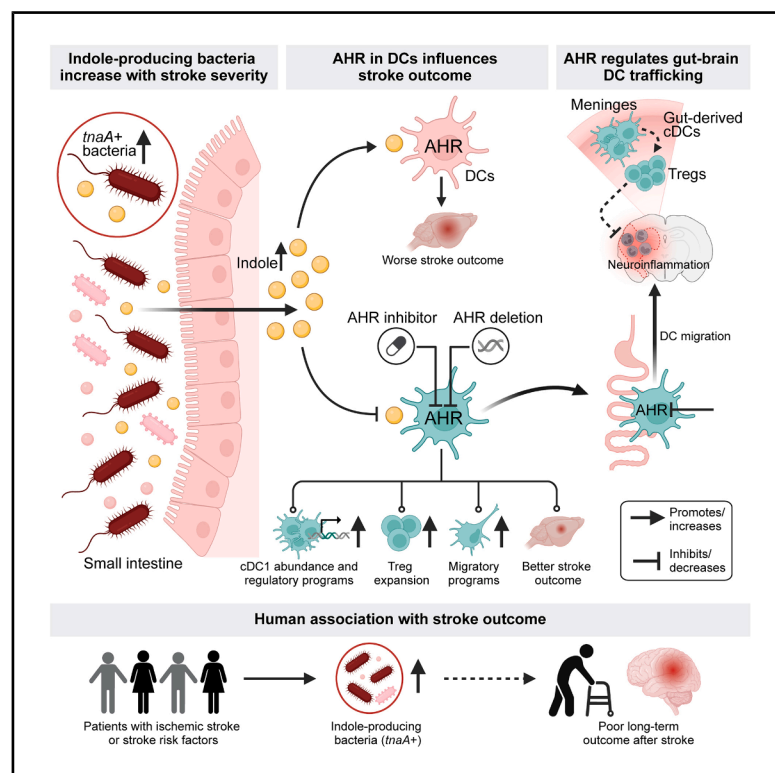


# Gut microbiota primes stroke severity via the AHR in intestinal dendritic cells

## Graphical abstract



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## In brief

Indole-producing *Escherichia coli* worsens ischemic stroke through the activation of aryl hydrocarbon receptor signaling in dendritic cells. Limiting AHR signaling in DCs promotes gut-derived DC migration to the meninges, expands meningeal regulatory T cells, and reduces neuroinflammation. This microbiota-AHR axis offers a promising therapeutic target.

## Highlights

- Microbial indole worsens stroke through AHR signaling in DCs
- AHR loss promotes regulatory and migratory programs in intestinal DCs
- AHR inhibition enhances gut-derived DC trafficking to the meninges
- *tnaA*-encoding bacteria are associated with poor outcomes in patients with stroke

Article

# Gut microbiota primes stroke severity via the AHR in intestinal dendritic cells

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## SUMMARY

The gut microbiota modulates systemic immunity and neurological diseases, yet the underlying mechanisms remain unclear. Here, we identify the enrichment of indole-producing *Escherichia coli* being associated with worse outcomes in mice and patients with ischemic stroke. Microbial indole triggers an aryl hydrocarbon receptor (AHR)-dependent response in dendritic cells (DCs). DC-specific AHR deletion improves stroke outcomes and preserves intestinal conventional type 1 DCs. AHR deletion also promotes immunoregulatory and migratory transcriptional signatures and enhances CCR7-dependent migration *in vitro*. Pharmacological AHR inhibition favors gut-derived DC accumulation in the meninges, accompanied by increased regulatory T cells (Tregs) and reduced neuroinflammation, while Treg depletion abolishes the neuroprotection conferred by AHR inhibition. Detrimental effects of indole-producing *Escherichia coli*, indole administration, and microbiota transplantation from patients with stroke are attenuated in DC-specific AHR-deficient mice. These findings define a microbiota-AHR axis that programs intestinal immune responses before brain injury and high-light microbial tryptophan metabolism as a tractable therapeutic target.

## INTRODUCTION

The gut microbiota shapes systemic immunity, with effects extending beyond the intestine.<sup>1,2</sup> In ischemic stroke, glial and peripheral immune responses critically influence infarct development and functional outcome.<sup>3,4</sup> These inflammatory processes can support tissue repair but, when excessive or dysregulated, amplify neuronal injury.<sup>5</sup> The microbiota may modulate both peripheral immunity and neuroinflammation through immune-rich compartments, particularly the gut and meninges.<sup>6</sup> At the host-microbiota interface, intestinal dendritic cells (DCs) integrate microbial cues and direct T cell priming.<sup>7</sup> Accordingly, altering microbiota composition before ischemia can shift DC-T cell responses linked to neuroprotection.<sup>6,8</sup> Among T cell subsets shaped by host-microbiota interactions, regulatory T cells (Tregs) restrain post-stroke inflammation and promote repair,<sup>9,10</sup> whereas their depletion exacerbates neuroinflammation and worsens outcome.<sup>11</sup> Yet, how the microbiota shapes DC function to modulate Treg responses and brain-border inflammation remains poorly defined.

Microbial metabolites may mediate these immune responses.<sup>12–14</sup> Tryptophan-derived metabolites are central regulators of immune homeostasis with divergent immunomodulatory effects.<sup>15</sup> Bacterial tryptophanase converts dietary tryptophan to indole, which can engage the aryl hydrocarbon receptor (AHR), which is a transcription factor integrating environmental and microbial cues to shape DC function and T cell polarization at barrier sites.<sup>16,17</sup> AHR also modulates central nervous system (CNS) inflammation through glial cells and has been linked to ischemic brain injury.<sup>18–21</sup> Whether microbiota-derived AHR ligands directly shape the immune responses that influence stroke severity remains unclear. This question may extend beyond stroke, as microbiota-dependent modulation of immune responses has been implicated in experimental autoimmune encephalomyelitis and models of neurodegenerative diseases.<sup>2,22,23</sup> We therefore asked whether, in ischemic stroke, microbial tryptophan metabolism links the gut microbiota to stroke outcome through AHR-dependent regulation of intestinal immune responses. We identify tryptophanase A (*tnaA*)-encoding *Escherichia coli* (*E. coli*) and microbial indole as features associated with severe stroke in mice and patients. Indole-producing *E. coli* worsened ischemic injury through AHR in CD11c<sup>+</sup> cells. Deletion of AHR in DCs or pharmacological AHR inhibition preserved intestinal conventional type 1 DCs (cDC1s), promoted immunoregulatory and migratory DC states, and was associated with increased meningeal Tregs and reduced ischemic brain injury. AHR inhibition further enhanced gut-to-meninges trafficking of CD11b<sup>+</sup>CD103<sup>+</sup> cDCs, and Treg depletion abolished the neuroprotection conferred by AHR inhibition. Furthermore, microbiota from patients with severe strokes reproduced the associated immune phenotype in mice, while its detrimental effect was attenuated in DC-specific AHR-deficient mice. In patients with ischemic stroke, enrichment of *tnaA*-encoding bacteria was linked to poor outcomes and was also observed in individuals with stroke risk factors. These findings identify a microbiota-driven axis linking microbial tryptophan metabolism to the severity of ischemic brain injury.

## RESULTS

### Indole-producing *E. coli* drives stroke severity

To identify gut bacteria associated with stroke severity, we performed metagenomic analysis after transient middle cerebral artery occlusion (tMCAO), a mouse model of ischemic stroke. We stratified mice as moderate or severe stroke based on a composite score integrating infarct size, weight loss, and sensorimotor deficits (neuroscore) assessed 3 days after stroke (Figure S1A). Overall, 43% of mice developed severe stroke (Figures 1A and S1B). Given our prior evidence implicating the distal small intestine (ileum) in stroke,<sup>6</sup> we focused on this compartment. Ileal microbial diversity and community composition shifted with severity, whereas stool microbial communities remained largely stable (Figures 1B and 1C). In the ileum, severe stroke was associated with depletion of several obligate anaerobes, including Muribaculaceae and Akkermansiaceae, and enrichment of *E. coli* relative to sham. *E. coli* was also enriched in severe compared with moderate stroke, although this was not significant (Figures 1D and S1C).

Whole-genome sequencing and selective culture confirmed ileal *E. coli* enrichment on day 3 after stroke (Figures 1E and S1D) but not at 16 h (Figure S1E). The *E. coli* tryptophanase enzyme encoded by *tnaA* converts tryptophan to indole.<sup>24</sup> We found that ileal *tnaA* abundance, including *E. coli*-derived *tnaA*, increased in severe stroke (Figure 1F). Bacterial isolates from these mice produced indole from tryptophan *in vitro*, comparable to the positive control *E. coli* wild type (*E. coli* WT), whereas the *tnaA*-deficient strain (*E. coli*  $\Delta$ *tnaA*), lacking tryptophanase activity, did not (Figure S1F), thus indicating that the *E. coli* enriched in the ileum were competent indole producers. Using the semi-quantitative Kovács assay, we found increased ileal indole in severe stroke (Figure 1G), whereas stool, plasma, and urine levels were unchanged (Figure S1G). Mass spectrometry provided specific quantification of indole and confirmed its increase in severe stroke (Figure S1H). Because indole production also depends on substrate availability, we found that ileal tryptophan decreased 3 h after stroke, transiently increased at 16–48 h, and returned near sham levels by 72 h (Figure S1I). These changes occurred alongside later increase in ileal indole and *tnaA*-encoding *E. coli*, which suggests that altered tryptophan availability may contribute to temporal differences in indole production.

To directly test whether *E. coli*-derived indole causally contributes to stroke severity, mice were colonized before stroke with indole-producing *E. coli* WT or indole-deficient *E. coli*  $\Delta$ *tnaA* (Figure 1H). Both strains colonized mice comparably, whereas *tnaA* expression was absent and urinary indole was reduced in *E. coli*  $\Delta$ *tnaA*-colonized mice (Figures S1J–S1M). We found that infarct volume and sensorimotor deficits were reduced in mice colonized with indole-deficient *E. coli*  $\Delta$ *tnaA* (Figure 1I). To determine whether indole alone is sufficient to worsen stroke outcome independently of bacterial colonization, we administered indole to mice for 2 weeks before stroke induction (Figure 1J) and confirmed an increase of urinary indole levels compared with vehicle-treated mice (Figure S1N). Indole-treated mice exhibited increased infarct volumes and higher neuroscores than vehicle-treated controls, whereas the downstream

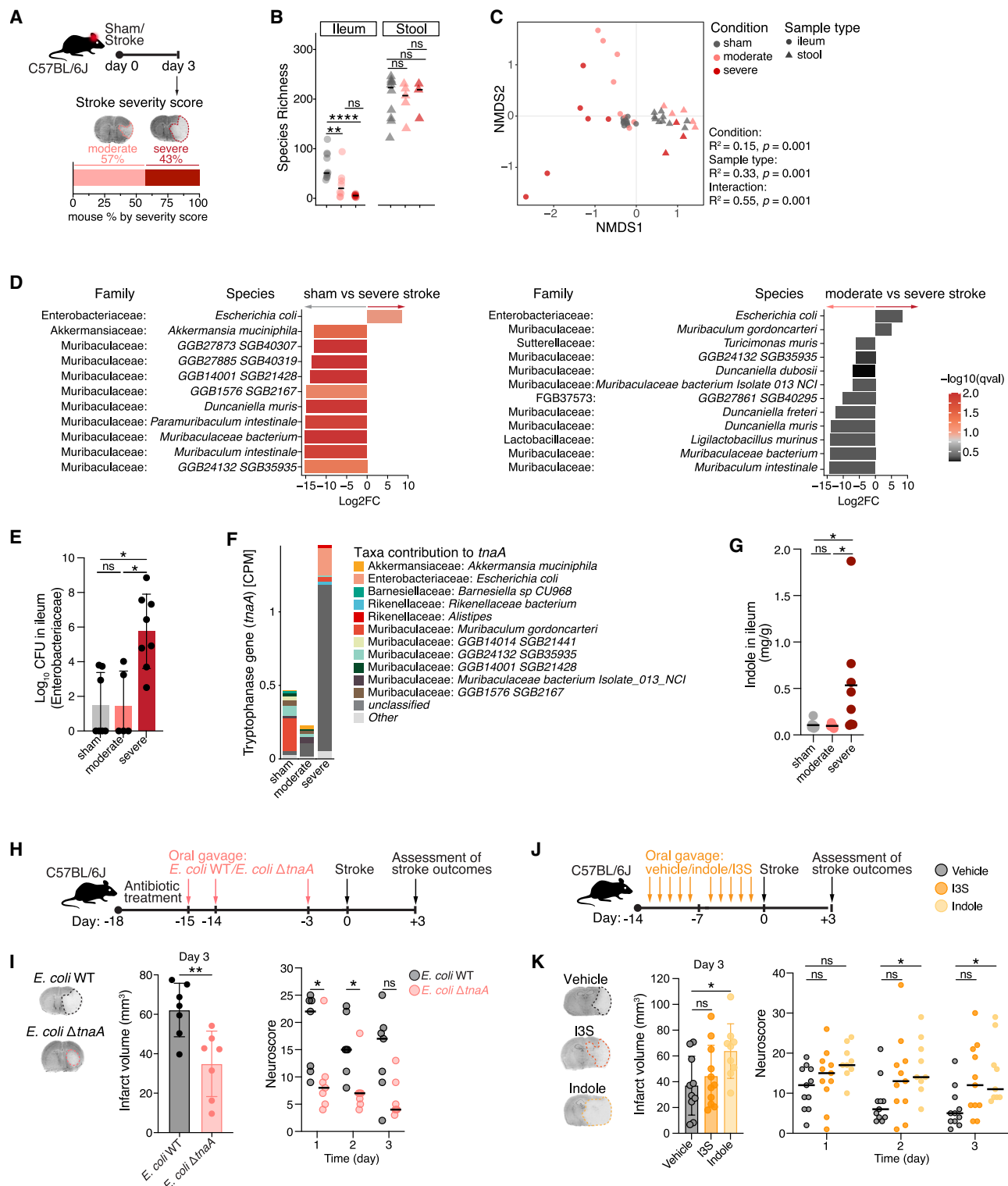


**Figure 1. Indole-producing *E. coli* links the gut microbiota to stroke severity**

(A) Experimental design and severity stratification 3 days after stroke based on infarct volume, body weight loss, and neurological score ( $n = 17$ ).

(B and C) Species richness (B, median) and Bray-Curtis dissimilarity (C) in ileum and stool (sham,  $n = 10/10$ ; moderate,  $n = 5/8$ ; severe,  $n = 3/6$ , stool/ileum). NMDS: non-metric multidimensional scaling.

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tryptophan metabolite indoxyl-3 sulfate (I3S) had no significant effect (Figure 1K). Enrichment of indole-producing *E. coli* prior to stroke worsens outcome, thus identifying microbiota-derived indole as a sufficient driver of stroke severity.

### AHR-expressing DCs contribute to stroke severity

Given that indole is an AHR ligand, we asked whether AHR signaling links microbial indole to post-stroke immune changes. Across the intestinal tract and liver, *Ahr* expression increased in the proximal small intestine toward the colon and liver but was unaffected by stroke (Figure S2A; Table S1). In contrast, the canonical AHR target genes *Cyp1a1* and *Ahr* were transiently reduced throughout the intestine and in the liver 16 h after stroke, returning to baseline by 72 h, whereas *Cyp1b1* selectively increased in the ileum at 72 h (Figure S2A). CYP1B1 protein also increased in the ileum, whereas CYP1A1 did not mirror the transcriptional changes (Figure S2B). Thus, stroke induced limited and transient changes in canonical AHR target genes, with a localized increase in ileal *Cyp1b1* and protein levels 72 h post-stroke—the temporal and anatomical site where we observed an expansion of indole-producing *E. coli* associated with stroke severity.

We next mapped AHR expression across immune subsets and organs using AHR-TdTomato reporter mice<sup>25</sup> (Figure 2A). Across tissues, AHR expression was generally highest in ileal lamina propria (LP) immune cells, particularly monocytes, macrophages/monocyte-derived DCs (moDCs), and natural killer cells (Figures 2B, S2C, and S2D), consistent with previous report.<sup>25</sup> In the meninges and brain, cDC1s showed the strongest AHR expression, increasing in the brain at 72 h post-stroke (Figures 2C and S2E). Published single-cell RNA sequencing (scRNA-seq) data confirmed the highest *Ahr* expression in brain cDC1s among CD45<sup>+</sup> cells in sham and stroke conditions (Figure 2D). In gut-associated tissues, our scRNA-seq showed *Ahr* enrichment in monocytes and DC subsets but lower expression in most lymphocytes (Figure 2E). Consistent with early accumulation of myeloid cells in the ischemic brain,<sup>26,27</sup> the proportion of AHR<sup>+</sup> cDCs among the total CD45<sup>+</sup> cells increased in the brain after stroke, along with AHR<sup>+</sup> monocytes and macrophages/moDCs, whereas AHR<sup>+</sup> microglia were unchanged (Figures 2F and S2F). Similar accumulation occurred in the meninges, while peripheral cDC frequencies declined at 72 h (Figures 2F and S2G). In the ileum LP, the cDC1 subset XCR1<sup>+</sup>CD11b<sup>+</sup>CD103<sup>+</sup>AHR<sup>+</sup> transiently increased at 16 h, while decreasing in the brain and meninges (Figure 2G). Thus, cDC1s are a major AHR-expressing population in the brain and

meninges and reveal temporal, compartment-specific changes in AHR<sup>+</sup> cDC1s after stroke.

We next sought to determine the cell-specific contribution of AHR signaling to disease outcome. At acute time points post-stroke (16–72 h), brain-invading immune cells are predominantly composed of monocytes, neutrophils, and DCs, with lymphocytes present in smaller numbers.<sup>29</sup> We therefore induced stroke in mice lacking AHR in distinct immune cell populations (Figure 2H). Rag1<sup>−/−</sup> mice (deficient in lymphocytes) reconstituted with AHR-deficient T cells, as well as LysM<sup>Cre/+</sup>; *Ahr*<sup>fl/fl</sup> mice targeting granulocytes, macrophages, and monocytes (hereafter referred to as LysM-Ahr knockout [KO] mice) showed only minor, non-significant reductions in infarct volume (Figure 2I). In contrast, mice lacking AHR expression in CD11c<sup>+</sup> cells (*Itgax*<sup>Cre/+</sup>; *Ahr*<sup>fl/fl</sup>, hereafter CD11c-Ahr-KO) exhibited markedly smaller infarcts at both 16 and 72 h compared with *Itgax*<sup>+/+</sup>; *Ahr*<sup>fl/fl</sup> littermate control mice (Figure 2J). These findings identify AHR in CD11c<sup>+</sup> cells as a key determinant of early brain injury.

### Indole-producing *E. coli* drives stroke severity via the AHR in DCs

To determine whether microbial indole directly induces canonical AHR-associated target genes in DCs, purified CD11c<sup>+</sup> cells were exposed to increasing concentrations of indole or I3S (Figure 3A). Indole dose-dependently induced AHR-responsive genes *Ahr* and *Cyp1a1* with clear induction at 500 μM (Figure 3B). This concentration was within the range estimated in standardized ileal-content extracts from mice with severe stroke (Figure 3C). These responses were reduced by the AHR inhibitor CH-223191<sup>30</sup> and in CD11c-Ahr-deficient cells, confirming AHR-dependent signaling (Figure 3B). In contrast, I3S induced *Ahr* and *Cyp1a1* only at concentrations exceeding levels measured in the mouse ileum, and these effects were not reduced in CD11c-Ahr-deficient cells (Figures S3A and S3B) suggesting that, in this setting, I3S acts independently of AHR in CD11c<sup>+</sup> cells.

We next tested whether detrimental effects of indole-producing *E. coli* on stroke required AHR in DCs. In C57BL/6J mice, colonization with *tnaA*-encoding *E. coli* (*E. coli* WT) or indole treatment worsened stroke outcomes (Figures 1I and 1K). In contrast, the detrimental effect of *E. coli* WT was abolished in CD11c-Ahr-KO mice, as infarct volumes did not differ between *E. coli* WT- and *E. coli* Δ*tnaA*-colonized animals (Figure 3D), thus indicating that bacterial tryptophanase exacerbates stroke through AHR in CD11c<sup>+</sup> cells. Likewise, indole administration

(D) Differentially abundant species in sham versus severe, and moderate versus severe stroke, ranked by absolute log2 fold change. Bar direction indicates enrichment; color,  $-\log_{10}(q\text{val})$ ;  $q\text{val} < 0.1$ , significant.

(E) Ileal Enterobacteriaceae colony-forming units (CFUs) 72 h after sham or stroke, (mean ± SD,  $n = 5$ –8).

(F) Ileal *tnaA* abundance (counts per million [CPM]) and species contributions by severity. “Unclassified” denotes *tnaA* not assigned to a MetaPhlAn-detected species (sham:  $n = 10$ ; moderate:  $n = 8$ ; severe:  $n = 6$ ).

(G) Ileal indole 72 h after sham or stroke by Kovács assay (mean,  $n = 5$ –8).

(H) After 3 days of antibiotics, mice received  $1 \times 10^8$  *E. coli* WT or *E. coli* Δ*tnaA* by gavage 2 weeks before stroke.

(I) Nissl-stained sections, infarct volume (mean ± SD), and neuroscore (median) after stroke ( $n = 7$ /group); dashed lines indicate infarct.

(J) Mice received vehicle, indole, or I3S daily by gavage for 2 weeks before stroke.

(K) Nissl-stained sections, infarct volumes (mean ± SD), and neuroscore (median) after stroke ( $n = 9$ –11); dashed lines indicate infarct.

Unpaired *t* test (I, left), Mann-Whitney (I, right), or Kruskal-Wallis with Dunn’s correction (B), (E), (G), and (K). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ ; ns, not significant. See also Figure S1.

