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## Roles of Toll-like receptor 2 (TLR2) and superantigens on adaptive immune responses during CNS staphylococcal infection

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### Abstract

*Staphylococcus aureus* is a common etiologic agent of brain abscesses and possesses numerous virulence factors that manipulate host immunity. One example is superantigens (SAG) that clonally expand T cell subsets bearing specific V $\beta$  receptors. Toll-like receptor 2 (TLR2) is one receptor implicated in *S. aureus* recognition. However, the interplay between TLR2, SAG, and adaptive immunity during brain abscess formation has not yet been investigated and could reveal novel insights into host-pathogen interactions for regulating protective immunity. A comprehensive analysis of abscess-associated T cell populations in TLR2 KO and WT mice was performed following infection with a *S. aureus* clinical isolate. Both natural killer T (NKT) and  $\gamma\delta$  T cell infiltrates were increased in brain abscesses of TLR2 KO mice and produced more IL-17 and IFN- $\gamma$  compared to WT populations, which could have resulted from elevated bacterial burdens observed in these animals. Analysis of SAG-reactive T cells revealed a predominant V $\beta_{8,1,8,2}$  infiltrate reactive with staphylococcal enterotoxin B (SEB), whereas SEA-reactive V $\beta_{11}$  T cells were less numerous. Brain abscesses of TLR2 KO mice had fewer V $\beta_{8,1,8,2}$  and V $\beta_{11}$  T cells and produced less TNF- $\alpha$  and IFN- $\gamma$  compared to WT animals. Treatment of primary microglia with purified SEB augmented TNF- $\alpha$  production in response to the TLR2 ligand Pam3Cys, which may serve to amplify proinflammatory cascades during CNS *S. aureus* infection. Collectively, these studies demonstrate that TLR2 impacts adaptive immunity to *S. aureus* infection and modulates SAG responses.

### Keywords

Toll-like receptor 2; *Staphylococcus aureus*; superantigen; staphylococcal enterotoxin B; microglia; natural killer T cells;  $\gamma\delta$  T cells

## INTRODUCTION

Brain abscesses continue to be associated with significant morbidity and mortality, despite recent therapeutic advances (Greenberg, 2008). In addition, long-term morbidity issues arise in patients recovering from these infections as a result of the extensive parenchymal damage typically associated with brain abscess formation, which can manifest as seizures, cognitive

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deficits, and/or hemiparesis (Davis and Baldwin, 1999; Greenberg, 2008; Lu et al., 2006). Due to the emergence of multi-drug resistant isolates such as methicillin-resistant *S. aureus* (MRSA) and the ubiquitous nature of bacteria, these CNS infections are likely to persist (Jones et al., 2004; Naesens et al., 2009). Indeed, epidemiological studies have identified *S. aureus* as one of the most common isolates associated with brain abscesses in humans, with streptococcal species being another frequent etiologic agent of infection (Carpenter et al., 2007; Jones et al., 2004; Prasad et al., 2006). This fact substantiates our need to understand the complexities related to host-pathogen immune dynamics in the brain if we are to be successful at developing novel therapeutics to combat these devastating infections.

Brain abscesses form in response to a parenchymal infection with pyogenic bacteria (Mathisen and Johnson, 1997; Townsend and Scheld, 1998). When pathogenic organisms enter the CNS, an acute edematous response ensues typified by localized microglial and astrocyte activation (Baldwin and Kielian, 2004; Kielian, 2004). The infection culminates in the formation of a mature abscess characterized by extensive necrosis and surrounded by a fibrous capsule. Our laboratory has established a mouse model of experimental brain abscess, which has helped define the basic innate immune pathways involved in abscess pathogenesis and served as a tool to query novel therapeutic agents (Kielian et al., 2001; Kielian et al., 2008). Although we better understand innate immune responses elicited during brain abscess development (Garg et al., 2009; Kielian et al., 2004a; Kielian et al., 2007b), relatively little is known about the impact of adaptive immunity in bacterial clearance and tissue injury.

Antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells exist at very low frequencies in naïve hosts. After infection, antigen-reactive naïve T cells undergo clonal expansion resulting in a higher frequency of antigen-specific cells with enhanced effector functions. Upon activation, naïve CD4<sup>+</sup> T cells differentiate into Th1, Th2, or Th17 cells depending on the cytokine milieu (Bettelli et al., 2007). In general, with regard to infectious diseases, Th1 cells are required for controlling intracellular infections, Th2 cells combat parasitic infections, and Th17 cells have been implicated in regulating infections caused by extracellular pathogens (Dong, 2008). In contrast, activated CD8<sup>+</sup> T cells are cytotoxic and mediate their effector functions through the production of cytokines such as TNF- $\alpha$  as well as perforin granules (Seder, 1999). NKT cells possess semi-invariant TCRs, which are more like the conserved pattern recognition receptors of innate immune cells and participate in anti-microbial defense (Balato et al., 2009; Godfrey et al., 2010; Tupin et al., 2007).  $\gamma\delta$  T cells are a unique subset that have been postulated to link innate and adaptive immunity by using their TCR as a pattern recognition receptor, which recognizes microbial peptide antigens eliciting IL-17 and IFN- $\gamma$  release (Born et al., 2010; Deknuydt et al., 2009). To date, the relative frequencies of these various T cell populations and their dynamics of entry/egress into brain abscesses have not yet been investigated.

Activation of the adaptive immune response during bacterial infections leads to a skewing of the V $\beta$  TCR repertoire (Li et al., 1999; Papageorgiou and Acharya, 2000). The V $\beta$  repertoire that is expanded can be predicted based upon preferential TCR affinity of microbial superantigens (SAG). Superantigens are bacterial or viral protein toxins with potent immunostimulatory properties. In contrast to conventional TCR antigens, SAG bind to MHC class II molecules on antigen presenting cells outside the binding groove where they directly cross-link T cell subsets expressing preferential V $\beta$  genes (Li et al., 1999; Papageorgiou and Acharya, 2000). This non-classical mode of T cell activation allows SAGs to activate large fractions of the T cell population (i.e. polyclonal response involving as many as 1 in 5 cells), a significantly higher frequency compared to conventional T cell activation (i.e. monoclonal activation; one in 10<sup>5</sup>–10<sup>6</sup> cells). Superantigen-induced T cell activation leads to the heightened release of proinflammatory cytokines such as TNF- $\alpha$  that play a role in the

induction of toxic shock syndromes often associated with bacterial infections. *S. aureus* strains can produce several well characterized SAGs including staphylococcal enterotoxin A (SEA) and SEB, which preferentially lead to the expansion of  $V\beta_{11}$  and  $V\beta_{8.1,8.2}$  T cell subsets, respectively (Li et al., 1999; Papageorgiou and Acharya, 2000). With regard to human disease, many *S. aureus* clinical isolates are high toxin producers (including SEA and SEB)(Dinges et al., 2000; Monk et al., 2004), indicating that the evaluation of SAG involvement during CNS *S. aureus* infection is clinically relevant.

