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TLR4 signaling drives tissue inflammation, Claudin-5 internalization, and vascular barrier breakdown in a mouse model of neonatal meningitis

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eLife Assessment

This study demonstrates that endothelial toll-like receptor 4 is a central regulator of leptomeningeal inflammation in neonatal *E. coli* meningitis. The data are derived from cell-type-specific gene knockouts in mice and cultured endothelial cells and are **convincing**. This work is **important** as it advances our understanding of host cellular processes and molecular pathways underlying meningitis pathogenesis and specifically expands the knowledge of how breakdown of the blood brain barrier contributes to the pathogenesis.

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Abstract

Neonatal bacterial meningitis is a leading cause of infant morbidity and mortality, yet the molecular and cellular basis of the leptomeningeal response to infection remains poorly defined. Here, we study a mouse model of neonatal *E. coli* meningitis, combining conditional gene knockouts, leptomeningeal single-nucleus RNA sequencing, and endothelial cell culture to explore the role of Toll-like receptor 4 (TLR4) signaling in the host response to infection. Deletion of *Tlr4* in non-myeloid cells dramatically reduced the inflammatory response in all leptomeningeal cell types and abrogated the infection-associated increase in vascular permeability. In a brain endothelial cell line (bEnd.3 cells), exposure to *E. coli* triggered NF-κB activation, selective internalization of Claudin-5, and increased monolayer permeability, responses that were eliminated by *Tlr4* knockout. RNA-seq showed that TLR4 controls an NF-κB-driven transcriptional program that orchestrates the endothelial response to *E. coli*. These findings reveal multiple TLR4-dependent host responses to neonatal Gram-negative bacterial meningitis.

Introduction

Bacterial meningitis is a major global health challenge, causing ~250,000 deaths annually (Brouwer, 2010 [↗](#); GBD 2016 Neurology Collaborators, 2019 [↗](#)). Among neonates, Group B Streptococcus (*Streptococcus agalactiae*, GBS) and *Escherichia coli* K1 are the predominant causes of meningitis and are responsible for roughly 40% and 30% of cases, respectively (Ouchenir et al., 2017 [↗](#); Bundy et al., 2023 [↗](#)). While vaccination has markedly reduced mortality in childhood meningitis, neonatal infections continue to carry high case-fatality rates, especially in low-income regions, and roughly half of the survivors experience lasting neurological deficits (Edmond et al.,

2010 [Guarnera et al., 2024](#)). The high susceptibility of neonates likely reflects the immaturity of both the adaptive immune system and multiple CNS barriers, including the blood–brain barrier (BBB), the blood–cerebrospinal fluid (CSF) barrier, and the leptomeninges (Saunders et al., 2012 [Yang et al., 2023](#)). Understanding host–pathogen interactions in this developmental window is essential for designing strategies to protect the neonatal brain (Kim, 2003 [Kim, 2003](#)).

The leptomeninges form a surface barrier enveloping the brain and spinal cord. It contains a dense vascular network and large numbers of immune cells within the CSF-filled subarachnoid space. Within the leptomeninges, endothelial cells (ECs) possess BBB properties, including expression of Claudin-5 (Cldn5), Occludin, and transporters characteristic of CNS microvessels (Mastorakos et al., 2019 [Mastorakos et al., 2019](#)). At present, the response of leptomeningeal ECs to bacterial infection, including alterations in barrier properties, is largely unexplored.

The present study focuses on the role of one innate immune receptor, Toll-like receptor 4 (TLR4), in regulating the leptomeningeal response to bacterial meningitis. TLR4 is a pattern-recognition receptor that detects lipopolysaccharide (LPS), the major outer membrane component of Gram-negative bacteria such as *E. coli* (Medzhitov, 2001 [Kawai and Akira, 2007](#)). Activation of TLR4 initiates MyD88- and TRIF-dependent signaling cascades, leading to the induction of proinflammatory cytokines, chemokines, and adhesion molecules that orchestrate leukocyte recruitment to infected tissues (Vallières and Rivest, 1997 [Pålsson-McDermott and O’Neill, 2004](#) [Nagyoszi et al., 2010](#) [Casanova et al., 2011](#)). In the context of bacterial meningitis, TLR4 has been shown to mediate both host-protective and pathological responses, including vascular inflammation and leukocyte infiltration (Van Well et al., 2013 [Doran et al., 2016](#) [Too et al., 2019](#) [Wang et al., 2023](#)). While these studies established TLR4 as a key driver of meningitis-associated inflammation, the molecular and cellular events associated with the TLR4-dependent host response during bacterial meningitis remain largely unexplored.

Claudin-5 (Cldn5) is a prominent tight-junction protein in brain ECs. Originally identified as an endothelial-specific component of tight junctions (Morita et al., 1999 [Morita et al., 1999](#)), Cldn5 was later shown to be indispensable for restricting paracellular permeability *in vivo*, as *Cldn5*-deficient mice exhibit size-selective leakage of small molecules across the BBB (Nitta et al., 2003 [Nitta et al., 2003](#)). Under physiological conditions, Cldn5 is localized to endothelial cell–cell contacts, but its distribution and stability are dynamically regulated by endocytosis, recycling, and degradation pathways that respond to inflammatory stimuli (Engelhardt and Sorokin, 2009 [Coisne and Engelhardt, 2011](#) [Hashimoto et al., 2023](#)). During CNS inflammation and infection, pro-inflammatory cytokines and innate immune receptor activation disrupt tight-junction continuity and alter endothelial barrier properties. This junctional remodeling includes Cldn5 internalization, but the upstream signaling pathways controlling Cldn5 trafficking remain incompletely understood.

A recent single-cell transcriptome study defined the genomic response of the leptomeninges in a mouse model of neonatal *E. coli* meningitis (Wang et al., 2023 [Wang et al., 2023](#)). Leptomeningeal ECs exhibited a clear inflammatory transcriptional signature, implicating the leptomeningeal vasculature as a locus of immune signaling during *E. coli* infection. Whether these signals directly control tight junction remodeling and vascular leak was not tested.

Here, we study a mouse neonatal meningitis model and cultured CNS ECs (bEnd.3 cells) to test whether non-myeloid TLR4 signaling orchestrates the multi-cellular response of the leptomeninges to infection and regulates Cldn5 trafficking and vascular barrier function. In the present work, we (i) used single nucleus (sn)RNA-seq to profile cell-type-specific responses in the leptomeninges from WT mice, endothelial and fibroblast (i.e., non-myeloid) *Tlr4* conditional knockout mice (*Tlr4*^{VEKO}), and myeloid *Tlr4* conditional knockout mice (*Tlr4*^{MKO}); (ii) quantified intravascular tracer leakage and immune pathway activation *in vivo*; (iii) tracked Cldn5 redistribution and NF-κB nuclear translocation in bEnd.3 cells exposed to *E. coli*; (iv) generated CRISPR *Tlr4*^{KO} bEnd.3 lines to assess the TLR4 dependence of NF-κB activation, tight-junction dynamics, and barrier permeability; (v) quantified Cldn5 colocalization with junctional, plasma-membrane, and endosomal/lysosomal markers; and (vi) defined TLR4-dependent transcriptional programs in bEnd.3 cells exposed to *E. coli*. The results of these experiments establish a

framework for understanding the pathophysiology of neonatal meningitis that establishes TLR4 signaling as a central regulator of both the multi-cellular leptomeningeal response to infection and Cldn5 trafficking and junctional integrity.

Results

Cell-type-specific transcriptional responses of the leptomeninges from WT, *Tlr4*^{VEKO}, and *Tlr4*^{MKO} mice to neonatal *E. coli* infection

Moving from brain to skull, the meninges consist of four distinct layers: (1) the pia, a thin, semi-permeable layer that mediates solute exchange between the CNS and CSF; (2) the subarachnoid space, a highly vascularized region that contains a high density of immune cells; (3) the arachnoid and its barrier layer, demarcating the boundary between CNS and non-CNS territories; and (4) the dura, a fibrous layer outside of the CNS with a high density of blood vessels, lymphatics, and immune cells (Figure 1A). The first three layers constitute the leptomeninges. Taking advantage of a natural cleavage plane between dura and leptomeninges, we have optimized whole mount immunostaining and confocal imaging of the leptomeninges together with the adjacent cortex, preserving the anatomical integrity of the pia and subarachnoid space (Figure 1B).

In the *E. coli* meningitis model used here, postnatal day (P)5 mice were given a subcutaneous injection of 20 µl of phosphate buffered saline (PBS) with 120,000 colony forming units of *E. coli* RS218 (O18:K1:H7), a clinical isolate from the CSF of a neonate with bacterial meningitis (Zhu et al., 2020; Wang et al., 2023) or, as a control, 20 µl of phosphate buffered saline (PBS). This *E. coli* strain was additionally transformed with a plasmid encoding a red fluorescent protein (RFP) to facilitate detection of bacteria in infected tissues. Infected and control mice were sacrificed at P6, 24 hours after inoculation. P5 mice were chosen to approximate the developmental stage of the human newborn BBB and immune system, as described in Wang et al. (2023). This model recapitulates the main temporal sequence of events in human neonates with bacterial meningitis: peripheral entry of bacteria (e.g., via skin abrasions during birth), hematogenous spread of bacteria (bacteremia), and bacterial seeding of the meninges.

To assess cell type-specific roles of TLR4 signaling, we used two conditional knockout lines: *Tlr4*^{VEKO} (= *Cdh5-CreER*; *Tlr4*^{fl/fl}) and *Tlr4*^{MKO} (= *Lyz2*^{Cre/+}; *Tlr4*^{fl/fl}). *Cdh5* encodes Vascular Endothelial (VE) Cadherin, and the *Cdh5-CreER* line carries a *Cdh5* promoter-*CreER* transgene (Monvoisin et al., 2006). *Lyz2*, also referred to as *LysM*, encodes a myeloid-specific lysozyme, and the *Lyz2*^{Cre} line is a *Cre* knock-in at the *Lyz2* locus (Clausen et al., 1999). The cell type specificities of *Cdh5-CreER* and *Lyz2*^{Cre} were assessed using a *Rosa26 loxP-stop-loxP* reporter (*Rosa26-LSL-tdT-2A-nlsGFP*; Wang et al., 2018). The specificity of *Cdh5-CreER* was additionally assessed using a *Rosa26-LSL-SUN1-sfGFP* reporter (Mo et al., 2015).

Throughout most of the CNS, the *Cdh5-CreER* line directs Cre-mediated recombination specifically to ECs (e.g., Wang et al., 2025b). However, in the meninges, only ~75% of the cells recombined by the *Cdh5-CreER* transgene are ECs and ~25% are neither ECs nor myeloid cells (Figure 1 – figure supplement 1). Quantification of DAPI stained sections of leptomeninges shows that ECs (ERG+ cells) constitute 31+/-9% of leptomeningeal cells, and myeloid cells (PU.1+ cells) constitute 14+/-5% of leptomeningeal cells. Therefore, dural border cells, arachnoid barrier cells, and fibroblasts together constitute the remaining ~55% of leptomeningeal cells. Since the non-ECs and non-myeloid cells that express *Cdh5-CreER* are one-third as numerous as ECs, they constitute ~10% of leptomeningeal cells, equivalent to ~18% of the combined population of dural border cells, arachnoid barrier cells, and fibroblasts. These data imply that at P2, when 4HT was administered, the *Cdh5-CreER* transgene produced Cre-mediated recombination in all or nearly all ECs, in a minority of dural border cells, arachnoid barrier cells, and fibroblasts, and not in myeloid cells, consistent with the pattern of *Cdh5* expression in leptomeninges described by others (Mapunda et al., 2023; Pietilä et al., 2023; Smyth et al., 2024). Extrapolating these data to *Tlr4*, *Tlr4*^{VEKO} mice should be considered to have complete or nearly complete *Tlr4* knockout in ECs, with *Tlr4*

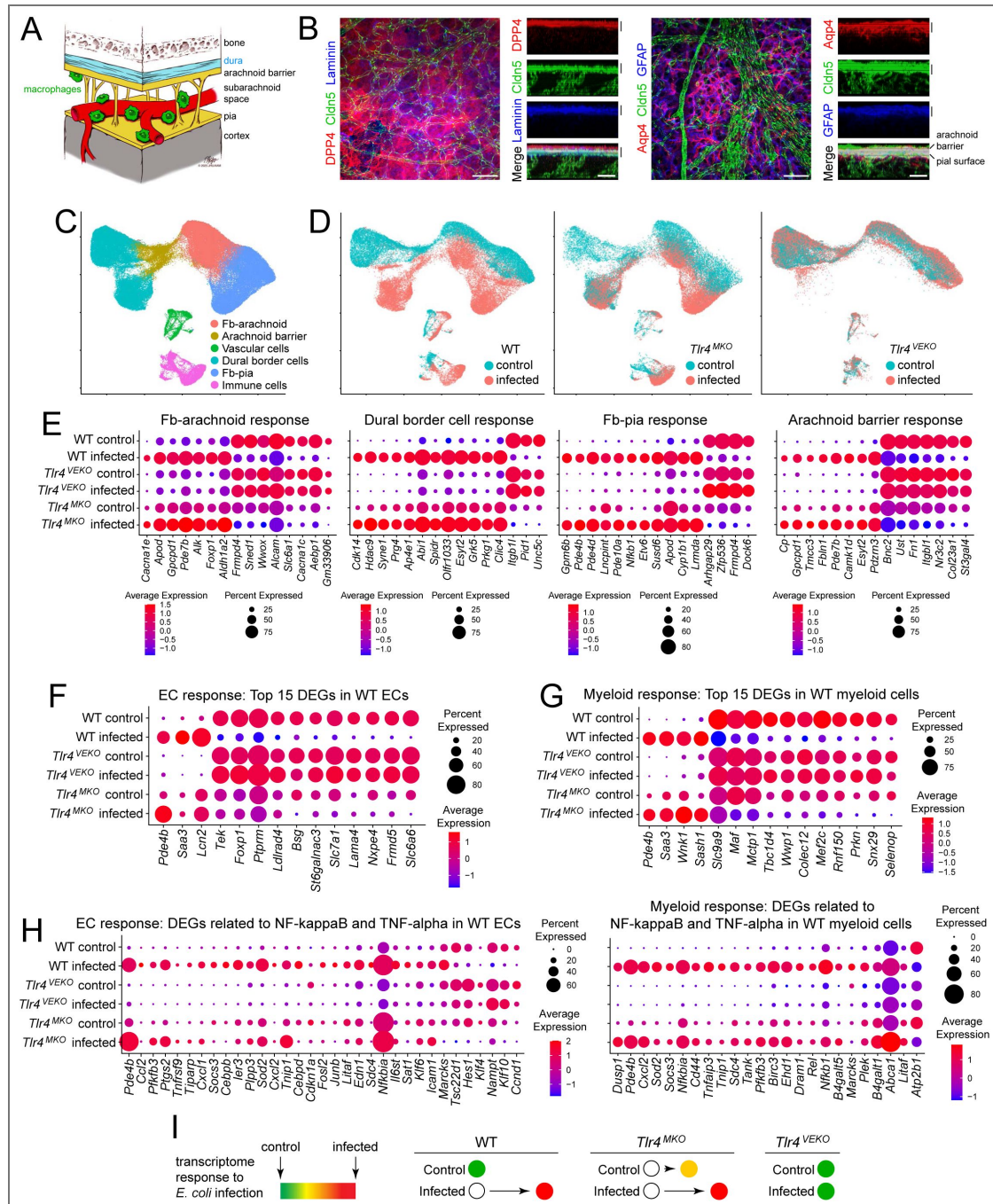


Figure 1. Cell-type-specific transcriptional responses of leptomeninges from WT, *Tlr4*^{VEKO}, and *Tlr4*^{MKO} mice to neonatal *E. coli* infection.

(A) Schematic of the leptomeninges (pia, subarachnoid space, and arachnoid barrier) and adjacent structures. (B) Immunostaining of P6 mouse leptomeninges shown as a whole mount (left) or as a transverse section through a series of Z-planes (right). The vertical black bars indicate the extent of the leptomeninges. Scale bars, 20 μ m. (C) UMAP plot of leptomeninges snRNA-seq from WT, *Tlr4*^{VEKO}, and *Tlr4*^{MKO} mice at P6, from uninfected and *E. coli*-infected mice, with cell clusters identified. N=126,235 nuclei. Fb, fibroblast. (D) UMAP comparison of P6 leptomeningeal snRNA-seq from control vs. 24-hour *E. coli*-infected mice of the indicated genotypes. (E) snRNA-seq from four leptomeninges cell clusters (Fibroblast-arachnoid, Dural border cells, Fibroblast-pia, and Arachnoid barrier cells) showing differentially expressed genes in control vs. *E. coli*-infected mice of the indicated genotypes at P6. For each cell cluster, DEGs were chosen based on a comparison of infected vs. uninfected WT cells. (F and G) As in panel (E) except for ECs (F) and myeloid cells (G). DEGs, differentially expressed genes. (H) As in panel (F) and (G) except DEGs were related to NF- κ B and TNF- α signaling. (I) Summary of transcriptional responses of leptomeninges from WT, *Tlr4*^{VEKO}, and *Tlr4*^{MKO} mice to *E. coli* infection.

additionally knocked out in a fraction of the other non-myeloid cell types. For simplicity, we will refer to the *Tlr4*^{VEKO} KO pattern as “non-myeloid” KO of *Tlr4*, although the data in [Figure 1 – figure supplement 1](#) imply that it is predominantly an EC KO.

Expression of the *Lyz2*^{Cre} knock-in allele was restricted to myeloid cells within the leptomeninges as determined by immunostaining for three partially overlapping myeloid markers, CD206, ASC, and PU.1 ([Figure 1 – figure supplement 2](#)). *Lyz2*^{Cre} directed Cre-recombination with 90-100% efficiency in CD206+ cells and with 50-70% efficiency in ASC+ and PU.1+ cells, the range depending on whether tdTomato or GFP colocalization was being scored ([Figure 1 – figure supplement 2](#)). In the text that follows, we will refer to *Lyz2*^{Cre}-recombined cells simply as “myeloid cells”, although they should be understood as CD206+ myeloid cells. The low abundance of TLR4 and the limitations of commercial anti-TLR4 antibodies precluded a direct immunohistochemical assessment of TLR4 loss in *Tlr4*^{VEKO} and *Tlr4*^{MKO} mice.

To profile the transcriptional landscape of the leptomeninges in WT, *Tlr4*^{VEKO}, and *Tlr4*^{MKO} mice, with and without meningitis, we performed single-nucleus RNA sequencing (snRNA-seq) on dissected leptomeninges ([Supplementary file 1](#)). A uniform manifold approximation and projection (UMAP) analysis of the dataset revealed six major cell classes: Fb (fibroblast)-arachnoid1, Fb-pia, arachnoid barrier cells, dural border cells, vascular cells, and immune/myeloid cells ([Figure 1C](#); [Figure 1 – figure supplement 3A–C](#)), consistent with prior studies ([Pietilä et al., 2023](#); [Wang et al., 2023](#); [Smyth et al., 2024](#); we note that the cell cluster referred to in [Wang et al., 2023](#) as “fibroblasts dura 3” corresponds to dural border cells). The same set of cell clusters was observed with both PIP-seq ([Clark et al., 2023](#)) and 10X Genomics platforms, which additionally confirmed expression of *Cdh5* in non-myeloid and non-EC leptomeningeal cell types and expression of *Lyz2* exclusively in myeloid cells ([Figure 1 – figure supplement 4](#)).

Comparison of infected and uninfected conditions showed that *Tlr4*^{VEKO} mice exhibited a global attenuation of infection-induced transcriptional responses across all major leptomeningeal cell types, as judged by the positions of cell clusters in the UMAP ([Figure 1D](#)). Similar trends were observed in principal component analysis (PCA), where infected and uninfected WT samples were widely separated along the PC2 axis, whereas infected and uninfected *Tlr4*^{VEKO} samples clustered closely together ([Figure 1 – figure supplement 5](#)). Uninfected *Tlr4*^{MKO} transcriptomes localized roughly midway between infected and uninfected WT samples along PC2.

At the level of individual genes, all major leptomeningeal cell types displayed infection-associated transcriptional changes in which the most differentially expressed genes, based on a comparison between infected and uninfected WT mice, exhibited similarly responses in WT and *Tlr4*^{MKO} mice but little or no response in *Tlr4*^{VEKO} mice ([Figure 1E–G](#)). In WT and *Tlr4*^{MKO} mice, infection upregulated NF-κB and TNF-α transcriptional responses, whereas *Tlr4*^{VEKO} mice were largely devoid of these responses ([Figure 1H](#) and [Figure 1 – figure supplement 3D](#)). Similar trends were observed in dot plots showing transcriptional changes associated with JAK-STAT and Interferon (IFN)-γ signaling pathways ([Figure 1 – figure supplement 5](#)). Interestingly, leptomeningeal cells in uninfected *Tlr4*^{MKO} mice exhibit a transcriptional state that partially mimics the infection response ([Figure 1D–H](#) and [Figure 1 – figure supplements 5](#) and [6](#)). We speculate that this *Tlr4*^{MKO} phenotype could represent an increase in the baseline level of innate immune signaling in uninfected *Tlr4*^{MKO} mice. Comparing snRNA-seq data sets for individual mice and individual genes confirms for ECs and myeloid cells the conclusions obtained from aggregated data ([Figure 1 – figure supplement 7](#)). Together, these findings define an *E. coli* meningitis-associated transcriptional program controlled by TLR4 signaling that drives inflammatory responses in all of the major leptomeningeal cell types ([Figure 1I](#)).

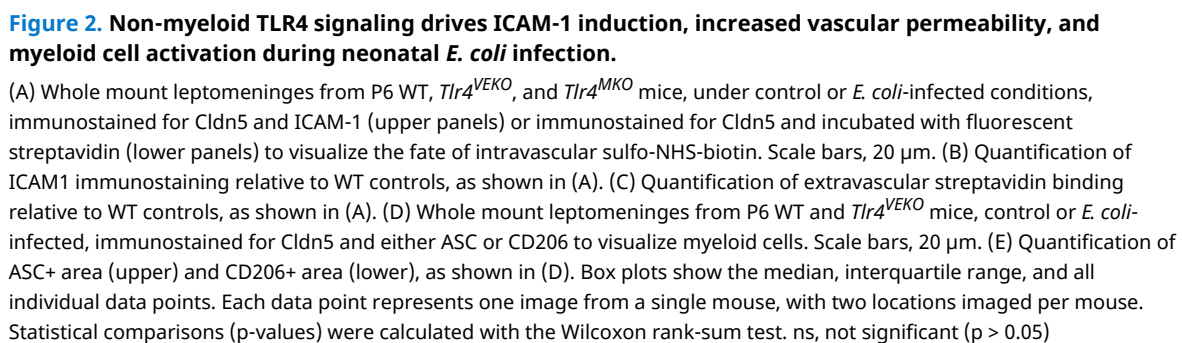
TLR4 signaling drives ICAM1 induction, increased vascular permeability, and myeloid activation during neonatal *E. coli* infection

To examine the role of TLR4 signaling in meningitis-associated responses within the leptomeninges, we used whole mount imaging to assess markers of vascular permeability and immune activation. Intercellular adhesion molecule-1 (ICAM1) serves as a well-established marker of endothelial activation during infection and inflammation (Bui et al., 2020 [↗](#); Singh et al., 2023 [↗](#)). Whole mount leptomeninges from P6 uninfected and *E. coli*-infected WT, *Tlr4*^{VEKO}, and *Tlr4*^{MKO} mice revealed robust ICAM1 upregulation in WT and *Tlr4*^{MKO} mice, whereas *Tlr4*^{VEKO} mice showed little change in ICAM1 levels (Figure 2A–B [↗](#)). These protein-level changes mirrored the genotype-specific transcriptional differences detected by snRNA-seq (Figure 2 – figure supplement 1A [↗](#)). TLR4-dependent ICAM1 upregulation was also observed in underlying cortical capillaries (Figure 2 – figure supplement 1B–C [↗](#)). For these and all other immunostaining experiments with mice or with cultured cells, the numbers of mice and the numbers of images used in the analyses are listed in Supplementary file 2.

To assess BBB integrity within the leptomeninges, we performed intraperitoneal (IP) injections of sulfo-NHS-biotin, a low molecular weight intravascular tracer. In this assay, perivascular streptavidin staining serves as a readout of vascular leakage (Wang et al., 2019 [↗](#)). WT and *Tlr4*^{MKO} mice exhibited marked increases in sulfo-NHS-biotin extravasation upon *E. coli* infection, whereas *Tlr4*^{VEKO} mice showed no change from the uninfected state (Figure 2A and C [↗](#)). The simplest interpretation of these data is that the site of sulfo-NHS biotin leakage is primarily vascular. However, we cannot exclude some contribution from increased arachnoid barrier permeability.

In addition to these endothelial responses, we examined alterations in myeloid cells within the leptomeninges and the underlying cortex. The ASC protein (Apoptosis-associated speck-like protein containing a CARD; ASC/PYCARD), an inflammasome adaptor that changes its oligomeric state and subcellular localization in response to inflammatory stimuli (Sester et al., 2016 [↗](#); Franklin et al., 2018 [↗](#)), localizes to leptomeningeal myeloid cells and brain microglia (Figure 2D [↗](#)). Quantification of the area occupied by ASC+ cells revealed significant increases in infected WT mice within both the leptomeninges and cortex (Figure 2D and E [↗](#); Figure 2 – figure supplement 1D–E [↗](#)), whereas infected *Tlr4*^{VEKO} mice exhibited a more modest response. To further characterize myeloid morphology, we stained for CD206 (Figure 2D and E [↗](#); Figure 2 – figure supplement 1F [↗](#)). Within the leptomeninges, and similar to ASC staining, the area occupied by CD206+ myeloid cells increased in infected WT mice but showed substantially smaller changes in infected *Tlr4*^{VEKO} mice (Figure 2D–E [↗](#)). In the brain, CD206+ cells were too sparse to reliably quantify (Figure 2 – figure supplement 1F [↗](#)). The increase in area of ASC and CD206 immunostaining in the infected leptomeninges relative to controls largely reflects a shift of myeloid cells from a compact to a more extended morphology (Wang et al., 2023 [↗](#)). These findings imply that non-myeloid TLR4 signaling promotes myeloid activation and associated morphologic changes during *E. coli* meningitis.

Immunostaining for cleaved Caspase-3+ cells in leptomeninges+cortex flat mounts revealed increased numbers of cleaved Caspase-3+ cortical cells within ~30 um beneath the pial surface following *E. coli* infection in WT and *Tlr4*^{MKO} mice, but not in *Tlr4*^{VEKO} mice (Figure 2 – figure supplement 2A [↗](#) and B [↗](#)). Similar analyses in coronal sections extending ~750 um into the cortex showed ~2-fold greater numbers of cleaved Caspase-3+ cortical cells in WT mice compared to *Tlr4*^{VEKO} mice (Figure 2 – figure supplement 2C [↗](#) and D [↗](#)). In coronal sections, the number of cleaved Caspase-3+ cells in the leptomeninges correlated with the number in the cortex (R=0.79) in both WT mice and *Tlr4*^{VEKO} mice (Figure 2 – figure supplement 2E [↗](#)). Co-staining with neuronal (NeuN), glial (GFAP), EC (Cldn5), and myeloid (PU.1) markers revealed that cleaved Caspase-3 in the cortex localized to myeloid cells (Figure 2 – figure supplement 2F [↗](#)). Immunostaining for Iba1, a marker of microglial activation, showed increased numbers of cortical Iba1+ cells in both WT and *Tlr4*^{VEKO} mice in response to infection, with a ~2-fold greater number in WT mice compared to



Tlr4^{VEKO} mice (Figure 2 – figure supplement 3 [↗](#)). The infection-dependent appearance of cleaved Caspase-3+ myeloid cells in the cortex could reflect the previously described non-apoptotic role of caspases in immune cell differentiation and activation (Sordet et al., 2002 [↗](#); Santambrogio et al., 2005 [↗](#)). Together, these results indicate that non-myeloid TLR4 signaling drives both increased vascular permeability and myeloid cell activation during neonatal *E. coli* meningitis.

The loss of TLR4 signaling in *Tlr4*^{VEKO} mice had little or no effect on the clinical course of the *E. coli* infection. The presence of *E. coli* in blood cultures and its dissemination to brain, liver, and lung occurred indistinguishably in infected WT and *Tlr4*^{VEKO} mice (Figure 2 – figure supplement 4A–C [↗](#)). The most obvious clinical manifestation of *E. coli* infection is a failure to gain weight, an observation consistent across WT, *Tlr4*^{VEKO}, *Tlr4*^{MKO}, and *Tlr4*^{-/-} mice (Figure 2 – figure supplement 4D [↗](#); Wang et al., 2023 [↗](#)).

Claudin-5 redistribution in response to *E. coli* in leptomeningeal endothelial cells and in bEnd.3 cells

In our previous description of meningitis-associated responses in the leptomeninges, we observed a redistribution of Cldn5 independent of changes in total Cldn5 levels (Wang et al., 2023 [↗](#)). Given the central role of Cldn5 in maintaining BBB integrity, we investigated whether TLR4-mediated inflammatory signaling contributed to these changes. Consistent with prior findings (Wang et al., 2023 [↗](#)), both WT and *Tlr4*^{MKO} mice showed an increase in the area occupied by Cldn5 in the leptomeninges following infection, likely referable to both increased vessel diameter and a redistribution of Cldn5 within ECs (Figure 3A–B [↗](#); Figure 3 – figure supplement 1C [↗](#)). Similar to the effects of global *Tlr4* KO (Wang et al., 2023 [↗](#)), *Tlr4*^{VEKO} mice showed minimal redistribution of Cldn5 (Figure 3A–B [↗](#)). The area occupied by Zonula Occludens-1 (ZO-1), a tight junction scaffold protein, showed a modest but statistically significant increase in WT leptomeningeal vessels but no significant change in *Tlr4*^{VEKO} leptomeningeal vessels during infection (Figure 3 – figure supplement 1A [↗](#) and B [↗](#)). In sum, these data suggests that non-myeloid TLR4 signaling regulates EC tight-junction organization.

To directly test the link between inflammatory signaling and Cldn5 redistribution, we turned to cultured mouse brain ECs (bEnd.3), which express both TLR4 and Cldn5 at baseline (Williams et al., 1988 [↗](#); Montesano et al., 1990 [↗](#); Sikorski et al., 1993 [↗](#)). Although they are brain-derived, bEnd.3 cells lack many BBB-specific attributes and, therefore, they likely exhibit generalized endothelial responses rather than brain-specific responses to bacterial exposure. Time-course imaging of bEnd.3 cells exposed to living or heat-killed *E. coli* or Group B *Streptococcus* (GBS) revealed rapid redistribution of Cldn5 from the plasma membrane to intracellular compartments within one hour of bacterial exposure under all four conditions (Figure 3C, 3D [↗](#), and 3E [↗](#) first and third plots). Nuclear translocation of NF-κB was greatest following exposure to live *E. coli*, beginning within 1 hour and progressively increasing over the 6-hour duration of the experiment, consistent with strong TLR4 activation (Figure 3C [↗](#) left half, and 3E second plot). Heat-killed *E. coli* produced a more modest nuclear translocation of NF-κB, peaking at 3–6 hours (Figure 3D [↗](#) left half, and 3E fourth plot). Despite the robust internalization of Cldn5 in response to either live or heat-killed GBS, the NF-κB response to GBS was minimal, suggesting that TLR2-mediated NF-κB signaling is weaker than TLR4-mediated signaling in bEnd.3 cells (Figure 3C and D [↗](#) right halves, and 3E second and fourth plots). Together, these findings indicate that Cldn5 redistribution is a rapid endothelial response to bacterial exposure that can occur in the absence of detectable NF-κB nuclear translocation, suggesting partial or complete independence from NF-κB-driven transcriptional responses.

TLR4 signaling controls NF-κB activation, tight junction dynamics, and barrier integrity in bEnd.3 cells exposed to *E. coli*

To directly test the role of TLR4 signaling in bEnd.3 cells exposed to *E. coli*, we generated a CRISPR *Tlr4* knockout line (Figure 4 [↗](#)). *Tlr4* knockout lines were generated using Cas9 and sgTrack-mCherry-mediated sgRNA delivery (see Methods; Hulton et al., 2020 [↗](#)).