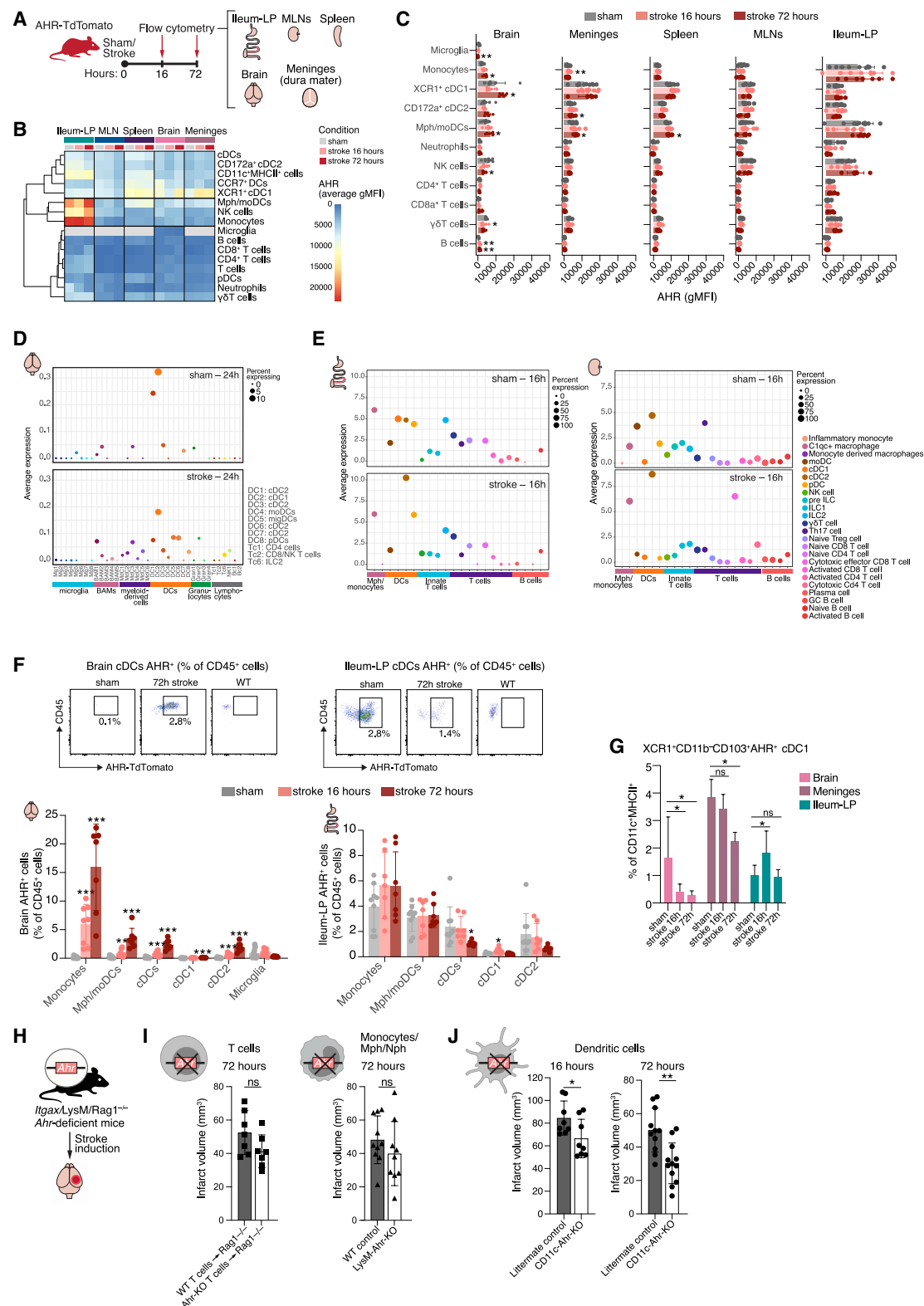


**Figure 2. AHR is enriched in cDC1 and AHR deletion in CD11c<sup>+</sup> cells limits stroke injury**

(A) Flow cytometric analysis of AHR-TdTomato reporter mice in sham and 16 or 72 h after stroke. Ileum LP, ileal lamina propria; MLNs, mesenteric lymph nodes.

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did not worsen injury in CD11c-Ahr-KO mice, as infarct volumes were similar between vehicle- and indole-treated animals (Figure 3E). Collectively, these findings show that indole induces a dose-dependent, AHR-dependent transcriptional response in DCs *in vitro*, and they establish that the detrimental effects of bacterial tryptophanase and indole on stroke severity require AHR in CD11c<sup>+</sup> cells.

### Increased Tregs at brain-border sites in mice lacking AHR in DCs

To determine how AHR signaling in CD11c<sup>+</sup> cells influences stroke outcome, we examined the brain myeloid compartment early after stroke. At 16 h, CD11c-Ahr-KO mice displayed a significantly increased number and proportion of cDC subsets, whereas monocyte and macrophages/moDCs cells were not significantly affected (Figures 4A, 4B, and S4A). In the meninges, cDCs increased after stroke, but their abundance was not affected by AHR deletion (Figures S4B and S4C). Thus, AHR loss in CD11c<sup>+</sup> cells selectively promotes early cDC accumulation in the injured brain.

Because cDCs respond rapidly to tissue injury and can shape subsequent adaptive immune responses,<sup>27,31</sup> we next assessed whether this early increase was accompanied by transcriptional changes in brain cDCs at a later stage, when T cells begin to accumulate in the brain.<sup>3</sup> We performed scRNA-seq of CD11c<sup>+</sup> cells isolated from ischemic brain tissue three days after stroke. Uniform manifold approximation and projection (UMAP) analysis of 1,461 control and 1,312 CD11c-Ahr-KO CD11c<sup>+</sup> cells identified eight clusters comprising four DC subsets (*Fit3*)-cDC2 (cluster 0; *Irf4*, *Cd209a*, and *H2-Ab1*), cDC1 (cluster 4; *Xcr1*, *Naaa*, and *Itgae*), migratory DCs (cluster 5; *Ccr7*), and plasmacytoid DCs (cluster 6; *It3*, *Siglech*, *Ccr9*, and *Ly6d*), and four macrophage-associated clusters (1, 2, 3, and 7) including inflammatory macrophages (cluster 1; *Ms4a7*, *Adgre1*, *Apoe*, and *Msr1*) and microglia (cluster 2; *Sall1*) (Figures 4C, S4D, and S4E). Consistent with the flow cytometry, CD11c-Ahr-KO mice showed increased proportions of cDC1 and cDC2 subsets (clusters 0 and 4) and fewer inflammatory macrophages (cluster 1), whereas microglia were unchanged (Figure 4D). The cDC1 cluster (cluster 4), which expressed the highest *Ahr* (Figure 4E), exhibited relatively few differentially expressed genes (164 DEGs) (Figure 4F), which suggests that AHR deletion primarily affects cDC1 abundance in the brain rather than extensively altering

their transcriptional program. In contrast, cDC2 and inflammatory macrophages showed broader transcriptional changes, including the reduced expression of inflammatory genes such as *Apoe*, *Lyz2*, and *Trem2* (Figures 4F, 4G, and S4F), which is consistent with an attenuated inflammatory environment and reduced infarct volume in CD11c-Ahr-KO mice.

We next asked whether changes in cDC abundance mediated by AHR were associated with the modulation of T cell responses. At 3 days, CD11c-Ahr-KO mice had fewer brain IL-17<sup>+</sup>CD4<sup>+</sup> T helper (Th17) cells (Figure 4H), while brain IL-17<sup>+</sup>γδT cells and Foxp3<sup>+</sup> Tregs were unchanged (Figure S4G). Interestingly, we observed a higher frequency of Foxp3<sup>+</sup> Tregs in the meninges and mesenteric lymph nodes (MLNs) but not in the gut or spleen, while other meningeal T cell populations were unaffected (Figures 4I, S4H, and S4I). These findings suggest that AHR deletion in CD11c<sup>+</sup> cells promotes Treg accumulation. Systemic cytokines showed modest, non-significant trends toward lower TNFα and IFNγ levels and higher IL-10 in CD11c-Ahr-KO mice (Figure S4J). Overall, AHR deficiency in CD11c<sup>+</sup> cells is associated with increased brain cDC1s, an attenuated pro-inflammatory T cell response, and enhanced Treg accumulation in secondary lymphoid organs and brain-border sites.

### Loss of the AHR promotes intestinal cDC1 abundance and regulatory and migratory transcriptional programs in DC subsets

To better understand how AHR loss affects DC states and contributes to increased Tregs, we examined microbiota-exposed compartments.<sup>32</sup> In the ileal LP, total DC frequencies declined after stroke in both genotypes (Figures S5A and S5B). However, while macrophages/moDC and cDC2 frequencies were comparable, intestinal cDC1s were significantly enriched in CD11c-Ahr-KO mice at baseline (sham) and remained elevated at both 16 and 72 h after stroke (Figures 5A and S5C). Lymphocytes and neutrophils in the ileal LP, MLNs, and spleen were unaffected under sham conditions and 16 h after stroke (Figure S5D), thus indicating a selective effect for AHR on intestinal cDC1 abundance.

We then compared scRNA-seq profiles of CD11c<sup>+</sup> cells from the ileum LP, spleen, and brain 3 days after stroke. Canonical AHR-related genes *Ahr*, *Cyp1b1*, and *Nqo1* were reduced mainly in ileum LP CD11c<sup>+</sup> cells from CD11c-Ahr-KO mice, with inconsistent changes in spleen and brain CD11c<sup>+</sup> cells (Figure S5E). Principal-component analysis (PCA) based on

(B) Unsupervised clustering of mean AHR geometric fluorescence intensity (gMFI) across immune subsets, organs, and time points (cDCs, conventional dendritic cells; mph, macrophages; moDCs, monocytic-derived dendritic cells; NK, natural killer cells; pDCs, plasmacytoid dendritic cells; sham: *n* = 9–10, stroke 16 h: *n* = 8–9, stroke 72 h: *n* = 7–8).

(C) AHR gMFI in indicated immune populations and organs (mean ± SD, *n* as in B); \* indicates differences versus sham.

(D) *Ahr* expression across brain CD45<sup>+</sup> populations 24 h after sham or stroke in published scRNA-seq data.<sup>28</sup> Dot size indicates percentage of expressing cells; cluster annotations follow Garcia-Bonilla et al.<sup>28</sup>: Mg, microglia; BAM, border-associated macrophages; MdC, myeloid-derived cells; DC, dendritic cells; Gran, granulocytes; Tc, T cells; NK, natural killer cells; Bc, B cells; migDCs, migratory DCs; and ILC2, innate lymphocyte type 2.

(E) *Ahr* expression across ileum LP and MLN CD45<sup>+</sup> populations 16 h after sham or stroke; dot size indicates the percentage of expressing cells (*n* = 4/condition).

(F) Frequencies and representative plots of indicated AHR<sup>+</sup> populations in brain and ileum LP (mean ± SD; *n* as in B).

(G) XCR1<sup>+</sup>CD11b<sup>+</sup>CD103<sup>+</sup>AHR<sup>+</sup> cDC1 among CD11c<sup>+</sup>MHCII<sup>+</sup> cells in brain, meninges, and ileum LP (mean ± SD; *n* as in B).

(H) Stroke induction in mice lacking AHR in CD11c<sup>+</sup> cells, neutrophils/monocytes, or CD4<sup>+</sup> T cells.

(I and J) Infarct volumes after stroke in LysM-Ahr-KO and WT C57BL/6J mice, Rag1<sup>−/−</sup> mice reconstituted with WT or AHR-deficient CD4<sup>+</sup> T cells, CD11c-Ahr-KO, and littermate control mice (mph: macrophages, Nph: neutrophils, mean ± SD, *n* = 7–12).

Unpaired *t* test (I) and (J) or Kruskal-Wallis with Dunn's correction versus sham (C), (F), and (G). \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001; ns, not significant. See also Figure S2.

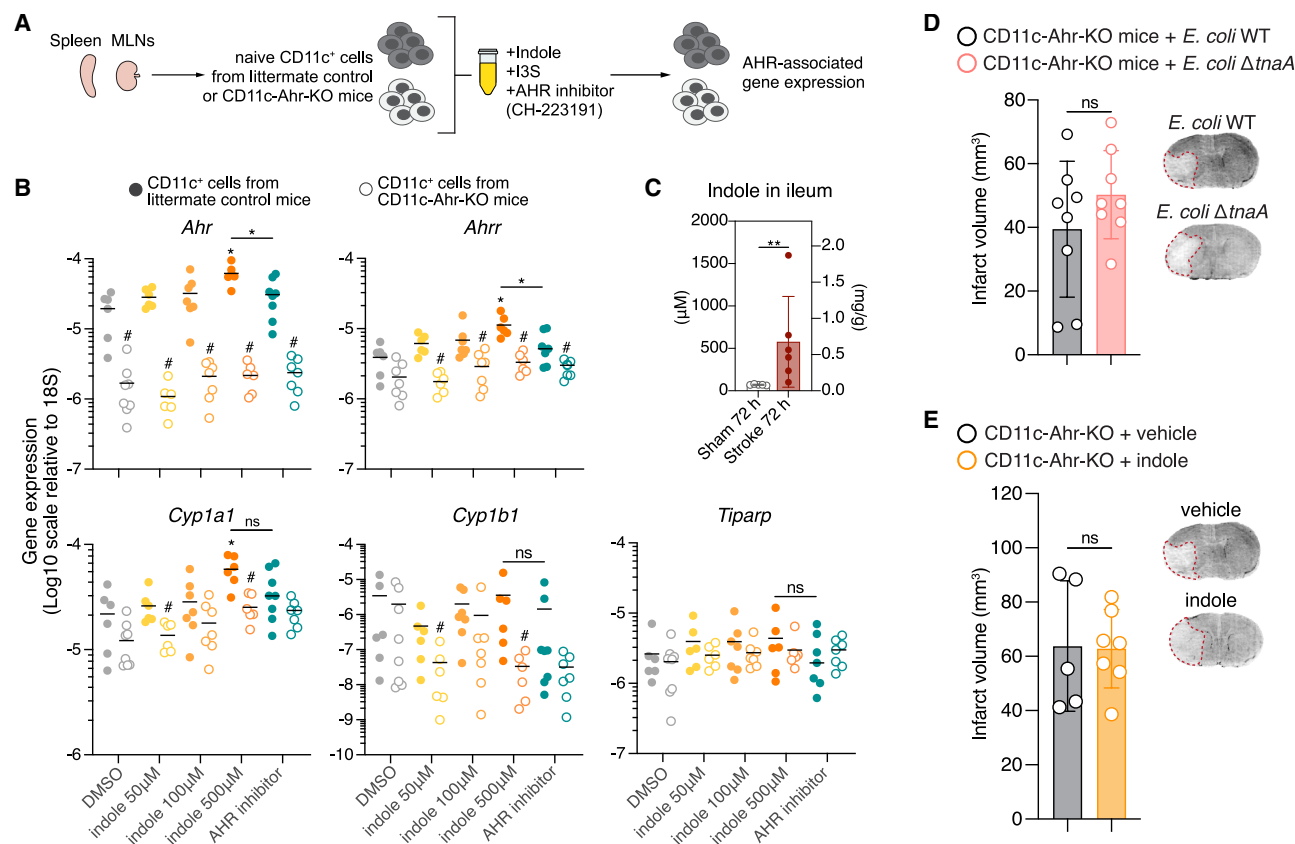


**Figure 3. Microbial indole affects stroke outcomes via the AHR in CD11c<sup>+</sup> cells**

(A) Ex vivo treatment of CD11c<sup>+</sup> cells from littermate control and CD11c-Ahr-KO mice with indole, I3S, or CH-223191. (B) *Ahr* and AHR-associated gene expression after vehicle, increasing indole concentrations, or CH-223191 (mean,  $n = 6-8$ ); \*, versus vehicle-treated controls; #, genotype difference within condition. (C) Ileal indole after sham or severe stroke by semi-quantitative Kovács assay, expressed as  $\mu\text{M}$  (left axis) and mg/g wet weight (right axis) (mean  $\pm$  SD,  $n = 5-6$ ). (D and E) Nissl-stained sections and infarct volumes 3 days after stroke in CD11c-Ahr-KO mice colonized with *E. coli* WT or *E. coli*  $\Delta\text{tnaA}$  (D,  $n = 7-8$ ) or treated with vehicle or indole (E;  $n = 5-7$ ) (mean  $\pm$  SD). Mann-Whitney (B)–(E) or Kruskal-Wallis with Dunn's correction (B). # $p < 0.05$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ; ns, not significant. See also Figure S3.

DEGs in CD11c<sup>+</sup> cells indicated that genotype-associated transcriptional differences were greatest in the ileum LP (Figure 5B). We therefore investigated how DC-intrinsic AHR loss alters transcriptional programs in intestinal CD11c<sup>+</sup> cell populations (Figures 5C–5E). *Ahr* expression was enriched in several DCs (clusters 0, 1, 3, and 8) and *Cx3cr1*<sup>+</sup> resident macrophages (cluster 2) (Figure 5F). CD11c-Ahr-KO mice showed increased proportions of cDC1 expressing *Xcr1* and *Itgae* (for CD103) (cluster 3) and *Ccr7*<sup>+</sup> migratory DCs (cluster 8), with reduced *Cx3cr1*<sup>+</sup> and *Apoe*<sup>+</sup> macrophage populations (Figure 5G), consistent with flow cytometry (Figures 5A and S5C). Immune pathway enrichment analysis identified Th17-, tolerance-, and migration-associated programs across DC and macrophage clusters in both genotypes (Figure 5H). Comparison between conditions across the entire ileal CD11c<sup>+</sup> compartment identified tolerance-associated genes as the most enriched pathway in CD11c-Ahr-KO mice (Figure 5I). To better know which cell subsets were driving the effect, we analyzed each cluster using a predefined

gene set associated with tolerance and regulatory programs. *Trem1*<sup>+</sup> and *Rorc*<sup>+</sup> cDC2s from CD11c-Ahr-KO mice (clusters 0 and 1, respectively) showed increased in maturation markers (*Cd80* and *Cd86*) together with inhibitory and negative-feedback regulators (*Havcr2*, *Phlpp1*, *Cblb*, and *Tnfrsf3*), whereas few genes were upregulated in control cDC2s, including antigen-processing genes (Figure 5J). TGF- $\beta$ -associated regulatory genes were more prominent in *Cx3cr1*<sup>+</sup> macrophages and pDCs (Figure S5F). *Xcr1*<sup>+</sup> cDC1s (cluster 3) from CD11c-Ahr-KO mice also showed increased maturation and costimulatory molecules, while retinoic-acid- and TGF- $\beta$ -associated mucosal regulatory features (*Aldh1a2*, *Itgb8*, *Tgfb1*, and *Tgfb2*) were most evident in cDC1s and *Ccr7*<sup>+</sup> DCs (Figure 5J), the latter consistent with a mature regulatory DC (mregDC) signature.<sup>33</sup> Thus, AHR deletion in CD11c<sup>+</sup> cells was associated with shared mature and immunoregulatory programs across subsets, with additional upregulated genes associated with mucosal tolerance in cDC1s and *Ccr7*<sup>+</sup> mregDCs. Although interpretation of *Xcr1*<sup>+</sup>

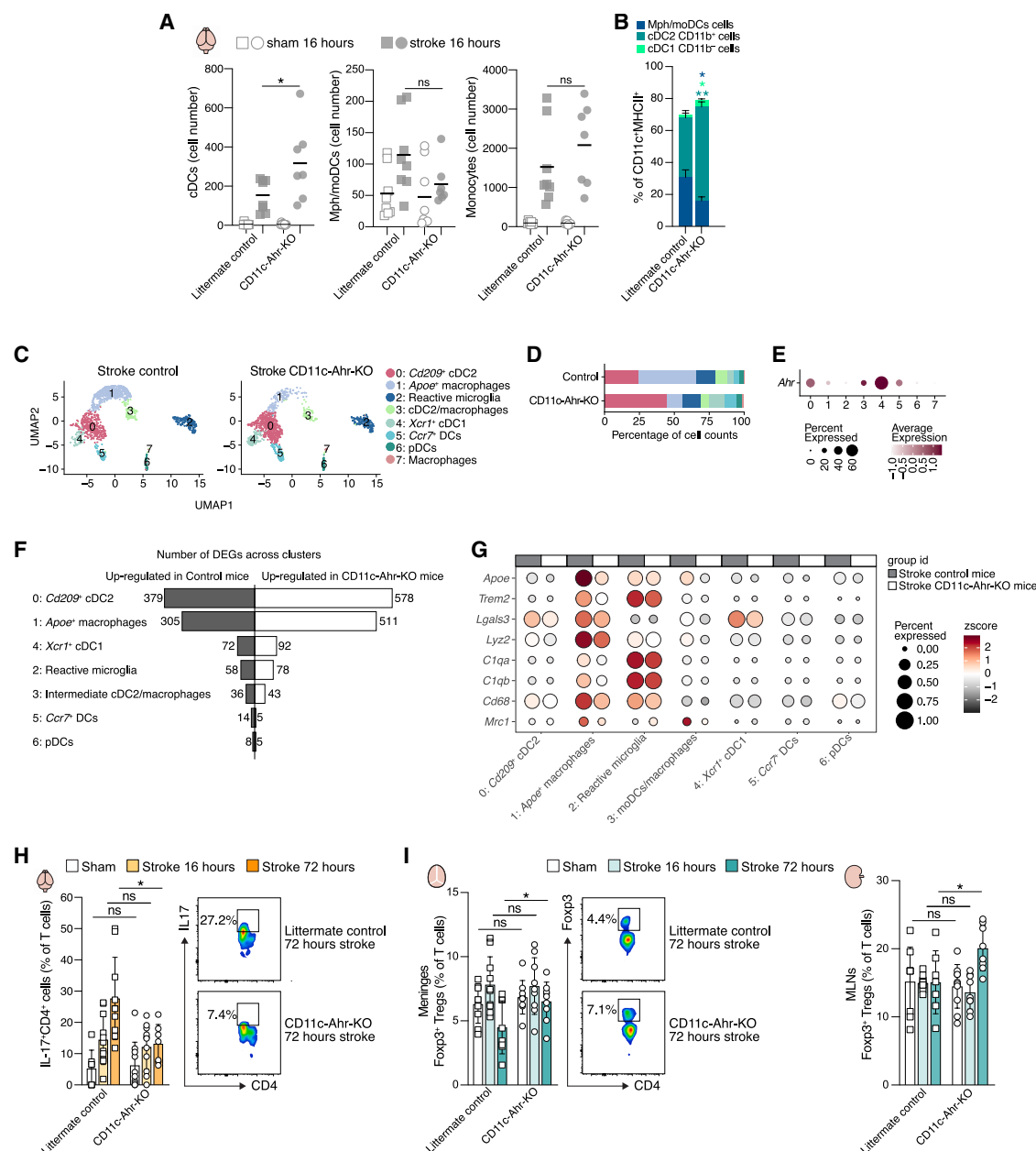


**Figure 4. CD11c-specific AHR deletion promotes Treg accumulation in the meninges and MLNs**

(A) Numbers of indicated brain immune populations 16 h after sham or stroke in littermate control and CD11c-Ahr-KO mice (mean,  $n = 7-8$ ).  
 (B) Proportions of indicated brain immune populations 16 h after stroke (mean + SEM,  $n = 7-8$ ), colored  $p$  values indicate genotype differences for the corresponding population.  
 (C) UMAP of brain CD11c<sup>+</sup> cells from littermate control and CD11c-Ahr-KO mice 72 h after stroke.  
 (D) Proportions of annotated CD11c<sup>+</sup> populations by genotype.  
 (E) *Ahr* expression across CD11c<sup>+</sup> populations in controls; dot size indicates the percentage of expressing cells.  
 (F) Number of DEGs between genotypes within each CD11c<sup>+</sup> population.  
 (G) Selected macrophage inflammatory-response genes across populations and genotypes; color indicates scaled expression and dot size percentage of expressing cells.  
 (H) Frequency and representative gating of brain IL17<sup>+</sup>CD4<sup>+</sup> T cells (mean  $\pm$  SD,  $n = 7-11$ ).  
 (I) Frequency and representative gating of Foxp3<sup>+</sup> Tregs in meninges and MLNs (mean  $\pm$  SD,  $n = 8-10$ ).  
 Mann-Whitney (A), (B), (H), and (I). \* $p < 0.05$ ; \*\* $p < 0.01$ ; ns, not significant.  
 See also Figure S4.

