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Signaling through MyD88 Regulates Leukocyte Recruitment after Brain Injury[1]

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Injury to the CNS provokes an innate inflammatory reaction that engages infiltrating leukocytes with the capacity to repair and/or exacerbate tissue damage. The initial cues that orchestrate leukocyte entry remain poorly defined. We have used flow cytometry to investigate whether MyD88, an adaptor protein that transmits signals from TLRs and receptors for IL-1 and IL-18, regulates leukocyte infiltration into the stab-injured entorhinal cortex (EC) and into sites of axonal degeneration in the denervated hippocampus. We have previously established the kinetics of leukocyte entry into the denervated hippocampus. We now show that significant leukocyte entry into the EC occurs within 3–12 h of stab injury. Whereas T cells showed small, gradual increases over 8 days, macrophage infiltration was pronounced and peaked within 12–24 h. MyD88 deficiency significantly reduced macrophage and T cell recruitment to the stab-injured EC and the denervated hippocampus at 5 days post-injury. Whereas macrophage and T cell entry remained impaired into the denervated hippocampus of MyD88-deficient mice at 8 days, leukocyte infiltration into the stab-injured EC was restored to levels observed in wild-type mice. Transcripts for TNF-α, IL-1β, and CCL2, which increased >50-fold after stab injury in C57BL/6 mice at the time of peak expression, were severely reduced in injured MyD88 knockout mice. Leukocyte recruitment and gene expression were unaffected in TLR2-deficient or TLR4 mutant mice. No significant differences in gene expression were observed in mice lacking IL-1R or IL-18R. These data show that MyD88-dependent signaling mediates proinflammatory gene expression and leukocyte recruitment after CNS injury. *The Journal of Immunology,* **2008, 181: 6481–6490.**

Injury to the CNS provokes inflammation. Glial cells residing in the damaged tissue are poised to initiate an inflammatory response that recruits leukocytes to the injured CNS. Infiltrating leukocytes may promote repair but retain the capacity to exacerbate injury-induced neuropathology (1–3). From a therapeutic standpoint, it is important to understand the signaling mechanisms that guide leukocytes to the damaged CNS. The initial cues that orchestrate leukocyte entry remain ill defined.

CNS-resident glial cells are activated by brain injury (4–10). Microglia, the "brain macrophages," react quickly to damage, consistent with their status as the resident innate myeloid cell in the CNS. Glial cells produce cytokines and chemokines that regulate leukocyte infiltration (4, 8–10). The mechanisms used by glia to actively direct neuroinflammatory responses, which share qualitative and temporal properties with innate immune responses to pathogens, have been collectively termed CNS innate responses (4–7).

Innate roles for both microglia and astrocytes have been emphasized by numerous demonstrations that these cells express TLR protein in vitro (11–13) and, more recently, in vivo (11, 14–20). TLRs are an important class of pattern-recognition receptors that guard against infection by recognizing conserved pathogen-associated motifs. This triggers a signaling cascade that initiates an innate immune response. Most TLRs are dependent on signaling through the adaptor protein MyD88, which also associates with the receptors for IL-1 and IL-18. TLRs are additionally becoming recognized as an important class of receptors in innate responses to endogenous damage or "danger signals" (21–25). In the CNS, sterile inflammation and injury increase MyD88, TLR2, and TLR4 expression (15–18, 20, 26–33), and endogenous roles for TLRs have been identified in the regulation of innate responses independent of adjuvant or infection. We have previously shown that TLR2 signaling regulates T cell infiltration and microglial expansion in response to axonal degeneration in the hippocampus (15). Others have identified roles for endogenous TLR2 and/or TLR4 signaling in neuropathic pain (26, 34), Alzheimer's disease (35, 36), injury (20, 30, 37), and cerebral ischemia (16–19, 27, 38). MyD88 signaling has also been implicated in CNS response to neurodegeneration or injury (32, 33, 37, 39). Although MyD88 is known to impact leukocyte infiltration induced by adjuvant or infection (40–42), its regulatory effects on the kinetics of macrophage and T cell recruitment after sterile injury are not known.

We have assessed the impact of MyD88 signaling on the innate response to stab injury in the entorhinal cortex (EC)[3] of mice and to the resulting axonal degeneration in the denervated hippocampus. This was achieved by using a stereotactic surgical model to localize mechanical damage to the EC of mice (15, 43–45). This stab lesion provokes junctional breakdown of the blood brain barrier in the EC, but not in the adjacent hippocampal regions of the brain where there is anterograde degeneration of transected axons

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[3] Abbreviations used in this paper: EC, entorhinal cortex; ADAM, a disintegrin and metalloproteinase; EAE, experimental autoimmune encephalomyelitis; KO, knockout; MMP, matrix metalloproteinase; qPCR, quantitative real-time RT-PCR.

and localized glial response (46, 47). This lesion therefore allows direct comparison of mechanisms involved in initiating glial response to different types of injury. Our findings suggest that signaling through the MyD88 adaptor protein is a key event for proinflammatory gene expression and leukocyte recruitment to the injured brain.

Materials and Methods

Mice

A total of 326 mice were used in this study. C57BL/6 mice were purchased from Charles River Canada or Taconic and were used as control mice unless otherwise stated. C57BL/6 backcrossed TLR2-deficient mice (48), MyD88-deficient mice (49), and TNFR1-deficient mice (50) were bred and maintained under specific pathogen-free conditions. C57BL/6 backcrossed mice lacking CCL2 (51), IL-1R (52), or IL-18R (53) were purchased from The Jackson Laboratory. TLR4-defective C3H/HeJ mice, which have a point mutation that renders this receptor hyporesponsive (54), were also purchased from The Jackson Laboratory, and C3H/HeN mice (Charles River Canada) were used as wild-type controls. CCR2-deficient mice (F2 C57BL/6 × 129/Ola (55)) were bred in our facility and B6.129P F2/J mice purchased from The Jackson Laboratory were used as background controls. All animal experiments were conducted according to Canadian Council on Animal Care guidelines, as administered by the McGill University Animal Care Committee, or according to Danish law and protocols approved by the Danish Ethical Animal Care Committee.

Surgery

Under anesthesia, animals were placed into a stereotactic apparatus (Kopf Instruments) for wire knife transection of axons in the EC, as previously described (15, 43). This produces a mechanical stab injury to the EC and provokes axonal degeneration in the denervated hippocampus (15, 43–45, 56, 57). At various times post-injury (specified below), mice were perfused with PBS and the affected brain regions were carefully dissected for flow cytometry or RNA analyses. For studies of leukocyte response to stab injury in the EC, ~2 mm of tissue surrounding the wire knife lesion was collected (56). For uninjured controls, EC were dissected from either the contralateral hemisphere of the brain or else from unmanipulated mice (56). Leukocyte entry into sites of axonal degeneration was examined in isolated whole hippocampi after careful removal of the choroid plexus (15) and was compared with basal levels of leukocyte proportions in unmanipulated or contralateral hippocampus. For histological analyses, mice were perfused at 5 days post-lesion with PBS, followed by 4% paraformaldehyde. Brains were removed, embedded in OCT (Electron Microscopy Sciences), and frozen in 2-methylbutane cooled on dry ice.

