IL-17 expression was quantitated by ELISA. Results were normalized based on the amount of total protein recovered from abscesses to correct for differences in tissue sampling size. Significant differences in IL-17 expression between brain abscesses TLR2 KO and WT mice are indicated by asterisks (* $p < 0.05$). Results are representative of two independent experiments.
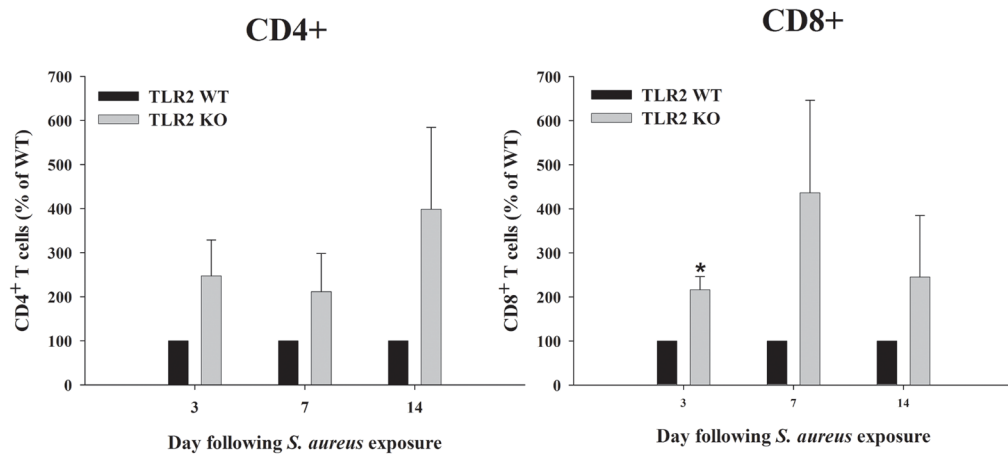
Figure 2. CD4$^+$ and CD8$^+$ T cell infiltrates are enhanced in TLR2 KO mice
Abscess-associated cells were recovered from TLR2 KO and WT mice at the indicated days post-infection (n = 4 to 6 animals per group), whereupon CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells were quantitated by FACS. Significant differences between TLR2 KO and WT mice are denoted with asterisks (*, $p < 0.05$). Results represent the mean ± SEM from 3–4 independent experiments.
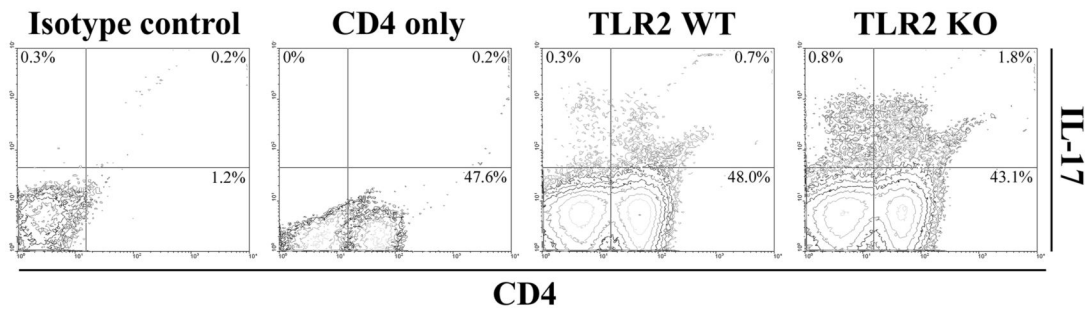
Figure 3. The frequency of CD4⁺ Th17 cells is increased in brain abscesses of TLR2 KO mice

T cells were recovered from brain abscesses of TLR2 KO and WT mice at day 14 following *S. aureus* exposure using CD90-conjugated magnetic beads and stimulated with a cocktail of PMA + ionomycin for 12 h in the presence of GolgiBlock™, whereupon IL-17 production was quantitated by intracellular cytokine staining and FACS. The results presented are representative of three independent experiments.
