# Microbial tryptophan metabolites regulate gut barrier function via the aryl hydrocarbon receptor

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**Inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, are associated with dysbiosis of the gut microbiome. Emerging evidence suggests that small-molecule metabolites derived from bacterial breakdown of a variety of dietary nutrients confer a wide array of host benefits, including amelioration of inflammation in IBDs. Yet, in many cases, the molecular pathways targeted by these molecules remain unknown. Here, we describe roles for three metabolites—indole-3-ethanol, indole-3-pyruvate, and indole-3-aldehyde—which are derived from gut bacterial metabolism of the essential amino acid tryptophan, in regulating intestinal barrier function. We determined that these metabolites protect against increased gut permeability associated with a mouse model of colitis by maintaining the integrity of the apical junctional complex and its associated actin regulatory proteins, including myosin IIA and ezrin, and that these effects are dependent on the aryl hydrocarbon receptor. Our studies provide a deeper understanding of how gut microbial metabolites affect host defense mechanisms and identify candidate pathways for prophylactic and therapeutic treatments for IBDs.**

gut microbiome | intestinal epithelium | colitis | inflammatory bowel disease

Inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, afflict millions worldwide and greatly affect patient quality of life. These diseases are due to chronic inflammation in the intestines caused by a breakdown in immune homeostasis within the gut (1, 2). Although the exact etiology of this disease remains unknown (3), several factors have been identified that regulate intestinal homeostasis, including the integrity of the gut epithelium (4). This single layer of cells provides a physical and immunological barrier to the intestinal luminal contents, which include the gut microbiota and dietary nutrients. Many IBDs are characterized by compromised gut epithelial barrier function that enables these antigens access to the underlying host tissue in which the immune cells reside. This epithelial leakiness leads to activation of the host immune response and increased inflammation that drives disease pathology.

The gut microbiome, or collection of microorganisms that reside in the intestines, plays a major role in regulating immune homeostasis in the intestines (5, 6). These microbes include bacteria, fungi, archaea, and viruses, which are estimated to rival the number of cells in the human body (7). The gut microbiota collectively contains ∼100 times the number of genes in the human genome and encompasses a wide variety of species with microbial densities ranging from $10^9$ cells/mL in the small intestine and $10^{12}$ cells/g of luminal content in the large intestine.

The gut microbiome modulates myriad physiological functions that greatly affect the overall health of the host, including metabolism and the gut–brain axis (8–10). For example, the gut microbiota break down indigestible dietary fiber and biosynthesize essential nutrients that provide numerous benefits to the host. In addition, these microbes regulate the host immune system by modulating the activities of various immune cell types, ultimately leading to changes in inflammation. Despite increasing evidence that the gut microbiota are critical elements in modulating intestinal immune homeostasis, the mechanisms that regulate many of these pathways remain unknown.

Small-molecule metabolites that are produced by the gut microbiota are emerging as important factors in maintaining intestinal immune homeostasis (11–14). These molecules are biosynthesized by gut microbes and secreted into the intestinal lumen, where they modulate the activities of many host cell types, including intestinal epithelial cells (IECs), and, for metabolites that are able to penetrate the gut epithelium, immune cells within the lamina propria. One of the most widely characterized classes of metabolites are short-chain fatty acids such as acetate, propionate, and butyrate, which derive from bacterial anaerobic fermentation of complex polysaccharides within dietary fiber (15). These metabolites exhibit pleiotropic effects on many different immune cell types, including macrophages, dendritic cells (DCs), B and T lymphocytes, and IECs, and contribute to an antiinflammatory environment within the gut (11–14).

Dietary nutrients, including essential amino acids such as tryptophan (Trp), also serve as substrates for enzymes within the gut microbiota. Bacterial catabolism of Trp produces many metabolites, several of which can modulate aspects of the host immune response, including differentiation of regulatory $CD4^+$ T cells (Tregs), maintenance of intestinal epithelial lymphocytes, activation of natural killer T cells, and protection against mouse models of multiple sclerosis and colorectal cancer (16–20). Additional studies have examined the effects of Trp metabolites in

## Significance

**The gut microbiome comprises trillions of microorganisms that inhabit the mammalian intestines. These microbes regulate many aspects of host physiology, including defense mechanisms against factors that contribute to inflammatory bowel diseases (IBDs). Despite the abundance of the gut microbiota, little is known regarding how these microbes modulate these host processes, including the barrier function of the intestinal epithelium, which controls intestinal permeability associated with IBDs. Here, we discover that three gut microbially produced, small-molecule metabolites, which derive from dietary tryptophan, improve intestinal barrier integrity and protect against inflammation caused by IBDs. Our studies identify a host receptor and downstream targets of the metabolites, which could serve as potential pathways for prophylactic and therapeutic treatments for ameliorating morbidity in IBDs.**

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The authors declare no competing interest.

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colitis models and have focused on various cell types including DCs, Tregs, goblet cells, and innate lymphoid cells (ILCs) (21–25). Intriguingly, indole-3-pyruvate (IPyA) and indole-3-aldehyde (I3A) can ameliorate mouse models of colitis (24, 25), and these reports focus on the effects of these metabolites on modulating $CD103^+$ DCs, T regulatory 1 (Tr1) cells, and $Nkp46^+$ ILCs. Host-produced Trp metabolites such as kynurenine (Kyn) and the microbially produced Trp metabolites indole (IND) and indole-3-propionate (IPA) have been shown to regulate intestinal barrier function by modulating the gut epithelium via IL-10 receptor (IL-10R) expression (26) and alternative pathways, including those activated by Toll-like receptor 4 (TLR4) and the pregnane X receptor (PXR), respectively (27–30). Collectively, these studies have investigated only a limited number of Trp metabolites, and their physiological effects on function of the gut epithelial barrier, which is critical to host–microbiota immune homeostasis, remain largely unexplored for most Trp-derived metabolites. Thus, we were motivated to explore the effects on the gut epithelial barrier of a broader collection of Trp metabolites, whose activities toward intestinal barrier function remain largely uncharacterized.

In this study, we have identified and characterized new roles for three Trp metabolites—indole-3-ethanol (IEt) (also known as tryptophol), IPyA, and I3A—in modulating gut barrier integrity via tight junctions (TJs) and adherens junctions (AJs), which together comprise the apical junctional complex (AJC) (31), a major regulator of intestinal permeability. TJ proteins (e.g., ZO1 and occludin) form a barrier against free diffusion at the apical site of adjacent epithelial cell membranes to prevent paracellular transport of molecules between cells (32), and interactions of AJ proteins (e.g., E-cadherin and β-catenin), which are located basolaterally but subjacent to TJs, form adhesive forces between adjacent epithelial cells to seal the intestinal barrier. Here, we have demonstrated that a Trp-rich diet ameliorates morbidity and inflammation using a mouse model of colitis induced by dextran sodium sulfate (DSS) gut epithelial damage. We have determined that the active Trp metabolites produced in vivo are IEt, IPyA, and I3A, while antibiotic treatment to deplete Trp-metabolizing bacteria decreases their production. We also demonstrated that treatment of mice with IEt, IPyA, and I3A individually also improves disease outcomes in DSS colitis. At a mechanistic level, a major portion of the metabolites' effects is mediated by the aryl hydrocarbon receptor (AhR), which is known to recognize indole-containing Trp metabolites (33, 34). Finally, we determined the Trp metabolites affect AJC function by modulating the levels and activity of key proteins including myosin IIA and ezrin that regulate filamentous actin (F-actin). Collectively, these studies provide a mechanistic understanding of how small-molecule Trp metabolites produced by the gut microbiota modulate host processes, including gut barrier function, to benefit the host in diseases such as IBDs that negatively affect the intestinal epithelium.