Flow cytometry

For studies of the stab-injured EC, leukocyte infiltration was assessed at 3 h, 12 h, 24 h, 48 h, 5 days, and 8 days in C57BL/6 mice, at 24 h, 5 days, and 8 days in MyD88 knockout (KO) mice, and at 5 days for CCL2-deficient mice as well as for CCR2-deficient mice and wild-type controls. Stab-injured EC from TLR2-deficient mice were examined at 2, 5, and 8 days after stab injury, and those from TNFR1-deficient mice were examined at 2 and 5 days. The stab-injured EC of TLR4 defective C3H/HeJ mice and wild-type controls (C3H/HeN) were assessed at 24 h, 2 days, and 5 days. Leukocyte infiltration into the denervated hippocampus of MyD88-deficient and C57BL/6 mice was examined at 24 h, 5 days, and 8 days post-lesion. Except for the C57BL/6 mice lesioned in parallel with MyD88 KO mice, data obtained from the denervated hippocampi of C57BL/6 TLR2- and TNFR1-deficient mice, as well as TLR4-defective mice (and wild-type controls), have been published (15) and allow direct comparison between these injury models.

The EC and hippocampus were carefully dissected from individual mice and prepared as described previously (15, 43, 44, 56, 57). Briefly, a single cell suspension was prepared using the plunger from a 1-cc syringe and a 70-μm mesh (BD Biosciences). After pelleting the cells by centrifugation, 100 μl of blocking solution (24G2 supernatant containing 2% FBS, 50 μg/ml Syrian hamster IgG (Jackson ImmunoResearch), and 0.1% sodium azide) was added to prevent nonspecific binding. Abs to CD45 and CD11b or TCRβ were used to stain for microglia/macrophages or T cells, respectively (BD Biosciences). Macrophages were distinguished from microglia based on relative CD45 levels (58, 59). After forward/side scatter gating, positive staining was determined based on fluorescence levels compared with isotype and autofluorescence controls (BD Biosciences). Cells stained with CD45 only or CD45 in conjunction with isotype controls for TCRβ or CD11b were used to identify the background fluorescence and/or nonspecific binding of the CD45^{high} population. Data were collected on a FACScan or a FACSCalibur flow cytometer (BD Biosciences) and analyzed using CellQuest Pro software (BD Biosciences). In some instances, the sample volume was measured before and after data acquisition, which allowed estimation of total numbers of macrophages and T cells in cell suspensions (15, 57, 60).

Immunofluorescence staining

Horizontal cryostat sections (10- to 12-μm thick) were blocked and stained using a previously established avidin-biotin approach (15). All leukocytes and activated microglia were identified using rat anti-mouse CD45, and T cells were identified by staining with rat anti-human CD3 (Serotec). Sections were coverslipped in Prolong Gold anti-fade medium containing 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen) and analyzed using an Olympus BX51 fluorescence microscope. Control sections stained using rat IgG2b (Serotec) or rat IgG1 (Hybridomus) at the same concentration as the primary Abs showed no fluorescence.

RNA isolation and quantitative real-time RT-PCR (qPCR)

RNA was isolated from unmanipulated or stab-injured EC at 1 h, 3 h, 24 h, 48 h, 5 days, or 8 days post-injury and converted to cDNA as previously described (43). qPCR was done using either an ABI PRISM 7000 or a 7300 sequence detection system (Applied Biosystems) as previously described for IL-1β, CCL2, TNF-α, matrix metalloproteinase (MMP)12, a disintegrin and metalloproteinase (ADAM)12, IFN-γ, IL-17, and 18S (15, 61, 62). Intron-spanning primers and the FAM-MGB (minor groove binder) probe used to detect CD3ε mRNA were as follows: 5′-GCCTCCTAGCTGTTGGCACTT (forward), 5′-TGAGAT GGAGACTTTGTATTCAATGTT-3′ (reverse), and FAM-CCAGGAC GATGCCG-MGB-3′ (probe).

Statistics

Data are presented as mean ± SEM. Groups of matched contralateral and ipsilateral stab-injured EC or hippocampi were analyzed by two-tailed paired Student's *t* tests. Otherwise, data were analyzed using two-tailed Student's *t* test or one-way ANOVA with Bonferroni's post hoc analysis, as appropriate. Values of $p < 0.05$ were considered statistically significant and are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Results

Macrophage and T cell recruitment to the stab-injured EC

We used flow cytometry to measure leukocyte infiltration to the EC after stab injury. CD45 levels were used to distinguish macrophages (CD11b$^+$CD45^{high}; Fig. 1*A*, *upper right quadrants*) from CNS-resident microglia (CD11b$^+$CD45^{dim}; Fig. 1*A*, *lower right quadrants*) (58, 59). We have previously used flow cytometry to establish that macrophages and T cells are recruited to the denervated hippocampus in response to axonal degeneration (15, 43). Likewise, the small proportion of CD11b$^+$CD45^{high} macrophages found in the unmanipulated EC dramatically increased after injury (Fig. 1*A*). A statistically significant increase in macrophage proportions was detected in the stab-injured EC by 3 h, compared with contralateral uninjured EC (~14-fold; Table I). Macrophage proportions then rapidly reached a peak, showing a >60- to 100-fold increase at 12–24 h post-lesion (Table I). This represented ~7,000–10,000 macrophages vs only ~50 in control EC (Fig. 1*C*). Macrophage proportions declined slightly after 24 h but remained significantly elevated relative to contralateral controls even at 8 days, when proportions were still increased 30-fold (Table I). At this time, the number of macrophages dropped to only ~600 in the stab-injured EC (Fig. 1*C*). These data emphasize the robust infiltration of macrophages within 12–24 h after injury and the rapid decline beyond 5 days.

T cell recruitment was also monitored by flow cytometry after stab injury. A few TCRβ^+CD45^{high} T cells were found in the unmanipulated EC (Fig. 1*B*). As seen for macrophage entry, a

