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Commentary

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MyD88 expression by CNS-resident cells is pivotal for eliciting protective immunity in brain abscesses

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ABSTRACT

MyD88 KO (knockout) mice are exquisitely sensitive to CNS (central nervous system) infection with *Staphylococcus aureus*, a common aetiological agent of brain abscess, exhibiting global defects in innate immunity and exacerbated tissue damage. However, since brain abscesses are typified by the involvement of both activated CNS-resident and infiltrating immune cells, in our previous studies it has been impossible to determine the relative contribution of MyD88-dependent signalling in the CNS compared with the peripheral immune cell compartments. In the present study we addressed this by examining the course of *S. aureus* infection in MyD88 bone marrow chimaera mice. Interestingly, chimaeras where MyD88 was present in the CNS, but not bone marrow-derived cells, mounted pro-inflammatory mediator expression profiles and neutrophil recruitment equivalent to or exceeding that detected in WT (wild-type) mice. These results implicate CNS MyD88 as essential in eliciting the initial wave of inflammation during the acute response to parenchymal infection. Microarray analysis of infected MyD88 KO compared with WT mice revealed a preponderance of differentially regulated genes involved in apoptotic pathways, suggesting that the extensive tissue damage characteristic of brain abscesses from MyD88 KO mice could result from dysregulated apoptosis. Collectively, the findings of the present study highlight a novel mechanism for CNS-resident cells in initiating a protective innate immune response in the infected brain and, in the absence of MyD88 in this compartment, immunity is compromised.

Key words: bone marrow chimaera mice, brain abscess, central nervous system, MyD88, *Staphylococcus aureus*, Toll-like receptor.

INTRODUCTION

In the CNS (central nervous system), brain abscesses arise from a parenchymal infection by pyogenic bacteria and represent a serious life-threatening condition. Brain abscesses can form by bacterial perforation of the thin bony structures separating the brain from neighbouring sites of chronic infection occurring in the paranasal sinuses, middle ear, or upper molars. Other routes include seeding of the brain with bacterial emboli originating from systemic sites of infection (i.e. endocarditis or septicemia) (Mathisen and Johnson, 1997) where the frontal and temporal lobes are most commonly affected (McClelland et al., 1978; Carpenter et al., 2007), penetrating trauma to the head, or following neurosurgery (Tenney, 1986; Schliamser et al., 1988). Although brain abscesses ensue in response to a diverse array of pathogens, streptococcal species and *Staphylococcus aureus* represent the most common aetiological agents of infection in humans.

Innate immunity plays an essential role in the host response to bacterial infections. Among the central players

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Abbreviations: CFU, colony forming unit; CNS, central nervous system; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; Ier3/IEX, immediate early response 3; IL, interleukin; IL-1R etc., IL-1 receptor; KO, knockout; Lcn2, lipocalin-2; NF-κB, nuclear factor κB; Pacsin3, protein kinase C and casein kinase substrate in neurons 3; Pfc, complement factor properdin; qRT-PCR, quantitative real-time RT (reverse transcriptase)-PCR; ROS, reactive oxygen species; SOCS3, suppressor of cytokine signalling 3; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-α; WT, wild-type..

in anti-bacterial immunity are members of the TLR (Toll-like receptor) family of pattern recognition receptors (Akira et al., 2006; Trinchieri and Sher, 2007). These receptors recognize conserved motifs from a wide range of pathogens that are inherently resistant to mutation based on their essential nature for pathogen survival (Medzhitov and Janeway, 2002). When considering the array of TLRs that could be triggered during *S. aureus* infection in the brain, several candidates emerge, since the bacterium presents an arsenal of distinct immunostimulatory motifs. For example, bacterial lipoproteins and PGN (peptidoglycan) can trigger TLR1 and TLR2, whereas bacterial DNA can stimulate TLR9 in endosomal compartments (Akira et al., 2006; Trinchieri and Sher, 2007). Based on this complexity it is expected that numerous receptors are engaged following bacterial infection in the brain. This is supported by our previous studies demonstrating that brain abscess pathogenesis following *S. aureus* infection was not markedly affected by the loss of TLR2 (Kielian et al., 2005). Therefore a broader role for additional recognition molecules sensing bacterial infection was apparent.

MyD88 is a central adaptor molecule for the majority of TLRs, with the exception of TLR3 (Akira, 2006; O'Neill and Bowie, 2007). This molecule is also responsible for transducing activation signals emanating from the IL-1R [IL (interleukin)-1 receptor] and IL-18R (Wesche et al., 1997; Adachi et al., 1998; Burns et al., 1998; Medzhitov et al., 1998). Since IL-1 and IL-18 have been shown to have important roles in anti-bacterial immunity, coupled with the pivotal role of MyD88-dependent pathways in bacterial recognition and the induction of downstream cytokine signalling networks, MyD88 represents a central converging point in the innate inflammatory pathway. Indeed, recent studies from our laboratory have demonstrated the essential role of MyD88-dependent mechanism(s) in mounting a productive host innate immune response during the acute stage of brain abscess development (Kielian et al., 2007). Studies by other groups have also established the importance of MyD88-dependent pathways in the innate immune response to Gram-positive infections in both the CNS and periphery (Takeuchi et al., 2000; Koedel et al., 2004; Miller et al., 2006; Fremond et al., 2007).

Although our previous report demonstrated an essential and non-redundant role for MyD88 in eliciting an innate immune response during brain abscess development (Kielian et al., 2007), it remained unclear whether MyD88 expression was more important in CNS-resident compared with infiltrating immune cells since the molecule was globally absent in KO (knockout) mice. To address this question, we engineered radiation bone marrow chimaera mice where MyD88 was differentially expressed in the CNS compared with the peripheral immune cell compartments. Unexpectedly, the results demonstrated that MyD88 expression in the CNS was required to mount an innate immune response equivalent to WT (wild-type) during the acute stage of brain abscess development. The requirement for MyD88 in CNS-resident cells was reinforced by the finding that neutrophil influx into the infected brain was only achieved in chimaeric mice where MyD88 was present in the CNS. This is probably due to the fact that numerous neutrophil chemokines were restored to WT levels only in animals where MyD88 was expressed in the CNS compartment.

Curiously, our previous study demonstrated that, despite global defects in innate immunity, bacterial burdens remained relatively consistent between MyD88 KO and WT mice, suggesting that mechanisms other than bacterial burdens themselves were responsible for the enhanced susceptibility of MyD88 KO mice to CNS *S. aureus* infection (Kielian et al., 2007). In the present study, we performed transcriptional profiling with Illumina microarrays to identify pathways that were differentially regulated by MyD88 during brain abscess development that might provide some insights into why these animals succumb so quickly to infection, despite having relatively equivalent bacterial burdens as WT animals. These studies revealed a preponderance of differentially regulated genes involved in apoptotic pathways, suggesting that the extensive tissue damage characteristic of brain abscesses from MyD88 KO mice could result from dysregulated apoptosis. Collectively, these results reveal an essential role for MyD88 in CNS-resident cells that triggers a protective innate immune response within the first 24 h following intracerebral infection with *S. aureus*.

MATERIALS AND METHODS

Mice

All animal use protocols were approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health guidelines for the use of rodents. *MyD88* gene KO mice (originally from Dr Shizuo Akira, Osaka University, Japan) (Adachi et al., 1998) have been previously backcrossed with C57BL/6 mice for over ten generations (Kawai et al., 1999; Fremond et al., 2004). Age- and sex-matched C57BL/6 mice (Harlan Laboratories) were used as WT controls. For the generation of bone marrow chimaeras, B6/SJL mice that are congenic for the CD45 allele (CD45.1) on a C57BL/6 background were purchased from Jackson Laboratories. Mice were used between 10 and 12 weeks of age for all brain abscess studies.