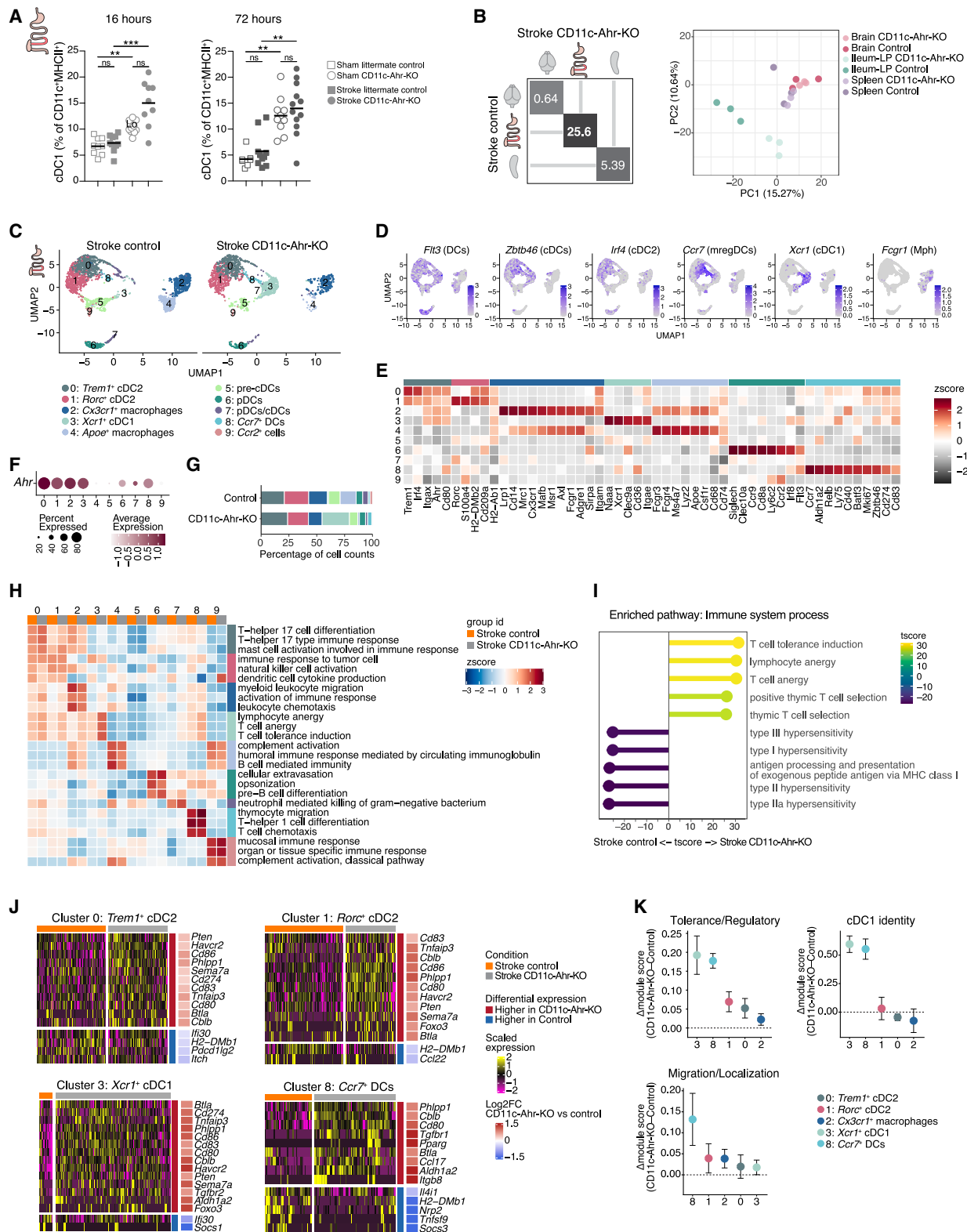


**Figure 5. CD11c-specific AHR deletion preserves intestinal cDC1s, and is associated with regulatory and migratory transcriptional features in DC subsets**

(A) Ileum LP cDC1 frequencies in littermate control and CD11c-Ahr-KO mice (mean,  $n = 7-12$ ).

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cDC1s and *Ccr7*<sup>+</sup> mregDCs was limited by the marked underrepresentation of cDC1s in control mice and the small number of mregDCs (Figure S5G), we assessed whether the gene-level patterns were consistent across biological replicates. A predefined tolerance/regulatory module score, averaged per mouse within each cluster, showed the largest positive differences between CD11c-Ahr-KO and control mice in *Xcr1*<sup>+</sup> cDC1s and *Ccr7*<sup>+</sup> mregDCs (Figure 5K). Because we found increased intestinal cDC1s in CD11c-Ahr-KO mice, we assessed whether this compositional difference was accompanied by reinforcement of a cDC1-associated gene signature. The cDC1 identity module showed its largest positive differences in *Xcr1*<sup>+</sup> cDC1s and *Ccr7*<sup>+</sup> mregDCs, which is consistent with shared cDC1-associated transcriptional features in these populations (Figure 5K). DC migration is essential for initiating T cell responses in secondary lymphoid organs,<sup>34</sup> we examined a migration/localization module, which showed its clearest positive difference in *Ccr7*<sup>+</sup> mregDCs (Figure 5K). Although none of the mouse-level module-score differences reached statistical significance (Figure S5H), their direction associated AHR deletion with immunoregulatory and migratory transcriptional programs alongside preservation of cDC1-associated markers and intestinal cDC1 abundance.

### AHR inhibition confers Treg-dependent neuroprotection and promotes gut-derived DC accumulation in the meninges

Having found that AHR deletion in DCs promotes regulatory and migratory DC programs, we asked whether these features contribute to neuroprotection. CD11c<sup>+</sup> cells isolated from CD11c-Ahr-KO mice 3 days after stroke increased Foxp3<sup>+</sup> Treg numbers in co-culture compared with littermate control cells, thus indicating an enhanced capacity to support Treg expansion (Figures 6A and 6B). We next tested whether Tregs are required for the neuroprotective effect of AHR inhibition. Because this could not be addressed directly in CD11c-Ahr-KO mice, we combined the AHR antagonist CH-223191 with Treg depletion in DREG (depletion of regulatory T cells) mice. In C57BL/6J WT mice, dietary CH-223191 treatment reduced infarct size and increased intestinal cDC1s and Treg proportions, recapitulating key features of CD11c-Ahr-KO mice (Figures 6C, S6A, and S6B). We then depleted Tregs in CH-223191-treated DREG mice before stroke (Figures 6D, 6E, and S6C). Infarct volume was assessed at 16 h to minimize survival bias or other off-target effects of Treg depletion at later time points. Treg depletion remained comparable between

treatment groups (Figures S6C and S6D). Following Treg depletion, AHR inhibition no longer conferred neuroprotection (Figure 6F), despite the preserved enrichment of ileal CD11b<sup>−</sup>CD103<sup>+</sup> cDC1 cells (Figure 6G). These findings indicate that Tregs are required for the early neuroprotective effect of AHR inhibition and suggest a model in which an increase in Treg frequency occurs downstream of AHR-dependent DC modulation.

Since AHR-deficient CD11c<sup>+</sup> cells showed enrichment of migration-associated genes, we first assessed DC migration *in vitro* (Figure 6H). Upon maturation, intestinal DCs upregulate the chemokine receptor CCR7 and migrate along CCL19/CCL21 gradients to secondary lymphoid organs for T cell priming.<sup>35</sup> Mature bone marrow-derived DCs (BMDCs) from CD11c-Ahr-KO mice migrated more efficiently toward CCL21 than control, with maximal responses at 10–50 ng/mL (Figure 6I). Naive AHR-deficient BMDCs also showed enhanced migration, although at higher CCL21 concentrations (Figure 6I). CCL19 elicited a similar but weaker effect (Figure S6E). These data indicate enhanced CCR7-dependent chemotaxis in AHR-deficient DCs.

To determine whether this augmented migratory capacity translates into altered DC trafficking *in vivo* we treated Kikume Green-Red (KikGR) mice with the AHR inhibitor for 2 to 3 weeks and performed photoconversion-based cell tracking. In this mouse model, intestinal cells exposed to violet light irreversibly convert from green (KikG<sup>+</sup>) to red fluorescence (KikR<sup>+</sup>), thus enabling the tracking of gut-derived cells (Figure 6J). CH-223191-mediated AHR inhibition in KikGR33 mice was confirmed by reduced intestinal CYP1A1 and CYP1B1 prior to inducing stroke, accompanied by increased CCR7 expression (Figure S6F). We analyzed intestinal-derived immune cells expressing KikR protein at baseline (sham surgery) and at 16- and 72-h post-stroke in MLNs, brain, and meninges (Figure 6J). Under sham conditions, gut-derived KikR<sup>+</sup> cell frequencies were similar between treatment groups (Figure S6G). To quantify the effect of stroke on cell migration, we calculated a migration index defined as the ratio of migrating (KikR<sup>+</sup>) cells in stroke versus sham condition, as previously published.<sup>36</sup> After stroke, AHR inhibition increased the accumulation of gut-derived cDCs in the meninges at 16 and 72 h in comparison with sham (Figure 6K). This effect was driven by gut-derived CD11b<sup>−</sup>CD103<sup>+</sup> cDC1s, which were enriched in the meninges at 16 h, whereas cDC2 and macrophage/moDC populations were unchanged (Figures 6L and S6H). In the brain, AHR inhibition did not significantly alter the proportion of intestinal-derived

(B) Euclidean distance and principal-component analysis (PCA) of CD11c<sup>+</sup> cell DEGs by organ and genotype (*n* = 3/condition).

(C) UMAP of ileum LP CD11c<sup>+</sup> cells 72 h after stroke (*n* = 3/condition).

(D and E) Selected DC/macrophage markers projected onto UMAP (D) and across clusters (E).

(F) *Ahr* expression across control ileum LP CD11c<sup>+</sup> populations; dot size indicates percentage of expressing cells.

(G) Cluster proportions by genotype.

(H) Three most enriched immune system pathways (GO:0002376) per cluster and genotype.

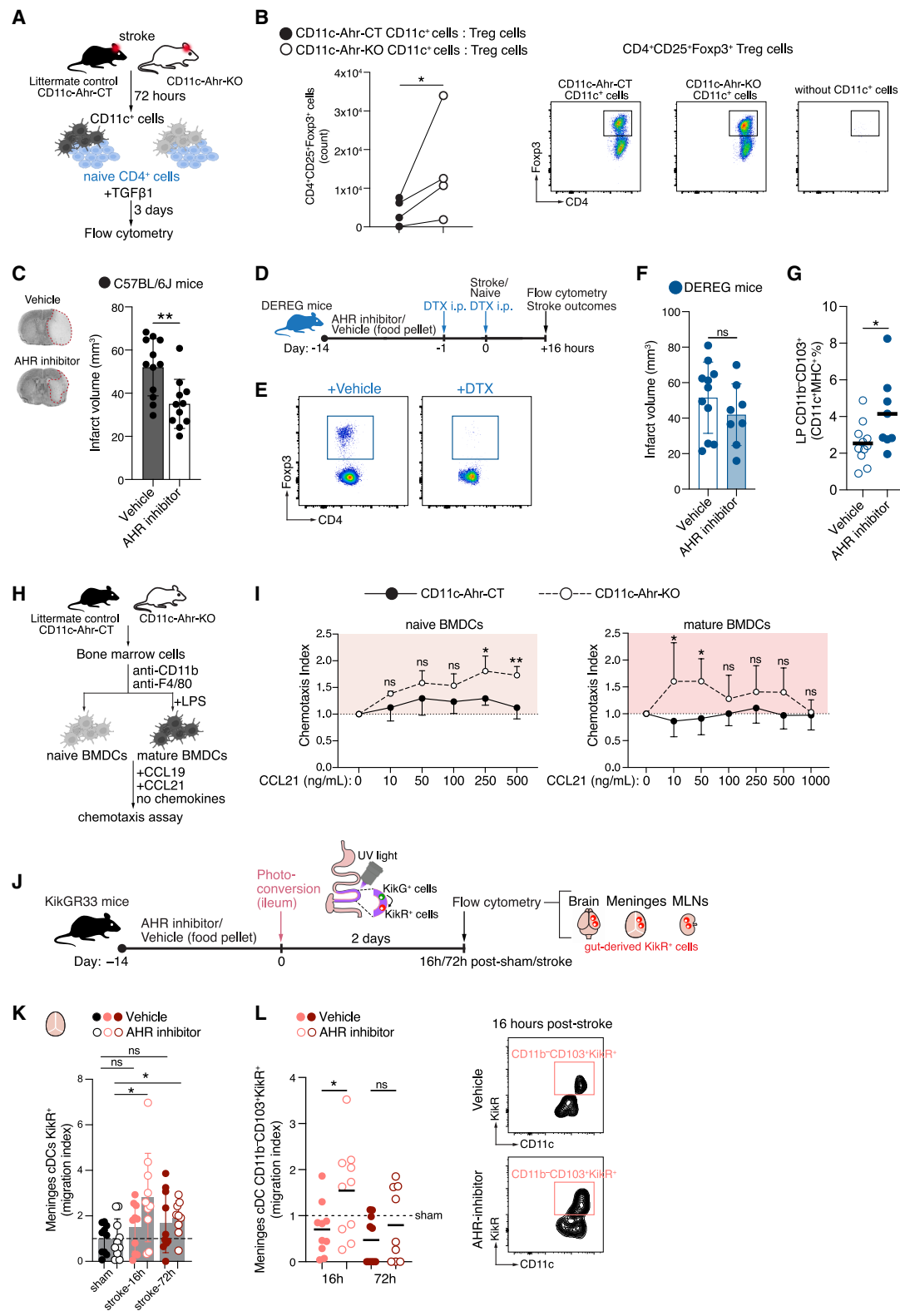
(I) Differential pathway enrichment (GO:0002376) between CD11c-Ahr-KO and control CD11c<sup>+</sup> cells; positive *t* scores indicate enrichment in CD11c-Ahr-KO cells and negative scores in controls.

(J) Within-cluster differential expression of predefined maturation, peripheral tolerance, and mucosal immune regulation genes.

(K) Differences ( $\Delta$ ) in module scores between genotypes by cluster (mean  $\pm$  SEM; *n* = 3/condition); dashed line, no difference.

Kruskal-Wallis with Dunn's correction (A). \*\**p* < 0.01; \*\*\**p* < 0.001; ns, not significant, gene sets are defined in STAR Methods.

See also Figure S5.



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CD11c<sup>+</sup>MHCII<sup>+</sup> cells at 72 h post-stroke, and gut-derived cDC1s were not detectable under the conditions tested (Figure S6I). While we did not find cDC1 cell trafficking to the spleen, gut-derived cDC1s were also enriched in the MLNs upon AHR inhibition (Figure S6J). Together, these findings indicate that AHR inhibition enhances CCR7-dependent DC migration and promotes gut-derived cDC1 accumulation in MLNs and meninges. Although pharmacological AHR inhibition affects multiple cell types, its ability to phenocopy key features of CD11c-Ahr-KO mice supports a central role for AHR in regulating cDC1 frequencies, DC migration, Treg responses, and stroke outcome.

### Microbiota from patients with stroke alters intestinal cDCs and stroke outcome after transfer to mice

In a small cohort of patients with stroke (Table S2), fecal indole was higher in moderate-to-severe stroke (National Institutes of Health Stroke Scale, NIHSS  $\geq 5$ ), than in mild stroke (NIHSS  $< 5$ ), and positively correlated with NIHSS score (Figure S7A), which suggests an association between indole and stroke severity. To assess whether microbiota from patients with stroke functionally influence DCs and stroke outcome, we utilized a reverse translational approach and performed fecal microbiota transplantation (FMT) from patients with mild (NIHSS  $\leq 3$ ,  $N = 3$ ) or moderate-to-severe neurological deficits (NIHSS  $\geq 13$ ,  $N = 3$ ) into antibiotic-treated recipient mice. Selection of patients for FMT was based on their NIHSS score (Figure 7A; Table S2). Recipients of severe-stroke donor microbiota were enriched in *tnaA*-encoding taxa, including Enterobacteriaceae and Akkermansiaceae (amplicon sequence variant ASV\_8 and ASV\_1, respectively) as analyzed by 16S rRNA sequencing. BLASTn identified the Enterobacteriaceae ASV\_8 as *E. coli* (Figures S7B and S7C). Whole-genome sequencing confirmed that human isolates clustered within the *E. coli* clade and were closely related to reference strains and our mouse isolate (Figure S7D), thus supporting cross-species colonization and conserved functional potential. However, urinary indole did not significantly correlate with ileal *tnaA*-encoding bacterial abundance (Figure S7E). These data confirm the transfer of *tnaA*-enriched microbiota from patients with severe stroke to recipient mice.

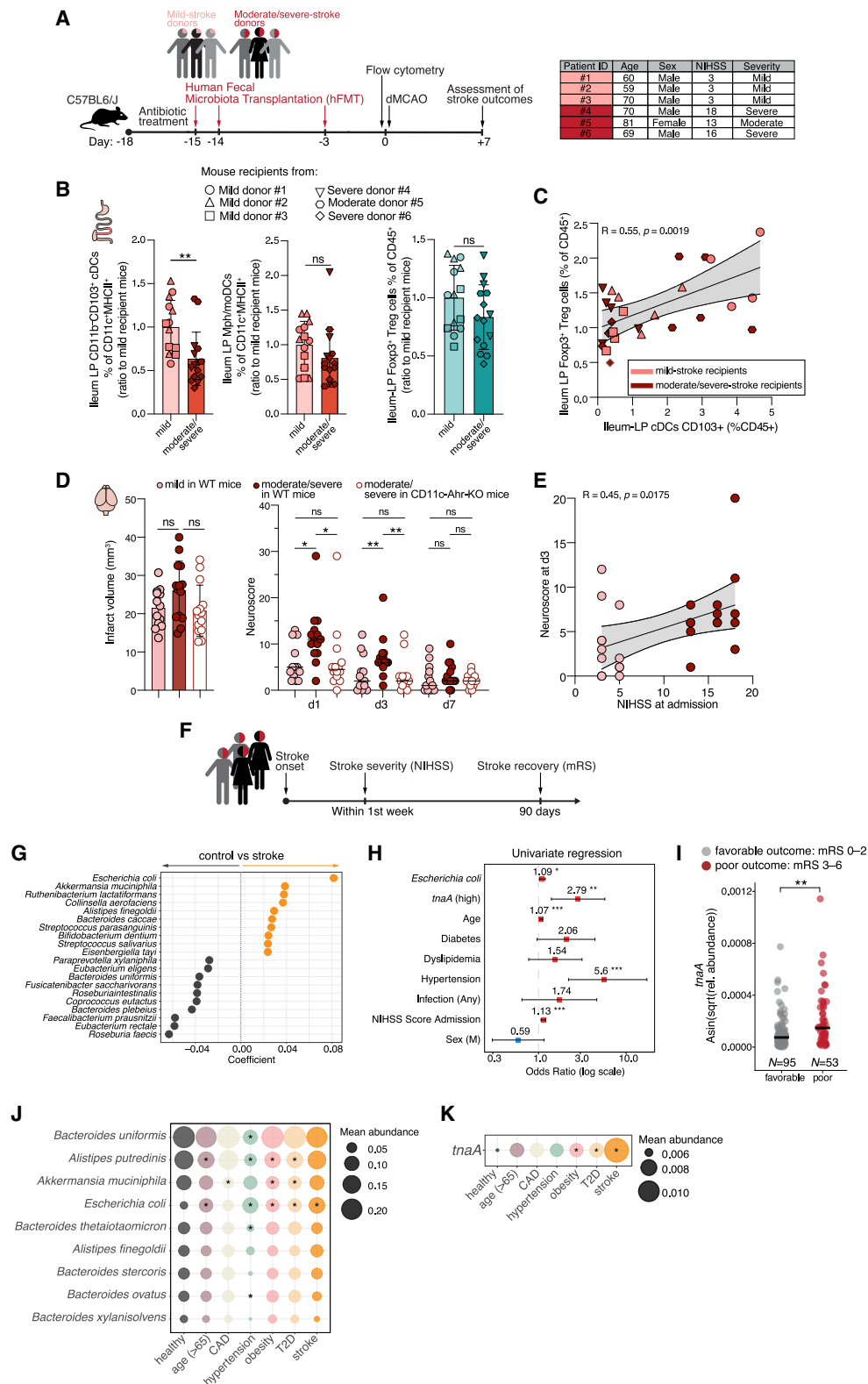
We next examined whether severe-stroke donor microbiota altered intestinal DC composition in recipient mice. Flow cytometric analysis revealed a significant reduction in ileal CD11b<sup>+</sup>CD103<sup>+</sup> cDC frequencies in mice colonized with microbiota from severe-stroke donors (Figure 7B). After accounting for donor-level clustering in mixed-effects models, the direction of this effect was preserved but no longer significant. This pattern was consistent with the reduction in ileal DCs observed after experimental stroke (Figures S5A and S5B). These alterations were restricted to the gut, as no comparable changes were observed in MLNs (Figure S7F), while macrophage/modC and intestinal Treg frequencies were unaffected (Figure 7B). Across recipients, CD11b<sup>+</sup>CD103<sup>+</sup> cDCs positively correlated with Treg frequencies independently of donor group (Figure 7C), thus linking microbiota-dependent changes in intestinal cDCs to Treg responses.

To determine whether the microbiota from patients with stroke influenced outcomes in mice, we subjected recipient mice to distal middle cerebral artery occlusion (dMCAO), a model that does not induce gut dysbiosis<sup>37</sup> and therefore isolates the effect of transferred microbiota (Figure 7A). Mice colonized with severe-stroke donor microbiota exhibited larger infarct volumes on average and increased neurological deficits than recipients of mild-stroke donor microbiota (Figure 7D). Notably, neurological deficits in recipient mice positively correlated with the NIHSS scores of the corresponding human donors, supporting cross-species transfer of disease severity (Figure 7E). The detrimental effect associated with severe-stroke donor microbiota was less pronounced in CD11c-Ahr-KO recipients than in WT mice colonized with the microbiota from severe-stroke donors, which suggests that CD11c-intrinsic AHR expression may contribute, at least in part, to microbiota-associated modulation of stroke severity (Figure 7D).

We next assessed AHR-associated gene expression in peripheral blood myeloid cells from patients with ischemic stroke at 24 and 72 h after onset using bulk RNA sequencing in a pilot cohort. Samples were segregated primarily by stroke severity rather than sampling time, thus indicating that transcriptional variation is driven more strongly by clinical severity than by temporal dynamics within the first 72 h (Figure S7G). Patients with higher NIHSS scores showed an increased expression of AHR-

### Figure 6. AHR inhibition promotes Treg expansion and gut-derived DC accumulation in the meninges linked to improved stroke outcome

- (A) Co-culture of induced Tregs with CD11c<sup>+</sup> cells isolated from littermate control or CD11c-Ahr-KO mice 72 h after stroke.  
(B) CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs numbers and representative plots of after co-culture with control or CD11c-Ahr-KO CD11c<sup>+</sup> cells or without CD11c<sup>+</sup> cells ( $n = 4$ ; lines connect paired experiments).  
(C) Nissl-stained sections and infarct volumes 72 h after stroke in mice fed vehicle- (control) or CH-223191- (AHR inhibitor) supplemented chow for 2 weeks before stroke (mean  $\pm$  SD,  $n = 11$ –12).  
(D) Treg depletion in DEREG mice treated with vehicle or CH-223191 before stroke (DTX, diphtheria toxin).  
(E) Representative plots confirming Treg depletion.  
(F) Infarct volumes 16 h after stroke in Treg-depleted DEREG mice treated with vehicle or CH-223191 (mean  $\pm$  SD,  $n = 8$ –11).  
(G) Ileum LP CD11b<sup>+</sup>CD103<sup>+</sup> cDC1 frequencies in mice from (F) (mean,  $n = 8$ –11).  
(H) CCL19/CCL21 migration assay of naive and mature BMDCs from littermate control and CD11c-Ahr-KO mice.  
(I) Chemotaxis index toward CCL21 relative to migration without chemokine (mean  $\pm$  SD; four independent experiments).  
(J) Photoconversion and tracking of ileum-derived KikRed<sup>+</sup> (KikR<sup>+</sup>) cells in vehicle- or CH-223191-treated KikGR33 mice after sham or stroke.  
(K) Migration index of gut-derived meningeal KikR<sup>+</sup> cDCs, calculated as stroke/sham ratios (mean  $\pm$  SD,  $n = 7$ –10).  
(L) Migration index and representative flow cytometry plots of gut-derived CD11b<sup>+</sup>CD103<sup>+</sup>KikR<sup>+</sup> cells in meninges (mean,  $n = 9$ –10).  
Unpaired *t* test (C), (F), and (G); Mann-Whitney (L); ratio paired *t* test (B); Kruskal-Wallis test with Dunn's correction (K); or repeated-measures two-way ANOVA with Šidák's correction (I). \* $p < 0.05$ ; \*\* $p < 0.01$ ; ns, not significant.  
See also Figure S6.