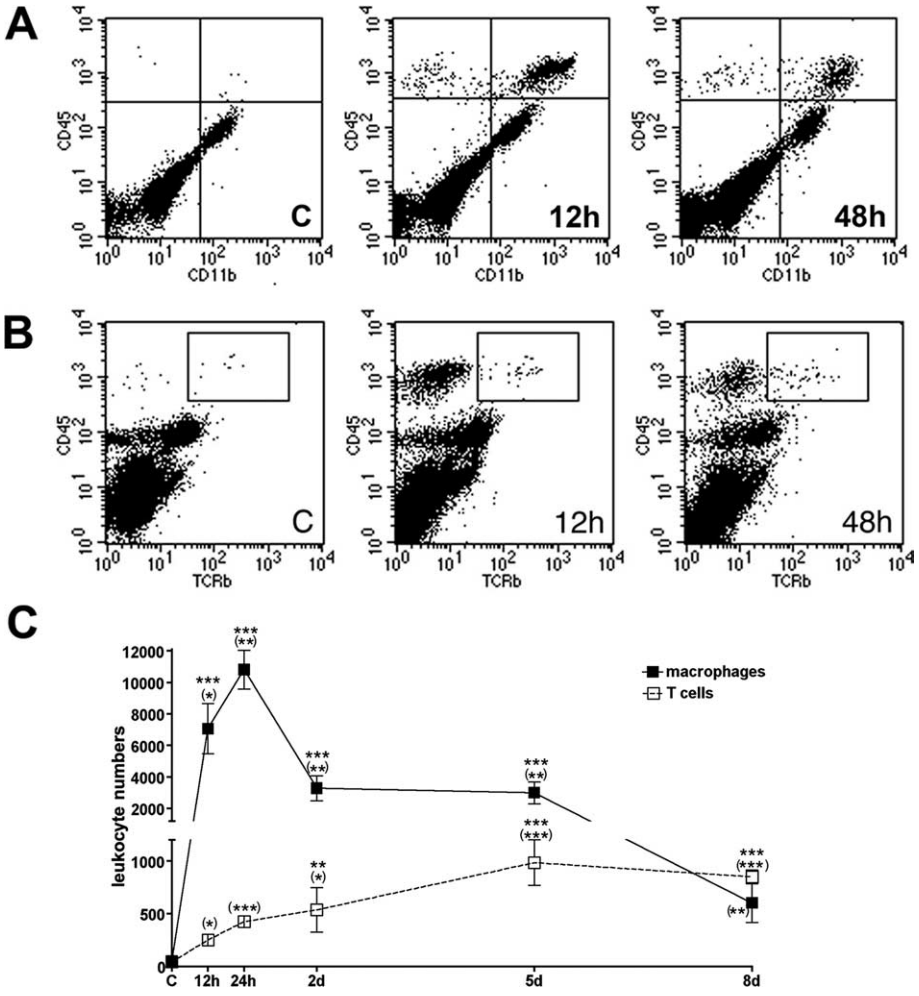

FIGURE 1. Flow cytometric evaluation of macrophage and T cell recruitment to the stab-injured EC. *A*, Flow cytometry profiles showing $CD11b^{+}CD45^{high}$ macrophages (*upper right quadrants*) in uninjured control (C) EC or 12 and 48 h after stab injury in C57BL/6 mice. *B*, Flow cytometry profiles showing $TCR\beta^{+}CD45^{high}$ T cells (boxes) in uninjured control (C) EC or 12 and 48 h after stab injury in C57BL/6 mice. *C*, Estimates of numbers of macrophages and T cells recruited to the EC after stab injury in C57BL/6 mice generated by flow cytometry, as outlined in *Materials and Methods*; $n = 4–13$ per group, except control EC, where $n = 32–36$. Asterisks indicated statistically significant differences vs uninjured EC. Asterisks in parentheses indicate statistically significant differences vs paired contralateral uninjured EC. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. d, Day.

small increase in T cell proportions was detected within 3 h of stab injury (~5-fold, $p = 0.07$; Table I). Unlike macrophage numbers and proportions, which rapidly peaked and then began to decline, T cell proportions showed a continued gradual increase after injury. Compared with uninjured contralateral controls, there was only a 7- to 13-fold increase in T cell proportions between 12 h and 2 days, with proportions increasing >35-fold at 5 days (Table I). Thus, whereas only ~40 T cells were detected in uninjured contralateral controls, there were ~250 T cells by 12 h, ~500 T cells by 1–2 days, and roughly 900 T cells at 5–8 days (Fig. 1*C*). These data demonstrate that macrophages and T cells show different patterns of recruitment after a mechanical injury that disrupts the blood-brain barrier. Whereas macrophage entry is an acute and pronounced response to stab injury, T cell recruitment occurs more progressively with a much smaller magnitude.

To strengthen our flow cytometric observations of macrophage and T cell infiltration to the stab-injured EC, we performed immunofluorescence on tissue sections to detect $CD3^{+}$ T cells and $CD45^{+}$ leukocytes. Histologically, both single cells and clusters of $CD3^{+}$ T cells were located throughout the stab-injured EC of C57BL/6 mice at 5 days (Fig. 2*A*). CD45 staining, which revealed cells with round and ramified morphologies, identified infiltrating leukocytes and activated

Table I. *Macrophage and T cell proportions (mean ± SEM) in paired contralateral and stab-injured EC at various times post-lesion*

	3 h	12 h	24 h	2 Days	5 Days	8 Days
Macrophages						
Number (*n*)	3	4	5	13	12	7
Statistical significance[a]	*	**	**	***	***	*
Stab-injured	0.14 ± 0.02	1.87 ± 0.26	2.26 ± 0.34	1.16 ± 0.13	1.18 ± 0.26	0.30 ± 0.10
Contralateral	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
T cells						
Number (*n*)	3	4	3	13	9	7
Statistical significance[a]	NS	**	**	***	***	***
Stab-injured	0.05 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.13 ± 0.01	0.36 ± 0.04	0.40 ± 0.00
Contralateral	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.00