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) that recognize invariant pathogen motifs and elicit downstream inflammatory mediator production (Akira, 2006). In addition, TLR signaling is critical for the initiation of adaptive immunity in response to infectious pathogens (Akira et al., 2001; Pasare and Medzhitov, 2004). With regard to *S. aureus*, TLR2 has been identified as a critical sensor of numerous bacterial motifs including lipoteichoic acids, lipoproteins, and peptidoglycan. We and others have previously demonstrated alterations in innate immune parameters in TLR2 KO mice during brain abscess development (Kielian et al., 2005b; Stenzel et al., 2008); however, relatively little information is currently available regarding the impact of TLR2 on adaptive immunity and whether this PRR participates in the response to bacterial SAGs. The current study demonstrates that the loss of TLR2-dependent signaling enhances NKT and  $\gamma\delta$  T cell recruitment into brain abscesses, which may be an attempt to facilitate *S. aureus* clearance that is delayed in TLR2 KO mice. In addition, *S. aureus* infection induced  $V\beta_{8.1,8.2}$  T cell entry into brain abscesses, which are reactive with staphylococcal enterotoxin B (SEB), whereas few  $V\beta_{11}$  T cells were detected. Interestingly, the number of both  $V\beta$  subsets was lower in abscesses from TLR2 KO mice and produced less TNF- $\alpha$  and IFN- $\gamma$  compared to  $V\beta$  T cells recovered from WT animals. Finally, SEB was found to augment TNF- $\alpha$  production in response to the TLR2 ligand Pam3Cys, suggesting a potential mechanism for SAG to augment CNS innate immune responses in addition to its ability to clonally expand  $V\beta_{8.1,8.2}$  subsets. Collectively, these findings demonstrate the complexity of the CNS response to a *S. aureus* clinical isolate and reveal a link between TLR2, SAG, and  $V\beta_{8.1,8.2}$  and  $V\beta_{11}$  T cells during brain abscess development.

## 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 minimum of eight generations prior to use in these studies. Age- and sex-matched C57BL/6 mice (Charles River, Frederick, MD) were used as WT controls. For all brain abscess studies, TLR2 KO and WT mice were used between 10 and 12 weeks of age.

### Generation of experimental brain abscesses

Brain abscesses were induced by the stereotactic intracerebral injection of a methicillin-resistant *S. aureus* (MRSA) USA300 strain encapsulated in agarose beads as previously described (Kielian et al., 2007b). This USA300 isolate was recovered from an otherwise healthy individual that died from a brain abscess (Sifri et al., 2007). 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 striatum 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 10  $\mu$ l Hamilton syringe fitted with a 26-gauge needle was used to slowly deliver 2  $\mu$ l of *S. aureus*-laden beads ( $2 \times 10^3$  to  $5 \times 10^3$

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 abscess-associated T cell subsets

To determine whether the loss of TLR2-dependent signals impacted the influx of various T cell subsets into the infected CNS, abscess-associated T cell subsets were quantitated by FACS analysis as previously described (Carson et al., 1998; Ford et al., 1995; Kielian et al., 2007b; Renno et al., 1995). 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  $\mu$ m nylon mesh cell strainer using a rubber policeman. At this point, an aliquot of tissue homogenate from each animal was collected to quantitate bacterial burdens. Next, the resulting slurry was digested for 30 min at 37° C in HBSS supplemented with 2 mg/ml collagenase type I and 28 U/ml DNase I (both from Sigma-Aldrich, St. Louis, MO) 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 multi-color FACS including CD4-PECy5, CD8-APC, NK1.1-FITC, and TCR  $\gamma\delta$ -PE to detect CD4<sup>+</sup> Th, CD8<sup>+</sup>, NKT (CD4<sup>+</sup>, NK1.1<sup>+</sup>) and  $\gamma\delta$  T cells. Recent studies from our laboratory have established that NK1.1<sup>+</sup> cells infiltrating brain abscesses represent NKT and not NK cells (Mariani and Kielian, manuscript in preparation). 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 and correction for autofluorescence with unstained cells. During analysis, events were gated based on a total lymphocyte gate. Controls included cells stained with isotype control antibodies to assess the degree of non-specific staining. Results are presented as the percentage of each cell population recovered from brain abscesses of TLR2 KO or WT mice as well as the absolute number of T cells recovered, with normalization to adjust for the recovery of different cell numbers from the two mouse strains.

### Quantitation of SAG-reactive V $\beta$ <sub>8.1,8.2</sub> and V $\beta$ <sub>11</sub> T cells in brain abscesses and intracellular cytokine staining

To assess the number of V $\beta$ <sub>8.1,8.2</sub> and V $\beta$ <sub>11</sub> T cells reactive with SEB and SEA, respectively, and whether the frequency of either population was altered in TLR2 KO mice, abscess-associated V $\beta$ <sub>8.1,8.2</sub> and V $\beta$ <sub>11</sub> T cells were collected and evaluated by intracellular cytokine staining. 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. Abscess-associated cells were stained with CD4-AlexaFluor700 and CD4<sup>+</sup> T cells purified using a FACSAria (BD Biosciences). FACS-purified CD4<sup>+</sup> T cells were immediately stimulated with a cocktail

of PMA + ionomycin (Leukocyte Activation Cocktail) in the presence of GolgiPlug™ for 4 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 Vβ<sub>8,1,8,2</sub> and Vβ<sub>11</sub> (both conjugated to FITC; BD Biosciences), fixed and permeabilized with BD Cytotfix/Cytoperm Fixation and Permeabilization Solution Kit (BD Bioscience), and stained for intracellular TNF-α or IFN-γ using anti-TNF-α eFluor450® (eBioscience, San Diego, CA) and anti-IFN-γ-PE-Cy7 (BD Biosciences) antibodies. Cells were analyzed using a BD LSRII with compensation set based on the staining of each individual fluorochrome alone 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. During analysis, events were collected within the CD4<sup>+</sup> gate and results are reported as the absolute number of Vβ<sub>8,1,8,2</sub> and Vβ<sub>11</sub> T cells recovered from brain abscesses of TLR2 KO or WT mice, with normalization to adjust for the recovery of different cell numbers from the two mouse strains.

### Quantitation of abscess-associated bacterial burdens

To quantitate the numbers of viable bacteria associated with brain abscesses of TLR2 KO and WT mice, serial 10-fold dilutions of abscess homogenates were plated onto blood agar plates. Titers were calculated by enumerating colony growth and are expressed as colony forming units (CFU) per gram wet tissue weight.

