CELIAC DISEASE

Aryl hydrocarbon receptor ligand production by the gut microbiota is decreased in celiac disease leading to intestinal inflammation

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Metabolism of tryptophan by the gut microbiota into derivatives that activate the aryl hydrocarbon receptor (AhR) contributes to intestinal homeostasis. Many chronic inflammatory conditions, including celiac disease involving a loss of tolerance to dietary gluten, are influenced by cues from the gut microbiota. We investigated whether AhR ligand production by the gut microbiota could influence gluten immunopathology in nonobese diabetic (NOD) mice expressing DQ8, a celiac disease susceptibility gene. NOD/DQ8 mice, exposed or not exposed to gluten, were subjected to three interventions directed at enhancing AhR pathway activation. These included a high-tryptophan diet, gavage with *Lactobacillus reuteri* that produces AhR ligands or treatment with an AhR agonist. We investigated intestinal permeability, gut microbiota composition determined by 16*S* rRNA gene sequencing, AhR pathway activation in intestinal contents, and small intestinal pathology and inflammatory markers. In NOD/DQ8 mice, a high-tryptophan diet modulated gut microbiota composition and enhanced AhR ligand production. AhR pathway activation by an enriched tryptophan diet, treatment with the AhR ligand producer *L. reuteri*, or pharmacological stimulation using 6-formylindolo (3,2-b) carbazole (Ficz) decreased immunopathology in NOD/DQ8 mice exposed to gluten. We then determined AhR ligand production by the fecal microbiota and AhR activation in patients with active celiac disease compared to nonceliac control individuals. Patients with active celiac disease demonstrated reduced AhR ligand production and lower intestinal AhR pathway activation. These results highlight gut microbiota-dependent modulation of the AhR pathway in celiac disease and suggest a new therapeutic strategy for treating this disorder.

INTRODUCTION

Celiac disease is a chronic inflammatory enteropathy that develops as a consequence of breakdown of oral tolerance to gluten in people who carry HLA-DQ2 or HLA-DQ8 genes (*1*). Although 40% of the worldwide population expresses one or more celiac disease susceptibility genes, only 1% will develop this disorder (*2*). It is currently accepted that additional environmental factors are needed to trigger the inflammatory response that culminates in villous atrophy, and these may include gut microbial factors and nongluten proteins (*3*, *4*).

The gut microbiota fosters intestinal homeostasis by modulating host immunity and physiology (*5*–*7*). Disruption of gut microbiota composition and function, which are influenced by a variety of factors including lifestyle choices and environment, has been suggested as a key contributor to the worldwide epidemic of chronic illnesses, including celiac disease (*2*, *3*, *7*, *8*). Recent studies in mice have demonstrated mechanisms through which bacterial opportunistic pathogens from patients with celiac disease and enteric viral infections directly or indirectly influence host immune responses to gluten (*4*, *9*, *10*). However, only some patients with celiac disease have a clinical history of enteric infections, suggesting that other mechanisms linking microbial cues and immunological pathways may be relevant.

By-products of microbial metabolism in the gut, such as those produced through metabolism of tryptophan from dietary sources, are emerging as key players in host-gut microbiota cross-talk (*11*–*14*). In the gastrointestinal tract, tryptophan can be metabolized by the gut microbiota into ligands of the aryl hydrocarbon receptor (AhR) (*14*–*17*), by host cells into kynurenine via indoleamine 2,3-dioxygenase 1 (IDO1) (*13*, *18*) or into 5-hydroxytryptamine via tryptophan hydroxylase 1 (*13*, *19*). AhR is a ligand-activated nuclear transcription factor widely expressed in the body and retained as an inactive complex in the cytoplasm. After ligand binding, AhR translocates to the nucleus and regulates the expression of target genes like *Cyp1a1*, *Il22*, and *Il17* (*14*, *15*). In this manner, AhR signaling orchestrates immune responses at mucosal barrier sites such as the gut mucosa, modulating intraepithelial lymphocytes, T cells, and group 3 innate lymphoid cells that produce interleukin-17 (IL-17) and IL-22 (*14*), all of which may affect celiac disease pathogenesis. A recent study showed that AhR expression is down-regulated in the gut mucosa of patients with active celiac disease; however, the relationship of celiac disease to the gut microbiota and mechanisms of AhR regulation

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remain unknown (*20*). Differences in composition and metabolic function of the gut microbiota between patients with celiac disease and healthy controls have been described, including decreases in core bacterial groups known to metabolize tryptophan to AhR ligands such as *Lactobacillus* (*21*–*23*). This prompted us to investigate the general hypothesis that the gut microbiota in active celiac disease has impaired capacity to stimulate the AhR pathway.