Generation of MyD88 radiation bone marrow chimaera mice

For generating MyD88 bone marrow chimaeras, CD45 congenic B6/SJL mice were used where only one allele, CD45.1, originates from the SJL strain, whereas the remainder of the genome is derived from C57BL/6 mice. These animals

represent the WT strain since they express functional MyD88. MyD88 KO mice are on a C57BL/6 background and express the CD45.2 allele, allowing for the discrimination between donor- compared with recipient-derived leucocytes based on staining with antibodies specific for CD45.1 and CD45.2. The following radiation chimaeras were generated in these experiments (donor bone marrow→irradiated recipient): WT→WT, KO→KO, WT→KO and KO→WT. The experimental chimaeras were WT→KO and KO→WT, whereas the other two groups (WT→WT and KO→KO) represented controls to rule out any non-specific effects of irradiation on immune activation. The latter could be verified by confirming that the inflammatory profiles of irradiated control groups obtained in the present study (WT→WT and KO→KO) were similar to those observed in non-irradiated MyD88 WT and KO mice as reported recently (Kielian et al., 2007). CNS parenchymal cells are radiation-resistant and maintain the host phenotype following radiation exposure (Hickey and Kimura, 1988; Ajami et al., 2007). Reconstitution of irradiated MyD88 KO animals with bone marrow from WT recipients ensured that cells derived from the bone marrow expressed this molecule along with CNS perivascular cells (Hickey and Kimura, 1988; Bechmann et al., 2001). In contrast, parenchymal microglia would not express MyD88 as a result of their radioresistance. The procedure for bone marrow chimaera generation was based on previously published studies with minor modifications (Hickey and Kimura, 1988; Byram et al., 2004; Zehntner et al., 2004; Chakravarty and Herkenham, 2005). Briefly, bone marrow donor mice were euthanized with an overdose of inhaled isoflurane and marrow was isolated from the long bones by flushing with sterile $1 \times$ PBS. Recipient mice were placed on antibiotic-supplemented water (1 g/l neomycin and 125 mg/l polymyxin) for 4 days prior to bone marrow transfer and subjected to irradiation (1000 rad) using a J.L. Shepherd Model i45 caesium irradiator to destroy the marrow. Within 4 h following irradiation, recipient mice received an i.v. (intravenous) injection of 2×10^7 bone marrow cells supplemented with 1×10^7 cells from the spleen to serve as an immediate source of immune cells. Engraftment was allowed to take place over a 6–8 week period and chimaeric animals were maintained on antibiotic-supplemented water for the first 2 weeks to provide protection during transient immunocompromise. At 6–8 weeks post-transplant, chimaeric mice were bled retro-orbitally and cells were stained for flow cytometric analysis using CD45.1 and CD45.2. Only animals that displayed chimaerism of greater than 90% were used in brain abscess studies. Animals were evaluated for their responses to an intracerebral inoculation of *S. aureus* at approx. 10–12 weeks following bone marrow transfer, a period that we, and others (Hassan-Zahraee et al., 2000; Becher et al., 2001; Zehntner et al., 2004), have established to be sufficient for establishing chimaerism. We found that bone marrow chimaeras generated with B6/SJL and MyD88 KO mice did not exhibit any evidence of graft versus host disease, indicating that this was not a confounding issue.

Generation of experimental brain abscess

Live *S. aureus* (strain RN6390) was encapsulated in agarose beads prior to implantation in the brain as previously described (Kielian et al., 2001). Previous studies from our laboratory have established that the introduction of sterile agarose beads does not induce overt inflammation or peripheral immune cell infiltrates (Kielian et al., 2001; Baldwin and Kielian, 2004). To induce brain abscesses, mice were anaesthetized with 2.5% avertin i.p. (intraperitoneally) and a 1 cm longitudinal incision was made along the vertex of the skull extending from the ear to the eye. A rodent stereotaxic apparatus equipped with a Cunningham mouse adaptor (Stoelting) was used to implant *S. aureus*-encapsulated beads into the caudate/putamen region using the following co-ordinates 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 bevelled needle was used to slowly deliver 2 μl of beads [10^4 CFUs (colony forming units)] 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 incision was closed using surgical glue.

Preparation of brain abscess homogenates

To prepare brain abscess homogenates for downstream protein and RNA analysis, lesion sites were visualized by the stab wound created during injections and were sectioned within 1–2 mm on all sides. Upon recovery, brain abscesses were homogenized in 500 μl of PBS supplemented with a Complete™ protease inhibitor cocktail tablet (Roche) and 160 units/ml RNAse inhibitor (Promega) using a Polytron homogenizer (Brinkmann Instruments). At this point, a 20 μl aliquot of abscess homogenate was removed for quantitative culture of viable bacteria as described below. Subsequently, homogenates were centrifuged at 21 000 ***g*** for 15 min at 4°C to pellet membrane material, and supernatants were removed and stored at −70°C until ELISA and multiplex cytokine microbead array analysis as described below.

Quantification of viable bacteria from brain abscesses

To quantify the numbers of viable bacteria associated with brain abscesses, serial 10-fold dilutions of abscess homogenates were plated on to blood agar plates. Titres were calculated by enumerating colony growth and were expressed as CFUs per ml of homogenate.

ELISA

Protein levels of CXCL2 were quantified in brain abscess homogenates using an ELISA kit according to the manufacturer's instructions (DuoSet, R&D Systems; level of sensitivity=15.6 pg/ml). Results were normalized to the amount of

total protein extracted from tissues to correct for differences in sampling size as previously described (Baldwin and Kielian, 2004; Kielian et al., 2004a).

Multi-analyte microbead array to detect pro-inflammatory mediator expression

To expand the analysis of inflammatory mediators differentially expressed between the various MyD88 bone marrow chimaera mice, a mouse 20-plex cytokine microbead array system was used according to the manufacturer's instructions (BioSource International). This microbead array allowed for the simultaneous detection of 20 individual inflammatory molecules in a single 50 μl brain abscess homogenate sample including IL-1α, IL-1β, TNF-α (tumour necrosis factor-α), IFN-γ (interferon-γ), IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 p40/p70, IL-13, IL-17, IP-10 (chemokine CXCL10), MIG (monokine induced by IFN-γ), MCP-1 (monocyte chemotactic protein-1), KC (chemokine CXCL1), GM-CSF (granulocyte/macrophage colony-stimulating factor), VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor). Results were analysed using a Bio-Plex Workstation (Bio-Rad) and adjusted based on the amount of total protein extracted from abscess tissues for normalization. The level of sensitivity for each microbead cytokine standard curve ranged from 1 to 35 pg/ml.