## Results

**Trp Feeding Ameliorates DSS Colitis in Mice and Is Dependent on the Gut Microbiota.** We began by examining the effects of a Trp-rich diet on morbidity and inflammation using a mouse model of colitis (Fig. 1*A*). As measured by changes in weight loss, colon length, disease activity index, and histopathology, we found that Trp feeding exhibited protective effects (Fig. 1 *B* and *D*–*G*). A major feature of DSS colitis that leads to this morbidity and inflammation is an increase in permeability of the gut epithelium. Importantly, Trp feeding during DSS colitis also attenuated the increase in gut permeability, as measured by flux of the fluorescent tracer fluorescein isothiocyanate (FITC)-dextran from the intestinal lumen to the blood (Fig. 1*C*). We further found that the improvement in intestinal permeability is likely due to modulation of TJ and AJ by immunofluorescence (IF) staining of key protein components of these structures within the intestinal tissues (Fig. 1 *H*–*M* and *SI Appendix*, Fig. S1). Aspects of these experiments confirm previously reported findings that Trp feeding improves disease outcomes in DSS colitis (35–38). In those studies, however, effects of Trp feeding were attributed to immune pathways, including production of prostaglandin E2 (PGE2), and gut permeability due to changes in AJC expression was not addressed (36). To examine whether the effects of the Trp-rich diet are due to bacterial catabolism of Trp, we also pretreated certain groups of mice with a mixture of antibiotics containing ampicillin, metronidazole, neomycin, and vancomycin (ABX) to deplete bacteria that contribute to Trp metabolite production. For all of the above-described readouts of physiological function during DSS colitis, we found that ABX treatment eliminated the effects of Trp feeding (Fig. 1).

**Specific Trp Metabolites Improve Intestinal Barrier Function In Vitro.** To determine the identities of the Trp metabolites that mediate this protective effect, we performed an in vitro screen of Trp and 12 of its metabolites using the human Caco-2 IEC cell line, which forms a polarized monolayer that resembles the gut epithelium (39). We found that IEt, IPyA, and I3A (50 μM to 1 mM) all attenuated the increase in epithelial permeability caused by treatment with a proinflammatory cytokine, TNFα, an inflammatory factor associated with IBDs, in a dose-dependent manner as measured by changes in trans-epithelial electrical resistance (TEER) and FITC-dextran flux (*SI Appendix*, Fig. S2). Upon examination of TJ protein localization by IF, we also found that TNFα treatment causes a striking sinuous phenotype wherein the Caco-2 cells become jagged, as opposed to the typical smooth, chicken-wire–like appearance of monolayers (*SI Appendix*, Fig. S3). This sinuous phenotype, which was previously reported (40), is likely due to an increased contractility of the actin cytoskeleton as indicated by increased colocalization of F-actin with TJ proteins (*SI Appendix*, Fig. S3 *C* and *H*). Importantly, IEt, IPyA, and I3A all prevent this effect, which does not depend on changes in messenger RNA transcript or protein levels of the TJ and AJ proteins (*SI Appendix*, Figs. S3 and S4). The remaining Trp metabolites that were tested did not have any effects on these phenotypes, so it is unlikely that they are the active Trp metabolites for this phenotype (*SI Appendix*, Figs. S5 and S6).

**Trp Metabolites Improve Disease Outcomes in DSS Colitis in Mice.** Based on these in vitro studies, we hypothesized that IEt, IPyA, and I3A would ameliorate DSS colitis in mice. We first verified that these three metabolites are indeed produced from Trp catabolism during DSS colitis by targeted mass spectrometry (MS)-based metabolomics using liquid chromatography-MS (LC-MS) (*SI Appendix*, Fig. S7). Importantly, these studies enabled us to establish therapeutically relevant doses. We then administered the same ABX treatment to deplete the gut microbiota and prevent further microbial metabolism of IEt, IPyA, and I3A, followed by giving each metabolite to mice by oral gavage prior to the induction of DSS colitis and examined changes in weight loss, colon length, disease activity index, and histopathology as above (Fig. 2). All three of these metabolites ameliorated each of these measures of disease severity in mice. Furthermore, IEt, IPyA, and I3A also prevented increased intestinal permeability (Fig. 2*C*) and disassembly of the AJC during DSS colitis, as established by IF staining of the TJ and AJ proteins (*SI Appendix*, Figs. S8–S10). We also examined the effects of IEt, IPyA, and I3A on TNFR1 expression in IECs, which increases in DSS colitis, and found that these metabolites inhibit expression of this receptor (*SI Appendix*, Fig. S11 *A*–*C*). We further examined an important downstream target of TNFR1, NF-κB activation, using a reporter cell line, and found that these metabolites inhibit activity of this important transcription factor that is responsible for the transcription of many inflammatory mediators (*SI Appendix*, Fig. S11*D*). In addition, we determined the effects of IEt, IPyA, and I3A on IL-10R expression in IECs, which decreases in DSS colitis, and found that these

