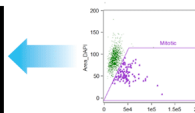
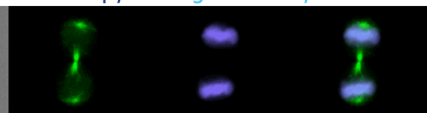
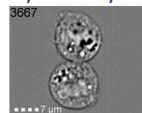




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TRIL Is Involved in Cytokine Production in the Brain following *Escherichia coli* Infection

Paulina Wochal,* Vijay A. K. Rathinam,[†] Aisling Dunne,* Thaddeus Carlson,[‡] Wen Kuang,[§] Katherine J. Seidl,[‡] J. Perry Hall,[‡] Lih-Ling Lin,[‡] Mary Collins,[‡] Stefan A. Schattgen,[†] Christopher R. MacKay,[†] Caio T. Fagundes,* Susan Carpenter,*^{†,1} Katherine A. Fitzgerald,^{†,1} and Luke A. J. O'Neill*,¹

TLR4 interactor with leucine-rich repeats (TRIL) is a brain-enriched accessory protein that is important in TLR3 and TLR4 signaling. In this study, we generated *Tril*^{-/-} mice and examined TLR responses in vitro and in vivo. We found a role for TRIL in both TLR4 and TLR3 signaling in mixed glial cells, consistent with the high level of expression of TRIL in these cells. We also found that TRIL is a modulator of the innate immune response to LPS challenge and *Escherichia coli* infection in vivo. *Tril*^{-/-} mice produce lower levels of multiple proinflammatory cytokines and chemokines specifically within the brain after *E. coli* and LPS challenge. Collectively, these data uncover TRIL as a mediator of innate immune responses within the brain, where it enhances neuronal cytokine responses to infection. *The Journal of Immunology*, 2014, 193: 1911–1919.

Acute systemic inflammatory responses to severe infections may lead to chronic inflammatory processes in the CNS. Septic shock is associated with a spectrum of brain dysfunction and damage, which leads to increased morbidity and mortality (1–3).

Despite its anatomical sequestration from the circulating blood by the blood–brain barrier (BBB), lack of a lymphatic system, and low MHC expression, the brain remains an active player in the inflammatory processes that occur elsewhere in the body (4, 5). In fact, the interplay between the peripheral immune system and the CNS has a reciprocal effect on both systems. Dysregulation of the CNS impacts on the outcome of an acute systemic infection. Equally, however, severe systemic infection often leads to destructive brain inflammation (6, 7).

The systemic inflammatory response is initiated by the recognition of microbial pathogen-associated molecular patterns (PAMPs) or endogenous damage-associated molecular patterns, by evolutionarily conserved pathogen recognition receptors (8). TLRs are a family of pathogen recognition receptors that recognize a wide range of

PAMPs triggering innate immunity. To date, 10 human and 13 mouse members of the TLR family have been identified, which recognize a wide variety of PAMPs (9–11). Upon activation by PAMPs, TLRs initiate downstream signaling cascades leading to the activation of transcription factors such as NF- κ B and/or IFN-regulatory factors, which, in turn, induce the production of proinflammatory cytokines and chemokines, as well as type I IFNs (12).

TLR4 is the most extensively studied member of the TLR family. It is responsible for the recognition of LPS, which is a major component of the outer membrane of Gram-negative bacteria and a key player in the pathogenesis of Gram-negative sepsis (13, 14). TLR4 is constitutively expressed within the CNS and can be found in the parenchymal glial cells, microglia, and astrocytes, as well as in neurons (15–19). TLR4 is also expressed in the meninges, choroid plexus, and circumventricular organs (CVOs) of the brain. These structures are highly vascularized and, despite the presence of peculiar epithelial barriers, lack a characteristic BBB; thus, they are more exposed to invading pathogens, allowing for the cross talk between the periphery and the CNS (20–23).

Binding of LPS and subsequent TLR4 activation is facilitated by a number of accessory molecules including the LPS-binding protein, glycoprotein CD14, and myeloid differentiation protein-2 (24), all of which are central for LPS sensing by TLR4. CD14 exists in a soluble form and as a GPI-linked protein in the plasma membrane (25). Similar to TLR4, it is constitutively expressed within the CNS. In fact, CD14 is found in the meninges, choroid plexus, and CVOs, mirroring the expression of TLR4 within the brain (26). In addition, CD14 is also present in microglia but is absent in astrocytes (27). Interestingly, circulating LPS causes a sequential increase in the expression of CD14, first within the highly vascularized CVOs and then in the brain parenchyma (27, 28).

TLR4 interactor with leucine-rich repeats (TRIL) was initially characterized as a novel component of the TLR4 signaling pathway, highly expressed in the brain (29). It was shown to be required for TLR4-mediated responses in vitro via direct interaction with TLR4 and its ligand, LPS (30). In subsequent in vitro studies, TRIL was also shown to play a role in the regulation of TLR3-mediated signaling. TRIL is, therefore, similar to CD14, which can also regulate TLR3 signaling (31).

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The online version of this article contains supplemental material.

Abbreviations used in this article: A.U., arbitrary unit; BBB, blood–brain barrier; BMDM, bone marrow–derived macrophage; CVO, circumventricular organ; PAMP, pathogen-associated molecular pattern; Poly(I:C), polyinosinic-polycytidylic acid; TRIL, TLR4 interactor with leucine-rich repeats; WT, wild type.

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In this study, we have generated *Tril*^{-/-} mice to further investigate the role of TRIL. We confirmed the role of TRIL in mixed glial cells in TLR4 and TLR3 signaling. *Tril*^{-/-} mice also produced less cytokines in the brain, after intracranial LPS challenge and i.p. infection with *Escherichia coli*. These results confirm a specific role for TRIL in the regulation of TLR4 and TLR3 signaling primarily within the brain.

Materials and Methods

Animals

C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) and generated *Tril*^{-/-} mice were bred at the University of Massachusetts Medical School. Mouse strains were maintained under specific pathogen-free conditions in the animal facilities at the University of Massachusetts Medical School. Mice studies were carried out in strict accordance with guidelines set forth by the American Association for Laboratory Animal Science. The animal protocols for this work were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School (permit no. A-2258-11).