Immunofluorescence staining and confocal microscopy

Neutrophil and microglia/macrophage accumulation in brain abscesses from MyD88 bone marrow chimaera mice were evaluated by immunofluorescence staining using Gr-1 and Iba-1 respectively. For these studies, mice were infected with 10^4 CFUs of *S. aureus* RN6390 engineered to express GFP (green fluorescent protein) under the control of the RNAIII promoter (a gift of Dr Ambrose Cheung, Dartmouth Medical School, Hanover, NH, U.S.A.) to visualize bacterial dissemination in tissue sections. At the indicated time points post-infection (i.e. 18 or 24 h), MyD88 bone marrow chimaera mice were perfused transcardially to eliminate leucocytes from the vasculature, whereupon brains were removed and immediately flash-frozen on dry ice. These early time intervals were required since the survival period of MyD88 KO mice did not extend much beyond this time point (Kielian et al., 2007). Prior to cryostat sectioning, brain tissues were embedded in OCT (optimal cutting temperature) medium, whereupon serial 10 μm sections were made throughout the entire lesioned tissue, mounted on to SuperFrost Plus slides (Fisher Scientific), air-dried, and stored at −80°C until use. For Gr-1 staining, fresh frozen tissues were analysed; however, to detect Iba-1 immunoreactivity, tissue sections were post-fixed in 4% (w/v) paraformaldehyde for 1 h, since strong reactivity with this antibody was not detected in freshly frozen sections. Tissues were incubated with either rat anti-mouse Gr-1 (BD Biosciences) or rabbit anti-mouse Iba-1 (Biocare Medical) antibodies overnight at 4°C in a humidified chamber. Following numerous rinses in PBS, Gr-1 staining was detected with a mouse anti-rat IgG-HRP (horseradish peroxidase)-conjugated antibody (Invitrogen) for 1 h at room temperature (25°C) and visualized using a TSA-Alexa Fluor® 594 kit (Invitrogen). For Iba-1 detection, a rabbit ImmPRESS kit (Vector Laboratories) was used in conjunction with a TSA-Alexa Fluor® 594 kit. Upon completion of the staining protocols, slides were coverslipped using the Prolong anti-fade reagent (Invitrogen) and sealed using nail polish. Slides were imaged using a Zeiss laser-scanning confocal microscope (LSM 510; Carl Zeiss Microimaging). Specific staining of antibodies was confirmed by the absence of fluorescence signal following incubation of brain abscess tissues with secondary antibodies alone (results not shown).

RNA isolation and Illumina oligonucleotide microarray

Total RNA from brain abscesses of MyD88 KO and WT mice was isolated using TRIzol® reagent (Invitrogen) and subjected to DNAse treatment prior to use in microarray studies. RNA concentrations and integrity were determined with an Agilent 2100 bio-analyser using Agilent RNA6000 Nano kits.

Transcriptional profiling of changes in gene expression between *S. aureus*-infected MyD88 KO and WT mice was determined using Illumina Sentrix MouseRef-8 Expression BeadChips (Illumina). For these experiments, RNA samples from four individual animals/group/time point were analysed to account for biological variability. Total RNA was used to generate biotin-labelled cRNA using the Illumina TotalPrep RNA Amplification Kit (Ambion, catalogue number IL1791). Briefly, 0.5 μg of total RNA was first converted into single-stranded cDNA with reverse transcriptase using an oligo-dT primer containing the T7 RNA polymerase promoter site and then copied to produce double-stranded cDNA molecules. The double-stranded cDNA was cleaned and concentrated with the supplied columns and used in an overnight *in vitro* transcription reaction where single-stranded RNA (cRNA) was generated and labelled by incorporation of biotin-16-UTP. A total of 0.75 μg of biotin-labelled cRNA was hybridized at 58°C for 16 h to Illumina's Sentrix MouseRef-8 Expression BeadChips. Each BeadChip has 24 000 well-annotated RefSeq transcripts with approx. 30-fold redundancy. The arrays were washed, blocked, and the labelled cRNA was detected by staining with streptavidin-Cy3. The arrays were scanned using an Illumina BeadStation 500X Genetic Analysis Systems scanner and the image data was extracted using the Illumina BeadStudio software, version 3.0.

Microarray data analysis and statistical methods

The expression data were filtered to include only probes with a consistent signal on each chip; the probe original signal filter value was established at a detection P value<0.02. The resulting dataset was next analysed with DIANE 6.0, a

spreadsheet-based microarray analysis program. An overview of DIANE can be found online at http://www.grc.nia.nih.gov/branches/rrb/dna/diane_software.pdf. Using DIANE, the results were normalized with a *Z*-score transformation (Cheadle et al., 2003). *Z*-normalized data were then analysed with PCA (principal component analysis). To determine the gene expression changes caused by each specific RNA comparison, *Z*-scores for paired treatment groups were compared using the Z-ratio statistic (Cheadle et al., 2003):

$$Z\text{-ratio} = \frac{Z\text{-score}_{\text{LPS}} - Z\text{-score}_{\text{vehicle}}}{\sigma[Z\text{-score}_{\text{LPS}} - Z\text{-score}_{\text{vehicle}}]}$$

Expression changes for individual genes were considered significant if they met four criteria: *Z*-ratio above 1.5 or below −1.5; FDR (false detection rate) (Tusher et al., 2001) of less than 0.30; a *P* value statistic for *Z*-score replicability below 0.05; and mean background-corrected signal intensity greater than zero.

qRT-PCR [quantitative real-time RT (reverse transcriptase)-PCR]

To confirm a subset of differentially expressed genes from microarray studies, qRT-PCR was performed as previously described (Kielian et al., 2004b). ABI Assays on Demand kits were used to examine Lcn2 (lipocalin-2), Pacsin3 (protein kinase C and casein kinase substrate in neurons 3), Ier3/IEX (immediate early response 3), Pfc (complement factor properdin) and SOCS3 (suppressor of cytokine signalling 3) expression, whereas GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers and the TAMRA TaqMan probe were synthesized by ABI based on previously published sequences (Esen et al., 2004; Tanga et al., 2005).

Statistics

Significant differences between the various MyD88 bone marrow chimaera experimental groups were determined using one-way ANOVA followed by the Holm–Sidak method for multiple pair-wise comparisons with Sigma Stat (SPSS Science). For comparisons in gene expression profiles between MyD88 KO and WT mice by qRT-PCR the Student's *t* test was used.

RESULTS

MyD88 expression in the CNS compartment is essential for achieving maximal inflammatory responses to intracerebral *S. aureus* infection

Recent studies from our laboratory have identified an essential role for MyD88-dependent pathways in initiating innate immune responses to *S. aureus* during the acute stage of brain abscess formation (Kielian et al., 2007). However, the relative importance of MyD88 within the CNS compartment compared with infiltrating peripheral immune cells was uncertain, since prior studies utilized MyD88 KO mice where this adaptor was globally absent. To address this question, we generated chimaeric animals using B6.SJL congenic mice that express the CD45.1 allele, allowing the discrimination between donor and recipient cells based on the fact that MyD88 KO mice are on a C57BL/6 background and express CD45.2. In pilot studies, we carefully titrated the dose of ionizing radiation administered to recipient mice to ablate the bone marrow without inducing toxicity to the gastrointestinal tract (results not shown). Mice were evaluated at 8 weeks following bone marrow transfer to assess the degree of chimaerism by examining the extent of CD45.1 and CD45.2 expression on peripheral blood leucocytes by FACS. A representative FACS screen of bone marrow chimaera mice is provided in Figure 1. For these experiments, a total of four bone marrow treatment groups were compared. The two experimental groups consisted of mice where MyD88 was present in the CNS but not in bone marrow-derived cells (i.e. KO→WT; Group 1) and vice versa (i.e. WT→KO; Group 2), whereas bone marrow transfers into genetically identical mice (i.e. KO→KO and WT→WT; Groups 3 and 4 respectively) were also performed to rule out any non-specific effects of ionizing radiation on the responses obtained. The latter could be verified by confirming that the inflammatory profiles of irradiated control groups obtained in the present study (WT→WT and KO→KO) were similar to those observed in non-irradiated MyD88 WT and KO mice as reported recently (Kielian et al., 2007). Although chimaeric mice were screened at 8 weeks following bone marrow transfer, animals were not infected with *S. aureus* until 10–12 weeks after transplantation. Any mice that demonstrated incomplete chimaerism (i.e.>10% residual recipient phenotype) were excluded from the study.