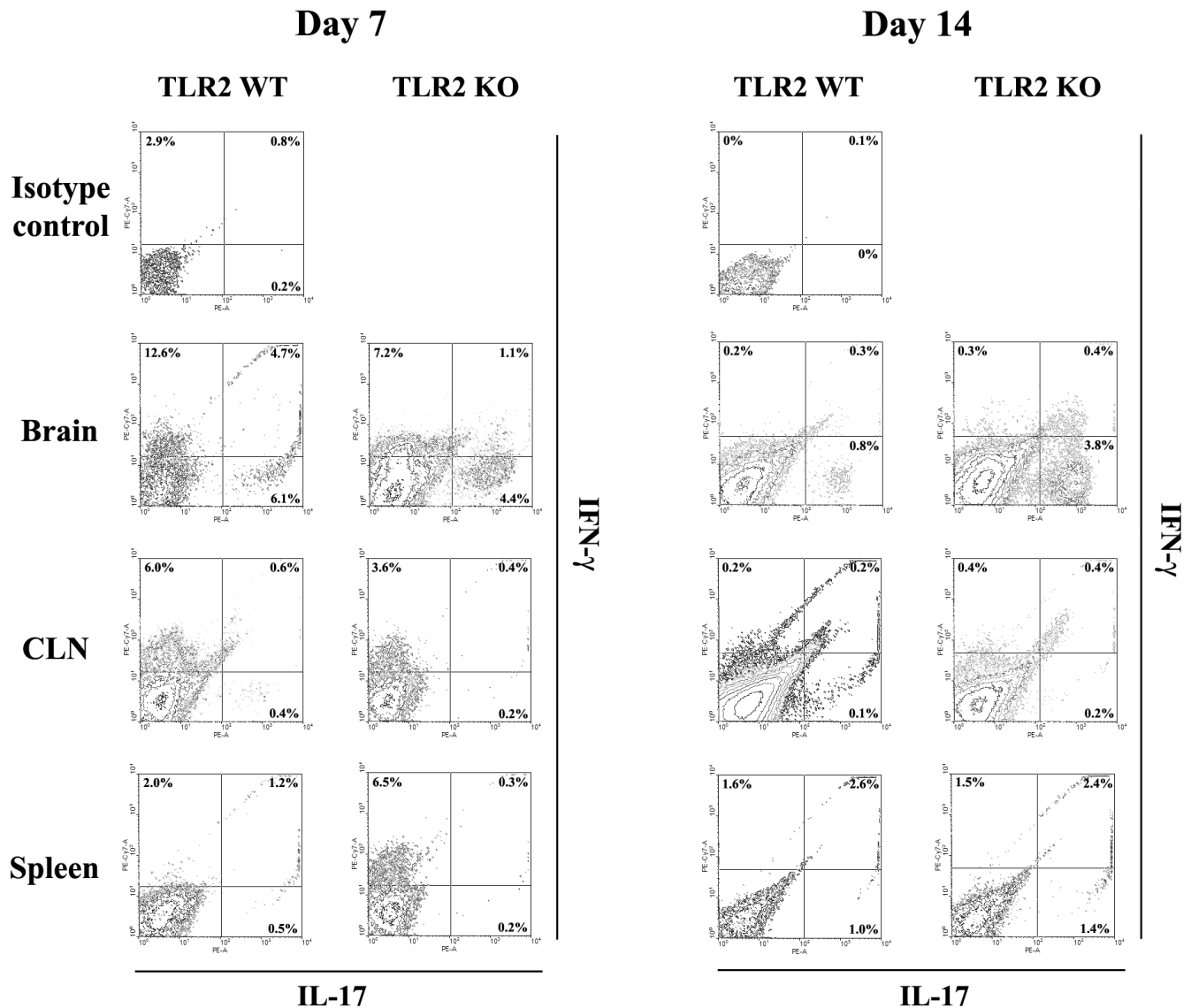
**Figure 4. IL-17-producing cells are more frequent in brain abscesses of TLR2 KO mice**
T cells were recovered from brain abscesses, draining cervical lymph nodes (CLN), and spleens
of TLR2 KO and WT mice at days 7 and 14 following *S. aureus* exposure using CD90-
conjugated magnetic beads and stimulated with a cocktail of PMA + ionomycin for 12 h in the
presence of GolgiBlock™, whereupon IL-17 and IFN-γ production was quantitated by
intracellular cytokine staining and FACS. The results presented are representative of three
independent experiments.