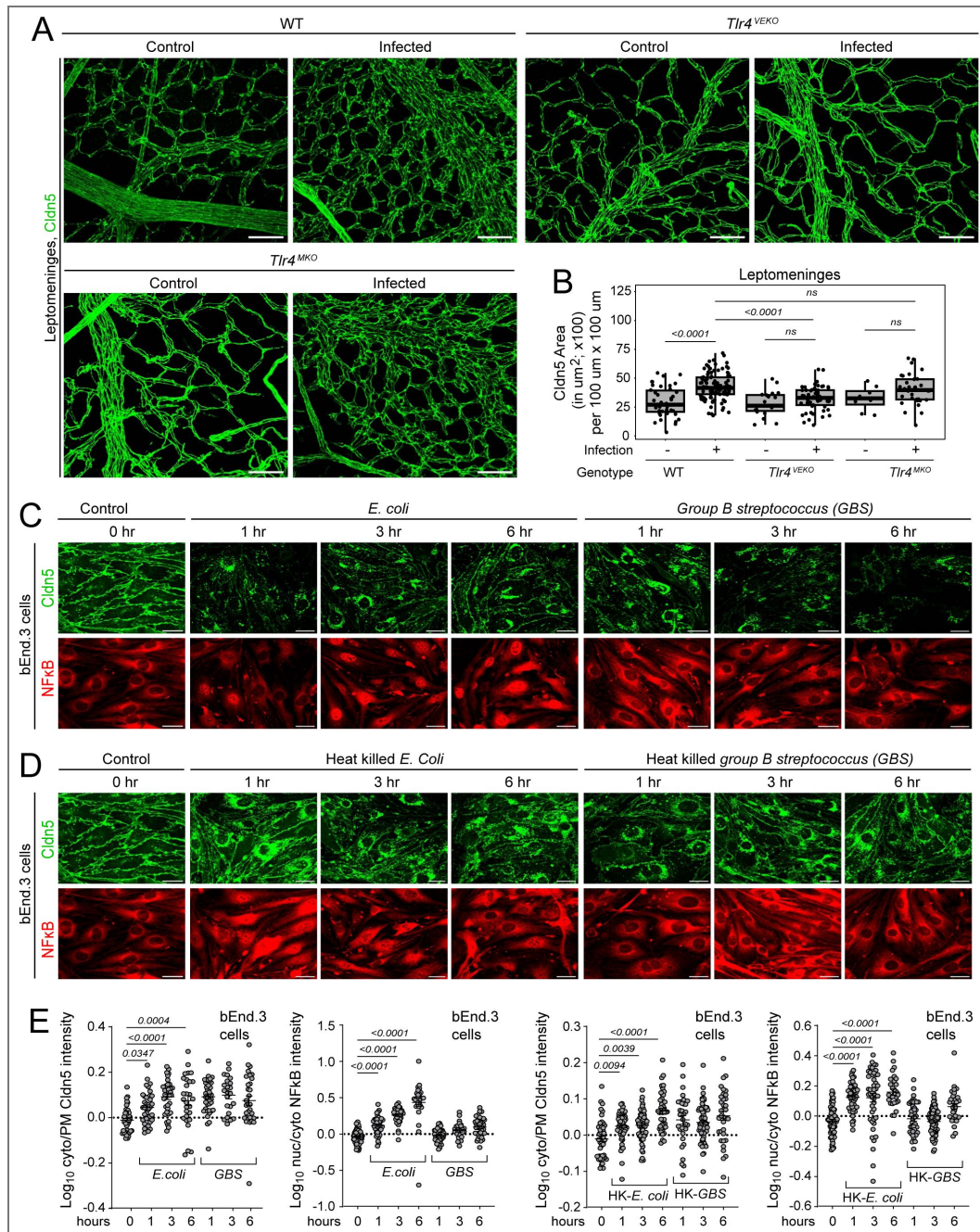


Figure 3. Claudin-5 redistribution in response to *E. coli* in leptomeningeal endothelial cells and in bEnd.3 cells.

(A) Whole mount leptomeninges from P6 WT, *Tlr4*^{VEKO}, and *Tlr4*^{MKO} mice, control or *E. coli*-infected, immunostained for Cldn5. Scale bars, 20 μ m. (B) Quantification of Cldn5+ area in the leptomeninges, as shown in (A). (C) Control bEnd.3 cells and bEnd.3 cells exposed to live *E. coli* or live group *B Streptococcus* (GBS) for 1–6 hours were immunostained for Cldn5 and NF- κ B. Scale bars, 20 μ m. (D) Control bEnd.3 cells and bEnd.3 cells exposed to heat-killed *E. coli* or heat-killed group *B Streptococcus* (GBS) for 1–6 hours were immunostained for Cldn5 and NF- κ B. Scale bars, 20 μ m. (E) Quantification of experiments shown in (C) and (D). First plot, quantification of log₁₀-transformed cytoplasmic-to-plasma membrane (Cyto/PM) intensity ratio for Cldn5 in bEnd.3 cells exposed to live *E. coli* for the indicated time in hours. Second plot, quantification of log₁₀-transformed nuclear-to-cytoplasmic (Nuc/Cyto) intensity ratio for NF- κ B in bEnd.3 cells exposed to live GBS for the indicated time in hours. The third and fourth plots are analogous to the first and second plots, except that bEnd.3 cells were exposed to heat-killed (HK) *E. coli* or GBS. Box plot in (B) shows median, interquartile range, and all individual data points. Each *in vivo* data point represents one image from a single mouse, with two locations imaged per mouse. For plots in (E), each data point represents a single bEnd.3 cell analyzed from representative images in three biological replicates. Statistical comparisons (p-values) were calculated with the Wilcoxon rank-sum test. ns, not significant (p > 0.05).

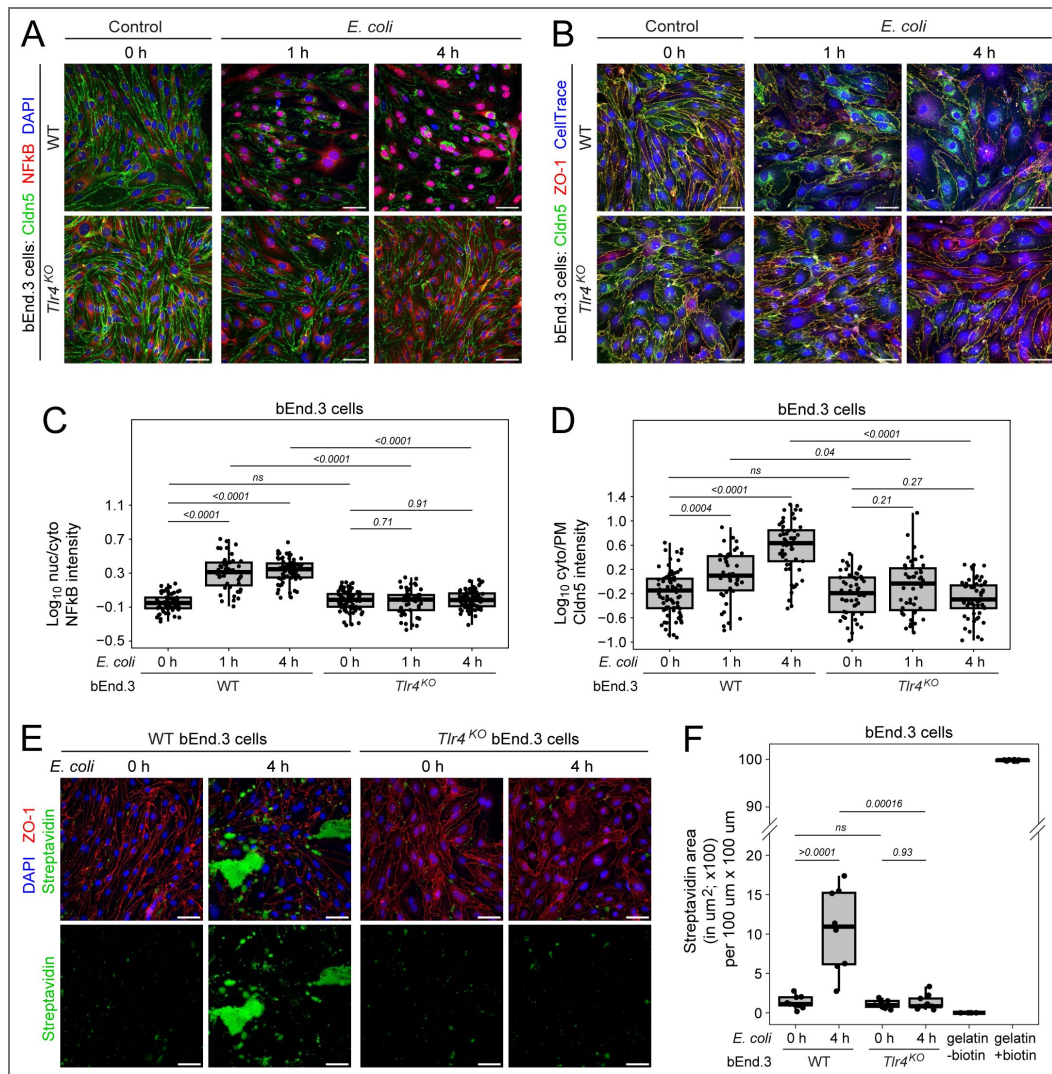


Figure 4. TLR4 signaling controls NF-κB activation, tight junction dynamics, and barrier integrity in bEnd.3 cells exposed to *E. coli*.

(A) WT and *Tlr4*^{KO} bEnd.3 cells, either not exposed to *E. coli* (control) or exposed to *E. coli* for 1 hour or 4 hours, were fixed and immunostained for Cldn5 and NF-κB and stained with DAPI. Scale bars, 20 μm. (B) WT and *Tlr4*^{KO} bEnd.3 cells, either not exposed to *E. coli* (control) or exposed to *E. coli* for 1 hour or 4 hours, were fixed and immunostained for Cldn5 and ZO-1 and stained with CellTrace. Scale bars: 20 μm. (C) Quantification of log₁₀-transformed nuclear-to-cytoplasmic (Nuc/Cyto) NF-κB intensity ratio in WT and *Tlr4*^{KO} bEnd.3 cells, with or without exposure to *E. coli*, as shown in (A). (D) Quantification of log₁₀-transformed cytoplasmic-to-plasma membrane (Cyto/PM) Cldn5 intensity ratio in WT and *Tlr4*^{KO} bEnd.3 cells, with or without exposure to *E. coli*, using ZO-1 localization to define the plasma membrane region, as shown in (B). (E) WT and *Tlr4*^{KO} bEnd.3 cells were grown to confluence on biotinylated gelatin-coated coverslips, with or without *E. coli* exposure for 4 hours, were then incubated with fluorescent streptavidin, and were finally fixed and immunostained for ZO-1. Representative images are shown with merged (upper) and streptavidin-only (lower) channels. Scale bars, 20 μm. (F) Quantification of streptavidin+ area in WT and *Tlr4*^{KO} bEnd.3 cells with or without exposure to *E. coli*, as shown in (E). Wells coated with gelatin alone or biotinylated gelatin (without cultured cells) served as negative and positive controls, respectively. Box plots show median, interquartile range, and all individual data points. Each data point represents a single cell (C and D) or one image field (F) from representative images in three biological replicates. Statistical comparisons (p-values) were calculated with the Wilcoxon rank-sum test. ns, not significant (p > 0.05).

Exons 1 and 3 of the mouse *Tlr4* locus were targeted with sgRNAs and clonal lines were isolated by fluorescence-activated cell sorting. Functional loss of TLR4 was confirmed by quantifying NF- κ B nuclear translocation following bacterial exposure (Figure 4 – figure supplement 1A [↗](#)). Sanger sequencing confirmed frameshift mutations in all *Tlr4* alleles in several exon 3-targeted clones, one of which (*Tlr4*^{KO}-m1) was selected for further study (Figure 4 – figure supplement 1B [↗](#) and C [↗](#)). Immunostaining for Cldn5 and NF- κ B in WT and *Tlr4*^{KO} bEnd.3 cells showed that deletion of TLR4 prevented both NF- κ B nuclear translocation and Cldn5 internalization in response to *E. coli* (Figure 4A–D [↗](#)).

To test whether TLR4 signaling directly regulates endothelial barrier permeability, we used a cell culture assay to quantify barrier permeability across confluent bEnd.3 monolayers (Dubrovskiy et al., 2013 [↗](#)). In this assay, bEnd.3 cells were grown to confluence on biotinylated gelatin and breaks in the monolayer of cells were visualized by adding fluorescent streptavidin to the medium. Cldn5-containing tight junctions were observed only in confluent bEnd.3 monolayers (Figure 4 – figure supplement 2A [↗](#)). Control experiments showed that the streptavidin signal depended on coating the plate with biotinylated gelatin and on gaps in the cell monolayer (Figure 4 – figure supplement 2B [↗](#)). When bEnd.3 cells were grown to confluence, the monolayer effectively restricted streptavidin access to the underlying biotinylated gelatin substrate (Figure 4 – figure supplement 2B [↗](#)). Application of this assay to WT and *Tlr4*^{KO} bEnd.3 monolayers that were exposed to *E. coli* showed that loss of TLR4 signaling prevented streptavidin access to the underlying biotinylated gelatin substrate and preserved tight-junction integrity (Figure 4E and F [↗](#)). Together, these data indicate that TLR4 signaling in cultured brain ECs drives Cldn5 internalization and increases monolayer permeability *in vitro*, mirroring TLR4's role *in vivo*.

Comparisons of Cldn5 localization with junctional, plasma membrane, and trafficking markers in WT and *Tlr4*^{KO} bEnd.3 cells with or without *E. coli* exposure

Next, we examined the subcellular localization and specificity of Cldn5 redistribution in WT and *Tlr4*^{KO} bEnd.3 cells. We examined several tight junction-associated and integral membrane proteins expressed in bEnd.3 cells – ZO-1, β -catenin, glucose transporter 1 (GLUT1), and platelet endothelial cell adhesion molecule-1 (PECAM1) – to assess changes in their colocalization with Cldn5 during *E. coli* exposure (Figure 5A–5C [↗](#)). This analysis was conducted using confocal microscopy with a 40X objective; thus, the designation of “colocalization” is at the resolution conferred by this objective. Cellular colocalization was quantified using percent-overlap analysis in ImageJ (see Methods). At baseline, ZO-1 and β -catenin showed an average of ~75% overlap with Cldn5, consistent with their colocalization at endothelial tight junctions (Figure 5D [↗](#), Figure 5 – figure supplement 1A [↗](#)). Both ZO-1 and β -catenin remained plasma membrane-localized after 4 hours of *E. coli* exposure, while Cldn5 was redistributed to cytoplasmic compartments in WT but not *Tlr4*^{KO} cells (Figure 5A, 5D [↗](#), Figure 5 – figure supplement 1A [↗](#)).

We next analyzed non-tight junction membrane proteins. The glucose transporter GLUT1 showed relatively little overlap with Cldn5 at baseline and localized primarily to the plasma membrane (Figure 5B [↗](#)). In these images, GLUT1 immunostaining was present across the cell but it did not reveal a silhouette of the nucleus, implying that it was localized to the plasma membrane rather than the cytoplasm. During *E. coli* exposure, GLUT1 was internalized alongside Cldn5, showing ~75% overlap within cytoplasmic compartments and revealing the silhouette of the nucleus (Figure 5D [↗](#), Figure 5 – figure supplement 1A [↗](#)). In contrast, PECAM1, another non-tight junction surface marker, retained its plasma-membrane localization during the 4-hour exposure to *E. coli* in both WT and *Tlr4*^{KO} cells (Figure 5C, 5D [↗](#), Figure 5 – figure supplement 1A [↗](#)). Taken together, these findings reveal distinct specificities of internalization among membrane and membrane-associated proteins dependent on TLR4 signaling in cultured brain endothelial cells.

To identify the intracellular compartments involved in Cldn5 trafficking, we co-stained WT and *Tlr4*^{KO} cells for Early Endosome Antigen 1 (EEA1), Lysosome-associated Membrane Protein 2 (LAMP2), Rab7, and Rab11 to label endosomal and lysosomal vesicles, and for Protein Disulfide

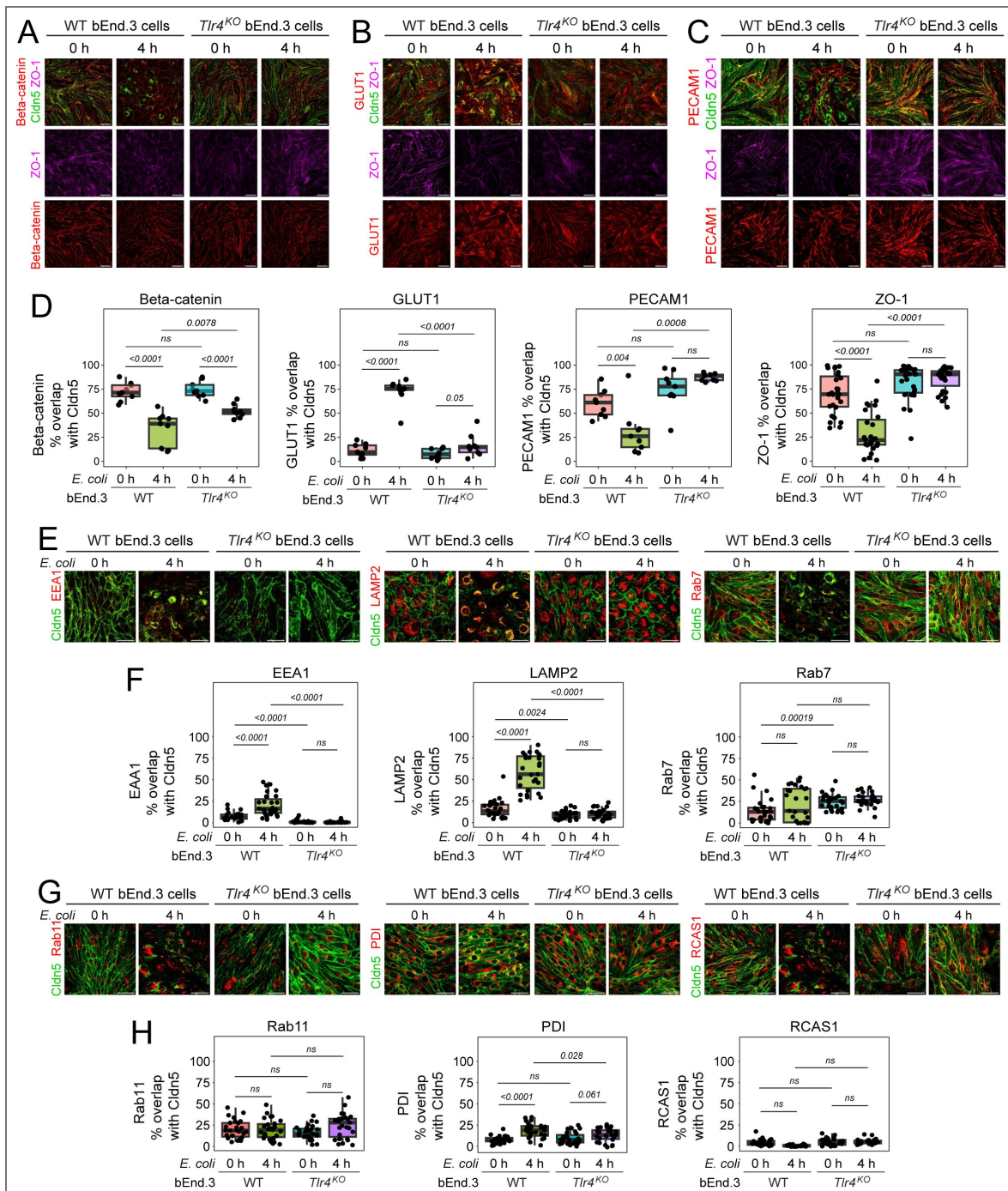


Figure 5. Comparisons of Cldn5 localization with junctional, plasma membrane, and trafficking markers in WT and *Tlr4*^{KO} bEnd.3 cells with or without *E. coli* exposure.

(A-C) WT and *Tlr4*^{KO} bEnd.3 cells, either not exposed to *E. coli* (control) or exposed to *E. coli* for 4 hours, were fixed and immunostained for Cldn5 and the indicated markers. Scale bars, 20 μ m. (D) Quantification of overlap between Cldn5 and β -catenin, GLUT1, PECAM1, and ZO-1, with each data point representing a 100 μ m $\times$ 100 μ m region of interest (ROI). (E) WT and *Tlr4*^{KO} bEnd.3 cells, either not exposed to *E. coli* (control) or exposed to *E. coli* for 4 hours, were fixed and immunostained for Cldn5 and the indicated markers. Scale bars, 20 μ m. (F) Quantification of overlap between Cldn5 and EEA1, LAMP2, and Rab7, as in (D). (G) WT and *Tlr4*^{KO} bEnd.3 cells, either not exposed to *E. coli* (control) or exposed to *E. coli* for 4 hours, were fixed and immunostained for Cldn5 and the indicated markers. Scale bars, 20 μ m. (H) Quantification of overlap between Cldn5 and Rab11, PDI, and RCAS1, as in (D). Box plots show median, interquartile range, and all individual data points. Each data point represents one image from representative images in three biological replicates. Statistical comparisons (p-values) were calculated with the Wilcoxon rank-sum test. ns, not significant (p > 0.05)

Isomerase (PDI) and Receptor-binding Cancer Antigen Expressed on SiSo Cells (RCAS1) to mark the endoplasmic reticulum and Golgi apparatus, respectively (Figure 5E-H) (Stamatovic et al., 2009; 2017; Scott et al., 2014; Shearer and Petersen, 2019; MacDonald et al., 2020). After 4 hours of exposure to *E. coli*, Cldn5 colocalization with LAMP2⁺ vesicles increased to ~50% overlap in a TLR4-dependent manner (Figure 5E and 5F). *E. coli* exposure also produced small increases in Cldn5 colocalization with EEA1 and PDI, to ~20% overlap, which was similarly dependent on TLR4 (Figure 5E-H). *E. coli* exposure produced little change in the low-level colocalization of Cldn5 with Rab7, Rab11, or RCAS1, and there was little or no effect of TLR4 loss (Figure 5E-H). We interpret the TLR4-dependent increase in Cldn5-EEA1 and Cldn5-LAMP2 overlap as representing the endocytic internalization of Cldn5 and its delivery to lysosomes. We interpret the modest increase in Cldn5-PDI as likely arising from the close proximity of endosomes and lysosomes to the ER, which is present throughout the cytoplasm.

Further insights into the mechanism of Cldn5 endocytosis came from visualizing the internalization of transferrin, cholera toxin subunit B (CTB), and 10-kDa dextran, well-established tracers for clathrin-mediated endocytosis, caveolin-mediated endocytosis, and macropinocytosis, respectively (Figure 6A; Johnson and Spence, 2010). Prior to *E. coli* exposure, these tracers and Cldn5 exhibited ~80% overlap at the plasma membrane (Figure 6B). Following *E. coli* exposure, reduced overlap with transferrin and CTB, together with persistent overlap with 10-kDa dextran, suggested a macropinocytosis-like mechanism of Cldn5 internalization (Figure 6B). The persistence of intracellular Cldn5 immunostaining with up to at least 6 hours of *E. coli* exposure, together with washout experiments showing restoration of plasma membrane Cldn5 localization within 3 hours of *E. coli* removal, suggest little or no degradation of internalized Cldn5 during this time frame (Figure 6C and D). Together, these results indicate that TLR4 signaling triggers a highly specific and reversible internalization of Cldn5, providing a potential mechanistic link between inflammatory activation and increased vascular permeability.

To test the possible role of NF- κ B signaling in Cldn5 internalization, we measured Cldn5 co-localization with Lamp2 in bEnd.3 cells after one hour of exposure to *E. coli* with or without the I κ B kinase inhibitor ACHP (2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidiny)-3 pyridinecarbonitrile) at 1 μ M (Figure 6E and F). Cldn5 internalization and co-localization with Lamp2 were insensitive to ACHP-mediated inhibition of NF- κ B signaling under conditions that inhibited NF- κ B nuclear translocation (Figure 6G and H). These data imply that the TLR4-dependent internalization of Cldn5 operates via an arm of the TLR4 pathway that is both rapid and distinct from NF- κ B mediated changes in gene expression.

Role of TLR4 signaling in the transcriptional response to *E. coli* in bEnd.3 cells

To define the TLR4-dependent transcriptional response to *E. coli* exposure in ECs, we performed bulk RNA-seq on WT and *Tlr4*^{KO} bEnd.3 cells with or without 3 hours of *E. coli* exposure (Figure 7). Principal component analysis revealed separation of datasets based on both bacterial exposure and genotype (WT vs. *Tlr4*^{KO}) (Figure 7 – figure supplement 1A and B). Interestingly, WT and *Tlr4*^{KO} bEnd.3 cells that were not exposed to *E. coli* clustered separately, suggesting a basal level of TLR signaling even in the absence of *E. coli* exposure. Differential expression analysis in WT bEnd.3 cells showed robust induction of proinflammatory transcripts upon *E. coli* exposure, including *Il6*, *Cxcl2*, *Tnf*, *Icam1*, and *Ccl2* (Figure 7A), whereas this response was largely absent in *Tlr4*^{KO} bEnd.3 cells (Figure 7B; note the change in axis scales between Figures 7A and 7B). Pathway analysis showed that inflammatory signaling was highly TLR4-dependent: TNF- α signaling via NF- κ B, IL6–JAK–STAT signaling, and general inflammatory response pathways showed strong transcriptional activation in WT but not in *Tlr4*^{KO} bEnd.3 cells (Figure 7C–F).

We next quantified transcripts for representative cytokines, chemokines, and adhesion molecules that define endothelial activation. *Il6*, *Cxcl10*, *Ccl2*, *Cxcl5*, *Cd83*, *Ccl20*, and *Icam1* transcripts were all significantly upregulated in WT bEnd.3 cells following *E. coli* exposure but remained unchanged in *Tlr4*^{KO} bEnd.3 cells (Figure 7G). Heatmap visualization of the Hallmark “TNF- α signaling via NF- κ B” gene set confirmed that NF- κ B-responsive genes, including *Nfkbia*, *Ccl2*, *Il6*,

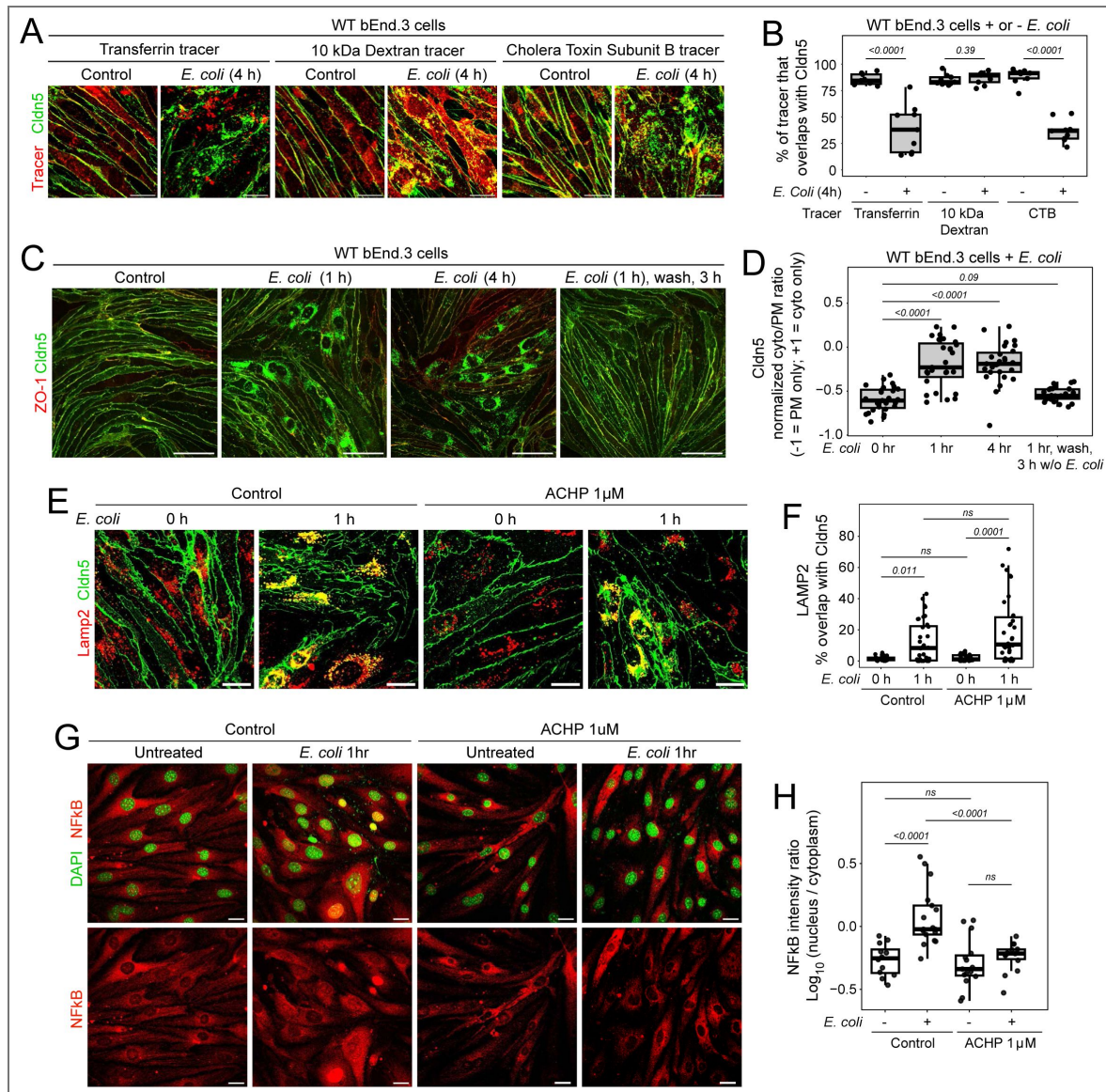


Figure 6. In bEnd.3 cells, pharmacologic inhibition of NF-κB nuclear translocation and signaling does not impede rapid internalization of Cldn5 in response to *E. coli* exposure.

(A) bEnd.3 cells were pre-incubated for 30 minutes with the indicated fluorescent tracer, then either not exposed to *E. coli* (control) or exposed to *E. coli* for 4 hours and then fixed and immunostained for Cldn5. Scale bars, 20 μm. (B) Quantification of % overlap between tracer and Cldn5 from the experiment shown in (A). (C) Cldn5 internalization and recovery during 1 hour of *E. coli* exposure, followed by washout of *E. coli*, and then an additional 3 hours of incubation in medium without bacteria. Scale bars, 50 μm. (D) Quantification of the experiment shown in (C). The metric on the y-axis is (cytoplasmic signal - plasma membrane signal)/(cytoplasmic signal + plasma membrane signal). 100% cytoplasmic localization corresponds to 1.0. 100% plasma membrane localization corresponds to -1.0. 50% cytoplasmic localization and 50% plasma membrane localization corresponds to 0.0. (E) The subcellular localization of Lamp2 and Cldn5 were determined in bEnd3 cells in the presence or absence of 1 μM of the IKK inhibitor ACHP before *E. coli* exposure or after 1 hour of *E. coli* exposure. Scale bars, 20 μm. (F) Quantification of the experiment shown in (E). Each symbol represents a 100 μm x 100 μm region of interest (ROI). (G) NF-κB translocation from cytoplasm to nucleus was visualized in the presence or absence of 1 μM of ACHP before *E. coli* exposure or after 1 hour of *E. coli* exposure. Scale bars, 20 μm. (H) Quantification of the experiment shown in (G). Each symbol represents an individual cell. Statistical comparisons (p-values) were calculated with the Wilcoxon rank-sum test. ns, not significant (p > 0.05).