[a] *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

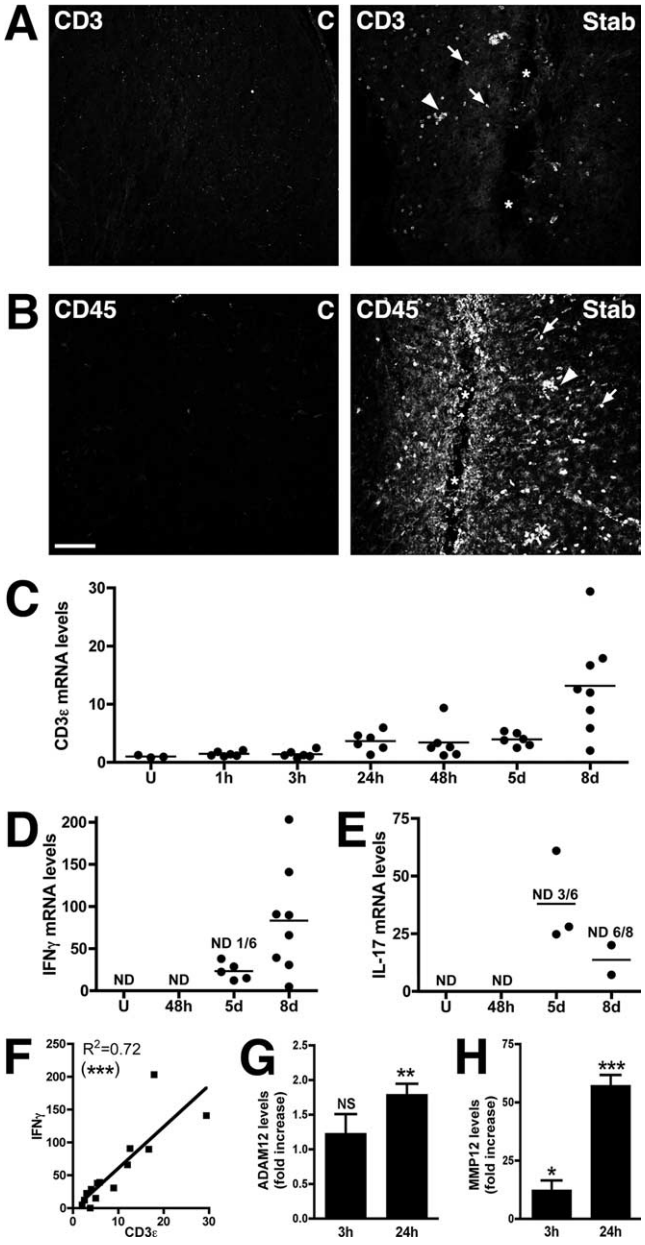

FIGURE 2. Immunofluorescence staining and qPCR analysis of leukocyte recruitment after stab injury. *A* and *B*, Immunofluorescence staining identified CD3$^+$ T cells (*A*) and CD45$^+$ leukocytes/activated microglia (*B*) in the stab-injured EC of C57BL/6 mice at 5 days, whereas very little staining was observed in contralateral (C) uninjured EC. Arrows point to examples of single stained cells and arrowheads to clusters of CD3 or CD45$^+$ cells. Asterisks identify the site of the lesion. Images are representative of $n = 4$. Scale bar = 100 μm. *C–H*, qPCR analysis of CD3ε (*C*), IFN-γ (*D*), IL-17 (*E*), IFN-γ (*F*), ADAM12 (*G*), and MMP12 (*H*) mRNA levels in C57BL/6 mice, normalized to 18S, at times (d, Day) indicated after stab injury to the EC ($n = 5$–9). A significant correlation was observed between mRNA levels for IFN-γ and CD3ε. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs uninjured (U) controls.

microglia in the stab-injured EC at 5 days (Fig. 2*B*). As with CD3 staining, single cells and clusters of round CD45$^+$ cells were observed (Fig. 2*B*). Generally, more leukocytes were identified by CD45 than by CD3, which is consistent with our flow cytometry data showing significant infiltration by both CD11b$^+$CD45^{high} macrophages and TCRβ^+CD45^{high} T cells at 5 days.

We also measured levels of transcripts expressed by CNS-infiltrating leukocytes by qPCR. Levels of CD3ε mRNA, expressed by all T cells, were increased nearly 4-fold in the stab-injured EC of C57BL/6 mice between 24 h and 5 days, and by 8 days CD3ε mRNA levels were increased 13-fold (Fig. 2*C*). The cytokines IFN-γ and IL-17, which are expressed by activated T cells in the CNS autoimmune inflammatory disease experimental autoimmune encephalomyelitis (EAE) (58, 62, 63), were detected only sporadically after stab injury. Whereas IFN-γ mRNA was detected in most samples 5 and 8 days after stab injury (Fig. 2*D*), and levels were significantly correlated with CD3ε mRNA levels (Fig. 2*F*), most samples had undetectable IL-17 (Fig. 2*E*). IFN-γ and IL-17 mRNA levels were 50- to 200-fold lower than in EAE (data not shown). Neither IFN-γ nor IL-17 were detected by 40-cycle qPCR in the stab-injured EC of C57BL/6 mice before 5 days (Fig. 2, *D* and *E*, and data not shown). We did detect a small but significant fold increase in message for ADAM12 by 24 h (Fig. 2*G*), which is also expressed by T cells within CNS infiltrates in EAE (61). We also found that transcript levels of the macrophage-expressed (61) MMP12 were significantly increased by 3 h, when significant macrophage infiltration into the stab-injured EC had begun (Fig. 2*H*). Similar to the case with macrophage proportions, MMP12 expression was dramatically increased by 24 h, when a nearly 60-fold increase in mRNA levels was detected (Fig. 2*H*).

MyD88 signaling regulates leukocyte entry

Macrophage and T cell infiltration were examined in the stab-injured EC and in the denervated hippocampus of MyD88-deficient mice at 24 h, 5 days, and 8 days to determine whether signaling through this adaptor protein had any regulatory effect on leukocyte entry to the injured brain. As expected (15, 43), lower proportions of macrophages and T cells were observed in the denervated hippocampus compared with the stab-injured EC of C57BL/6 mice (Fig. 3*B* vs Fig. 5*C*; Fig. 4*B* vs Fig. 5*D*). Still, occasional CD3$^+$ T cells and CD45$^+$ leukocytes could be observed in the denervated zones of the hippocampus (Fig. 5, *A* and *B*).

Stab injury-induced leukocyte entry was strikingly lower in MyD88-deficient mice than in stab-injured C57BL/6 mice (Figs. 3*A* and 4*A*). In the absence of MyD88 signaling, macrophage recruitment was 45–80% lower than that in C57BL/6 mice at 24 h and 5 days (Fig. 3*B*). T cell entry was also significantly reduced in stab-injured MyD88-deficient mice (by 40–55%; Fig. 4*B*). By 8 days, however, macrophage and T cell infiltration were no longer significantly reduced in MyD88-deficient mice vs C57BL/6 mice (Figs. 3*B* and 4*B*). Leukocyte proportions in uninjured contralateral EC, which showed no statistically significant differences at various times post-injury (Table I) and were thus pooled for further analysis, were not significantly different between C57BL/6 and MyD88-deficient mice (Figs. 3*B* and 4*B*).

In the denervated hippocampus of MyD88-deficient mice there was no statistically significant injury-induced recruitment of T cells at 24 h, 5 days, or 8 days as compared with contralateral hippocampi (Fig. 5*D*). Macrophage infiltration was not significantly increased at 24 h or 5 days either, although a small but significant increase was observed at 8 days (Fig. 5*C*). By comparison, significant macrophage and T cell recruitment was observed in C57BL/6 mice at all times examined (Fig. 5, *C* and *D*), as we have found previously (15, 43). Proportions of macrophages and T cells were significantly higher in injured