As previously reported, MyD88 KO mice do not survive very long following intracerebral *S. aureus* infection, with the majority of animals succumbing within 24 h after bacterial exposure with the infectious inoculum used in the earlier and present studies (i.e. 10^4 CFUs) (Kielian et al., 2007). This fact dictated the time course for analysing the current experiments, since we were restricted by the survival time of mice where MyD88 KO bone marrow was transferred into irradiated MyD88 KO recipients (KO→KO). Therefore we examined the entire cohort of bone marrow chimaera animals at two acute intervals, namely 18 and 24 h following *S. aureus* infection. As shown in Figure 2, numerous pro-inflammatory cytokines and chemokines were already detected in WT control mice (WT→WT) at these early time points and were significantly attenuated in MyD88 KO control mice (KO→KO) in agreement with our previous report using non-irradiated animals (Kielian et al., 2007), suggesting that the irradiation paradigm itself did not significantly alter the inflammatory phenotype of MyD88

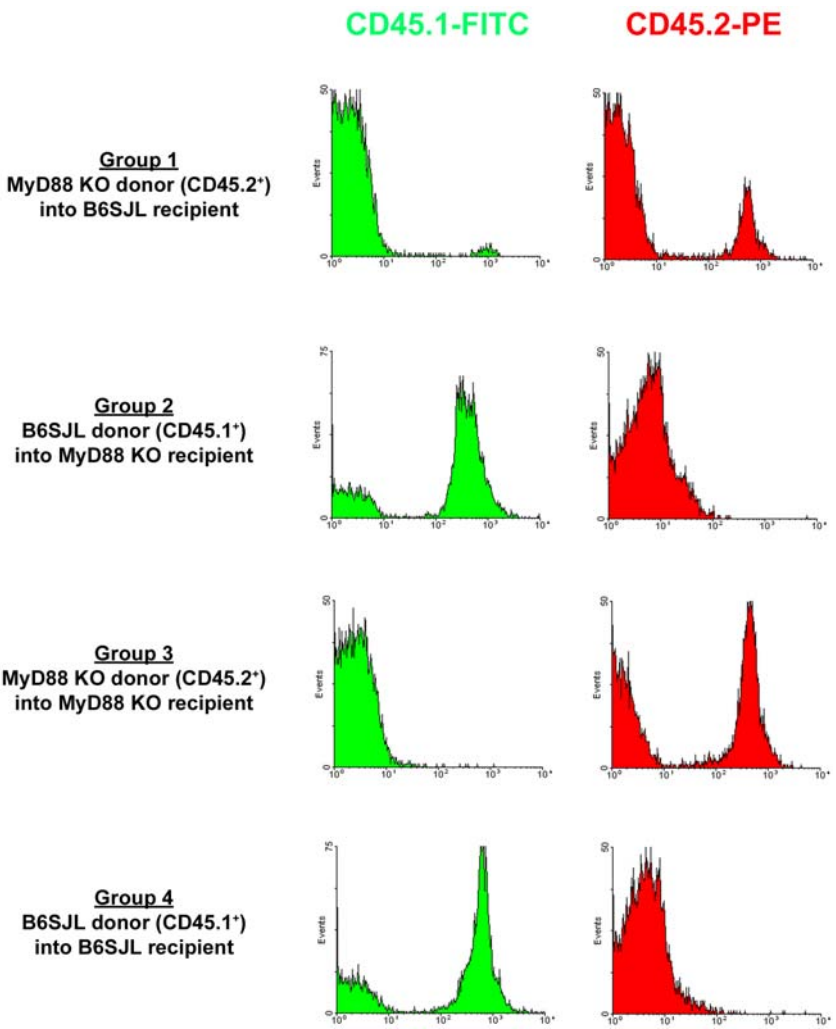

Figure 1 **Validation of MyD88 bone marrow chimaera mice by FACS analysis**
Peripheral blood leucocytes were recovered from MyD88 bone marrow chimaeric mice at 8 weeks following irradiation and bone marrow reconstitution, whereupon CD45.1 (B6/SJL origin) and CD45.2 (MyD88 KO origin) expression was evaluated by FACS. Results are presented from one bone marrow chimaera study and are representative of five independent experiments.

KO and WT mice. Next we assessed which of the experimental chimaeras achieved cytokine expression profiles that were equivalent to WT levels, which would implicate a compartmental-specific contribution for MyD88-dependent signals. As shown in Figure 2, chimaeras where MyD88 was present in the CNS but not in bone marrow-derived cells (KO→WT) were able to mount pro-inflammatory mediator expression profiles equivalent to or exceeding those detected in WT mice at both 18 and 24 h post-infection. This finding implicates CNS MyD88 as essential in eliciting the initial wave of inflammation during the acute response to parenchymal infection. In contrast, the inflammatory phenotype of chimaeric mice where MyD88 was absent in the CNS compartment but present in infiltrating bone marrow-derived cells (WT→KO) was basically identical with MyD88 KO control mice (KO→KO; Figure 2). This finding reinforces the importance of MyD88 in the brain to elicit an effective innate immune response to *S. aureus* and that MyD88 originating from peripheral immune cells alone is not sufficient to recover inflammatory mediator expression to WT levels.

CNS MyD88 dictates the degree of neutrophil influx into *S. aureus*-induced brain abscesses

Our previous study with MyD88 KO mice demonstrated that neutrophil influx into brain abscesses was significantly attenuated in these animals (Kielian et al., 2007). In the present study, we examined whether MyD88 in the CNS or peripheral immune cell compartment was important for dictating neutrophil entry into the infected brain. For these studies we utilized immunofluorescence staining and confocal microscopy with an *S. aureus* strain that constitutively expresses GFP. Importantly, this *S. aureus*-GFP strain is