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associated genes and a reduced expression of genes related to T cell activation (Figures S7H and S7I). Although this analysis was performed in a small set of patients, these findings suggest an association between stroke severity and an AHR-related myeloid program.

To evaluate the clinical relevance of microbiota-derived indole for stroke outcome in patients, we analyzed fecal metagenomic data collected within the first week after ischemic stroke (Figure 7F). Consistent with our experimental findings, *E. coli* was among the most enriched taxa in patients with stroke, and *tnaA* abundance was higher than in controls (Figures 7G and S7J). In univariate analyses, both *E. coli* abundance and high *tnaA* levels were associated with an unfavorable 90-day outcome (modified Rankin Scale, mRS) (Figure 7H). After adjustment for clinical covariates, age, hypertension, and NIHSS at admission remained independent predictors, whereas *E. coli* was no longer associated with outcome. High *tnaA* nevertheless retained a strong trend toward poor outcomes (odds ratio [OR] = 2.23, 95% confidence interval [CI] 0.97–5.25,  $p = 0.061$ , Table S3). In line with these results, patients with an unfavorable outcome (mRS 3–6) had greater *tnaA* abundance than those with a favorable recovery (mRS 0–2) (Figure 7I). These data suggest that enrichment of indole-producing bacteria may be linked to stroke progression and recovery. We next asked whether the enrichment of *tnaA*-encoding taxa was also present in individuals with common stroke-associated comorbidities. Notably, *E. coli* and *tnaA* were also enriched in individuals with obesity or type 2 diabetes, similar to the pattern detected in patients with stroke (Figures 7J and 7K). Thus, indole-producing bacterial communities may already be expanded in conditions associated with increased stroke risk.

Collectively, these findings identify *tnaA*-encoding bacteria as a microbiota signature associated with poor stroke outcomes. Transfer of microbiota from patients with severe stroke altered intestinal cDC composition and worsened ischemic injury in recipient mice, whereas this effect was attenuated in CD11c-Ahr-KO animals, thus supporting a microbiota-indole-AHR-DC axis linking human microbiota composition to stroke severity.

## DISCUSSION

We identify microbial tryptophan metabolism as a preconditioning mechanism that influences stroke severity through AHR-dependent programming of intestinal DCs, thus highlighting DCs as a link between microbial metabolic cues, regulatory immune programs, and gut-to-brain-border trafficking after stroke (Figure S7K). Although whether indole directly suppresses a cDC1-Treg response remains unresolved, limiting AHR signaling or microbial tryptophan metabolism before stroke reduces disease severity and may have preventive potential.

Microbial tryptophan metabolites can act as AHR ligands with distinct biological effects.<sup>15,17,38,39</sup> In stroke, indole-3-carboxaldehyde and indole-3-propionic acid (IPA) exert protective effects through microglial AHR,<sup>21</sup> while other studies report a beneficial role for IPA or indole lactic acid.<sup>40,41</sup> By contrast, our findings identify indole as a microbial metabolite that exacerbates stroke outcome through AHR in DCs, which is consistent with reports linking indole or colonization with indole-producing *E. coli* to anxiety- and depression-like behaviors.<sup>42</sup> Thus, structurally related microbial indoles can have divergent effects depending on metabolite and biological context, thus highlighting the importance of resolving ligand-specific AHR responses.<sup>43</sup> Ligand specificity may be particularly relevant in intestinal DCs, which integrate microbial cues and direct T cell differentiation.<sup>44</sup> CD103<sup>+</sup> DCs promote Treg differentiation in response to vitamin A through TGF- $\beta$  and retinoic acid,<sup>45</sup> whereas ROR $\gamma$ t<sup>+</sup> antigen-presenting cells support tolerance to dietary and microbial antigens.<sup>46</sup> Tryptophan metabolites also modulate DC function.<sup>47</sup> Host-derived kynurenine induces Tregs through AHR in DCs,<sup>48,49</sup> and IL-10-conditioned DCs require AHR to maintain regulatory functions.<sup>50</sup> Our finding that AHR loss in CD11c<sup>+</sup> cells is associated with increased Treg responses contrasts with these reports.<sup>17,51</sup> This divergence may reflect ligand identity, DC subset, tissue and disease context, and the timing of AHR activation. In our model, AHR deletion in CD11c<sup>+</sup> cells preserves cDC1s, enhances immunoregulatory and migratory programs across several DC subsets, and increases Treg abundance in

### Figure 7. Fecal microbiota transplantation from patients with stroke into recipient mice affects CD11b<sup>+</sup>CD103<sup>+</sup> cell frequencies and stroke severity in mice

(A) Human fecal microbiota transplantation (hFMT) from patients with mild or severe stroke into antibiotic-treated mice, followed 2 weeks later by flow cytometry or dMCAO.  
(B) Ileum LP CD11b<sup>+</sup>CD103<sup>+</sup> cDCs, mph/moDCs, and Foxp3<sup>+</sup> Tregs in hFMT recipient mice, relative to mice receiving mild-stroke donor microbiota (mean  $\pm$  SD,  $n = 14$ –15).  
(C) Correlation between ileum LP Treg and CD11b<sup>+</sup>CD103<sup>+</sup> cDCs in recipients.  
(D) Infarct volumes 7 days after dMCAO (mean  $\pm$  SD) and neurological scores at days 1, 3, and 7 (median) in C57BL/6J mice receiving mild- or severe-stroke donor microbiota ( $p = 0.0683$ ), and CD11c-Ahr-KO mice receiving severe-stroke donor microbiota ( $p = 0.1139$ ) ( $n = 13$ –14).  
(E) Correlation between recipient neuroscore at day 3 and donor NIHSS.  
(F) Metagenomic analysis of patients with ischemic stroke and age-matched controls (see STAR Methods). NIHSS and mRS were assessed during the first week and at 90 days, respectively.  
(G) Ten taxa most enriched in patients with stroke (orange) and control patients (gray).  
(H) Univariate regression analysis of *tnaA*, *E. coli*, and clinical risk factors with unfavorable 90-day outcomes (mRS;  $N = 148$ ).  
(I) Arcsin-square-root-transformed *tnaA* abundance (median) in favorable (mRS: 0–2;  $N = 95$ ) and unfavorable outcomes (mRS: 3–6;  $N = 53$ ).  
(J and K) Relative abundance of *tnaA*-encoding species (J) and *tnaA* (K) in healthy individuals, patients with stroke, and individuals with stroke risk factors (T2D: type 2 diabetes, CAD: coronary artery disease; healthy:  $N = 116$ ; age > 65:  $N = 214$ ; CAD:  $N = 75$ ; hypertension:  $N = 34$ ; obesity:  $N = 208$ ; T2D:  $N = 344$ ; stroke:  $N = 275$ ); \*, versus healthy controls.  
Mann-Whitney test (B) and (I), Kruskal-Wallis with Dunn's correction (D), and Spearman's test (C) and (E). Statistical inference for (B) and (D) used mixed-effects models with donor as random intercept. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, not significant.  
See also Figure S7.

the MLNs and meninges under inflammatory stroke conditions. Although AHR-deficient DCs promoted Tregs *in vitro*, our *in vivo* data do not establish direct *de novo* Treg induction by DCs. Increased Treg abundance may instead reflect differentiation, expansion, maintenance, or recruitment supported by preserved and migrating intestinal DCs. Treg depletion abolished CH-223191-mediated neuroprotection, thus demonstrating that Tregs are required for the beneficial effect of pharmacological AHR inhibition. However, because CH-223191 inhibits AHR in multiple cell types, this does not demonstrate that Tregs act exclusively downstream of AHR in CD11c<sup>+</sup> cells; direct effects on Tregs or other cells may also contribute.<sup>52,53</sup> Although our experiments do not establish DCs as the sole inducers of Tregs, they link AHR loss of function to tolerance-supporting DC states, increased Treg responses, and reduced brain injury. How Tregs improve outcomes remains unclear, but preservation of the Treg pool is likely important for limiting excessive post-stroke inflammation. Tregs suppress pro-inflammatory myeloid and T cell responses, including through IL-10,<sup>6,10,54,55</sup> while meningeal T cells restrain immune activation at brain borders and pathogenic immune cell recruitment into the CNS.<sup>56</sup> Microbiota-driven DC programming may therefore support Treg responses at brain borders, thus contributing to reduced neuroinflammation and improved stroke outcome.

The microbiota also shapes immune cell trafficking along the gut-brain axis. After stroke, immune cells migrate from the gut to the brain and meninges,<sup>36</sup> and antibiotic-induced microbiota alterations restrict the meningeal accumulation of IL-17-producing  $\gamma\delta$ T cells and reduce neuroinflammation.<sup>6</sup> Although the meninges contain both locally derived and circulating immune cells,<sup>57–60</sup> how microbial signals influence intestinal DC states that subsequently traffic to brain borders remains poorly understood. We address this gap by linking AHR-dependent intestinal DC programming to a migratory state and increased meningeal accumulation after stroke. CCR7<sup>+</sup> mregDCs represent a conserved maturation-associated state in which migratory and immunoregulatory programs are shared across cDC1 and cDC2 lineages.<sup>33</sup> Consistent with this, intestinal scRNA-seq analysis showed the strongest increase in migration-associated genes in mregDCs upon AHR deletion, alongside enhanced cDC1 identity-associated genes. AHR inhibition increased the accumulation of gut-derived CD11b<sup>+</sup>CD103<sup>+</sup> cDCs in the MLNs and meninges associated with better stroke outcome, and AHR-deficient DCs displayed enhanced chemotaxis toward the CCR7 ligands CCL19/CCL21 *in vitro*. Whether CCR7 mediates meningeal entry and microbial indole regulates intestinal DC trafficking remains to be established *in vivo*.

Previous reports describe post-stroke expansion of Enterobacteriaceae,<sup>61,62</sup> which is potentially driven by intestinal inflammation, impaired gastrointestinal motility,<sup>37</sup> altered diet and substrate availability. We extend these observations by linking the *E. coli*-tryptophanase-indole metabolism to stroke severity and show its manipulation before stroke modifies outcomes in mice. Our data showed enrichment of *E. coli* and *tnaA* in patients with stroke and individuals with common stroke risk factors, particularly obesity and diabetes, thus suggesting that pre-existing differences in microbial tryptophan metabolism may influence stroke susceptibility and severity. Human microbiota ana-

lyses and transplantation experiments support the translational relevance of this pathway: mice receiving microbiota from patients with severe neurological deficits had fewer CD11b<sup>+</sup>CD103<sup>+</sup> DCs in the gut, which correlated with Treg frequencies, and stroke severity was attenuated in CD11c-Ahr-KO mice. Together with clinical observations linking low circulating DC numbers to stroke severity,<sup>63</sup> our findings support a model in which the pre-stroke microbiota shapes DC-dependent immune responses and neurological outcome through AHR in CD11c<sup>+</sup> cells across species. If similar regulation occurs in humans, then targeting microbial tryptophan metabolism through microbiota manipulation,<sup>64</sup> or AHR-directed interventions,<sup>51</sup> could complement vascular risk management and may have broader relevance to other neurological diseases in which pre-existing microbiota state linked with indole-producing bacteria may prime the gut immune system and influences disease development.

### Limitations of the study

Although we identify indole as a key microbial metabolite regulating AHR-dependent responses in DCs and exclude a major role for the host-conjugated indole metabolite I3S, other AHR ligands may modulate gut immune cell function and contribute to stroke outcome. Our data support the small intestine as a relevant site of microbiota-AHR crosstalk after stroke, and so future studies should determine whether similar mechanisms occur in other intestinal compartments where tryptophan metabolism is relevant. In addition, while we demonstrate CCR7-dependent DC migration *in vitro*, whether CCR7-dependent trafficking of intestinal cDCs is required for AHR-mediated neuroprotection will require *in vivo* loss-of-function studies in defined DC subsets. Because CH-223191 inhibits AHR systemically, we cannot exclude direct effects on Tregs or other immune and non-immune cells, in addition to DC-mediated effects. The mechanistic mouse experiments were performed exclusively in males. Sex-dependent differences in AHR-regulated transcriptional and biological responses have been reported in other tissues and disease contexts.<sup>65–67</sup> Whether microbial indole-AHR signaling in intestinal DCs similarly affects stroke outcome in females remains to be determined. Finally, human microbiota analyses are correlative and require validation in larger prospective cohorts.

### RESOURCE AVAILABILITY

#### Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Corinne Benakis ([corinne.benakis@med.uni-muenchen.de](mailto:corinne.benakis@med.uni-muenchen.de)).

#### Materials availability

This study did not generate any new, unique reagents.

#### Data and code availability

Metabolomic data are available in MetaboLights: MTBLS10296. Murine 16S rRNA sequencing and metagenomic data are deposited in ENA: PRJEB76125, and human metagenomic data in ENA: PRJEB112493. RNA-seq datasets are available in GEO: GSE242793 (WT) and GEO: GSE335318 (KO) (CD11c<sup>+</sup> cell scRNA-seq), GEO: GSE333893 (CD45<sup>+</sup> cell scRNA-seq), and GEO: GSE331113 (human PBMC bulk RNA-seq). Processed MetaPhlAn

and HUMAnN profiles and metadata are available on Zenodo: <https://zenodo.org/records/20266213>. Analysis code is available at [https://github.com/BenakisLab/AhR\\_stroke](https://github.com/BenakisLab/AhR_stroke) and software is listed in the [key resources table](#). Additional information is available from the [lead contact](#) upon request.

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## AUTHOR CONTRIBUTIONS

Conceptualization, C.B. and A.L.; methodology, R.D.J., A. Ruggles, M.A.A., M. Gelderblom, V.R., T.T., S.K., J. Huehn, and C.O.; investigation and formal analysis, R.D.J. (lead experimental work), A. Ruggles and M.A.A. (critical experiments added during revision), and D.F., L.D.-M., K.P., R.S., A. Ricci, O.C., and S.R.; 16S rRNA-seq, metagenomic, and bulk RNA-seq analysis, A. Sorbie; scRNA-seq and analysis, A. Simats, C.D., M.V., and K.S.; western blotting, S.L.; *in vitro* migration assays, J.B. and Y.H.; targeted LC-MS/MS, M.Z. and V.P.; indole UHPLC-MS/MS, M. Gigl; AHR expertise, V.R., T.T., S.K., J. Huehn, and C.O.; resources, S.M.M. and L.H. (patient samples and expertise), P.M. and A.L. (patient samples), M.L. and I.F.-C. (patient metagenomic data), A.-M.T., C.W., and L.K. (patient bulk RNA-seq data), A.C. and J. Herkel (AHR-genetic mouse models), and J.B., M.Z., M. Gigl, and M.D.; funding acquisition, C.B., A.L., and M.D.; project administration and supervision, C.B.; formal analysis, visualization, and writing, C.B.; review and editing, C.B., A.L., V.R., T.R., R.S., R.D.J., D.F., A. Sorbie, A. Ruggles, and A. Simats; critical review of the revised manuscript, A.L., R.D.J., A. Ruggles, A. Sorbie, T.R., C.O., J. Huehn, J.B., S.L., M.A.A., A. Simats, D.F., M.Z., V.P., I.F.-C., L.H., T.T., and M.V.; all authors read and approved the manuscript.

## DECLARATION OF INTERESTS

M.D. received personal fees from Odyssey Therapeutics outside the submitted work.

## DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the author(s) used ChatGPT 5.3 Instant and Perplexity to improve language and readability and to generate R scripts for flow cytometry data plotting. After using this tool or service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Permanent distal cerebral artery occlusion model
  - Small intestine photoconversion in KikGR33 mice
  - Fecal microbiota transplantation
  - *Escherichia coli* colonization in mice
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  - Measurement of infarct volume
  - Stroke severity score
  - Organ and tissue collection
  - Gut immunohistochemistry and CD11c-eYFP quantification
  - Cell isolation
  - Flow cytometry
  - Cytometric Bead Array (CBA)
  - *In vitro* CD11c<sup>+</sup> cell incubation with indole derivatives
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  - Single-cell RNA sequencing
  - Single-cell RNA sequencing analysis
  - Bacterial DNA extraction
  - Quantification of 16S ribosomal (r16S) gene DNA copy numbers
  - Metagenomic sequencing
  - 16S rRNA gene amplicon sequencing
  - Targeted LC-MS/MS quantification for tryptophan-derived metabolites
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  - Sample preparation for Kovács and Indican assays
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  - Human metagenomic profiling
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- [QUANTIFICATION AND STATISTICAL ANALYSIS](#)

## SUPPLEMENTAL INFORMATION

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                                    | SOURCE        | IDENTIFIER                              |
|--------------------------------------------------------|---------------|-----------------------------------------|
| <b>Antibodies</b>                                      |               |                                         |
| CD45 eFluor450 (30-F11)                                | eBioscience   | Catalog# 48-0451-82; RRID: AB_1518806   |
| CD45 Brilliant Ultra Violet™ 737 (30-F11)              | eBioscience   | Catalog # 367-0451-82; RRID: AB_2895963 |
| CD16/CD32 Monoclonal Antibody (93)                     | eBioscience   | Catalog# 14-0161-85; RRID: AB_467134    |
| CD3 FITC (17A2)                                        | eBioscience   | Catalog# 11-0032-82; RRID: AB_2572431   |
| CD3 APC (17A2)                                         | eBioscience   | Catalog# 17-0032-82; RRID: AB_10597589  |
| CD3 Brilliant Violet 605™ (OKT3)                       | Biolegend     | Catalog# 317322; RRID: AB_2561911       |
| CD3 Brilliant Violet 605                               | Biolegend     | Catalog# 100237; RRID: AB_2562039       |
| CD3e PE (145-2C11)                                     | Invitrogen    | Catalog# A14714; RRID: AB_2534230       |
| Purified NA/LE Hamster Anti-Mouse CD3e (145-2C11)      | BDBiosciences | Catalog# 553057; RRID: AB_394590        |
| CD4 PerCP/Cy5.5 (RM4-5)                                | eBioscience   | Catalog# 45-0042-82; RRID: AB_1107001   |
| CD4 APC/Fire™ 810 (GK1.5)                              | Biolegend     | Catalog# 100479; RRID: AB_2860583       |
| CD25 PE/Cy7 (PC61.5)                                   | eBioscience   | Catalog# 25-0251-82; RRID: AB_469608    |
| CD25 BB515 (PC61)                                      | BDBiosciences | Catalog# 564424; RRID: AB_2738803       |
| CD19 APC-eFluor™ 780 (eBio1D3)                         | eBioscience   | Catalog# 47-0193-82; RRID: AB_10853189  |
| CD19 PE-Cyanine7 (HIB19)                               | eBioscience   | Catalog# 25-0199-41; RRID: AB_1582279   |
| CD19 PE (eBio1D3)                                      | eBioscience   | Catalog# 12-0193-82; RRID: AB_657659    |
| CD19 APC-eFluor™ 780 (eBio1D3)                         | eBioscience   | Catalog# 47-0193-82; RRID: AB_10853189  |
| FOXP3 PE (NRRF-30)                                     | eBioscience   | Catalog# 12-4771-82; RRID: AB_529580    |
| TCR $\gamma/\delta$ Brilliant Violet 605 (GL3)         | Biolegend     | Catalog# 118129; RRID: AB_2563356       |
| TCR $\gamma/\delta$ BV750 (GL3)                        | BDBiosciences | Catalog# 746962; RRID: AB_2871745       |
| CD11c Brilliant Violet 650 (N418)                      | Biolegend     | Catalog# 117339; RRID: AB_2562414       |
| CD172a (SIRP alpha) PE-Cyanine7 (P84)                  | eBioscience   | Catalog# 25-1721-82; RRID: AB_2762640   |
| MHCII (I-A/I-E) BV786 (2G9)                            | BDBiosciences | Catalog# 743875; RRID: AB_2741826       |
| MHCII (I-A/I-E) FITC (M5/114.15.2)                     | eBioscience   | Catalog# 11-5321-82; RRID: AB_465232    |
| Ly6G (Gr1) PE-eFluor™ 610 (1A8-Ly6g)                   | eBioscience   | Catalog# 61-9668-82; RRID: AB_2574679   |
| CD11b BD Horizon™ R718 (M1/70)                         | BDBiosciences | Catalog# 567469; RRID: AB_2916612       |
| CD11b PerCP-Cyanine5.5 (M1/70)                         | eBioscience   | Catalog# 45-0112-82; RRID: AB_953558    |
| CD11b Brilliant Violet 570™ (M1/70)                    | Biolegend     | Catalog# 101233; RRID: AB_10896949      |
| CD64 (Fc $\gamma$ RI) Brilliant Violet 711 (X54-5/7.1) | Biolegend     | Catalog# 139311; RRID: AB_2563846       |
| CD64 (Fc $\gamma$ RI) PE/Cyanine7 (X54-5/7.1)          | Biolegend     | Catalog# 139313; RRID: AB_2563903       |
| F4/80 Brilliant Violet 605 (BM8)                       | Biolegend     | Catalog# 123133; RRID: AB_2562305       |
| F4/80 PE-Cyanine7 (BM8)                                | eBioscience   | Catalog# 25-4801-82; RRID: AB_469653    |
| CD11c APC (N418)                                       | eBioscience   | Catalog# 17-0114-82; RRID: AB_469346    |
| CD11c Super Bright™ 436 (N418)                         | eBioscience   | Catalog# 62-0114-82; RRID: AB_2722928   |
| CD103 (Integrin alpha E) APC-eFluor™ 780 (2E7)         | eBioscience   | Catalog# 47-1031-82; RRID: AB_2811820   |
| CD103 (Integrin alpha E) Brilliant Violet™ 480 (2E7)   | eBioscience   | Catalog# 414-1031-82; RRID: AB_2942166  |
| IFN gamma FITC (XMG1.2)                                | eBioscience   | Catalog# 11-7311-82; RRID: AB_465412    |
| IL-17A APC (eBio17B7)                                  | eBioscience   | Catalog# 17-7177-81; RRID: AB_763580    |
| LAP (Latency Associated peptide) PE (TW7-16B4)         | eBioscience   | Catalog# 12-9821-82; RRID: AB_2572734   |
| Ly-6C RB705 (AL-21)                                    | BDBiosciences | Catalog# 570264; RRID: AB_3685625       |
| CD8a APC-eFluor™ 780 (53-6.7)                          | eBioscience   | Catalog# 47-0081-82; RRID: AB_1272185   |
| CD8a Brilliant Violet 510™ (53-6.7)                    | Biolegend     | Catalog# 100751; RRID: AB_2561389       |