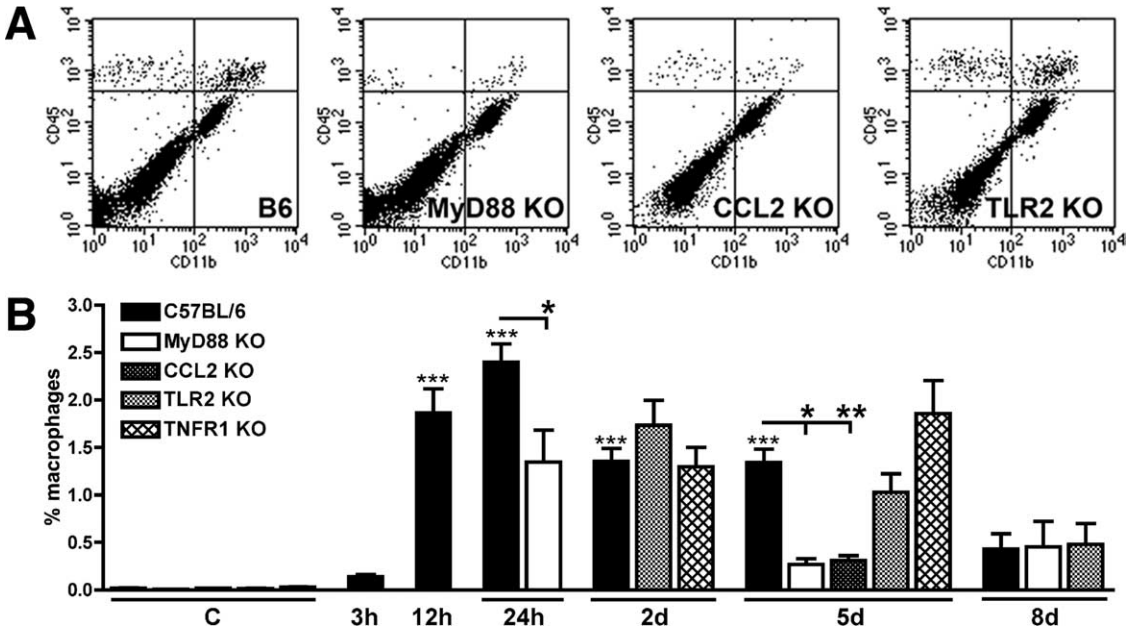

FIGURE 3. Reduced macrophage recruitment after stab injury in MyD88-deficient mice. *A*, CD11b/CD45 flow cytometry profiles of C57BL/6 mice and MyD88 KO, CCL2 KO, and TLR2 KO mice shown 5 days after stab injury illustrate reduced macrophage recruitment in mice lacking MyD88 and CCL2 (*upper right quadrants*). *B*, Quantification of proportions of $CD11b^{+}CD45^{high}$ cells showed rapid accumulation of macrophages after stab injury in C57BL/6 mice above basal proportions in control (C) EC ($n = 3$–26 per group, except for control, where $n = 57$), and the impact of TLR2, MyD88, CCL2, and TNFR1 deficiency on macrophage recruitment ($n = 3$–11 per group). Asterisks directly above black bars indicate statistical significance in injured C57BL/6 mice vs pooled control C57BL/6 EC. Otherwise, asterisks denote statistical significance in KO mice vs C57BL/6 mice at 24 h and 5 days (d). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

C57BL/6 hippocampi than in injured MyD88-deficient hippocampi at 5 days and 8 days post-lesion, although not at 24 h, when T cell infiltration had just begun (43) and when a large variation in macrophage proportions was observed in MyD88 KO mice. Taken together, these results show that signaling through the MyD88 adaptor protein is an important regulator of leukocyte recruitment both after stab injury in the EC and in response to degenerating axons in the hippocampus.

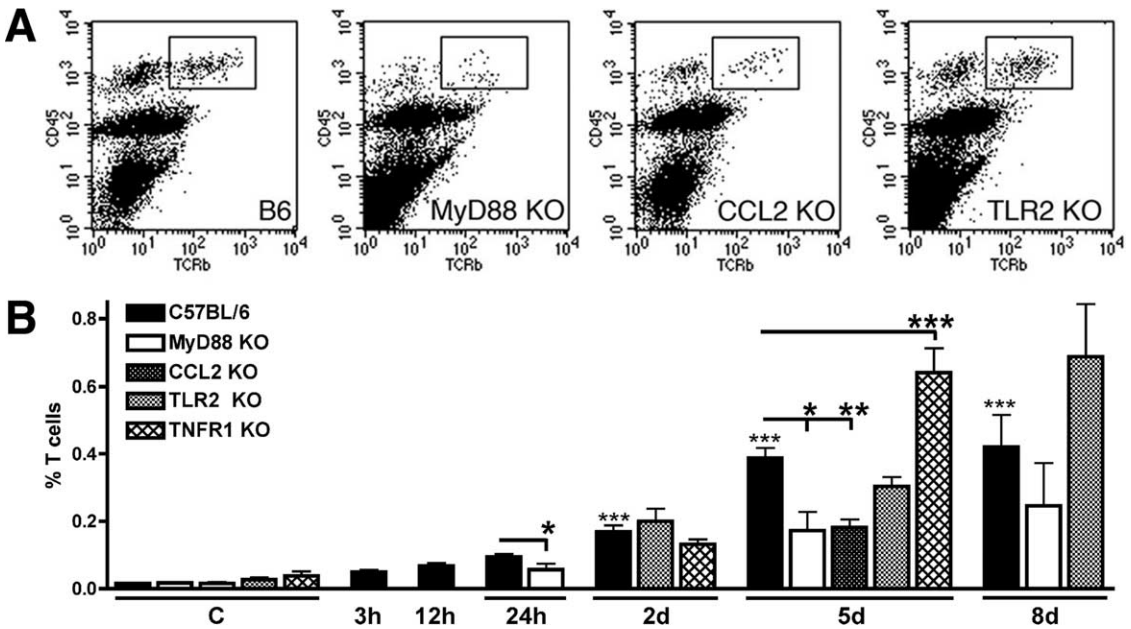

FIGURE 4. Reduced T cell recruitment after stab injury in MyD88-deficient mice. *A*, Flow cytometry profiles showing $TCR\beta^{+}CD45^{high}$ T cells (boxes) in C57BL/6 mice and MyD88 KO, CCL2 KO, and TLR2 KO mice 5 days after stab injury illustrate reduced T cell recruitment in mice lacking MyD88 and CCL2. *B*, Quantification of proportions of $TCR\beta^{+}CD45^{high}$ cells showed progressive T cell accumulation after stab injury in C57BL/6 mice above basal levels in control (C) EC ($n = 3$–22 per group, except for control, where $n = 44$), and the impact of TLR2, MyD88, CCL2, and TNFR1 deficiency on T cell recruitment ($n = 3$–11 per group). Asterisks directly above black bars indicate statistical significance in injured C57BL/6 mice vs pooled control C57BL/6 EC. Otherwise, asterisks denote statistical significance in KO mice vs C57BL/6 mice at 24 h and 5 days 9d). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