### Western blotting

To demonstrate whether the superantigens SEA or SEB were expressed by *S. aureus* during brain abscess development, Western blots were performed as previously described (Kielian et al., 2005a). A total of 20 μg protein from brain abscess homogenates was run on 10% SDS-PAGE gels and following transfer, blots were probed with antibodies specific for SEA (Sigma) and SEB (Santa Cruz, San Diego, CA). Purified SEA and SEB (both from Sigma) were included in Western blots as positive controls to demonstrate antibody specificity.

### Effects of *S. aureus* superantigens on microglial activation

Primary microglia were isolated from the brains of neonatal C57BL/6 mice (2–4 days of age) as previously described (Esen and Kielian, 2007). To determine the effects of staphylococcal SAG on microglial activation, microglia were exposed to purified SEA or SEB in the presence or absence of the TLR2 ligand Pam3Cys (Invivogen, San Diego, CA) to assess whether SAG exhibited any additive/synergistic actions with a PAMP relevant to *S. aureus*. In some experiments, microglia were pre-treated with 100 ng/ml recombinant mouse IFN-γ (BD Biosciences) for 24 h prior to SAG exposure to augment MHC Class II expression, to examine whether SAG effects would be enhanced. Microglia were exposed to Pam3Cys ± SEA/SEB for 24 h, whereupon conditioned supernatants were collected for downstream ELISA analysis. The effects of SEA/SEB on microglial viability were evaluated by the ability of mitochondria to convert the substrate (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MTT) into formazan crystals as previously described (Kielian et al., 2004b).

### Enzyme linked immunosorbent assay (ELISA)

Quantitation of TNF-α levels in conditioned supernatants from microglia treated with Pam3Cys ± SEA/SEB was performed using a standard sandwich ELISA kit according to the manufacturer's instructions (mouse OptEIA, BD Biosciences; lower limit of sensitivity = 15.6 pg/ml).



## Statistics

Significant differences in bacterial titers and T cell infiltrates between TLR2 KO and WT mice at a particular time point were determined using a paired Student's *t*-test (SPSS Science, Chicago, IL). For studies examining the effects of SEA/SEB on TLR2-mediated signaling in response to Pam3Cys, a one-way analysis of variance (ANOVA) followed by the Holm-Sidak method for multiple pair-wise comparisons was performed. For all analyses, a *p*-value of less than 0.05 was considered to be statistically significant.

## RESULTS

### TLR2 loss influences the frequency of specific T cell subsets during *S. aureus* brain abscess development

To examine whether the loss of TLR2 affected the influx of various T cell subsets into brain abscesses, FACS analysis was performed in TLR2 KO and WT mice. Although a recent study from our laboratory had examined abscess-associated CD4<sup>+</sup> and CD8<sup>+</sup> T cell infiltrates in TLR2 KO mice, only limited time points were examined and NKT and  $\gamma\delta$  T cells were not included in the analysis (Nichols et al., 2009). In addition, our earlier report utilized a *S. aureus* laboratory adapted strain (RN6390) that is easily amenable to genetic manipulation and possesses a deletion in *rsbU*, which encodes a positive regulator essential for activation of the stress response sigma factor B (Kullik et al., 1998). In contrast, the current study utilized a *S. aureus* USA300 isolate with important clinical origins (i.e. recovered from an otherwise healthy patient who died from a brain abscess)(Sifri et al., 2007), which is likely more reminiscent of strains causing natural CNS infections. CD4<sup>+</sup> T cell infiltrates were first detectable on day 3 and progressively increased until day 10 after *S. aureus* exposure, representing the most numerous T cell population associated with brain abscesses (Fig. 1A and Supplemental Fig. 1). There were no significant differences in the relative percentages of CD4<sup>+</sup> T cells in brain abscesses of TLR2 KO versus WT mice (Fig. 1A). CD8<sup>+</sup> T cells were the next most frequent population and were significantly elevated at day 7 post-infection in TLR2 KO animals (Fig. 1B and Supplemental Fig. 1; *p* < 0.05, paired *t*-test). Interestingly, both NKT and  $\gamma\delta$  T cell infiltrates were increased in brain abscesses of TLR2 KO mice at early time points following *S. aureus* infection (i.e. days 3 and 7; *p* < 0.05, paired *t*-test), but were roughly equivalent at later intervals (i.e. days 10 and 14; Fig. 1C and D and data not shown). Similar relationships were observed between TLR2 WT and KO mice when absolute numbers of each T cell population were determined (Supplemental Table 1).

Quantitation of bacterial burdens associated with brain abscesses of TLR2 KO and WT mice revealed an important role for TLR2 in *S. aureus* clearance. Specifically, bacterial burdens were significantly increased in TLR2 KO animals at day 7 post-infection (*p* < 0.05, paired *t*-test) and remained elevated at day 14 compared to WT mice, although *S. aureus* titers had also begun to decline in the KO group over time (Fig. 2). Collectively, these results demonstrate the heightened accumulation of NKT and  $\gamma\delta$  T cells during the early stages of brain abscess formation in TLR2 KO mice, two cell types that span innate and adaptive immunity. It is possible that the enhanced recruitment of these cells is an attempt to compensate for the loss of critical signal(s) that would normally be elicited by TLR2 in response to *S. aureus* challenge.

We next characterized cytokine secretion profiles of the various abscess-associated T cell populations to determine whether alterations in key proinflammatory cytokines were apparent between TLR2 KO and WT mice. Intracellular cytokine staining revealed that the majority of CD4<sup>+</sup> T cells infiltrating brain abscesses were Th17 and Th1 subtypes producing IL-17 and IFN- $\gamma$ , respectively (Fig. 3). In addition, a population of IL-17 and IFN- $\gamma$  double-

positive cells was demonstrated (Supplemental Fig. 2). In general, the percentages of IL-17 producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells were reduced in TLR2 KO mice at days 7 and 10, respectively (Fig. 3A and C;  $p < 0.05$ , paired  $t$ -test). In contrast, the numbers of cells producing IFN- $\gamma$  was increased in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in TLR2 KO mice at day 10 post-infection compared to WT animals (Fig. 3B and D). Similar to the increase in NKT infiltrates in TLR2 KO mice, a higher percentage of abscess-associated NKT cells from TLR2 KO animals produced IL-17 and IFN- $\gamma$  compared to NKT cells from WT mice (Fig. 3E and F; Supplemental Fig. 2). These data indicate that IL-17 and IFN- $\gamma$  production are elevated in select T cell subsets in the absence of TLR2-dependent signaling, which may represent an attempt to augment the CNS immune response to contain bacterial infection.