In this study, we demonstrate that an enriched tryptophan diet modulated the intestinal microbiota of nonobese diabetic (NOD) mice expressing the DQ8 celiac disease susceptibility gene (NOD/DQ8). Gut microbiota modulation led to increased AhR ligand production and AhR pathway activation, which reduced immunopathology after exposure to gluten in NOD/DQ8 mice. Reduced immunopathology was also observed after giving NOD/DQ8 mice exposed to gluten either *Lactobacillus reuteri* with a high capacity to produce AhR ligands (*24*, *25*) or an AhR agonist. Last, patients with active celiac disease exhibited a reduction in AhR ligands in stool and a decreased capacity of the gut microbiota to activate AhR compared to nonceliac control individuals.

RESULTS

Enriched tryptophan diet modulates the gut microbiota to produce AhR ligands

We first investigated the capacity of an enriched tryptophan diet to shape the gut microbiota after 3 weeks of dietary intervention in NOD/DQ8 mice before gluten treatment. Mice were fed a diet containing either low or high tryptophan (Fig. 1A) for 3 weeks, and fecal microbiota composition was analyzed. The principal coordinate analysis revealed a difference in fecal microbiota profiles between mice fed a low- and enriched tryptophan diet ($P = 0.001$; Fig. 1B), but no difference in alpha diversity was observed (fig. S1A). Mice fed the enriched tryptophan diet had a lower relative abundance of bacteria belonging to the Proteobacteria phylum such as *Bilophila* ($P = 0.002$), *Desulfovibrio* ($P = 0.004$), and Enterobacteriaceae ($P = 0.0007$), and higher abundance in bacteria belonging to Firmicutes phylum such as *Lactobacillus* ($P = 0.002$), *Aerococcus* ($P = 0.002$), *Facklamia* ($P = 0.0003$), *Jeotgalicoccus* ($P = 0.0009$), and *Staphylococcus* ($P < 0.0001$; Fig. 1, C and D, and fig. S1B). These data suggested that a high-tryptophan diet in gluten-naïve NOD/DQ8 mice favored the growth of bacteria considered beneficial, such as *Lactobacillus*, that metabolize tryptophan into AhR ligands (*15*, *17*, *24*) at the expense of potentially proinflammatory bacteria belonging to Proteobacteria (*26*, *27*). The tryptophan concentration in feces was higher in mice fed the enriched tryptophan diet ($P = 0.05$; Fig. 1E). This was associated with increased concentrations of AhR agonists including tryptamine ($P = 0.006$), indole-3-aldehyde ($P = 0.009$), and indole-3-lactic acid ($P = 0.01$) (Fig. 1E and fig. S1, C to E). A higher concentration of tryptophan and AhR agonists was also observed in the serum of mice fed the enriched tryptophan diet (fig. S1, F to I). In contrast, the concentration of kynurenine, a tryptophan metabolite produced mainly by the IDO1 enzyme in host cells and implicated in chronic inflammation (*28*), was higher in feces of mice fed the low-tryptophan diet ($P = 0.02$; Fig. 1F). Last, the IDO activity, determined by the kynurenine-to-tryptophan ratio, was higher in mice fed the low-tryptophan diet ($P = 0.05$; Fig. 1F). Thus, the metabolomic profile indicated that an enriched tryptophan diet may shape the gut microbiota to produce AhR ligands and potentially decrease intestinal IDO1 activity.

To investigate the functional relevance of these findings, we determined the capacity of the gut microbiota to activate AhR. Fecal and small intestinal contents from mice fed the enriched tryptophan diet induced greater activation of AhR than did contents from mice fed the low-tryptophan diet (Fig. 1G). Expression of *Cyp1a1*, an AhR target gene (*14*), was higher in the duodenum of mice fed the enriched tryptophan diet ($P = 0.006$; Fig. 1H). Fecal lipocalin-2, duodenal *Il6* expression, intraepithelial lymphocyte counts, ion transport, and paracellular permeability were lower in mice fed the enriched tryptophan diet, suggesting an anti-inflammatory effect of the diet (Fig. 1, I to L). The production of AhR agonists by the gut microbiota of gluten-naïve NOD/DQ8 mice fed a high-tryptophan diet activated AhR.