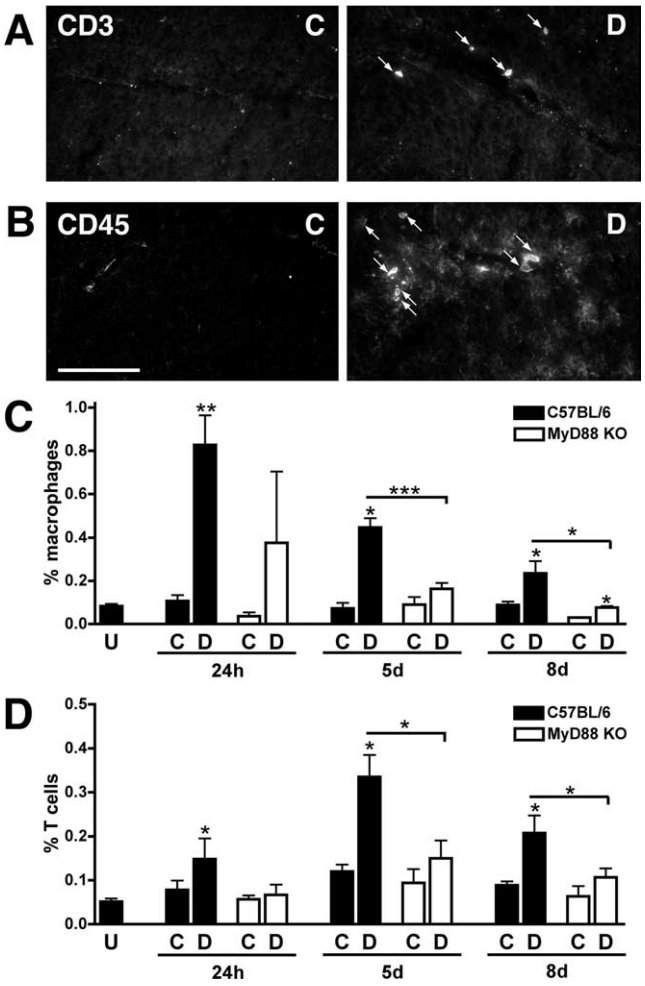

FIGURE 5. Leukocyte recruitment to the denervated hippocampus is MyD88-dependent. *A* and *B*, Immunofluorescence staining identified $CD3^+$ T cells (*A*) and $CD45^+$ leukocytes/activated microglia (*B*) in the denervated hippocampus (D) of C57BL/6 mice 5 days after lesion, whereas little staining was observed in the unlesioned contralateral hippocampus (C). Arrows point to $CD3^+$ or $CD45^+$ leukocytes. Images are representative of $n = 4$. Scale bar = 100 μm. *C* and *D*, Proportions of $CD11b^+CD45^{high}$ macrophages and $TCR\beta^+$ $CD45^{high}$ T cells were increased in the denervated hippocampus of C57BL/6 mice at 24 h, 5 days, and 8 days post-lesion, compared with the unlesioned contralateral hippocampus ($n = 4$–7, asterisks directly above filled bars). Leukocyte proportions were similar between contralateral hippocampi and hippocampi from unmanipulated (U) mice. Leukocyte recruitment was not significantly different between contralateral and denervated hippocampi of MyD88 KO mice, except for macrophage recruitment at 8 days ($n = 3$–5, asterisk directly above open bar). Macrophage and T cell proportions in denervated hippocampi were significantly lower in MyD88-deficient mice vs C57BL/6 mice at 5 and 8 days (d) post-lesion. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

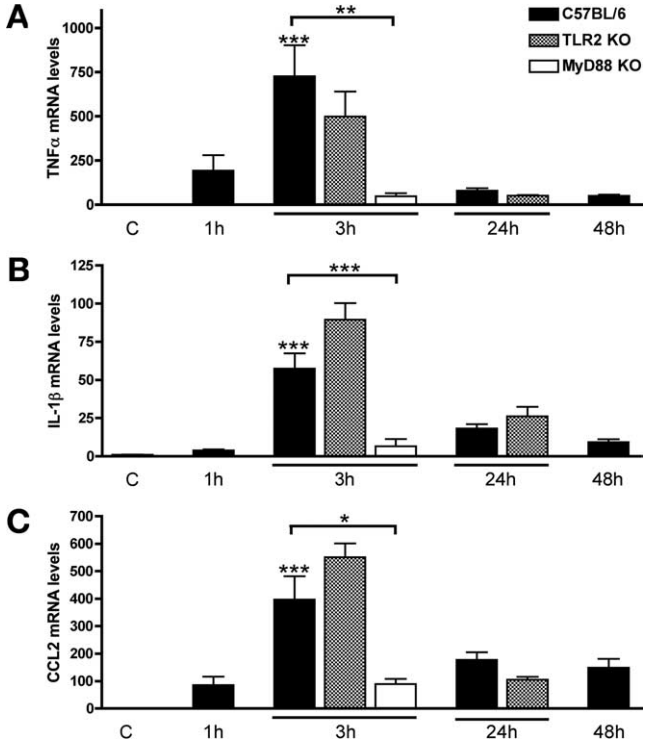

FIGURE 6. MyD88 signaling drives proinflammatory gene expression after stab injury. qPCR analysis of TNF-α (*A*), IL-1β (*B*), and CCL2 (*C*) mRNA levels, normalized to 18S, in control uninjured EC (C) and after stab injury in C57BL/6, as well as MyD88 KO and TLR2 KO mice ($n = 4$–11). Values are shown as fold increase vs the mean of the control group, which was arbitrarily set to 1. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs uninjured controls or C57BL/6 mice at 3 h, as indicated.

Regulation of proinflammatory mediators

To clarify the mechanism by which MyD88 deficiency regulates leukocyte entry, we analyzed effects of MyD88 signaling on stab injury-induced proinflammatory gene expression. TNF-α, IL-1β, and CCL2 were examined as possible candidates because they were previously implicated in MyD88-signaled inflammatory response (39, 42, 49), and we have previously shown that CCL2 signaling through its receptor, CCR2, drives leukocyte entry (43) and that TNF acting via TNFR1 affects T cell recruitment (15) to the denervated hippocampus. We confirmed that CCL2 had a significant effect on leukocyte recruitment to the stab-injured EC. Both macrophage (Fig. 3*B*) and T cell (Fig. 4*B*) proportions were significantly reduced in CCL2-deficient mice at 5 days post-injury compared with those in injured C57BL/6 controls. By contrast, macrophage and T cell proportions in the stab-injured EC of CCR2-deficient mice were not statistically different from those in stab-injured B6.129 control mice at 5 days (data not shown). Enhanced T cell recruitment was observed 5 days after stab injury in TNFR1-deficient mice, but not at 2 days, and macrophage recruitment was unaffected (Figs. 3*B* and 4*B*).

IL-1β, CCL2, and TNF-α mRNA levels were expressed at very low levels in the unmanipulated EC (Fig. 6). Transcripts for IL-1β, CCL2, and TNF-α were elevated 4-, 85-, and 190-fold, respectively, within 1 h of stab injury in wild-type mice relative to uninjured EC and were even more dramatically increased by 3 h (Fig. 6, filled bars). At this time, there was a >700-fold increase in TNF-α levels, a 400-fold increase in levels of CCL2, and a 50-fold increase in IL-1β levels. Levels of all three transcripts then declined but remained elevated even at 48 h (50-, 150-, and 10-fold, respectively; Fig. 6).

These data identified a clear peak in proinflammatory gene expression and led us to investigate expression levels in mice lacking MyD88 at 3 h. Injury-induced increases in IL-1β, TNF-α, and CCL2 mRNA were significantly lower in the stab-injured EC of MyD88-deficient mice compared with injured C57BL/6 controls. After stab injury, MyD88-deficient mice expressed only 7–22% of the cytokine and chemokine mRNA levels seen in C57BL/6 mice (Fig. 6).