Tril^{-/-} mice generation

The targeting vector was designed to encode a 19-kb fragment of mouse genomic *Tril* DNA together with the flippase recombination target–neomycin resistance cassette, flanked by two *LoxP* sites. Generated construct was used to transfect the embryonic stem cells in the C57BL/6 mice, to enable homologous recombination. After a number of rounds of selection with neomycin, random embryonic stem cells clones were chosen and conditional *Tril*^{-/-} mice were generated. Female founders were next crossed with C57BL/6 males expressing Protamine-Cre, resulting in permanent deletion of *LoxP*-flanked *Tril* alleles and generation of global *Tril*^{-/-} mice.

Genotyping of *Tril*^{-/-} mice

The genotypes of *Tril*^{-/-} mice were determined by PCR analysis of genomic DNA, from tail biopsies. The genomic DNA was isolated using the Genomic DNA isolation Kit (Lamda Biotech) according to the manufacturer's instructions. Isolated genomic DNA was next used for genotyping by PCR with specific oligonucleotide primers for the *Tril* wild type (WT) and targeted allele (*Tril*-F, 5'-TTC ACT TAC CAC CCT GCC AGG TTC -3', *Tril*-R1, 5'-GTC TGT ATG GGA AGA GAG GCA CAC TG-3', *Tril*-R2, 5'-CAC CAG AGC GTT CTG GTC ATG C-3'). Primers F and R1 amplified WT allele and primers F and R2 targeted one. The three primers were used in a PCR using GoTaq (Promega) with the following amplification conditions: 95°C for 5 min and 30 cycles of 95°C for 30 s, 58°C for 30 s, and a 5-min incubation at 72°C at the end of the run. Amplification products were resolved on a 2% agarose gel.

Cell culture and stimulations

Primary murine bone marrow–derived macrophages (BMDMs) and bone marrow–derived dendritic cells (BMDCs) were generated from WT and age/sex-matched *Tril*^{-/-} mice. BMDMs were cultured in DMEM with 10% FBS and 20% L929 supernatants, and BMDCs were maintained in RPMI 1640 (with 10% FCS, L-glutamine [2 mM], 50 μ M 2-ME, 1% penicillin-streptomycin solution [v/v], supplemented with GM-CSF [20 ng/ml]). Primary murine mixed glial cells were prepared from 1- to 3-d-old neonatal brains of WT and age/sex-matched *Tril*^{-/-} mice. Cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin solution (v/v). All cells were used at 10 days, in vitro plated out, and stimulated the next day. The cellular composition of primary mixed glial cells was assessed by FACS analysis using markers specific for astrocytes (GLAST-allophycocyanin; Miltenyi Biotec), microglia (Cd11b-PE; eBioscience), and neurons (β -3-Tubulin; Biolegend), indicating >83% of astrocytes, ~2–3% of microglia, and only trace amounts of neurons within the primary mixed glial cell population. Cultured primary hippocampal neurons were generated from embryonic day 15 to 17 embryos using a previously described method (32) and maintained in serum-free Neurobasal media supplemented with B27 and GlutaMAX (Invitrogen). Primary microglia and astrocytes were isolated from mixed glial cells cultures. Generated as described earlier, primary mixed glial cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin solution (v/v), in the presence of 5 ng/ml M-CSF (R&D Systems) until fully confluent. Primary microglia were then separated from astrocyte monolayers by agitation on a rotary shaker at 125

rpm for 4 h. Primary astrocytes were isolated from the same cultures by trypsinization after microglia were removed as previously described (33). Obtained cells were maintained in DMEM with 10% FCS and 1% penicillin-streptomycin solution (v/v). Microglial cultures were additionally supplemented with 5 ng/ml M-CSF. The purity of primary microglia and astrocytes populations was assessed by FACS analysis using specific markers for astrocytes and microglia, GLAST-allophycocyanin (Miltenyi Biotec) and CD11b-PE (eBioscience), revealing >98% purity of isolated microglia and 97% purity of isolated astrocytes (data not shown). In addition, morphological assessment of primary mixed glial cells and isolated populations of microglia and astrocytes was carried out using phase-contrast microscopy (data not shown). Immortalized microglia cells were generated by infecting primary mixed glial cells with a recombinant retrovirus J2 encoding viral oncogenes v-myc and v-raf, based on the method previously described by Blasi et al. (34). Characterization of the immortalized clonal microglial cells using FACS technique revealed them to be CD11b-PE⁺ and GFAP-allophycocyanin⁻ (data not shown). For quantitative RT-PCR analysis, cells were stimulated for 5 h with 100 ng/ml LPS (Sigma-Aldrich) or 25 μ g/ml polyinosinic-polycytidylic acid [Poly(I:C)] (Sigma-Aldrich) before RNA isolation. For ELISA assay, cells were treated with 10 or 100 ng/ml LPS, 25 or 50 μ g/ml Poly(I:C), 100 nM Pam3CSK4 (Invivogen), or 1 μ g/ml R848 (Invivogen) before harvesting supernatants.

ELISA

Cell culture supernatants were assayed by ELISA for CCL5/RANTES (R&D Systems), TNF- α (eBioscience), and/or IL-6 (eBioscience), in accordance with the manufacturer's instructions. A sandwich ELISA for mouse IFN- β was used as previously described (35).

Nanostring and quantitative RT-PCR

Primary murine mixed glial cells were treated as described earlier followed by the RNA extraction with an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Brain and spleen tissues were isolated from WT and *Tril*^{-/-} mice 6 h post-*E. coli* infection, and RNA was purified using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from total RNA using the iScript Select cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR was performed by using iQ SYBR green supermix (Bio-Rad) and specific primers for murine *Tril* (forward, 5'-ACG TGC TCA CCT ACA GCC TA-3', reverse, 5'-CAG GAC GGT CTT ACC CTT TCC-3'), *Il6* (forward, 5'-AAC GAT GAT GCA CTT GCA GA-3', reverse, 5'-GAG CAT TGG AAA TTG GGG TA-3'), and *Ccl5* (forward, 5'-GCC CAC GTC AAG GAG TAT TTC TA-3', reverse, 5'-ACA CAC TTG GCG GTT CCT TC-3'). Relative quantification was performed using standard curve analysis. The mRNA in samples was normalized to that of β -Actin or *Gapdh*, and represented as the mRNA levels in arbitrary units (A.U.) or as a ratio of gene copy number per 100 copies of β -Actin or *Gapdh*. The SEMs were calculated using the Student *t* test. For the Nanostring analysis, total RNA was hybridized to a custom gene expression CodeSet and analyzed on an nCounter Digital Analyzer. Counts were normalized to endogenous controls per Nanostring Technologies' specifications. Values were log-transformed and displayed as a heat map (Euclidean clustering) generated using the ggplot package within the open source R software environment.