### **CNS *S. aureus* infection leads to the preferential accumulation of V $\beta$ <sub>8.1,8.2</sub> T cells**

*S. aureus* is known to express numerous secreted virulence factors, including superantigens (SAG). Depending on the isolate, *S. aureus* has been reported to harbor at least 20 distinct SAGs (Fraser and Proft, 2008). Among the best characterized are SEA and SEB, which trigger the clonal expansion of T cells bearing the V $\beta$ <sub>11</sub> and V $\beta$ <sub>8.1,8.2</sub> TCRs, respectively (Dinges et al., 2000; Foster, 2005). Upwards of 30% T cell expansion can occur following SAG stimulation and two prominent cytokines produced by SAG-reactive V $\beta$  T cells are TNF- $\alpha$  and IFN- $\gamma$  (Papageorgiou and Acharya, 2000). Currently, the frequency of SEA- and SEB- reactive V $\beta$  T cell subsets associated with CNS *S. aureus* infection and the relationship between TLR2 and V $\beta$  T cells have not yet been explored. Since CD4<sup>+</sup> T cells have been reported to express TLR2 and respond to pathogen-associated molecular patterns relevant to *S. aureus* (Kabelitz, 2007; Komai-Koma et al., 2004; Reynolds et al., 2010), it is possible that TLR2 deficiency may alter the numbers of SAG-reactive V $\beta$  populations in brain abscesses.

In terms of frequency, abscess-associated V $\beta$ <sub>8.1,8.2</sub> T cells were dramatically higher compared to V $\beta$ <sub>11</sub> cells (Fig. 4). This finding could be explained by SEB production by *S. aureus* during brain abscess formation, whereas SEA was not detectable (Fig. 5 and data not shown, respectively). In addition, both V $\beta$  T cell populations were reduced in brain abscesses of TLR2 KO mice (Fig. 4). Because V $\beta$ <sub>8.1,8.2</sub> T cells outnumbered V $\beta$ <sub>11</sub> cells in brain abscesses, we evaluated the cytokine expression profiles of the former in TLR2 KO and WT mice. Upon re-stimulation with PMA/ionomycin, intracellular cytokine staining demonstrated that the number of abscess-associated V $\beta$ <sub>8.1,8.2</sub> T cells producing TNF- $\alpha$  was reduced in TLR2 KO mice compared to WT animals at both 7 and 14 days post-infection (Fig. 6). Similar effects were observed with IFN- $\gamma$  producing V $\beta$ <sub>8.1,8.2</sub> T cells, where the number of cytokine secreting cells was lower in TLR2 KO mice compared to WT animals (Fig. 6). A subpopulation of total CD4<sup>+</sup> and V $\beta$ <sub>8.1,8.2</sub> T cells were found to produce both TNF- $\alpha$  and IFN- $\gamma$  (Supplemental Fig. 3). Although the percentages of TNF- $\alpha$  and IFN- $\gamma$  expressing cells were roughly equivalent between TLR2 WT and KO mice (Supplemental Fig. 3), calculation of absolute cell numbers revealed dramatic decreases in TLR2 KO animals based on the fact that total cellular infiltrates were increased in these mice. Collectively, these results indicate that TLR2 influences V $\beta$ <sub>8.1,8.2</sub> T cell frequency and cytokine release during brain abscess development.

### **Staphylococcal enterotoxins augment microglial inflammatory mediator release in response to a TLR2 ligand**

Previous reports have demonstrated that SAGs are capable of augmenting TLR4-mediated signaling in response to LPS in dendritic cells and monocytes (Hopkins et al., 2005; Rossi et al., 2004). However, to the best of our knowledge, no studies have investigated the ability of SAG to augment TLR-mediated signaling in response to PAMPs in microglia. This is a relevant question to address since microglia are exposed to both PAMPs and SAG during

brain abscess development. Exposure of primary microglia to purified SEB did not elicit TNF- $\alpha$  production at any of the doses examined (Fig. 7A). However, SEB was capable of augmenting TNF- $\alpha$  production in combination with the TLR2 agonist Pam3Cys, compared to cytokine levels observed with Pam3Cys alone (Fig. 7A;  $p < 0.05$ , one-way ANOVA). Similar effects were observed following microglial treatment with SEA  $\pm$  Pam3Cys (data not shown). Importantly, the combination of SEB with Pam3Cys did not induce any evidence of cytotoxicity (Fig. 7B). These findings indicate that besides their ability to induce the clonal expansion of V $\beta$  reactive T cell subsets, SAGs are also capable of enhancing microglial activation in response to TLR2 ligands, which may serve as an amplification loop for antibacterial immunity during *S. aureus*-induced brain abscess formation.

## DISCUSSION

Despite advances in defining innate immune pathways elicited during brain abscess development, little information is currently available regarding the functional impact of adaptive immunity in bacterial clearance. Innate immune responses must be tightly regulated to effectively eradicate bacteria while limiting the extent of bystander damage to surrounding brain tissue. This is essential since the CNS is not capable of robust regeneration that would be required to resolve such widespread parenchymal damage characteristic of a brain abscess. It is anticipated that adaptive immune mechanisms are required during the later stages of infection to tailor ongoing innate anti-bacterial responses towards effective pathogen resolution. Therefore, understanding the role that adaptive immunity plays during brain abscess development may identify novel pathways that could be targeted for potential therapeutic interventions.

The loss of TLR2-dependent signaling was found to impact various T cell populations in opposing manners. For example, TLR2 KO mice exhibited enhanced NKT,  $\gamma\delta$ , and CD8<sup>+</sup> T cell infiltrates into brain abscesses, whereas the recruitment of V $\beta_{8,1,8,2}$  and V $\beta_{11}$  T cells was reduced compared to WT animals. With regard to NKT cells, these cells become rapidly activated in response to IL-12 even when their invariant TCR is not engaged by a lipid antigen (Tupin et al., 2007). We have previously reported exaggerated IL-12 production in TLR2 KO microglia (Kielian et al., 2005a); therefore, heightened IL-12 release may be one trigger to augment NKT cells in the absence of TLR2 signaling, since IL-12 is a potent activator of these cells (Balato et al., 2009; Godfrey et al., 2010). The ability to become activated in response to the local cytokine milieu makes NKT cells able to rapidly respond to a wide array of infectious pathogens, similar to other cells of the innate immune system. Activated NKT cells produce large amounts of IL-17 and a higher percentage of NKT cells isolated from brain abscesses of TLR2 KO mice were IL-17-positive compared to WT animals. NKT cells can also express V $\beta_{8,2}$  (Tupin et al., 2007); therefore, they may also be subject to the mitogenic effects of SEB, although this remains speculative. Indeed, a recent study demonstrated that NKT cells may regulate cytokine responses following SEB exposure (Ragin et al., 2006). Finally, enhanced NKT cell accumulation in brain abscesses of TLR2 KO mice may be a consequence of the elevated bacterial burdens seen in these animals. We also observed an increase in  $\gamma\delta$  T cells in brain abscesses of TLR2 KO mice. Recent studies have demonstrated that  $\gamma\delta$  T cells can be induced to express MHC Class II and assume antigen presenting cell functions (Born et al., 2010; Brandes et al., 2005; Cheng et al., 2008). Based on this knowledge, it is possible that infiltrating  $\gamma\delta$  T cells could substitute for the loss of TLR2 signaling by antigen presenting cells; however, this possibility remains highly speculative. Also, the percentages of IL-17 secreting  $\gamma\delta$  T cells from TLR2 KO mice was elevated compared to WT animals, which may indirectly serve to recruit additional PMN in an attempt to contain *S. aureus* infection (Witowski et al., 2000; Ye et al., 2001).