A high-tryptophan diet sustains gut microbial AhR activation in gluten-treated mice

After 3 weeks of tryptophan dietary intervention, NOD/DQ8 mice were sensitized to gliadin once a week for 3 weeks and then challenged with gluten three times a week for 3 weeks before sacrifice at day 59 (Fig. 2A). During gluten sensitization and challenge, mice continued to receive a diet containing either low or enriched tryptophan (Fig. 2A). Gluten treatment of NOD/DQ8 mice, regardless of dietary intervention, shifted fecal microbiota composition ($P = 0.001$; fig. S2A) and alpha diversity (fig. S2, B and C). There was an increased abundance of bacteria from the Firmicutes phylum and lower abundance of bacteria belonging to the Bacteroidetes phylum (fig. S2, D to G). This was accompanied by higher AhR agonist production in mice fed the enriched tryptophan diet ($P = 0.04$; fig. S3). Compared with the low-tryptophan diet, the enriched tryptophan diet did not affect alpha diversity (fig. S4A), but principal coordinate analysis revealed different clustering between the low- and the enriched tryptophan diet groups ($P = 0.001$; Fig. 2B) following gluten treatment. Differential analysis confirmed this effect with a relative increase in bacteria belonging to the Bacteroidetes and Firmicutes phyla at the expense of bacteria from the Proteobacteria and Verrucomicrobia phyla (Fig. 2, C and D). At the species level, abundance of *Bilophila*, *Desulfovibrio*, *Suturella*, and *Erwinia*, from the Proteobacteria phylum, was lower in the mice fed the enriched tryptophan diet (Fig. 2C). Among Firmicutes, *Ruminococcus gnavus* ($P = 0.006$; Fig. 2C), known to produce AhR ligands (*17*, *29*), was increased in mice treated with the enriched tryptophan diet. Accordingly, tryptophan and AhR agonist concentrations in feces and serum were higher in mice fed an enriched tryptophan diet (Fig. 2E and fig. S4, B and C). We also observed a decrease in kynurenine concentration and IDO activity in feces of mice fed an enriched tryptophan diet compared to mice fed a low-tryptophan diet (Fig. 2F). Together, these results suggest that exposure to gluten was a major driver of gut microbiota shifts that were independent of tryptophan content in the diet. However, the enriched tryptophan diet maintained the functional capacity to produce AhR ligands and promoted a gut microbiota structural profile that was associated with beneficial bacteria capable of AhR ligand production. The capacity of small intestine and colon contents to activate AhR was higher in mice fed a tryptophan-enriched diet compared with mice fed a low-tryptophan diet (Fig. 2G). This finding was paralleled by higher duodenal expression of *Ahr* and AhR target genes, such as *Cyp1a1* and *Il22* (Fig. 2H). The enriched tryptophan diet was also associated with higher villus-to-crypt ratios, lower intraepithelial lymphocyte counts, and lower intestinal paracellular permeability, fecal lipocalin-2, and