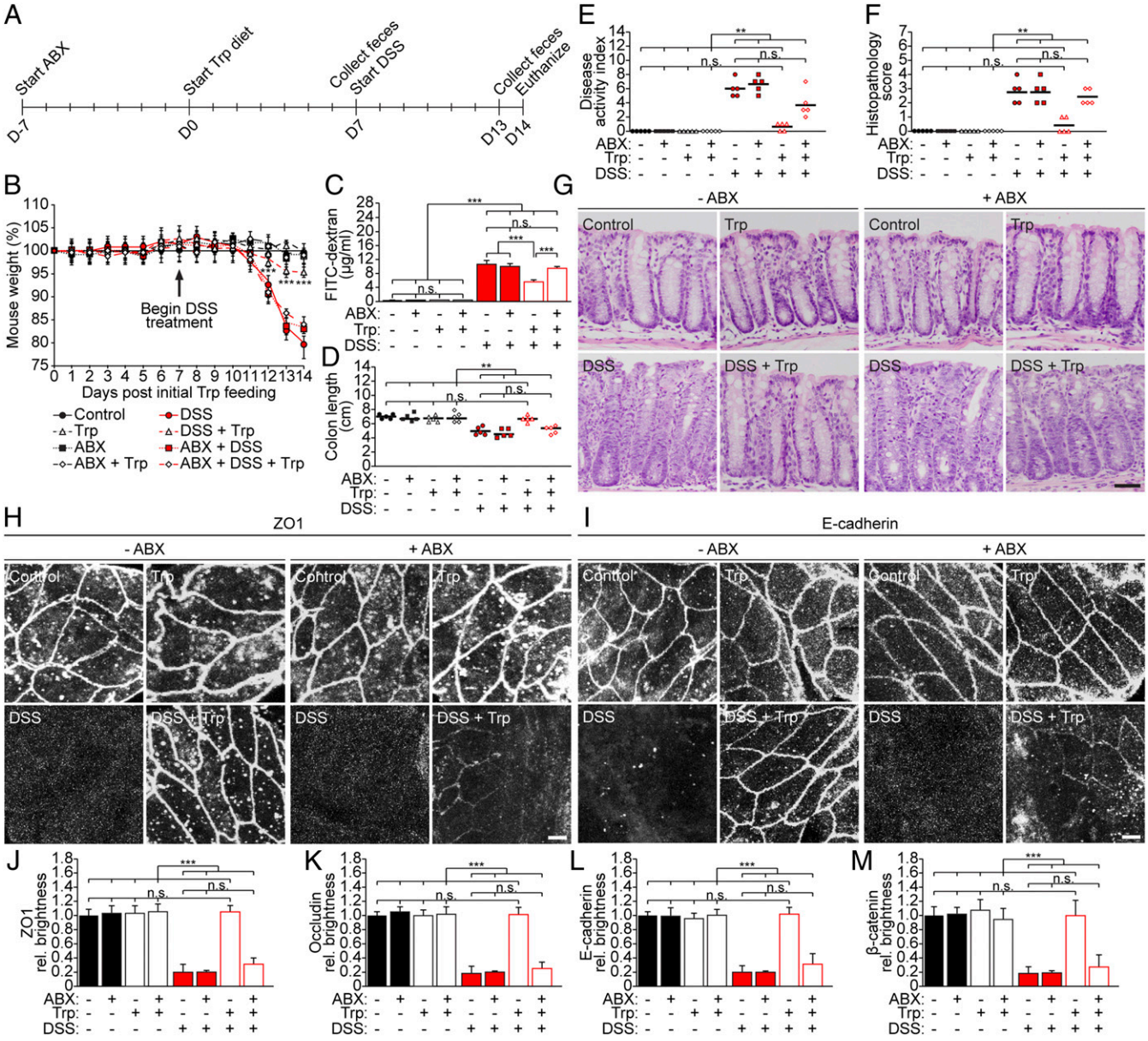


**Fig. 1.** Dietary Trp ameliorates a mouse model of colitis in a microbiome-dependent manner. (*A*) C57BL/6 mice were pretreated with antibiotics (ABX: ampicillin [9 mg/kg], metronidazole [9 mg/kg], neomycin [9 mg/kg], and vancomycin [4.5 mg/kg], intragastrically) for 7 d and then fed a Trp-rich diet (42 g Trp/kg diet) or standard chow (2 g Trp/kg diet) for 7 d, followed by administration of DSS (3%, wt/vol) or vehicle for 7 d (ad libitum) with continued antibiotic treatment and Trp feeding. (*B*) The mice were weighed daily. (*C*) Mice were orally gavaged with FITC-dextran (900 mg/kg) on day 14, and serum levels of FITC-dextran were measured 4 h later. (*D*) On day 14, the mice were killed, and colon lengths were measured, (*E*) disease activity index was measured, and (*F* and *G*) the distal colon was stained with hematoxylin and eosin (H&E) and blindly scored (0 = none, 1 = very mild, 2 = mild, 3 = moderate, 4 = severe) for epithelial damage, mononuclear and polymorphonuclear infiltrate, and submucosal edema. (Scale bar: 50 μm.) (*H–M*) Colon sections were stained for TJ and AJ proteins and imaged by confocal microscopy (see also *SI Appendix*, Fig. S1, for occludin and β-catenin). (Scale bars: 20 μm.) (*J–M*) Relative (rel.) brightness of images with error as SD from the mean was calculated ($n = 15$). (*H* and *J*) ZO1. (*I* and *L*) E-cadherin. (*K*) Occludin. (*M*) β-catenin. Data are representative of at least three independent experiments; $n = 5$ mice per group. One-way ANOVA followed by post hoc Tukey's test: $^{**}P < 0.01$, $^{***}P < 0.001$, n.s.: not significant.

metabolites increase expression when administered alone and in combination with DSS in vivo (*SI Appendix*, Fig. S12). We also found that oral gavage of IEt, IPyA, and I3A led to sustained levels of each metabolite within the intestinal lumen during DSS colitis by LC-MS analysis (*SI Appendix*, Figs. S13–S15).

**Trp Metabolites Are Ligands of the AhR.** We next set out to establish the molecular mechanism by which the Trp metabolites exert their protective effects on gut permeability in DSS colitis. We hypothesized that IEt, IPyA, and I3A are recognized by AhR, a host receptor that is activated in several cell types by many indole-containing ligands, including IPyA and I3A (24, 25), and that AhR may mediate the effects of these metabolites during DSS colitis. We tested this hypothesis by first assessing whether IEt, IPyA, and I3A activate AhR and found that all three metabolites activate this receptor, using a luciferase reporter assay in HEK 293T cells (*SI Appendix*, Fig. S16*A*). We then examined effects of these metabolites on AhR-dependent gene expression and found increased transcription of AhR and canonical downstream target genes in polarized Caco-2 monolayers (*SI Appendix*, Fig. S16 *B–F*).

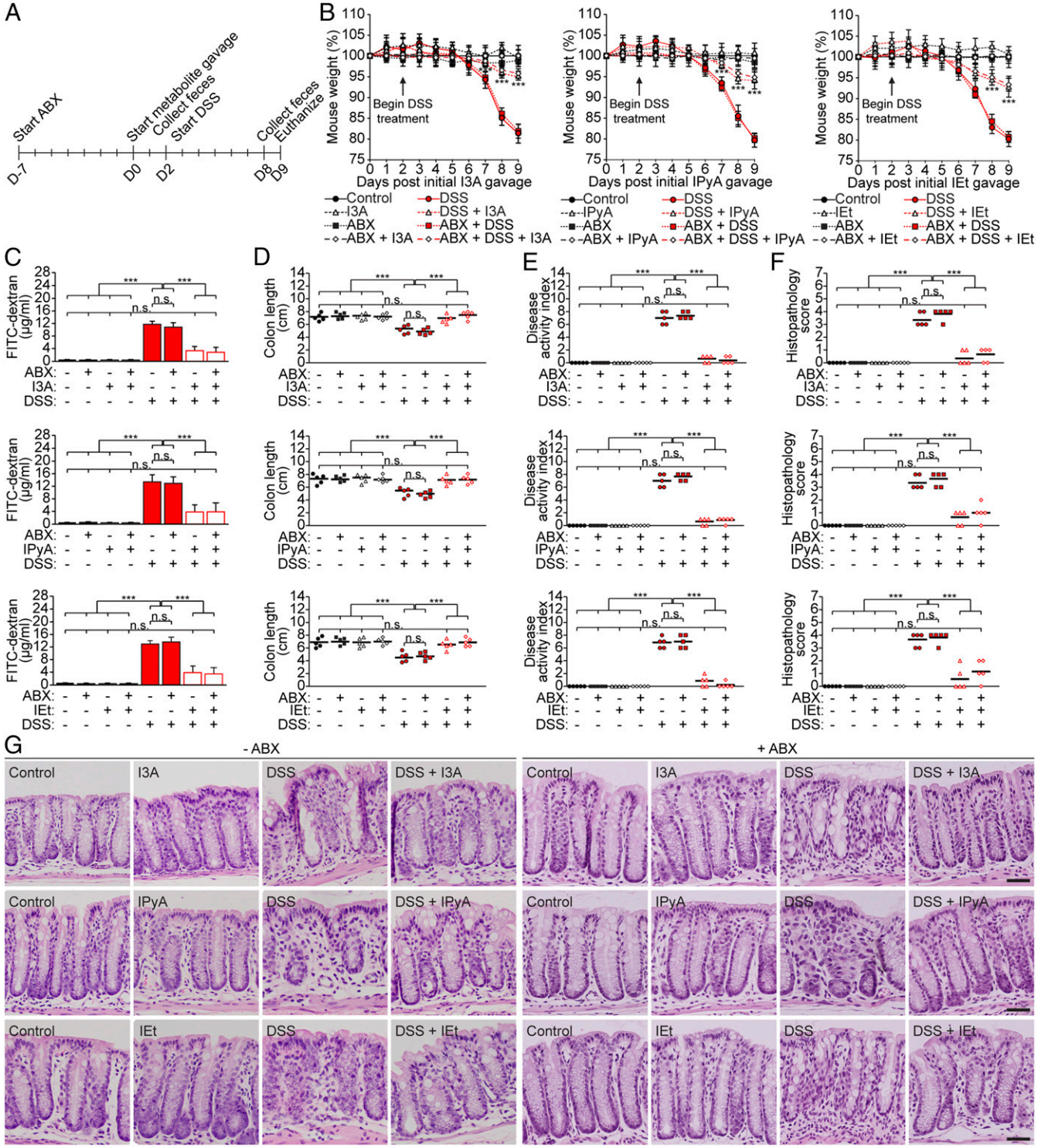


**Fig. 2.** Tryptophan metabolites I3A, IPyA, and IEt ameliorate a mouse model of colitis. (*A*) C57BL/6 mice were pretreated with antibiotics (ABX: ampicillin [9 mg/kg], metronidazole [9 mg/kg], neomycin [9 mg/kg], and vancomycin [4.5 mg/kg], intragastrically) for 7 d, followed by I3A (1,000 mg/kg), IPyA (2,900 mg/kg), or IEt (600 mg/kg) for 2 d and then administered DSS (3%, wt/vol) for 7 d (ad libitum) with continued antibiotic and metabolite treatment. (*B*) The mice were weighed daily. (*C*) Mice were orally gavaged with FITC-dextran (900 mg/kg) on day 9, and serum levels of FITC-dextran were measured 4 h later. (*D*) On day 9, the mice were killed, and colon lengths were measured, (*E*) disease activity index was measured, and (*F* and *G*) the distal colon was stained with H&E and blindly scored (0 = none, 1 = very mild, 2 = mild, 3 = moderate, 4 = severe) for epithelial damage, mononuclear and polymorphonuclear infiltrate, and submucosal edema. (Scale bar: 50 μm.) Data are representative of at least three independent experiments; $n = 5$ mice per group. One-way ANOVA followed by post hoc Tukey's test: $^{***}P < 0.001$, n.s.: not significant.