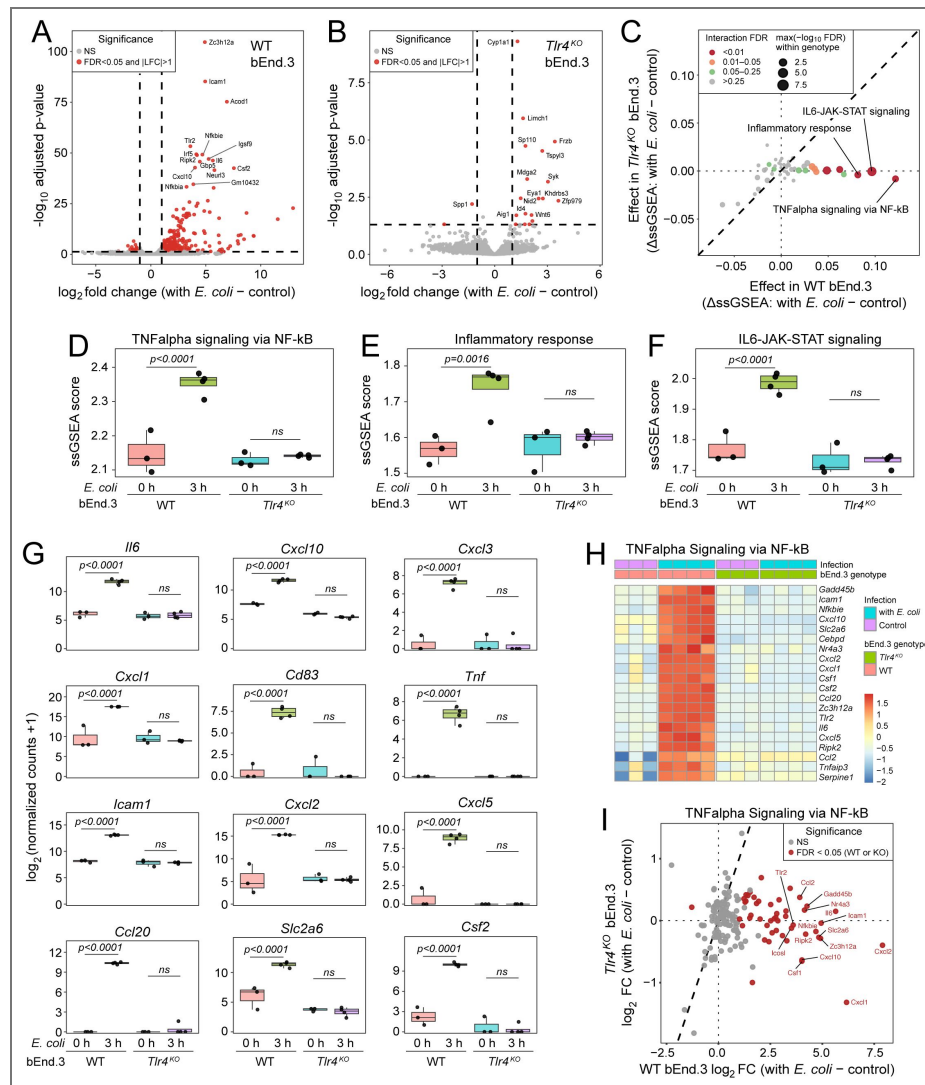


Figure 7. In bEnd.3 cells, TLR4 signaling plays a central role in the transcriptional response to *E. coli* exposure.

(A) Volcano plot of differential transcript abundances in WT bEnd.3 cells, with or without exposure to *E. coli* for 3 hours. The plot shows transcript-level log₂ fold change (LFC) on the horizontal axis versus -log₁₀(false discovery rate; FDR) on the vertical axis. Red points mark significantly changed transcripts (FDR < 0.05 and |LFC| ≥ 1); grey points are not significant. (B) Volcano plot of differential transcript abundances in *Tlr4*^{KO} bEnd.3 cells, with or without exposure to *E. coli* for 3 hours, plotted as in (A). Note the change of scale of the vertical and horizontal axes between (A) and (B). (C) Pathway effect map from ssGSEA Hallmark scores. Each symbol is a pathway. The horizontal axis is the effect of *E. coli* exposure for 3 hours on WT bEnd.3 cells (ΔssGSEA = *E. coli* exposed - not exposed) and the vertical axis is the effect of *E. coli* exposure for 3 hours on *Tlr4*^{KO} bEnd.3 cells. Symbol size encodes the within-genotype significance [max(-log₁₀ FDR) across both genotypes], and the color code indicates the genotype × *E. coli* exposure interaction FDR. The 45-degree diagonal line represents equal effects for the two genotypes. (D–F) ssGSEA pathway activity based on genotype and *E. coli* exposure for three Hallmark immune response pathways: TNF-α signaling via NF-κB (D), Inflammatory response (E), and IL6-JAK-STAT signaling (F). Symbols represent ssGSEA scores for individual samples. The vertical axis shows the interaction FDR score from a linear model for genotype × *E. coli* exposure. (G) Transcript abundance changes for 12 immune system genes in WT and *Tlr4*^{KO} bEnd.3 cells, with or without exposure to *E. coli* for 3 hours. (H) Heatmap of transcript abundance changes in WT and *Tlr4*^{KO} bEnd.3 cells, with or without exposure to *E. coli* for 3 hours, for TNF-α signaling via NF-κB. Rows correspond to transcripts with the greatest changes; values are variance-stabilized transformation (VST) z-scores derived from DESeq2-normalized counts. Columns are arranged by genotype and condition. (I) Scatterplot showing transcript abundance changes in WT and *Tlr4*^{KO} bEnd.3 cells, with or without exposure to *E. coli* for 3 hours for TNF-α signaling via the NF-κB pathway. Each symbol represents one gene. Red points indicate genes significant for either genotype (FDR < 0.05). The top 15 genes by effect size are labeled. Note the different scales for the horizontal and vertical axes. The diagonal line represents equal effects for the two genotypes.

and *Tnfrsf3*, were selectively induced in WT cells by *E. coli* exposure (Figure 7H). Direct fold change comparisons between genotypes showed that induction of NF- κ B target genes was strongly attenuated in *Tlr4*^{KO} cells (Figure 7I).

To assess TLR4-dependent pathway activation following *E. coli* exposure, we performed single-sample gene set enrichment analysis (ssGSEA) across 50 Hallmark pathways. The pathways most strongly affected by genotype $\times$ exposure interactions included IL6–JAK–STAT3 signaling, allograft rejection, inflammatory response, TNF- α signaling via NF- κ B, and the interferon- γ response (Figure 7 – figure supplement 1C). Heatmaps of Leading-Edge genes in these pathways further highlighted the broad suppression of cytokine and cell adhesion transcript induction in *Tlr4*^{KO} bEnd.3 cells compared to WT (Figure 7 – figure supplement 1D–F).

Together, the *in vivo* and bEnd.3 transcriptional analyses identify TLR4 as a dominant regulator of inflammatory gene expression in response to *E. coli* exposure.

Discussion

This study demonstrates that, in a mouse model of neonatal *E. coli* meningitis, non-myeloid TLR4 signaling is a key determinant of the inflammatory response in all major leptomeningeal cell types and of the infection-associated increase in vascular permeability. Using conditional *Tlr4* knockout models, leptomeningeal single-nucleus RNA sequencing, and *Tlr4* knockout in bEnd.3 cells, we show that TLR4 activation directs molecular, cellular, and transcriptional reprogramming of the vascular barrier. Our quantitative analysis of *Cdh5-CreER*-mediated recombination, showing recombination in all leptomeningeal ECs but in only a minority of other nonmyeloid cells, implies that the blunted infection response in the *Tlr4*^{VEKO} leptomeninges is likely referable to loss of TLR4 signaling in ECs. In cell culture, endothelial TLR4 activation triggers rapid, selective, and reversible Cldn5 internalization, coincident with increased endothelial permeability. At the transcriptional level, endothelial TLR4 controls NF- κ B and IL6/JAK/STAT inflammatory programs and cytokine gene expression. These findings establish TLR4 as the central leptomeningeal sensor of gram-negative bacteria, linking innate immune recognition to barrier dysfunction and to the tissue-wide inflammatory response.

The leptomeningeal response to bacterial infection

Our snRNA-seq analyses revealed a dramatic transcriptome response to neonatal *E. coli* infection across all major leptomeningeal cell types that was broadly attenuated with non-myeloid deletion of *Tlr4*. Non-myeloid TLR4 signaling was required for the induction of canonical immune activation markers, including ICAM1 and leukocyte-recruiting chemokines. In contrast, myeloid-specific inactivation of *Tlr4* with *Lyz2-Cre* had minimal effect in the mouse meningitis model. Taken together, these data point to innate immune sensing as the gatekeeper of immune escalation in the leptomeninges. It is possible that myeloid TLR4 signaling in the leptomeninges may augment non-myeloid signaling or become relevant later in the course of the infection. We note that these experiments do not distinguish between local vs. distal anatomic sources of LPS or downstream effector molecules, such as cytokines, as activators of the leptomeningeal inflammatory response (Huang et al., 2021). Histologic observations of RFP-expressing *E. coli* in the brain, liver, and lungs, together with positive blood cultures, indicate substantial systemic dissemination of *E. coli* in this model. Thus, the inflammatory responses of leptomeningeal cells likely reflect exposure to bacterial products and inflammatory mediators derived from both local and distal sources.

In bEnd.3 cells, TLR4 activation triggers a rapid (≤ 1 hour) and reversible internalization of Cldn5, and *Tlr4*^{KO} in bEnd.3 cells prevents both NF- κ B translocation from cytoplasm to nucleus and Cldn5 redistribution. With TLR4 activation, ZO-1 and β -catenin remain junctional, PECAM1 remains at the plasma membrane, and GLUT1 co-internalizes with Cldn5, implying protein-specific re-localization. Percent-overlap and tracer analyses support endocytic routing of Cldn5, with prominent LAMP2+ (i.e., endosomal/lysosomal) localization, in a pattern that argues against canonical clathrin/caveolin pathways and is most consistent with macropinocytosis-like uptake. Rapid Cldn5 internalization occurred independently of NF- κ B nuclear translocation, suggesting regulation through a non-transcriptional arm of TLR4 signaling. Strikingly, washout of *E. coli*

restores Cldn5 to cell-cell junctions within three hours. These observations are broadly aligned with prior work showing cytokine/chemokine-evoked endocytosis of tight-junction proteins (including claudins and occludin) during EC barrier opening, via Rho/ROCK or caveolae-dependent routes (Stamatovic et al., 2006 [DOI](#), 2009 [DOI](#), 2012 [DOI](#)). Functionally, Cldn5 internalization was accompanied by increased barrier permeability, as measured by streptavidin passage across bEnd.3 monolayers or by Sulfo-NHS-biotin leakage *in vivo*. These findings reveal an EC-intrinsic process linking TLR4 activation to tight-junction remodeling and increased vascular permeability. In classic BBB tight-junction biology, Cldn5 is considered a gatekeeper of size-selective permeability (Morita et al., 1999 [DOI](#)). These findings extend that picture by identifying Cldn5 internalization as a disease-induced process that is rapid, selective, and reversible.

Meningitis and vascular permeability

Mouse models of neonatal *E. coli* meningitis have shown conflicting effects of global TLR signaling on the clinical course of infection. Krishnan et al. (2013) [DOI](#) reported that global loss of TLR4 resulted in a more rapid downhill course, whereas Jia et al. (2025) [DOI](#) reported that global loss of TLR4 conferred substantial protection. Interestingly, Krishnan et al. (2013) [DOI](#) reported that global loss of TLR2 conferred substantial protection in the same early postnatal *E. coli* meningitis model. The observations reported here are intermediate between those of Krishnan et al. (2013) [DOI](#) and Jia et al. (2025) [DOI](#), as we observed no obvious differences in two measures of disease progression (bacterial burden and body weight) between infected WT and *Tlr4*^{VEKO} mice at 24 hours post-infection, despite substantial blunting of infection-associated gene expression and permeability changes in the leptomeninges of *Tlr4*^{VEKO} mice. These divergent findings may reflect differences in experimental models and/or the fact that global TLR deletion affects multiple compartments simultaneously, including immune cell pathogen sensing, systemic inflammatory responses, and vascular barrier regulation.

Multiple pathways and mechanisms have been proposed to explain the increase in vascular permeability in the leptomeninges following bacterial infection. In an EC monolayer model of *E. coli* infection, activation of protein kinase C- α (PKC- α) led to disassembly of VE-cadherin mediated cell-cell junctions and a concomitant increase in monolayer permeability (Sukumaran and Prasadarao, 2003 [DOI](#)). In a similar EC monolayer model of *E. coli* infection, NOD-like receptor family pyrin domain containing 6 (NLRP6), a regulator of caspase, NF- κ B, and inflammasome signaling, was found to reduce *E. coli*-induced tight junction disruption while also increasing *E. coli*-induced cell death (Jia et al., 2026 [DOI](#)). Wang, et al. (2025a) [DOI](#) found that RIPK1, a kinase and scaffolding protein that integrates signals from inflammatory and cell death pathways, plays a central role in endothelial cell death and BBB breakdown during *E. coli* meningitis. Huang et al. (2024) [DOI](#) showed that Wnt/beta-catenin signaling, which controls BBB-specific gene expression and is essential for maintaining BBB integrity, is inhibited by NF- κ B signaling; augmenting Wnt/beta-catenin signaling blunted LPS-induced vascular leakage while reducing Wnt/beta-catenin signaling had the opposite effect.

Yang et al. (2024) [DOI](#) observed IL22-mediated degradation of occludin, ZO-1, and Cldn5 in cultured ECs, and showed that IL22 acts via a VEGF-STAT3 pathway to increase vascular permeability; in a mouse model of *E. coli* meningitis, this effect was largely abrogated by knocking out *Il22*. Finally, models of LPS-induced and *Klebsiella pneumoniae*-induced BBB breakdown implicate a Caspase-11 pathway that leads to the induction of Gasdermin D pores in CNS EC plasma membranes, leading to BBB breakdown and eventual pyroptotic cell death of ECs (Wei et al., 2024 [DOI](#)). Thus, the emerging picture is one of multiple inflammatory and cell death pathways converging on endothelial hyper-permeability.

We speculate that increased endothelial permeability in the leptomeninges could represent a double-edged response, deleterious when sustained and/or excessive, yet potentially protective when transient, facilitating the regulated passage of immune mediators and immune cells that are needed to combat infection. More generally, increases in vascular permeability may represent a general response to CNS stress or injury. For instance, others have observed reduced BBB integrity

in brain regions remote from a focal optic-nerve injury, following transient global ischemia, in ischemic stroke, and in LPS-driven systemic inflammation (Banks et al., 2015 [↗](#); Ju et al., 2018 [↗](#); Smith et al., 2018 [↗](#); Cottarelli et al., 2025 [↗](#)).

Activation of TLR4 signaling and the resulting increase in vascular permeability has direct clinical implications for neonatal meningitis. In particular, the data are consistent with a model in which endothelial TLR4 signaling contributes causally to CNS injury by promoting loss of barrier integrity, thereby permitting inflammatory cytokines and bacterial products to diffuse into the CNS parenchyma. These findings align with clinical observations in infants with bacterial meningitis, where elevated CSF cytokines (e.g., IL-6, TNF- α) and albumin level correlate with poor neurologic outcomes (Garges et al., 2006 [↗](#); Xu et al., 2019 [↗](#); Gao and Hu, 2024 [↗](#)). By demonstrating the centrality of endothelial-intrinsic TLR4 signaling, our data highlight the endothelium not merely as a passive target but as a primary effector of neuropathology. These observations raise the possibility that selective modulation of endothelial TLR4 or more proximate regulators of Cldn5 trafficking could preserve vascular integrity, offering a potential avenue for neuroprotection in neonatal meningitis.

Limitations of the study

Several limitations of the present study should be noted. While bEnd.3 cells provide a useful endothelial model, they do not exhibit the distinctive structural and molecular complexity of the blood–brain barrier (Garcia-Gallardo and Campbell, 2025 [↗](#)). In particular, the bEnd.3 culture system lacks pericytes and astrocytes. Additionally, studying fixed cells and tissues does not permit detailed analyses of molecular and cellular dynamics. Going forward, live cell imaging of CNS ECs in culture (cell lines and/or acutely isolated cells) and *in vivo* two-photon imaging of the leptomeninges would complement the present studies by revealing dynamic responses in real time. Finally, the present study focused on *E. coli* K1, the dominant Gram-negative neonatal pathogen. Future work could assess TLR signaling in response to other bacterial pathogens, such as Group B *Streptococcus*.

Concluding remarks

In sum, this study shows that TLR4 signaling couples innate immune recognition to inflammatory responses in multiple leptomeningeal cell types, inducing endothelial Cldn5 internalization and increasing vascular permeability. The rapid and reversible nature of the Cldn5 internalization response implies that tight-junction remodeling is a dynamically regulated process. These results highlight potential opportunities to preserve vascular barrier properties by reducing endothelial TLR4 signaling, stabilizing Cldn5 in tight junctions, or dampening IL6–JAK–STAT and NF- κ B signaling in endothelial cells (Freitas et al., 2018 [↗](#); Gadina et al., 2018 [↗](#); Zaffaroni and Peri, 2018 [↗](#); Hashimoto et al., 2021 [↗](#)).

Methods

Mouse Models and *E. coli* Infection

Mice were maintained on a C57BL/6J background. Non-myeloid *Tlr4* conditional knockout mice (referred to as *Tlr4*^{VEKO}) were generated by crossing *Tlr4*^{fl/fl} (JAX, stock #024872) females with *Cdh5-CreER*^{+/+};*Tlr4*^{-/-} males, yielding *Cdh5-CreER*^{+/+};*Tlr4*^{fl/-} offspring. Myeloid-specific conditional knockout mice (*Tlr4*^{MKO}), were generated by crossing *Tlr4*^{fl/fl} females with *Lyz2*^{Cre/+};*Tlr4*^{-/-} males, yielding *Lyz2*^{Cre/+};*Tlr4*^{fl/-} offspring. We note that for both conditional knockout mouse lines, only a single Cre-mediated recombination event was required to convert *Tlr4*^{fl/-} heterozygous cells to *Tlr4*^{-/-} homozygous cells. For both sets of crosses, littermates lacking Cre were used as controls. The *Cdh5-CreER* line (Monvoisin et al., 2006 [↗](#)) was the same line used in Wang. et al. (2025a). The *Lyz2*^{Cre} line (JAX, stock #004781) was described in Clausen et al. (1999) [↗](#). [For the present study, the *Lyz2*^{Cre} line was chosen based on the observations of Abram et al (2014) [↗](#) that it gives the most efficient myeloid-specific Cre-recombination among twelve Cre lines tested. We note that McKinsey et al (2020) [↗](#) have found that *PF4*^{Cre} also recombines LoxP targets efficiently in

leptomeningeal myeloid cells.] The *Rosa26-LSL-tdT-2A-nlsGFP* reporter line (JAX, stock #030867) was described in Wang et al. (2018) [\[1\]](#). The *Rosa26-LSL-SUN1-sfGFP* reporter line (JAX, stock #021039) was described in Mo et al. (2015) [\[2\]](#). Neonatal mice were genotyped by PCR and used for meningitis experiments without sex selection. Meninges snRNA-seq experiments and histological studies used postnatal day 6 (P6) mice with age-matched controls. To activate Cre-mediated recombination in progeny from the cross with *Cdh5-CreER*, 4-hydroxytamoxifen (4HT; Sigma-Aldrich H7904) was dissolved at a final concentration of 2 mg/ml in 1:10 vol:vol ethanol:sunflower seed oil (Sigma-Aldrich S5007), stored in aliquots at -80°C, and administered by intraperitoneal (IP) injection of 40-50 µl at P2. Mice were housed and handled according to the approved Institutional Animal Care and Use Committee protocol of the Johns Hopkins Medical Institutions (protocol MO22M375).

Neonatal *E. coli* K1 infection model

To model neonatal meningitis, postnatal day 5 (P5) mice were used, approximating the developmental stage of the human newborn blood-brain barrier and immune system as described in Wang et al. (2023) [\[3\]](#). Litters were randomized to receive a subcutaneous inoculation in the dorsal skin of 1.2×10^5 colony-forming units (CFU) of *E. coli* RS218 (O18:K1:H7) with an RFP-expression plasmid (Zhu et al., 2020 [\[4\]](#)) in 20 µL PBS or 20 µL PBS alone. Twenty-four hours later (at P6), mice were euthanized for tissue collection. Brains, meninges, and relevant tissues were harvested, fixed, and processed for immunofluorescence and tracer leakage assays.

Antibodies for tissue staining

Leptomeningeal and cortical whole mounts were immunostained with the following primary antibodies: mouse anti-Claudin-5 (Alexa Fluor™ 488, ThermoFisher #352588), mouse anti-ZO-1 (Alexa Fluor™ 594, ThermoFisher #339194), rabbit anti-ICAM-1 (CD54, eBioKAT-1, ThermoFisher #14-0542-85), rat anti-CD206 (Bio-Rad MCA2235), rabbit anti-ASC/TMS1 (Cell Signaling Technology #67824S), rat anti-PECAM-1 (BD Pharmingen #553370), rabbit anti-GLUT1 (ThermoFisher MA5-31960), rabbit anti-cleaved Caspase-3 (Cell Signaling Technology, #9664). Alexa dye-conjugated secondary antibodies were from ThermoFisher.

Sulfo-NHS-biotin leakage

For analysis of vascular leakage, mice were injected intraperitoneally with Sulfo-NHS-biotin (30 µl of 20 mg/mL Sulfo-NHS-biotin in PBS per mouse; ThermoFisher 21217) 15 minutes before sacrifice. After IP injection, the tracer rapidly equilibrates into the systemic circulation.

Immunostaining of leptomeninges whole mounts

For whole mount imaging of the leptomeninges and adjacent cortex, a preparation was developed to preserve these structures in their native configuration. After euthanasia, brains (with leptomeninges attached) were fixed by immersion in 1% paraformaldehyde in PBS overnight at 4°C. The forebrain was then dissected and hemisected, and a thin slice of tissue from the surface of the brain was obtained by making a 300 µm vibratome section parallel to the surface of the cortex. Tissue slices were permeabilized in 1% Triton X-100/PBS before immunostaining, and blocked with 5% goat or donkey serum, depending on the secondary antibody host species. Primary antibodies were applied at 1:400 dilution, followed by fluorophore-conjugated secondary antibodies. Vascular leakage in mice injected with Sulfo-NHS-biotin was visualized by staining with streptavidin-Alexa Fluor 488 (Thermo Fisher, #S11223).

Confocal microscopy and image acquisition

All images were acquired on a Zeiss LSM 780 laser-scanning confocal microscope controlled by Zeiss Zen software using 20x, 40x, or 63x/1.4 NA oil-immersion objectives. Fluorophores were excited with 405 nm, 488 nm, 561 nm, and 633 nm laser lines, and channels were collected sequentially to minimize spectral overlap. Z-stacks were acquired with step sizes optimized for each staining set; thinner stacks were used for cultured cell monolayers and deeper stacks for

leptomeningeal whole mounts. The pinhole was set to 1 Airy unit. Laser power, gain, and offset settings were maintained at constant values within each experimental cohort (e.g. comparisons across genotypes). For leptomeninges imaging, two fields were imaged per hemisphere. Maximum intensity projections or single optical sections were exported from Zen for quantification and figure preparation.

Blood bacterial burden (CFU assay)

Neonatal mice (P6; 24 hours post-infection) were euthanized on ice and whole blood was collected by cardiac puncture into tubes containing EDTA to prevent coagulation. Samples were kept on ice, and 20 μ L of whole blood was diluted in 100 μ L of LB broth, briefly vortexed, and plated onto LB agar plates. Plates were incubated overnight at 37°C, and colonies (which were visibly red due to RFP expression) were counted the following day. Bacterial burden was reported as colony-forming units (CFUs) per 20 μ L of blood. Plates with colony numbers exceeding the reliable counting range were assigned a value of 2,000 CFUs.

Quantifying RFP-expressing *E. coli* in brain sections

Sections were stained with DAPI to label nuclei and imaged for RFP-expressing *E. coli*. Fluorescence images were acquired as maximum intensity projections under identical imaging settings across experimental groups. For brain analyses, four anatomically matched territories from coronal sections of cortex were imaged and quantified per mouse. Quantification was performed using fixed threshold-based image analysis in Fiji, where the RFP signal was segmented to generate binary masks representing bacterial area. To define the corresponding cortical tissue area, DAPI images were thresholded and morphologically processed (including smoothing and hole filling) to generate continuous masks of the imaged cortical region. RFP signal was normalized to this DAPI-defined cortical area and reported as the ratio of RFP-positive area to DAPI-defined cortical area.

bEnd.3 cell culture and maintenance

The mouse brain endothelial cell line bEnd.3 (ATCC CRL-2299) was cultured according to ATCC recommendations. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; high glucose, 4.5 g/L) supplemented with 10% fetal bovine serum (FBS). Cultures were kept at 37°C in a humidified 5% CO₂ incubator. Cells were passaged at ~80–90% confluence using 0.05% trypsin–EDTA and reseeded at a split ratio of 1:4–1:6.

CRISPR-Cas9 generation of bEnd.3 *Tlr4*^{KO} cell lines

A bEnd.3-Cas9 monoclonal line was first established by transducing bEnd.3 cells with a lentivirus carrying *Streptococcus pyogenes* Cas9 (Addgene #52962) (Sanjana et al., 2014 [DOI](#)). Transduced cells were serially diluted to generate monoclonal populations, and Cas9 expression was validated by western blot. Using this Cas9-expressing line, *Tlr4* knockouts were generated by transduction with sgRNAs targeting exon 3 of *Tlr4* delivered with the sgTrack-mCherry lentivirus (Addgene #114013) (Hulton et al., 2020 [DOI](#)). Transduced cells were sorted by flow cytometry for mCherry expression, seeded in 96-well trays at one cell per well, and individual colonies were expanded. Genomic DNA was analyzed by PCR amplification across the mutated region in *Tlr4* exon 3, followed by subcloning of PCR products into a plasmid vector and sequencing of individual plasmids following colony PCR. A cloned bEnd.3 derivative cell line was considered a candidate *Tlr4*^{KO} if none of its cloned PCR products showed the WT sequence and all cloned PCR products showed frameshift mutations in *Tlr4* exon 3. Functional loss of TLR4 was confirmed by quantifying NF- κ B nuclear translocation after *E. coli* exposure compared to parental controls.

bEnd.3 immunofluorescence

For all experiments involving responses to bacterial exposure, bEnd.3 cells were seeded onto glass coverslips at ~80% confluency two days prior to the start of the experiment to allow them to form a confluent monolayer with tight junctions. After exposure to *E. coli* or GBS or mock exposure,

cells were fixed in 1% paraformaldehyde in PBS for 15 min at room temperature, permeabilized in 0.1% Triton X-100/PBS, and blocked with 5% goat or donkey serum depending on the secondary antibody host species. Primary antibodies were applied at 1:400 dilution overnight at 4°C, followed by fluorophore-conjugated secondary antibodies. Coverslips were mounted in antifade medium and imaged by confocal microscopy.

Antibodies for bEnd.3 cell immunostaining

Cultured bEnd.3 cells were immunostained with the following primary antibodies: mouse anti-Claudin-5 (Alexa Fluor™ 488, Thermo Fisher #352588), mouse anti-ZO-1 (Alexa Fluor™ 594, Thermo Fisher #339194), rabbit anti-NF-κB p65 (Cell Signaling Technology #8242S), rabbit anti-β-catenin (Cell Signaling Technology #8480), rat anti-PECAM-1 (Thermo Fisher #63-0311-82), rabbit anti-GLUT1 (Thermo Fisher MA5-31960), mouse anti-EEA1 (Cell Signaling Technology #48453T), rabbit anti-LAMP2 (Cell Signaling Technology #34141T), rabbit anti-Rab7 (Cell Signaling Technology #9367T), rabbit anti-Rab11 (Cell Signaling Technology #5589T), rabbit anti-PDI (Cell Signaling Technology #3501T), and rabbit anti-RCAS1 (Cell Signaling Technology #12290T). Primary antibodies were diluted 1:400 in PBS with 1% Triton X-100 and applied overnight at 4 °C, followed by fluorophore-conjugated secondary antibodies (Thermo Fisher). Coverslips were mounted and imaged by confocal microscopy.

bEnd.3 streptavidin leak assay

Barrier permeability was assayed using biotin-conjugated gelatin substrates as described by Dubrovskiy et al. (2013) [\[13\]](#). Bovine gelatin (Sigma, G1393) was diluted to 1% in D-PBS, prewarmed to 37°C, and dissolved with stirring in a 70°C water bath. EZ-Link NHS-LC-Biotin (Thermo Fisher, Cat. No. 21336) dissolved in DMSO (5 mg/mL) was added to the gelatin at a final concentration of 0.5 mg/mL and reacted for 1 hour at room temperature with constant stirring. The solution was clarified by centrifugation (10,000 x g, 5 minutes), aliquoted, and stored at -20°C. For coating, biotinylated gelatin was thawed, diluted in 0.1 M bicarbonate buffer (pH 8.3) to 0.25 mg/mL, sterilized by 0.22 μm filtration, and applied to culture substrates (e.g., 50 μl per well of a 96-well plate, 2 ml per 35 mm dish). Adsorption was performed overnight at 4°C, followed by PBS washing. Unconjugated gelatin was used as a control. Incorporation of biotin into gelatin was validated using the Pierce Biotin Quantitation Kit (Thermo Fisher, Cat. No. 28005) according to the manufacturer's instructions. bEnd.3 monolayers were grown to confluence on coated coverslips, and control experiments included (i) gelatin without cells, (ii) biotinylated gelatin without cells, (iii) low-density cells on gelatin, and (iv) low- or high-density cells on biotinylated gelatin. To quantify the integrity of the bEnd.3 monolayer, streptavidin-FITC tracer (Thermo Fisher, #11-4317-87) at 2 μg/mL in DMEM was added to the cell culture medium for 5 min, followed by extensive PBS washing and fixation in 1% paraformaldehyde. Samples were immunostained for ZO-1, nuclei were visualized with DAPI, and the coverslips were mounted in antifade solution and imaged by confocal microscopy. Quantification of streptavidin signal was measured as area fraction per field of view. Each condition was repeated in three independent biological replicates, with multiple image fields analyzed per replicate. To ensure comparability across conditions, all images were collected using identical confocal acquisition settings, and exposure times and intensity scaling were held constant within each experiment.

bEnd.3 and *E. coli* washout experiments

bEnd.3 cells were exposed to *E. coli* K1 (at a 5:1 ratio of *E.coli*:bEnd.3 cells) in serum-containing DMEM for 1 hour at 37°C, 5% CO₂. For washout, the medium was removed, and cells were washed extensively with pre-warmed PBS, then returned to fresh complete medium for a 3-hour recovery period. Parallel groups were fixed at 1 hour (no washout) or after the 3-hour washout. Cells were fixed in 1% paraformaldehyde, permeabilized, blocked, and immunostained (e.g., for Cldn5 and ZO-1) as described above, then mounted for confocal imaging. Acquisition settings (laser power, gain, offset, and channel order) were held constant across conditions within each experiment. Quantification of Claudin-5 cytoplasm/plasma membrane ratios followed the CellProfiler pipeline

(Stirling et al., 2021 [↗](#)), and results were analyzed as $\log_{10}$ -transformed single-cell values with statistical significance assessed using the Wilcoxon rank-sum test. Three independent biological replicates were performed, with multiple fields analyzed per replicate. For inhibition of NF- κ B signaling, ACHP (2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3 pyridinecarbonitrile; Tocris #4547) was added to the cell culture medium at a final concentration of 1 μ M.

bEnd.3 tracer experiments

This procedure was performed as described (Stamatovic et al., 2006 [↗](#), 2017 [↗](#)). Briefly, bEnd.3 monolayers on glass coverslips were preloaded with plasma-membrane-binding/uptake tracers prior to bacterial exposure: Cholera Toxin Subunit B, Alexa Fluor™ 647 conjugate (CTB-AF647; Thermo Fisher, C34778), Transferrin from human serum, Alexa Fluor™ 647 conjugate (Tf-AF647; Thermo Fisher, T23366), or Fixable Dextran, 10,000 MW, Alexa Fluor™ 647 (Dextran-AF647; Thermo Fisher, D22914). Tracers were added in ice-cold complete medium for 30 minutes at 4°C to allow accumulation at the plasma membrane. Excess tracer was removed by gentle washes with PBS, and cells were returned to tracer-free complete medium at 37°C. Cultures were then exposed to *E. coli* K1 (at a 5:1 ratio of *E. coli*:bEnd.3 cells) for the indicated intervals (e.g., 4 hours) or left unexposed as controls. Cells were fixed in 1% paraformaldehyde, permeabilized, blocked, and immunostained (e.g., for Cldn5 and ZO-1) as described above. Colocalization/overlap with Cldn5 was quantified in Fiji using fixed 100 x 100 μ m ROIs and AND-masking of binarized channels (see “Percent overlap quantification”), with all acquisition parameters (laser power, gain, offset, channel order) held constant within each experiment. Three independent biological replicates were performed, with multiple fields analyzed per replicate.