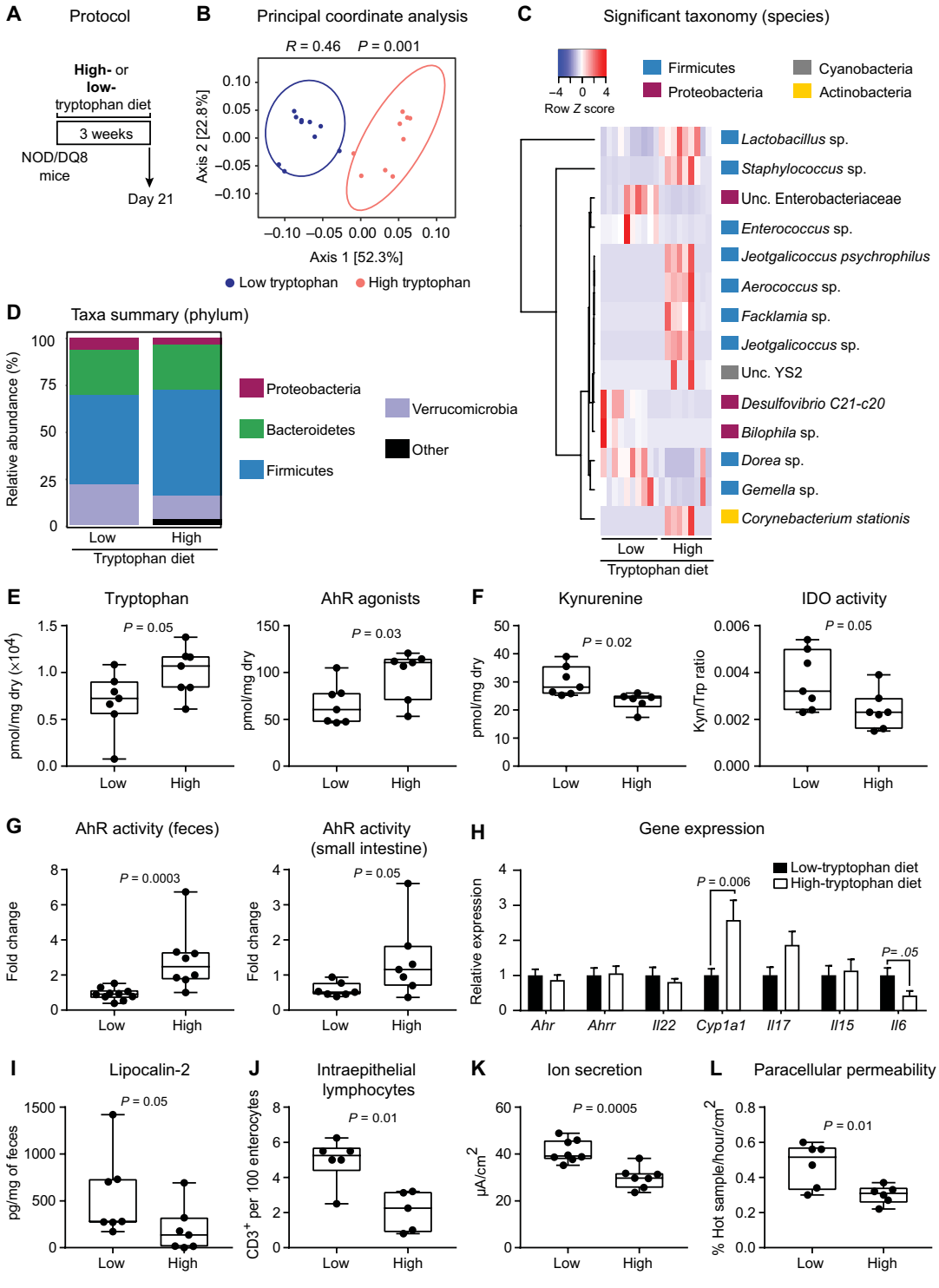

Fig. 1. Dietary tryptophan alters the gut microbiota composition of naïve NOD/DQ8 mice and activates the AhR pathway. (**A**) The protocol for testing the effects of a 3-week dietary tryptophan supplement in NOD/DQ8 mice is presented. (**B**) Shown is principal coordinate analysis for bacterial *16S rRNA* gene sequence abundance (based on UniFrac distance matrices) in the feces of NOD/DQ8 mice fed either a low-tryptophan ($n = 10$) or high-tryptophan ($n = 9$) diet. (**C**) Shown is a heat map of the significantly different fecal microbial species for NOD/DQ8 mice fed a low-tryptophan ($n = 10$) or high-tryptophan ($n = 9$) diet, based on total relative abundance (Unc, unclassified). (**D**) Shown is fecal relative abundance, at the phylum level, for NOD/DQ8 mice fed a low-tryptophan ($n = 10$) or high-tryptophan ($n = 9$) diet. (**E**) Shown is quantification of tryptophan and total AhR agonists (tryptamine, indole-3-aldehyde, and indole-3-lactic acid) in feces of NOD/DQ8 mice fed a low-tryptophan ($n = 7$) or high-tryptophan ($n = 7$) diet. (**F**) Shown is quantification of kynurenine and IDO activity (calculated as the kynurenine-to-tryptophan ratio) in feces of NOD/DQ8 mice fed a low-tryptophan ($n = 7$) or high-tryptophan ($n = 6$ to 7) diet. (**G**) Shown is AhR activity in the feces and small intestine contents of NOD/DQ8 mice fed a low-tryptophan ($n = 7$ to 10) or high-tryptophan ($n = 7$ to 8) diet. (**H**) Shown is expression of genes in the AhR pathway in the proximal small intestine of NOD/DQ8 mice fed a low-tryptophan ($n = 6$ to 7) or high-tryptophan ($n = 5$ to 7) diet. (**I**) Fecal lipocalin-2 was quantified for NOD/DQ8 mice fed a low-tryptophan ($n = 7$) or high-tryptophan ($n = 7$) diet. (**J**) $CD3^+$ intraepithelial lymphocytes were counted ($CD3^+$ per 100 enterocytes) in the small intestine of NOD/DQ8 mice fed a low-tryptophan ($n = 6$) or high-tryptophan ($n = 5$) diet. (**K** and **L**) Shown is small intestinal barrier function assessed by (K) ion secretion (microamperes per square centimeter) and (L) paracellular permeability to ^{51}Cr-EDTA (% hot sample per hour per square centimeter) in NOD/DQ8 mice fed a low-tryptophan ($n = 6$ to 8) or high-tryptophan ($n = 6$ to 7) diet. Each dot represents an individual mouse. Data from one independent experiment are presented as median with interquartile range and whiskers extending from minimum to maximum or as mean ± SEM. Differences in microbial composition were calculated by PERMANOVA using unweighted UniFrac distance. Significant differences between taxa were evaluated by two-tailed Student's *t* test or Wilcoxon test, and multiple testing was corrected via false discovery rate (FDR) estimation. Statistical significance for other parameters was determined by Student's two-tailed *t* test (E, tryptophan; H, *Il6*; and F, K, and L), or Mann-Whitney test (E, AhR agonists; H, *Cyp1a1*; and G, I, and J).