We also found that an inhibitor of AhR, GNF351, partially blocks the effects of IPyA in Caco-2 monolayers challenged with TNFα, based on an examination of TEER, FITC-dextran flux, and IF of the AJC proteins (*SI Appendix*, Fig. S16 *G–P*).

**Effect of Trp Metabolites Is Partially Dependent on AhR In Vivo.** To examine whether the physiological effects of IEt, IPyA, and I3A toward gut barrier integrity are mediated by AhR, we treated $Ahr^{-/-}$ or $Ahr^{+/-}$ littermate control mice with a Trp-rich diet and then challenged the mice with DSS colitis (Fig. 3*A*). We found that Trp feeding decreases morbidity and inflammation in $Ahr^{+/-}$ mice but not in $Ahr^{-/-}$ mice, as determined by changes in weight loss, colon length, disease activity index, and histopathology (Fig. 3 *B* and *D–G*). The improvements in gut permeability (Fig. 3 *C* ) and disassembly of the AJC within intestinal tissues conferred by these metabolites during DSS colitis were also abrogated in the $Ahr^{-/-}$ mice compared to their littermate controls (Fig. 3 *H–Q* and *SI Appendix*, Fig. S17). Importantly, we found no difference in the effects of Trp feeding during DSS colitis in $Ahr^{+/+}$ compared to $Ahr^{+/-}$ mice, validating the use of $Ahr^{+/-}$ mice as controls for the above studies (*SI Appendix*, Fig. S18).

To assess the extent to which IEt, IPyA, and I3A are the active Trp metabolites produced in vivo during DSS colitis, we preadministered these metabolites to the mice and found that the effects of each metabolite are partially dependent on AhR (Fig. 4 and *SI Appendix*, Fig. S19, IEt; *SI Appendix*, Fig. S20, IPyA; and *SI Appendix*, Fig. S21, I3A). In these studies, we administered IEt at therapeutically relevant levels as determined in *SI Appendix*, Fig. S7 (Fig. 4) to $Ahr^{+/-}$ and $Ahr^{-/-}$ mice and found that it ameliorated morbidity and inflammation in $Ahr^{+/-}$ mice but not in $Ahr^{-/-}$ mice, as determined by changes in weight loss, colon length, disease activity index, and histopathology (Fig. 4 *A, B,* and *D–G*). We also determined that IEt improved intestinal permeability (Fig. 4*C*) via modulation of the AJC in intestinal tissues within these mice, and these effects were also dependent on AhR (Fig. 4 *H–Q* and *SI Appendix*, Fig. S19). In parallel experiments, we found similar effects with therapeutically relevant amounts of IPyA and I3A (as determined in *SI Appendix*, Fig. S7) in improving disease outcomes during DSS colitis and intestinal permeability in these mice due to modulation of the TJ and AJ proteins (*SI Appendix*, Figs. S20 and S21). Together, these studies suggest that these metabolites are the active ones produced during Trp supplementation that confer the majority of the effects on intestinal epithelial barrier function that are dependent on AhR in a mouse model of DSS colitis.

**Trp Metabolites Inhibit Activation of Myosin IIA In Vivo in an AhR-Dependent Manner.** The previous studies implicate AhR as an upstream target of the Trp metabolites. We next set out to elucidate downstream mechanisms that would directly explain the metabolites' effects on gut permeability in the context of TNFα treatment of Caco-2 monolayers as an in vitro model and DSS colitis in vivo. Because TNFα triggers increased permeability by opening up the AJC with mechanical force via contraction of the actin cytoskeleton (41, 42), we hypothesized that IEt, IPyA, and I3A might modulate the activity of actin regulatory proteins, such as nonmuscle myosin IIA (MyoIIA), a motor protein that generates force to alter the actin skeleton, to decrease intestinal permeability (43). Thus, we examined the activation state of MyoIIA in DSS-treated mice fed with Trp-rich or standard diets. MyoIIA is phosphorylated at serine 19 (p-MLC, for phospho-myosin light chain), an activation mark, by myosin light-chain kinase (MLCK). We found, by Western blotting and IF staining of intestinal tissue, that a Trp-rich diet reversed the increases in p-MLC and MLCK levels induced in vivo in DSS colitis (Fig. 5 *A–D*, *Q*, and *R*). Importantly, we examined the effects in $Ahr^{-/-}$ mice and found that they were attenuated, indicating that the effects on MyoIIA are downstream of and dependent upon AhR activation by Trp metabolites (Fig. 5). Furthermore, we also assessed the effects of the three relevant Trp metabolites—IEt, IPyA, and I3A—and found that treatment of mice with each of these metabolites, individually, led to similar effects for each metabolite in inhibiting MyoIIA activation that are also AhR dependent, suggesting that these metabolites contribute to the effects of Trp feeding (Fig. 5 *E–Q* and *S–U*).

**Trp Metabolites Inhibit Activation of Ezrin In Vitro.** Ezrin, a founding member of the ezrin/radixin/moesin protein family, is a major upstream regulator of MyoIIA-mediated actin assembly that physically links the actin cytoskeleton to the plasma membrane upon activation, which is marked by phosphorylation of threonine 567 (p-ezrin) and leads to mechanical opening of the AJC and increased permeability (44). We hypothesized that activation of ezrin might serve as a critical event that is inhibited by IEt, IPyA, and I3A, ultimately leading to the effects of these metabolites in preventing the increase of epithelial permeability caused by TNFα. To test this mechanistic hypothesis, we retrovirally transduced into Caco-2 monolayers either wild-type (WT) ezrin or one of two mutant forms of ezrin, one of which could not be activated (T567A, phosphodeficient) and the other of which was constitutively active (T567E, phosphomimetic) (45). We then treated these monolayers with IEt, IPyA, and I3A, subsequently challenged them with TNFα, and then evaluated epithelial permeability by TEER and FITC-dextran flux, and the sinuous phenotype by IF for AJC markers. We found that T567E- and T567A-expressing monolayers exhibited, respectively, exaggerated and attenuated phenotypes relative to WT-expressing or control monolayers, indicating the expected functionality of these T567 mutants of ezrin (*SI Appendix*, Figs. S22–S25). Interestingly, metabolite treatment attenuated the increased epithelial permeability in WT- and T567A-expressing monolayers (which retain endogenous WT ezrin); however, the effects of the metabolites were largely abrogated in T567E-expressing monolayers (*SI Appendix*, Fig. S22). We observed similar effects on the sinuous phenotype caused by TNFα treatment of Caco-2 monolayers (*SI Appendix*, Figs. S23–S25). These data indicate that constitutively active ezrin can override the effects of the Trp metabolites and strongly suggest that the mechanism of action of these metabolites is via the activity of the key actin regulatory protein ezrin.

**Trp Metabolites Inhibit Activation of Ezrin In Vivo in an AhR-Dependent Manner.** Finally, to ascertain whether the metabolites inhibit ezrin activation in a physiologically relevant context, we fed DSS-treated mice a Trp-rich diet and found that, relative to a standard diet, Trp feeding attenuates the increase in p-ezrin in intestinal tissues caused by DSS treatment by Western blotting and IF (Fig. 6 *A–C*, *M*, and *N*). We also found that treatment of mice with each of the active Trp metabolites (IEt, IPyA, or I3A) also inhibits ezrin activation, suggesting that these metabolites, which are produced in vivo from bacterial catabolism of Trp, may mediate the effects of Trp feeding during DSS colitis (Fig. 6 *D–M* and *O–Q*). Finally, we found that the effects of both the Trp-rich diet and treatment with the individual metabolites IEt, IPyA, and I3A exhibited a substantial, but not complete, dependence upon AhR during DSS challenge because the activation of ezrin was decreased in $Ahr^{+/-}$ mice, but not in $Ahr^{-/-}$ mice (Fig. 6).