In vivo intracranial LPS challenge

Six- to 8-wk-old age- and sex-matched C57BL/6 and *Tril*^{-/-} mice were anesthetized with isoflurane and then injected intracranially with 20 μ l LPS (100 ng/ μ l, 2 μ g LPS per mouse) or saline (control). Twenty-four hours post-LPS injection, the expression of proinflammatory cytokines *Il6* and *Ccl5* was examined within the brain. Generated data were analyzed by unpaired two-tailed Student *t* test with Prism software. The *p* values <0.05 were considered significant.

In vivo *E. coli* infection

Age- and sex-matched C57BL/6 and *Tril*^{-/-} mice were infected with 10⁹ CFUs *E. coli* BL21 strain via the i.p. route. Cytokine levels at the mRNA and the protein levels were analyzed 6 h postinfection in the brain and spleen. Data from in vivo experiments were analyzed by unpaired two-tailed Student *t* test with Prism software. The *p* values <0.05 were considered significant.

Statistical analysis

Differences between groups were analyzed for statistical significance with Student *t* test using Prism 6 Software (GraphPad, San Diego, CA). A *p* value <0.05 was considered statistically significant.

Results

Generation and characterization of *Tril*^{-/-} mice

A targeting vector to generate *Tril*^{-/-} mice was constructed as outlined in Fig. 1A. Conditional *Tril*^{-/-} female founders were crossed with C57BL/6 males expressing Protamine-Cre, resulting in permanent deletion of *LoxP*-flanked *Tril* alleles. Mice homozygous for the targeted allele were confirmed by PCR using genomic DNA isolated from tail biopsies (Fig. 1B). Because TRIL is a brain-enriched protein, the cDNA derived from brain lysates was used to conduct RT-PCR analysis of *Tril* expression in WT (^{+/+}), heterozygous (^{+/-}), and homozygous *Tril*^{-/-} mice. As expected, *Tril* expression was absent in *Tril*^{-/-} mice, whereas moderate to high expression was observed in heterozygous and WT mice, respectively (Fig. 1C). These data confirm successful deletion of *Tril* in generated knockout mice, which were used in subsequent experiments.

General characteristics of *Tril*^{-/-} mice

Tril^{-/-} mice are viable and fertile, and are born at expected Mendelian ratios. To address whether *Tril* deficiency affects any behavioral characteristics, we subjected *Tril*^{-/-} mice and their WT littermates to a comprehensive set of behavioral tests. *Tril*^{-/-} mice appeared healthy and displayed no phenotypic differences in general behavior, as well as in motor coordination (as determined via the rotarod), anxiety (elevated zero maze, stress-induced hyperthermia, four-plate test), and pain (tail-flick, thermal sensitivity in response to inflammation; Supplemental Fig. 1A).

Test scores demonstrate no significant differences between WT, heterozygous, or *Tril*^{-/-} mice in the acute pain model represented by tail-flick assay (Supplemental Fig. 1B), as well as in the behavioral studies evaluating the impact of TRIL on stress-induced hyperthermic responses (Supplemental Fig. 1C) and locomotor activity (Supplemental Fig. 1D).

TRIL does not impact TLR4- and TLR3-mediated responses in peripheral immune cells

Our previous studies characterized a role for TRIL in mixed glial cells and in the astrocytoma cell line U373 in which we knocked down *Tril* using small interfering RNA and short hairpin RNA (31). Using cells from *Tril*^{-/-} mice, we analyzed TRIL function in multiple cell types and confirmed a high level of *Tril* expression

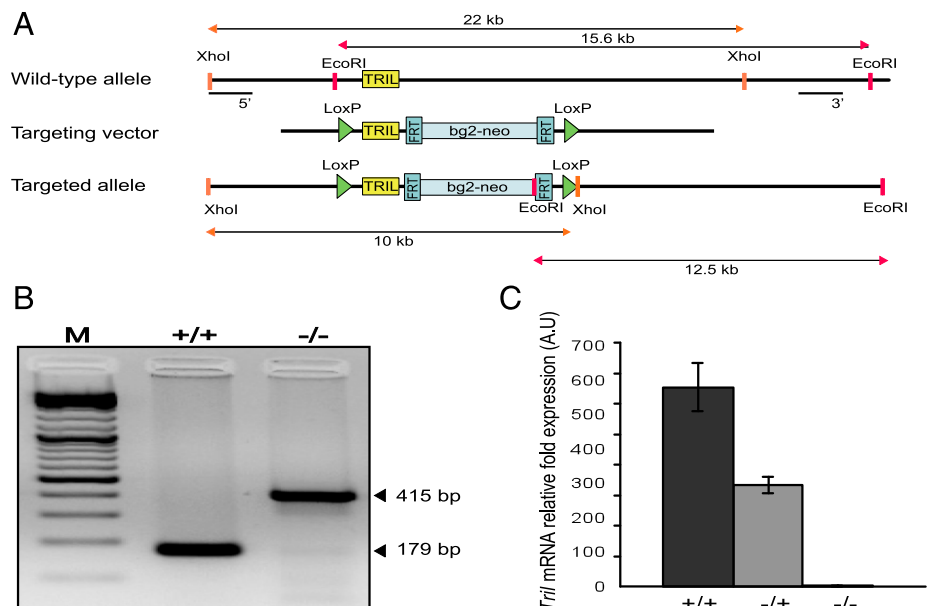
in astrocytes, cerebellar granule neurons, and microglia, with lower expression evident in a range of peripheral immune cells (Fig. 2A). We next examined responses to various TLR agonists in primary BMDMs and BMDCs isolated from *Tril*^{-/-} and WT mice. We analyzed cytokine expression after stimulation with the respective TLR4 and TLR3 ligands, LPS and Poly(I:C). Treating BMDCs with LPS led to an increase in mRNA for *Il6* (Fig. 2B) and *Ccl5* (Fig. 2C), and *Tril* deficiency had no effect on these responses, consistent with the low expression level of *Tril* in these cells. Poly(I:C) did not induce a strong response in BMDCs. In BMDMs, lack of *Tril* also had no effect on the induction of *Il6* (Fig. 2D) and *Ccl5* (Fig. 2E) mRNA in response to stimulation with both LPS and Poly(I:C). Similar results were seen with LPS and Poly(I:C) when IL-6 (Fig. 2F, 2I), TNF- α (Fig. 2G, 2J), and CCL5 (Fig. 2H, 2K) production was measured by ELISA (Fig. 2F–K). *Tril* deficiency also had no effect on induction of IL-6, TNF- α , and CCL5 by the TLR2 ligand Pam3CSK4 and TLR7/8 ligand R848, in either BMDCs (Fig. 2F–H) or BMDMs (Fig. 2I–K).