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**Continued**

| REAGENT or RESOURCE                                                        | SOURCE                           | IDENTIFIER                                                   |
|----------------------------------------------------------------------------|----------------------------------|--------------------------------------------------------------|
| CD45R/B220 PE-Cy <sup>TM</sup> 5 (RA3-6B2)                                 | BDBiosciences                    | Catalog# 561879; RRID: AB_10898014                           |
| CD45R/B220 PE (RA3-6B2)                                                    | eBioscience                      | Catalog# 12-0452-82; RRID: AB_465671                         |
| CD45R/B220 Brilliant Violet 785 (RA3-6B2)                                  | Biolegend                        | Catalog# 103245; RRID: AB_11218795                           |
| PE/Cyanine5 anti-mouse CD197 (CCR7)                                        | Biolegend                        | Catalog#120113; RRID: AB_493571                              |
| CD197 (CCR7) PE-Cyanine5.5 (3D12)                                          | eBioscience                      | Catalog# 35-1979-42; RRID: AB_2815128                        |
| CD172a (SIRPα) Alexa Fluor® 700 (P84)                                      | Biolegend                        | Catalog# 144022; RRID: AB_2650813                            |
| NK1.1 PE (PK136)                                                           | eBioscience                      | Catalog# 12-5941-82; RRID: AB_466050                         |
| NK1.1 Brilliant Violet 650 <sup>TM</sup> (PK136)                           | Biolegend                        | Catalog# 108735; RRID: AB_11147949                           |
| XCR1 Brilliant Violet 421 (ZET)                                            | Biolegend                        | Catalog# 148216; RRID: AB_2565230                            |
| XCR1 APC/Cyanine7 (ZET)                                                    | Biolegend                        | Catalog# 148224; RRID: AB_2783118                            |
| Phalloidin-iFluor 647                                                      | Abcam                            | Catalog# ab176759                                            |
| Rabbit anti-CYP1A1 (Cytochrome P450, family 1, subfamily A, polypeptide 1) | Proteintech                      | Catalog# 13241-1-AP; RRID: AB_2877928                        |
| Mouse anti-CYP1B1 (Cytochrome P450, family 1, subfamily B, polypeptide 1)  | Proteintech                      | Catalog# 67033-1-Ig; RRID: AB_2882348                        |
| Rat anti-CCR7                                                              | Thermo Fisher                    | Catalog# MA1-163; RRID: AB_2536868                           |
| Rabbit anti-beta-actin                                                     | Cell Signaling                   | Catalog# 12620S; RRID: AB_2797972                            |
| Goat anti-rabbit                                                           | Vector Laboratories              | Catalog# PI-1000; RRID: AB_2336198                           |
| Goat anti-rat                                                              | R&D Systems/Biotechne            | Catalog# HAF005; RRID: AB_1512258                            |
| Goat anti-mouse                                                            | Dako                             | Catalog# P0447; RRID: AB_2617137                             |
| TotalSeq <sup>TM</sup> -B0301 anti-mouse Hashtag 1                         | Biolegend                        | Catalog# 155831; RRID: AB_2814067                            |
| TotalSeq <sup>TM</sup> -B0302 anti-mouse Hashtag 2                         | Biolegend                        | Catalog# 155833; RRID: AB_2814068                            |
| TotalSeq <sup>TM</sup> -B0303 anti-mouse Hashtag 3                         | Biolegend                        | Catalog# 155835; RRID: AB_2814069                            |
| TotalSeq <sup>TM</sup> -B0304 anti-mouse Hashtag 4                         | Biolegend                        | Catalog# 155837; RRID: AB_2814070                            |
| <b>Bacterial and virus strains</b>                                         |                                  |                                                              |
| <i>E. coli</i> BW25113                                                     | PMID: 16738554, ME Collection    | ME9062                                                       |
| <i>E. coli</i> Δ <i>tnaA</i> JW3686                                        | PMID: 16738554, Keio Collection  | JW3686-KC                                                    |
| <b>Biological samples</b>                                                  |                                  |                                                              |
| Stool samples from patients with stroke                                    | CHUV (Switzerland), LMU (Munich) | (Please see details in <a href="#">STAR Methods</a> section) |
| <b>Chemicals, peptides, and recombinant proteins</b>                       |                                  |                                                              |
| Ampicillin sodium salt                                                     | Sigma-Aldrich                    | Catalog# A9518-5G                                            |
| Metronidazole                                                              | Sigma-Aldrich                    | Catalog# 46461-250MG                                         |
| Neomycin trisulfate salt hydrate                                           | Sigma-Aldrich                    | Catalog# N5285-25G                                           |
| Vancomycin                                                                 | Sigma-Aldrich                    | Catalog# SBR00001                                            |
| Penicillin-Streptomycin (10,000 U/mL)                                      | Gibco <sup>TM</sup>              | Catalog# 15140122                                            |
| Human TGF-beta 1 Recombinant Protein                                       | PeproTech                        | Catalog# 100-21C-10UG                                        |
| Mouse CCL19 (MIP-3 beta) Recombinant Protein                               | PeproTech                        | Catalog# 250-27B-20UG                                        |
| Mouse CCL21 (6Ckine) Recombinant Protein                                   | PeproTech                        | Catalog# 250-13-20UG                                         |
| Tamoxifen                                                                  | Sigma Aldrich                    | Catalog# 85256                                               |
| Histopaque®-1077                                                           | Sigma Aldrich                    | Catalog# 10771                                               |
| Propidium Iodide                                                           | eBiosciences                     | Catalog# 00-6990-50                                          |
| Dimethylsulfoxid                                                           | Sigma-Aldrich                    | Catalog# D2650-100ML                                         |
| Indole                                                                     | Sigma-Aldrich                    | Catalog# I3408                                               |
| Indoxyl sulfate potassium salt                                             | Sigma-Aldrich                    | Catalog# I3875                                               |
| CH-223191                                                                  | MedChemTronica                   | Catalog# HY-12684                                            |
| RIPA Lysis and Extraction Buffer                                           | Thermo Fisher                    | Catalog# 89901                                               |
| Halt <sup>TM</sup> Protease Inhibitor Cocktail (100X)                      | Thermo Fisher                    | Catalog# 78429                                               |

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**Continued**

| REAGENT or RESOURCE                                       | SOURCE                       | IDENTIFIER             |
|-----------------------------------------------------------|------------------------------|------------------------|
| Halt™ Phosphatase Inhibitor Single-Use Cocktail           | Thermo Fisher                | Catalog# 78426         |
| NuPAGE™ Bis-Tris Mini Protein Gels, 4%–12%, 1.0–1.5 mm    | Thermo Fisher                | Catalog# NP0336BOX     |
| Immobilon Western Chemiluminescent HRP Substrate          | Merck Millipore              | Catalog# WBKLS0500     |
| Bovine Serum Albumin                                      | Merck Millipore              | Catalog# A3912-1KG     |
| Dulbecco's Phosphate Buffered Saline                      | Sigma Aldrich                | Catalog# D8537-500ML   |
| Fetal calf serum (FBS)                                    | GIBCO                        | Catalog# 10500064      |
| MacConkey Agar                                            | Roth                         | Catalog# X922.1        |
| Collagenase D                                             | Roche                        | Catalog# 11088882001   |
| Cresyl-violet acetate                                     | Sigma-Aldrich                | Catalog# C5042         |
| Collagen type-I gel, rat tail                             | Merck                        | Catalog# C3867         |
| 1,4-Dithiothreitol (DTT)                                  | Roche                        | Catalog# DTT-RO        |
| DMEM                                                      | Gibco™                       | Catalog# D6429         |
| DNAse I, from bovine pancreas                             | Roche                        | Catalog# 11284932001   |
| EDTA ≥99%, p.a., ACS                                      | Carl Roth®                   | Catalog# 8043.1        |
| Ethanol Absolute (200 Proof)                              | Fisher BioReagents           | Catalog# BP2818100     |
| Ethanol 80%                                               | EMPROVE® EXPERT              | Catalog# 5435431000    |
| Paraformaldehyde                                          | Morphisto                    | Cat# 11762.00100       |
| Ehrlich's reagent                                         | Sigma-Aldrich                | Catalog# 03891-250ML   |
| FACS Flow sheath fluid                                    | BDBioscience                 | Catalog# 342003        |
| Diphtheria Toxin                                          | Merck                        | Catalog# D0564-1MG     |
| NaCl 0.9% B. Braun                                        | Braun                        | Catalog# FREU910       |
| Isoflurane                                                | Merck                        | Catalog# 792632        |
| GM-CSF Protein (AA 18–141)                                | Antibodies-online            | Catalog# ABIN2017856   |
| Brefeldin A from <i>Penicillium brefeldianum</i>          | Sigma-Aldrich                | Catalog# B6542-5MG     |
| Ionomycin from <i>Streptomyces conglobatus</i>            | Sigma-Aldrich                | Catalog# I9657-1MG     |
| Phorbol-12-myristat-13-acetat                             | Sigma-Aldrich                | Catalog# P1585-1MG     |
| HBSS (1X) w/o CaCl <sub>2</sub> /MgCl <sub>2</sub>        | Gibco                        | Catalog# 14175053      |
| HBSS (10X) w/o CaCl <sub>2</sub> /MgCl <sub>2</sub>       | Gibco                        | Catalog# 14185045      |
| HBSS (1X) w CaCl <sub>2</sub> /MgCl <sub>2</sub>          | Gibco                        | Catalog# 14025092      |
| IMDM (Iscove's Modified Dulbecco's Medium)                | Merck                        | Catalog# 51471C-1000ML |
| Heparin                                                   | Sigma-Aldrich                | Catalog# H0200000      |
| HEPES Buffer 1M Solution                                  | Corning                      | Catalog# 25-060-CI     |
| Hystopaque-1077                                           | Sigma-Aldrich                | Catalog# 10771         |
| ULTRA PURE LPS from <i>Salmonella minnesota</i> R595 (Re) | List Biological Laboratories | Catalog# 434-1mg       |
| RPMI 1640 Medium, HEPES                                   | Gibco                        | Catalog# 52400025      |
| Recombinant Human IL-4 Protein                            | R&D Systems                  | Catalog# 204-IL-020/CF |
| Dynabeads™ Oligo(dT)25                                    | ThermoFisher Scientific      | Catalog# 61005         |
| BD Horizon™ Brilliant Stain Buffer                        | BD Biosciences               | Catalog# 563794        |
| Flow Cytometry Staining Buffer                            | Invitrogen                   | Catalog# 00-4222-26    |
| Percoll®                                                  | Sigma-Aldrich                | Catalog# GE17-0891-01  |
| DNAse I                                                   | Roche                        | Catalog# 11284932001   |
| eBioscience™ IC Fixation Buffer                           | Invitrogen                   | Catalog# 00-8222-49    |
| Intracellular Staining Permeabilization Wash Buffer (10X) | Biolegend                    | Catalog# 421002        |
| SPRIselect, 5 mL                                          | Beckman Coulter              | Catalog# B23317        |

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| REAGENT or RESOURCE                                            | SOURCE                  | IDENTIFIER           |
|----------------------------------------------------------------|-------------------------|----------------------|
| Kovács reagent                                                 | Merck                   | Catalog# 60983-100ML |
| Lipopolysaccharides from <i>Escherichia coli</i> O111:B4 (LPS) | Sigma Aldrich           | Catalog# L2630-10MG  |
| DAPI (4',6-diamidino-2-phenylindole)                           | ThermoFisher Scientific | Cat# D3571           |
| Tissue-Tek® O.C.T. Compound                                    | Sakura Finetek          | #4583                |

### Critical commercial assays

|                                                                    |                     |                       |
|--------------------------------------------------------------------|---------------------|-----------------------|
| Pan Dendritic Cell Isolation Kit, mouse                            | Miltenyi Biotec     | Catalog# 130-100-875  |
| CD11c MicroBeads UltraPure, mouse                                  | Miltenyi Biotec     | Catalog# 130-125-835  |
| Anti-F4/80 MicroBeads UltraPure, mouse                             | Miltenyi Biotec     | Catalog# 130-110-443  |
| CD45 MicroBeads, mouse                                             | Miltenyi Biotec     | Catalog# 130-052-301  |
| Naive CD4 <sup>+</sup> T Cell Isolation Kit, mouse                 | Miltenyi Biotec     | Catalog# 130-104-453  |
| T cell Enrichment Kit, mouse                                       | Invitrogen          | Catalog# 8804-6820-74 |
| LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation | Invitrogen          | Catalog# L34966       |
| Foxp3 / Transcription Factor Staining Buffer Set                   | eBioscience         | Catalog# 00-5523-00   |
| Zombie NIR™ Fixable Viability Kit                                  | Biolegend           | Catalog# 423106       |
| CellTrace™ CFSE Cell Proliferation Kit                             | Invitrogen          | Catalog# C34570       |
| Dneasy PowerLyzer PowerSoil Kit                                    | QIAGEN              | Catalog# 12855-50     |
| FACS Research CS&T Beads                                           | BDBioscience        | Catalog# 650622       |
| Pierce™ BCA Protein Assay Kits                                     | Thermo Fisher       | Catalog# 23227        |
| Indican Assay Kit                                                  | Merck               | Catalog# MAK128-1KT   |
| Agilent High Sensitivity DNA kit                                   | Agilent             | Catalog# 5067-4626    |
| 3' Feature Barcode Kit, 16 rxns                                    | 10x Genomics        | Catalog# 1000262      |
| Chromium Next GEM Chip G Single Cell Kit                           | 10x Genomics        | Catalog# 1000127      |
| Chromium Next GEM Single Cell 3' Kit v3.1, 4 rxns                  | 10x Genomics        | Catalog# 1000269      |
| PicoPure™ RNA-Isolation Kit                                        | Thermo Fisher       | Catalog# KIT0204      |
| Dual Index Kit TT, Set A                                           | 10x Genomics        | Catalog# 1000215      |
| Luna® Universal qPCR Master Mix                                    | New England Biolabs | Catalog# M3003L       |
| QuantiNova SYBR Green Master Mix                                   | Qiagen              | Catalog# 208052       |

### Deposited data

|                                              |                        |                                                |
|----------------------------------------------|------------------------|------------------------------------------------|
| Metabolomic peak intensities                 | This paper             | MetaboLights: MTBLS10296                       |
| Raw 16S rRNA sequencing data (Mouse)         | This paper             | ENA: PRJEB76125                                |
| Raw metagenomic data (Mouse)                 | This paper             | ENA: PRJEB76125                                |
| Raw metagenomic data (Human)                 | Available upon request | ENA: PRJEB112493                               |
| Raw scRNAseq data (CD11c <sup>+</sup> cells) | This paper             | GEO: GSE242793 (WT) and<br>GEO: GSE335318 (KO) |
| Raw scRNAseq data (CD45 <sup>+</sup> cells)  | This paper             | GEO: GSE333893                                 |
| Bulk RNA sequencing human PBMCs              | Available upon request | GEO: GSE331113                                 |

### Experimental models: Organisms/strains

|                                          |                                                                                            |                                                    |
|------------------------------------------|--------------------------------------------------------------------------------------------|----------------------------------------------------|
| Mouse: C57BL/6J                          | Charles River                                                                              | Strain #:000664                                    |
| Mouse: KikGR (Tg(CAG-KikGR)33Hadj)       | Weill Cornell Medical College, New York, USA, Dr. Anrather                                 | MGI:J:153627                                       |
| Mouse: Ahrfl/flItgaxcr <sup>+</sup> /cr- | University Medical Center Hamburg-Eppendorf, Hamburg, Germany, Dr. Herkel and Dr. Carambia | Ahr <sup>flx</sup> : #006203<br>Cd11c-Cre: #008068 |
| Mouse: Ahrfl/flILysMcr <sup>+</sup> /cr- | University Medical Center Hamburg-Eppendorf, Hamburg, Germany, Dr. Herkel and Dr. Carambia | Ahr <sup>flx</sup> : #006203<br>LysMcre: #004781   |

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| REAGENT or RESOURCE                                                                                                                                                                                                                                         | SOURCE                                                                | IDENTIFIER               |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|--------------------------|
| Mouse: DERE mice                                                                                                                                                                                                                                            | Institute for Stroke and Dementia Research, Munich, Germany, Dr. Korn | MMRRC Strain #032050-JAX |
| Mouse: C57BL/6-Ahrem1Stck/J (Ahr-TdTomato)                                                                                                                                                                                                                  | The Jackson Laboratory                                                | Strain #:038111          |
| Mouse: Rag1 <sup>-/-</sup>                                                                                                                                                                                                                                  | The Jackson Laboratory                                                | Strain #:002216          |
| <b>Oligonucleotides</b>                                                                                                                                                                                                                                     |                                                                       |                          |
| Ahr:<br>Forward: 5' GCCCTTCCCGCAAGATGTTAT 3'<br>Reverse: 5' GCTGACGCTGAGCCTAAGAAC 3'                                                                                                                                                                        | Metabion                                                              | N/A                      |
| Ahr (Aryl hydrocarbon receptor repressor):<br>Forward: 5' CTGCCCAGGTACTCTGAACC 3'<br>Reverse: 5' ACTGTCCACAAAGCCTGACC 3'                                                                                                                                    | Metabion                                                              | N/A                      |
| Cyp1a1 (Cytochrome P450 family 1 subfamily A polypeptide 1):<br>Forward: 5' CGTTATGACCATGATGACCAAGA 3'<br>Reverse: 5' TCCCCAACTCATTGCTCAGAT 3'                                                                                                              | Metabion                                                              | N/A                      |
| Cyp1b1 (Cytochrome P450 family 1 subfamily B polypeptide 1):<br>Forward: 5' CCAGATCCCGCTGCTCTACA 3'<br>Reverse: 5' TGGACTGTCTGCACTAAGGCTG 3'                                                                                                                | Metabion                                                              | N/A                      |
| Tiparp (TCDD-inducible poly-ADP-ribose polymerase):<br>Forward: 5' CCAATGATTCTCAGGAGCACTTG 3'<br>Reverse: 5' GTAGACAGCCTTCGTAGTTGGTC 3'                                                                                                                     | Metabion                                                              | N/A                      |
| 18S:<br>Forward: 5' AGAAACGGCTACCACATCCAA 3'<br>Reverse: 5' CCTGTATTGTTATTTTCGTCACTACCT 3'                                                                                                                                                                  | Metabion                                                              | N/A                      |
| Escherichia Coli:<br>Forward: 5' CATGCCGCGTGTATGAAGAA 3'<br>Reverse: 5' CGGGTAACGTCAATGAGCAAA 3'                                                                                                                                                            | Metabion                                                              | N/A                      |
| tnaA:<br>Forward: 5' CTTTAAACATCTCCCTGAACCGTTC 3'<br>Reverse: 5' GGCCTAACAGGAAAGAGCCA 3'                                                                                                                                                                    | Metabion                                                              | N/A                      |
| 16S:<br>Forward: 5' ACTCCTRCGGGAGGCAGCAG 3'<br>Reverse: 5' GGACTACCVGGGTATCTAAT 3'                                                                                                                                                                          | Metabion                                                              | N/A                      |
| Ahr (genotyping):<br>Forward: 5' GGTACAAGTGACATGCCTGC 3'<br>Reverse: 5' CAGTGGGAATAAGGCAAGAGTGA 3'                                                                                                                                                          | Metabion                                                              | N/A                      |
| Itgax-Cre (genotyping):<br>Forward (Itgax): 5' GCTTCTTCTGTGGTGACTTG 3'<br>Reverse (Cre): 5' GCGAACATCTCAGGTTCTG 3'                                                                                                                                          | Metabion                                                              | N/A                      |
| KikGR33 (genotyping):<br>Forward: 5' GAAGATCGAGCTGCGTATGGA<br>Reverse: 5' ACCCTTCAGCACTCCATCACG                                                                                                                                                             | Metabion                                                              | N/A                      |
| DEREG (genotyping):<br>Forward (Transgene): 5' CCCAGGTTA<br>CCATGGAGAGA<br>Reverse (Mutant oIMR9402): 5' GAAC<br>TTCAGGGTCAGCTTGC<br>Forward: (wild-type oIMR8744): 5' CAA<br>ATGTTGCTTGTCTGGTG<br>Reverse (wild-type oIMR8745): 5' GTCA<br>GTCGAGTGACAGTTT | Metabion                                                              | N/A                      |

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| REAGENT or RESOURCE                                                                                                                                                                               | SOURCE                             | IDENTIFIER                                                                                                                                                      |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Rag1 <sup>-/-</sup> (genotyping):<br>Forward (Mutant): 5' TGGATGTGGAA<br>TGTGTGCGAG 3'<br>Forward (Wild type): 5' TCTGGACTT<br>GCCTCCTCTGT 3'<br>Reverse (common): 5' CATTCCATC<br>GCAAGACTCCT 3' | Metabion                           | N/A                                                                                                                                                             |
| Ahr-TdTomato (genotyping):<br>Reverse (Transgene): 5' CCTTGGAG<br>CCGTACATGA 3'<br>Reverse (Wild type): 5' GCATGGGA<br>GCTACAG GAATC 3'<br>Forward (Common): 5' TTGAATGAC<br>GAAAGACTTAGCC 3'     | Metabion                           | N/A                                                                                                                                                             |
| <b>Software and algorithms</b>                                                                                                                                                                    |                                    |                                                                                                                                                                 |
| GraphPad Prism v11                                                                                                                                                                                | Graphpad Inc.                      | <a href="https://www.graphpad.com/">https://www.graphpad.com/</a>                                                                                               |
| Image J 1.54g                                                                                                                                                                                     | NIH                                | <a href="https://imagej.net/ij/">https://imagej.net/ij/</a>                                                                                                     |
| Illustrator                                                                                                                                                                                       | Adobe                              | <a href="https://www.adobe.com/">https://www.adobe.com/</a>                                                                                                     |
| Cell Ranger v.7.1.0                                                                                                                                                                               | 10x Genomics                       | N/A                                                                                                                                                             |
| FlowJo v.10.6                                                                                                                                                                                     | Treestar Inc.                      | <a href="https://www.flowjo.com/flowjo/overview">https://www.flowjo.com/flowjo/overview</a>                                                                     |
| nf-core/rnaseq v3.2                                                                                                                                                                               |                                    | <a href="https://nf-co.re/rnaseq/3.23.0/">https://nf-co.re/rnaseq/3.23.0/</a>                                                                                   |
| STAR aligner v 2.7.1a                                                                                                                                                                             | Dobin et al. <sup>68</sup>         | N/A                                                                                                                                                             |
| samtools v1.22.1                                                                                                                                                                                  | Li et al. <sup>69</sup>            | N/A                                                                                                                                                             |
| bamsignals v1.28.0                                                                                                                                                                                | Mammana et al. <sup>70</sup>       | <a href="https://github.com/lamortenera/bamsignals">https://github.com/lamortenera/bamsignals</a> .                                                             |
| DeSeq2                                                                                                                                                                                            | Love et al. <sup>71</sup>          | N/A                                                                                                                                                             |
| EnrichR                                                                                                                                                                                           | Chen et al. <sup>72</sup>          | N/A                                                                                                                                                             |
| nf-core/bacass v2.5                                                                                                                                                                               |                                    | <a href="https://nf-co.re/bacass/2.5.0/">https://nf-co.re/bacass/2.5.0/</a>                                                                                     |
| GTDB-Tk v2.4.0                                                                                                                                                                                    | Chaumeil et al. <sup>73</sup>      | N/A                                                                                                                                                             |
| Mashtree 1.4.6                                                                                                                                                                                    | Katz et al. <sup>74</sup>          | N/A                                                                                                                                                             |
| MetaPhlAn 3 version 3.0                                                                                                                                                                           | Beghini et al. <sup>75</sup>       | N/A                                                                                                                                                             |
| MetaPhlAn4 version 4.1                                                                                                                                                                            | Blanco-Míguez et al. <sup>76</sup> | N/A                                                                                                                                                             |
| HUMAnN3 version q3.0.0.alpha.3                                                                                                                                                                    | Beghini et al. <sup>75</sup>       | N/A                                                                                                                                                             |
| HUMAnN4 version 4.0.0.alpha.1                                                                                                                                                                     | Beghini et al. <sup>75</sup>       | N/A                                                                                                                                                             |
| Atria                                                                                                                                                                                             | Chuan et al. <sup>77</sup>         | N/A                                                                                                                                                             |
| DADA2                                                                                                                                                                                             | Callahan et al. <sup>78</sup>      | N/A                                                                                                                                                             |
| FastTree2                                                                                                                                                                                         | Price et al. <sup>79</sup>         | N/A                                                                                                                                                             |
| immundeconv                                                                                                                                                                                       | Sturm et al. <sup>80</sup>         | N/A                                                                                                                                                             |
| curatedMetagenomicData                                                                                                                                                                            | Pasolli et al. <sup>81</sup>       | N/A                                                                                                                                                             |
| R v4.5.1                                                                                                                                                                                          | R Core Team, 2025                  | <a href="https://www.R-project.org/">https://www.R-project.org/</a>                                                                                             |
| R package Seurat 4.2.0                                                                                                                                                                            | Stuart et al. <sup>82</sup>        | N/A                                                                                                                                                             |
| Bioconductor scDblFinder v1.18.0                                                                                                                                                                  |                                    | <a href="https://bioconductor.org/packages/release/bioc/html/scDblFinder.html">https://bioconductor.org/packages/release/bioc/html/scDblFinder.html</a>         |
| IntegrateData in Seurat v5.0.3                                                                                                                                                                    |                                    | <a href="https://satijalab.org/seurat/articles/seurat5_integration">https://satijalab.org/seurat/articles/seurat5_integration</a>                               |
| R package pheatmap                                                                                                                                                                                |                                    | <a href="https://CRAN.R-project.org/package=pheatmap">https://CRAN.R-project.org/package=pheatmap</a>                                                           |
| Bioconductor EnhancedVolcano v.1.6.0                                                                                                                                                              |                                    | <a href="https://bioconductor.org/packages/release/bioc/html/EnhancedVolcano.html">https://bioconductor.org/packages/release/bioc/html/EnhancedVolcano.html</a> |
| SeuratExtend package v1.2.1                                                                                                                                                                       | Hua et al. <sup>83</sup>           | <a href="https://huayc09.github.io/SeuratExtend/index.html">https://huayc09.github.io/SeuratExtend/index.html</a>                                               |