One question that arises from this work relates to the major cellular target of TLR2 signaling *in vivo*. This is a difficult issue to address since there are numerous cell types associated with brain abscesses that express TLR2, including both CNS resident and infiltrating leukocytes. In particular, microglia and astrocytes (CNS intrinsic) as well as neutrophils, macrophages, CD4<sup>+</sup> T cells, and even NKT and  $\gamma\delta$  T cells have recently been reported to express TLR2 (Akira, 2006; Kielian, 2006; Martin et al., 2009; Mokuno et al., 2000). However, a plausible scenario of action can be proposed based on the infiltration kinetics and activation of responding cell types. For example, immediately following *S. aureus* infection in the CNS, TLR2 is likely pivotal for microglial activation and the production of numerous chemokines and cytokines critical for the recruitment of peripheral immune cells into the site of infection and their subsequent activation (Kielian et al., 2005a). Astrocytes may also participate in this initial recognition event, which are an important source of chemokines following *S. aureus* infection and can be triggered via TLR2-dependent signaling (Esen et al., 2004). Recent studies from our laboratory using bone marrow chimeras with MyD88 KO mice revealed an essential role for CNS intrinsic cells, presumably microglia and astrocytes, in eliciting a wild type inflammatory response during the early stage of brain abscess development (Garg et al., 2009). However, this phenotype cannot be solely attributed to TLR2 since numerous other TLRs relevant to *S. aureus* recognition, such as TLR9 and the IL-1R, are both driven by MyD88-dependent pathways and could conceivably participate in the CNS antibacterial response. Nonetheless, this study clearly demonstrated a key role for resident microglia and/or astrocytes in the initial recognition of *S. aureus* and eliciting downstream inflammatory cascades (Garg et al., 2009).

Within 12–24 h following infection, the importance of TLR2 may shift to include newly recruited immune cells from the peripheral circulation, primarily neutrophils, followed by macrophages that accumulate at significant levels within 48–72 h post-infection (Kielian, 2004; Kielian et al., 2001). At this juncture, TLR2 engagement by *S. aureus* would provide critical signals for amplification of the CNS immune response targeted at *S. aureus* clearance. Extensive studies in the experimental brain abscess model have established that the innate immune response is not effective at containing bacterial replication until approximately 5–7 days following *S. aureus* exposure (Kielian et al., 2001; Kielian et al., 2004a; Kielian et al., 2007a). At this time, T and NKT cell infiltrates are beginning to be prevalent and appear to play an important role in effective bacterial clearance and maintenance of maximal innate immune responses (Mariani and Kielian, manuscript in preparation). In addition, unpublished data from our laboratory has revealed an important role for both CD4<sup>+</sup> and NKT cells in effective bacterial clearance and maintaining maximal innate immune responses during the later stages of brain abscess development (Mariani and Kielian, manuscript in preparation). TLR2 may also be engaged on these infiltrating T cell populations to impact later stages of infection. In summary, TLR2 expression is likely important for triggering activation of both CNS resident and the various infiltrating innate and adaptive immune populations throughout the course of brain abscess development. This widespread utilization of TLR2-dependent signaling is evident by the increased mortality rates and altered T cell responses observed in TLR2 KO mice during the current study. A schematic depicting these multiple potential targets of TLR2 signaling are presented in Figure 8.

Previous studies from our laboratory have evaluated the functional importance of TLR2 in microglial activation to *S. aureus* comparing responses of TLR2 KO and WT microglia (Kielian et al., 2005a). These experiments revealed that TLR2 loss leads to the exaggerated production of IL-12 cytokine family members in response to intact bacteria; however, this effect was selective in that the expression of numerous other mediators (including chemokines) was not affected. Therefore, our *in vitro* data with TLR2 KO microglia does

not point towards altered chemokine expression (Kielian et al., 2005a); however, it is possible that T cell chemokine production is affected in TLR2 KO mice *in vivo* to account for the differential recruitment of various T cell populations into brain abscesses of TLR2 KO mice. This possibility is being explored in ongoing studies in our laboratory that will be the subject of a future publication and is beyond the scope of the current report.

The complexity of brain abscess development, both in terms of responding cell types and divergent cytokine production by each, makes it very difficult to assign the role of IL-17 production to any particular cell population. However, when considering the percentages of each cell type associated with brain abscesses, an overall impression in terms of IL-17 can be envisioned. For example, we have previously reported that IL-17 production is higher in brain abscesses of TLR2 KO mice (Kielian et al., 2005b), which corresponds to the heightened numbers of IL-17 producing NKT cells from these animals. In addition,  $\gamma\delta$  T cell infiltrates were increased in TLR2 KO mice and these cells are also capable of IL-17 production (Cua and Tato, 2010; Martin et al., 2009). However, we did not examine cytokine production in  $\gamma\delta$  T cells in the current study due to limiting cell numbers recovered from brain abscesses. In addition to IL-17 release from NKT and  $\gamma\delta$  T cells, IL-17 has been reported to be produced by astrocytes and microglia (Das Sarma et al., 2009; Kang et al., 2010; Tzartos et al., 2008). Therefore, it is possible that all of these mechanisms serve to augment IL-17 production within brain abscesses of TLR2 KO mice. In contrast, the percentages of CD4 and CD8 T cells producing IL-17 were reduced in TLR2 KO animals; however, this was only noted at one time point for each cell population (i.e. days 7 and 14, respectively). Therefore, based on the fact that IL-17 expression is elevated in brain abscesses of TLR2 KO mice, it is evident that this transient reduction in IL-17 production from CD4 and CD8 T cells is not significant enough to impact overall IL-17 levels associated with brain abscesses of TLR2 KO animals.