TRIL modulates TLR4- and TLR3- but not TLR2- or TLR7/8-mediated responses in primary murine mixed glial cells

Tril is highly expressed within brain cells, notably in astrocytes and neurons compared with microglia (Fig. 3A). We therefore next investigated TLR-mediated responses in mixed glial cells (which primarily consist of astrocytes, >83% astrocytes and ~2–3% of microglia; Fig. 3B, histogram) derived from WT and *Tril*^{-/-} mice. As shown on the bar graph in Fig. 3B, *Tril*^{-/-} cells are indeed devoid of *Tril* expression as expected; high basal level of *Tril* mRNA in the untreated WT mixed glial cells was further boosted after stimulation with both LPS and Poly(I:C), consistent with our previous studies (29, 31). We next analyzed the mRNA levels of 50 murine genes in WT and *Tril*^{-/-} primary mixed glial cells before and after 5-h stimulation with LPS (100 ng/ml) and Poly(I:C) (50 μ g/ml; Fig. 3C) using a nonenzymatic RNA profiling technology that uses bar-coded fluorescent probes to simultaneously analyze mRNA expression levels of differentially regulated genes (nCounter; Nanostring). We found that the expression of a number of proinflammatory cytokines and chemokines were reduced in *Tril*-deficient cells in response to LPS and Poly(I:C) (Fig. 3C). The mRNA levels of *Il6*, *Ccl5*, *Tnfa*, *Il1a*,

FIGURE 1. Generation of *Tril*^{-/-} mice.

(A) Gene-targeting strategy for generation of *Tril*^{-/-} mice. The structures of WT allele, targeting vector, and targeted allele are represented. (B) PCR analysis of genomic DNA prepared from tail biopsies using primers *Tril*-F1, *Tril*-R1, and *Tril*-R2, detecting both WT (179 bp) and mutant (415 bp) alleles. (C) RT-PCR analysis of *Tril* expression in the brain of WT (^{+/+}), heterozygous (^{+/-}), and mutant (^{-/-}) mice. *Tril* mRNA levels were normalized against *Gapdh* and expressed relative to the lowest detectable sample. Data are presented as the mean $\pm$ SD of one experiment representative of three independent experiments. FRT, flippase recombination target.