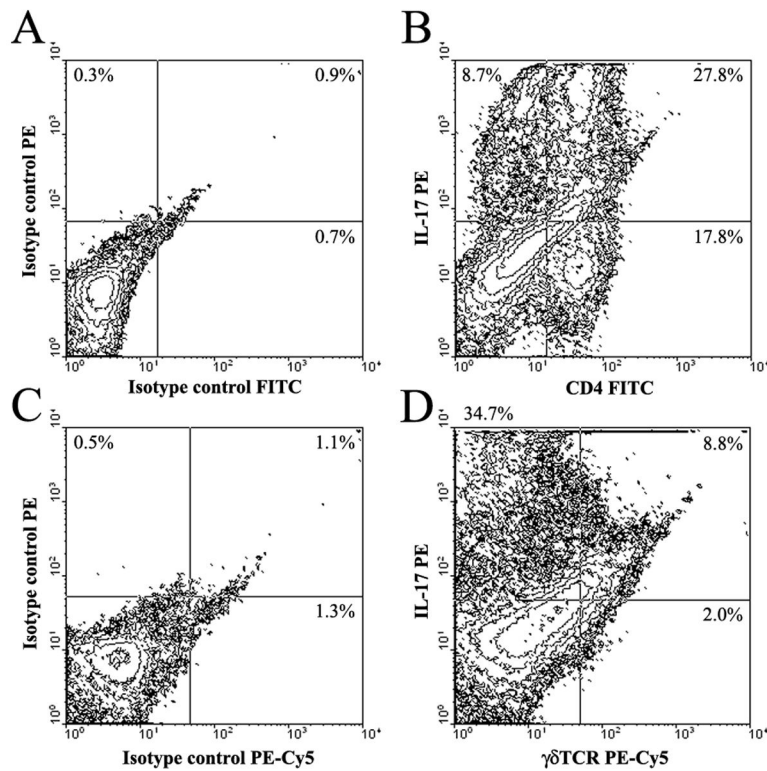
Figure 5. γδ T cells are a source of IL-17 in brain abscesses
Cells were recovered from brain abscesses of C57BL/6 wild type mice at day 14 following *S. aureus* exposure using CD90-conjugated magnetic beads and stimulated with a cocktail of PMA + ionomycin for 12 h in the presence of GolgiBlock™, whereupon IL-17 production in CD4$^+$ Th17 or γδ T cells was quantitated by intracellular cytokine staining. The results presented are representative of two independent experiments.
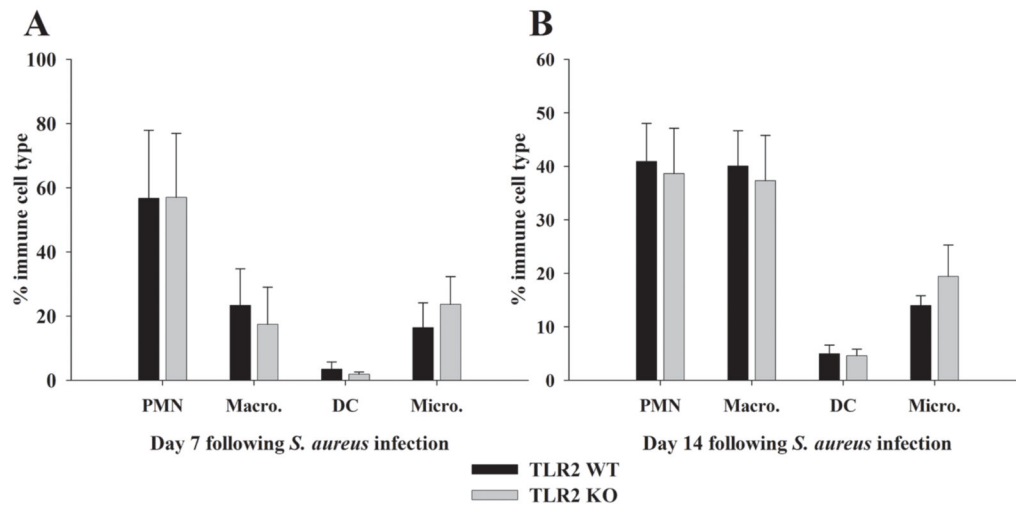
Figure 6. Innate immune cell infiltrates into brain abscesses of TLR2 KO and WT mice are equivalent

Abscess-associated cells were recovered from TLR2 KO and WT mice at days 7 and 14 following *S. aureus* infection using a Percoll gradient method and analyzed by FACS. Results are presented as the percent positive cells for each population pooled from three independent experiments (mean ± SEM).