## Discussion

Small-molecule metabolites produced by the gut microbiota are being increasingly appreciated to modulate numerous aspects of host physiology during health and disease (11–14). Despite this growing evidence, the identities of the majority of these molecules, their precise effects on the host, and the mechanisms by which they act remain largely uncharacterized. In this study, we focused on the effects of specific Trp metabolites that are produced by the gut microbiota in improving intestinal barrier

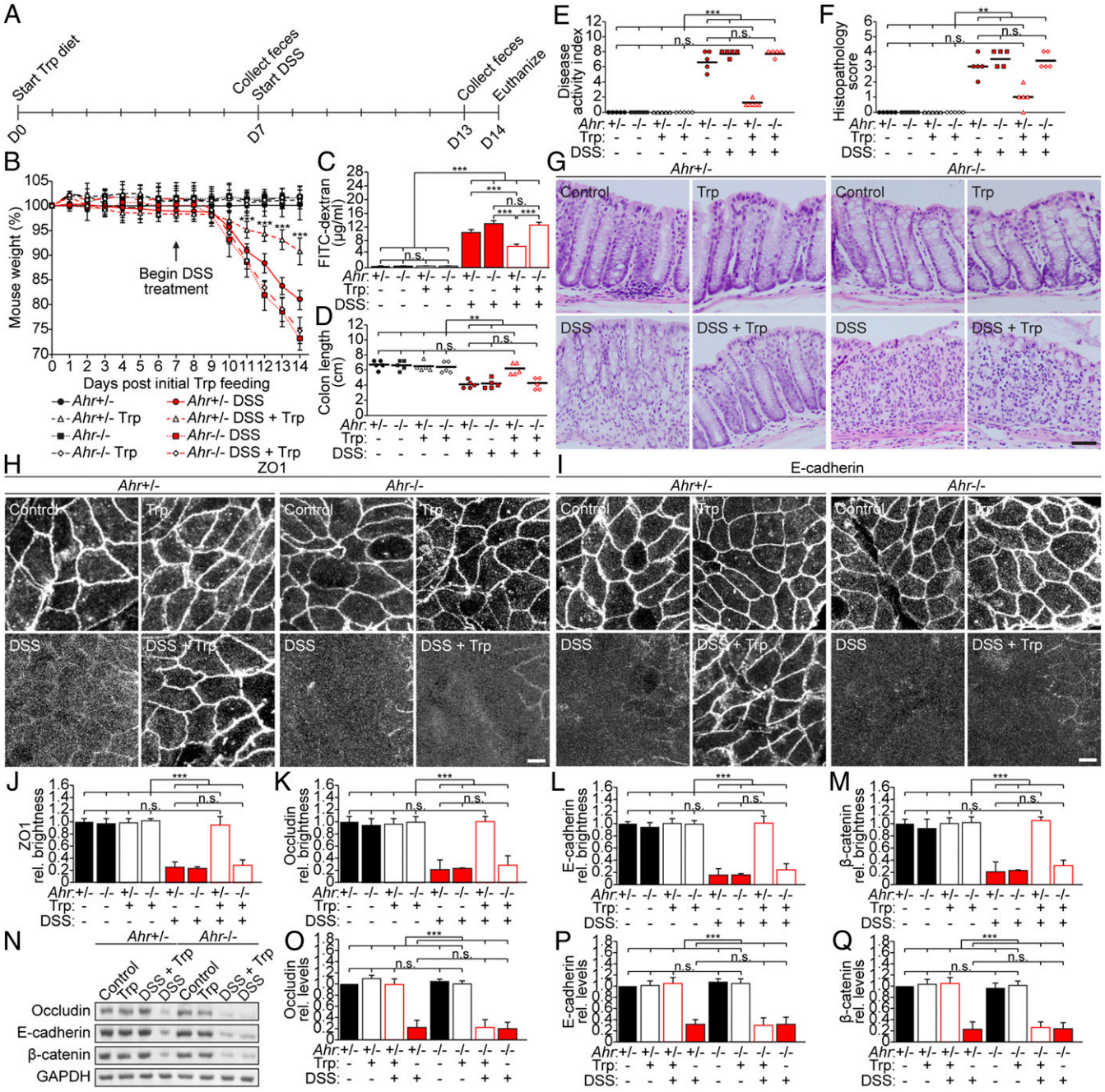


**Fig. 3.** Effect of a high-Trp diet in a mouse model of colitis is dependent on AhR. (*A*) $Ahr^{+/-}$ or $Ahr^{-/-}$ mice were fed a Trp-rich diet (42 g Trp/kg diet) or standard chow (2 g Trp/kg diet) for 7 d, followed by administration of DSS (3%, wt/vol) or vehicle for 7 d (ad libitum) with continued Trp feeding. (*B*) The mice were weighed daily. (*C*) Mice were orally gavaged with FITC-dextran (900 mg/kg) on day 14, and serum levels of FITC-dextran were measured 4 h later. (*D*) On day 14, the mice were killed, and colon lengths were measured, (*E*) disease activity index was measured, and (*F* and *G*) the distal colon was stained with H&E and blindly scored (0 = none, 1 = very mild, 2 = mild, 3 = moderate, 4 = severe) for epithelial damage, mononuclear and polymorphonuclear infiltrate, and submucosal edema. (Scale bar: 50 μm.) (*H–M*) Colon sections were stained for TJ and AJ proteins and imaged by confocal microscopy (see also *SI Appendix*, Fig. S17, for occludin and β-catenin). (Scale bars: 20 μm.) (*J–M*) Relative (rel.) brightness of images with error as SD from the mean was calculated ($n$ = 15). (*H* and *J*) ZO1. (*I* and *L*) E-cadherin. (*K*) Occludin. (*M*) β-catenin. (*N–Q*) TJ and AJ protein levels were determined by Western blotting with the indicated antibodies and (*O–Q*) quantified by densitometry ($n$ = 3). Data are representative of at least three independent experiments; $n$ = 5 mice per group. One-way ANOVA followed by post hoc Tukey's test: $^{**}P < 0.01$, $^{***}P < 0.001$, n.s.: not significant.

function. In particular, we identified roles for three Trp metabolites—IEt, IPyA, and I3A—in increasing gut barrier integrity during challenge with a proinflammatory cytokine in vitro and in vivo using a mouse model of DSS colitis (Fig. 7). In addition, we demonstrated that the effects of these metabolites were largely dependent on AhR, a receptor that provides many diverse functions for the host (33, 34) and inhibits activation of the actin regulatory proteins MyoIIA and ezrin.

Supplementation of diets with Trp, which could be replicated by protein-rich diets, has been shown to be beneficial to the host