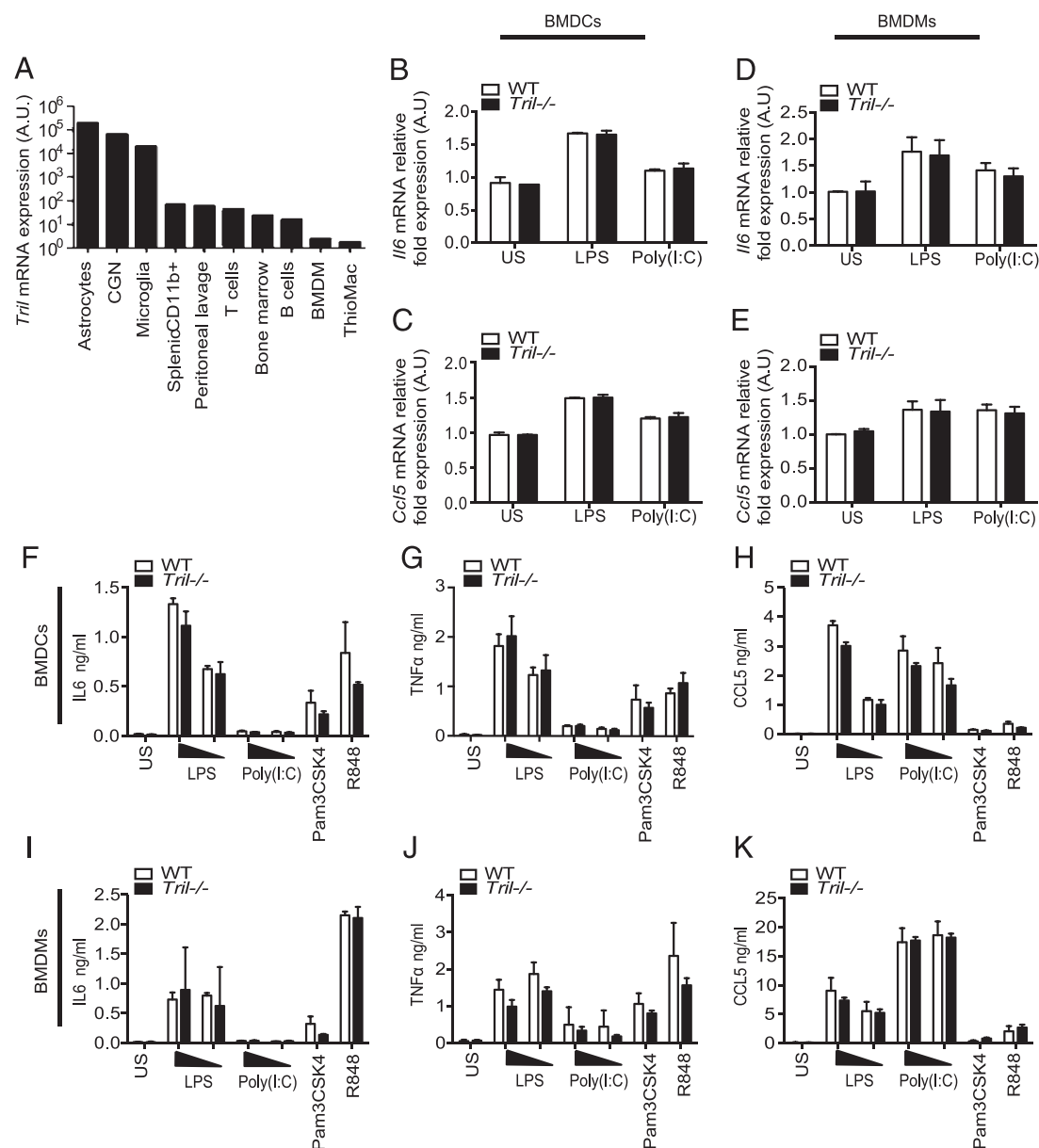


FIGURE 2. TRIL does not impact TLR-mediated responses in BMDMs and dendritic cells (BMDCs). **(A)** *Tril* expression in various mouse cell populations measured by RT-PCR. *Tril* expression levels were normalized to *Gapdh* and expressed relative to the lowest detectable sample. **(B–E)** Expression of *Il6* (B and D) and *Ccl5* (C and E) in primary BMDCs (left panel) and BMDMs (right panel) isolated from WT and *Tril*^{-/-} mice untreated or stimulated for 5 h with LPS (100 ng/ml) or Poly(I:C) (25 μg/ml). mRNA levels were normalized to *β-Actin* and represented in A.U. relative to unstimulated cells. **(F–K)** ELISA for IL-6 (F and I), TNF-α (G and J), and CCL5 (H and K) measured in primary BMDCs (top panel) and BMDMs (bottom panel) derived from WT and *Tril*^{-/-} mice stimulated for 24 h with LPS (10 and 100 ng/ml), Poly(I:C) (25 and 50 μg/ml), Pam3CSK4 (100 nM), or R848 (1 μg/ml). Data are presented as the mean ± SEM of two independent experiments carried out in triplicates.

Il1b, and *Ifnb1* were all decreased in *Tril*^{-/-} cells. In addition, the expression levels of chemokines such as *Cxcl2* and *Ccl4* were also found to be significantly reduced in *Tril*^{-/-} upon ligand activation. Following on from the gene expression studies, we also examined cytokine production by ELISA in both WT and *Tril*-deficient primary mixed glial cells after stimulation with TLR agonists (Fig. 3D–G). In agreement with the gene expression data, after 24-h treatment with two different doses of LPS (10 and 100 ng/ml) and Poly(I:C) (25 and 50 μg/ml), a statistically significant decrease in the IL-6 and CCL5 production was observed in primary mixed glial cells derived from *Tril*^{-/-} mice compared with WT controls (Fig. 3D, 3E). In addition, lack of *Tril* affected TNF-α and IFN-β protein levels in response to LPS and Poly(I:C), respectively (Fig. 3F, 3G). No major differences in the responses of

Tril^{-/-} and WT cells were seen after treatment with the TLR2 agonist Pam3CSK4 and TLR7/8 ligand R848 (Fig. 3D–G). Taken together, these data strongly indicate that TRIL affects both TLR3 and TLR4 signaling pathways in glial cells, but it does not impact responses of TLR2 and TLR7/8, consistent with our previous studies on the U373 cell line (31).

As a control to confirm that LPS and Poly(I:C) were acting via TLR4 and TLR3, respectively, in mixed glial cells, we also examined the TLR-mediated responses in cells from *Tlr4*^{-/-}, *Tlr3*^{-/-}, and *Trif*^{-/-} mice, both at the mRNA and the protein level, and observed attenuated response to LPS in *Tlr4*- and *Trif*-deficient cells, and Poly(I:C) in cells lacking *Trif* and *Tlr3* (data not shown).

To evaluate in more detail the cell type responsible for detected differences in the cytokine production between WT and *Tril*^{-/-}