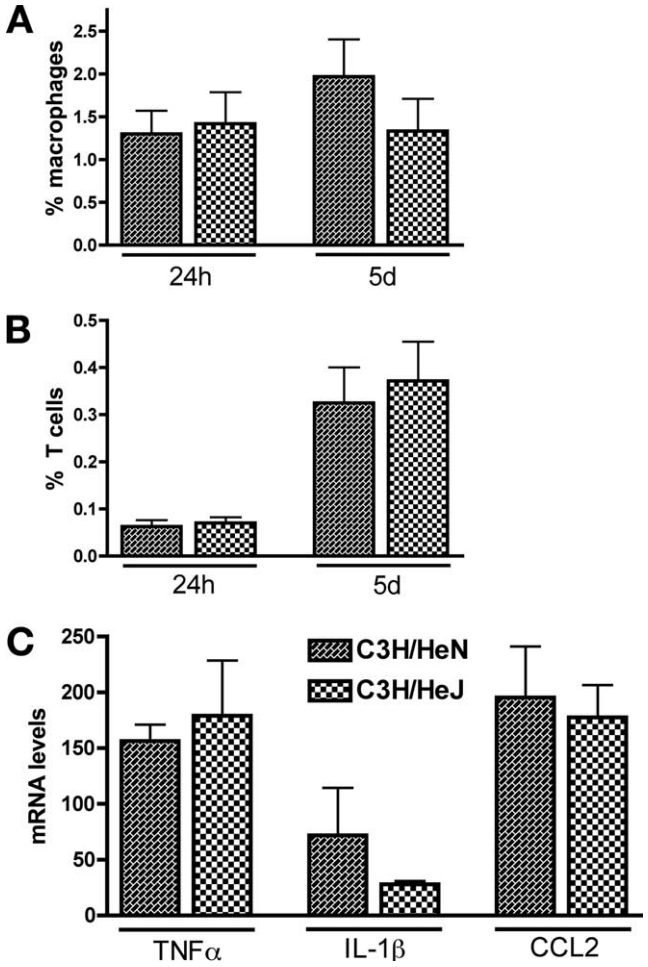

FIGURE 7. Response to stab injury in TLR4 mutant mice. *A* and *B*, Proportions of $CD11b^{+}CD45^{high}$ macrophages (*A*) and $TCR\beta^{+}$ $CD45^{high}$ T cells (*B*) were not significantly different between TLR4 mutant C3H/HeJ mice and background controls (C3H/HeN) 24 h or 5 days (d) after stab injury to the EC. *C*, Levels of TNF-α, IL-1β, and CCL2 mRNA were measured by qPCR and normalized to 18S. qPCR data are expressed as arbitrary values and levels cannot be compared between cytokines. $n = 4-8$ per group. No statistically significant differences were observed.

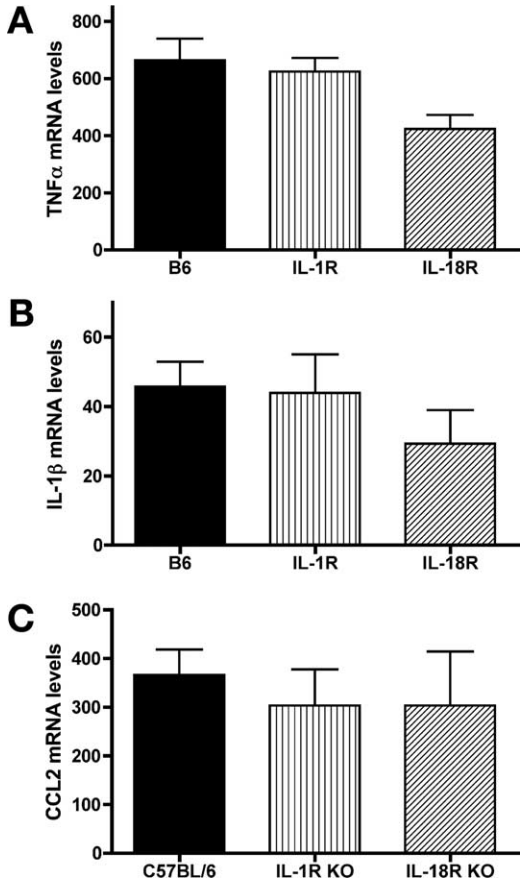

FIGURE 8. Proinflammatory gene expression is independent of IL-1R and IL-18R. qPCR analysis of TNF-α (*A*), IL-1β (*B*), and CCL2 (*C*) mRNA levels in C57BL/6 mice, normalized to 18S, at 3 h after stab injury to the EC, as well as mice lacking IL-1R and IL-18R (n = 3–10). No statistically significant differences were observed.

Inflammation induced by stab injury is independent of TLR2 and TLR4 signaling

We then focused our attention to the role of TLR2 and TLR4 in guiding MyD88-mediated leukocyte entry to the stab-injured EC. We previously identified a specific role for TLR2 signaling in T cell (but not macrophage) recruitment to the denervated hippocampus, but found no effect of defective TLR4 signaling on leukocyte entry (15).

Interestingly, unlike in the denervated hippocampus, there was no significant reduction in T cell entry into the stab-injured EC of TLR2-deficient mice, relative to C57BL/6 controls, at 2 days, 5 days, or 8 days after injury (Fig. 4). Macrophage recruitment was also unaffected by TLR2 deficiency (Fig. 3). We also saw no significant difference in leukocyte entry after stab injury to TLR4 mutant C3H/HeJ mice (relative to C3H/HeN wild-type controls, Fig. 7, *A* and *B*). Thus, leukocyte entry occurred independently of TLR2 and TLR4 signaling. Consistent with our flow cytometry data, we did not observe any reduction in stab injury-induced mRNA levels of TNF-α, IL-1β, and CCL2 in TLR2-deficient mice or TLR4 mutant mice (Figs. 6 and Fig. 7*C*). We have previously shown that mRNA for TNF-α, but not CCL2 or IL-1β, is reduced in the denervated hippocampus of TLR2-deficient mice vs C57BL/6 mice (15).

Importantly, because these data in the stab-injured EC were obtained from the same groups of TLR2-deficient and TLR4 mutant mice (and controls) used in our studies of denervated hippocampi that identified a role for TLR2 in regulating T cell entry and microglial expansion (15), direct comparison between these injury models can be made. Together, our results suggest that involvement of specific TLRs may depend on the type and/or location of the injury sustained by the CNS.

Stab injury-induced gene expression is unaffected by IL-1R or IL-18R deficiency

Because MyD88-deficiency had such dramatic effects on proinflammatory gene expression that could not be attributed to TLR2 or TLR4 signaling, we asked whether non-TLR pathways were regulating this response by screening TNF-α, IL-1β, and CCL2 levels 1 and 3 h after stab injury in IL-1R and IL-18R knockout mice. However, no statistically significant differences were observed (3 h, Fig. 8; 1 h, data not shown).

Discussion

We have monitored the impact of MyD88 signaling on leukocyte infiltration to the stab-injured EC and to sites of axonal injury in the denervated hippocampus. Our findings identify a regulatory

role for MyD88-signaled response in the recruitment of macrophages and T cells and in the regulation of early proinflammatory gene expression.