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| REAGENT or RESOURCE                 | SOURCE                                         | IDENTIFIER                                  |
|-------------------------------------|------------------------------------------------|---------------------------------------------|
| Other                               |                                                |                                             |
| Silicone rubber-coated monofilament | Doccot                                         | Cat# 602223PK10Re,<br>Catalog# 602212Pk10Re |
| Laser Doppler flowmetry             | PF 5010 LDPM, Periflux<br>System 5000, Perimed | N/A                                         |
| UV405 laser                         | BerlinLasers                                   | SKU# 405VLLMG                               |

## EXPERIMENTAL MODEL AND STUDY PARTICIPANTS DETAILS

### Clinical patient population

Fecal samples and clinical data from six patients with stroke recruited within the Centre Hospitalier Universitaire Vaudois (CHUV) Lausanne (Switzerland) and the LMU University Hospital, Munich, Germany were obtained to perform fecal microbiota transplantation into mice. Demographical and clinical characteristics of the six patients are shown below.

| Patient ID | Severity score | Sex | Age | TOAST Mechanism   | NIHSS at admission | Intervention type                 | Days between sample acquisition and stroke onset |
|------------|----------------|-----|-----|-------------------|--------------------|-----------------------------------|--------------------------------------------------|
| #1         | Mild           | M   | 60  | CE <sup>a</sup>   | 3                  | IVT <sup>c</sup>                  | < 7                                              |
| #2         | Mild           | M   | 59  | ESUS <sup>b</sup> | 3                  | IVT <sup>c</sup>                  | < 7                                              |
| #3         | Mild           | M   | 70  | CE <sup>a</sup>   | 3                  | No reperfusion therapy            | 3                                                |
| #4         | Severe         | M   | 70  | ESUS <sup>b</sup> | 18                 | IVT <sup>c</sup> EVT <sup>d</sup> | < 7                                              |
| #5         | Moderate       | F   | 81  | ESUS <sup>b</sup> | 13                 | IVT <sup>c</sup>                  | 2                                                |
| #6         | Severe         | M   | 69  | CE <sup>a</sup>   | 16                 | No reperfusion therapy            | 3                                                |

All study protocols and informed consent for human subjects were approved by the local ethics committees, and the study was conducted in accordance with the Declaration of Helsinki as well as institutional guidelines (protocol number: CER-VD 2023-01654 and 24-0830 LMU Munich). Written and informed consent was obtained from all patients.

<sup>a</sup>CE: cardioembolic

<sup>b</sup>ESUS: Embolic stroke of undetermined source

<sup>c</sup>IVT: intravenous thrombolysis

<sup>d</sup>EVT: endovascular thrombectomy

### Animal experiments, husbandry and dietary interventions

All animal procedures were performed in accordance with the guidelines for the use of experimental animals and were approved by the respective governmental committees (Regierungspresidium Oberbayern, the Rhineland Palatinate Landesuntersuchungsamt Koblenz). Wild-type C57BL/6J mice were purchased from Charles River. Rag1<sup>−/−</sup> mice (NOD.129S7[B6]-Rag-1tm1Mom/J) and AHR-TdTomato (C57BL/6-Ahrem1Stck/J) were purchased from the Jackson Laboratory (US), bred and housed in the animal core facility of the Center for Stroke and Dementia Research (CSD, Munich, Germany).

KikGR33 mice (Tg(CAG-KikGR)33Hadj/J) were given by Dr. Josef Anrather, Weill Cornell Medical College, New York. DERE mice (C57BL/6-Tg(Foxp3-HBEGF/EGFP)23.2Spar/Mmjax) were given by Dr. Thomas Korn, TUM, Munich. CD11c-Ahr deficient mice (*Itgax*<sup>Cre/+</sup>; *Ahr*<sup>fl/fl</sup>, hereafter CD11c-Ahr-KO) littermate controls (*Itgax*<sup>+/+</sup>; *Ahr*<sup>fl/fl</sup>, hereafter littermate controls) and LysM-Ahr deficient mice (*LysM*<sup>Cre/+</sup>; *Ahr*<sup>fl/fl</sup>, hereafter LysM-Ahr-KO) were generated by Dr. Antonia Carambia and Dr. Johannes Herkel, Eppendorf University of Hamburg. The *Ahr*<sup>fl/fl</sup> mouse line was provided by Dr. Francisco Quintana (Harvard Medical School, Boston; mice originally generated by Dr. Christopher Bradfield; Jackson Laboratory strain #006203) and was backcrossed for more than 10 generations onto a C57BL/6J background. To generate conditional AHR-deficient mice and littermate controls, *Ahr*<sup>fl/fl</sup> mice were crossed with mice heterozygous for Cre recombinase under the control of the *Itgax* or *LysM* promoter. This breeding strategy yielded approximately 50% Cre-positive (AHR-deficient) and 50% Cre-negative (control) littermates. For CD11c-specific *Ahr* deletion, *Itgax*<sup>Cre/+</sup>; *Ahr*<sup>fl/fl</sup> and *Itgax*<sup>+/+</sup>; *Ahr*<sup>fl/fl</sup> littermates were generated using the same strategy at the animal facility of the CSD, Munich, Germany.

Transgenic mice were bred and housed in the animal core facility of the Center for Stroke and Dementia Research (Munich, Germany). For *in vivo* experiments, only male mice were used, except for AHR-TdTomato mice, for which both sexes were included. For *in vitro* experiments, both male- and female-derived cells were used.

Mice of both genotypes were co-housed prior to experiments and then randomly assigned to experimental groups to minimize microbiota differences between genotypes. Consistent with this, 16S rRNA gene sequencing of stool samples revealed no detectable

differences in microbial community composition between CD11c-Ahr-KO and littermate controls prior to experimental manipulation (Figure S8). Wild-type C57BL/6 mice from Charles River were acclimated for 1–2 weeks prior to performing experiments. Mice were fed a standard chow diet (Ssniff, Soest, Germany) ad libitum and housed under SPF conditions (12-hour light-dark cycles at  $22 \pm 2^\circ\text{C}$ ). In specific experiments, mice were fed for two to three weeks prior to inducing stroke with a mouse chow supplemented with an AHR inhibitor (CH-223191, 62 mg/kg, Hycultec, HY-12684) or the corresponding control chow without AHR inhibitor (Ssniff, Soest). Stroke- and sham-operated mice were housed separately, because sham-operated mice tend to become aggressive towards stroke-operated mice. 2 to 5 mice were housed together by cage. Animals were randomly assigned to treatment groups, and all analyses were performed by investigators blinded to group allocation. Detailed exclusion criteria are described below. All animal experiments were performed and reported in accordance with the ARRIVE guidelines.<sup>84</sup>

## METHOD DETAILS

### Transient proximal cerebral artery occlusion (intraluminal filament model)

Transient middle cerebral artery occlusion (tMCAO) was induced in mice aged 8–12 weeks with monitoring cerebral blood flow by transcranial laser Doppler flowmetry as previously described.<sup>85,86</sup> Mice were anesthetized with 1.5%–2% isoflurane in medical air (78% nitrogen and 21% oxygen), and rectal temperature maintained at  $37 \pm 0.5^\circ\text{C}$  for the duration of surgery. A silicon-coated filament was inserted into the external carotid artery and advanced until it obstructed the MCA together with the ligation of the common carotid artery for 45 minutes. Regional cerebral blood flow (CBF, bregma coordinates: 2-mm posterior, 5-mm lateral) was recorded by transcranial laser Doppler flowmetry (PF 5010 LDPM, Periflux System 5000, Perimed) at induction of ischemia, before filament removal and at reperfusion. When MCA occlusion was confirmed (a residual CBF <20%) animals were placed in a recovery chamber at  $37^\circ\text{C}$  until filament removal. To allow reperfusion, mice were again anesthetized, and the filament removed. Following tMCAO, mice were placed in a temperature-controlled recovery chamber for 2 hours to prevent post-surgery hypothermia. Sham surgery was performed as described above including ligation of the external and common arteries, but the filament was not inserted. The overall mortality rate of animals subjected to MCA occlusion was approximately 15%. All animals in the sham group survived the procedure. Exclusion criteria: 1) Insufficient MCA occlusion and reperfusion (a reduction in blood flow to >20% of the baseline value and CBF recovery <80% after 10 minutes of reperfusion); 2) Death during the surgery; 3) Lack of brain ischemia as quantified post-mortem by histological analysis or by visual assessment. In some experiments, neurological deficits were assessed using the neuro-score scale.<sup>86,87</sup>

### Permanent distal cerebral artery occlusion model

Permanent focal cerebral ischemia was induced in 8–12-week-old mice using a distal middle cerebral artery occlusion (dMCAO) model, as previously described.<sup>88</sup> In brief, a small temporal craniotomy was performed to access the distal middle cerebral artery, which was subsequently coagulated by a bipolar microsurgical electrocoagulation generator. Following dMCAO, mice were transferred to a temperature-controlled recovery chamber for 10 minutes to prevent postoperative hypothermia. The overall mortality rate was below 5%, attributable mainly to intraoperative anesthesiologic complications or euthanasia due to fulfillment of exclusion criteria. Exclusion criteria: 1) intraoperative subarachnoid hemorrhage; 2) Surgical time exceeding 15 minutes; 3) MCA recanalization after two electrocoagulation attempts, resulting in failure to achieve permanent occlusion.

### Small intestine photoconversion in KikGR33 mice

KikGR33 mice were anesthetized with 1.5%–2% isoflurane delivered in medical air, and maintained at  $37^\circ\text{C}$  throughout the procedure. Photoconversion was performed with a defocused (1.5-cm beam diameter) violet laser source (405 nm) essentially as described before.<sup>6</sup> Briefly, mice were placed in a supine position and covered with an aluminum foil blanket. A 3-cm incision was made in the abdominal wall, exposing the distal part of the small intestine (ileum). The exposed gut segment (~6 cm) was illuminated through an opening in the aluminum foil covering the animal, for 20 minutes on each of the anterior and posterior surfaces. Intestinal tissue was kept hydrated by applying saline throughout the procedure. The intestine was returned to the abdominal cavity, and the peritoneum and skin were sutured closed. Two days after photoconversion mice were anesthetized with pentobarbital intraperitoneally and transcardially perfused with 20 mL cold saline containing heparin (2 U/mL). Cell suspensions were isolated for flow cytometric analysis, and the percentage of photoconverted cells (KikR<sup>+</sup>) was analyzed in the appropriate cell populations.

### Fecal microbiota transplantation

C57BL/6J and CD11c-Ahr-KO mice male mice were treated for 3 days with an antibiotic solution containing ampicillin 1 g/L, neomycin sulfate 1 g/L and vancomycin 0.5 g/L provided ad libitum in the drinking water. Antibiotics were added to autoclaved tap water and water bottles containing antibiotic mix were changed once. In addition, 200  $\mu\text{L}$ /mouse of the antibiotic solution ampicillin 1 g/L, neomycin sulfate 1 g/L, vancomycin 0.5 g/L and metronidazole 1 g/L was orally gavaged once per day for 3 days. After 3 days the antibiotic treatment was stopped overnight and on day 4, day 5, and week 2, the mice were orally gavaged with 200  $\mu\text{L}$  of human fecal suspension. Human fecal suspension was prepared by adding 100 mg of stool in 1 mL sterile of NaCl 0.9% (BBraun), followed by vigorously vortexing, centrifugation at 500 g for 3 minutes at  $4^\circ\text{C}$ . The supernatant was collected (500–600  $\mu\text{L}$ /sample)

and 200  $\mu$ L were administered by oral gavage into mice. In a set of mice, microbiota depletion by the antibiotic treatment and recolonization were assessed by qPCR for 16S ribosomal DNA copy number.

### **Escherichia coli colonization in mice**

After antibiotic treatment following the protocol mentioned in the section above “fecal microbiota transplantation”, mice were orally gavaged with 200  $\mu$ L of a  $10^8$  bacterial suspension of the *E. coli* BW25113 (ME Collection, ME9062) or *E. coli*  $\Delta$ tnaA JW3686-KC strains (Keio Collection<sup>89</sup>) in mid-exponential phase for each strain. Colonization was performed on day 4, day 5, and week 2. Stool and urine (whenever possible) were collected to validate colonization via measurement of total bacterial load and *E. coli* load by qPCR, tryptophanase gene (*tnaA*) presence by endpoint PCR and quantitative indole measurement by Kovács assay.

### **Drug administrations**

#### **Indole, I3S and vehicle (DMSO/saline)**

Mice were treated daily for two weeks by oral gavage with 200  $\mu$ L of indole (20 mg/kg) or indoxyl-3-sulfate (10 mg/kg) prepared in 5% DMSO in 95% sterile saline (vehicle). Control animals received an equal volume of vehicle solution only.

#### **Diphtheria toxin**

DEREG mice were treated intraperitoneally with 0.5  $\mu$ g of diphtheria toxin (DT) on the day before and the day of the tMCAO surgery to deplete regulatory T cells.

### **Measurement of infarct volume**

Mice were anesthetized and perfused transcardially with ice-cold saline containing heparin (2 U/mL). Brains were then immediately frozen on dry ice and serially sectioned (400  $\mu$ m intervals, 20  $\mu$ m thick) for cresyl-violet staining. Sections were scanned at a resolution of 600 dpi, and infarct volumes, corrected for swelling,<sup>90</sup> were quantified using ImageJ software (NIH, US) by an investigator blinded to the treatment. For dMCAO, infarct volumes were not corrected for swelling.

### **Stroke severity score**

Severity score (Figure 1A) was calculated as a composite score combining infarct size, weight loss, and neurological deficit at 3 days post-stroke, based on the following criteria:

| Weight loss score                                                                                                                     | Score 1              | Score 2                   | Score 3    |
|---------------------------------------------------------------------------------------------------------------------------------------|----------------------|---------------------------|------------|
| % of weight loss at day 3 relative to baseline                                                                                        | < 5%                 | $\geq$ 5%                 | $\geq$ 15% |
| Infarct volume score                                                                                                                  | Score 1              | Score 2                   |            |
| Infarct volume at day 3                                                                                                               | < 50 mm <sup>3</sup> | $\geq$ 50 mm <sup>3</sup> |            |
| Neuroscore (0–48)                                                                                                                     | Score 1              | Score 2                   | Score 3    |
| at 3 days post-stroke                                                                                                                 | < 9                  | $\geq$ 9                  | $\geq$ 28  |
| The composite severity score corresponds to the sum of individual scores: Moderate severity: total score 3–6, Severe: total score 7–8 |                      |                           |            |

### **Organ and tissue collection**

Stool pellets were collected directly into a pre-labeled sterile 1.5 mL tube, and immediately frozen on dry ice. Mice were terminally anesthetized with ketamine (120 mg/kg) and xylazine (16 mg/kg). In a subset of experiments, mice were injected intra-venously (i.v.) with 3 mg CD45-eFluor450 or CD45-APC-Cy7, 3 min before transcardiac perfusion, to exclude blood contamination. Urine was collected from the bladder using a 1-mL syringe (Omnican 100). Blood was drawn via cardiac puncture and collected in 50 mM EDTA-coated tubes, kept on ice, and centrifuged at  $2,000 \times g$  for 10 min at 4°C. Plasma was isolated and stored at  $-80^{\circ}\text{C}$  until further use. Immediately after cardiac puncture, mice were transcardially perfused with ice-cold 0.9% NaCl containing heparin (2 U/mL) and the entire intestinal tract was removed and placed in ice-cold  $1 \times$  PBS. The small intestine, measured from the stomach to the cecum, was divided into three regions: the proximal 9.5 cm as duodenum, the central portion as jejunum, and the terminal 15 cm proximal to the cecum as ileum. The colon was defined as a 2-cm segment immediately proximal to the cecum. Luminal content from each small and large intestinal region and the cecum were gently expelled into 2-mL Eppendorf tubes and immediately frozen on dry ice and stored at  $-80^{\circ}\text{C}$  until further use. After content removal, 1-cm tissue segments from the proximal end of each small intestinal region and the colonic segment were excised, opened longitudinally, and cleaned. All tissue samples were immediately frozen on dry-ice and stored at  $-80^{\circ}\text{C}$  until use.

### Gut immunohistochemistry and CD11c-eYFP quantification

Ileal samples from stroke or sham operated CD11c-eYFP transgenic animals were prepared using the Swiss roll method<sup>91</sup> and fixed overnight in 4% paraformaldehyde at room temperature. Samples were then transferred to 30% sucrose for at least 5 days before embedding in cryostat temperature medium (OCT TM, Tissue-Tek®). Tissue sections (8  $\mu$ m) were counterstained (1:1000 dilution) with DAPI to detect cell nuclei and phalloidin i-Fluor 647 (Abcam, ab176759) to detect actin filaments. At least 60 pictures per mouse were acquired from 3 consecutive intestinal sections with an inverted fluorescence microscope (Zeiss Axio Observer Z1, 20x magnification). To calculate the number of CD11c-eYFP<sup>+</sup> cells per villi a minimum of 120 villi per mouse were counted. Images were analyzed using Image J software, and the researcher was blinded to the analysis. Images with the single channel of 647 (for villi detection) were first transformed into 8-bit type, and the threshold was adjusted to define villi shape. The image was then transformed into a mask, and the villi area was defined with the tool *fill holes*. The “villi mask” was applied to the homologue image with CD11c-eYFP positive cells. Results are expressed as the number of pixels of eYFP signal per villi area.

### Cell isolation

Mice were deeply anaesthetized with ketamine (120 mg/kg) and xylazine (16 mg/kg) and perfused transcardially with ice-cold saline containing heparin (2 U/mL).

#### Isolation of intestinal lamina propria cells

The small intestine was removed and collected in RPMI (Gibco, 1640). Lamina propria cell suspension was generated as essentially as previously described.<sup>6</sup> Briefly, intestines were cleaned of mesenteric fat and intestinal contents, and Peyer's patches were excised. Intestines were opened longitudinally, washed with 1X PBS, cut into ~2-cm pieces and placed into 20 mL of RPMI-1640 containing 8% fetal bovine serum (FBS), 3 mM EDTA, 0.5 mM DDT. Tissue pieces were placed in a shaking incubator set at 250 rpm and 37°C for 20 minutes. Tissue suspension was vortexed for 20 seconds, and the supernatant containing the intraepithelial lymphocytes was discarded. Remaining tissue pieces were washed with Ca<sup>2+</sup>Mg<sup>2+</sup> 1X PBS to remove EDTA, minced thoroughly, and placed into 5 mL RPMI-1640 containing 5% FBS, 10  $\mu$ g/mL of DNase I (Roche) and 0.2 mg/mL collagenase D (Sigma). Tissue was digested at 37°C for 30 minutes with constant agitation (250 rpm), then vigorously vortexed for 30 seconds. The resulting cell suspension containing lamina propria cells was filtered through a 40- $\mu$ m cell strainer and washed with 10 mL 1X PBS. Cell suspensions were washed with 1X PBS through a 30- $\mu$ m strainer and collected at 500 g for 7 minutes at 4°C. Cells were stained for flow cytometry analysis.

#### Isolation of cells from mesenteric lymph nodes and spleen

Mesenteric lymph nodes (MLNs) and spleen were isolated and minced thoroughly in digestion media containing 2 mg/mL collagenase D (Sigma) in HBSS<sup>+</sup>/+ (Sigma) and incubated for 30 minutes at 37°C. Spleen and MLNs were homogenized with the end of a 1-mL syringe plunger on a 40  $\mu$ m strainer, and cells were eluted from the strainer with 10–20 mL 1X PBS. The spleen was washed with 10 mL erythrocyte lysis buffer (150 mM NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, 0.1 mM EDTA; pH 7.2), and eluted cells were incubated for 7 minutes at room temperature and washed with 10–20 mL 1X PBS. Cell suspensions were centrifuged at 500 g for 7 minutes at 4°C and subsequently stained for flow cytometry analysis.

#### Isolation of brain leukocytes

Brains were removed, olfactory bulbs and cerebellum excised, and hemispheres separated. One hemisphere was placed in a Dounce homogenizer containing 3 mL RPMI-1640 medium with phenol red (Sigma) and gently homogenized. Then 4 mL of RPMI-1640 and 3 mL of 100% Percoll (final concentration 30 % Percoll) were added and overlaid over 2 mL of 70% Percoll. Gradients were centrifuged at 500 g, for 30 minutes at 18°C. Cells were recovered from the interface, washed with 1X PBS through a 30- $\mu$ m strainer and stained for flow cytometry analysis.

#### Isolation of meningeal (dura mater) leukocytes

All steps were performed on ice to maintain tissue quality. After perfusion, the ventral surface of the skull was removed to expose the brain parenchyma, and the brain was carefully extracted. Skull cap with intact dura mater were stored in cold RPMI 1640 medium (Thermo Fisher Scientific) with 5% fetal bovine serum (FBS) until dissection. The dura mater was carefully separated from the skull under a dissecting microscope and digested in 5 mL of RPMI with 5% FBS, deoxyribonuclease I (DNase I; 10  $\mu$ g/mL), and collagenase D (0.5 mg/mL) at room temperature (RT) with shaking (250 rpm) for 10 minutes. Digested samples were filtered through a 70- $\mu$ m strainer and washed with 20 mL 1X PBS. Cell suspensions were centrifuged at 500 g for 7 minutes at 4°C. Cells were stained for flow cytometry analysis.