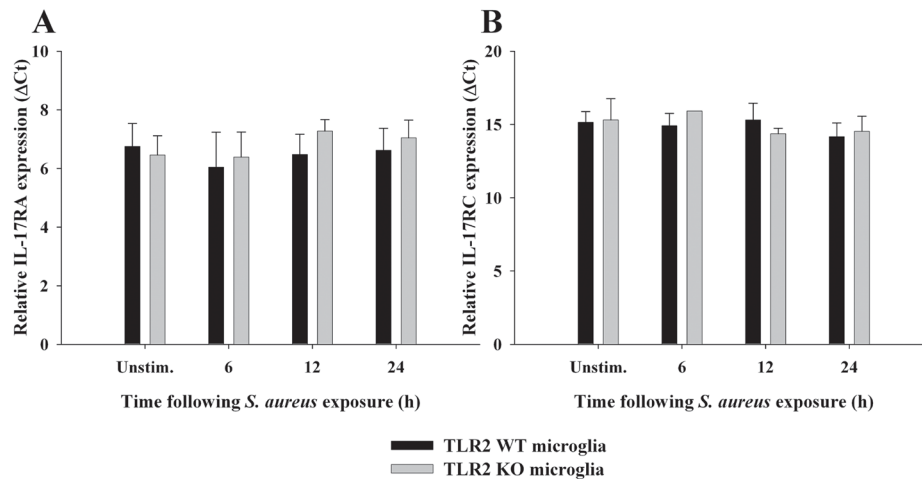
Figure 7. IL-17R isoform expression in microglia
TLR2 KO and WT microglia were seeded at 2×10^6 cells per well in 6-well plates and incubated overnight. The following day, cells were stimulated with 10^7 heat-inactivated *S. aureus* for 6, 12, or 24 h, whereupon total RNA was isolated and examined for IL-17RA (A) and IL-17RC (B) expression by qRT-PCR. Gene expression levels were calculated after normalizing IL-17R signals against GAPDH and are presented in relative mRNA expression units (mean ± SEM of two independent experiments).