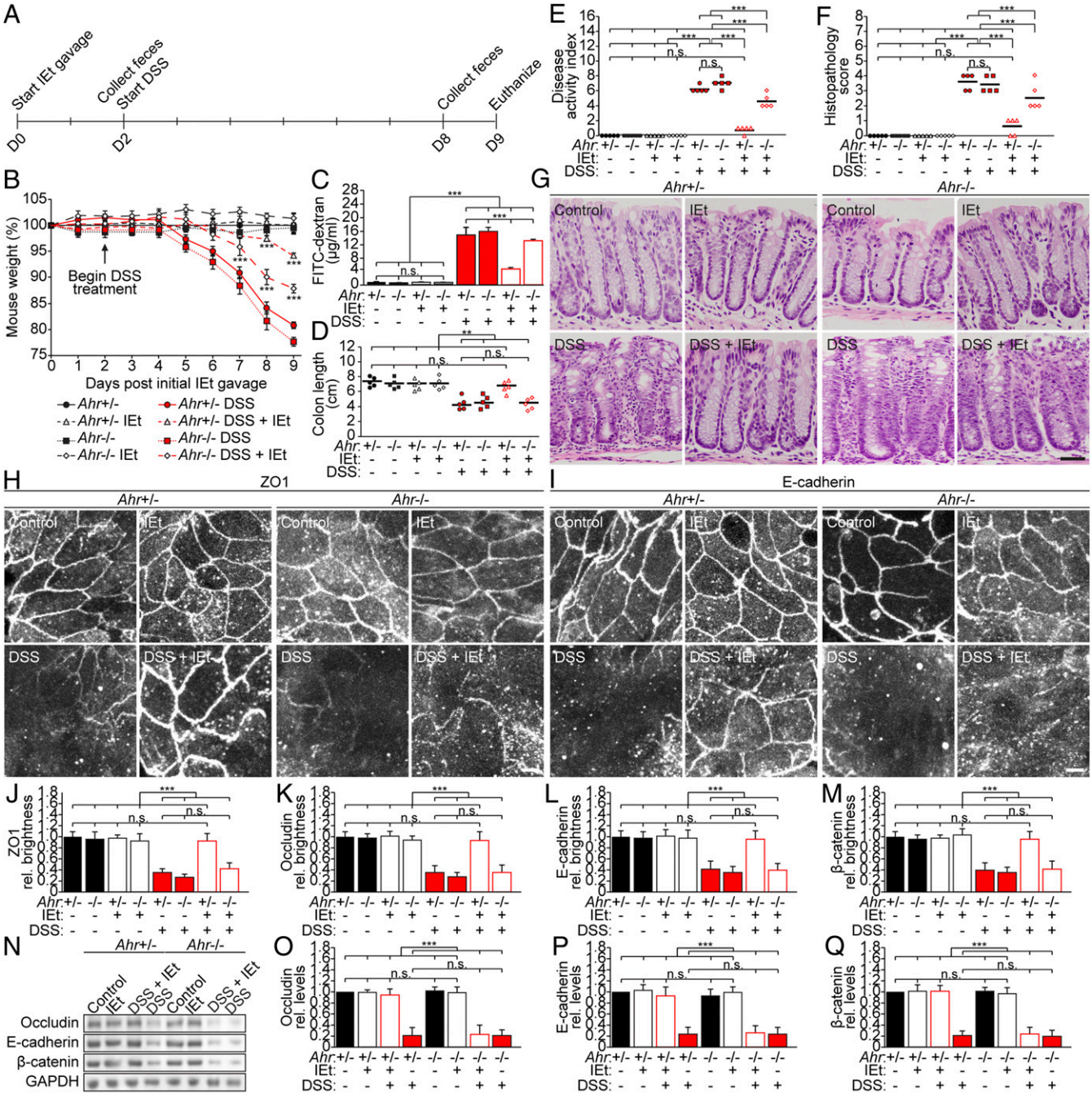


**Fig. 4.** Effect of tryptophan metabolite IEt in mouse model of colitis is dependent on AhR. (*A*) $Ahr^{+/-}$ or $Ahr^{-/-}$ mice were pretreated with IEt (600 mg/kg) for 2 d and then administered DSS (3%, wt/vol) for 7 d (ad libitum) with continued metabolite treatment. (*B*) The mice were weighed daily. (*C*) Mice were orally gavaged with FITC-dextran (900 mg/kg) on day 9, and serum levels of FITC-dextran were measured 4 h later. (*D*) On day 9, the mice were killed, and colon lengths were measured, (*E*) disease activity index was measured, and (*F* and *G*) the distal colon was stained with H&E and blindly scored (0 = none, 1 = very mild, 2 = mild, 3 = moderate, 4 = severe) for epithelial damage, mononuclear and polymorphonuclear infiltrate, and submucosal edema. (Scale bar: 50 μm.) (*H*–*M*) Colon sections were stained for TJ and AJ proteins and imaged by confocal microscopy (see also *SI Appendix*, Fig. S19 for occludin and β-catenin). (Scale bars = 20 μm.) (*J*–*M*) Relative (rel.) brightness of images with error as SD from the mean was calculated ($n = 15$). (*H* and *J*) ZO1. (*I* and *L*) E-cadherin. (*K*) occludin. (*M*) β-catenin. (*N*–*Q*) TJ and AJ protein levels were determined by Western blotting with the indicated antibodies and (*O*–*Q*) quantified by densitometry ($n = 3$). Data are representative of at least three independent experiments; $n = 5$ mice per group. One-way ANOVA followed by post hoc Tukey's test: $^{**}P < 0.01$, $^{***}P < 0.001$, n.s.: not significant.

by improving disease outcomes in various DSS colitis models (35–38). Critically, the identities of the metabolites and the affected host pathways were not fully characterized. We found that a Trp-rich diet provides the host with microbiota-derived metabolites, including IEt, IPyA, and I3A, which ameliorate morbidity using a mouse model of DSS colitis. These metabolites are produced by mouse and human gut bacteria, including *Lactobacillus reuteri* and *Clostridium sporogenes*, although to our knowledge, the amounts of these metabolites have not been quantified within humans (16, 25, 46). Our data suggest that a mechanism by which