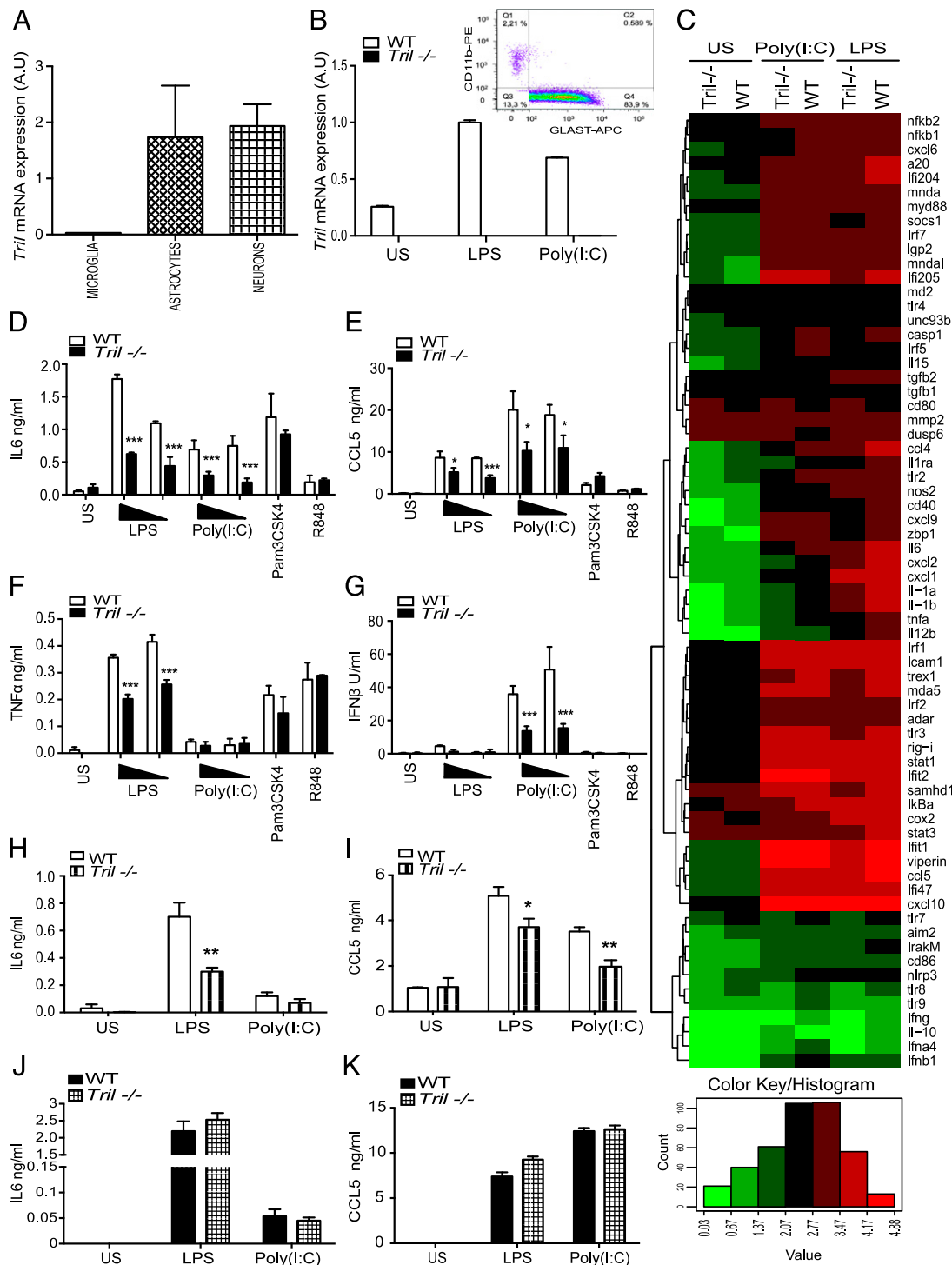


FIGURE 3. TRIL modulates TLR4- and TLR3-mediated response in the primary murine mixed glial cells and astrocytes. **(A)** RT-PCR analysis of *Tril* expression in cultured microglia, astrocytes, and neuron cell populations; mRNA levels of *Tril* were normalized to β -Actin and expressed in A.U. **(B)** Expression of *Tril* at the basal level and after 5 h of stimulation with LPS (100 ng/ml) or Poly(I:C) (25 μ g/ml) in murine mixed glial cells derived from WT and *Tril*^{-/-} mice; mRNA levels of *Tril* were normalized to β -Actin and expressed in A.U. **(B, histogram)** FACS analysis of microglia and astrocyte composition within primary mixed glial cells. Isolated primary mixed glial cells were stained using markers specific for astrocytes (GLAST-allophycocyanin; Miltenyi Biotech), microglia (Cd11b-PE; eBioscience), and neurons (β -3-Tubulin; Biolegend; data not shown). Graph demonstrates two main populations of cells among mixed glial cells; astrocytes and microglia; data are representative of three individual experiments. **(C)** Gene expression analysis of primary murine mixed glial cells derived from WT and *Tril*^{-/-} mice untreated or after 5 h of stimulation with LPS (100 ng/ml) or Poly(I:C) (25 μ g/ml). Gene expression profiles are displayed as a heat map (log10 transformed) with hierarchical clustering indicated by dendrogram. Upregulated genes are shown in red, downregulated genes are represented in green. **(D–G)** ELISA for IL-6 (D), CCL5 (E), TNF- α (F), and IFN- β (G), production in primary murine mixed glial cells derived from WT and *Tril*^{-/-} mice, untreated or stimulated for 24 h with LPS (10 and 100 ng/ml), Poly(I:C) (25 and 50 μ g/ml), Pam3CSK4 (100 nM), or R848 (1 μ g/ml). Data are represented as the mean $\pm$ SD of one experiment representative of three independent experiments, all carried out in triplicate. **(H and I)** ELISA for IL-6 (H) and CCL5 (I), production in the purified primary astrocyte population isolated from primary murine mixed glial cells derived from WT and *Tril*^{-/-} mice, untreated or stimulated for 24 h with LPS (100 ng/ml) or Poly(I:C) (25 μ g/ml). Data are represented as the mean $\pm$ SEM of two independent experiments, a total of three mice were pooled in each of three experiments carried out. (Figure legend continues)

mixed glial cells, we next examined purified populations of astrocytes and microglia. In agreement with the data obtained with primary mixed glial cells composed largely of astrocytes, deficiency of *Tril* in the purified astrocyte population strongly affected both IL-6 and CCL5 production after LPS and Poly(I:C) stimulation (Fig. 3H, 3I). Because of technical difficulties in obtaining sufficient number of purified microglia and strongly restricted number of *Tril*^{-/-} mice available, we were unable to examine the responses to LPS and Poly(I:C) stimulation among these cells. However, analysis of immortalized mixed glial cell population composed primarily of microglia and completely devoid of astrocytes revealed no significant differences in the IL-6 and CCL5 production upon LPS and Poly(I:C) stimulation (Fig. 3J, 3K). These data clearly demonstrate that astrocytes and not microglia are primarily responsible for the reduced cytokine production in *Tril*-deficient primary mixed glial cells after stimulation with LPS and Poly(I:C), which is in agreement with the high expression of *Tril* within these cells.

Investigation into the in vivo role of TRIL in E. coli-induced sepsis and upon intracranial LPS challenge

Finally, we addressed the role of TRIL in vivo using an *E. coli* challenge model, which is dependent on TLR recognition pathways in vivo. We first examined the expression of *Tril* before and after 6 h of infection with 10⁹ CFU of the *E. coli* via the i.p. route. The expression of *Tril* was analyzed within the spleen and brain tissues by RT-PCR. As can be seen in Fig. 4A, the basal expression of *Tril* was significantly higher in the brain compared with spleen, and it was further enhanced post-*E. coli* infection. *Tril* expression was not detected in the spleen; no increase in expression was observed upon infection. Bacterial load measured within the spleen and brain revealed that both tissues contain a high number of bacteria ranging from 10⁸ to 10⁴ CFU/ml in the spleen and brain, respectively (Fig. 4B). As shown in Fig. 4C, analysis of gene expression profile generated using total RNA isolated from the brain of WT (+/+) and *Tril*^{-/-} (-/-) mice post-i.p. infection with *E. coli* revealed reduced expression levels of proinflammatory cytokines such as *Il6*, *Tnfa*, *Il1a*, and *Il1b*, as well as chemokines *Ccl4*, *Cxcl2*, *Cxcl10*, in the *Tril*^{-/-} mice. Interestingly, a number of genes involved in the antiviral response, such as *Viperin* and *Rig-I*, were also dramatically reduced in *Tril*^{-/-} mice (Fig. 4C). An additional RT-PCR analysis using cDNA generated from spleen and brain of *Tril*^{-/-} and WT mice infected with *E. coli* demonstrated that the mRNA levels of *Il6* and *Ccl5* were significantly decreased in the brain samples derived from *Tril*^{-/-} mice (Fig. 4D, 4E). RT-PCR of cDNA from the spleens of WT and *Tril*^{-/-} mice showed no significant change in *Il6* and *Ccl5* mRNA levels (Fig. 4F, 4G).