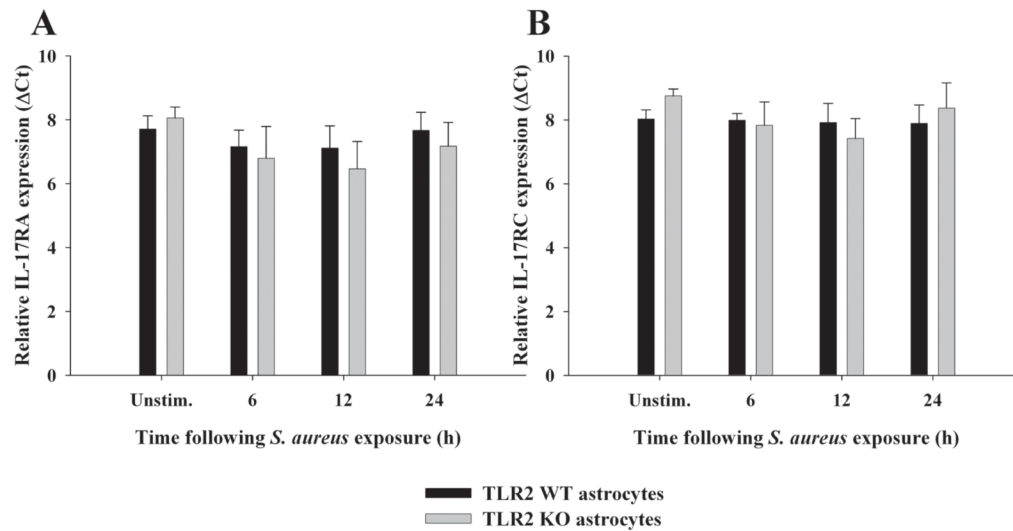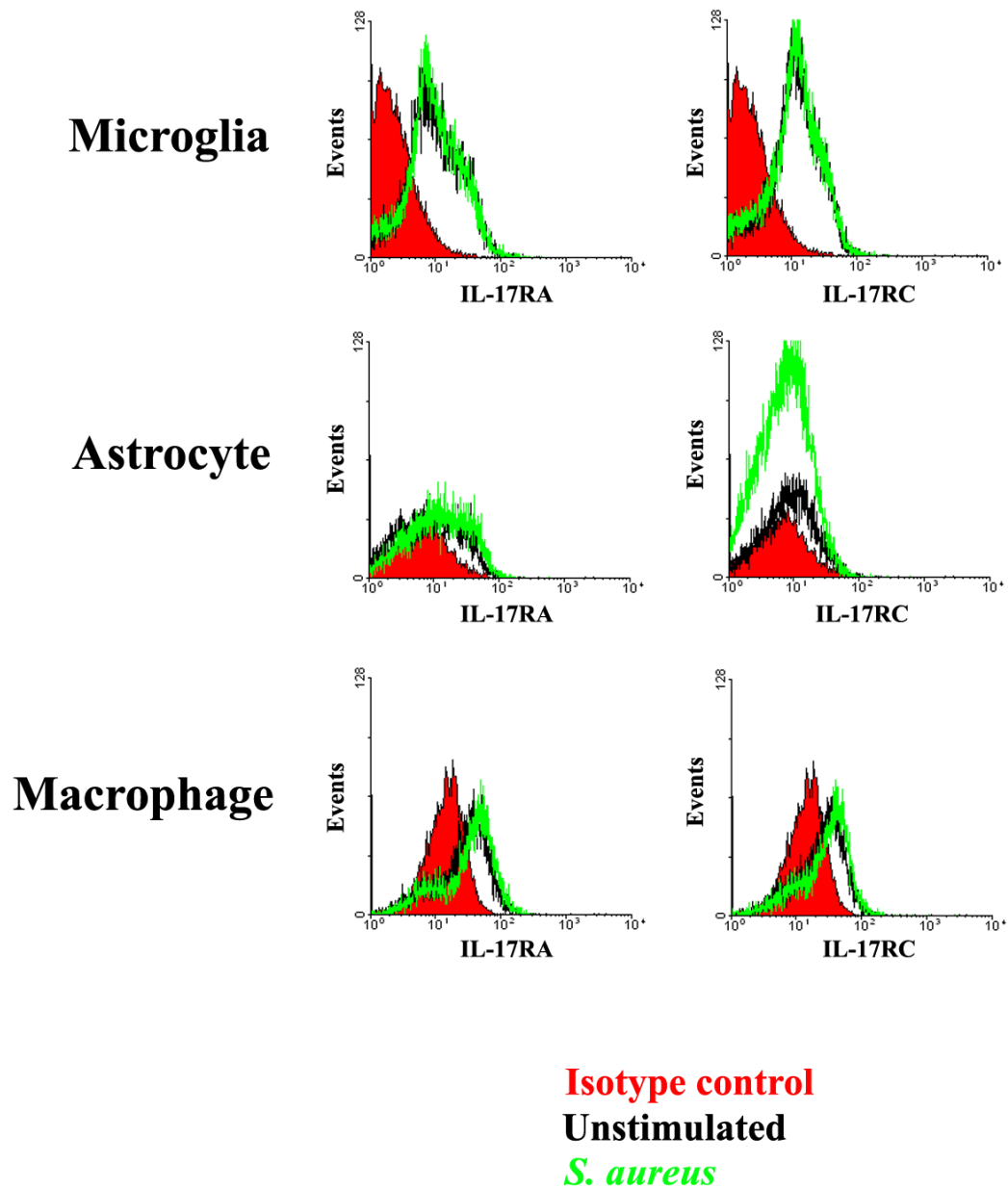
Figure 8. IL-17R isoform expression in astrocytes
TLR2 KO and WT astrocytes were seeded at 1×10^6 cells per well in 6-well plates and incubated overnight. The following day, cells were stimulated with 10^7 heat-inactivated *S. aureus* for 6, 12, or 24 h, whereupon total RNA was isolated and examined for IL-17RA (A) and IL-17RC (B) expression by qRT-PCR. Gene expression levels were calculated after normalizing IL-17R signals against GAPDH and are presented in relative mRNA expression units (mean ± SEM of two independent experiments).