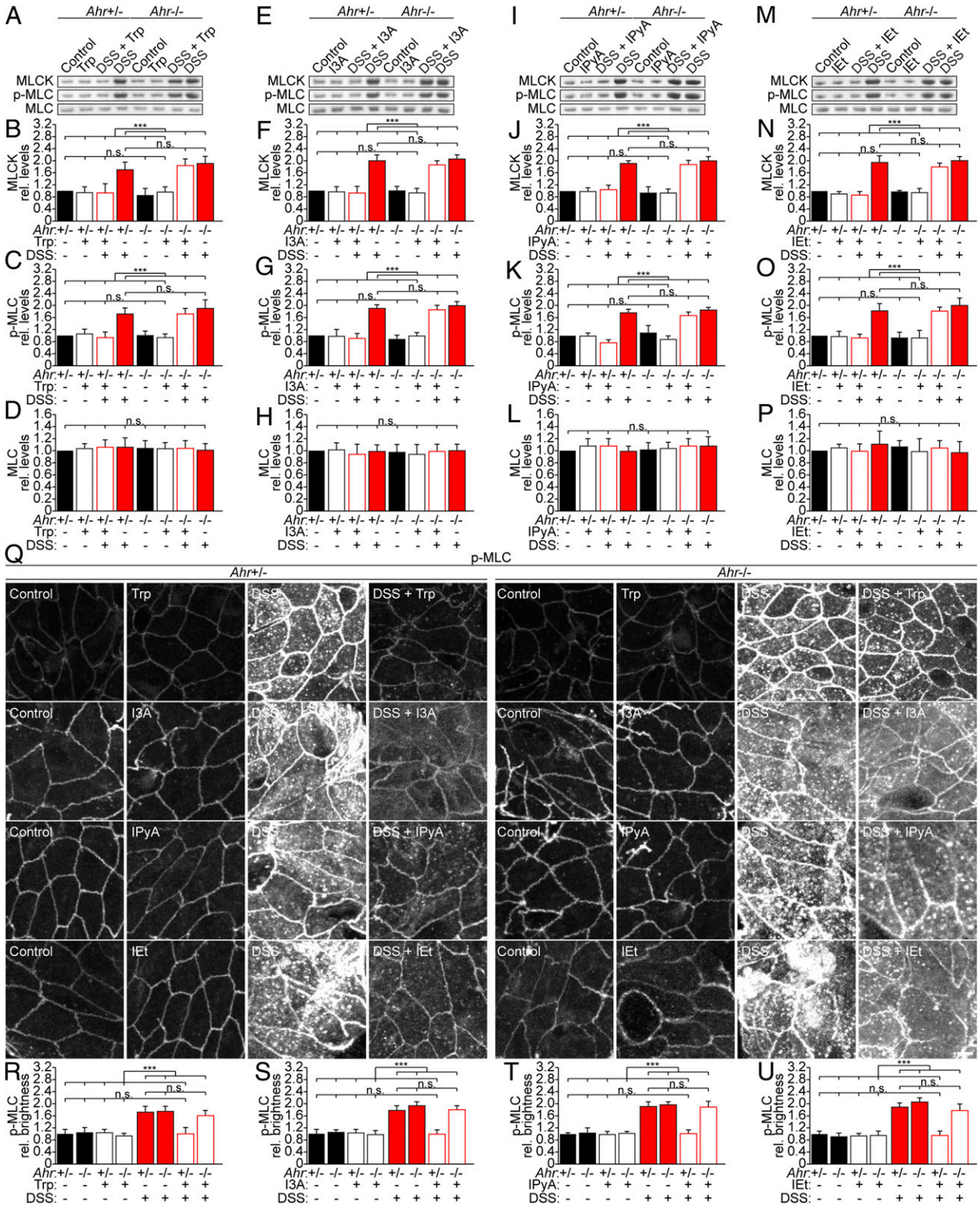


**Fig. 5.** Trp feeding and metabolites prevent myosin IIA activation during mouse model of colitis, which is AhR dependent. (*A–D*, *Q*, and *R*) $Ahr^{+/-}$ or $Ahr^{-/-}$ mice were fed a Trp-rich diet (42 g Trp/kg diet) or standard chow (2 g Trp/kg diet) for 7 d, followed by administration of DSS (%, wt/vol) or vehicle for 7 d (ad libitum) with continued Trp feeding. (*E–Q* and *S–U*) Alternatively, $Ahr^{+/-}$ or $Ahr^{-/-}$ mice were pretreated with I3A (1,000 mg/kg), IPyA (2,900 mg/kg), or IEt (600 mg/kg) for 2 d and then administered DSS (3%, wt/vol) for 7 d (ad libitum) with continued metabolite treatment. Activated myosin levels within intestinal tissue were determined by (*A–P*) Western blotting against MLCK, p-MLC, and myosin light chain (MLC) and quantified by densitometry ($n = 3$) or (*Q–U*) immunofluorescence, followed by confocal microscopy and quantification of relative (rel.) brightness ($n = 15$). (Scale bar: 20 μm.) Data are representative of at least three independent experiments; $n = 5$ mice per group. One-way ANOVA followed by post hoc Tukey's test: $^{***}P < 0.001$, n.s.: not significant.

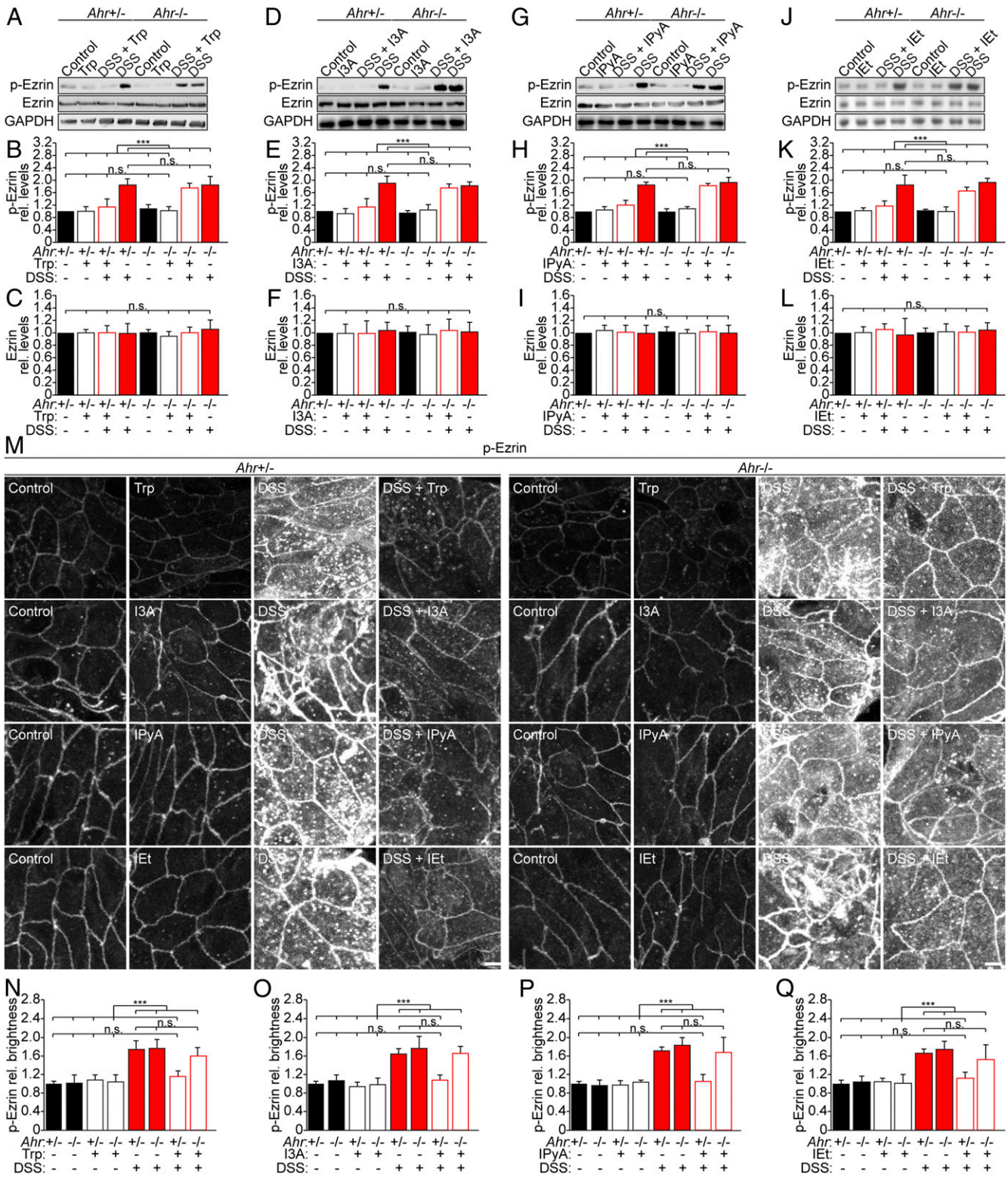


**Fig. 6.** Trp feeding and metabolites prevent ezrin activation in mouse model of colitis, which is AhR dependent. (*A–C*, *M*, and *N*) $Ahr^{+/-}$ or $Ahr^{-/-}$ mice were fed a Trp-rich diet (42 g Trp/kg diet) or standard chow (2 g Trp/kg diet) for 7 d, followed by administration of DSS (3%, wt/vol) or vehicle for 7 d (ad libitum) with continued Trp feeding. (*D–M* and *O–Q*) Alternatively, $Ahr^{+/-}$ or $Ahr^{-/-}$ mice were pretreated with I3A (1,000 mg/kg), IPyA (2,900 mg/kg), or IEt (600 mg/kg) for 2 d and then administered DSS (3%, wt/vol) for 7 d (ad libitum) with continued metabolite treatment. Activated ezrin levels within intestinal tissue were determined by (*A–L*) Western blotting against p-ezrin and quantified by densitometry ($n = 3$) or (*M–Q*) immunofluorescence, followed by confocal microscopy and quantification of relative (rel.) brightness ($n = 15$). (Scale bar: 20 μm.) Data are representative of at least three independent experiments, $n = 5$ mice per group. One-way ANOVA followed by post hoc Tukey's test: $***P < 0.001$, n.s. = not significant.

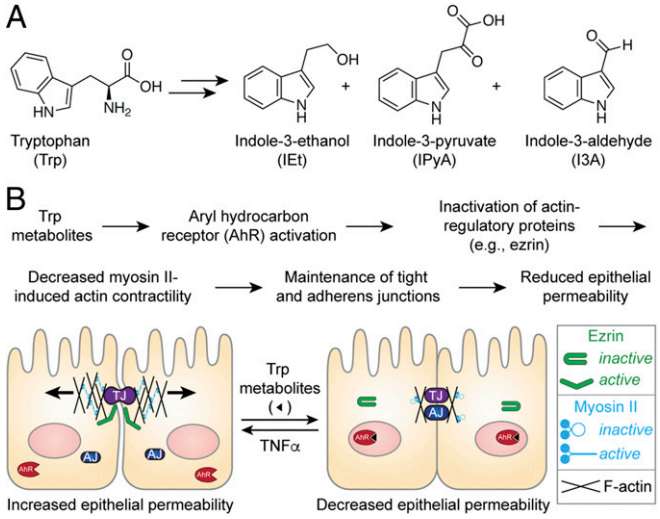


**Fig. 7.** Proposed model of Trp-derived metabolite function in regulating gut epithelial permeability. (*A*) Trp metabolites, indole-3-ethanol, indole-3-pyruvate, and indole-3-aldehyde, produced by the gut microbiota, decrease intestinal permeability caused by proinflammatory cytokines (e.g., TNFα). (*B*) Trp metabolites decrease epithelial permeability through activation of AhR and modulation of TJs and AJs via actin regulatory proteins (e.g., ezrin and nonmuscle myosin II). Cartoon schematic of the model. F-actin: filamentous actin.