Although MyD88 deficiency clearly impacts on leukocyte recruitment, the receptor(s) that signal through MyD88 have yet to be identified. Macrophage and T cell recruitment to the stab-injured EC were unaffected in TLR2-deficient and TLR4-defective mice. We have previously shown that signaling through TLR2, but not TLR4, regulated T cell entry into the denervated hippocampus, whereas neither TLR2 nor TLR4 affected macrophage recruitment (15). Thus, macrophage infiltration into either injury site occurred independently of TLR2 or TLR4 signaling, whereas T cell infiltration was differentially regulated by TLR2 (the current study and Ref. 15). By comparison, MyD88 deficiency reduced both macrophage and T cell entry to both injury sites, although recruitment had rebounded to match values in the stab-injured EC of control mice at 8 days. Also, MyD88-deficiency had a greater impact on T cell infiltration into the denervated hippocampus than could be accounted for by TLR2 signaling alone, because T cell infiltration was delayed by TLR2 deficiency, returning to control levels in injured C57BL/6 mice by 5 days (15) but blocked at 5 and 8 days by MyD88-deficiency (the current study).

Our data suggest that multiple signals may converge at the MyD88 adaptor protein for a full-blown response. TLR2-dependent signals may exert a proportionally stronger effect in the denervated hippocampus than in the stab-injured EC. This may provide a simple explanation for the observed differences in requirements for MyD88/TLR2 signaling in T cell recruitment to the stab-injured EC and the denervated hippocampus. Response to anterograde axonal degeneration in the hippocampus occurs without junctional disruption of the blood-brain barrier (46, 47). By contrast, blood-brain barrier damage after tissue trauma allows the entry of serum proteins and other factors that might contribute to the onset of neuroinflammatory pathways. The type or location of the injury may therefore determine which of a number of possible recruitment mechanisms are initiated. Along this line, we found that innate responses in the stab-injured EC and denervated hippocampus (15) occurred independently of TLR4, whereas recent studies in other systems identified roles for TLR4 signaling (26, 30, 35, 37, 38). However, in the absence of definitive information on cell type-specific up-regulation of individual TLRs and downstream signaling in each tissue, it can only be speculated whether different biological outcomes reflect signaling through different TLRs or signaling through the same TLR in different cells.

There are a number of potential ligands for the MyD88-signaled response. Heat shock proteins, matrix proteins, and other potential endogenous ligands for TLRs (22, 24) may be released after injury by dying cells or by leakage through a damaged blood-brain barrier. Although the majority of endogenous ligands so far described have been proposed to signal through TLR2 and TLR4, they are not restricted to them. For instance, mRNA may activate TLR3 (64), RNA sequences within small nuclear ribonucleoprotein particles may trigger TLR7/8-expressing cells (65), and chromatin-Ig complexes have been shown to signal through TLR9 (66). TLR3 ligands, however, are unlikely to account for inflammation induced by stab injury, because TLR3 signaling is MyD88 independent (21, 22). Generation of response to stab injury may therefore rely on signaling by TLRs other than TLR2 or TLR4. Although macrophage and T cell recruitment were not specifically investigated, response to cold-induced cortical injury was recently also shown to be MyD88 dependent but TLR2/TLR4 independent (39). MyD88 is also implicated in the signaling for response to IL-1β and IL-18 (22, 67). IL-1β is rapidly induced after injury and is implicated in the generation of early response in other systems (68, 69). However, we found that stab injury-induced TNF-α expression was not reduced by IL-1R deficiency, which is consistent with previous work (69). IL-18 is constitutively expressed in the brain and was not up-regulated after optic nerve crush injury (70, 71). Up-regulation of IL-18 after ischemic brain injury was delayed relative to IL-1β induction, and infarct volume was unaffected by IL-18 deficiency at 24 h (72, 73). IL-18 therefore appears more likely to regulate response in later stage inflammation. Consistent with this, we found no effect of IL-18R deficiency on early gene expression. Whether MyD88 can mediate response to injury independently of the Toll/IL-1R (TIR) domain (74) is an interesting possibility that has yet to be investigated.

Despite a shared dependence on MyD88 signaling, the kinetics and magnitudes of response by macrophages and T cells were strikingly different after stab injury. The observation that peak macrophage infiltration precedes peak T cell entry is a pattern we have also observed in the denervated hippocampus (15, 43) and after cuprizone-induced demyelination (60). It is unlikely that initial increases in T cell and macrophage proportions may have simply reflected efflux due to physical damage of the blood-brain barrier by the stab injury, because T cells outnumber monocytes in peripheral blood (75). It is even more apparent that inherent mechanisms control leukocyte recruitment to the injured brain beyond 3 h, because the patterns of response were temporally and quantitatively different. This may reflect the activation of different MyD88-associated receptors. Analogously, T cell entry to the denervated hippocampus after axonal lesion was specifically regulated by TLR2 signaling, whereas macrophage infiltration was not (15).

A number of chemokines (CCL2, CCL3, CCL4, CCL5, CXCL2, and CXCL10) are expressed in the stab-injured EC (43) that could differentially regulate macrophage and T cell recruitment. Although macrophage and T cell proportions were both reduced by CCL2 deficiency, only T cell entry was affected in TNFR1 KO mice where, similar to what we observed in the denervated hippocampus (15), a significantly higher proportion of T cells was observed 5 days after stab injury. The mechanism for enhanced T cell recruitment to the stab-injured EC of TNFR1-deficient mice is currently not known but appears to be independent of TLR2 signaling, unlike the response in the denervated hippocampus (15). Enhanced recruitment might reflect increased stabilization of cytokine/chemokine mRNA by the association of MyD88 with IFN-γR1 (74), because IFN-γ was expressed at detectable levels 5–8 days after stab injury. Residual levels of TNF-α in MyD88-deficient mice might trigger negative feedback mechanisms via TNFR1 that are unavailable in TNFR1-deficient mice. Indeed, elevated cytokine and chemokine levels have been observed in TNFR1-knockout mice with EAE (62).

T cells are thought to play a neuroprotective role in CNS repair, however there is no consensus yet whether myelin-reactive T cells confer "protective autoimmunity" after injury (1–3). It is interesting that the T cell-expressed cytokines IFN-γ and IL-17 are expressed in autoimmune inflammatory disease (58, 62, 63), but not immediately after traumatic injury. Only by 5–8 days did IFN-γ become detectable at low levels in the stab-injured EC, and even then IL-17 was detected in only one-third of the samples. IFN-γ was not produced in response to anterograde axonal degeneration or after cortical implant injury (45, 76), nor was it found to be up-regulated in the stab-injured EC at 24 h (56). Innate CNS mechanisms that are intended to elicit T cell repair responses may be exploited by an aggressive autoreactive T cell repertoire that can exacerbate neuropathology (77). Autoimmune inflammation may only occur if injury delivers appropriate signals that promote the switch to adaptive immunity. TLR signaling is implicated as a

mediator of this process (78–80). The possibility that injury in the CNS might selectively recruit subsets of T cells that are geared toward promoting repair vs harmful inflammation opens possibilities for therapeutic manipulation. Our findings point to the MyD88 pathway as having a physiological role in such processes, in addition to its well-documented effects on response to infection.

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Disclosures

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