E. coli and GBS preparation and exposure of bEnd.3 cells

Escherichia coli K1 (RS218) and its RFP-expressing derivative (ampicillin-resistant) were cultured in LB broth and on LB agar plates with or without ampicillin, depending on the strain. *Streptococcus agalactiae* (GBS, ATCC 13813) was cultured in brain heart infusion (BHI) broth and on BHI agar plates. For tissue culture experiments, a single colony was grown overnight in the appropriate broth, and a 200 μ l aliquot of the overnight culture was diluted into fresh medium and grown for 1 hour at 37°C. Bacterial density was estimated by optical density at 600 nm, and bacteria were added to cultured bEnd.3 cells (at a 5:1 ratio of bacteria to bEnd.3 cells for all experiments). Heat-killed bacteria were prepared in parallel by heating suspensions to 95°C for 30 min, and bacterial killing was confirmed by plating aliquots to check for the absence of growth. For bacterial exposure experiments, bEnd.3 cells were cultured in the absence of antibiotics in serum-containing medium, and bacteria remained in the medium for the duration of the experiment, except in designated washout assays.

Image Analysis: Iba1 quantification

Immunostained Iba1 image quantification was performed as described above for quantifying RFP-expressing *E. coli* in brain sections, except that for Iba1 the superficial cortical territory beneath the leptomeninges was manually delineated in coronal sections based on the pattern of Cldn5 immunostaining. Iba1-positive signal was quantified using fixed threshold-based segmentation and normalized to the defined cortical area.

Image Analysis: nuclear-to-cytoplasmic (Nuc/Cyto) intensity ratio

NF- κ B and Ki-67 nuclear translocation was quantified using a custom CellProfiler pipeline (Stirling et al., 2021 [↗](#)). Nuclei were segmented by DAPI, cytoplasmic regions were defined as the cell body excluding the nucleus, and mean intensities were measured in both compartments. Ratios of nuclear-to-cytoplasmic (Nuc/Cyto) intensity were computed per cell and $\log_{10}$ -transformed for analysis. Output spreadsheets from CellProfiler were imported into R (v4.3) and annotated by parsing file names to assign genotype, clone, treatment, and timepoint metadata. Data from replicate experiments were combined, and box plots were generated to show distributions of

single-cell values. Statistical comparisons between conditions were performed using the Wilcoxon rank-sum test. For validation, replicate-level plots were generated, outliers were defined by interquartile range ($1.5 \times \text{IQR}$) and excluded from simplified representative plots, and a subset of clones and timepoints (e.g., 0, 1, and 3 hours) were displayed for final figures.

Image Analysis: cytoplasmic-to-plasma membrane (Cyto/PM) intensity ratio

Claudin-5 redistribution between the plasma membrane and cytoplasm was quantified in CellProfiler (Stirling et al., 2021 [\[4\]](#)) using a custom pipeline. Plasma membrane regions were segmented using ZO-1 as a boundary, while cytoplasmic regions were defined as the cell body excluding the nucleus and plasma membrane masks. Integrated mean intensities of Claudin-5 were measured in both regions, and cytoplasmic-to-plasma membrane (Cyto/PM) ratios were calculated for each cell and $\log_{10}$ -transformed for analysis. Output spreadsheets from CellProfiler were imported into R (v4.3) for downstream analysis. File names were parsed to assign genotype, treatment, timepoint, and replicate metadata, and data from replicate experiments were combined. Box plots were generated to display distributions of single-cell values, with statistical comparisons performed using the Wilcoxon rank-sum test. For representative figures, a single replicate per group was shown, and outliers were removed based on the interquartile range ($1.5 \times \text{IQR}$).

Image Analysis: percent overlap quantification (ImageJ)

Colocalization between Cldn5 and trafficking/endothelial markers (e.g., EEA1, Rab7, Rab11, LAMP2, ZO-1) or tracers was quantified in Fiji (Schindelin et al., 2012 [\[5\]](#)) using a scripted workflow. For each field, maximum-intensity-projected channels were processed as follows: images were Gaussian-blurred ($\sigma = 2$), converted to 8-bit, and binarized using fixed thresholds chosen per channel and experiment. Binary masks were generated with “BlackBackground” enabled and saved for Cldn5 and the comparison marker. Overlap masks were created with Image Calculator (logical AND) between the Cldn5 mask and the marker mask (and, when needed, between Cldn5 and ZO-1). An ROI set comprising nine $100 \times 100 \mu\text{m}$ squares was loaded via the Region of Interest (ROI) Manager and applied uniformly to all images. For each ROI, we recorded raw mean intensities (non-binary images) and, on the binary masks, measured area and mean intensity. “Signal area” was computed as $(\text{ROI area} \times \text{mask mean})/255$ for Cldn5 and for the overlap image, providing an area-weighted signal metric. Percent overlap with Cldn5 was then calculated per ROI as $100 \times (\text{overlap signal area} \div \text{Cldn5 signal area})$. Identical steps were repeated for each endosomal marker or tracer by substituting the corresponding channel and threshold. Outputs were written to CSV and pooled across images/replicates for plotting; statistical comparisons were performed using Wilcoxon rank-sum tests as described in the Statistics section. All acquisition and analysis parameters (thresholds, ROIs, and export settings) were held constant within a given experiment.

Image Analysis: statistics

All image quantifications were performed on raw data exported directly from Fiji or CellProfiler without manual exclusion of individual cells or ROIs, unless otherwise noted for representative plots. For Cyto/PM and Nuc/Cyto ratios, values were $\log_{10}$ -transformed prior to statistical testing. Percent overlap measurements were expressed as the fraction of Cldn5-positive signal area colocalizing with the comparison marker within fixed $100 \times 100 \mu\text{m}$ ROIs. For extravascular tracer (Sulfo-NHS-biotin) and ICAM-1 analyses, fluorescence intensity or area fractions were normalized to the ROI area or expressed as fold change relative to WT uninfected controls. For all datasets, each dot represents a single measurement (one cell *in vitro* or one image field *in vivo*), and biological replicates correspond to independent mice or independent cell culture experiments. Statistical comparisons between two groups were performed using the two-sided Wilcoxon rank-sum test (Mann–Whitney U). Where multiple groups were compared, pairwise Wilcoxon rank-sum

tests were performed, and p-values are reported in figure panels. Box plots display the median and interquartile range, with all individual data points overlaid. All statistical analyses were conducted in R (v4.3) using ggplot2 and ggpubr packages, with significance set at $p < 0.05$.

snRNA-seq libraries and sequencing

For single-nucleus RNA sequencing, leptomeninges were collected as previously described (Wang et al., 2023 [\[1\]](#)). In brief, P6 mice were euthanized 24 hours after infection, brains were rapidly removed, and the leptomeninges were dissected into ice-cold Hibernate-A medium. The leptomeninges were frozen at -80°C .

For the PIP-seq protocol, single nucleus (sn)RNA-seq libraries were prepared using the PIP-seq T20 3' Single Cell RNA Kit v4.0 PLUS (Fluent BioSciences; Clark et al., 2023 [\[2\]](#)). Frozen tissue was suspended in homogenization buffer (0.25 M sucrose, 25 mM KCl, 5 mM MgCl_2 , 20 mM Tricine-KOH, pH 7.8) supplemented with 1 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, EDTA free protease inhibitor (Roche 11836 170 001), 0.5% IGEPAL-630, and 40 U/mL Protector RNase Inhibitor (Sigma, 03335402001). Samples were homogenized in a 2 mL Dounce homogenizer, using 15 strokes with a loose-fitting pestle followed by 30 strokes with a tight-fitting pestle. The sample was filtered through a $10\ \mu\text{m}$ filter (CellTrix, Sysmex, 04-004-2324), and nuclei were pelleted in Low Retention 1.5 mL microcentrifuge tubes (Thermo Scientific, 3451) for 5 min at $500 \times g$ at 4°C . Nuclei were washed twice with Nuclei Suspension Buffer (supplied in the Fluent BioSciences kit) supplemented with 1% BSA and 40 U/mL Protector RNase Inhibitor. Nuclei were counted, and $\sim 40,000$ nuclei (for the T20 kit) or $\sim 20,000$ nuclei (for the T10 kit) were used for library production following the Fluent BioSciences protocol. The resulting snRNA-seq libraries were sequenced on an Illumina NovaSeq X Plus sequencer (Supplementary file 1).

For the 10X genomics protocol, snRNA-seq libraries were constructed from suspended nuclei (prepared as described above) using the 10x Genomics Chromium single-cell 3' v3 kit following the manufacturer's protocol. Libraries were sequenced on an Illumina NovaSeq 6000.

Analysis of snRNA-seq data

Reads were aligned with the PIPseeker program (Fluent BioSciences, version 3.3.0) using a STAR index based on the GRCh38 mouse genome with addition of Cre sequences (STAR version 2.7.9a). The CellBender program was used to detect empty droplets and remove background (version 0.3.2; Fleming et al., 2023 [\[3\]](#)). The SOLO program was used to remove doublets (scVI-tools 1.2.2-post2) (Bernstein et al., 2020 [\[4\]](#)). Manual curation was used to further remove potentially mixed (doublet) nuclei. Clusters representing various brain cells were removed from the final data set. The data were normalized using a regularized negative binomial regression algorithm implemented with the SCTransform function (Hafemeister and Satija, 2019 [\[5\]](#)). UMAP dimensional reduction was performed using the R uwot package (<https://github.com/jlmelville/uwot> [\[6\]](#)) integrated into the Seurat R package (Melville, 2022 [\[7\]](#)). Data for the various scatter plots were extracted using the Seurat AverageExpression function, and differential gene expression was analyzed using the Seurat FindMarkers function. The Wilcoxon rank sum test was used to calculate p-values. The p-values were adjusted with a Bonferroni correction using all genes in the dataset. Data exploration, analysis, and plotting were performed using RStudio (RStudio Team, 2020 [\[8\]](#)), the tidyverse collection of R packages (Wickham, 2017 [\[9\]](#)), and ggplot2 (Wickham, 2009 [\[10\]](#)).

Dotplots were generated with the default settings, including default normalization. For Figure 1E-H [\[11\]](#) and Figure 1 – figure supplement 5 [\[12\]](#), differential expression between infected and control samples was calculated using Seurat FindMarkers function using only the WT data. The results were filtered for statistically significant difference per cell type. Differentially expressed genes from the indicated Hallmark gene sets are presented in the dot plots for individual cell clusters.

GSEA analysis

For Gene Set Enrichment Analysis (GSEA; Subramanian et al., 2005 [\[13\]](#)), genes were ranked by the fold expression change between control and mutant datasets. The ranked gene list was used to detect enriched gene sets within the Broad Institute Hallmark Gene Sets using the fgsea R package

(<https://github.com/ctlab/fgsea>; Korotkevich et al., 2019).

RNA-seq on WT and *Tlr4*^{KO} bEnd.3 cells: libraries and sequencing

bEnd.3 mouse brain endothelial cells were cultured in 10-cm tissue culture dishes and grown to confluency under standard conditions (37°C, 5% CO₂) and either exposed or not exposed to *E. coli* for 3 hours. Total RNA was isolated using the Zymo Research RNA purification kit according to the manufacturer's instructions. Briefly, culture medium was aspirated, cells were rinsed once with ice-cold PBS, and 600 µL of the provided lysis buffer with β-mercapto-ethanol was added directly to the dish. The lysate was collected by scraping and transferred to a microcentrifuge tube, briefly mixed, and applied to the Zymo spin column. After binding, the column was washed with the supplied wash buffers, including an on-column DNase I treatment step to remove contaminating genomic DNA, and RNA was eluted in 50 µL of DNase/RNase-free water. RNA concentration and purity were assessed by spectrophotometry (A₂₆₀/A₂₈₀), and samples were stored at -80°C. Library construction was performed with the Illumina mRNA stranded kit following the manufacturer's protocol, and sequencing was performed on an Illumina NovaSeqX with a read length of 300 nucleotides.

RNA-seq on WT and *Tlr4*^{KO} bEnd.3 cells: RNA-seq processing and normalization

Gene-level count matrices from bEnd.3 cells were analyzed in R (v4.5) with Bioconductor (v3.21). The study included two genotypes (WT and *Tlr4*^{KO}) under two conditions (control and exposed to *E. coli* for 3 hours), with biological replication per group (WT: 3 control samples and 4 exposed to *E. coli* samples; *Tlr4*^{KO}: 3 control samples and 4 exposed to *E. coli* samples). Counts were normalized using DESeq2's median-of-ratios size-factor method to control for library size/composition effects. A variance-stabilizing transform (VST) was calculated for quality control and visualization only (e.g., PCA, sample-distance heatmaps). All samples were retained for analysis after QC review.

RNA-seq on WT and *Tlr4*^{KO} bEnd.3 cells: Differential expression

To quantify infection responses within each genotype, DESeq2's negative-binomial generalized linear model was fit separately to WT and in *Tlr4*^{KO} data and then control vs. exposed to *E. coli* data were compared. The reported log₂ fold changes are the control vs. exposed to *E. coli* estimates from these genotype-specific analyses. P-values were adjusted for multiple testing with the Benjamini–Hochberg procedure. False-discovery rates (FDR or q-values) are cited throughout. Gene-level WT vs. *Tlr4*^{KO} effect comparisons were visualized by plotting the two genotype-specific fold changes against one another.

RNA-seq on WT and *Tlr4*^{KO} bEnd.3 cells: Pathway activity scoring and statistics

Pathway analyses used the MSigDB Hallmark collection for mouse, retrieved via msigdb. Single-sample gene-set enrichment (ssGSEA) was performed with GSVA to obtain an activity score for each pathway in each sample. Scores were computed from log₂-scaled normalized counts (log₂ (normalized counts + 1)), using only genes present in both the RNA-seq data and the gene set. For statistical inference, we modeled pathway scores with a linear model that included genotype, infection status, and their interaction. From this model we reported three quantities per pathway: the *E. coli* exposure effect in WT, the *E. coli* exposure effect in *Tlr4*^{KO}, and the genotype x exposure interaction (difference-in-response between genotypes). P-values were adjusted across pathways with the Benjamini–Hochberg procedure. Adjusted values are reported as q-values (FDR).

RNA-seq on WT and *Tlr4*^{KO} bEnd.3 cells: Visualization

Heatmaps were drawn from VST values after centering each gene by its mean (raw z-scores). Within each Hallmark pathway, genes were ranked by the magnitude of the *E. coli* exposure effect (difference of condition means within each genotype), and the top genes were displayed to show the pattern and directionality across groups. Boxplots summarize ssGSEA scores by group. Per-facet annotations show FDRs for WT and *Tlr4*^{KO} infection effects and the interaction term. Figure 7C [plots](#) the pathway-level infection responses in WT (x-axis) versus *Tlr4*^{KO} (y-axis), with point size reflecting within-genotype significance and point color indicating interaction FDR. All figures were exported as vector PDFs.

RNA-seq on WT and *Tlr4*^{KO} bEnd.3 cells: Software

Analyses used DESeq2, limma (for pathway-score modeling), SummarizedExperiment, GSVA, msigdb, pheatmap, ggplot2, and ggrepel within the R/Bioconductor ecosystem.

Figure supplements

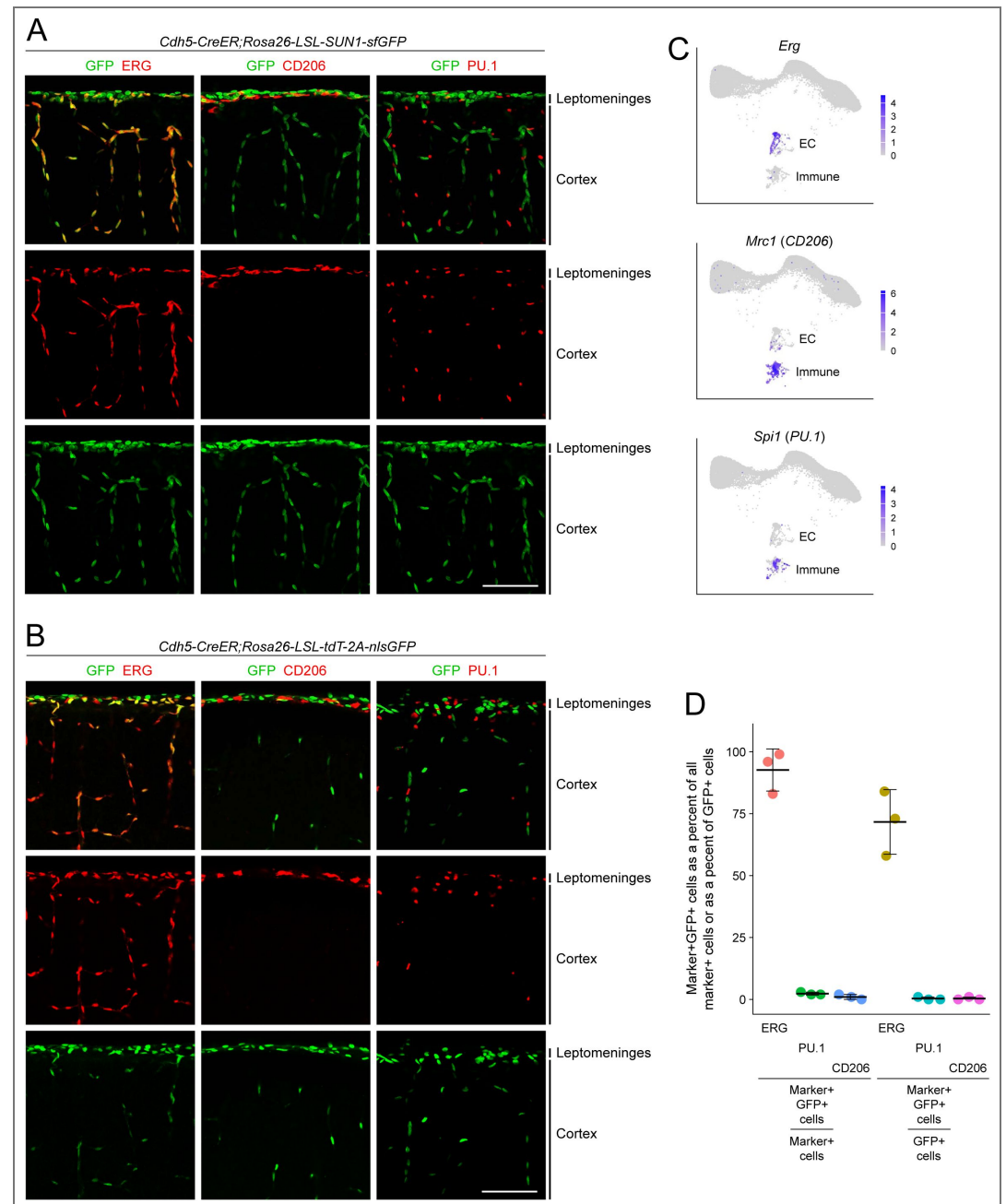


Figure 1 – figure supplement 1. Specificity of *Cdh5-CreER* in leptomeninges. (A and B) *Cdh5Cre*-mediated recombination of the *loxP-stop-loxP* (*LSL*) reporters *Rosa26-LSL-SUN1-sfGFP* (A) and *Rosa26-LSL-tdT-2A-nlsGFP* (B). Immunostaining for markers ERG, CD206, and PU.1 was performed on coronal sections of P6 brains following 4HT administration at P2. Nuclear-localized GFP is observed in nearly all ECs, as visualized with ERG immunostaining, but not in myeloid cells, as visualized with PU.1 and CD206 immunostaining. The region corresponding to the leptomeninges is labeled on the right. Scale bars, 100 μ m. (C) WT uninfected leptomeninges UMAPs showing the expression of the genes encoding the markers used in (A) and (B): *Erg* (ECs), *Mrc1* (CD206; myeloid cells); *Spi1* (PU.1; myeloid cells). (D) Quantification of the co-localization of nuclear GFP from *Rosa26-LSL-tdT-2A-nlsGFP* with cell-type-specific markers in the leptomeninges. The left set of three data points show the percent of marker+ cells that express GFP. The right set of three data points show the percent of GFP+ cells that express each of the three

cell-type specific markers. Approximately 75% of GFP+ cells are ECs and approximately 0% are myeloid cells. Therefore, the remaining 25% of GFP+ cells correspond to some combination of other leptomeningeal cell types. Each data point represents one mouse. A mean of 158 cells was scored per datapoint.

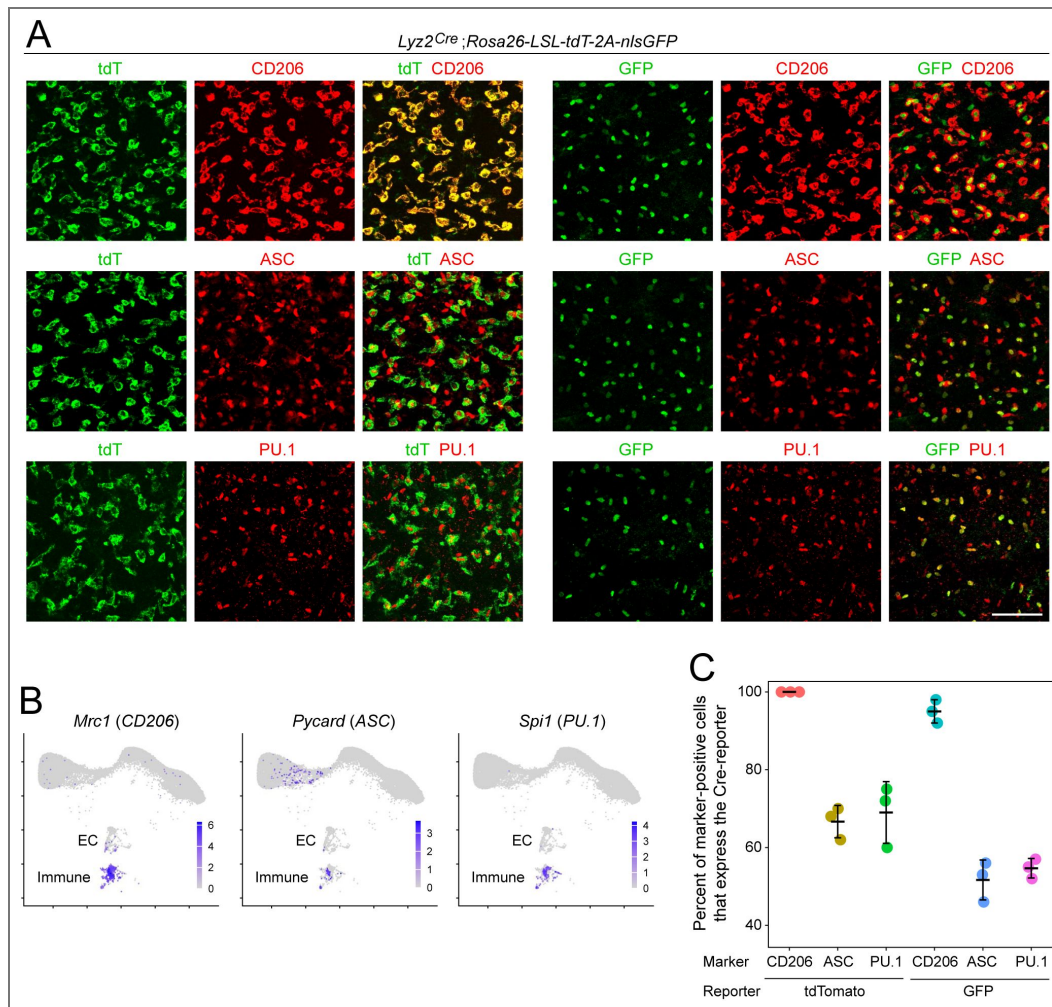


Figure 1 – figure supplement 2. Specificity of *Lyz2^{Cre}* in leptomeninges.

(A) *Lyz2^{Cre}*-mediated recombination of the *loxP-stop-loxP* (LSL) reporter *Rosa26-LSL-tdT-2A-nlsGFP*, with the tdTomato reporter (false-colored green) shown in the left set of nine panels and the nuclear-localized GFP reporter (green) shown in the right set of nine panels. Immunostaining for markers CD206, PU.1, and ASC was performed on P6 leptomeninges+cortex whole mounts, as described for Figure 1B. The leptomeninges region is shown. Scale bar, 200 μ m. (B) WT uninfected leptomeninges UMAPs showing the expression of the genes encoding the markers used in (A): *Mrc1* (CD206; myeloid cells), *Pycard* (ASC; myeloid plus arachnoid barrier cells), and *Spi1* (PU.1; myeloid cells). (C) Quantification of co-localization data, as shown in (A). Each data point represents one mouse. A mean of 136 cells was scored per datapoint. The Cre reporters, tdTomato and GFP, are expressed in 90-100% of CD206+ myeloid cells, in 40-70% of ASC+ cells, and in 50-80% of PU.1+ cells.

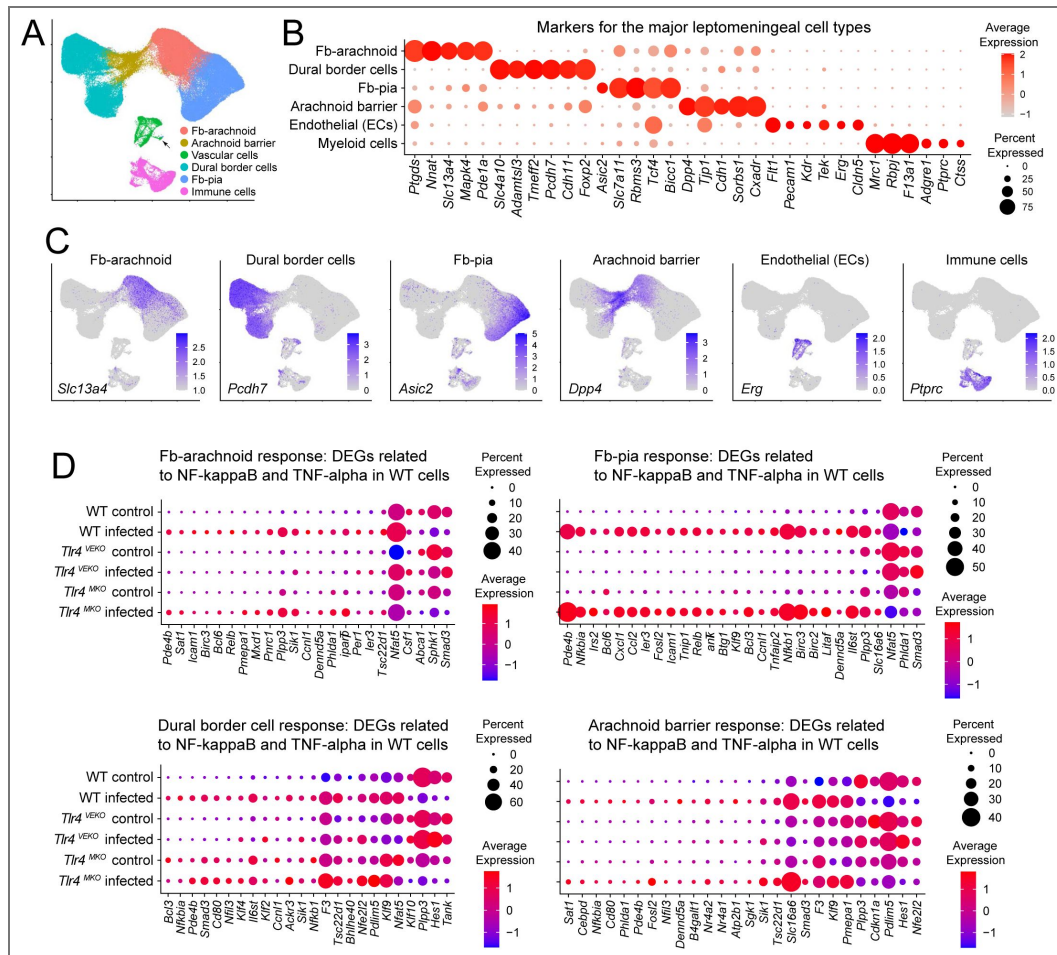


Figure 1 – figure supplement 3. snRNA-seq cell cluster assignments and analysis of differentially expressed genes related to NK-κB and TNF-α responses.

(A) UMAP plot of leptomeninges snRNA-seq data from WT, *Tlr4^{VEKO}*, and *Tlr4^{MKO}* mice at P6, either uninfected control or with *E. coli* infection, with cell clusters identified. N=126,235 nuclei. Fb, fibroblast. (B) snRNA-seq transcript abundances among the six major leptomeninges cell clusters for a set of 33 genes, for which the transcript abundances distinguish these clusters. We note that the dural border cell cluster was originally referred to as “fibroblasts dura3” in Wang et al. (2023). (C) UMAP plots of leptomeninges snRNA-seq showing the abundances of transcripts that are highly enriched in each of the six major leptomeningeal cell clusters. (D) snRNA-seq from four leptomeningeal cell clusters showing differentially expressed genes related to NK-κB and TNF-α responses in control vs. *E. coli*-infected mice of the indicated genotypes at P6. The corresponding dot plots for ECs and myeloid cells are shown in Figure 1H.



Figure 1 – figure supplement 4. Comparison of WT P6 leptomeninges snRNA-seq using the PIP-seq and 10X Genomics platforms.

(A) WT P6 leptomeninges snRNA-seq determined with the PIP-seq platform. Expression of 24 genes is illustrated to highlight: *Cdh5* (upper left), EC and immune cell genes (left column), and, the four principal cell clusters (right two columns; from left to right within each UMAP these are Dural border cells (DB), Arachnoid barrier cells (AB), Fibroblast arachnoid (Fb-ar.), and Fibroblast-pia (Fb-pia)). (B) As for (A), except that the WT P6 leptomeninges snRNA-seq was determined with the 10X Genomics platform. Note that the yields of immune cells and ECs differ between the two datasets.