In contrast to the increased frequency of NKT and  $\gamma\delta$  T cells infiltrating brain abscesses of TLR2 KO mice, the recruitment of SEA- and SEB-reactive  $V\beta_{11}$  and  $V\beta_{8.1,8.2}$  T cells, respectively, was attenuated compared to WT animals. The mechanism(s) responsible for this finding are not known but could be explained by: 1) reduced chemokine or altered adhesion molecule expression at the blood-brain barrier; 2) limited expansion of  $V\beta_{8.1,8.2}$  and  $V\beta_{11}$  T cells within the infected CNS; or 3) increased apoptosis of  $V\beta$  subsets in TLR2 KO mice following SAG expansion. In addition, the frequency of  $V\beta_{8.1,8.2}$  T cells producing either TNF- $\alpha$  or IFN- $\gamma$  was reduced in TLR2 KO mice, which correlated with the inability to rapidly clear *S. aureus* from the CNS in these animals. It is also plausible that the loss of TLR2 directly impacts CD4<sup>+</sup> T cell behavior since these cells have been reported to express functional TLR2 (Kabelitz, 2007; Komai-Koma et al., 2004). In this regard, the loss of TLR2-dependent signaling may diminish a stimulatory signal required for the clonal expansion of SEB-reactive  $V\beta_{8.1,8.2}$  T cells, resulting in reduced accumulation, as was observed in brain abscesses of TLR2 KO mice in this study. However, this possibility remains highly speculative and awaits future study. Nonetheless, our results do indicate that SEB is capable of augmenting TNF- $\alpha$  production by microglia in combination with the TLR2 ligand Pam3Cys. This may represent a possible mechanism to amplify inflammation during brain abscess development by SAG actions on antigen presenting cells in addition to  $V\beta_{8.1,8.2}$  T cells.

It is important to note that a few findings in the current study differed from a previous report from our laboratory with TLR2 KO mice (Nichols et al., 2009). In particular, our earlier paper demonstrated that Th17 infiltrates were enhanced in TLR2 KO mice, whereas in the current study we did not observe any dramatic differences in CD4<sup>+</sup> Th17 cells. However, we show here that NKT and  $\gamma\delta$  T cells from TLR2 KO mice did produce more IL-17, in agreement with our previously proposed mechanism whereby heightened IL-17 release may

represent a compensatory response in an attempt to contain *S. aureus* infection. This discrepancy is likely explained by the fact that the current studies were performed with a *S. aureus* clinical isolate that was recovered from a patient who died from a brain abscess, whereas our earlier report utilized a laboratory adapted strain of *S. aureus*. In fact, this point was noted by Stenzel et al. who used a *S. aureus* clinical isolate to demonstrate higher mortality rates and bacterial burdens in TLR2 KO mice compared to WT animals (Stenzel et al., 2008). With our use of a new brain abscess clinical isolate, our results are more similar to Stenzel et al. and point to the important issue of distinct *S. aureus* strains being able to elicit diverse immunological effects. Nonetheless, our studies have extended these initial observations to characterize the effects of TLR2 loss on other T cell populations, SAG-reactive T cell subsets, and their cytokine secretion patterns, where novel differences were identified.

In summary, we demonstrated that TLR2-dependent signals are important for the accumulation of SAG-reactive  $V\beta_{8.1,8.2}$  and  $V\beta_{11}$  T cells into brain abscesses and their subsequent activation. In contrast, TLR2 either delivers a negative regulatory signal to attenuate NKT and  $\gamma\delta$  T cell influx into the infected CNS, or alternatively, the heightened accumulation of these subsets may have resulted from the inability to effectively clear *S. aureus* from the brain. The latter may be more plausible as a higher proportion of abscess-associated NKT and  $\gamma\delta$  T cells infiltrating TLR2 KO mice produced IFN- $\gamma$  and IL-17, two key anti-bacterial cytokines. Collectively, these studies highlight the impact of TLR2 on adaptive immune responses and point to the important role this pattern recognition receptor plays during CNS bacterial infection.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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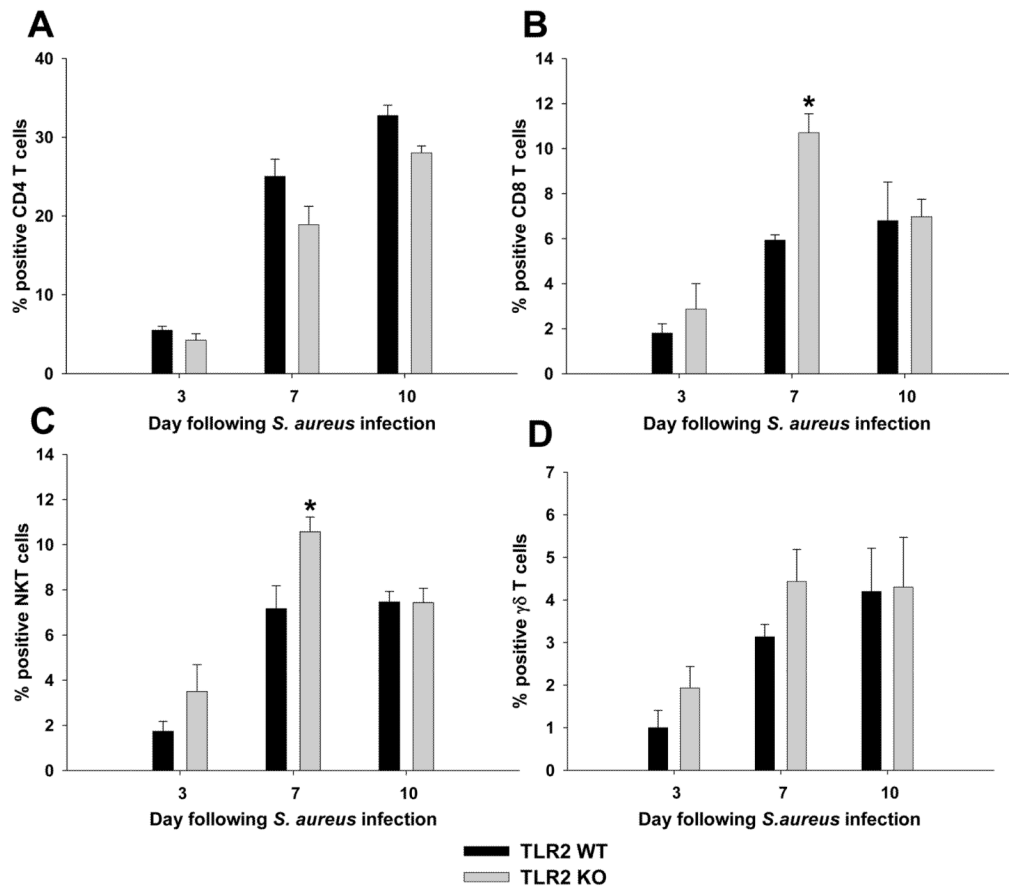
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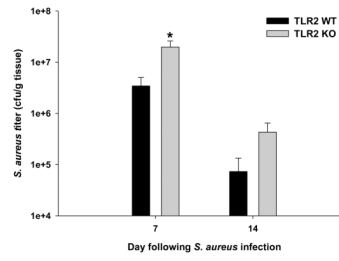


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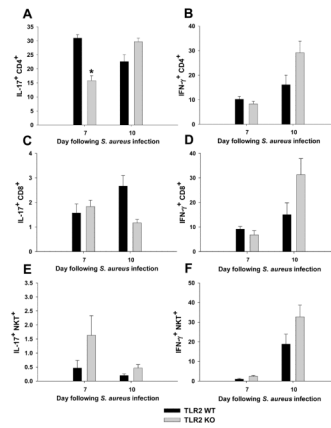
**Figure 1. TLR2 loss induces transient increases in NKT,  $\gamma\delta$ , and CD8<sup>+</sup> T cell recruitment into brain abscesses**

Abscess-associated cells from TLR2 KO and WT mice ( $n = 4-6$  per group) were recovered at the indicated time points post-infection and subjected to FACS analysis to identify CD4<sup>+</sup> (A), CD8<sup>+</sup> (B), NKT (C), and  $\gamma\delta$  T cell (D) infiltrates. Results are presented as the average percentage of each population recovered from a total of three independent experiments (mean  $\pm$  SEM). Similar trends were obtained when absolute T cell numbers were determined based on the total number of cells recovered from the brains of TLR2 KO or WT mice. Significant differences in abscess-associated T cell subsets between TLR2 KO and WT mice are denoted by asterisks (\*,  $p < 0.05$ ).