Despite the i.p. administration of bacteria in the in vivo *E. coli* infection model tested, the significant differences in the proinflammatory cytokines production between WT and *Tril*^{-/-} were detected in the brain, but not the spleen. We decided, therefore, to further examine whether the observed differences were caused by the direct effect of TRIL on TLR4-mediated response within the brain. As shown in Fig. 4H and 4I, direct intracranial injection of 2 µg LPS into the brain of WT and *Tril*^{-/-} mice leads to significant decrease in the *Il6* and *Ccl5* expression in the brain of *Tril*^{-/-} compared with WT controls (Fig. 4H, 4I). TRIL is therefore involved in the modulation of TLR4-mediated responses directly within the brain.

These results indicate that TRIL functions primarily within the brain, where in response to intracranial LPS challenge and i.p. infection with *E. coli*, it mediates cytokine production from glial cells.

Discussion

Interaction between the peripheral immune system and the CNS plays a fundamental role in mounting an appropriate response to acute systemic infections (36). Brain dysfunction often actively contributes to deterioration of systemic infections; however, prolonged brain inflammation is frequently a consequence of the systemic inflammatory response.

TRIL was characterized as a novel accessory protein for TLR4 and TLR3 highly expressed in the brain (29, 31). In a series of in vitro studies, TRIL was shown to play a positive role in the regulation of TLR4 and TLR3 signaling pathways. However, its function in vivo has never been tested. The aim of this study was to further investigate TRIL in vitro and more importantly in vivo using *Tril*^{-/-} mice.

Primary BMDMs and BMDCs derived from *Tril*^{-/-} and WT mice produced the same level of cytokines in response to LPS or Poly(I:C). TRIL possesses a similar structure and function to CD14; therefore, we previously speculated that TRIL might act as a substitute for CD14 in cells where CD14 is expressed at low levels. CD14 is abundantly expressed on BMDMs and BMDCs (37–39). However, among glial cells, CD14 is highly expressed within microglia and is not present in astrocytes or neurons (15, 16). We demonstrated that mRNA levels of various proinflammatory genes induced in response to LPS or Poly(I:C) were strongly reduced in primary mixed glial cells derived from *Tril*^{-/-} mice when compared with WT controls. Because primary mixed glial cells are ~83–85% astrocytes and 2–3% microglia, it is possible that TRIL may indeed fulfill the role of CD14 in astrocytes, which is further supported by differences in the IL-6 and CCL5 production between WT and *Tril*^{-/-} purified astrocytes, but not immortalized microglia cells after LPS and Poly(I:C) stimulation. In addition, no difference was observed in response to Pam3CSK4 or R848, consistent with the lack of a role for TRIL in signaling mediated by TLR2 and TLR7/8 (29, 31).

Nearly one third of all cases of sepsis are caused by Gram-negative bacterial infection, among which *E. coli* is considered the most causative pathogen (40). Thus, aiming to address the in vivo role of TRIL, we used i.p. infection with *E. coli*. Expression of *Tril* was enhanced post-*E. coli* infection; however, this occurred exclusively in the brain and not spleen, consistent with the results indicating the significance of *Tril* expression in mixed glial cells compared with macrophages and dendritic cells. Further analysis of the cytokine expression profile in the brain after *E. coli* challenge revealed reduced levels of proinflammatory cytokines and chemokines in samples derived from *Tril*^{-/-} mice when compared with littermate controls. Similar to our earlier observation in the in vitro studies using primary mixed glial cells, levels of many inflammatory cytokines such as *Il6*, *Tnfa*, *Il1a*, and *Il1b* and chemokines *Ccl4*, *Cxcl2*, *Cxcl10* were all reduced in the *Tril*^{-/-} mice upon bacterial infection. Analysis of the gene expression panel also revealed a dramatic difference in some of the IFN-stimulated genes involved in viral recognition and the antiviral response, such as *Rig-I* and *Viperin*, respectively, in the LPS-treated, *Tril*^{-/-} mice. TRIL is implicated in the regulation of TLR4-mediated signaling; therefore,

(J and K) ELISA for IL-6 (J) and CCL5 (K), production examined in immortalized microglia cells derived from WT and *Tril*^{-/-} mice, untreated or stimulated for 24 h with LPS (100 ng/ml) or Poly(I:C) (25 µg/ml). Data are represented as the mean ± SEM of three independent experiments, all carried out in triplicates. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