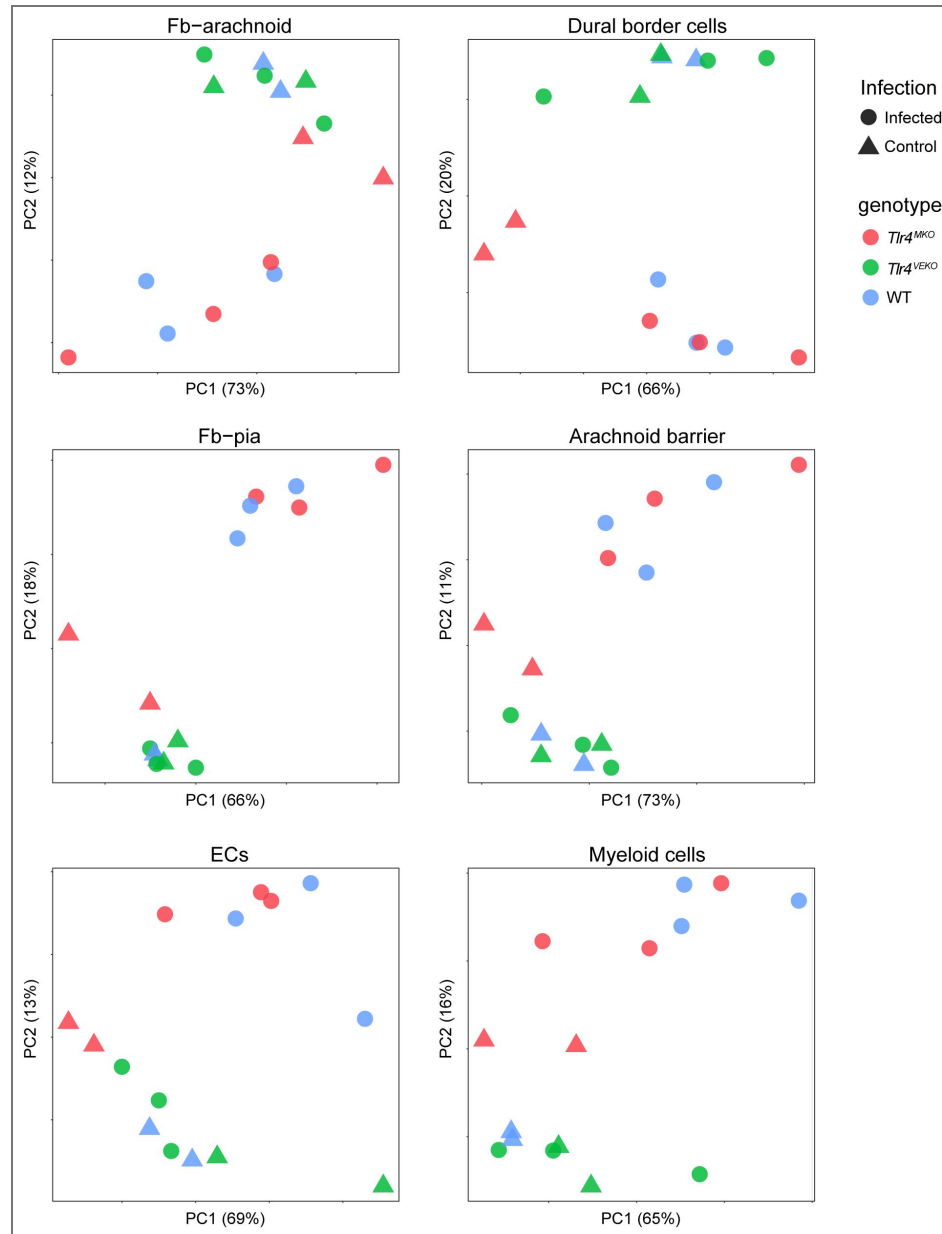


Figure 1 - figure supplement 5. Principal component analysis of snRNA-seq-derived transcriptomes for each of the six major leptomeningeal cell clusters divided by genotype and infection status.

The two experimental conditions (control vs. infected) and the three genotypes (WT, *Tlr4*^{VEKO}, and *Tlr4*^{MKO}) are indicated by the colors and symbols in the upper right. The datapoints represent the same leptomeningeal snRNA-seq datasets analyzed in Figure 1C-H, from control vs. *E. coli*-infected mice at P6.

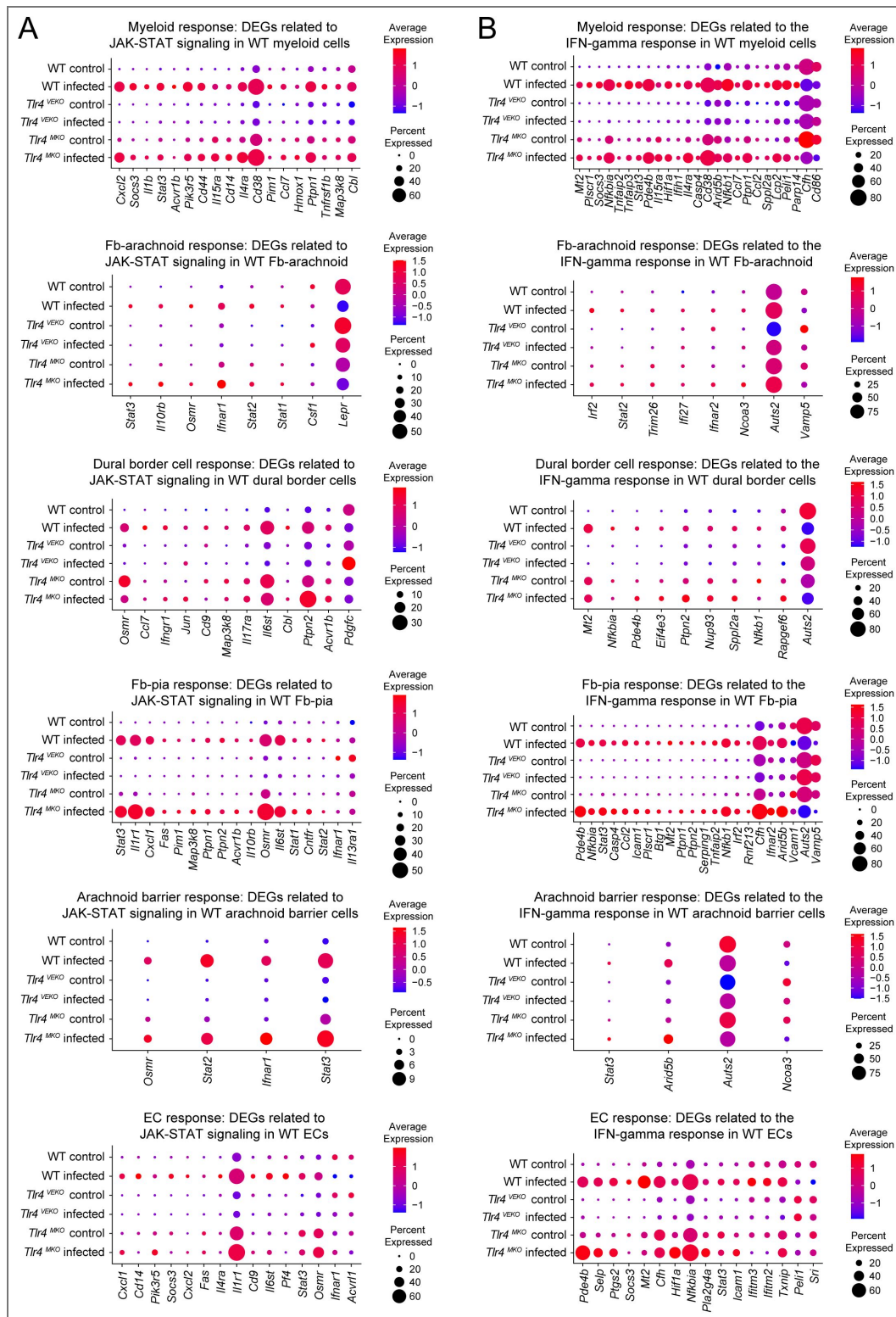


Figure 1 – figure supplement 6. Transcriptome changes by cell cluster for the JAK-STAT and IFN-γ pathways.

(A and B) snRNA-seq from each of the major leptomeninges cell clusters showing differentially expressed genes that are related to the JAK-STAT pathway (A) or the IFN-γ pathway (B). Dot plots are based on the same leptomeningeal snRNA-seq datasets analyzed in Figure 1C-H, from control vs. *E. coli*-infected mice at P6.

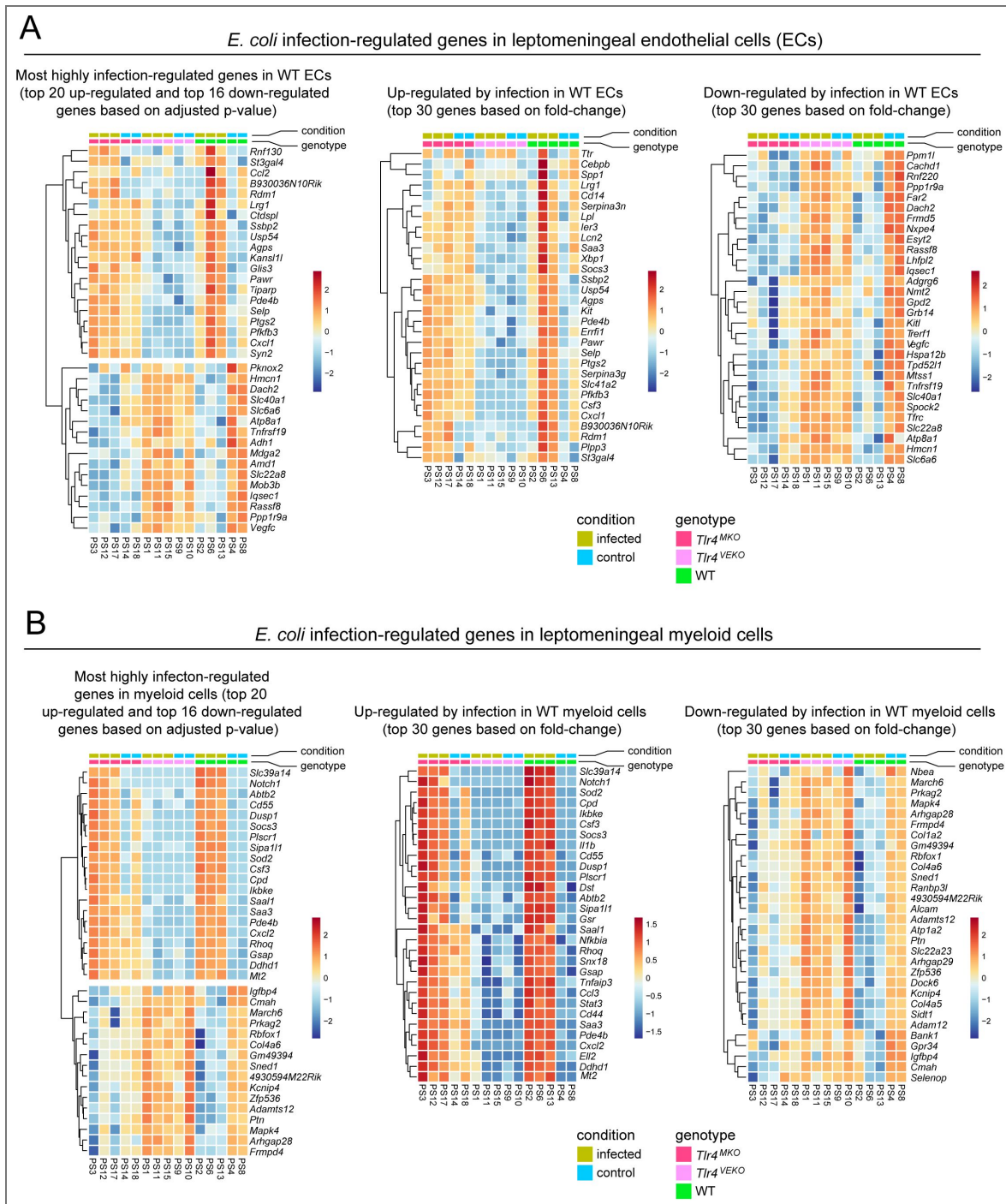


Figure 1 – figure supplement 7. Genes regulated by *E. coli* infection in leptomeningeal endothelial cells and myeloid cells analyzed at the level of individual mice and individual genes.

(A) Genes regulated by *E. coli* infection in leptomeningeal ECs. Left panel, most differentially regulated genes based on adjusted p-value. Right two panels, most differentially regulated genes based on fold change. (B) Genes regulated by *E. coli* infection in leptomeningeal myeloid cells. Left panel, most differentially regulated genes based on adjusted p-value. Right two panels, most differentially regulated genes based on fold change. The data are derived from the same leptomeningeal snRNA-seq datasets analyzed in Figure 1C-H, from control vs. *E. coli*-infected mice at P6.

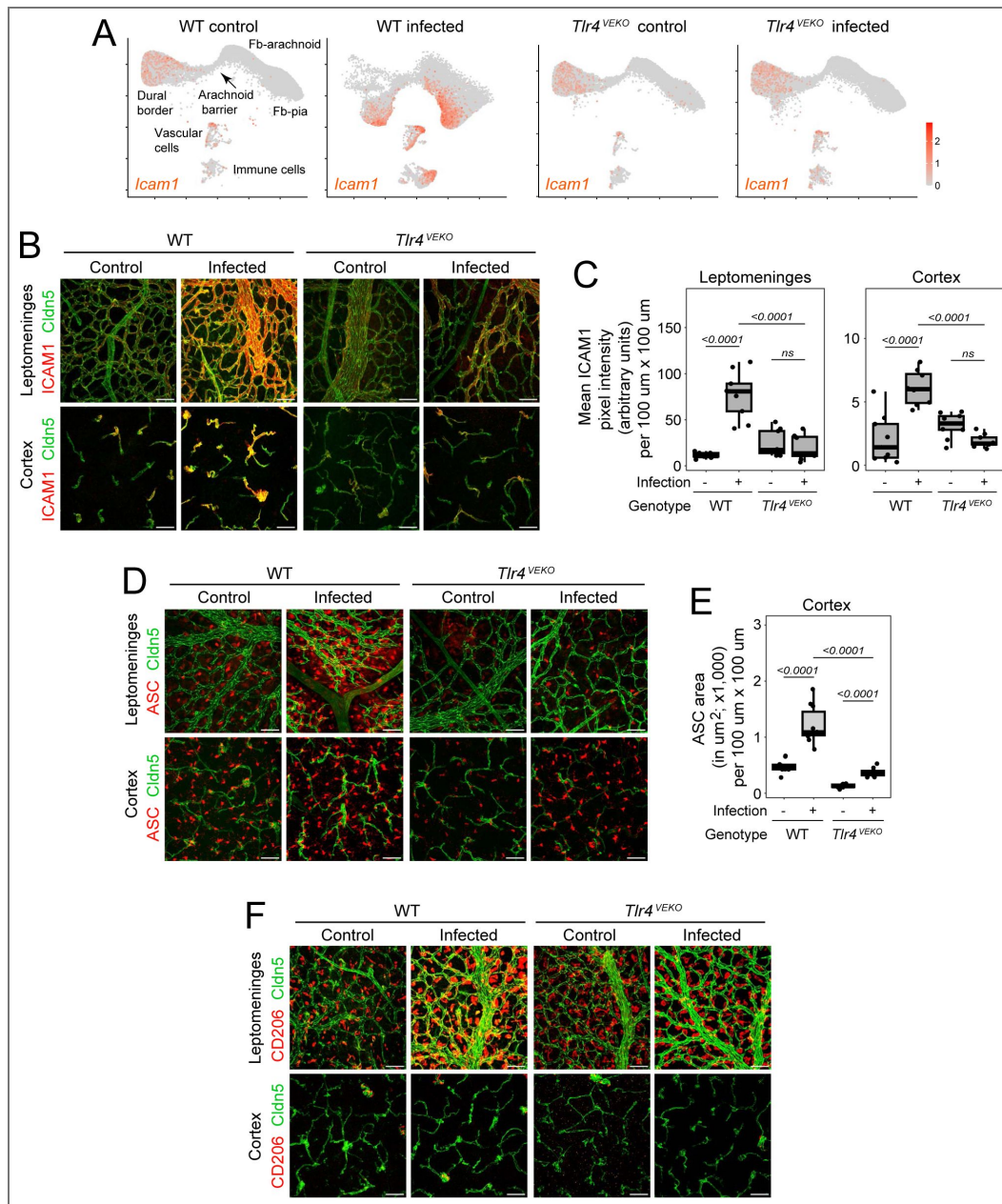


Figure 2 - figure supplement 1. Infection responses in the leptomeninges and adjacent cerebral cortex in WT and *Tlr4*^{VEKO} mice.

(A) UMAP plots of leptomeninges snRNA-seq data from WT and *Tlr4*^{VEKO} mice at P6, either uninfected control or with *E. coli* infection, showing *Icam1* transcripts. (B) Whole mount cortical surface, with attached leptomeninges, from P6 WT and *Tlr4*^{VEKO} mice, control or *E. coli*-infected, immunostained for Cldn5 and ICAM1. Upper panels, stacked Z-planes at the level of the leptomeninges; lower panels, stacked Z-planes at the level of the adjacent cerebral cortex. Scale bars, 20 μ m. (C) Quantification of ICAM1 immunostaining in the leptomeninges and adjacent cerebral cortex, as shown in (B). Note the different scales for the vertical axis in the two plots. (D) As in (B), except immunostained for Cldn5 and ASC. These are the same four leptomeninges+cortex flat mounts as shown in Figure 2D: the upper row of four images shown here reproduces the upper row of four images shown in Figure 2D, and the lower row of four images shown here correspond to the regions in the top row but were captured at a greater depth to visualize the underlying cortex. (E) Quantification of ASC immunostaining in the cerebral cortex, as shown in the lower row of images in (D). (F) As in (B), except immunostained for Cldn5 and CD206. These are the same four leptomeninges+cortex flat mounts as shown in Figure 2D: the upper row of four images shown here reproduces the lower row of four images shown in Figure 2D, and the lower row of four images shown here correspond to the regions in the top row but were captured at greater depth to visualize the underlying cortex. These data are not quantified because CD206 cells were extremely rare in the cortex in all samples.

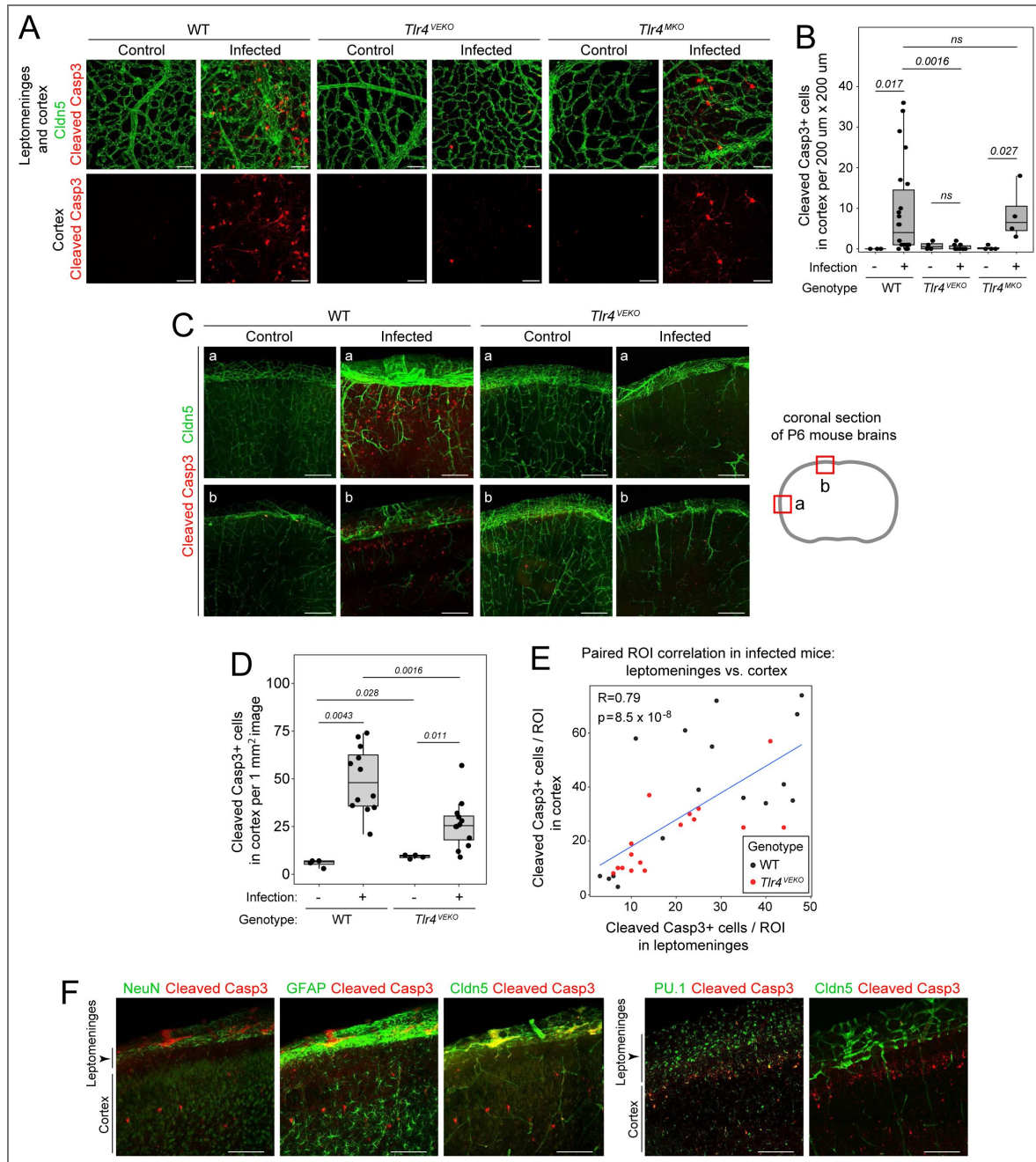


Figure 2 – figure supplement 2. Cleaved Caspase-3+ cells accumulate in the cortex in *E. coli*-infected WT and *Tlr4^{MKO}* mice but not in *Tlr4^{VEKO}* P6 mice.

(A) Flat mount images showing the meninges and adjacent cortex for the indicated genotypes and conditions. Scale bar, 50 μm. (B) Quantification of cleaved Caspase-3+ cells from flat mount images like the ones shown in (A). The region of cortex analyzed is adjacent to the leptomeninges. (C) Coronal sections showing meninges (upper) and cortex (lower). For quantification, two regions were imaged per hemisphere, as shown in the schematic on the right and labeled 'a' and 'b'. Examples of 'a' and 'b' images are shown at left. Scale bar, 200 μm. (D) The number of cleaved Caspase-3+ cells in leptomeninges and cortex were quantified from 1 mm x 1 mm images like those shown in (C). The different planes of sectioning and the different cortical depths in the images quantified in (B) and (D) account for the different numbers of cleaved caspase3+ cells. (E) Correlation between cleaved Caspase-3+ cells in the leptomeninges and cortex for individual coronal images as shown in (C). (F) Cleaved Caspase-3+ cells in cortex are NeuN-, GFAP-, Cldn5-, and PU.1+, implying a myeloid identity. Scale bar, 100 μm.

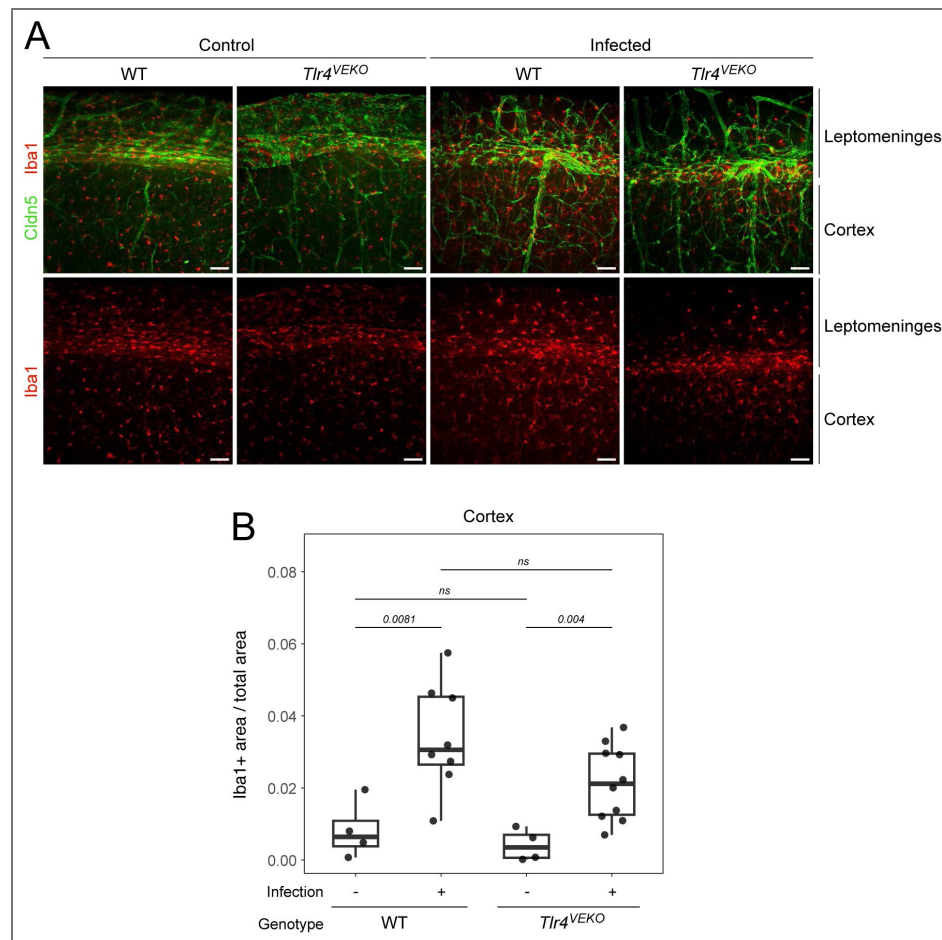


Figure 2 – figure supplement 3. An increase in cortical Iba1+ immunostaining with *E. coli* infection.

(A) Coronal sections of P6 cortex and leptomeninges showing Iba1 immunostaining with or without *E. coli* infection in P6 WT and *Tlr4*^{VEKO} mice. Scale bars, 50 μ m. (B) Quantification of the relative area occupied by Iba1+ pixels in the cortex of WT and *Tlr4*^{VEKO} mice with or without *E. coli* infection at P6, as shown in (A).

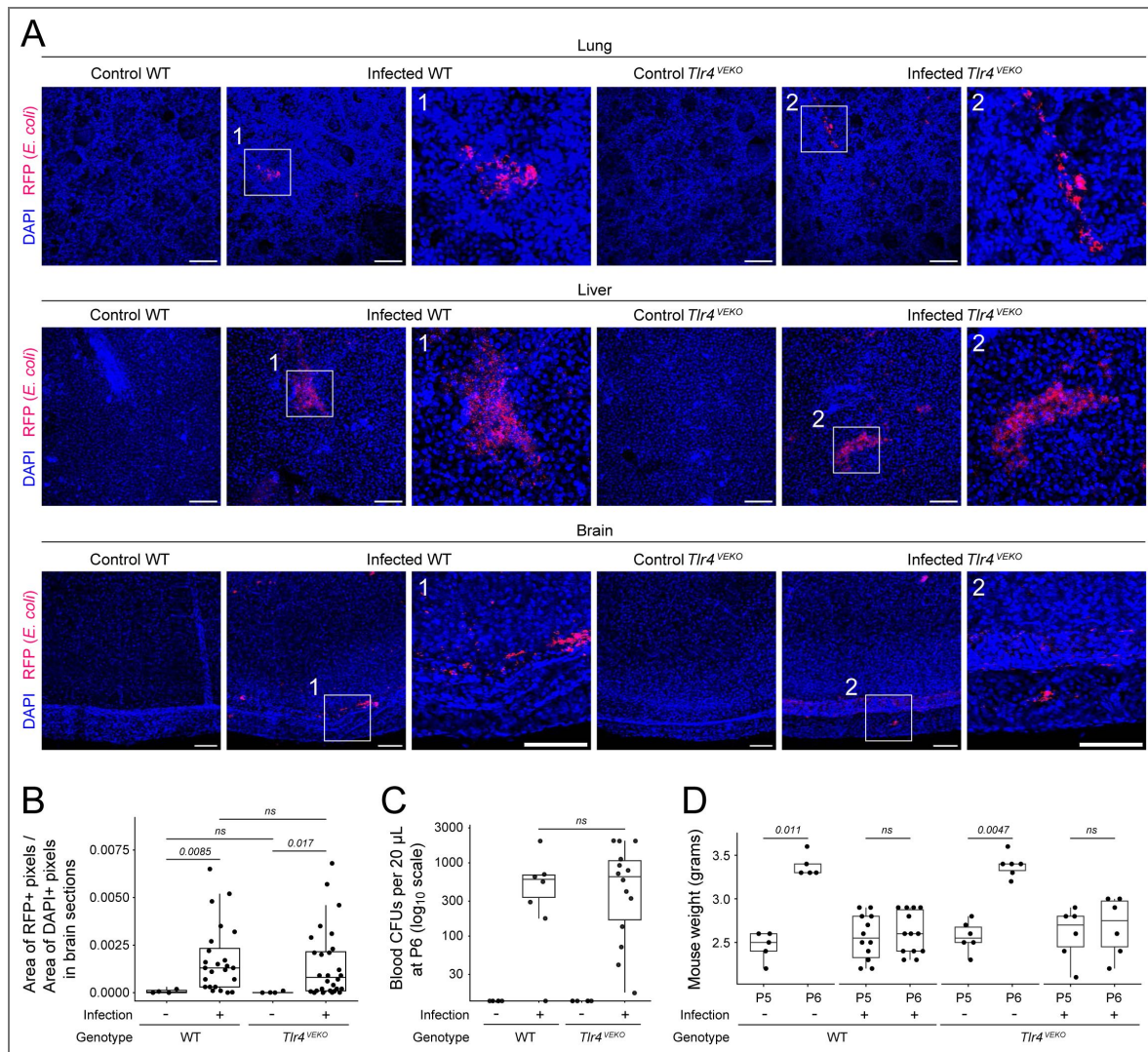


Figure 2 - figure supplement 4. Assessment of clinical progression at 24 hours in WT and *Tlr4*^{VEKO} mice following *E. coli* infection.

(A) Representative maximum intensity projection images of RFP-expressing *E. coli* (red) and DAPI (blue) in lung, liver, and brain from control (uninfected) and *E. coli*-infected WT and *Tlr4*^{VEKO} mice at 24 hours post-infection. Boxed regions (marked 1 and 2) indicate areas of bacterial accumulation. All scale bars, 100 μm. (B) Quantification of bacterial burden in the brain normalized to tissue area, expressed as the ratio of RFP-positive area to DAPI-positive area for each image. Each point represents an individual field of view. Boxes indicate median and interquartile range (IQR); whiskers represent 1.5 x IQR. (C) Bacterial titers in blood measured as colony-forming units (CFUs) per 20 μL of blood at P6 (log₁₀ scale). Each point represents an individual animal. Plates with colony numbers exceeding the reliable counting range were assigned a value of 2,000 CFUs. (D) Body weight measurements at P5 (pre-infection) and P6 (24 hours post-infection). Each point represents an individual animal. For (B-D), statistical comparisons (p-values) were calculated with the Wilcoxon rank-sum test. ns, not significant.