Microglia

Astrocyte

Macrophage

Isotype control
Unstimulated
S. aureus

Figure 9. Comparison of IL-17R isoform expression in microglia, astrocytes, and macrophages
Primary microglia, astrocytes, and macrophages from C57BL/6 wild type mice were stimulated with 10^7 heat-inactivated *S. aureus* for 24 h, whereupon cell surface expression of IL-17RA and IL-17RC was analyzed by FACS.
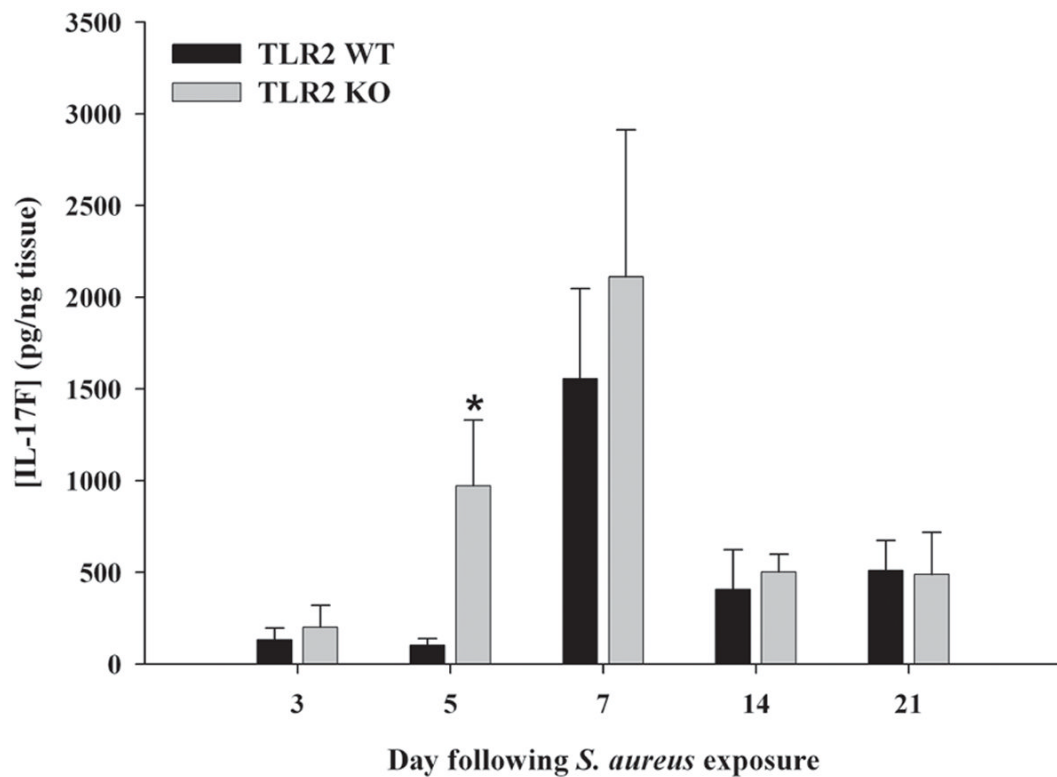
Figure 10. IL-17F production is transiently elevated in brain abscesses of TLR2 KO mice
TLR2 KO and WT mice (n = 3 to 5 per group) were infected with *S. aureus* intracerebrally as described in the Materials and Methods. Animals were euthanized at the indicated time points following bacterial exposure, whereupon IL-17F expression was quantitated by ELISA. Results were normalized based on the amount of total protein recovered from abscesses to correct for differences in tissue sampling size. Significant differences in IL-17F expression between brain abscesses TLR2 KO and WT mice are indicated by asterisks (* $p < 0.05$). Results are representative of two independent experiments.