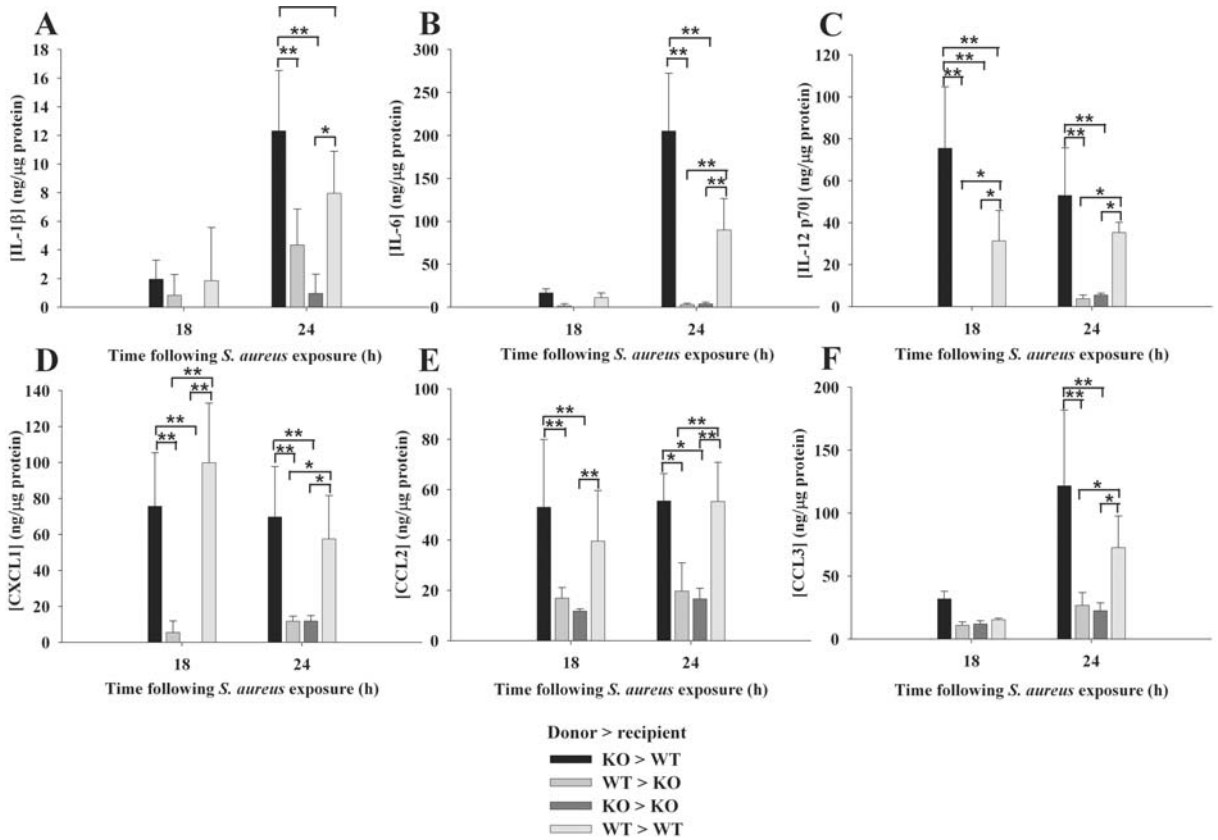

Figure 2 **MyD88 in the CNS compartment is important for regulating pro-inflammatory mediator expression in brain abscesses**
Abscess homogenates from MyD88 bone marrow chimaeras (n=4–6 mice per group) were prepared at 18 or 24 h following *S. aureus* infection, whereupon inflammatory mediator expression was analysed at the protein level using multi-plex microbead arrays. Mediator levels were normalized to the amount of total protein to account for differences in tissue sampling size. Significant differences between chimaera groups were determined by one-way ANOVA followed by the Holm–Sidak method for multiple pair-wise comparisons and are denoted with asterisks (*$P<0.05$; **$P<0.001$). Results are representative of three independent experiments.

identical with the isolate used in our previous studies with the exception of the GFP construct. As shown in Figure 3, neutrophil influx was readily apparent in abscesses of WT control mice (WT→WT), whereas very few cells could be visualized in lesions of MyD88 KO control animals (KO→KO), in agreement with our previous report (Kielian et al., 2007). In concordance with the finding that chemokine expression was restored to WT levels in bone marrow chimaera where MyD88 was present in the CNS compartment (KO→WT), significant numbers of neutrophils were associated with brain abscesses of these chimaeras, reaching levels that were equivalent to WT mice (WT→WT; Figure 3). In contrast, minimal neutrophil influx was detected in chimaeras where MyD88 was only present in bone marrow-derived cells and absent in the CNS (WT→KO; Figure 3). We did not perform quantitative measurements of PMN (polymorphonuclear cell) infiltrates by FACS in the various chimaera groups owing to limiting numbers of mice. Collectively, these findings indicate that MyD88 expression in resident CNS cells is critical for dictating subsequent neutrophil efflux into the infected brain. Importantly, these findings mirror chemokine expression profiles (Figure 2), providing additional supportive evidence for the important role of central MyD88 in initiating the inflammatory cascade required for CNS amplification of inflammatory networks to recruit peripheral anti-microbial effector cells.

Neutrophil and microglia/macrophages occupy distinct anatomical niches during *S. aureus* infection in the CNS parenchyma

The use of a *S. aureus*-GFP isolate enabled us to evaluate the cell type(s) intimately associated with bacteria in the CNS parenchyma. A consistent trend surfaced where neutrophils were in direct contact with bacteria (Figure 4), whereas microglia/macrophages were never found associated with *S. aureus*, but rather were physically removed from the bacteria and localized along the abscess margins. The physical locales of these cell types are probably attributed to their effector functions during brain abscess development. For example, neutrophils are essential for controlling bacterial burdens and the extent of brain abscess dissemination (Kielian et al.,

Table 1 **Differentially expressed genes between MyD88 KO and WT mice harbouring brain abscesses**

(a) Ratios of MyD88 KO compared with MyD88 WT at 12 h post-infection

Gene name	Common name	GenBank® accession number	*Z*-ratio
Lcn2	Lipocalin 2	NM_008491.1	−11.13
Pacsin3	Protein kinase C and casein kinase substrate in neurons 3	NM_028733.1	−3.25
Erdr1	Erythroid differentiation regulator 1	NM_133362.1	−2.62
SOCS3	Suppressor of cytokine signaling 3	NM_007707.2	−2.40
CXCL1	C-X-C motif ligand 1	NM_008176.1	−2.25
Ier3	Immediate early response 3	NM_133662.1	−2.02
Pdap1	PDGFA-associated protein 1	XM_132501.2	−1.93
Calm2	Calmodulin 2	NM_007589	−1.65
Glul	Glutamine synthase	NM_008131.2	−1.65
Hspca	Heat-shock protein 1α	NM_010480.3	−1.65
Mtch1	Mitochondrial carrier homologue 1	NM_019880.2	1.95
Bach	Brain acyl-CoA hydrolase	NM_133348.1	1.93
Hba-a1	Haemoglobin α, adult chain 1	NM_008218.1	1.63
Ndufs2	NADH dehydrogenase Fe-S protein 2	NM_153064.3	1.55

(b) Ratios of MyD88 KO compared with MyD88 WT at 24 h post-infection

Gene name	Common name	GenBank® accession number	*Z*-ratio
Ifitm2	Interferon-induced transmembrane protein 2	NM_030694	−3.26
Lcn2	Lipocalin 2	NM_008491.1	−2.39
Pfc	Properdin factor, complement	XM_135820.3	−2.05
Serpina3n	Serine (cysteine) proteinase inhibitor, clade A, member 3N	NM_009252.1	−1.87
Socs3	Suppressor of cytokine signaling 3	NM_007707.2	−1.68
IL-1β	Interleukin-1β	NM_008361	−1.67
Ifitm1	Interferon-induced transmembrane protein 1	NM_026820.2	−1.54
Egr4	Early growth response 4	NM_020596.1	1.81
Pacsin3	Protein kinase C and casein kinase substrate in neurons 3	NM_028733.1	1.73

2001), whereas microglia/macrophages are typically localized along the abscess margins (Flaris and Hickey, 1992; Kielian et al., 2008).