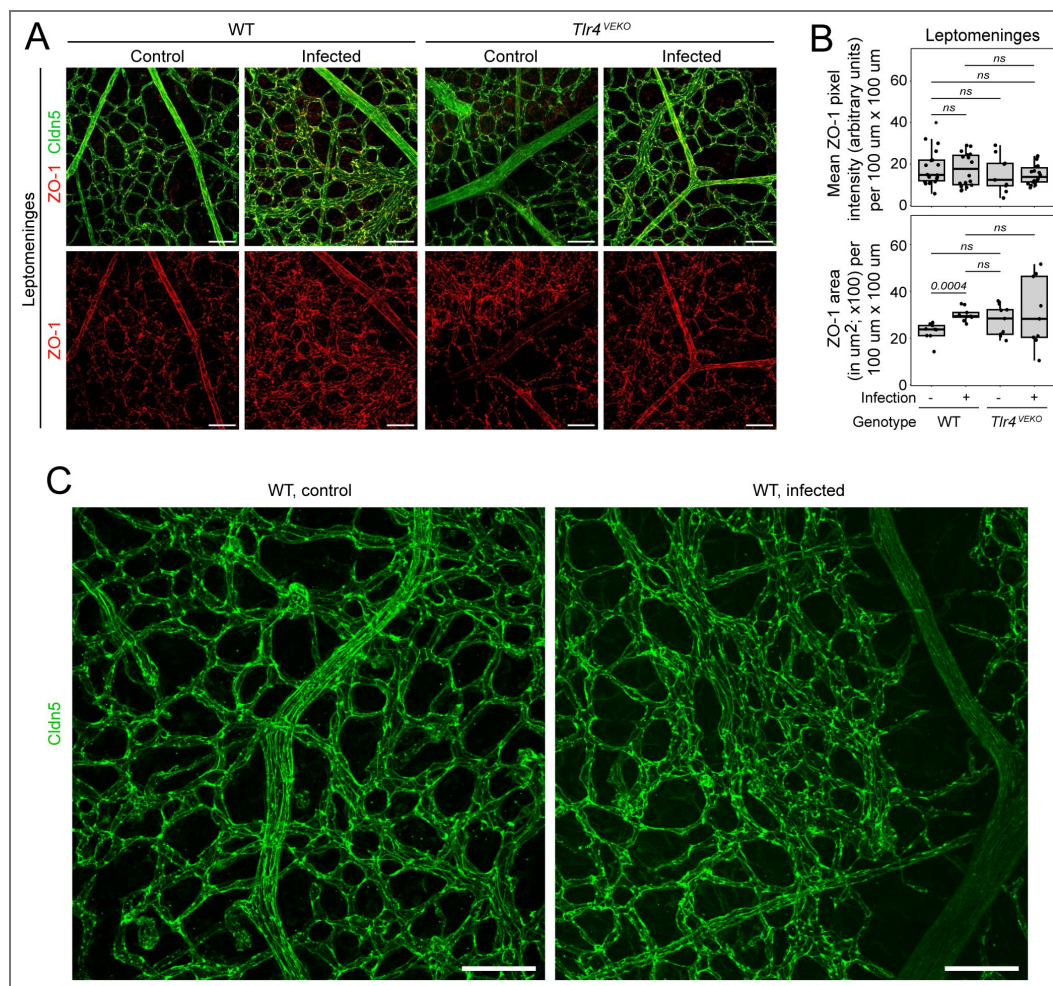


Figure 3 – figure supplement 1. ZO-1 in leptomeningeal endothelial cells with or without *E. coli* infection.

(A) Whole mount leptomeninges from P6 WT and *Tlr4*^{VEKO} mice, control or *E. coli*-infected, immunostained for Cldn5 and ZO-1. Scale bars, 20 μ m. (B) Quantification of ZO-1 immunostaining in the leptomeninges, as shown in (A). (C) Enlarged images of whole mount leptomeningeal vasculature immunostained for Cldn5. Visual inspection reveals a modest elevation in non-junctional Cldn5 in *E. coli*-infected (right) compared to control (left). Scale bar, 50 μ m.

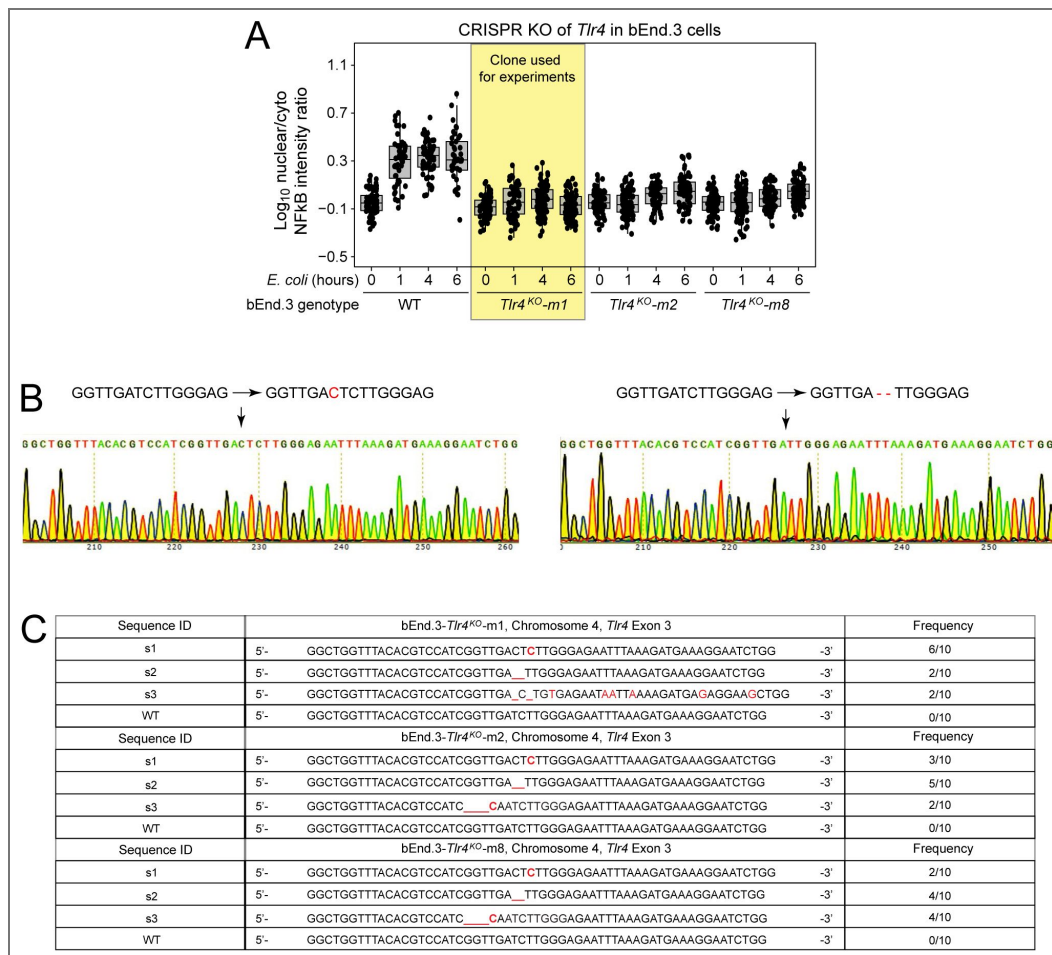


Figure 4 – figure supplement 1. CRISPR knockout of *Tlr4* in bEnd.3 cells

(A) Functional testing of three bEnd.3 clones following CRISPR KO of *Tlr4*. For each clone, the TLR4-mediated NF-κB response to *E. coli* exposure (migration of NF-κB from cytoplasm to nucleus) was quantified by immunostaining, as shown in Figure 3C-E. (B) Sanger sequencing from two plasmid subclones carrying genomic PCR products encompassing the region targeted by the *Tlr4* guide RNA from bEnd.3-*Tlr4*^{KO}-m1 cells. Each sequencing run shows a distinctive frameshift mutation. The sequences show the WT sequence on the left and the mutant sequence on the right (with the altered nucleotides in red). Left, a single nucleotide insertion. Right, a two-nucleotide deletion. (C) Compendium of ten plasmid subclone sequences from each of the three bEnd.3 clones shown in (A). Each clone shows three distinct alleles, all of which differ from WT and all of which produce a frameshift. No WT sequences were observed in any subclone. These data imply the *Tlr4* gene is present at three copies in bEnd.3 cells, and that each subclone is likely null for *Tlr4* function.

Figure 4 – figure supplement 2. The streptavidin leak assay requires a confluent monolayer of bEnd.3 cells.

(A) Among bEnd.3 cells, junctional localization of Cldn5 requires cell-cell contact as shown by immunostaining of cells grown at different densities. Scale bars, 20 μ m. (B) bEnd.3 cells were grown at either low or high density on biotinylated gelatin-coated coverslips, incubated with fluorescent streptavidin, and then fixed and immunostained for ZO-1. In regions where the bEnd.3 cells had not formed a contiguous network of tight junctions, the streptavidin gained access to the underlying biotinylated gelatin. Scale bars, 20 μ m.

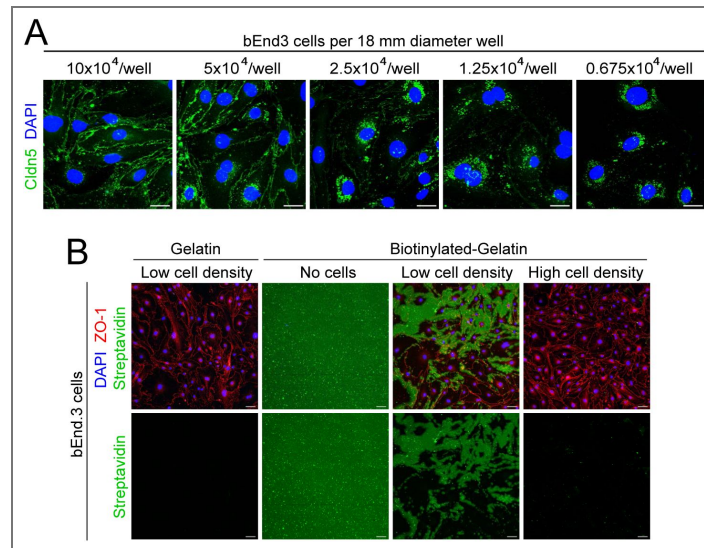
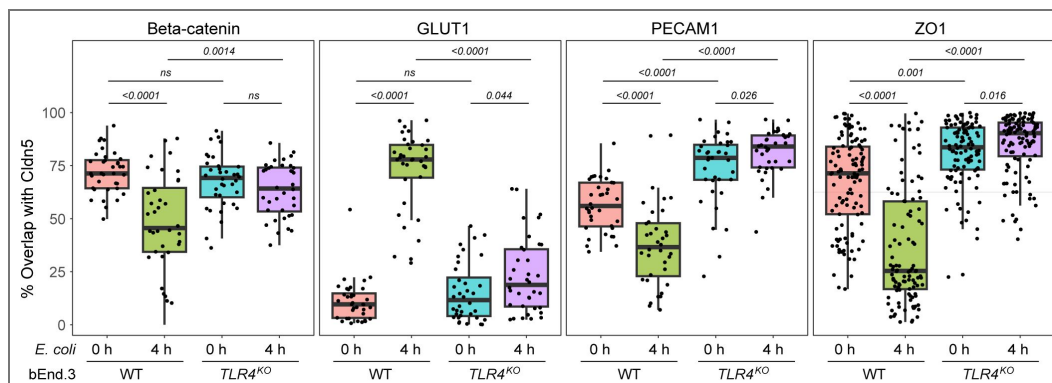


Figure 5 – figure supplement 1. Cldn5 dynamics in bEnd.3 cells following exposure to *E. coli*.

The overlap between Cldn5 and β -catenin, GLUT1, PECAM1, and ZO-1, in WT and *Tlr4*^{KO} bEnd.3 cells, either not exposed to *E. coli* (control) or exposed to *E. coli* for 4 hours. Shown here is a more granular quantification of the experiment shown in Figure 5A-D, with each datapoint representing a 100 μ m x 100 μ m region of interest (ROI). Box plots show median, interquartile range, and all individual data points. Each data point represents one image from representative images in three biological replicates. Statistical comparisons (p-values) were calculated with the Wilcoxon rank-sum test.



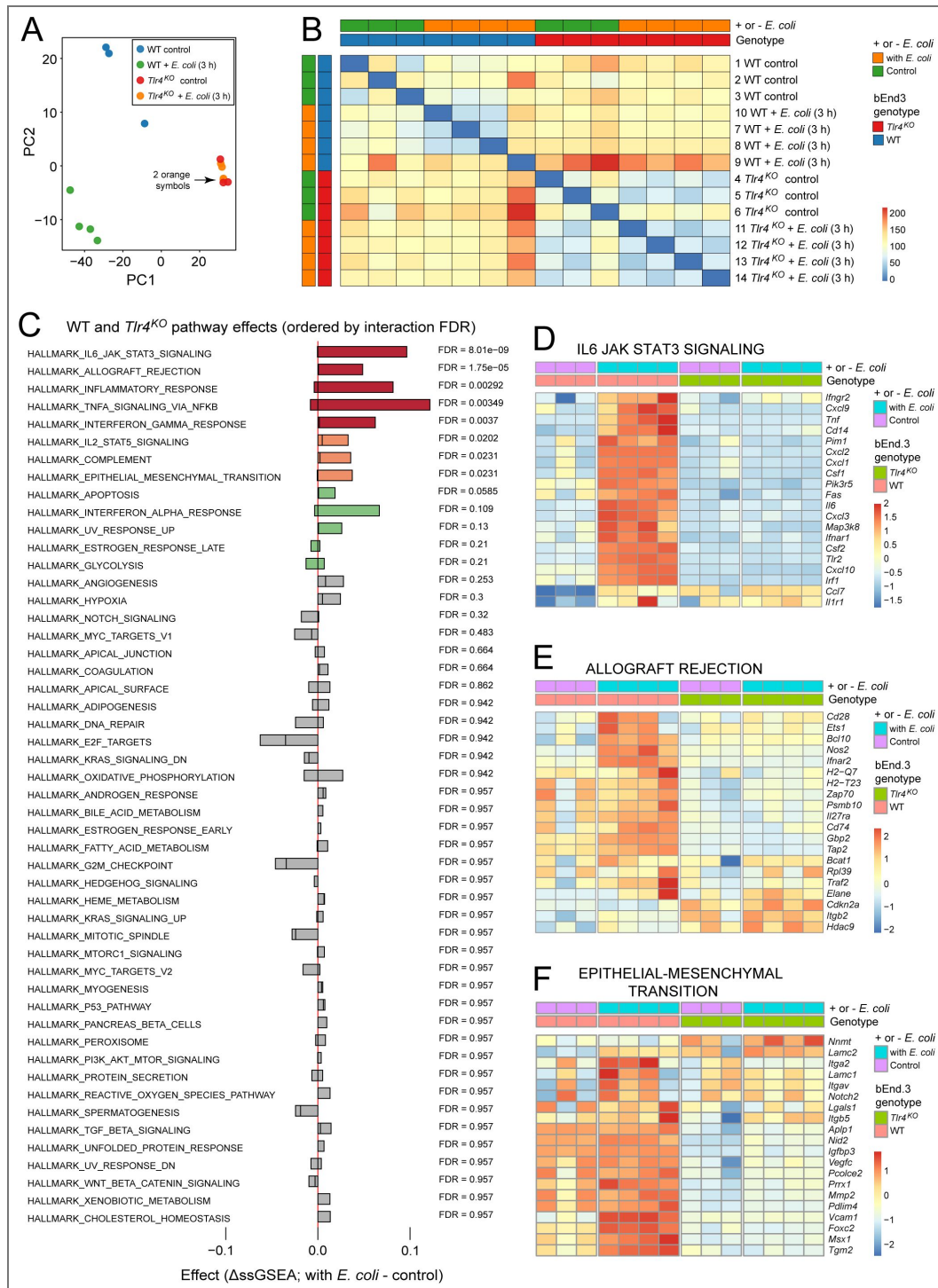


Figure 7 – figure supplement 1. Transcriptome analysis of WT and *Tlr4*^{KO} bEnd.3 cells, with or without exposure to *E. coli*.

(A) Principal component analysis (PCA) showing the relatedness of the 14 bEnd.3 RNAseq samples. Two of the four orange symbols are nearly superimposed. (B) Pearson correlations for the 14 bEnd.3 RNAseq samples. (C) Pathway-level differences between WT and *Tlr4*^{KO} bEnd.3 cells under control and *E. coli*-exposed conditions. Red, false discovery rate (FDR) less than 0.01. Orange, FDR between 0.01 and 0.05. Green, FDR between 0.05 and 0.25. Grey, FDR greater than 0.25. (D-F) Individual transcript abundances in the 14 bEnd.3 RNAseq samples for three GSEA categories. The transcriptome responses to infection are minimal in *Tlr4*^{KO} bEnd.3 cells.

Data availability

All RNA sequencing data were deposited in GEO (accession number GSE319556).

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Additional files

Supplementary file 1. Overview of snRNA-seq on leptomeninges.

Supplementary file 2. Numbers of mice and cells used in various experiments.

Additional information

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References

- Abram CL**, Roberge GL, Hu Y, Lowell CA (2014) Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. *J Immunol Methods* **408**:89-100 <https://doi.org/10.1016/j.jim.2014.05.009> | PubMed
- Banks WA**, Gray AM, Erickson MA, Salameh TS, Damodarasamy M, Sheibani N, Meabon JS, Wing EE, Morofuji Y, Cook DG, *et al.* (2015) Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. *J Neuroinflammation* **12**:223 <https://doi.org/10.1186/s12974-015-0434-1> | PubMed
- Bernstein NJ**, Fong NL, Lam I, Roy MA, Hendrickson DG, Kelley DR (2020) Solo: Doublet Identification in Single-Cell RNA-Seq via Semi-Supervised Deep Learning. *Cell Syst* **11**:95-101. <https://doi.org/10.1016/j.cels.2020.05.010> | PubMed
- Brouwer MC**, Tunkel AR, van de Beek D (2010) Epidemiology, diagnosis, and antimicrobial treatment of acute bacterial meningitis. *Clin Microbiol Rev* **23**:467-492 <https://doi.org/10.1128/cmr.00070-09> | PubMed
- Bui TM**, Wiesolek HL, Sumagin R (2020) ICAM-1: A master regulator of cellular responses in inflammation, injury resolution, and tumorigenesis. *J Leukoc Biol* **108**:787-799 <https://doi.org/10.1002/jlb.2mr0220-549r> | PubMed
- Bundy LM**, Rajnik M, Noor A (2023) Neonatal Meningitis. In: *StatPearls* StatPearls Publishing Company.

- Casanova JL, Abel L, Quintana-Murci L (2011) Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. *Annu Rev Immunol* **29**:447-491 <https://doi.org/10.1146/annurev-immunol-030409-101335> | PubMed
- Clark IC, Fontanez KM, Meltzer RH, Xue Y, Hayford C, May-Zhang A, D'Amato C, Osman A, Zhang JQ, Hettige P, et al. (2023) Microfluidics-free single-cell genomics with templated emulsification. *Nat Biotechnol* **41**:1557-1566 <https://doi.org/10.1038/s41587-023-01685-z> | PubMed
- Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* **8**:265-277 <https://doi.org/10.1023/a:1008942828960> | PubMed
- Coisne C, Engelhardt B (2011) Tight junctions in brain barriers during central nervous system inflammation. *Antioxid Redox Signal* **15**:1285-1303 <https://doi.org/10.1089/ars.2011.3929> | PubMed
- Cottarelli A, Jamoul D, Tuohy MC, Shahriar S, Glendinning M, Prochilo G, Edinger AL, Arac A, Agalliu D (2025) Rab7a is required to degrade select blood-brain barrier junctional proteins after ischemic stroke. *Acta Neuropathol Commun* **13**:203 <https://doi.org/10.1186/s40478-025-02125-6> | PubMed
- Doran KS, Fulde M, Gratz N, Kim BJ, Nau R, Prasadaraio N, Schubert-Unkmeir A, Tuomanen EI, Valentin-Weigand P (2016) Host-pathogen interactions in bacterial meningitis. *Acta Neuropathol* **131**:185-209 <https://doi.org/10.1007/s00401-015-1531-z> | PubMed
- Dubrovskiy O, Birukova AA, Birukov KG (2013) Measurement of local permeability at subcellular level in cell models of agonist- and ventilator-induced lung injury. *Lab Invest* **93**:254-263 <https://doi.org/10.1038/labinvest.2012.159> | PubMed
- Edmond K, Clark A, Korczak VS, Sanderson C, Griffiths UK, Rudan I (2010) Global and regional risk of disabling sequelae from bacterial meningitis: a systematic review and meta-analysis. *Lancet Infect Dis* **10**:317-28 [https://doi.org/10.1016/s1473-3099\(10\)70048-7](https://doi.org/10.1016/s1473-3099(10)70048-7) | PubMed
- Engelhardt B, Sorokin L (2009) The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. *Semin Immunopathol* **31**:497-511 <https://doi.org/10.1007/s00281-009-0177-0> | PubMed
- Fleming SJ, Chaffin MD, Arduini A, Akkad AD, Banks E, Marioni JC, Philippakis AA, Ellinor PT, Babadi M (2023) Unsupervised removal of systematic background noise from droplet-based single-cell experiments using CellBender. *Nat Methods* **20**:1323-1335 <https://doi.org/10.1038/s41592-023-01943-7> | PubMed
- Franklin BS, Latz E, Schmidt FI (2018) The intra- and extracellular functions of ASC specks. *Immunol Rev* **281**:74-87 <https://doi.org/10.1111/imr.12611> | PubMed
- Freitas RHCN, Fraga CAM (2018) NF-κB-IKKβ Pathway as a Target for Drug Development: Realities, Challenges and Perspectives. *Curr Drug Targets* **19**:1933-1942 <https://doi.org/10.2174/1389450119666180219120534> | PubMed
- Gadina M, Johnson C, Schwartz D, Bonelli M, Hasni S, Kanno Y, Changelian P, Laurence A, O'Shea JJ (2018) Translational and clinical advances in JAK-STAT biology: The present and future of jakinibs. *J Leukoc Biol* **104**:499-514 <https://doi.org/10.1002/jlb.5ri0218-084r> | PubMed
- Gao Y, Hu F (2024) Predictive role of PAR and LAR in refractory suppurative meningitis in infants. *BMC Pediatr* **24**:462 <https://doi.org/10.1186/s12887-024-04898-6> | PubMed
- Garcia-Gallardo A, Campbell M (2025) Understanding the Blood-Brain Barrier: From Physiology to Pathology. *Adv Exp Med Biol* **1477**:1-33 https://doi.org/10.1007/978-3-031-89525-8_1 | PubMed
- GBD 2016 Neurology Collaborators (2019) . . Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* **18**:459-480 [https://doi.org/10.1016/S1474-4422\(18\)30499-X](https://doi.org/10.1016/S1474-4422(18)30499-X)
- Garges HP, Moody MA, Cotten CM, Smith PB, Tiffany KF, Lenfestey R, Li JS, Fowler VG, Benjamin DK (2006) Neonatal meningitis: what is the correlation among cerebrospinal fluid cultures, blood cultures, and cerebrospinal fluid parameters?. *Pediatrics* **117**:1094-1100 <https://doi.org/10.1542/peds.2005-1132> | PubMed

- Guarnera A, Moltoni G, Dellepiane F, Lucignani G, Rossi-Espagnet MC, Campi F, Auriti C, Longo D (2024) Bacterial Meningoencephalitis in Newborns. *Biomedicines* **12**:2490 <https://doi.org/10.3390/biomedicines12112490> | PubMed
- Hafemeister C, Satija R (2019) Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biol* **20**:296 <https://doi.org/10.1186/s13059-019-1874-1> | PubMed
- Hashimoto Y, Campbell M, Tachibana K, Okada Y, Kondoh M (2021) Claudin-5: A Pharmacological Target to Modify the Permeability of the Blood-Brain Barrier. *Biol Pharm Bull* **44**:1380-1390 <https://doi.org/10.1248/bpb.b21-00408> | PubMed
- Hashimoto Y, Greene C, Munnich A, Campbell M (2023) The CLDN5 gene at the blood-brain barrier in health and disease. *Fluids Barriers CNS* **20**:22 <https://doi.org/10.1186/s12987-023-00424-5> | PubMed
- Huang X, Hussain B, Chang J (2021) Peripheral inflammation and blood-brain barrier disruption: effects and mechanisms. *CNS Neurosci Ther* **27**:36-47 <https://doi.org/10.1111/cns.13569> | PubMed
- Huang X, Wei P, Fang C, Yu M, Yang S, Qiu L, Wang Y, Xu A, Hoo RLC, Chang J (2024) Compromised endothelial Wnt/ β -catenin signaling mediates the blood-brain barrier disruption and leads to neuroinflammation in endotoxemia. *J Neuroinflammation* **21**:265 <https://doi.org/10.1186/s12974-024-03261-x> | PubMed
- Hulton CH, Costa EA, Shah NS, Quintanal-Villalonga A, Heller G, de Stanchina E, Rudin CM, Poirier JT (2020) Direct genome editing of patient-derived xenografts using CRISPR-Cas9 enables rapid in vivo functional genomics. *Nat Cancer* **1**:359-369 <https://doi.org/10.1038/s43018-020-0040-8> | PubMed
- Jia K, Du Y, Lin W, Cao X, Zhang J, Peng L, Li Z, Fang R (2025) Dual role of TLR4 in bacterial meningitis through regulating endothelial pyroptosis and inflammatory response during extraintestinal pathogenic *Escherichia coli* infection. *Front Immunol* **16**:1581696 <https://doi.org/10.3389/fimmu.2025.1581696> | PubMed
- Jia K, Du Y, Cao X, Shen X, Ran J, Lu Y, Peng L, Li Z, Fang R (2026) Meningitic *Escherichia coli* disrupts the blood-brain barrier through pyroptosis and tight junction degradation and NLRP6 deficiency aggravates infection outcomes. *Microbiol Res* **303**:128388 <https://doi.org/10.1016/j.micres.2025.128388> | PubMed
- Johnson I, Spence MTZ (2010) Probes for Endocytosis, receptors, and ion channels. In: *The Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies* Life Technologies. pp. 739-772
- Ju F, Ran Y, Zhu L, Cheng X, Gao H, Xi X, Yang Z, Zhang S (2018) Increased BBB Permeability Enhances Activation of Microglia and Exacerbates Loss of Dendritic Spines After Transient Global Cerebral Ischemia. *Front Cell Neurosci* **12**:236 <https://doi.org/10.3389/fncel.2018.00236> | PubMed
- Ju F, Ran Y, Zhu L, Cheng X, Gao H, Xi X, Yang Z, Zhang S (2018) Increased BBB Permeability Enhances Activation of Microglia and Exacerbates Loss of Dendritic Spines After Transient Global Cerebral Ischemia. *Front Cell Neurosci* **12**:236 <https://doi.org/10.3389/fncel.2018.00236> | PubMed
- Kawai T, Akira S (2007) TLR signaling. *Semin Immunol* **19**:24-32 <https://doi.org/10.1016/j.smim.2006.12.004> | PubMed
- Kim KS (2003) Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. *Nat Rev Neurosci* **4**:376-385 <https://doi.org/10.1038/nrn1103> | PubMed
- Korotkevich G, Sukhov V, Sergushichev A (2019) Fast gene set enrichment analysis. *bioRxiv* <https://doi.org/10.1101/060012>
- Krishnan S, Chen S, Turcatel G, Arditi M, Prasadara NV (2013) Regulation of Toll-like receptor 2 interaction with Ecgp96 controls *Escherichia coli* K1 invasion of brain endothelial cells. *Cell Microbiol* **15**:63-81 <https://doi.org/10.1111/cmi.12026> | PubMed
- MacDonald E, Savage B, Zech T (2020) Connecting the dots: combined control of endocytic recycling and degradation. *Biochem Soc Trans* **48**:2377-2386 <https://doi.org/10.1042/bst20180255> | PubMed

- Mapunda JA**, Pareja J, Vladymyrov M, Bouillet E, Hélie P, Pleskač P, Barcos S, Andrae J, Vestweber D, McDonald DM, *et al.* (2023) VE-cadherin in arachnoid and pia mater cells serves as a suitable landmark for in vivo imaging of CNS immune surveillance and inflammation. *Nat Commun* **14**:5837 <https://doi.org/10.1038/s41467-023-41580-4> | PubMed
- Mastorakos P**, McGavern D (2019) The anatomy and immunology of vasculature in the central nervous system. *Sci Immunol* **4**:eaav0492 <https://doi.org/10.1126/sciimmunol.aav0492> | PubMed
- McKinsey GL**, Lizama CO, Keown-Lang AE, Niu A, Santander N, Larphaveesarp A, Chee E, Gonzalez FF, Arnold TD (2020) A new genetic strategy for targeting microglia in development and disease. *eLife* **9**:e54590 <https://doi.org/10.7554/eLife.54590> | PubMed
- Medzhitov R** (2001) Toll-like receptors and innate immunity. *Nat Rev Immunol* **1**:135-145 <https://doi.org/10.1038/35100529> | PubMed
- Melville J** (2022) Uvot: the uniform manifold approximation and projection (UMAP) method for Dimensionality reduction. 0.1.14. <https://cran.r-project.org/web/packages/uvot/index.html>
- Mo A**, Mukamel EA, Davis FP, Luo C, Henry GL, Picard S, Urich MA, Nery JR, Sejnowski TJ, Lister R, *et al.* (2015) Epigenomic Signatures of Neuronal Diversity in the Mammalian Brain. *Neuron* **86**:1369-1384 <https://doi.org/10.1016/j.neuron.2015.05.018> | PubMed
- Montesano R**, Pepper MS, Möhle-Steinlein U, Risau W, Wagner EF, Orci L (1990) Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. *Cell* **62**:435-445 [https://doi.org/10.1016/0092-8674\(90\)90009-4](https://doi.org/10.1016/0092-8674(90)90009-4) | PubMed
- Monvoisin A**, Alva JA, Hofmann JJ, Zovein AC, Lane TF, Iruela-Arispe ML (2006) VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. *Dev Dyn* **235**:3413-3422 <https://doi.org/10.1002/dvdy.20982> | PubMed
- Morita K**, Sasaki H, Furuse M, Tsukita S (1999) Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *J Cell Biol* **147**:185-194 <https://doi.org/10.1083/jcb.147.1.185> | PubMed
- Nagyoszi P**, Wilhelm I, Farkas AE, Fazakas C, Dung NT, Haskó J, Krizbai IA (2010) Expression and regulation of toll-like receptors in cerebral endothelial cells. *Neurochem Int* **57**:556-564 <https://doi.org/10.1016/j.neuint.2010.07.002> | PubMed
- Nitta T**, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, Tsukita S (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* **161**:653-660 <https://doi.org/10.1083/jcb.200302070> | PubMed
- Ouchenir L**, Renaud C, Khan S, Bitnun A, Boisvert AA, McDonald J, Bowes J, Brophy J, Barton M, Ting J, *et al.* (2017) The Epidemiology, Management, and Outcomes of Bacterial Meningitis in Infants. *Pediatrics* **140**:e20170476 <https://doi.org/10.1542/peds.2017-0476> | PubMed
- Pålsson-McDermott EM**, O'Neill LA (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* **113**:153-162 <https://doi.org/10.1111/j.1365-2567.2004.01976.x> | PubMed
- Pietilä R**, Del Gaudio F, He L, Vázquez-Liébanas E, Vanlandewijck M, Muhl L, Mocci G, Bjørnholm KD, Lindblad C, Fletcher-Sandersjö A, *et al.* (2023) Molecular anatomy of adult mouse leptomeninges. *Neuron* **111**:3745-3764. <https://doi.org/10.1016/j.neuron.2023.09.002> | PubMed
- RStudio Team** (2020) RStudio. <https://rstudio.com>
- Sanjana NE**, Shalem O, Zhang F (2014) Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* **11**:783-784 <https://doi.org/10.1038/nmeth.3047> | PubMed
- Saunders NR**, Liddelow SA, Dziegielewska KM (2012) Barrier mechanisms in the developing brain. *Front Pharmacol* **3**:46 <https://doi.org/10.3389/fphar.2012.00046> | PubMed
- Schindelin J**, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, *et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**:676-682 <https://doi.org/10.1038/nmeth.2019> | PubMed