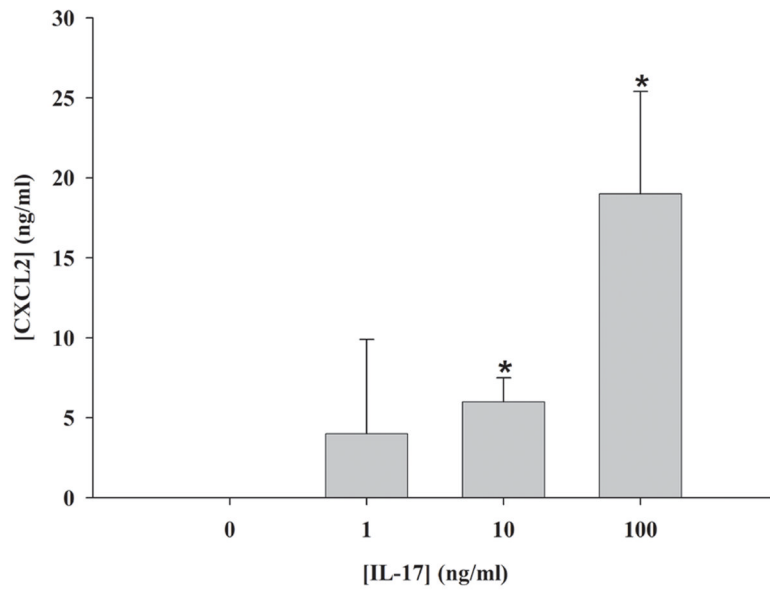
Figure 11. IL-17 induces expression of the PMN chemokine CXCL2 in astrocytes
C57BL/6 wild type astrocytes were treated with the indicated concentrations of recombinant
mouse IL-17 for 24 h, whereupon CXCL1 (KC) levels were quantitated by ELISA. Significant
differences between unstimulated and IL-17-treated astrocytes are denoted with asterisks (*,
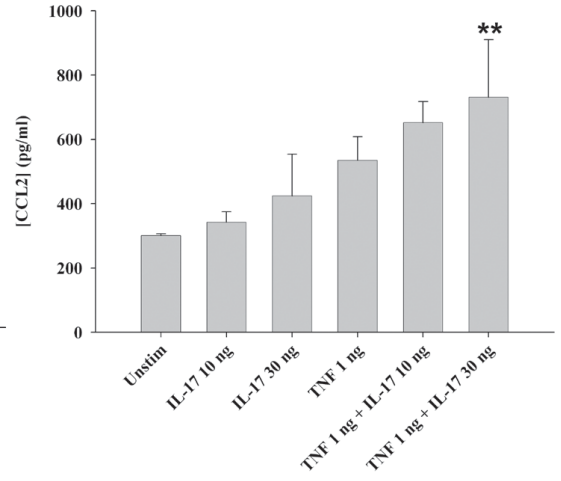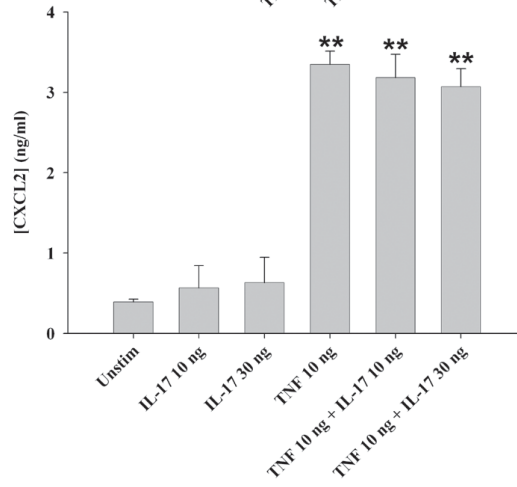$p < 0.05$). Results are representative of two independent experiments.