Lack of MyD88 expression in the CNS compartment leads to elevated *S. aureus* burdens

We next examined whether MyD88 expression in CNS parenchymal compared with infiltrating immune cells influences *S. aureus* survival in the brain. Interestingly, although no significant differences in bacterial burdens were observed at 18 h post-infection, a significant increase in *S. aureus* titres was detected in chimaeric animals where MyD88 was absent in the CNS but present in bone marrow-derived cells (WT→KO) at 24 h following bacterial exposure (Figure 5). This finding is in agreement with the fact that pro-inflammatory mediator production was depressed in MyD88 WT→KO chimaeras compared with KO→WT and

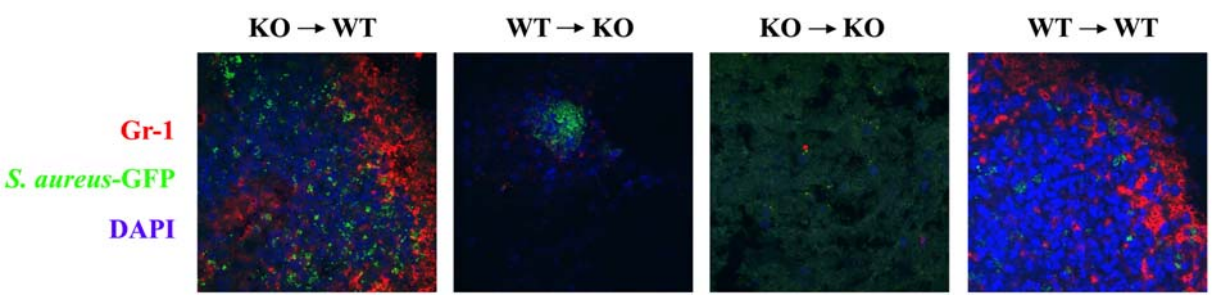

Figure 3 **MyD88 expression in the CNS dictates the degree of neutrophil influx into the infected CNS**

Bone marrow chimaera mice (n=4–6 per group) received an intracerebral infection with a *S. aureus*-GFP strain (green) and were euthanized 24 h later, whereupon brain tissues were flash-frozen on dry ice for subsequent cryostat sectioning. Serial 10 μm thick sections were prepared throughout the entire abscess, subjected to immunofluorescence staining for the neutrophil marker Gr-1 (red), and imaged by confocal microscopy (magnification, 40×). Nuclei were visualized by DAPI (4′,6-diamidino-2-phenylindole) staining (blue). Significant numbers of neutrophils can be visualized infiltrating brain abscesses of WT→WT and KO→WT chimaeras. Results are representative of two independent experiments.

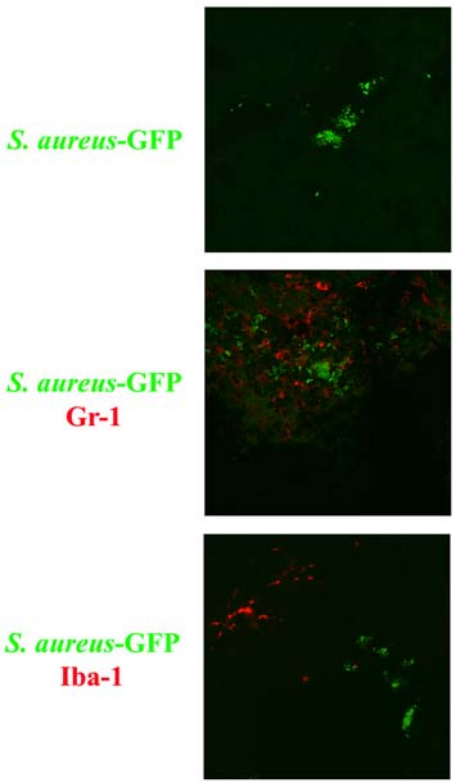

Figure 4 Neutrophils and microglia/macrophages occupy distinct anatomical niches during acute *S. aureus* infection in the brain parenchyma
WT mice received an intracerebral infection with a *S. aureus*-GFP strain (green) and were euthanized 24 h later, whereupon brain tissues were flash-frozen on dry ice for subsequent cryostat sectioning. Serial 10 μm thick sections were prepared throughout the entire abscess, subjected to immunofluorescence staining for the neutrophil or microglia/macrophage markers Gr-1 and Iba-1 respectively (red), and imaged by confocal microscopy (magnification, 40×). Neutrophils are found to directly interact with bacteria, whereas microglia/macrophage staining did not overlap with *S. aureus*. Results are representative of two independent experiments.

WT→WT animals; however, it is intriguing that elevated bacterial burdens were not also observed in MyD88 KO controls, although the latter is in agreement with our previous findings (Kielian et al., 2007). It is not clear what mechanism(s) are responsible for enhanced bacterial burdens in MyD88 WT→KO chimaeras, but one possibility could relate to the recent observation that some bacterial species possess homologues of the TIR (Toll/IL-1 receptor) domain to subvert host defences (Cirl et al., 2008), and it is conceivable that this mechanism could result in the failure to contain bacterial burdens in the presence of MyD88 WT leucocytes. An alternative explanation is that MyD88-positive leucocytes infiltrating the brain parenchyma do not receive a requisite signal(s) from the brain microenvironment in the absence of MyD88, culminating in ineffective bacterial neutralization. These possibilities remain highly speculative at the present time. Collectively, these results indicate that CNS-derived MyD88 signals influence the efficacy of ensuing bactericidal effector mechanisms.

Transcriptional profiling of MyD88 KO mice following intracerebral *S. aureus* infection reveals alterations in apoptosis regulatory pathways

Previous studies with MyD88 KO mice in the experimental brain abscess model revealed that, although these animals were incapable of mounting a significant innate immune response and experienced high mortality rates, paradoxically, these mice did not exhibit significantly elevated bacterial burdens as compared with WT animals (Kielian et al., 2007). This finding demonstrated that bacterial burdens themselves are not the sole determinant in dictating the extent of tissue injury and/or mortality during brain abscess development. To begin to dissect the potential mechanism(s) responsible for the rapid decline of MyD88 KO mice following intracerebral *S. aureus* infection, transcriptional profiling was performed. Interestingly, only a small subset of genes was found to be differentially expressed by microarray analysis and many of these have been implicated in controlling apoptotic pathways (i.e. Erdr1, Ier3, Pdap1, Calm2, Hspca, Mtch1, and Ifitm1 and 2; Table 1). Dysregulation of apoptotic pathways could conceivably be responsible, in part, for the excessive tissue destruction observed in MyD88 KO mice (Kielian et al., 2007), where a combination of rampant necrotic and apoptotic death leads to the destruction of infected and neighbouring non-infected brain parenchyma respectively. In addition, several genes detected by microarray analysis (i.e. CXCL1, IL-1β) have previously been shown to be down-regulated in brain abscesses of MyD88 KO mice at the protein level (Kielian et al., 2007), confirming the validity of the microarray findings to accurately distinguish changes in gene expression between the groups. However, to further confirm the differential expression patterns detected by microarray analysis, we selected a subset of genes for validation by qRT-PCR. As shown in Figure 6, the expression patterns of the five genes examined (Lcn2, SOCS3, Ier3, Pfc and Pascin3) were in agreement with the microarray findings, indicating that these genes may impact the differential outcome of *S. aureus* infection and widespread parenchymal destruction in the brains of MyD88 KO mice. It should be noted that the relatively low number of differentially expressed genes detected between MyD88 KO and WT mice is probably the result of our experimental design. Namely, we elected to perform microarrays on brain abscess RNA collected from individual animals at each time point rather than pooling RNA from a group of animals. We felt this was important to account for biological variability between individual mice and, as such, it is highly likely that the breadth of differentially expressed genes was underestimated by this approach. Collectively, these transcriptional profiling studies suggest dysregulation of pro-inflammatory and apoptotic genes in acute brain abscesses of MyD88 KO mice. The functional role for each of these molecules in contributing to the excessive parenchymal destruction observed following *S. aureus* infection in MyD88 KO mice remains to be determined.