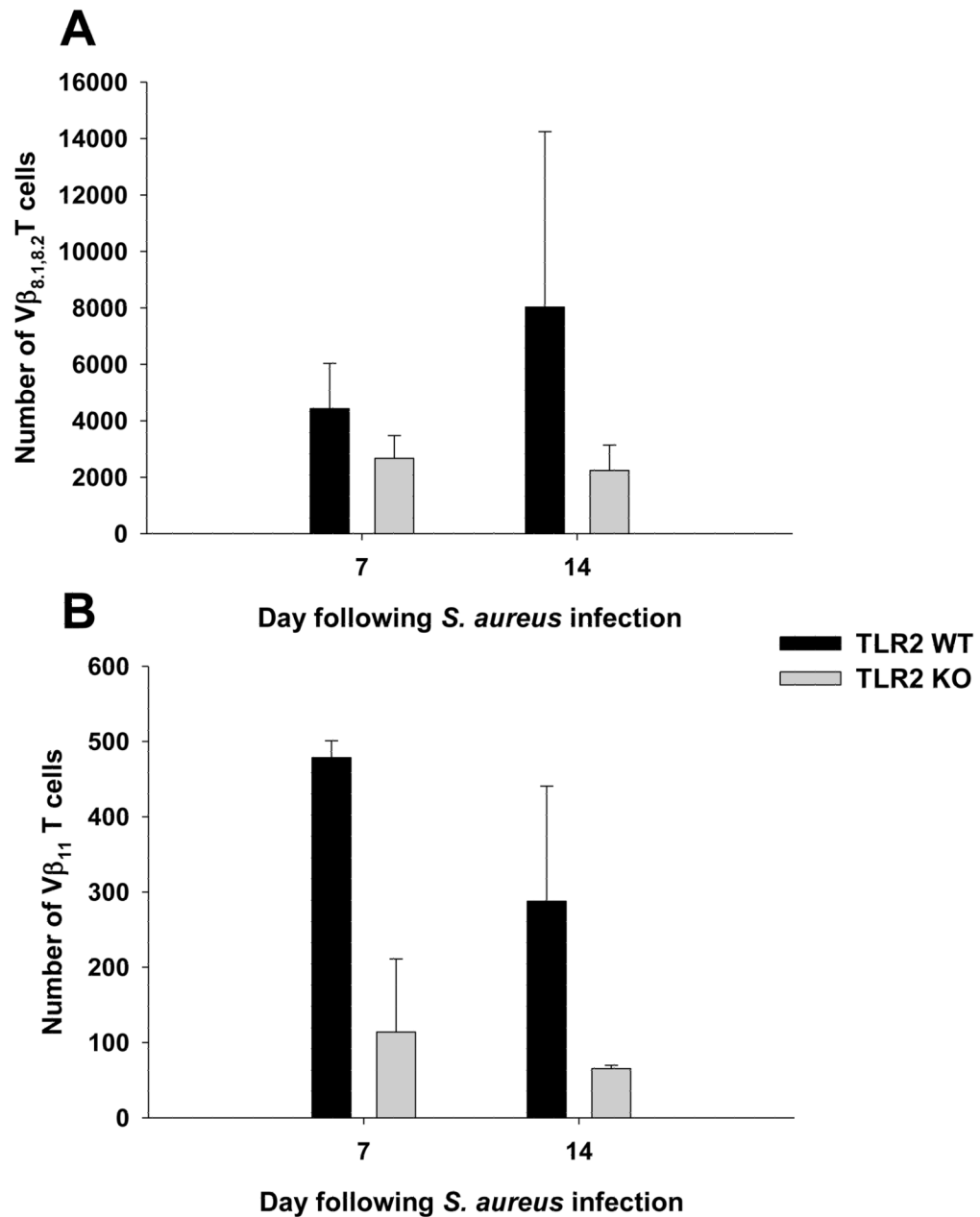
**Figure 2. TLR2 plays an important role in *S. aureus* clearance from brain abscesses**

Brain abscess tissues from TLR2 KO and WT mice (n = 4–6 per group) were collected at the indicated time points post-infection and bacterial burdens determined by quantitative culture. Results are expressed as the colony forming units (cfu) of *S. aureus* per gram of wet tissue weight and were combined from four independent experiments. Significant differences in bacterial titers between TLR2 KO and WT mice are denoted by asterisks (\*,  $p < 0.05$ ).



**Figure 3. Loss of TLR2-dependent signaling leads to alterations in T cell cytokine secretion profiles**

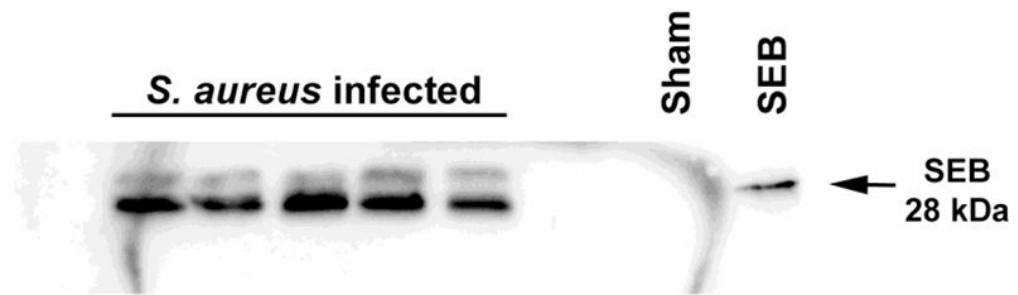
Abscess-associated CD4<sup>+</sup> (A and B), CD8<sup>+</sup> (C and D), and NKT (E and F) cells from TLR2 KO and WT mice (n = 4–6 per group) were recovered at the indicated time points post-infection and sorted by FACS. After sorting, cells were immediately activated with PMA/ionomycin/Golgi Plug for 4 h and processed for intracellular cytokine staining for IL-17 and IFN- $\gamma$ . Results are presented as the average percentage of each population recovered from a total of three independent experiments (mean  $\pm$  SEM). Similar trends were obtained when the absolute number of T cells producing cytokines was determined based on the total number of cells recovered from the brains of TLR2 KO or WT mice. Significant differences in cytokine production by abscess-associated T cell subsets from TLR2 KO and WT mice are denoted by asterisks (\*,  $p < 0.05$ ).