- Scott CC, Vacca F, Gruenberg J (2014) Endosome maturation, transport and functions. *Semin Cell Dev Biol* **31**:2-10 <https://doi.org/10.1016/j.semcdb.2014.03.034> | PubMed
- Sester DP, Thygesen SJ, Sagulenko V, Vajjhala PR, Cridland JA, Vitak N, Chen KW, Osborne GW, Schroder K, Stacey KJ (2015) A novel flow cytometric method to assess inflammasome formation. *J Immunol* **194**:455-462 <https://doi.org/10.4049/jimmunol.1401110> | PubMed
- Shearer LJ, Petersen NO (2019) Distribution and Co-localization of endosome markers in cells. *Heliyon* **5**:e02375 <https://doi.org/10.1016/j.heliyon.2019.e02375> | PubMed
- Sikorski EE, Hallmann R, Berg EL, Butcher EC (1993) The Peyer's patch high endothelial receptor for lymphocytes, the mucosal vascular addressin, is induced on a murine endothelial cell line by tumor necrosis factor-alpha and IL-1. *J Immunol* **151**:5239-5250 <https://doi.org/10.4049/jimmunol.151.10.5239> | PubMed
- Singh V, Kaur R, Kumari P, Pasricha C, Singh R (2023) ICAM-1 and VCAM-1: Gatekeepers in various inflammatory and cardiovascular disorders. *Clin Chim Acta* **548**:117487 <https://doi.org/10.1016/j.cca.2023.117487> | PubMed
- Smith NM, Giacci MK, Gough A, Bailey C, McGonigle T, Black AMB, Clarke TO, Bartlett CA, Swaminathan Iyer K, Dunlop SA, *et al.* (2018) Inflammation and blood-brain barrier breach remote from the primary injury following neurotrauma. *J Neuroinflammation* **15**:201 <https://doi.org/10.1186/s12974-018-1227-0> | PubMed
- Smyth LCD, Xu D, Okar SV, Dykstra T, Rustenhoven J, Papadopoulos Z, Bhasi K, Kim MW, Drieu A, Mamuladze T, *et al.* (2024) Identification of direct connections between the dura and the brain. *Nature* **627**:165-173 <https://doi.org/10.1038/s41586-023-06993-7> | PubMed
- Sordet O, Rébé C, Plenchette S, Zermati Y, Hermine O, Vainchenker W, Garrido C, Solary E, Dubrez-Daloz L (2002) Specific involvement of caspases in the differentiation of monocytes into macrophages. *Blood* **100**:4446-4453 <https://doi.org/10.1182/blood-2002-06-1778> | PubMed
- Santambrogio L, Potolicchio I, Fessler SP, Wong SH, Raposo G, Strominger JL (2005) Involvement of caspase-cleaved and intact adaptor protein 1 complex in endosomal remodeling in maturing dendritic cells. *Nat Immunol* **6**:1020-1028 <https://doi.org/10.1038/ni1250> | PubMed
- Stamatovic SM, Dimitrijevic OB, Keep RF, Andjelkovic AV (2006) Protein kinase Calpha-RhoA cross-talk in CCL2-induced alterations in brain endothelial permeability. *J Biol Chem* **281**:8379-8388 <https://doi.org/10.1074/jbc.m513122200> | PubMed
- Stamatovic SM, Keep RF, Wang MM, Jankovic I, Andjelkovic AV (2009) Caveolae-mediated internalization of occludin and claudin-5 during CCL2-induced tight junction remodeling in brain endothelial cells. *J Biol Chem* **284**:19053-19066 <https://doi.org/10.1074/jbc.m109.000521> | PubMed
- Stamatovic SM, Sladojevic N, Keep RF, Andjelkovic AV (2012) Relocalization of junctional adhesion molecule A during inflammatory stimulation of brain endothelial cells. *Mol Cell Biol* **32**:3414-3427 <https://doi.org/10.1128/mcb.06678-11> | PubMed
- Stamatovic SM, Johnson AM, Sladojevic N, Keep RF, Andjelkovic AV (2017) Endocytosis of tight junction proteins and the regulation of degradation and recycling. *Ann NY Acad Sci* **1397**:54-65 <https://doi.org/10.1111/nyas.13346> | PubMed
- Stirling DR, Swain-Bowden MJ, Lucas AM, Carpenter AE, Cimini BA, Goodman A (2021) CellProfiler 4: improvements in speed, utility and usability. *BMC Bioinformatics* **22**:433 <https://doi.org/10.1186/s12859-021-04344-9> | PubMed
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, *et al.* (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**:15545-15550 <https://doi.org/10.1073/pnas.0506580102> | PubMed
- Sukumaran SK, Prasadara NV (2003) Escherichia coli K1 invasion increases human brain microvascular endothelial cell monolayer permeability by disassembling vascular-endothelial cadherins at tight junctions. *J Infect Dis* **188**:1295-309 <https://doi.org/10.1086/379042> | PubMed

- Too LK, Yau B, Baxter AG, McGregor IS, Hunt NH (2019) Double deficiency of toll-like receptors 2 and 4 alters long-term neurological sequelae in mice cured of pneumococcal meningitis. *Sci Rep* **9**:16189 <https://doi.org/10.1038/s41598-019-52212-7> | PubMed
- Vallières L, Rivest S (1997) Regulation of the genes encoding interleukin-6, its receptor, and gp130 in the rat brain in response to the immune activator lipopolysaccharide and the proinflammatory cytokine interleukin-1beta. *J Neurochem* **69**:1668-1683 <https://doi.org/10.1046/j.1471-4159.1997.69041668.x> | PubMed
- van Well GT, Sanders MS, Ouburg S, Kumar V, van Furth AM, Morré SA (2013) Single nucleotide polymorphisms in pathogen recognition receptor genes are associated with susceptibility to meningococcal meningitis in a pediatric cohort. *PLoS One* **8**:e64252 <https://doi.org/10.1371/journal.pone.0064252> | PubMed
- Wang J, Rattner A, Nathans J (2023) Bacterial meningitis in the early postnatal mouse studied at single-cell resolution. *eLife* **12**:e86130 <https://doi.org/10.7554/eLife.86130> | PubMed
- Wang X, Zhang Y, Chen X, Fu K, Cui J, Wu J, Sun Y, Ren J, Xue F, Dai J, et al. (2025a) RIPK1 kinase drove brain microvascular endothelial cells death and blood-brain barrier disruption in neonatal Escherichia coli meningitis. *Nat Commun* **16**:7309 <https://doi.org/10.1038/s41467-025-62760-4> | PubMed
- Wang Y, Cho C, Williams J, Smallwood PM, Zhang C, Junge HJ, Nathans J (2018) Interplay of the Norrin and Wnt7a/Wnt7b signaling systems in blood-brain barrier and blood-retina barrier development and maintenance. *Proc Natl Acad Sci U S A* **115**:E11827-E11836 <https://doi.org/10.1073/pnas.1813217115> | PubMed
- Wang Y, Sabbagh MF, Gu X, Rattner A, Williams J, Nathans J (2019) Beta-catenin signaling regulates barrier-specific gene expression in circumventricular organ and ocular vasculatures. *eLife* **8**:e43257 <https://doi.org/10.7554/eLife.43257> | PubMed
- Wang Y, Rattner A, Li Z, Smallwood PM, Nathans J (2025b) Vascular endothelial-specific loss of TGF-beta signaling as a model for choroidal neovascularization and central nervous system vascular inflammation. *eLife* **14**:RP107018 <https://doi.org/10.7554/eLife.107018> | PubMed
- Wei C, Jiang W, Wang R, Zhong H, He H, Gao X, Zhong S, Yu F, Guo Q, Zhang L, et al. (2024) Brain endothelial GSDMD activation mediates inflammatory BBB breakdown. *Nature* **629**:893-900 <https://doi.org/10.1038/s41586-024-07314-2> | PubMed
- Wickham H (2009) *Ggplot2: Elegant Graphics for Data Analysis* New York: Springer-Verlag.
- Wickham H (2017) tidyverse: Easily Install and Load the 'Tidyverse'.. <https://CRAN.R-project.org/package=tidyverse>
- Williams RL, Courtneidge SA, Wagner EF (1988) Embryonic lethalties and endothelial tumors in chimeric mice expressing polyoma virus middle T oncogene. *Cell* **52**:121-31 [https://doi.org/10.1016/0092-8674\(88\)90536-3](https://doi.org/10.1016/0092-8674(88)90536-3) | PubMed
- Xu M, Hu L, Huang H, Wang L, Tan J, Zhang Y, Chen C, Zhang X, Huang L (2019) Etiology and Clinical Features of Full-Term Neonatal Bacterial Meningitis: A Multicenter Retrospective Cohort Study. *Front Pediatr* **7**:31 <https://doi.org/10.3389/fped.2019.00031> | PubMed
- Yang R, Wang J, Wang F, Zhang H, Tan C, Chen H, Wang X (2023) Blood-Brain Barrier Integrity Damage in Bacterial Meningitis: The Underlying Link, Mechanisms, and Therapeutic Targets. *Int J Mol Sci* **24**:2852 <https://doi.org/10.3390/ijms24032852> | PubMed
- Yang R, Chen J, Qu X, Liu H, Wang X, Tan C, Chen H, Wang X (2024) Interleukin-22 Contributes to Blood-Brain Barrier Disruption via STAT3/VEGFA Activation in Escherichia coli Meningitis. *ACS Infect Dis* **10**:988-999 <https://doi.org/10.1021/acsinfecdis.3c00668> | PubMed
- Zaffaroni L, Peri F (2018) Recent advances on Toll-like receptor 4 modulation: new therapeutic perspectives. *Future Med Chem* **10**:461-476 <https://doi.org/10.4155/fmc-2017-0172> | PubMed

Zhu N, Liu W, Prakash A, Zhang C, Kim KS (2020) Targeting E. coli invasion of the blood-brain barrier for investigating the pathogenesis and therapeutic development of E. coli meningitis. *Cell Microbiol* 22:e13231 <https://doi.org/10.1111/cmi.13231> | PubMed

Peer reviews

Reviewer #1 (Public review):

Summary:

In this manuscript, Seegren and colleagues demonstrate that in a mouse model of neonatal E. coli meningitis, loss of toll-like receptor 4 (TLR4) in VE-cadherin⁺ endothelial cells and a subset of meningeal fibroblasts leads to a marked decrease in transcriptional dysregulation across multiple leptomeningeal cell types, a decrease in vascular permeability, and a decrease in macrophage abundance. In contrast, loss of macrophage TLR4 had less pronounced effects. Using cultured wildtype and TLR4-knockout endothelial cells, the authors further demonstrate that TLR4 signaling leads to reversible internalization of the tight junction protein claudin-5, establishing a potential mechanism of increased vascular permeability. Authors also show that claudin-5 internalization is independent of NF- κ B. Finally, the authors use RNA-sequencing of wildtype and TLR4-knockout endothelial cells to define the TLR4-dependent cell-autonomous transcriptional response to E. coli.

Comments on revised version.

The authors have considerably improved and strengthened the work through the addition of new experimental data, new data analyses, and modifications to their interpretation. Notably, the authors used additional Cre-reporter mice to clarify that Cdh5-CreER is active in endothelial cells and some meningeal fibroblasts, and thus revised nomenclature and interpretation to acknowledge that the Tlr4^{fl/-};Cdh5-CreER cKO (Tlr4-VEKO) is not exclusively endothelial. The authors also demonstrated that Tlr4-VEKO does not affect peripheral E.coli burden, but acknowledge that changes to periphery-derived signals (e.g., cytokines) may contribute to observed leptomeningeal phenotypes.

The authors added PCA plots to show similarity in gene expression shifts across biological replicates (mice). This provides support for the claim that Tlr4-VEKO attenuates infection-associated transcriptional changes. With respect to differential expression analysis, I agree with authors that characteristics of individual cells (e.g. heterogeneity) are of interest. I remain concerned, however, that the formal differential analysis strategy appears to consider cells as independent experimental units, which they are not because a single cell cannot be randomly assigned to an experimental group (control or cKO, uninfected or infected). The mouse is the correct experimental unit for a comparison across these groups because it can be randomized. I appreciate that many of the gene expression changes appear consistent across mice (e.g. Figure 1 - Figure supplement 7) and that there are clear infection- and genotype-associated phenotypes in other assays. I would simply caution that the authors' analysis strategy likely leads to a larger number of type I errors (false positives) than is generally accepted; a mixed (hierarchical) model or pseudo-bulk approach would be more appropriate for future studies.

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Reviewer #2 (Public review):

Summary:

The authors use a postnatal mouse model of E. coli bacterial meningitis and a mouse brain endothelioma cell line combined with cell type specific gene deletion to study the function of

endothelial TLR4, a cell surface receptor that recognizes gram positive bacterial wall components, in the local leptomeningeal (LPM) response with a focus on endothelial barrier breakdown mediated by TLR4. Single cell transcriptional profiling and imaging studies using wholemount preps of the LPM support that LPM endothelial, CD206+ local macrophage and LPM fibroblast and arachnoid barrier cell inflammatory response and is abrogated in endothelial specific KO of TLR4, pointing to a role for endothelial TLR4 in local LPM response. Culture studies using Bend3.1 cells (a mouse brain endothelioma cell line) support a direct role for TLR4 in the bacteria-mediated inflammatory response and in internalization of Cldn5 via the endosomal-lysosomal pathway, resulting in loss of barrier integrity

Strengths:

The local LPM cell response in meningitis and the role of specific LPM cells in inflammation and CNS barrier breakdown has not been extensively studied, despite ample evidence for primary immune response in the meninges in human patients and in animal models. The authors employ a robust, multi-model approach using both in vivo and in vitro models with cell-type specific knockout to study the function of TLR4 in brain endothelial cell response. The authors nicely combine functional barrier assays with IF for junctional localization in their experimental design and they delve into potential mechanisms of Cldn5 internalization using markers of endosomal-lysosomal pathway localization. The authors also describe a new type of barrier assay using a streptavidin-coated plates upon which barrier forming cell cultures can be plated, this could be a very useful alternative or complement to other size-selective barrier assays and presumably could work for other barrier forming cell types, like epithelial cells.

Comments on revised version.

In their revision, the authors addressed prior noted weaknesses with new data and analysis. They now show that TLR4-VE-cad cKO mice have a largely similar disease progression as control mice, including increased bacterial burden in the LPM and brain. This underscores that the reduced vascular leakage and blunted inflammatory response is due to loss of TLR4 response to bacteria on VE-cad recombined cells and not because the mice are protected from meningitis. The authors also performed additional experiments to show that Cldn5 internalization via the endosomal-lysosomal pathway is independent of NFκB signaling. The authors also added in important discussion points about how their results fit into the broader literature on TLR4 in BBB endothelial cell junctional protein localization and prior work on meningitis in global TLR4.

<https://doi.org/10.7554/eLife.110458.2.sa2>

Reviewer #3 (Public review):

Summary:

This study investigates the molecular underpinnings of immune responses in the leptomeninges in neonatal bacterial meningitis. Bacterial meningitis is a major disease burden, particularly for neonates, and it has previously been noted that the meningeal immune environment in infants is permissive to opportunistic infection (Kim et al., *Sci Immunol*, 2023). There is less known about the contribution of the stromal compartment to meningeal immune responses. Seegren et al. interrogate the role of leptomeningeal endothelium in host defense in *E. coli* infected neonatal mice using mouse genetic tools to delete the LPS receptor Tlr4 from either endothelial cells/stromal cells (using *Cdh5-CreER*) or myeloid cells (using *LysM-Cre*). The authors use snRNAseq, cleared cortical mounts, and in vitro work to define the impact of *E. coli* infection on leptomeningeal endothelial cells. This study uses a range of innovative techniques to probe the role of the stromal compartment in meningitis. With additional experiments to confirm the specificity of their Cre models, this

strengthens the interpretation of the study significantly. The only major weakness is the inability to confirm TLR4 knockout in myeloid cells.

Strengths:

This study makes excellent use of cleared cortical mounts to examine the biology of the leptomeninges, in particular, changes to the endothelium, with unprecedented detail. In combination with high-quality sequencing data provide new insights into the impact of meningitis on the leptomeninges. The data presented by the authors is of very high quality.

The authors have also done substantial work to address my two major comments regarding 1) the specificity of their Cre systems and 2) peripheral impacts of the interventions.

(1) The authors identified and acknowledged some impacts in the leptomeningeal stroma (the relatively high level of recombination in ECs vs FBs presumably reflects a single low dose being given, where other groups have done more aggressive tamoxifen regimens that drive recombination in FBs as well). Given the incomplete recombination in the leptomeningeal FBs, I agree with their conclusion that it is probably endothelial driven. Acknowledging the contributions of other myeloid cells with the L. The Cre-NLS experiments with nuclear markers provided excellent data and had beautiful staining.

(2) The authors did not observe differences in bacterial burden in peripheral organs in either CKO model, suggesting that CNS impacts are not downstream of peripheral bacterial control.

Weaknesses:

(1) The inducible Cre lines used by the authors target peripheral tissues as well as CNS tissues. Although this is mollified by the lack of impact on peripheral disease burden.

(2) The authors were not able to confirm TLR4 knockout in myeloid cells, and this caveat is acknowledged. The lack of response in TLR4 VEKO mice strongly suggests successful conditional knockout.

(3) The cell line model (bEnd.3) is a relatively low fidelity model of BBB endothelial cells. The authors acknowledge this, and it is likely that endothelial cell responses to LPS are highly conserved.

(4) It is perhaps not surprising that Tlr4 is required for meningitis responses with *E. coli*. However, it is unclear if these findings can be generalised to other, more common, meningitis infections (streptococcal/pneumococcal).

<https://doi.org/10.7554/eLife.110458.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

*In this manuscript, Seegren and colleagues demonstrate that in a mouse model of neonatal *E. coli* meningitis, loss of endothelial toll-like receptor 4 (TLR4) leads to a marked decrease in transcriptional dysregulation across multiple leptomeningeal cell types, a decrease in vascular permeability, and a decrease in macrophage abundance. In contrast, loss of macrophage TLR4 had less pronounced effects. Using cultured wild-type*

and TLR4 knockout endothelial cells, the authors further demonstrate that TLR4-NF- κ B signaling leads to reversible internalization of the tight junction protein claudin-5, establishing a potential mechanism of increased vascular permeability. Finally, the authors use RNA sequencing of wild-type and TLR4-knockout endothelial cells to define the TLR4-dependent cell-autonomous transcriptional response to *E. coli*.

Strengths:

(1) The authors address an important, well-motivated hypothesis related to the cellular and molecular mechanisms of leptomeningeal inflammation.

(2) The authors use model systems (mouse conditional knockouts and cultured endothelial cells) that are appropriate to address their hypotheses. The data are of high quality.

Weaknesses:

(1) The authors perform single-nucleus RNA-seq on dissected leptomeninges from control and *E. coli*-infected mice across three genotypes (WT, Tlr4MKO, and Tlr4ECKO). A major discovery from this experiment, as summarized by the authors, is: "Tlr4ECKO mice exhibited a global attenuation of infection-induced transcriptional responses across all major leptomeningeal cell types, as judged by the positions of cell clusters in the UMAP." This conclusion could be considerably strengthened by improving the qualitative and quantitative analysis.

Thank you for this comment. We agree that the UMAP-based interpretation would benefit from additional qualitative and quantitative support. We have expanded the snRNA-seq analysis with additional images and supplemental figures (Figure 1 – figure supplement 3, Figure 1 – figure supplement 5, and Figure 1 – figure supplement 6). The first and third of these new supplemental figures show dot plots for each major leptomeningeal cell type, for each genotype, for the two experimental conditions (infected vs. uninfected), and for individual genes in three immune-related gene sets (NF- κ B and TNF- α , JAK-STAT, and IFN- γ), providing a more explicit comparison of infection-induced transcriptional responses across genotypes. The second of these new supplemental figure shows principal component analysis (PCA) of the individual snRNA-seq datasets (one mouse per dataset) for each genotype and experimental condition, demonstrating that the observed transcriptional shifts are consistent across biological replicates. Finally, Figure 1 – figure supplement 7, which was included in the original submission, shows changes in the most up- and down-regulated genes (based on adjusted p-value or fold change) in endothelial and myeloid cells across individual mice and genotypes/conditions, further supporting the genotype-dependent effects at the level of individual animals.

(2) The authors interpret *E. coli* infection-induced increases in leptomeningeal sulfo-NHSbiotin as evidence of compromised BBB integrity (i.e., extravasation from the vasculature) (Results, page 7), but another possible route in this context is sulfo-NHS-biotin entry from the dura across a compromised arachnoid barrier. The complete rescue in Tlr4ECKOs is strongly suggestive that the vascular route dominates, but it would strengthen the work if the authors could assess arachnoid barrier fidelity (e.g. via immunohistochemistry). At a minimum, authors should mention that the sulfo-NHS-biotin signal in this context may represent both vascular and arachnoid barrier extravasation.

Thank you for this comment. We agree that our data cannot rule out leakage across the arachnoid barrier during infection. While the rescue observed in Cdh5-CreER; Tlr4CKO (Tlr4^{VEKO}) mice strongly supports a dominant vascular contribution, we acknowledge that the sulfo-NHS-biotin signal may reflect permeability at both the vascular and arachnoid

barriers. We do not think there is a clear way to directly test this possibility functionally, since the arachnoid barrier appears intact by confocal microscopy. Subtle differences in barrier cell morphology might be detectable by electron microscopy, but this would not definitively address whether infection permits molecular passage across the arachnoid barrier. We have followed the reviewer's suggestion and revised the Results section to reflect this interpretation. Specifically, we added the following: "The simplest interpretation of these data is that the site of sulfo-NHS biotin leakage is primarily vascular. However, we cannot exclude some contribution from increased arachnoid barrier permeability."

(3) The authors state that "deletion of TLR4 prevented both NF- κ B nuclear translocation and Cldn5 internalization in response to E. coli (Figure 4A-D)" (Results, page 9). In Figures 4C and D, however, there is no indicator of a statistical test directly comparing the two genotypes. A comparison of within-genotype P-values should not be used to support a genotype difference (PMID: 34726155).

Thank you for pointing out this omission. We have updated the figures so that the between-genotype p-values are shown for those panels (including this panel) that had not previously shown them.

(4) In the first paragraph of the Results, the authors summarize the meningeal layers as (1) pia, (2) subarachnoid space, (3) arachnoid, and (4) dura, and then state "The second and third layers constitute the leptomeninges." This definition of leptomeninges seems to omit the pia, which is widely considered part of the leptomeninges (PMID: 37776854).

Thank you for pointing out this error, which has now been corrected.

(5) The Cdh5-CreER⁺;Tlr4 fl⁻ mouse lacks TLR4 in all endothelial cells (i.e., in peripheral organs as well as CNS/leptomeninges), and, as the authors note, the periphery is exposed to E. coli. It would be helpful if the authors could comment in the Discussion on the possibility that peripheral effects (e.g., peripheral endothelial cytokine production, changes to blood composition as a result of changes to peripheral endothelial permeability) may contribute to the observed leptomeningeal phenotypes.

Thank you for raising this point. We agree that peripheral responses could contribute to the observed leptomeningeal phenotypes in this model. We have added two sentences to the second paragraph of the Discussion to address this: "We note that these experiments do not distinguish between local vs. distal anatomic sources of LPS or downstream effector molecules, such as cytokines, that activate the leptomeningeal inflammatory response (Huang et al., 2021). Histologic observations of RFP-expressing *E. coli* in the brain, liver, and lungs, together with positive blood cultures, indicate substantial systemic dissemination in this model. Thus, the inflammatory responses of leptomeningeal cells likely reflect exposure to bacterial products and inflammatory mediators derived from both local meningeal and peripheral sources."

Reviewer #2 (Public review):

Summary:

The authors use a postnatal mouse model of E. coli bacterial meningitis and a mouse brain endothelioma cell line combined with cell-type-specific gene deletion to study the function of endothelial TLR4, a cell surface receptor that recognizes gram positive bacterial wall components, in the local leptomeningeal (LPM) response with a focus on endothelial barrier breakdown mediated by TLR4. Single-cell transcriptional profiling and imaging studies using whole-mount preps of the LPM support that LPM endothelial, CD206⁺ local macrophage and LPM fibroblast and arachnoid barrier cell inflammatory response and is abrogated in endothelial-specific KO of TLR4, pointing to a role for endothelial TLR4 in local LPM response. Culture studies using Bend3.1 cells (a mouse

brain endothelioma cell line) support a direct role for TLR4 in the bacteria-mediated inflammatory response and in internalization of Cldn5 via the endosomal-lysosomal pathway, resulting in loss of barrier integrity

Strengths:

The local LPM cell response in meningitis and the role of specific LPM cells in inflammation and CNS barrier breakdown have not been extensively studied, despite ample evidence for primary immune response in the meninges in human patients and in animal models. The authors employ a robust, multi-model approach using both in vivo and in vitro models with cell-type-specific knockout to study the function of TLR4 in brain endothelial cell response. The authors nicely combine functional barrier assays with IF for junctional localization in their experimental design, and they delve into potential mechanisms of Cldn5 internalization using markers of endosomal-lysosomal pathway localization. The authors also describe a new type of barrier assay using a streptavidin-coated plate upon which barrier-forming cell cultures can be plated, this could be a very useful alternative or complement to other size-selective barrier assays and presumably could work for other barrier forming cells types, likely epithelial cells.

Weaknesses:

(1) There are no measures of bacterial burden in peripheral organs, blood, in the LPM or brain in the TLR4 endothelial cKO mice. Lack of TLR4 in endothelial cells could prevent bacterial 'access' into the LPM and brain, essentially preventing meningitis and leading to a lack of inflammatory responses in the LPM-located cells simply because there is no bacteria present. Bacteremia may also be reduced, as might inflammatory responses in peripheral organs with TLR4-deficient peripheral endothelium. Bacterial counts and inflammatory measures in peripheral organs and blood are important to better understand the mechanism(s) underlying the reduced inflammatory profile in LPM cells and no LPM endothelial breakdown in the Tlr4 endothelial cKO mice. In other words, does deleting TLR4 in EC protect against the development of meningitis by somehow blocking bacteria access to the LPM (this would be supported by low or no CFU counts in infected Tlr4 endothelial cKO) or is it what the authors appear to propose in Figure 1J that TLR4 in EC is the only cell responding to the bacteria to trigger the immune cascade in the LPM? More data is needed to resolve this, as this is a major claim of the paper.

Thank you for this comment. We agree that it is important to distinguish whether the reduced inflammatory response in Cdh5-CreER; Tlr4CKO (*Tlr4*^{VEKO}) mice reflects altered bacterial burden versus altered host sensing. We have fleshed out these issues by conducting the following comparisons between infected and uninfected WT and infected and uninfected Cdh5-CreER; Tlr4CKO mice: (1) quantifying *E. coli* in the blood of infected mice by counting colonies on agar plates; (2) quantifying *E. coli* in the brain by measuring red fluorescent protein (RFP) signal (the infecting *E. coli* carry an RFP-expression plasmid); (3) histologically surveying liver and lung for RFP+ *E. coli*; (4) monitoring the weights of infected and uninfected mice. These data are presented in Figure 2 – figure supplement 4 and in the Results section, and they can be summarized as follows. (1) *E. coli* is consistently detectable in the blood, brain, and peripheral organs of infected mice and is not detectable in control mice; (2) there are no statistically significant differences between infected WT and infected Cdh5-CreER; Tlr4floxed mice in *E. coli* burden; (3) infected mice of both genotypes stop gaining weight between the time of infection (P5) and the time of sacrifice 24 hours later (P6). Our conclusion is that loss of TLR4 in endothelial cells and in a subset of other non-myeloid leptomeningeal cells does not alter the overall clinical course of the infection despite changes in leptomeningeal gene expression and vascular permeability.

(2) The authors look at the underlying cortical response (cerebral vasculature for ICAM and immune cells) but do not use markers that could identify microglia (*Iba1*), the

primary resident immune cell (CD206 is not useful, at this stage, in perivascular macrophages that are extremely sparse in the postnatal brain). This would be important to better study the impact on CNS resident immune cell morphological activation.

Thank you for this comment. In response, we have analyzed Iba1 staining in the cortex in infected vs. uninfected mice. This is shown in Figure 2 – figure supplement 3. These data demonstrate a several-fold increase in Iba1 immunostaining in infected compared to uninfected cortex, consistent with increased microglial activation in response to infection. There is no statistically significant difference between infected WT and infected Cdh5-CreER; Tlr4CKO mice in Iba1 staining in cortex.

(3) The authors suggest that Cldn5 junctional localization is selectively disrupted upon bacterial exposure, mediated by TLR4 - they suggest this based on studying PECAM, GLUT1, ZO-1 and B-catenin (all normally junction or cell surface located in cultured Bend3.1) in relationship to Cldn5 localization (normally high) - it is possibly these are also impact by bacteria exposure (maybe through different mechanisms?) - a better measure would be to use the similar cyto/PM measure they do for Cldn5 in Fig. 4D and to evaluate this or to use intensity measurements.

Thank you for this comment. As the reviewer noted, the analysis of Cldn5 localization with vs. without *E. coli* exposure and in WT vs. Tlr4KO bEnd.3 cells (shown in Figure 4B and D) – uses Cell Trace to partition the image into cytoplasmic vs. plasma membrane territories. For the analyses in Figure 5, we wanted to compare the localization (and potentially re-localization) behaviors of a variety of subcellular markers with the localization and re-localization of Cldn5 following *E. coli* exposure. By directly measuring the % overlap of the two immunostains, we get that data. We note that the goal of this analysis is to assess relative co-localization with Cldn5 rather than absolute subcellular partitioning of each marker. While this analysis could have been extended to include independent quantification of the subcellular localization of each of those other markers with respect to cytoplasmic vs. plasma membrane territories, it is clear by visual inspection of Figure 5A-C that beta-catenin, ZO-1, and PECAM1 remain plasma membrane-associated with *E. coli* exposure, and GLUT1 goes from the part of the plasma membrane not involved in cell-cell contact without *E. coli* exposure to cytoplasmic with *E. coli* exposure (as judged by the appearance of a nuclear “shadow” after *E. coli* exposure). Thus, we do not believe that additional cytoplasmic vs. plasma membrane quantification for these markers would alter the interpretation. The main reason that we did not extend this analysis to include independent quantification of the subcellular localization of each of those other markers with respect to cytoplasmic vs. plasma membrane territories is because that would introduce the Cell Trace localization as an additional variable.

(4) The discussion could benefit from delving more into the prior literature on E coli mediated breakdown of junctions in cultured human microvascular brain endothelial cell model and critical host-pathogen interactions of the bacteria with ECs (PMID: 14593586), and how this might involve TLR4.

Thank you for this comment. Two paragraphs addressing the prior literature have now been added to the discussion.

(5) It would be important to discuss how their results relate to earlier studies on TLR4^{-/-} and TLR2^{-/-} global knockout mice and protection vs vulnerability to development of meningitis (see PMCID: PMC3524395) - this paper showed that TLR4 global KO mice have increased susceptibility to die from meningitis and have much higher CFU counts in the CNS. In this manuscript and their prior work (Wang et al., 2023), this group shown that both global TLR4^{-/-} mutants and their EC-specific KO have reduced barrier permeability, but we don't have any information about CFU or susceptibility to death from meningitis in their models.

Thank you for these comments. The model we use – subcutaneous injection of *E. coli* (a clinical isolate from an infant with meningitis) at postnatal day (P)5 – results in the death of the infected mouse within 2 days (shown in Figure 1 – figure supplement 3 in Wang et al. 2023). Our analyses of infected mice were conducted 24 hours after infection. As noted in the reply to comment #1, in the revised manuscript we present a clinical assessment of WT vs. Cdh5-CreER; Tlr4CKO mice 24 hours after infection based on (1) a quantitative microscopic analysis of *E. coli* burden in the brain (visualized based on RFP fluorescence in the *E. coli* used here), (2) quantifying CFUs in blood and (3) mouse weights at P5 and P6, a sensitive indicator of overall health since this is a time when mice are normally gaining weight rapidly (~25% weight gain per day). These data (shown in Figure 2 figure supplement 4) indicate that bacterial burden and disease severity are similar between genotypes in our model. In Wang et al., 2023, we did not conduct a quantitative clinical assessment of WT vs. Tlr4^{-/-} mice following infection, but by visual inspection, infected WT and Tlr4^{-/-} mice appeared to have similar downhill clinical trajectories. We have expanded the Discussion to relate these findings to prior studies of global TLR4 and TLR2 knockout mice, noting that differences in experimental models and the distinction between global versus VECadCreER-specific deletion may account for the differing outcomes reported.

Comment on the paper listed by the reviewer (PMCID: PMC3524395).

The cited study demonstrates that global TLR4 deficiency leads to increased bacterial burden and mortality, indicating an essential role for TLR4 in host defense and bacterial clearance. In our study of Cdh5-CreER; Tlr4CKO mice, bacterial burden and disease severity at 24 hours post-infection are similar between WT and Cdh5-CreER; Tlr4CKO mice, indicating that Cdh5-CreER; Tlr4CKO does not alter the clinical course at this time point. This difference is noted in the Discussion section.