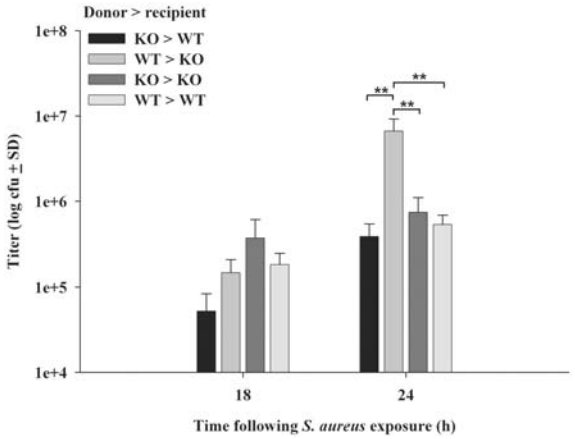

Figure 5 Lack of MyD88 expression in the CNS compartment leads to elevated *S. aureus* burdens

Abscess homogenates from MyD88 bone marrow chimaeras (n=4–6 mice per group) were prepared at 18 or 24 h following *S. aureus* infection, whereupon the number of viable bacteria were determined by quantitative plate assays. Significant differences between chimaera groups were determined by one-way ANOVA followed by the Holm–Sidak method for multiple pair-wise comparisons and are denoted with asterisks (**P<0.001). Results are representative of three independent experiments.

DISCUSSION

MyD88-dependent signalling plays a pivotal role in regulating the host innate immune response to bacterial infection (Akira et al., 2006; Trinchieri and Sher, 2007). Not only is this adaptor molecule central in the signalling of the majority of TLRs, but also mediates activation through the IL-1R and IL-18R (Wesche et al., 1997; Adachi et al., 1998; Burns et al., 1998; Medzhitov et al., 1998). The importance of MyD88 in the host response to infectious diseases has been highlighted by several laboratories in diverse infectious disease models (Takeuchi et al., 2000; Koedel et al., 2004; Miller et al., 2006; Fremond et al., 2007).

Our recent study demonstrated that MyD88 KO mice displayed global defects in innate immunity following intracerebral *S. aureus* infection as typified by the dramatic reduction in pro-inflammatory cytokine and chemokine expression and the inability to recruit significant numbers of neutrophils and macrophages from the periphery (Kielian et al., 2007). One main question that remained to be answered was how important is MyD88 in the CNS compartment? Does CNS MyD88 expression play a role in shaping the subsequent innate immune response following bacterial infection in the brain or are infiltrating leucocytes alone sufficient for establishing immunity? This issue could not be resolved in our previous study since MyD88 was globally absent in MyD88 KO mice and the fact that the brain abscess model is complicated by the involvement of both activated CNS-resident and infiltrating immune cells both of which express this adaptor molecule (Kielian, 2004; Stenzel et al., 2005; Kielian et al., 2007). This conundrum was addressed in the present study by generating radiation bone marrow chimaera mice where MyD88 was specifically expressed in either CNS-resident cells or peripheral bone marrow-derived leucocytes.

The results from MyD88 bone marrow chimaera mice were rather unexpected given the essential nature of MyD88-

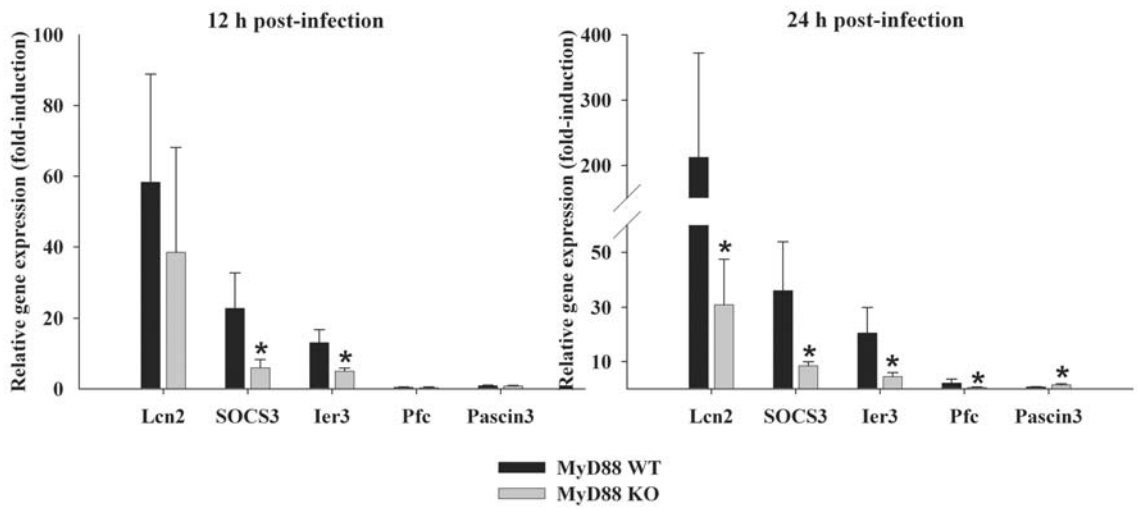

Figure 6 qRT-PCR confirms a subset of differentially expressed genes detected by microarray analysis

MyD88 KO and WT mice (n=4–5 per group) were infected with *S. aureus* intracerebrally and euthanized at 12 or 24 h, whereupon total RNA was isolated and evaluated for a subset of genes determined to be differentially expressed by microarray analysis using qRT-PCR. Genes analysed included Lcn2, SOCS3, Ier3, Pfc and Pascin3. Gene expression levels were calculated after normalizing target signals against the housekeeping gene GAPDH and are presented as the change in mRNA expression compared with uninfected animals (mean ± S.D.). Significant differences in gene expression levels between MyD88 KO and WT mice are denoted with asterisks (*P<0.05). Results are representative of two independent experiments examining a total of 8–10 individual animals.

dependent signalling in peripheral leucocyte activation and the fact that these cells represent the major infiltrate associated with evolving brain abscesses (Kielian, 2004). Indeed, studies by our group and others have shown that neutrophils constitute the main cell type associated with brain abscesses during the early stage of infection (Kielian et al., 2001; Stenzel et al., 2005; Kielian et al., 2007). In essence, our results demonstrated that MyD88 expression in CNS-resident cells, not bone marrow-derived cells, was essential for inducing maximal cytokine/chemokine expression since levels were restored to those observed in WT-infected animals only in MyD88 KO→WT chimaeras. In contrast, animals where MyD88 was present in bone marrow-derived cells, but not the CNS (i.e. WT→KO) did not display WT mediator expression. However, the finding that some inflammatory molecules were more highly expressed in WT→KO chimaeras compared with MyD88 KO controls (i.e. KO→KO) suggests a minor contribution for peripheral MyD88 in shaping the innate immune response during the first 24 h following *S. aureus* infection. It should be acknowledged that we cannot discount a potential role for MyD88 expression in the periphery during later stages of infection. In fact, this possibility appears likely since resident CNS cells (i.e. microglia and astrocytes), although immune competent, have relatively poor bactericidal activity compared with professional phagocytes (i.e. neutrophils and macrophages). In addition, since many gene products driven by MyD88 impact bacterial survival [i.e. iNOS (inducible NO synthase), ROI (reactive oxygen intermediates) and cytokines] it is likely that, with time, MyD88 signals originating from bone marrow-derived leucocytes may become more important in regulating anti-bacterial immunity in brain abscesses. However, this possibility could not be examined in the current study due to the short survival period of infected MyD88 KO mice.