#### Isolation and purification of BMDCs

Bone marrow-derived dendritic cells (BMDCs) were generated from tibia and femur of mice euthanized by cervical dislocation. Bone marrow was flushed out using a 30 mL syringe filled with sterile ice-cold RPMI complete medium (R10). The flushed bone marrow suspension was passed through a 100  $\mu$ m cell strainer to obtain a single-cell suspension. Cell suspension was cultured in IMDM (Modifiziertes Dulbecco-Medium nach Iscove, Merck) supplemented with 2  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 10% FBS, and 1% penicillin-streptomycin. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> and supplemented with 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, antibodies-online) and 20 ng/mL IL-4 (R&D Systems). Loosely adherent BMDCs were harvested after 7–10 days. For each biological replicate (n), bone marrow cells were pooled from 3–4

mice. Naïve BMDCs were purified using anti-F4/80 microbeads (Miltenyi Biotec) and anti-CD11c microbeads (Miltenyi Biotec). Purified BMDCs were then stimulated with 10 ng/mL UltraPure *Salmonella minnesota* LPS (434–1 mg, List Biological Laboratories) for 36 hours to generate activated DCs.

### T cell isolation for adoptive transfer

Single-cell suspensions were generated from the spleens of C57BL/6 or Ahr<sup>-/-</sup> mice by passing the tissue through a 70 µm cell strainer. CD4<sup>+</sup> T cells were obtained by pre-enrichment using a T cell negative selection kit following the manufacturer's instructions (Invitrogen). Then 10<sup>7</sup> purified T cells in 200 µL sterile saline were injected intraperitoneally into Rag1<sup>-/-</sup> mice 4 weeks before inducing stroke.

### Flow cytometry

Single-cell suspensions were prepared as described above. Cell suspensions were adjusted to a density of 1 × 10<sup>6</sup> cells in 1 mL FACS buffer (BDBiosciences) and incubated 30 minutes at 4°C with 1 µL of LIVE/DEAD™ Fixable aqua Dead Cell stain kit (ThermoFisher) or with Zombie Fixable Dye (BioLegend) for 10 min at room temperature. Cells were washed and nonspecific binding was blocked by incubation with anti-CD16/CD32 antibody (Invitrogen) for 10 minutes at 4°C. When appropriate, for CCR7 staining, cells were first incubated for 15 minutes at 37°C, then stained with γδTCR antibody for 10 minutes at room temperature, followed by staining with the surface anti-mouse antibody cocktail for 15 minutes at 4°C (see [key resources table](#)). For intracellular staining, cells were first stained for surface markers as detailed above and processed according to the manufacturer's instructions (Foxp3/Transcription Factor Staining Buffer Set, eBioscience). Briefly, cells were fixed for 30 minutes at room temperature, washed with permeabilization buffer, and incubated for 30 minutes at room temperature with the intracellular anti-mouse antibody cocktail (see [key resources table](#)) diluted in permeabilization buffer. Cells were then washed with FACS buffer, resuspended in 200 µL of FACS buffer, and counting beads were added (Counting Beads CountBright, Invitrogen). For cytokine staining, cells were first stimulated in flat bottom 48 well plates with 200 µL RPMI 1640 medium/well, supplemented with 10% FBS, 30 µM β-mercaptoethanol containing 50 ng/mL PMA (Sigma), 500 ng/mL ionomycin (Sigma) and 10 µg/mL Brefeldin A (Sigma) for 4 hours at 37°C. Then cells were washed and stained with surface antibody cocktail as described above and fixed with eBioscience™ IC Fixation Buffer (Invitrogen) for 30 minutes at room temperature, washed and incubated for 30 minutes at room temperature with the fluorochrome-conjugated anti-mouse IL-17 antibody in IC permeabilization Wash Buffer (BioLegend). Cells were then washed with FACS buffer, resuspended in 200 µL of FACS buffer, and counting beads were added. Flow cytometric data were acquired using a BD FACSVerser flow cytometer (BD Biosciences), BD FACSymphony™ A1 (BD Biosciences) or with a Fortessa FACS system (BD Biosciences) and data was analyzed using FlowJo software (Treestar). For some experiments, flow cytometry data was acquired using a Cytex Northern lights™ flow cytometer (Cytex Biosciences, US).

### Cytometric Bead Array (CBA)

Cardiac blood was taken from the anesthetized mouse through the left ventricle using a 23G needle (BD Microlance) in EDTA KE1/3 tubes (Sarstedt). Plasma was obtained after centrifuging the samples at 2000g, 4°C for 10 minutes. 50 µL of plasma were used for quantification of cytokines IL-2, IL-6, IL17A, IL-10, IL-1β, IL-4, TNF, RANTES and IFN-γ following the manufacturer's instructions (BD Cytometric Bead Array (CBA) Mouse/Rat Soluble Protein Master Buffer Kit, BD Biosciences). Samples were acquired using a BD FACSymphony™ A1 (BD Biosciences) and analyzed with CBA analysis software (BD Biosciences).

### In vitro CD11c<sup>+</sup> cell incubation with indole derivatives

Cells isolated from spleen and MLNs were stained with: CD45 eFluor450 (30-F11), MHCII FITC (M5/114.15.2), CD11c APC (N418). Stained samples were re-suspended in 1% BSA in 1X PBS and sorted using a BD FACSAria™ Fusion Flow Cytometer (yield mode). 1.5 × 10<sup>4</sup> cells of CD45<sup>+</sup>MHCII<sup>+</sup>CD11c<sup>+</sup> were sorted into a round-bottom 96 well plate containing 200 µL of cell culture medium (RPMI, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1% 2-mercaptoethanol). Cells were exposed to indole or indoxyl-3-sulfate (Sigma) at 10, 100, or 500 µM, AHR inhibitor (10 µM CH-223191) or with vehicle control (0.5% DMSO). Cultures were maintained at 37°C with 5% CO<sub>2</sub>. After 12 hours (overnight) non-adherent cells were first collected, and the wells were then washed with 0.5 mM EDTA for 5 minutes at room temperature to detach semi-adherent cells. The collected medium containing semi-adherent cells was pooled in the same collection tube as the non-adherent cells. For RNA isolation, samples were centrifuged at 3,000 g, for 10 minutes, at 4°C and the subsequent steps were done by following manufacturers protocol (PicoPure RNA isolation kit, KIT0204, ThermoFisher).

### 2D migration assay

Cell migration was assessed using Transwell cell culture inserts with of 5-µm pore size (Corning Costar; CLS3421-48EA, Sigma-Aldrich). Ahr-CD11c-KO and littermate control mice BMDCs were washed with PBS and preincubated for 1 hour in RPMI 1640 medium (Invitrogen) containing 0.1% BSA (Sigma-Aldrich, Buchs, Switzerland). Cells for each replicate were derived from pooled mice. A total of 5 × 10<sup>5</sup> cells were added to the upper chamber. The lower chamber contained medium alone or medium supplemented with recombinant CCL19 (MIP-3β; 250-27B-20UG, PeproTech) or CCL21 (6Ckine; 250-13-20UG, PeproTech). After 6 hours of incubation, cells that migrated to the lower chambers were collected and quantified by flow cytometry. Absolute cell numbers were determined using CountBright™ Absolute Counting Beads (C36950, Thermo Fisher Scientific) according to the manufacturer's instructions.

Counting beads were added to each sample at a defined volume, and the absolute number of migrated cells was calculated using the following equation: Absolute cell number = (Cell events acquired/Bead events acquired) x Number of beads per test. To allow comparison across experimental conditions, all absolute cell numbers were normalized to the control group and expressed relative to the mean cell number of the control condition. All statistical analyses were performed using GraphPad Prism (version 8.0.2). Normality of the data was assessed using the Shapiro–Wilk test. Parametric tests were applied when data were approximately normally distributed. Data were analyzed using two-way ANOVA with repeated measures where appropriate, followed by Sidak’s multiple comparisons test. For all analyses, each *n* represents cells pooled from 3–4 mice. Statistical significance was defined as *p* < 0.05.

### ***In vitro* induction of regulatory T cells**

CD11c<sup>+</sup> cells were isolated from the spleens and MLNs of littermate control and CD11c-Ahr-KO mice subjected to tMCAO using the Pan dendritic Cell Isolation Kit with 2 mg/mL collagenase D according to the manufacturer’s protocol (Miltenyi Biotec). Splenocyte CD4<sup>+</sup> T cells were purified from naive C57BL/6J mice using the naive CD4 T-cell Isolation Kit (Miltenyi Biotec). Purity was > 90% for CD4<sup>+</sup> cells, >65% for CD11c<sup>+</sup> cells as assessed by flow cytometry. 2.5 × 10<sup>4</sup> CD11c<sup>+</sup> cells and 2.5 × 10<sup>4</sup> CD4<sup>+</sup> cells (ratio 1:1) were resuspended and cultured in 200 μL RPMI 1640 medium/well, supplemented with 10% FBS, 2 mM L-glutamine, 30 μM β-mercaptoethanol, non-essential amino acids (NEAAs), 1 mM sodium pyruvate, penicillin/streptomycin (50 U/mL penicillin, 50 μg/mL streptomycin) in a round bottom pre-coated 96-well-plate for 3 days with TGF-β1 (1 ng/mL, PreproTech). Cells were washed with FACS buffer, blocked with CD16/CD32 for 10 minutes at 4°C and stained with CD45-eF450 and CD4-PerCP/Cy5.5 antibodies for 15 minutes at 4°C. Cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and then intracellularly stained with Foxp3-PE for 30 minutes at 4°C for flow cytometry analysis.

### **Single-cell RNA sequencing**

Cell suspensions were obtained as described above and further pre-enriched with Pan dendritic Cell Isolation Kit with 2 mg/mL collagenase D or CD45 MicroBeads according to the manufacturer’s protocol (Miltenyi Biotec) prior to CD11c<sup>+</sup> or CD45<sup>+</sup> cell sorting, respectively. Cells were stained with surface markers CD11c-APC or CD45 BUV-737, respectively. Unique cell hashtags antibodies were used to label cells from each organ individually. Propidium iodide (PI) was used as a cell viability marker. 7 × 10<sup>3</sup> live CD11c<sup>+</sup> cells (from 3 mice per genotype) or 5 × 10<sup>3</sup> live CD45<sup>+</sup> cells (from 4 mice per condition) were sorted according to their surface markers using a FACS Aria II cell sorter (BD Biosciences, Inc.) or BD FACSARIA™ Fusion Flow Cytometer (BD Biosciences, Inc.). Sorted and organ-specific labeled CD11c<sup>+</sup> or CD45<sup>+</sup> cells from the same animal were pooled together in the same collection tube. After sorting, all cells were centrifuged and cautiously resuspended to a final concentration of 800 cells/μL. Cells were then transferred to the NextGEM chip G according to the manufacturer’s instructions. scRNA-seq and cell hashing libraries were prepared using the 10x Chromium Next GEM Single Cell 3’ v3.1 (Dual Index, for CD11c<sup>+</sup> cell sequencing) and Chromium GEM-X Single Cell 5’ Kit (v3, for CD45<sup>+</sup> cell sequencing) combined with feature barcoding technology for Cell surface protein (cell hashing), as per established protocols. Single cell capture, barcoding and library preparation were performed with the 10x chromium system using version 2 chemistry, following the manufacturer’s instructions (10x Genomics). Approximately 25,000 cells/mouse were loaded onto the 10x chromium microfluidic chip. The generated libraries were checked for quality using a Bioanalyzer 2100 (Agilent Technologies), and then sequenced on an Illumina NextSeq 2000, aiming for 20,000 and 5,000 reads per cell for gene expression and cell-surface protein expression libraries, respectively.

### **Single-cell RNA sequencing analysis**

Cell Ranger software was used to demultiplex samples, process raw data, align reads to the mouse mm10 reference genome and summarize unique molecular identifier (UMI) counts. Filtered gene-barcode and hashing-barcode matrices containing only barcodes with UMI counts that passed the threshold for cell detection were used for further analysis.

#### **For CD11c<sup>+</sup> cells**

Filtered UMI count matrices were processed using R and the R package Seurat v.4.2.<sup>82</sup> As quality control steps, the following cells were filtered out for further analysis: (1) doublets originating from two cells from different samples (one cell positive for two hashtag oligos (HTO)); (2) cells with no HTO detected; (3) cells with <500 or >6,000 detected genes; (4) cells with >10 % of counts belonging to mitochondrial genes. Hereafter, raw gene counts in high-quality singlets were log normalized and submitted to the identification of highly variable genes by MeanVarPlot method. Data were scaled and regressed against the number of UMIs and mitochondrial RNA content per cell. Data were subjected to principal component analysis and unsupervised clustering by the Louvain clustering method. Mitochondrial, ribosomal, hemoglobin, gene model (Gm)-annotated, Rik-suffixed, and sex-associated genes (Tsix and Xist)<sup>28</sup> were excluded before recalculation of dimensionality reduction, clustering and differentially expressed gene analysis. Dimensionality reduction and clustering were performed using the first 10 Harmony-corrected dimensions, and clusters were identified at a resolution of 0.3 and visualized using uniform manifold approximation and projection (UMAP). Cell clusters were manually annotated based on cluster-enriched genes and visual inspection of the expression of established lineage and subset markers. Differentially expressed genes between conditions were calculated using the FindMarkers function. Genes were required to be detected in at least 10% of cells in either condition, with at least three cells per condition. Raw *p* values were adjusted within each cluster using the Benjamini–Hochberg method. Genes with an adjusted *p* value <0.05 and an absolute average log2 fold change ≥0.25 were retained for visualization. Heatmaps were generated using the ComplexHeatmap R package from scaled RNA expression values. Gene module

scores were calculated using the AddModuleScore function in Seurat. Module scores were subsequently averaged for each mouse within each cluster to enable comparisons at the biological-replicate level. Only clusters represented by at least three mice per genotype were included. Additional visualization and pathway enrichment analyses were performed using the SeuratExtend package v1.2.1.<sup>83</sup>

#### For CD45<sup>+</sup> cells

Quality control steps for UMI count data included – (1) doublet detection performed using the Bioconductor package scDblFinder v1.18.0. (2) Removing cells with >5% of counts attributed to mitochondrial genes. Additional filtering was applied on a per-sample basis using a dynamic threshold: cells were retained if their number of detected features fell within the range defined by the median  $\pm$  3  $\times$  median absolute deviation (MAD) of each sample. For data integration, anchors were identified across samples using the *FindIntegrationAnchors* function, followed by integration with *IntegrateData* in Seurat v5.0.3. Normalization was performed using the *SCTransform* method. The integrated data were then scaled, and highly variable genes were identified for downstream dimensionality reduction via principal component analysis (PCA). A nearest-neighbor graph was constructed based on the PCA space, and uniform manifold approximation and projection (UMAP) was used for visualization. Clustering was performed using the Louvain algorithm. Clusters were annotated based on established marker genes from the literature. Visualization, including dot plots and violin plots, was generated using Seurat functions in combination with ggplot2 v3.5.1.

Gene list (Figure 5)

| Category                                                                                                                                                | Genes                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|---------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Literature-curated genes associated with maturation, tolerance and regulatory DC function, and cDC identities (cDC1s and cDC2s) for Figures 5J and S5F. | <i>Aldh1a2, Batf3, Btla, Cadm1, Cblb, Ccl17, Ccl22, Ccr7, CD200, Cd200r1, CD209a, CD274, CD40, CD80, CD83, CD86, Clec10a, Clec4a2, Clec9a, Fas, Foxo3, Fscn1, H2-DMb1, Havcr2, Icosl, Ido1, Ifi30, Il10, Il10ra, Il27, Il4i1, Inhba, Irak3, Irf4, Irf8, Itch, Itgae, Itgb8, Lamp3, Maf, Nr2p, Pdcd1lg2, Phlpp1, pparg, Pten, Relb, Rorc, Sema7a, Sirpa, Socs1, Socs2, Socs3, Tgfb1, Tgfb2, Tgfb1, Tgfb2, Tnfaip3, Tnfrsf14, Tnfrsf4, Tnfrsf9, Xcr1, Zbtb46</i> |
| cDC1 identity-associated genes for module score in Figures 5K and S5H.                                                                                  | <i>Batf3, Cadm1, Clec9a, Irf8, Itgae, Xcr1</i>                                                                                                                                                                                                                                                                                                                                                                                                                 |
| Tolerance/regulatory-associated genes for module score in Figures 5K and S5H.                                                                           | <i>Aldh1a2, Btla, Cblb, Cd200, Cd200r1, Cd274, Foxo3, Havcr2, Ido1, Il10ra, Il4i1, Irak3, Itch, Itgb8, Maf, Pdcd1lg2, Phlpp1, Pparg, Pten, Socs1, Socs2, Socs3, Tgfb1, Tgfb1, Tgfb2, Tnfaip3</i>                                                                                                                                                                                                                                                               |
| DC migration/localization-associated genes for module score in Figures 5K and S5H.                                                                      | <i>Adam8, Arhgef6, Ccl5, Ccl17, Ccl22, Ccr2, Ccr5, Ccr6, Ccr7, Ccr9, Cdc42, Cd44, Cltc, Dock8, Flt3, Fscn1, Icam1, Itga4, Itgae, Itgam, Itgax, Itgb1, Itgb2, Itgb7, Marcks, Marcksl1, Mmp14, Mmp9, Myo1g, Net1, Plat, Plau, Rab11a, Rab27a, Rac1, Rhoa, Rock1, Rock2, S1pr1, S1pr3, Samsn1, Sell, Selplg, Serpine1, Tiam1, Vcam1, Wasf2, Cxcl10, Cxcl12, Cxcl16, Cxcr3, Cxcr4, Cxcr5</i>                                                                       |

#### Bacterial DNA extraction

100 mg of stool samples or 250 mg of ileal content were used to extract and purify microbial DNA using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN) according to the manufacturer's instructions. The amount of extracted microbial DNA was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher).

#### Quantification of 16S ribosomal (r16S) gene DNA copy numbers

Assessment of bacterial load was determined using qPCR employing the standard curve method to quantify total 16S copy number. Sequences were amplified from stool genomic DNA using 20  $\mu$ M of the universal primer pair 16S-F (5'-ACTCCTRCGGGAGGCAG-CAG-3') and 16S-R (5'-GGACTACCVGGGTATCTAAT-3'). Amplification was performed on a Roche LightCycler 480 (Roche) using a two-step cycling protocol consisting of: 50°C for 2 minutes, 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 1 minute. The standard was prepared by amplifying genomic DNA from the stool sample with the aforementioned primers, purifying the resulting product and generating a dilution series from 103 to 108 copies.

#### Metagenomic sequencing

Bacterial DNA was isolated from stool samples and ileal content as described above. Sequencing was performed at the Gene Center Munich (Laboratory for Functional Genome Analysis), according to standard procedures. Samples were sequenced on an Illumina NextSeq 2000 machine using 2x150bp paired-end reads, yielding an average depth of 5Gbp per sample.

### 16S rRNA gene amplicon sequencing

DNA was isolated from ileal-content samples as described above. Sequencing was performed by Novogene according to standard operating procedures. In brief, the V4 hypervariable region of the 16S rRNA gene was amplified using the 515F-GTGCCAGCMGCCGCGGTAA and 806R-GGACTACHVGGGTWTCTAAT primer pair and sequenced on an Illumina NovaSeq™ 6000 machine in paired-end mode.

### Targeted LC-MS/MS quantification for tryptophan-derived metabolites

#### Sample preparation

Ileal content was extracted using the cold organic solvent extraction protocol as previously described.<sup>92</sup> In brief, frozen material (10–600 mg) was combined with 100  $\mu$ L of 0.1-mm zirconia/silica beads (BioSpec) and 500  $\mu$ L acetonitrile:methanol (1:1, v/v) containing an internal standard mixture (final 500 nM) consisting of phenylalanine-d5, tryptophan-d5, ibuprofen-d4, tolafenamic acid-d4, estriol-d3, diclofenac-d4, warfarin-d5, oxfendazole-d3, chloramphenicol-d5, nafcillin-d5, and caffeine-d9. Samples were homogenized using a bead beater (BioSpec; 2 min 30 s, maximum speed, on ice), incubated at  $-20^{\circ}\text{C}$  for  $\geq 1$  h, and centrifuged ( $3,220 \times g$ , 15 min,  $4^{\circ}\text{C}$ ). Supernatants were stored at  $-80^{\circ}\text{C}$  until analysis. Fecal extracts were diluted 1:4 (sample:water), thawed on ice and centrifuged (10,000 rpm, 3 min). Between 4 and 20  $\mu$ L supernatants were transferred to a 96-well plate and spiked with 5  $\mu$ L internal standard solution (12.5  $\mu\text{M}$  stock; final 0.5  $\mu\text{M}$ ). Protein precipitation was performed using 100  $\mu$ L ice-cold acetonitrile:methanol (1:1, v/v). Samples were incubated at  $-20^{\circ}\text{C}$  for 1 h, centrifuged (3,000 rpm, 10 min,  $4^{\circ}\text{C}$ ), and 80  $\mu$ L supernatant was transferred to a fresh plate and stored at  $-80^{\circ}\text{C}$  until analysis.

#### LC-MS/MS acquisition

Samples were analyzed using HPLC (1290 Infinity II Multisampler, Agilent) coupled to a 6495 Triple Quadrupole mass spectrometer (Agilent) operating in electrospray ionization polarity-switching mode. Chromatographic separation was performed on an HSS T3 C18 column ( $2.1 \times 100$  mm,  $1.8 \mu\text{m}$ , TMS-end capped, Waters) maintained at  $30^{\circ}\text{C}$ . Mobile phase A: water + 0.1% formic acid, and mobile phase B: methanol + 0.1% formic acid, run at 0.37 mL/min using the following gradient: 0–0.5 min, 98% A/2% B; 0.5–13.0 min, linear to 5% A/95% B; 13.0–14.0 min, return to 98% A/2% B, followed by a 0.5-min pos-run time. Source parameters were: gas flow 15 L/min, sheath gas 12 L/min, nebulizer 25 psi, capillary voltage +3500 V/–3000 V, nozzle 0 V, gas temperature  $215^{\circ}\text{C}$ , and sheath gas temperature  $300^{\circ}\text{C}$ . The injection volume was 3  $\mu$ L. Acquisition include optimized metabolite-specific collision energies between 8–50 eV.

#### Data processing and quantification

Quantification was based on a 16-point calibration curve using authentic standards (100  $\mu\text{M}$ –10 pM) processed identically for all to samples. Peak integration was performed in MassHunter quantitative analysis for QQQ (Agilent, v12.1) using two MRM transitions per metabolite, (MRM transitions, retention times, collision energies, polarity, and analytical performance parameters for all monitored metabolites are provided in Table S4), with normalization to the internal standard tryptophan-d5 (primary normalization standard), with additional isotopically labeled compounds used for quality control. Peaks were accepted within a  $\pm 0.5$  min retention-time window, and quantitative acceptance thresholds were defined by calibration-derived limits of detection (LOD) and limits of quantification (LOQ). Final concentrations were calculated using a Python-based workflow incorporating calibration fits, normalization, and automated QC. Reported values represent matrix-appropriate units (nmol per mg for solid tissues).