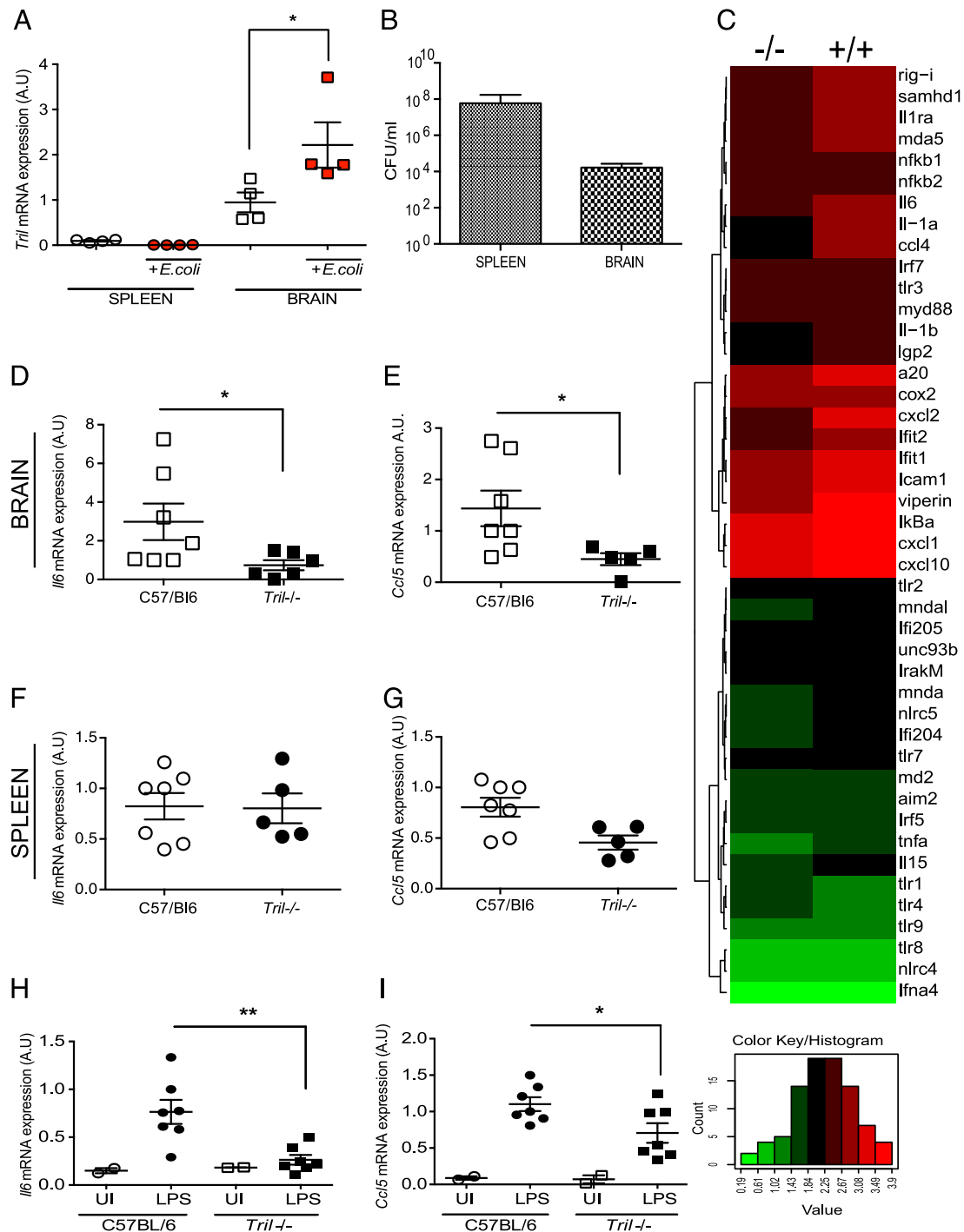


FIGURE 4. Investigation into the in vivo role of TRIL in the *E. coli*-induced Gram-negative sepsis model and after intracranial LPS challenge. **(A)** Expression of *Tril* measured by RT-PCR in both spleen and brain samples derived from WT and *Tril*^{-/-} mice infected with *E. coli* strain BL21 (10⁹ CFU) for 6 h (*n* = 4). mRNA levels of *Tril* were normalized to β -Actin and expressed in A.U. **(B)** Bacterial dissemination in the spleen and brain of WT mice after i.p. *E. coli* infection for 6 h. **(C)** Gene expression profile generated using RNA isolated from the brain of WT and *Tril*^{-/-} mice. Gene expression profile is displayed as a heat map (log10 transformed) with hierarchical clustering indicated by dendrogram. Upregulated genes are shown in red; downregulated genes are represented in green. **(D–G)** Cytokine expression measured by RT-PCR in brain (D and E) and spleen (F and G) of WT and *Tril*^{-/-} mice infected with *E. coli* (10⁹ CFU) for 6 h (*n* = 5–7). Data are presented as a mean $\pm$ SEM of two (A and B) or three (D–G) independent experiments, each carried out in triplicates. **(H and I)** Cytokine expression measured by RT-PCR in brain of WT and *Tril*^{-/-} mice after 24 h of intracranial LPS challenge (2 μ g LPS or saline control per mice; *n* = 2–7). Data are presented as mean $\pm$ SEM of two independent experiments, each carried out in triplicate. **p* < 0.05, ***p* < 0.01.

detected differences in IFN-stimulated gene expression could be explained by lower levels of IFN production in response to LPS. These data, together with the previously reported involvement of TRIL in the TLR3 signaling pathway, suggest a possible role for TRIL in the antiviral immune response. However, further studies are needed to verify these findings.

In our sepsis model, Gram-negative *E. coli* was administered via the i.p. route, but interestingly, the main effect of the lack of TRIL was observed in the brain. As mentioned earlier, TLR4 is highly expressed within the CNS. High levels of TLR4 can be found in the meninges, choroid plexus, and CVOs of rat brain (41). Constitutive expression of TLR4 and CD14 in the CVO and

meninges, sites of the brain with direct access to the circulation, provide for the possibility of direct TLR4-mediated LPS action in the CNS, which would also require TRIL (22, 41). Alternatively, brain inflammation can also be triggered by direct sensing of bacteria by resident glial cells, microglia, and astrocytes within the brain parenchyma after disruption of the BBB. In fact, we detected the presence of bacteria in the brain of WT mice post-infection with *E. coli*, which suggested that the integrity of the BBB was breached, allowing for bacteria to disseminate throughout the brain.

Astrocytes are one of the most abundant cell types in the CNS. They participate in the innate immune responses, provide support to neurons, regulate synaptic activity, and contribute to the formation of the BBB (42). Both human and murine astrocytes express a wide range of TLRs. Cultured human astrocytes were reported to constitutively express TLR2, TLR3, and TLR4 (43, 44), whereas mouse-derived astrocytes express TLR1-9, with particularly high levels of TLR3 and TLR4 (45). After activation, astrocytes produce a wide range of proinflammatory cytokines such as IL-6, TNF- α , and IFN- β , and chemokines CCL2, CCL5, CCL20, CXCL8, and CXCL10 (46–48). Our studies on primary mixed glial cells and purified primary astrocytes strongly suggest that TRIL, which is highly expressed in astrocytes, functions as a regulator of TLR-mediated responses within these cells.

Of note, astrocytes are also involved in the upregulation of inducible NO synthase and NO production (49). Thus, astrocytes participate in processes such as tissue damage and neurotoxicity. Given multiple functions of astrocytes, there is a possibility that TRIL might impact not only innate immune responses within these cells, but also BBB permeability and neuronal cell death.

In summary, our study clearly identifies a key role for TRIL in TLR4 responses in the brain. It also provides *in vivo* evidence that TRIL acts as a mediator of cytokine production in response to *E. coli* infection within the CNS.

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L.A.J.O. is a cofounder, minority shareholder, and member of the scientific advisory board of Opona Therapeutics Ltd., a university start-up company involved in the development of anti-inflammatory therapeutics.

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