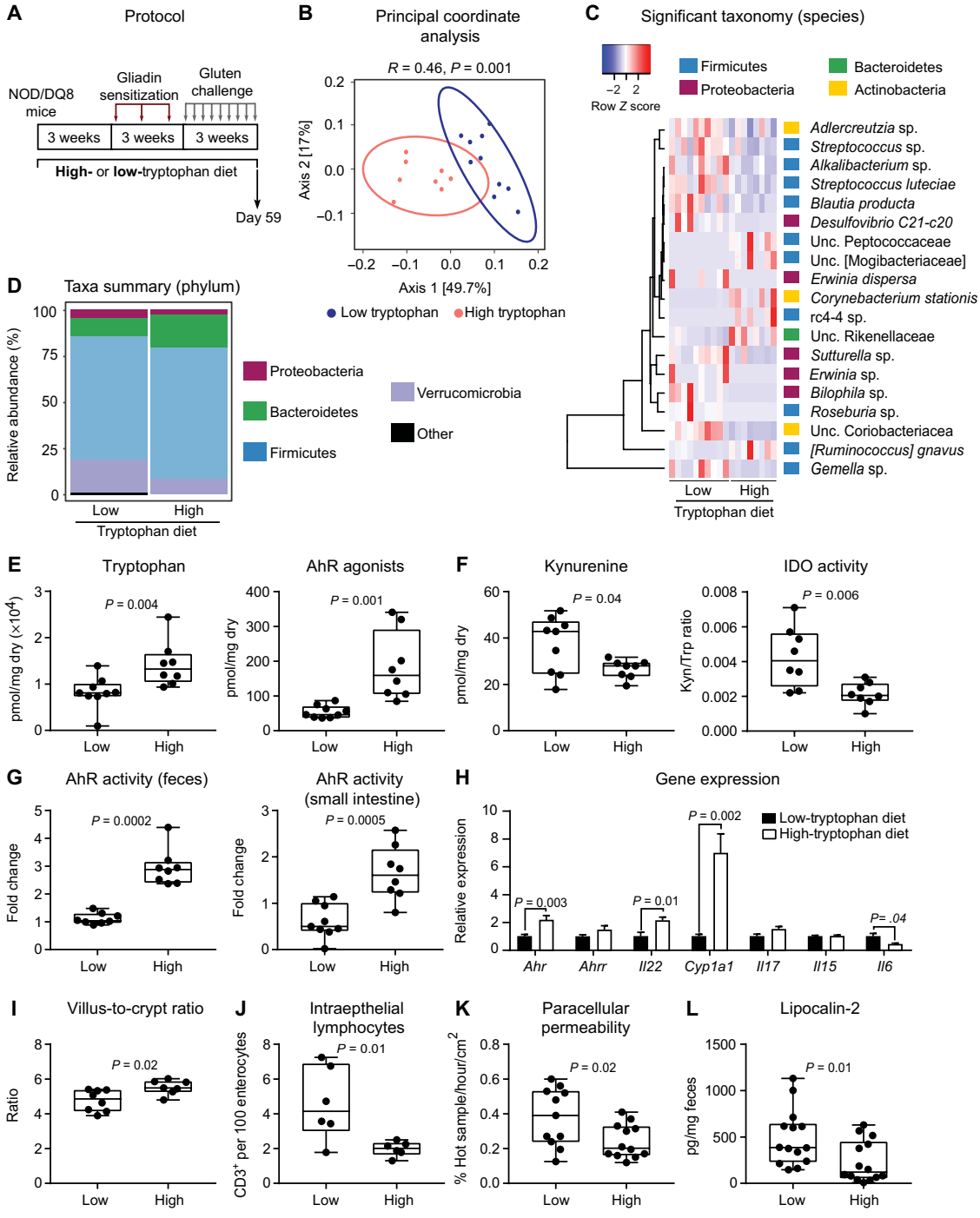

Fig. 2. Dietary tryptophan sustains AhR activation by the gut microbiota of gluten-treated NOD/DQ8 mice. (**A**) Gliadin sensitization scheme for testing the effects of tryptophan supplementation in gluten-treated NOD/DQ8 mice is presented. (**B**) Shown is principal coordinate analysis based on bacterial *16S rRNA* gene sequence abundance in the feces of gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 10$) or high-tryptophan ($n = 8$) diet, using UniFrac distance matrices. (**C**) Shown is a heat map of the significantly different fecal microbial species between gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 10$) or high-tryptophan ($n = 8$) diet, based on the total relative abundance. (**D**) Shown is the relative fecal microbial abundance at the phylum level for gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 8$) or high-tryptophan ($n = 8$) diet. (**E**) Tryptophan and total AhR agonists (tryptamine, indole-3-aldehyde, and indole-3-lactic acid) were quantified in feces of gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 9$) or high-tryptophan ($n = 8$) diet. (**F**) Kynurenine and IDO activity (calculated as kynurenine-to-tryptophan ratio) were quantified in feces of gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 8$ to 9) or high-tryptophan ($n = 8$) diet. (**G**) AhR activity in feces and small intestine contents of gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 8$ to 9) or high-tryptophan ($n = 8$) diet is shown. (**H**) Shown is expression of genes in the AhR pathway in the proximal small intestine of gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 5$ to 9) or high-tryptophan ($n = 6$ to 8) diet. (**I**) Shown are villus-to-crypt ratios in gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 8$) or high-tryptophan ($n = 7$) diet. (**J**) $CD3^+$ intraepithelial lymphocytes ($CD3^+$ per 100 enterocytes) were counted in the small intestine of gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 6$) or high-tryptophan ($n = 6$) diet. (**K**) Small intestinal paracellular