### Indole quantification by UHPLC-MS/MS

#### Sample preparation and derivatization procedure

Samples (ileal content) were weighed into bead beater tubes (2 mL, CKMix, Bertin Technologies, France), and 10  $\mu$ L of internal standard (indole-d6, 1  $\mu\text{M}$ ) and 1 mL of a mixture containing acetonitrile:methanol:water (4/4/2, v/v/v) were added. Homogenization and extraction were performed using a bead beater (Precellys Evolution Homogenizer, Bertin Technologies, France) at 9000 rpm for  $3 \times 30$  s with a 30 s pauses between intervals. Then, samples were centrifuged at  $5^{\circ}\text{C}$  for 15 min at 13000 rpm, and the supernatant was transferred to a new microcentrifuge tube and dried under a stream of nitrogen. After drying, derivatization was performed as described by Zhang et al. (2022) with modifications. Briefly, 100  $\mu$ L of dimethylaminocinnamaldehyde (2.5 mM in methanol) and 100  $\mu$ L of hydrochloric acid (6 mol/L) were added to the extract and the mixture was shaken at  $40^{\circ}\text{C}$  for 9 hours, then the mixture was dried under nitrogen. The dried sample was reconstituted with 100  $\mu$ L acetonitrile:water (1/1, v/v) before UHPLC-MS/MS analysis.

#### UHPLC-MS/MS analysis

Derivatized samples were analyzed using a QTRAP 6500 mass spectrometer (AB Sciex, Darmstadt, Germany) operated in ESI<sup>+</sup> mode connected to a Shimadzu Nexera X2 UHPLC system (Shimadzu, Duisburg, Germany). Aliquots of samples (5  $\mu$ L) were subjected to UHPLC-MS/MS analysis using a Kinetex phenylhexyl column ( $100 \times 2.1$  mm,  $1.7 \mu\text{m}$  i.d., Phenomenex, Aschaffenburg, Germany). After optimizing the instrument settings for indole and indole-d6, the derivatized compounds were analyzed using the mass transitions Q1/Q3 of m/z 275.2/260.2 (DP = 11 V, CE = 35 V, CXP = 28 V) for indole, and Q1/Q3 of m/z 281.2/266.2 (DP = 11 V, CE = 35 V, CXP = 24 V) for indole-d6. Chromatographic separation was achieved with a flow rate of 0.4 mL/min at  $40^{\circ}\text{C}$ . Starting with 10% aqueous formic acid (0.1%) as eluent A and acetonitrile (0.1% formic acid) as eluent B, the content of B was kept isocratic for 1.5 min, then raised to 60% B by 5 min, increased to 100% B within 2 min, and kept isocratic at 100% B for 1 min. Quantification was carried out using a scheduled UHPLC-MS/MS method, in which the specific mass transitions of all compounds were recorded

within a 30-second window of each analyte's retention time. The stable isotope dilution assay methodology was used to determine indole concentrations with calibration curves prepared from reference compounds.

### RT-qPCR

Total RNA was isolated from samples following the manufacturers protocol (PicoPure RNA isolation kit, KIT0204, ThermoFisher). Frozen tissue was first homogenized using the gentleMACS Dissociator (Miltenyi Biotec) followed by RNA isolation with the RNeasy mini-kit (Qiagen). cDNA was synthesized from 100 ng total RNA by MultiScribe Reverse Transcriptase (Invitrogen). Real-time quantitative PCR (RT-qPCR) was performed using QuantiNova SYBR Green Master Mix (Qiagen) or Luna Universal qPCR Master Mix (New England Biolabs) on the LightCycler 480 II. Each sample was measured in triplicate for all primers. Parameters for amplification were as follows: 95°C for 2 minutes, followed by 40 cycles at 95°C for 5 seconds, then at 59°C, 60°C or 61°C for 30 seconds. The annealing temperatures for primers were as follows: house-keeping gene *18S* (61°C), *Ahr* (61°C), *Cyp1a1* (61°C), *Cyp1b1* (61°C), *Ti-parp* (61°C), and *Ahr* (61°C). Raw data was obtained on the LightCycler 480 1.5.1 software. Relative changes in gene expression levels were normalized to the *18S* gene expression levels by using the  $2(-\Delta\Delta CT)$  method. Melting curve analysis and agarose gel electrophoresis were performed to verify the specificity of PCR amplicons.

### Protein extraction and Western Blot assay

Protein lysates were prepared using RIPA buffer (Thermo Fisher, Cat. 89901) supplemented with protease (Thermo Fisher, Cat. 78429) and phosphatase inhibitor cocktails (Thermo Fisher, Cat. 78426). Tissue samples were homogenized with a TissueLyser LT (Qiagen), and protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher, Cat. 23227). Equal amounts of protein were resolved on NuPAGE™ 4%–12% bis-Tris gels (Thermo Fisher, Cat. NP0336BOX) and electrophoretically transferred onto PVDF membranes. Membranes were blocked in 5% milk in TBST for 1 h at room temperature, followed by incubation with the primary antibodies: CYP1A1 (ProteinTech, Cat. 13241-1-AP), CYP1B1 (ProteinTech, Cat. 67033-1-Ig), CCR7 (Thermo Fisher, Cat. MA1-163), beta-actin (Cell Signaling, Cat. 12620S). After washing, membranes were incubated with HRP-conjugated secondary antibodies: goat anti-rabbit (Vector Laboratories, Cat. PI-1000), goat anti-rat (Biotecne, Cat. HAF005), goat anti-mouse (Dako, Cat. P0447) for 1 h at room temperature. Signal detection was performed using Immobilon Western HRP Substrate (Millipore, Cat. WBKLS0500) according to the manufacturer's instructions, and chemiluminescent signals were acquired with a Fusion FX7 imaging system. Band intensities were quantified using ImageJ. The sections of interest were cropped from the full blot images using ImageJ, and a gap was left in the figure to indicate that lanes originate from different membranes. No contrast, brightness, or other adjustments were applied to the images. Full blot images are available upon request. Protein expression in stroke samples was normalized to sham samples and expressed as fold change relative to sham.

### Sample preparation for Kovács and Indican assays

Ileal contents from the terminal region (15 cm) of the small intestine, stool, and urine were collected, snap-frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Blood was obtained via cardiac puncture into EDTA-coated tubes using a 1-mL Inject-F syringe, inverted 20 times, maintained on ice, and centrifuged at  $2,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ; plasma was isolated and stored at  $-80^{\circ}\text{C}$ . Stool and ileal contents were weighed and resuspended in sterile 1X PBS at 100 mg/mL. Samples were homogenized in 2-mL round-bottom tubes containing a 5-mm steel bead for 2 min at 40 Hz using a TissueLyser (QIAGEN). Homogenates were centrifuged at  $16,000 \times g$  for 10 min at room temperature, and supernatants were collected and passed through 0.22- $\mu\text{m}$  filters. Filtered supernatants were stored at  $-80^{\circ}\text{C}$  until further processing.

### Kovács assay (semi-quantitative indole measurement)

For indole detection, stool and ileal-content extracts were diluted 1:2 in 1X PBS; blood and urine samples were diluted 1:10 and 1:8, respectively, in ddH<sub>2</sub>O. Samples were analyzed in duplicate. 30  $\mu\text{L}$  of diluted samples were incubated with 45  $\mu\text{L}$  of Kovács reagent for 30 minutes at room temperature in the dark, according to the manufacturer's instructions (Merck, CAS 100-10-7). Absorbance was measured at 530 nm on a spectrophotometer (EnSpire Multimode Plate Reader 2300, Perkin – Elmer). Indole concentrations were calculated from an indole standard curve and corrected for the respective dilution factor. For stool and ileal contents, values were converted to mass-normalized indole equivalents based on the fixed extraction ratio of 100 mg sample per mL PBS and the molecular weight of indole (117.15 g/mol) and expressed as mg/g wet weight. For plasma and urine, concentrations were expressed as  $\mu\text{mol/L}$  or  $\mu\text{mol/mL}$ .

### Indican assay (semi-quantitative indoxyl-3 sulfate measurement)

Ileal-content extracts were diluted 1:4 in 1x PBS, and combined with 140  $\mu\text{L}$  of Reagent A, followed by 10  $\mu\text{L}$  of Reagent B. After incubation for 5 minutes at room temperature in the dark, 10  $\mu\text{L}$  of the provided standard were added to each well, and samples were analyzed in duplicate according to the manufacturer's instructions (MAK128, Sigma-Aldrich). Absorbance was measured at 480 nm after each reagent addition. Indican concentrations were calculated using the conversion factor 1 mg/dl = 39.8  $\mu\text{mol/L}$  and corrected for the dilution factor. Values were expressed both as  $\mu\text{mol/L}$  in the standardized ileal-content extracts and as mg/g wet weight after conversion based on the fixed extraction ratio of 100 mg sample per mL PBS and the molecular weight of potassium indoxyl sulfate (213.21 g/mol).

### Enumeration of Enterobacteriaceae from ileal content

To enumerate Enterobacteriaceae in ileal content, samples were diluted in equal volumes glycerol and weighed, before being homogenized by vortexing. 10  $\mu$ l of homogenized sample was then used to generate a dilution series ( $10^{-1}$ – $10^{-5}$ ), of which 10  $\mu$ l was then plated onto MacConkey agar and incubated at 37°C overnight. Colonies were counted and colony-forming units calculated using the following formula:  $c = w \times v / (v \times d)$ , where  $w$  = weight per volume,  $c$  = number of colonies counted,  $v$  = volume plated and  $d$  = dilution counted.

### Isolation of Enterobacteriaceae from ileal content

To enrich for facultative anaerobes, ileal content samples from sham and stroke-operated mice, stored in 20% glycerol, were homogenized and incubated for 2 hours in LB medium at 37°C with shaking (200 rpm). A loopful of medium was streaked onto MacConkey agar plates and incubated at 37°C overnight. To generate pure cultures, single-colonies from each plate were re-streaked onto MacConkey agar and again incubated overnight at 37°C.

### Measurement of indole production from Enterobacteriaceae

Ileal-content bacterial isolates as described above from sham and stroke-operated mice were cultivated for 24 hours in autoclaved tryptophan-peptone broth containing: 10 g casein peptone, 5 g sodium chloride, 10 g tryptophan in 1 L distilled water along with an uninoculated control (culture medium), a negative control (e.g. *tnaA*-deficient *E. coli* strain, Keio Collection, JW3686-KC) and a positive control (*E. coli* BW25113, ME Collection, ME9062) at 37°C with shaking (200 rpm) for 24 hours. A positive indole test is indicated by the formation of a pink to red ring on top of the medium within seconds of adding, while a yellow ring represents a negative result.

### Isolation and whole genome sequencing of *Escherichia coli*

To identify presumptive *E. coli*, a single colony from a severe stroke sample used in the Enterobacteriaceae enumeration experiment above, was re-streaked onto MacConkey agar (Roth) using the complex streak technique and incubated at 37°C overnight. A single colony from the resulting plate was then inoculated into 5 mL of LB broth (10g/L peptone, 5g/L yeast extract, 5g/L NaCl) and incubated in a shaking incubator at 37°C, 200 rpm overnight. The culture was then centrifuged at 8000  $\times$  g for 5 minutes and the supernatant removed. Human samples were isolated from fresh stool of a patient with severe stroke using the same procedure, with an additional initial step of plating fresh stool diluted 1:10 in PBS on MacConkey agar. DNA extraction was then performed. Whole genome sequencing was performed on an Illumina NovaSeqTM 6000 in paired-end mode (150bp) at a depth of 2Gbp. Assembly was performed using the nf-core/bacass nextflow pipeline v2.5, with the following parameters<sup>93</sup>: nextflow run nf-core/bacass -profile singularity—input sample\_sheet.csv -r 2.5.0—assembly\_type ‘short’—assembler ‘unicycler’—skip\_kraken2—skip\_kmerfinder—outdir bacass\_output. A phylogenomic tree was generated from assembled genomes using Mashtree version 1.4.6.<sup>74</sup> Human and mouse isolates were downloaded from the NCBI genome database, using *Salmonella enterica* or *Salmonella typhimurium* as an out-group. Species identity was confirmed using GTDB-Tk v2.4.0, database release version 226.<sup>73,94</sup>

### Mouse metagenomic profiling

Raw reads were concatenated before quality filtering and trimming using Atria,<sup>77</sup> (parameters: –length-range 75:200 -q 20 -n 2 -g NO—polyG), removing all reads with a quality score of less than 20 and shorter than 75 base pairs, and trimming polyG tails. Trimmed reads were then aligned against the C57BL/6J laboratory mouse genome (GRCm39) using bowtie2 with the “-very-sensitive” option enabled to remove host-derived sequences. Taxonomic profiles were generated from cleaned reads using MetaPhlAn 4 (version 4.1, parameters: –min\_mapq\_val 2—stat\_q 0.05—perc\_nonzero 0.2). Alterations in microbial composition were detected using Bray-Curtis dissimilarity on arcsine square-root transformed taxonomic profiles. Differential abundance was assessed by calculating log2 fold-changes and performing unpaired Wilcoxon rank-sum tests. *P*-values were corrected for multiple comparisons using the Benjamini-Hochberg procedure, and the resulting false discovery rate (FDR)-adjusted *p*-values are reported as *q*-values. Taxa with *q* < 0.1 were considered significant. Log2 fold changes were calculated on relative abundances after adding a pseudo-count of  $10^{-6}$  to accommodate zero values. Functional profiling was performed using HUMAnN4 (version 4.0.0.alpha.1, parameters: –nucleotide-identity-threshold 0—translated-identity-threshold 50—nucleotide-subject-coverage-threshold 30—translated-subject-coverage-threshold 30). For species-stratified analysis of *tnaA* abundance, gene-family abundances were assigned to species using the taxonomic stratification generated by HUMAnN. Species-level assignments were made when the species was detected by MetaPhlAn and the corresponding sequence mapped to its pangenome. *tnaA* contributions that could not be assigned to a MetaPhlAn-detected species were retained as unclassified. Resulting tables were regrouped into Level-4 enzyme commission (EC) categories, which were then re-mapped to KEGG orthologs.

### Human metagenomic profiling

Metagenomic data from patients with ischemic stroke were kindly provided by Dr. Israel Fernandez-Cadenas (Institut de Recerca Sant Pau, Barcelona), together with data from healthy control participants. Individuals who received antibiotics or probiotics in the month prior to ischemic stroke onset, or with recent infections were excluded.<sup>95,96</sup> Raw reads were processed in a similar manner, to murine data, except for minor alterations to enable comparison with curated Metagenomic data 3 data. Briefly, raw reads were concatenated before quality filtering and trimming using Atria, (parameters: –length-range 75:200 -q 20 -n 2 -g NO—polyG), removing

all reads with a quality score of less than 20 and shorter than 75 base pairs, and trimming polyG tails. Trimmed reads were then aligned against the human (GRCh38) and the PhiX genome for removal of contaminating sequences, using bowtie2 with the “-very-sensitive” option enabled. Taxonomic profiles were generated from cleaned reads using MetaPhlAn 3 (version 3.0, default parameters). Functional profiling was performed using HUMAnN3 (version q3.0.0.alpha.3, default parameters), aligning against the UniRef90 database. Resulting tables were regrouped into Level-4 enzyme commission (EC) categories.

| Characteristic               | N   | Control N = 100 <sup>a</sup> | Stroke N = 274 <sup>a</sup> | p-value <sup>b</sup> |
|------------------------------|-----|------------------------------|-----------------------------|----------------------|
| <b>Sex</b>                   | 371 |                              |                             | 0.041                |
| F                            |     | 57 (59%)                     | 128 (47%)                   |                      |
| M                            |     | 40 (41%)                     | 146 (53%)                   |                      |
| <b>Age</b>                   | 373 | 58 (55, 63)                  | 78 (66, 84)                 | <0.001               |
| <b>Hypertension</b>          | 197 |                              |                             | <0.001               |
| 0                            |     | 19 (79%)                     | 49 (28%)                    |                      |
| 1                            |     | 5 (21%)                      | 124 (72%)                   |                      |
| <b>Diabetes</b>              | 197 |                              |                             | 0.006                |
| 0                            |     | 24 (100%)                    | 130 (75%)                   |                      |
| 1                            |     | 0 (0%)                       | 43 (25%)                    |                      |
| <b>Dyslipidemia</b>          | 197 |                              |                             | 0.021                |
| 0                            |     | 16 (67%)                     | 72 (42%)                    |                      |
| 1                            |     | 8 (33%)                      | 101 (58%)                   |                      |
| <b>Atrial fibrillation</b>   | 187 |                              |                             | 0.014                |
| 0                            |     | 18 (100%)                    | 128 (76%)                   |                      |
| 1                            |     | 0 (0%)                       | 41 (24%)                    |                      |
| <b>Obesity</b>               | 374 | 0 (0%)                       | 9 (3.3%)                    | 0.12                 |
| <b>Outcome (mRS 90 days)</b> | 374 |                              |                             | <0.001               |
| mRS 0–2                      |     | N/A                          | 219 (80%)                   |                      |
| mRS 3–6                      |     | N/A                          | 55 (20%)                    |                      |
| <b>NIHSS score admission</b> | 167 | N/A                          | 6 (3, 12)                   |                      |
| <b>NIHSS 24h</b>             | 164 | N/A                          | 3 (1, 8)                    |                      |
| <b>TOAST</b>                 | 374 |                              |                             |                      |
|                              |     | N/A                          | 94 (34%)                    |                      |
| Cardioembolic                |     | N/A                          | 49 (18%)                    |                      |
| Large artery atherosclerosis |     | N/A                          | 30 (11%)                    |                      |
| Other etiology               |     | N/A                          | 6 (2.2%)                    |                      |
| Small Vessel Disease         |     | N/A                          | 19 (6.9%)                   |                      |
| Undetermined                 |     | N/A                          | 76 (28%)                    |                      |

<sup>a</sup>N (%); median (Q1, Q3)

<sup>b</sup>Pearson’s chi-squared test; Wilcoxon rank-sum test; Fisher’s exact test

### 16S rRNA gene amplicon profiling and analysis

Raw data was processed as previously described.<sup>97</sup> Briefly, read quality was assessed with MultiQC before trimming and adaptor removal with Cutadapt.<sup>98,99</sup> Amplicon sequence variants (ASVs) were inferred using DADA2, and spurious sequences filtered (0.25% relative abundance threshold).<sup>100</sup> ASVs remaining after filtering were assigned taxonomy using the SILVA database version 138.2 and a phylogenetic tree inferred using FastTree2 with the GTR+CAT model.<sup>78,79,101</sup> Beta diversity was assessed with compositional, abundance and phylogenetic metrics, employing Aitchison, Bray–Curtis, and generalized UniFrac distances. Distance matrices were visualized by non-metric multidimensional scaling, and differences between groups were assessed by permutational multivariate analysis of variance. Colonization efficiency of human donor microbiota in mouse recipients was calculated separately for each recipient as the percentage of ASVs detected in the corresponding donor that were also detected in the recipient. For each donor, colonization efficiency was summarized as the mean ± SD across the five corresponding recipient mice. Matched donor–recipient microbiota profiles were available for four of the six donors, with an overall mean donor-level colonization efficiency of 17.03% ± 8.45%.

### Bulk RNA sequencing of human blood cells

Bulk RNA-seq data derived from peripheral blood mononuclear cells (PBMCs) from patients with ischemic stroke were kindly provided by Prof. Dr. Luisa Klotz. Samples were aligned to the human reference genome GRCh38 using the STAR algorithm v 2.7.1a<sup>68</sup> with 10 parallel threads and default parameters otherwise. Files were sorted and low-quality reads were removed with samtools v1.22.1.<sup>69</sup> The R/Bioconductor package bamsignals v1.28.0 and R v4.2.1 (R Core Team, 2022) were used to create count matrices for all samples and reference genes. The resulting gene expression matrix was filtered to include LPS-stimulated monocytes from male patients only and transformed using the variance stabilizing transformation from the DESeq2 R package (Love et al., 2014).<sup>71</sup> To assess AHR associated gene regulation in these samples, we utilized the pan-AHR gene signature.<sup>102</sup> Deconvolution of bulk RNA seq data was performed using the immunodeconv R package, utilizing the quantiseq algorithm, with default parameters, identifying cells as mainly classically activated macrophages.<sup>80,103</sup>

### Analysis of metagenomic data from patients with stroke risk factors

To identify associations between indole-producing species and *tnaA* gene abundance and stroke risk factors, we utilized the publicly available R package curatedMetagenomicData (version 3.16.1, CMD3).<sup>81</sup> Indole-producing species were defined by searching the eggno database (v5.0) for “tryptophanase TNA” returning species encoding *tnaA* (COG3033). A list of group members encoding *tnaA* were downloaded and used to filter taxonomic profiles. We first filtered for data derived from adult stool samples removing samples which had been treated with antibiotics. The resulting dataset was further filtered to include only European patients, to limit region-specific differences (Austria, Luxembourg, Germany, Hungary, Slovakia and France). Taxonomic and functional profiles were downloaded with the returnSamples function. Functional profiles were regrouped to Enzyme-commission numbers / KEGG orthologs, using the humann\_regroup\_table function of HUMANN3 (version q3.0.0.alpha.3).

To facilitate comparison of data from patients with stroke, we selected either healthy controls (here defined as those with no known health conditions and under 65 years old), elderly samples (>65 years old) or those with the following health conditions: hypertension, diabetes (Type 1 or Type 2), coronary artery disease and obesity from the CMD3 datasets. Filtered datasets were then merged with data from patients with stroke. Indole-producing species were excluded if not present in at least one third of stroke and CMD3 samples. Mean arcsin-transformed abundances were then calculated separately for each risk factor. In cases where patients had multiple risk factors, samples were duplicated for each risk factor present and included in each calculation. An unpaired Wilcoxon rank-sum test was then used to compare the mean abundance of each species, in each disease condition to healthy controls. Resulting *p*-values were adjusted for multiple comparisons, using the Benjamini-Hochberg procedure. Functional profiles were filtered for *tnaA* (EC 4.1.99.1), and tested in the same manner.

### Association between *tnaA* and stroke outcome

Univariate and multivariate logistic regression was performed using the glm function from the stats R package to assess the association between binned *tnaA* (EC 4.1.99.1) relative abundance, known modifiers of stroke outcome (age, sex, hypertension, diabetes, stroke severity, dyslipidemia and infection) and stroke outcome based on modified Rankin scale (mRS) at 90 days. Abundance of *tnaA* was binned into high and low quantiles using the median as a threshold.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism version 11.0.0 and R where indicated. Summary data are shown as mean  $\pm$  SD unless otherwise specified. Normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated where appropriate. For comparisons between two independent groups, normally distributed data were analyzed using two-tailed unpaired Student’s *t* tests, whereas non-normally distributed data were analyzed using Mann–Whitney *U* tests. For comparisons among more than two groups, normally distributed data were analyzed using one-way or two-way ANOVA, as appropriate, followed by Tukey’s (all pairwise comparisons among groups), Šidák’s (selected planned comparisons), or Dunnett’s (comparisons against one control group) multiple-comparison test depending on the experimental design. Non-normally distributed data involving more than two groups were analyzed using Kruskal–Wallis tests followed by Dunn’s multiple-comparison test. Exact tests and correction methods are specified in the figure legends.

For fecal microbiota transplantation experiments, statistical analyses were performed using linear mixed-effects models with donor included as a random intercept to account for donor-level clustering and pseudoreplication. Exact statistical tests, sample sizes, and multiple-comparison procedures are indicated in the corresponding figure legends. *P* values < 0.05 were considered statistically significant.