A similar requirement for MyD88 expression in the CNS compartment was observed when evaluating neutrophil influx into the infected brain. Specifically, WT levels of neutrophil recruitment were only observed in chimaeras with MyD88 expression in the brain parenchyma (i.e. KO→WT). This finding probably stems from the fact that the expression of neutrophil chemokines was also restored to WT levels in these chimaeras. It is important to note that radiation bone marrow chimaera studies are not without potential caveats. For example, irradiation has been shown to induce transient blood–brain barrier compromise and the expression of several pro-inflammatory mediators (Belka et al., 2001; Diserbo et al., 2002; Li et al., 2004; Linard et al., 2004). The likelihood that these factors influenced the results obtained in the present study is minimized by the fact that chimaeras were not infected with *S. aureus* until 8 weeks post-transplant. Indeed, a recent report has demonstrated that pro-inflammatory molecule expression was similar between radiation chimaera and non-irradiated mice in response to intracerebral LPS (lipopolysaccharide) (Turrin et al., 2007). It has been suggested that irradiation serves to condition the CNS for subsequent colonization by bone marrow-derived cells that eventually transition into microglia-like cells, although some controversy with regard to this latter point still exists (Simard et al., 2006; Ajami et al., 2007; Mildner et al., 2007; Davoust et al., 2008). Therefore it is possible that a minor percentage of parenchymal microglia in the present study originated from bone marrow precursors since we did not shield the head during the irradiation procedure; nonetheless, our results clearly demonstrate an essential role for MyD88 expression by CNS-resident cells in eliciting protective immunity during early stages of brain abscess development.

To further investigate the potential pathway(s) affected by MyD88 loss during early brain abscess development, microarray analysis was performed. Interestingly, many genes that were differentially expressed in MyD88 KO mice were related to apoptosis, which may be due, in part, to the inability to trigger NF-κB (nuclear factor κB) activation, a well known survival signal (Van Antwerp et al., 1996; Li et al., 1999). One such gene was Ier3 (IEX-1), a stress-inducible gene that is rapidly up-regulated in response to a variety of factors including infection, inflammatory cytokines, and transcription factors such as NF-κB (Pietzsch et al., 1997; Domachowske et al., 2000; Arlt et al., 2008). Indeed, a previous study has demonstrated that IEX-1 expression is NF-κB- and TNF-α-dependent (Osawa et al., 2003) and, since both molecules are significantly attenuated in MyD88 KO mice, this is in agreement with the reduction in IEX-1 levels detected by microarray in these animals. Although IEX-1 has been shown to play a pivotal role in promoting cell survival in response to stress (Wu et al., 1998; Garcia et al., 2002; Mittal et al., 2006), its functional impact on cell survival remains controversial and probably depends on cell type, stimulus, and expression levels (Arlt et al., 2001; Schilling et al., 2001; Osawa et al., 2003). Interestingly, a previous study has demonstrated that IEX-1 reduces ROS (reactive oxygen species) production (Shen et al., 2006) and, by extension, attenuated IEX-1 levels in brain abscesses of MyD88 KO mice could conceivably exacerbate ROS accumulation contributing to the massive tissue damage observed in these animals. However, these possibilities remain speculative at the present time.

Another gene that was significantly down-regulated in brain abscesses of MyD88 KO mice is Lcn2 [also referred to as NGAL (neutrophil gelatinase-associated lipocalin)]. Similar to IEX-1, Lcn2 has also been implicated in regulating apoptosis and is produced by numerous cell types including macrophages and neutrophils in response to infection (Kjeldsen et al., 1993; Devireddy et al., 2001; Tong et al., 2005; Lee et al., 2007). Therefore, in the context of our brain abscess model, it is likely that reduced Lcn2 expression in MyD88 KO mice resulted from the fact that neutrophil and macrophage infiltrates are decreased approx. 85% and 15% respectively, compared with WT animals as we previously reported (Kielian et al., 2007). In addition, a previous study has demonstrated that Lcn2 is induced in macrophages in response to heat-killed group *B Streptococcus* via a TLR2-dependent manner,

implying a role for TLRs in Lcn2 induction (Draper et al., 2006). Obviously, TLR2-mediated signalling is ablated in MyD88 KO mice, suggesting that this may represent one mechanism by which Lcn2 expression is reduced during brain abscess formation in these animals. Another gene whose expression was decreased in brain abscesses of MyD88 KO animals was properdin. Properdin activates the alternative pathway of complement by assembling the C3 convertase on target surfaces (Kemper and Hourcade, 2008). Properdin is capable of binding to bacteria to engage the alternative complement pathway and its importance in host defence is illustrated by the enhanced sensitivity of properdin-deficient patients to meningococcal disease (Densen et al., 1987; Emonts et al., 2003). Unlike most complement components, properdin is produced by neutrophils and macrophages (Schwaeble et al., 1994; Wirthmueller et al., 1997) and the fact that the influx of both of these cell types is significantly attenuated in brain abscesses of MyD88 KO mice (Kielian et al., 2007) probably explains the reduction in properdin levels observed in these animals. Another gene that was attenuated in brain abscesses of MyD88 KO mice was SOCS3, which is a negative regulator of cytokine signalling elicited by JAK (Janus kinase)/STAT (signal transducer and activator of transcription), as well as TLR signalling pathways (Dimitriou et al., 2008). It is interesting to note that SOCS3 has been reported to block IL-10 production, as well as induce classical macrophage activation (Dimitriou et al., 2008; Liu et al., 2008). Our previous study demonstrated that macrophages and microglia isolated from brain abscesses of MyD88 KO mice expressed equivalent levels of IL-10 compared with WT cells, and this fact could be explained by the reduction in SOCS3 levels observed here. In addition, TNF-α has been reported to stabilize SOCS3 mRNA expression (Ehlting et al., 2007), and the failure to induce significant TNF-α expression in brain abscesses of MyD88 KO mice could be another factor contributing to reduced SOCS3 levels in these animals.

Importantly, our microarray analysis revealed several genes that we have previously reported to be significantly attenuated in brain abscesses of MyD88 KO mice at the protein level including IL-1β and CXCL1 (KC) (Kielian et al., 2007). This provides further confidence in the accuracy of our microarray analysis. Collectively the propensity of apoptosis-related genes that are differentially expressed in brain abscesses of MyD88 KO mice is highly suggestive that the expansive tissue damage that occurs in these animals is influenced by accelerated/dysregulated apoptotic pathways. This possibility remains to be directly tested in future studies with MyD88 KO mice in the brain abscess model.

One important point to emphasize is that, although we have identified a critical role for MyD88 in the CNS compartment during the early phase of *S. aureus* infection, several outstanding questions remain. The first is that we cannot determine whether MyD88 mediates its effects through TLRs or the IL-1R or IL-18R since all of these molecules utilize this signalling adaptor. This is important since earlier studies from our laboratory have revealed a pivotal role for IL-1 in innate immunity to CNS *S. aureus* infection (Kielian et al., 2004a). A second issue relates to the target cells in the brain where MyD88 expression is important. Likely candidates include microglia and astrocytes since both cell types are capable of recognizing *S. aureus* via a MyD88-dependent mechanism leading to pro-inflammatory mediator release (Esen and Kielian, 2006; T. Kielian, unpublished data). Regardless, the findings of the present study highlight a novel mechanism for CNS-resident cells in initiating a protective innate immune response in the infected brain and, in the absence of MyD88 in this compartment, immunity is compromised.

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