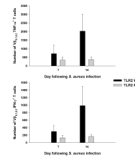
**Figure 4. SEB-reactive  $V\beta_{8.1,8.2}$  T cells are more frequent in CNS *S. aureus* infection compared to SEA-reactive  $V\beta_{11}$  T cells**

Abscess-associated cells from TLR2 KO and WT mice ( $n = 4-6$  per group) were recovered at the indicated time points post-infection and stained with CD4-AlexaFluor 700 and  $V\beta_{8.1,8.2}$ -FITC (A) or  $V\beta_{11}$ -FITC (B) to identify the numbers of infiltrating SEB- and SEA-reactive T cells, respectively. For analysis, the percentages of  $V\beta_{8.1,8.2}^+$  or  $V\beta_{11}^+$  T cells within the  $CD4^+$  gate were evaluated. Results are presented as the absolute number of  $V\beta$  T cell subsets combined from a total of three independent experiments.



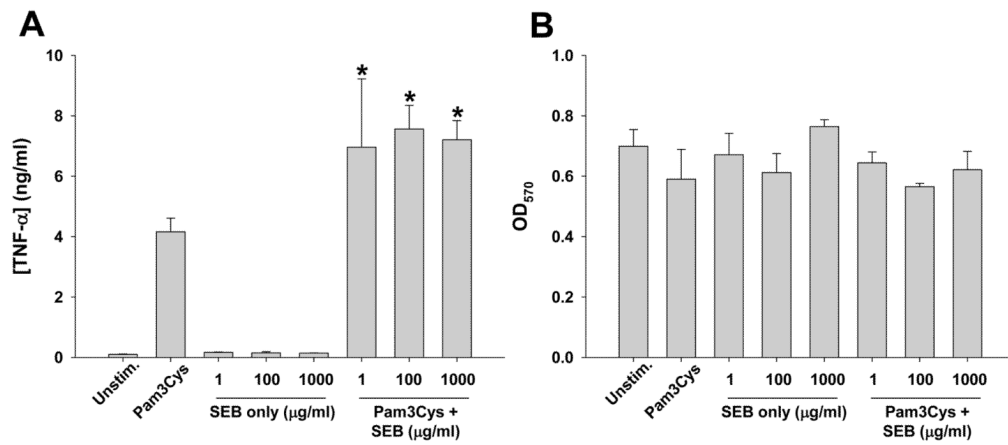


**Figure 5. Staphylococcal enterotoxin B (SEB) is expressed during brain abscess formation**  
Brain abscess homogenates were prepared from individual animals at day 7 post-infection, whereupon SEB expression was evaluated by Western blotting. A sample collected from a sham injected animal and purified SEB were included as negative and positive controls, respectively.



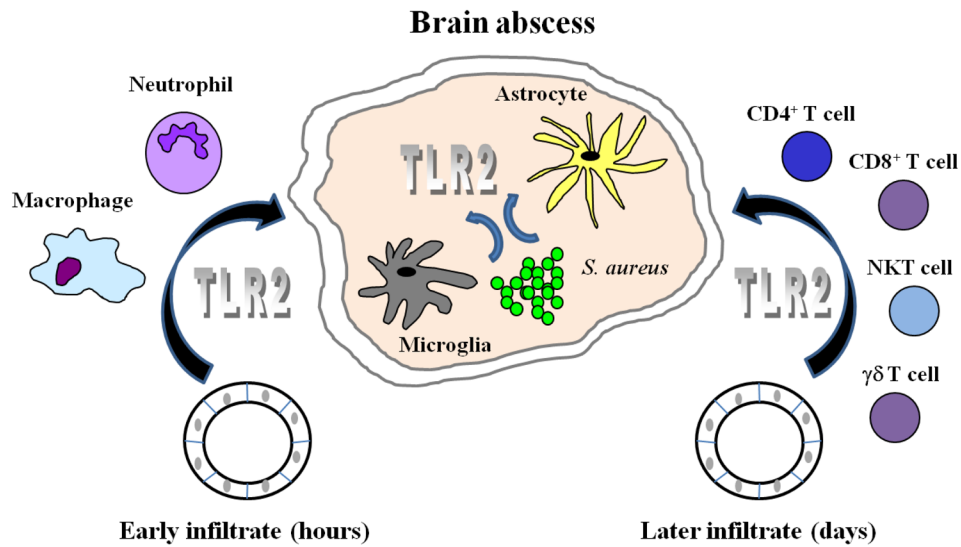
**Figure 6. TLR2-dependent signals influence cytokine release from V $\beta$ <sub>8.1,8.2</sub> T cells following *S. aureus* infection**

Abscess-associated CD4<sup>+</sup> T cells from TLR2 KO and WT mice (n = 4–6 per group) were recovered at the indicated time points post-infection by FACS, whereupon cells were immediately activated with PMA/ionomycin/Golgi Plug for 4 h. Cells were stained with V $\beta$ <sub>8.1,8.2</sub>-FITC and subsequently processed for intracellular cytokine staining for TNF- $\alpha$  and IFN- $\gamma$ . Results are presented as the absolute number of V $\beta$ <sub>8.1,8.2</sub><sup>+</sup> cytokine producing T cells combined from a total of three independent experiments.



**Figure 7. *S. aureus* SEB augments microglial TNF- $\alpha$  production in response to the TLR2 ligand Pam3Cys**

Primary microglia were pre-treated for 24 h with 100 ng/ml of recombinant mouse IFN- $\gamma$ , whereupon cells were exposed to various doses of purified SEB either alone or in combination with the TLR2 ligand Pam3Cys (10 ng/ml). Conditioned medium was collected at 24 h following microglial stimulation and TNF- $\alpha$  expression was quantitated by ELISA. Results presented are representative of three independent replicates for each treatment (mean  $\pm$  SD) and were replicated in three separate experiments. Significant differences between microglia stimulated with Pam3Cys alone versus Pam3Cys in combination with SEB are denoted by asterisks (\*,  $p < 0.05$ ).



**Figure 8. TLR2 has the potential to impact diverse cell populations throughout the course of CNS bacterial infection**

Immediately following *S. aureus* invasion in the brain parenchyma, resident microglia and astrocytes recognize the pathogen, in part, via TLR2. TLR2 signaling leads to the downstream production of inflammatory cytokines and chemokines that trigger the egress of neutrophils and monocytes (which differentiate into macrophages) from activated cerebral vasculature into the evolving abscess during the acute stage of infection. Within days, T cell populations begin to accumulate within the abscess proper, many of which have been shown to express TLR2 that could conceivably trigger cellular activation.