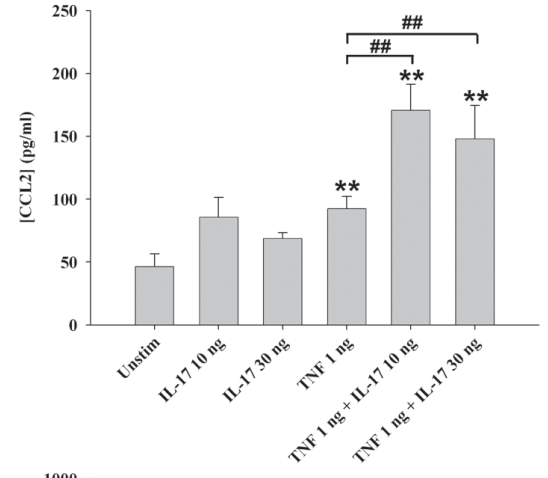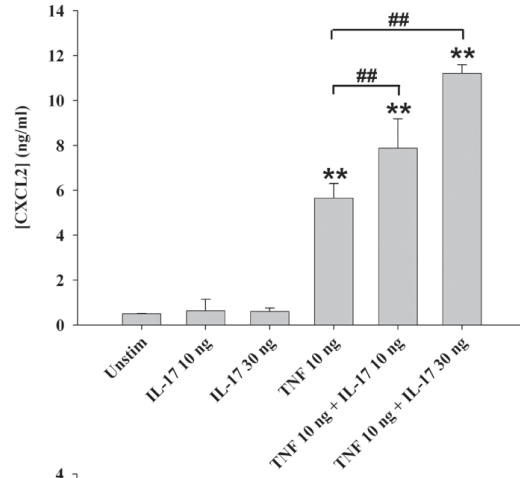
Figure 12. IL-17 potentiates TNF-α-induced chemokine expression in macrophages
Primary microglia or macrophages isolated from C57BL/6 wild type mice were treated with
the indicated concentrations of IL-17 or TNF-α or in combination for 24 h, whereupon CXCL2
and CCL2 production was quantitated by ELISA. Significant differences between unstimulated
versus cytokine-treated cells are indicated by asterisks (**, $p < 0.001$), whereas significant
differences between cells exposed to TNF-α versus TNF-α IL-17 are denoted by hatched signs
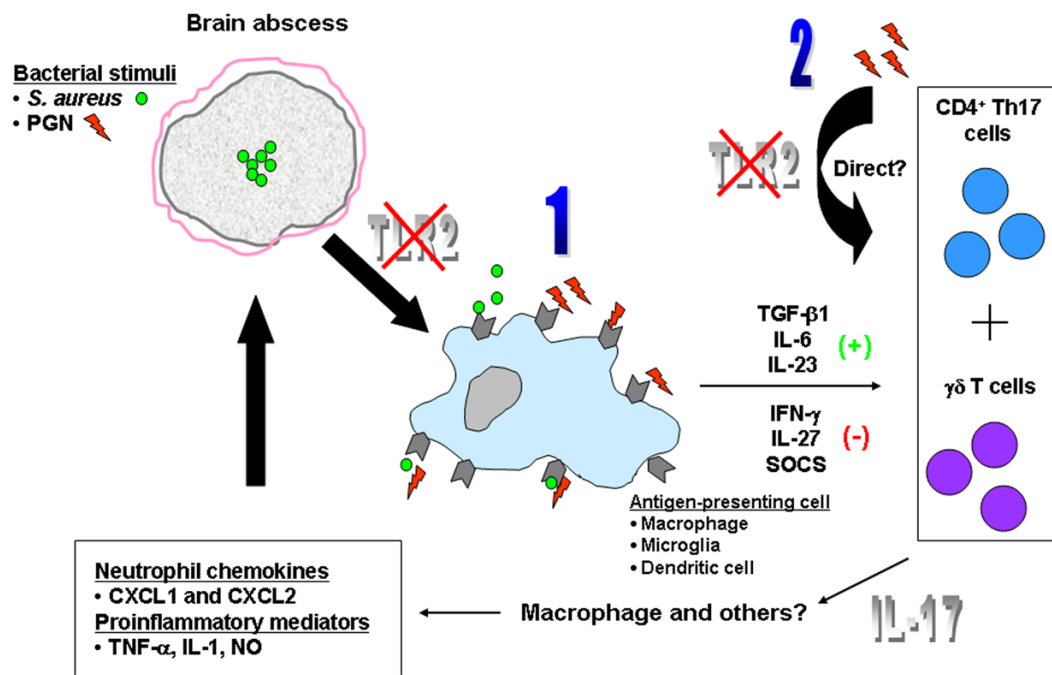(##, $p < 0.001$).

Figure 13. Potential mechanisms of exaggerated IL-17 expression in brain abscesses of TLR2 KO mice

The absence of TLR2 signaling could lead to elevated IL-17 expression in a number of potential ways including (1) changes in APC properties or (2) direct effects on T cell populations. Based on the pro-inflammatory effects of IL-17, we propose that this response compensates for the loss of TLR2 signaling to result in an effective host anti-bacterial immune response.