these metabolites help protect the host is to increase gut barrier integrity through maintenance of the AJC during challenge with TNFα and DSS, which both cause increased intestinal permeability (Fig. 7). We also showed that treatment of mice with IEt, IPyA, and I3A inhibits TNFR1 expression during DSS colitis and NF-κB activation by TNFα (*SI Appendix*, Fig. S11), which suggests that these metabolites inhibit TJ and AJ disassembly via the TNFα pathway characterized by Ma et al. and Wang et al. (47–49). We further demonstrated that these metabolites increase IL-10R expression in IECs during DSS colitis (*SI Appendix*, Fig. S12), which has been reported to protect against intestinal insults that target the AJC, including proinflammatory cytokines (50, 51). These results differ from previous studies in which Trp metabolites, including IPyA and I3A, were demonstrated to target alternative immune pathways, including induction of Tr1 and ILCs (24, 25), that also protect against colitis through their antiinflammatory activities and barrier protection, respectively. It is likely that these small molecules have multiple physiologically relevant targets in vivo and that their overall effects are synergistic based on interactions with receptors and pathways in multiple host-cell types. Nevertheless, the gut epithelium, the apical side of which directly faces the microbiota-rich lumen, is a highly relevant cell type to investigate, given that it likely experiences the highest concentrations of microbiota-derived metabolites and has major roles in controlling permeability and tissue homeostasis, as well as instruction of several immune cell types (4).

In our study, we identified several Trp metabolites in addition to IEt, IPyA, and I3A that also arise from Trp catabolism by the gut microbiota using mass spectrometry (MS)-based metabolomics (*SI Appendix*, Fig. S7). These include IND, indole-3-acetamide, tryptamine, indole-3-acetic acid, indole-3-lactate, indole-3-acrylate, and IPA. Although these metabolites are also produced in vivo, our data suggest that they likely do not modulate gut epithelial permeability in this context, based on our initial screen demonstrating that they do not change TEER or FITC-dextran flux in the presence of TNFα (*SI Appendix*, Figs. S5 and S6). Nevertheless, it is possible that these additional Trp metabolites may mediate alternative pathways in vivo that contribute to reduced morbidity in the DSS colitis model via immune-mediated pathways as described above. Notably, Mani and coworkers have shown that IPA improves intestinal barrier function in chemically induced colitis within mice; however, this metabolite targets pathways different from the ones we have identified here, including TLR4 and PXR activation (27). An interesting future direction would be to understand if these pathways synergize with metabolite-activated pathways identified in this study to ameliorate DSS colitis in mice. Trp-derived Kyn has been shown to improve DSS colitis by inducing IL-10R expression (26); however, we did not detect significant levels of this metabolite after Trp feeding (*SI Appendix*, Fig. S7). IND has also been shown to improve DSS colitis, most likely by increasing the expression of TJ and AJ proteins (28, 29); however, this metabolite did not prevent increased permeability caused by TNFα as determined by TEER and FITC-dextran flux (*SI Appendix*, Fig. S6 *B* and *L*). Our results may differ from these studies because the levels of certain Trp metabolites produced in vivo can vary due to the composition of gut microbiota. Also, these reports utilized in vitro model systems and murine colitis models different from those included in this study.

A major aspect of our study was the elucidation of the mechanism of action of Trp metabolites in the context of compromised gut barrier integrity during DSS-mediated epithelial damage. We demonstrated that the effects of IEt, IPyA, and I3A depend on AhR, a host receptor the traditional role of which is to recognize and detoxify foreign molecules within the host by up-regulating xenobiotic metabolizing enzymes (52). Recent studies have uncovered major roles for AhR in regulating many different aspects of the host immune response (33, 34). In the context of colitis, certain xenobiotic AhR ligands that are not microbial metabolites, such as 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester and 2,3,7,8-tetrachlorodibenzodioxin, have been shown to decrease inflammation via immunologically mediated pathways, including increased expression of Tregs and production of PGE2 and RegIIIγ within the colon (23, 36, 53); however, these studies did not address the effects of microbially derived AhR ligands in this disease model. Here, we demonstrate that the effects of Trp feeding and its resulting metabolites IEt, IPyA, and I3A in improving morbidity and intestinal permeability in DSS colitis via maintenance of gut-barrier function through the AJC are largely AhR-dependent. Thus, these data implicate AhR as a host receptor directly targeted by the Trp metabolites in this context. It is important to note that our study does not examine direct effects of the Trp-derived metabolites IEt, IPyA, and I3A on distinct immune cell types (e.g., $CD4^+$ T cell subsets) in DSS colitis. Nevertheless, our results suggest that Trp metabolite modulation of the AJC plays a role in strengthening gut epithelial barrier integrity in DSS colitis.

Finally, to understand the molecular mechanisms by which IEt, IPyA, and I3A ultimately attenuate pathological increases in intestinal permeability, we showed that either a Trp-rich diet or individual treatment with each of the three metabolites inhibits activation of ezrin, a key actin regulatory protein that controls and maintains the AJC in endothelial and epithelial cells (Figs. 6 and 7 and *SI Appendix*, Figs. S22–S25) (54, 55). Activation of ezrin is a two-step process involving recruitment of ezrin to the plasma membrane via binding to phosphatidylinositol-4,5-bisphosphate, rendering threonine (Thr) 567 more accessible to phosphorylation (44). This modification (p-Thr567) causes a dramatic conformational change that unmasks ezrin's binding site to F-actin, allowing p-ezrin to tether the cytoskeleton to the plasma membrane (Fig. 7). Ezrin's activation and association with the actin cytoskeleton leads to a mechanical opening of the AJC via the action of MyoIIA motors and, hence, to increased permeability (Fig. 7) (55, 56). Critically, we demonstrated that a Trp-rich diet or supplementation with IEt, IPyA, or I3A inhibits ezrin activation in vitro and in vivo and that a constitutively active form of ezrin could bypass the effects of the Trp metabolites (Fig. 6 and *SI Appendix*, Figs. S22–S25). Furthermore, we determined that

the effect of these metabolites in inhibiting ezrin is AhR-dependent in vivo (Fig. 6), suggesting that activation of AhR by the metabolites occurs upstream of effects on ezrin, MyoIIA, and AJC integrity (Fig. 7). Interestingly, a previous study demonstrated that ezrin protein levels are dependent on AhR in the retina (57), suggesting that the mechanism elucidated here may be generalizable to other tissues and physiological or pathological contexts.

In conclusion, we have identified and extensively characterized roles for several Trp metabolites—IEt, IPyA, and I3A—that are derived from the gut microbiota and that improve intestinal epithelial barrier function during challenges with inflammatory stimuli, including proinflammatory cytokines and DSS colitis. We have demonstrated that the effects of these metabolites are largely dependent on AhR in vivo, which suggests that the host possesses receptors that are capable of recognizing these small-molecule metabolites that act as signaling agents from the gut microbiota. We have also demonstrated that these Trp metabolites inhibit activation of actin regulatory proteins that control intestinal epithelial permeability by maintaining the AJC, including MyoIIA and ezrin. Critically, we found that inhibition of MyoIIA and ezrin activity by these metabolites is dependent upon AhR, supporting a model for the mechanism of action of these metabolites that takes into account a potential upstream receptor and downstream proteins that directly mediate effects on intestinal permeability. Thus, our study identifies how a class of gut microbial metabolites abundant in protein-rich diets can modulate host defense mechanisms such as the intestinal barrier by targeting specific host receptors and pathways. Interesting future directions include understanding the effects of these metabolites on alternative host pathways that include different immune cell types. We envision that the metabolites and host pathways that they affect may inspire or serve as potential targets for the development of prophylactic and preventative measures that could greatly improve patient outcomes in IBDs such as ulcerative colitis.

## Materials and Methods

For details, see *SI Appendix*.

**DSS Colitis and Histological Scoring.** Following treatment of mice with the appropriate combinations of antibiotics, DSS, and Trp-rich diet or metabolite, colon sections were prepared and analyzed in a blinded manner. The samples were given a score of 0 to 4 (where 0 = none; 1 = mild; 2 = moderate; 3 = severe; 4 = very severe) for epithelial injury, mononuclear infiltrate, and polymorphonuclear infiltrate.

**Data Availability.** Data are available in this paper and *SI Appendix*.

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