Reviewer #3 (Public review):

Summary:

This study investigates the molecular underpinnings of immune responses in the leptomeninges in neonatal bacterial meningitis. Bacterial meningitis is a major disease burden, particularly for neonates, and it has previously been noted that the meningeal immune environment in infants is permissive to opportunistic infection (Kim et al., Sci Immunol, 2023). There is less known about the contribution of the stromal compartment to meningeal immune responses. Seegren et al. interrogate the role of leptomeningeal endothelium in host defence in E. coli infected neonatal mice using mouse genetic tools to delete the LPS receptor Tlr4 from either endothelial cells (using Cdh5-CreER) or macrophages (using LysM-Cre). The authors use snRNAseq, cleared cortical mounts, and in vitro work to define the impact of E. coli infection on leptomeningeal endothelial cells. This study uses a range of innovative techniques to probe the role of the stromal compartment in meningitis.

Strengths:

This study makes excellent use of cleared cortical mounts to examine the biology of the leptomeninges, in particular, changes to the endothelium, with unprecedented detail. In combination with high-quality sequencing data provide new insights into the impact of meningitis on the leptomeninges. The data presented by the authors is of very high quality.

Weaknesses:

The weaknesses of the study were in terms of interpretation and perhaps study design.

(1) Most importantly, the authors need to provide additional validation of their conditional knockout models. The authors need to confirm that the Cdh5-CreER does not impact leptomeningeal fibroblasts and to confirm gene deletion in macrophages.

We are very grateful for this critique. After several years of using the Cdh5-CreER line in other parts of the CNS, where its expression is endothelial-specific, we applied it to the meninges without realizing that its specificity is broader in that tissue. Our initial analysis with a Cre reporter line that uses a membrane tdTomato appeared to confirm endothelial-specific recombination in the meninges. Following receipt of the reviews of this manuscript, we repeated this analysis with two Cre reporter lines that use a nuclearlocalized GFP, and we immunostained for each of several transcription factors to assess various meningeal cell types and quantified GFP co-localization (Figure 1 – figure supplements 1 and 2). This quantitative Cre reporter analysis shows CreER expression from the Cdh5-CreER transgene in all or nearly all endothelial cells and in a subset (~20%) of dural border cells and/or leptomeningeal fibroblasts, but not in myeloid cells. Additionally, our snRNA-seq analysis of *Cdh5* transcripts shows expression in endothelial cells, dural border cells, and leptomeningeal fibroblasts, but not in myeloid cells (Figure 1– figure supplement 4), which agrees with several recent publications (Mapunda et al., 2023; Pietilä et al., 2023; Smyth et al., 2024). Thus, our initial interpretation that the phenotypes in the Cdh5-CreER; Tlr4floxed mouse were a consequence of recombination exclusively in endothelial cells was not quite correct. The Results section of the revised manuscript includes an expanded description of Cre and CreER expression specificity analysis, with supporting data in Figure 1 – figure supplements 1 and 2. Throughout the text of the revised manuscript, we are careful to note that the Cdh5-CreER; Tlr4floxed mouse has Tlr4 deletion in a subset of dural border cells and leptomeningeal fibroblasts. To reflect this fuller understanding of the specificity of Cdh5-CreER, we have changed the name of the Cdh5-CreER; Tlr4floxed mice in the text and figures from TLR4ECKO (“endothelial cell KO”) to TLR4VEKO (“VE-cadherin CreER KO”).

(2) The authors could also strengthen the paper by providing data on the impact of these conditional knockout models on the course of meningitis and bacterial burden.

Thank you for this comment. We agree that these additional analyses strengthen the manuscript. We have fleshed out these issues by conducting the following comparisons between infected and uninfected WT and infected and uninfected Cdh5-CreER; Tlr4floxed mice: (1) quantifying *E. coli* in the blood of infected mice by counting colonies on agar plates; (2) quantifying *E. coli* in the brain by measuring the red fluorescent protein (RFP) signal (the infecting *E. coli* carry an RFP-expression plasmid); (3) histologically surveying liver and lung for RFP+ *E. coli*; (4) monitoring the weights of infected and uninfected mice. These data are presented in Figure 2 – figure supplement 4 and in the Results section, and they can be summarized as follows. (1) *E. coli* is consistently detectable in the blood, brain, and peripheral organs in infected mice and is not detectable in control mice; (2) there are no statistically significant differences in bacterial burden between infected WT and infected Cdh5-CreER; Tlr4floxed mice; (3) infected mice of both genotypes stop gaining weight between the time of infection (P5) and 24 hours later at the time of sacrifice (P6). Our conclusion is that loss of TLR4 in endothelial cells and in other non-myeloid cells in the leptomeninges does not alter

the overall clinical course of the infection despite changes in leptomeningeal gene expression and vascular permeability.

(3) Finally, it is perhaps not surprising that Tlr4 is required for meningitis responses with E. coli. However, it is unclear if these findings can be generalised to other, more common, meningitis infections (streptococcal/pneumococcal).

At present, it is an open question whether TLR4 plays as a large a role in meningitis caused by other gram-negative bacteria and whether TLR2 plays a similarly large role in meningitis caused by gram-positive bacteria. In the Discussion, the last two sentences under “Limitations of the study” summarize this point: “Finally, the present study focused on *E. coli* K1, the dominant Gram-negative neonatal pathogen. Future work could assess TLR signaling in response to other bacterial pathogens, such as Group B *Streptococcus*.”

(4) There are additional minor issues; for instance, the arachnoid fibroblast 2 population appears to closely resemble dural border cells.

Thank you for this comment. That is correct, and we have changed the nomenclature to “dural border cells”.

(5) The cell line model (bEnd.3) is a relatively low-fidelity model of BBB endothelial cells, and this should be acknowledged.

Thank you for this comment. That is correct. Despite being brain-derived, bEnd.3 cells have lost many BBB-specific attributes. Their responses might best be considered as generic endothelial responses rather than brain-specific endothelial responses. This is now stated in the Results section: “Although they are brain-derived, bEnd.3 cells lack many BBB-specific attributes and, therefore, they likely exhibit generalized endothelial responses rather than brain-specific responses to bacterial exposure.”

With these caveats, it is difficult to be certain that the endothelium alone is the driver of meningeal immune responses in meningitis, and what the impact of these is.

We agree with this critique. As noted above, the expression of Cdh5-CreER in essentially all endothelial cells and in a subset of dural border cells and leptomeningeal fibroblasts means that the comparison of TLR4 CKO with Cdh5-CreER vs. Lyz2-Cre is assessing phenotypes driven by TLR4 signaling in endothelial plus a subset of other non-myeloid cells vs. TLR4 signaling in myeloid cells. We have revised the text to reflect this more precise understanding of Cdh5-CreER specificity.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

(1) Transcriptomic analysis: The analysis and display of the single-nucleus RNA-seq data should be improved. The authors could perform a more granular, unbiased clustering of each cell class in the combined dataset and then compare the proportion of each experimental group (genotype x control/infected) in each cluster. At present, it appears the differentially-expressed genes (DEGs) shown in Figure 1 were identified using the Seurat FindMarkers function with default parameters (Methods). This considers each cell as an independent experimental unit and is therefore not appropriate for a comparison of control versus infected groups (see e.g., PMID 34584091, 35880426. The authors should implement a statistical analysis strategy that considers true biological replicates (mice, as shown in Supplementary File 1).

We do not fully agree with this critique. We agree that biological replication at the level of individual mice is important for interpreting these data, but within each mouse, the characteristics of individual cells is also of interest, including the degree of heterogeneity, the

sample size for a given cell cluster, and the statistical significance of any observed changes in transcript abundance. As requested, we have prepared a new supplemental figure (Figure 1 – figure supplement 5) showing a principal component analysis of the scRNA-seq data for each mouse (one mouse was used for each snRNA-seq dataset) and for each of the principal leptomeningeal cell types. This analysis shows, for example, that the three infected Cdh5-Cre; Tlr4flox/- mice have transcriptomes for each of the six cell clusters that are very similar to the transcriptomes of the two uninfected WT and the two uninfected Cdh5-CreER; Tlr4flox/- mice. Thus, the genotype- and condition-dependent effects are consistent across biological replicates. At the most granular level, Figure 1 – figure supplement 7, which was part of the original submission, shows for the most up- and down-regulated genes (based on adjusted p-value or based on fold-change) in endothelial cells and in myeloid cells how individual transcript abundances change for each mouse and for each genotype/condition.

(2) The authors use immunohistochemistry to assess claudin-5 "disorganization and redistribution" (Results, pages 7-8 and Figures 3A-B). They state that "Tlr4ECKO mice showed minimal changes in the distribution of Cldn5, implying that cell autonomous endothelial TLR4 signaling regulates tight-junction organization." It is not clear, however, that the quantified parameter (Cldn5+ area relative to total area) would be an accurate readout of claudin-5 organization/distribution (i.e., subcellular localization) as it would also be sensitive to claudin-5 expression, vascular density, and vessel diameter. The authors use a similar assessment of ZO-1 to suggest that changes to claudin-5 are not due to a "generalized disassembly of TJs" and could also use this to argue that the above potential confounds (vascular density, vessel diameter) do not change, but the data in Figure 3 - Figure Supplement 1B, lower panel, show that infection does cause an increase in ZO-1 area relative to total area (P = 0.0004). Thus, the statement in the results "Zonula Occludens-1 (ZO-1) [...] remained unchanged during infection (Figure 3 - figure supplement 1)" is not accurate. The authors should revise this section to ensure their conclusions are aligned with the presented data.

Thank you for this comment. The reviewer is correct that the Cldn5 area measurement is unable to deconvolve the various factors that might contribute to it (vessel density and diameter, and Cldn5 distribution). This part has been rewritten. "Consistent with prior findings (Wang et al., 2023), both WT and *Tlr4*^{MKO} mice showed an increase in the area occupied by Cldn5 in the leptomeninges following infection, likely referable to both increased vessel diameter and a redistribution of Cldn5 within ECs (Figure 3A-B; Figure 3 – figure supplement 1C)."

The reviewer is also correct about our initial description of the ZO-1 data. What we meant to write and what the revised manuscript now shows is: "The area occupied by Zonula Occludens-1 (ZO-1), a tight junction scaffold protein, showed a modest but statistically significant increase in WT leptomeningeal vessels but no significant change in *Tlr4*^{VEKO} leptomeningeal vessels during infection (Figure 3 – figure supplement 1A and B)."

(3) In the Methods, under Mouse Models and E. coli Infection, the authors state, "The Cdh5-CreER line (Monvoisin et al., 2023) was the same line used in Wang et al. (2023)." However, there is no mention of Cdh5-CreER in Wang et al. (2023). Could authors please clarify? Also, because this appears to be an inducible Cre, the authors must include details on the dose and timing of tamoxifen or 4-OHT used in this study.

Thank you for catching that error. We meant to reference Wang et al (2025), not Wang et al (2023). [Wang et al (2025) is: Wang Y, Rattner A, Li Z, Smallwood PM, Nathans J. (2025) Vascular endothelial-specific loss of TGF-beta signaling as a model for choroidal neovascularization and central nervous system vascular inflammation. Elife 14:RP107018.] This has now been corrected.

We have now included the details related to 4HT injection in the Methods section “Mouse Models and *E. coli* Infection”. These are intraperitoneal injection at P2 with 40- 50 μ L of 2 mg/ml 4HT.

(4) The legend for Figure 1A is “Schematic of the leptomeninges”, but the figure shows the entire brain-skull interface, including underlying cortex, leptomeninges, dura, and skull.

Thank you. Corrected.

(5) Page 7, typo: “In the brain, CD206+ cell were too sparse ...” Should be “cells”.

Thank you. Corrected.

(6) Page 14, typo: “... could represents a double-edged ...” Should be “represent”.

Thank you. Corrected.

Reviewer #2 (Recommendations for the authors):

(1) Perform CFU counts from LPM, dura, brain, peripheral organs (liver) in infected v mock mice from control v TLR4 EC-cKO.

Thank you for this comment, with which we agree. We have addressed this by quantifying bacterial burden and assessing disease severity in WT and Cdh5CreER; Tlr4floxed mice. Specifically, we performed CFU measurements in blood, monitored mouse weights at P5 and P6, and histologically surveyed the *E. coli*-RFP signal (i.e., *E. coli* burden) in brain, liver, and lung. These analyses show that bacterial burden and disease progression are comparable between WT and Cdh5-CreER; Tlr4floxed mice at 24 hours post-infection. These data are presented in Figure 2 – figure supplement 4 and described in the Results.

(2) *Lyz2Cre/+* is used to delete TLR4 from macrophages, but recombination efficiency (in LPM BAMs) is described as only partial, suggesting that TLR4-response in LPM BAMs (and potentially macrophages in the dura) is at least partially intact. It undercuts conclusions that can be made using this line.

Thank you for this comment. We have conducted a more detailed analysis of *Lyz2^{Cre}* specificity by immunostaining for multiple markers and quantifying the results (Figure 1 – figure supplement 2). We now think that the more cursory analysis in the original submission was inaccurate. The more in-depth analysis shows that *Lyz2^{Cre}* directed Cre-recombination with 90-100% efficiency in CD206+ cells and with 50-70% efficiency in ASC+ and PU.1+ cells, the range depending on whether tdTomato or GFP colocalization was being scored (Figure 1 – figure supplement 2). The Results section text now states: “In the text that follows, we will refer to *Lyz2^{Cre}*-recombined cells simply as “myeloid cells”, although they should be understood as CD206+ myeloid cells.”

Also, as noted in the reply to comment 4 below, a direct analysis of Tlr4 recombination efficiency is technically challenging due to the low abundance of TLR4 and the failure, in our hands, of commercial anti-TLR4 antibodies to produce clear immunostaining. We have added a comment in the results section noting that we do not have a measure of the efficiency of recombination of the floxed Tlr4 target *in vivo*: “The low abundance of TLR4 and the limitations of commercial anti-TLR4 antibodies precluded a direct immunohistochemical assessment of TLR4 loss in *Tlr4^{VEKO}* and *Tlr4^{MKO}* mice.”

(3) Inflammatory responses [qPCR] from peripheral organs and also physiological measures in the pups [weight post-infection, time to moribund or death curves] in control v TLR4 EC-cKO and TLR4 mac-cKO.

Thank you for this comment. We have not conducted a qPCR analysis of inflammatory gene expression in peripheral organs because (1) the dramatic upregulation of these transcripts in the leptomeninges, (2) the presence of *E. coli* in blood and peripheral organs, and (3) the clinical assessment (cessation of weight gain) all predict that such an analysis would reveal a large up-regulation of inflammatory gene expression throughout the body. More specifically, we have conducted the following comparisons between infected and uninfected WT and infected and uninfected Cdh5-CreER; Tlr4floxed mice: (1) quantifying *E. coli* in the blood of infected mice by counting colonies on agar plates; (2) quantifying *E. coli* in the brain by measuring the red fluorescent protein (RFP) signal (the infecting *E. coli* carry an RFP-expression plasmid); (3) histologically surveying liver and lung for RFP+ *E. coli*; (4) monitoring the weights of infected and uninfected mice. These data are presented in Figure 2 – figure supplement 4 and in the Results section, and they can be summarized as follows. (1) *E. coli* is consistently detectable in the blood, brain, and peripheral organs in infected mice and is not detectable in control mice; (2) there are no statistically significant differences between infected WT and infected Cdh5CreER; Tlr4floxed mice; (3) infected mice of both genotypes stop gaining weight between the time of infection (P5) and 24 hours later at the time of sacrifice (P6). Our conclusion is that loss of TLR4 in endothelial cells and in a subset of other non-myeloid cells in the leptomeninges does not alter the overall clinical course of the infection despite changes in leptomeningeal gene expression and vascular permeability.

(4) The conditional macrophage line is problematic due to the partial recombination. I question the utility of including this unless they can come up with a way resolve the response of recombined TLR4 macrophages vs ones that are not (could they use the single cell data to pick this a part? Are TLR4-null cells and TLR4 'wt' cells transcriptionally similar in the infected condition, suggesting TLR4 is not doing much in the macs, potentially due to alternate TLRs?). There are good BAM Cre lines that have been described [Lyve1-cre would be good for LPM BAMS, the other is Pf4-cre, see <https://pmc.ncbi.nlm.nih.gov/articles/PMC7375817/> - just as an FYI for the future].

Thank you for this comment. As noted in the reply to point 2 (above), we have conducted a more in-depth analysis of *Lyz2^{Cre}* specificity by immunostaining for multiple markers and quantifying the results (Figure 1 – figure supplement 2). We now think that the more cursory analysis in the original submission was inaccurate. The more in-depth analysis shows that *Lyz2^{Cre}* directed Cre-recombination with 90-100% efficiency in CD206+ cells and with 50-70% efficiency in ASC+ and PU.1+ cells, the range depending on whether tdTomato or GFP colocalization was being scored (Figure 1 – figure supplement 2). The text now states: “In the text that follows, we will refer to *Lyz2^{Cre}*-recombined cells simply as “myeloid cells”, although they should be understood as CD206+ myeloid cells.”

We agree that, based on Figure 6 in the cited paper [McKinsey et al (2020) A new genetic strategy for targeting microglia in development and disease eLife 9:e54590], the Pf4-Cre line may be superior to the *Lyz2^{Cre}* line that we used for recombination in leptomeningeal myeloid cells. Unfortunately, we missed this paper in our literature searches, probably because it focuses on a microglial CreER line, P2ry12-CreER, and the Pf4-Cre line is not mentioned in the title or abstract. Our decision to use the *Lyz2^{Cre}* line was based on an extensive comparison among myeloid Cre lines showing that *Lyz2^{Cre}* was the most efficient [Abram CL, Roberge GL, Hu Y, Lowell CA. 2014. Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. J Immunol Methods 408:89-100.] However, the Abram et al study did not look at the leptomeninges. Regarding the efficiency of recombination of the floxed Tlr4 target, a direct analysis is technically challenging due to the low abundance of TLR4 and the failure, in our hands, of commercial anti-TLR4 antibodies to produce clear immunostaining. We have added a comment in the results section noting that we do not have a measure of the efficiency of recombination of the floxed Tlr4 target: “The low abundance of TLR4 and the limitations of

commercial anti-TLR4 antibodies precluded a direct immunohistochemical assessment of TLR4 loss in *Tlr4*^{VEKO} and *Tlr4*^{MKO} mice.”

(5) Figure 1 - Figure Supplement 2 - the authors nicely break down the pathway response [NFKB and TNF] in EC and macs, it would be great to have similar information for the fibroblasts (in the main figure or the supplement). Does their inflammatory response show a similar pattern?

Thank you for this suggestion. We have now done that analysis and present it in Figure 1 – figure supplement 3. For completeness, we also performed the same type of analyses for JAK-STAT signaling and IFN-gamma response and these are shown in Figure 1 – figure supplement 6. The principal conclusion is that across all major leptomeningeal cell types, the *Cdh5*-CreER; *Tlr4* floxed samples (i.e., *Tlr4* KO'd in non-myeloid cells) show much reduced transcriptome changes with infection.

(6) What is ASC and Cd206 quantification measuring, and how does this relate to 'activation' - is this the intensity of signal or a morphological change? What is the precedence for using ASC (citations)? In their prior work, they showed no change in CD206 number, so a significant increase upon infection here, it's confusing exactly what is being studied. Also, loss of Lyve1 is a well-accepted measure of activation that they have previously used, adding that it could be helpful. This is not a major issue since they have robust data that the macrophages are not transcriptionally activated. Clarification of what exactly is being measured would be sufficient (in the text).

CD206 immunostaining, which reveals myeloid cell morphology, shows that, with *E. coli* infection, myeloid cells convert from a more compact morphology to a more expanded morphology. This is now explained more fully in the Results section.

Regarding ASC, changes in the state of ASC aggregation and ASC subcellular localization have been used by others to monitor immune cell responses to inflammatory signals (Sester et al., 2016; Franklin et al., 2018). While this change in subcellular localization may explain part of the increase in immunostained area in myeloid cells in the infected mice (Figure 2D), the increase in the area of ASC immunostaining largely reflects a shift of myeloid cells from a compact to a more extended morphology. This is now explained more fully in the Results section. We have also added two references (Sester et al., 2016; Franklin et al., 2018) that described how ASC distribution changes with inflammation.

Regarding LYVE1, we observe a decrease in LYVE1 transcript abundance in myeloid cells with infection, as predicted. Given the large amount of other data that document myeloid activation with infection, we have elected not to include this.

(7) The authors suggest the internalization of Cldn5 is not due to NFKB downstream signaling that includes transcriptional mechanisms because it happens as early as 1 hour, prior to NFKB localization to the nucleus. However, a lot of their experiments, including on endosomal-lysosomal protein co-localization are done at 4 hours, when their RNAseq data show robust NFKB-mediated gene upregulation and (though not tested) potentially protein production of factors that can act back on the cells, including to impact endo-lysosomal processing. Without studies at earlier timepoints post-bacteria exposure, separating these two mechanisms is difficult.

Thank you for this comment. We have explored this question by looking at Cldn5 internalization in bEnd.3 cells at 1 hour after *E. coli* exposure, and the data clearly show that internalization occurs within 1 hour. Additionally, we have conducted this experiment in the presence of 1 μ M ACHP, an IKK inhibitor that blocks NF- κ B migration to the nucleus. ACHP treatment shows no effect on the rapid internalization of Cldn5, implying a mechanism independent of NF- κ B control of gene expression. These data are shown in a new figure (Figure 6) in the revised manuscript.

(8) Figure 2 - CD206 are quite sparse however, Iba1 would work well to look at microglial activation.

Thank you for this suggestion, which we have followed. To assess microglial activation, we have immunostained for Iba1 and quantified the data. These are now included in Figure 2 – figure supplement 3. The data show that there is an increase in Iba1 immunostaining following *E. coli* infection in both WT and Cdh5-CreER; Tlr4 floxed mice, with more in the former than the latter, but the difference is not statistically significant.

(9) Suggest performing the LAMP+ co-localization experiment at <1hr, prior to NF κ B nuclear localization and transcriptional changes. This would better support it, this is (or is not) independent of the NF κ B. Could also test this with an NF κ B inhibitor, do they still see the CLDN5 internalization when NF κ B is blocked?

Thank you for these suggestions. We have done both of these analyses, and the results are presented in Figure 6. The results show that (1) Cldn5 is internalized within 1 hour and (2) its internalization is independent of NF- κ B signaling inhibition by 1 μ M ACHP. Since ACHP treatment shows no effect on the rapid internalization of Cldn5, that implies a mechanism independent of NF- κ B control for gene expression.

Reviewer #3 (Recommendations for the authors):

Major points

(1) The most important caveat is that the Cdh5-CreER model is known to recombine in leptomeningeal fibroblasts (10.1038/s41586-023-06993-7, 10.1101/2025.05.13.653681), and Cdh5 expression in these populations is now well described (10.1038/s41467-02341580-4, 10.1016/j.neuron.2023.09.002). Although the authors did not observe recombination in their reporter (details of the tamoxifen injection protocol should be provided), it is imperative to validate the specificity of their model to Tlr4 in endothelial cells, leveraging their sequencing data and providing additional IHC or ISH to confirm this. Alternatively, Tlr4 could be deleted in a more specific model, e.g., the Pdgfb-iCreERT2 or Slco1c1-CreERT2. It is also important to do the same with the LysM model, to confirm that the lack of impact of macrophage Tlr4 is not due to failure to delete the gene. This is again important to the interpretation of the study, since the authors propose that the endothelium, specifically, is the driver of the meningitis response.

We are very grateful for this critique. After several years of using the Cdh5-CreER line in other parts of the CNS, where its expression is endothelial-specific, we applied it to the meninges without realizing that its specificity is broader in that tissue. Our initial analysis with a Cre reporter line that uses a membrane tdTomato appeared to confirm endothelial-specific recombination in the meninges. Following receipt of the reviews of this manuscript, we repeated this analysis with two Cre reporter lines that use a nuclear-localised GFP, and we immunostained for each of several transcription factors to assess various meningeal cell types and quantified GFP co-localization (Figure 1 – figure supplements 1 and 2). This quantitative Cre reporter analysis shows CreER expression from the Cdh5-CreER transgene in all or nearly all endothelial cells and in a subset (~20%) of dural border cells and/or leptomeningeal fibroblasts, but not in myeloid cells. Additionally, our snRNA-seq analysis of

Cdh5 transcripts shows expression in endothelial cells, dural border cells, and leptomeningeal fibroblasts, but not in myeloid cells (Figure 1– figure supplement 4), which agrees with several recent publications (Mapunda et al., 2023; Pietilä et al., 2023; Smyth et al., 2024). Thus, our initial interpretation that the phenotypes in the *Cdh5*-CreER; Tlr4floxed mouse were a consequence of recombination exclusively in endothelial cells was not quite right. The Results section of the revised manuscript has an expanded description of Cre and CreER expression specificity analysis, with supporting data in Figure 1 – figure supplements 1 and 2. Throughout the text of the revised manuscript, we are careful to note that the *Cdh5*-CreER; Tlr4floxed mouse has Tlr4 deletion in a subset of dural border cells and leptomeningeal fibroblasts. To reflect this fuller understanding of the specificity of *Cdh5*-CreER, we have changed the name of the *Cdh5*-CreER; Tlr4floxed mice in the text and figures from TLR4ECKO (“endothelial cell KO”) to TLR4VEKO (“VE-cadherin CreER KO”).

We have also conducted a more detailed analysis of *Lyz2*^{Cre} specificity by immunostaining for multiple markers and quantifying the results (Figure 1 – figure supplement 2). We now think that the more cursory analysis in the original submission was inaccurate. The more in-depth analysis shows that *Lyz2*^{Cre}-directed Cre-recombination with 90-100% efficiency in CD206+ cells and with 50-70% efficiency in ASC+ and PU.1+ cells, the range depending on whether tdTomato or GFP colocalization was being scored (Figure 1 – figure supplement 2). The text in the Results section now states: “In the text that follows, we will refer to *Lyz2*^{Cre}-recombined cells simply as “myeloid cells”, although they should be understood as CD206+ myeloid cells.”

Regarding the efficiency of recombination of the floxed *Tlr4* target, a direct analysis is technically challenging due to the low abundance of TLR4 and the failure, in our hands, of commercial anti-TLR4 antibodies to produce clear immunostaining. The phenotype of *Cdh5*-CreER; Tlr4floxed mice – a dramatically reduced infection-associated transcriptional response – argues that the floxed *Tlr4* target was recombined at appreciable efficiency in those mice (Figure 1D and 1E). For *Lyz2*^{Cre}; Tlr4floxed mice the principal phenotype is an up-regulation of infection-associated transcripts in a subset of dural border cells in the absence of infection; the transcriptional response to infection was largely unaffected in all leptomeningeal cell types (Figure 1D and 1E). We have added a comment in the results section noting that we do not have a measure of the efficiency of recombination of the floxed *Tlr4* target *in vivo*: “The low abundance of TLR4 and the limitations of commercial anti-TLR4 antibodies precluded a direct immunohistochemical assessment of TLR4 loss in Tlr4VEKO and Tlr4MKO mice.”

(2) The authors did not examine the consequences of *Tlr4* cKO on the course of meningitis or bacterial burden. Knowing the impact of this would strengthen the paper and allow us to determine if the endothelial responses are helpful or harmful in meningitis progression.

For the revised manuscript, we have conducted the following comparisons between infected and uninfected WT and infected and uninfected *Cdh5*-CreER; Tlr4floxed mice: (1) quantifying *E. coli* in the blood of infected mice by counting colonies on agar plates; (2) quantifying *E. coli* in the brain by measuring the red fluorescent protein (RFP) signal (the infecting *E. coli* carry an RFP-expression plasmid); (3) histologically surveying liver and lung for RFP+ *E. coli*; (4) monitoring the weights of infected and uninfected mice. These data are presented in Figure 2 – figure supplement 4 and in the Results section, and they can be summarized as follows. (1) *E. coli* is consistently detectable in the blood, brain, and peripheral organs in infected mice and is not detectable in control mice; (2) there are no statistically significant differences between infected WT and infected *Cdh5*-CreER; Tlr4floxed mice; (3) infected mice of both genotypes stop gaining weight between the time of infection (P5) and 24 hours later at the time of sacrifice (P6). Our conclusion is that loss of TLR4 in endothelial cells and in a subset of other non-myeloid cells in the leptomeninges does not alter the overall clinical course of the infection despite changes in leptomeningeal gene expression and vascular permeability.

(3) TLR4 is a known receptor for LPS. It is unsurprising (especially in the in vitro experiments) that Tlr4 knockout reduces NF-κB signalling and other downstream changes to endothelial cells. Furthermore, it is uncertain if the infection was left to continue, similar changes to the endothelium would nonetheless occur through other mediators such as IL1B and TNFa.

We agree that it makes logical sense that *Tlr4* KO decreases NF-κB signaling. The interesting next question is: what are the mechanistic underpinnings of the responses that are downstream of TLR4 and NF-κB? The cell culture experiments with WT vs. *Tlr4*KO bEnd.3 cells identify one set of cell biological responses related to Cldn5 and junctional integrity, and the NF-κB inhibition experiment (Figure 6) implies that rapid internalization of Cldn5 occurs in the absence of NF-κB mediated transcriptional changes. Regarding the possibility that other mediators such as IL1B or TNFa might, at least partially, make up for the lack of TLR4 signaling later in the infection, that is an open question at present.

(3) The arachnoid fibroblast 2 cluster should be renamed to dural border cells based on their high expression of *Slc4a10*, *Adamtsl3*, *Tmeff2*, etc which are all highly enriched in dural border cells. I suspect this cluster is also highly enriched for *Slc47a1*, probably the most specific marker for these cells (10.1038/s41586-023-06993-7, 10.1016/j.neuron.2023.09.002).

Thank you for this comment. The reviewer is correct. These are dural border cells and they express *Slc47a1*, as seen in a new supplemental Figure 1 – figure supplement 4, which shows UMAP plots for many leptomeningeal cell type-specific genes. We have updated our cell cluster assignment to align with the assignments in Pietilä et al (2023).

(4) It would be helpful to provide higher resolution images of Cldn5 in the leptomeningeal mounts. At the current resolution, it is difficult to tell if there is a similar internalisation/disruption phenotype to what is observed in vitro. Notably, this finding is similar to another recent publication on Cldn5 recycling (in the context of stroke) (10.1186/s40478-025-02125-6).

Higher resolution images of Cldn5 in leptomeningeal vessels without or with *E. coli* infection are now shown in Figure 3 - figure supplement 1C. There is a visual impression of greater area occupied by Cldn5, which is confirmed by quantification (Figure 3A and B). This effect appears to be due to both an average increase in vessel diameter and a redistribution of some of the Cldn5 away from plasma membrane junctions. Thank you for pointing out the interesting and relevant Cottarelli et al (2025) paper, which we had not read. This is now referenced.

Minor points

(1) Typo: prominent should be spelled prominent.

Thank you for catching that one. It is now corrected.

(2) Strictly speaking, the arachnoid layer is not epithelial (despite *Cdh1* expression). They are fibroblasts that acquire barrier-forming properties.

Thank you for that comment. That appears to be the consensus view, and we will go along with it.

(3) Notably, *LyzM* Cre will also recombine in other myeloid populations, so I wouldn't describe it as a macrophage.

Thank you for this comment. We agree, and we have therefore changed the text and figure labels from “macrophage” to “myeloid”.

(4) It is interesting and notable that ICAM1 expression is observed in nonendothelial populations, in the IHC, too, perhaps.

We agree. ICAM1 may be a broader marker/mediator of inflammation than is generally recognized.

(5) In F1B, your labels on the right image to the arachnoid barrier and pial surface are presumably meant to refer to the image on the left with DPP4 and laminin labelling? The subarachnoid should be between the laminin and DPP4 layers (although it will be collapsed in your preparations).

Thank you for catching this error. The vertical bars were sized erroneously, and the labels were also placed erroneously. These have now been corrected.

(6) I would reference the papers that defined leptomeningeal cell type markers (10.1038/s41586-023-06993-7, 10.1016/j.neuron.2023.09.002) when you define your cell types.

Thank you. We have done that, and we have updated our cell cluster assignment to align with the assignments in Pietilä et al (2023).

(7) I would change references to the subarachnoid space in your figures to the leptomeninges (which include the SAS, but extend either side of it).

Thank you. The labels have been changed to “leptomeninges”.

(8) In Figure 2 - Supplement 1A, it looks like the populations are mislabelled.

Thank you. This has been corrected to be consistent with the assignments in Figure 1B

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