permeability was measured using ^{51}Cr-EDTA (% hot sample per hour per square centimeter) in gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 11$) or high-tryptophan ($n = 12$) diet. (**L**) Fecal lipocalin-2 was quantified in gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 14$) or high-tryptophan ($n = 14$) diet. Each dot represents an individual mouse. Data from one independent experiment are presented as median with interquartile range and whiskers extending from minimum to maximum or as mean ± SEM. Differences in microbial composition were calculated by PERMANOVA using unweighted UniFrac distance. Significant differences between taxa were evaluated by two-tailed Student's *t* test or Wilcoxon test, and multiple testing was corrected via FDR estimation. Statistical significance in other parameters was determined by Student's two-tailed *t* test (E, tryptophan; G, small intestine; and F and H to K) or Mann-Whitney test (E, AhR agonists; G, feces; and L).

duodenal *Il6* expression, but with no difference in ion secretion (Fig. 2, I to L, and fig. S4D). Collectively, these results suggest that an enriched tryptophan diet improved gluten immunopathology in NOD/DQ8 mice by inducing and maintaining an intestinal microbiota that was able to produce AhR agonists.

Activation of AhR reduces gluten immunopathology in mice

To investigate gut microbial modulation of AhR activity in the NOD/DQ8 mouse model, two previously isolated *L. reuteri* strains that naturally exhibited high AhR ligand production (*24*, *25*) (fig. S4E) were administered to gluten-treated NOD/DQ8 mice fed either a low- or enriched tryptophan diet (Fig. 3A). *L. reuteri* supplementation increased the capacity of the small intestinal microbiota to activate AhR in mice fed both diets, but the increase was only statistically significant in mice fed the enriched tryptophan diet ($P < 0.0001$; Fig. 3B). *L. reuteri* supplementation decreased intraepithelial lymphocyte counts in mice fed a low-tryptophan diet compared to mice treated with culture medium (vehicle control; $P = 0.03$; Fig. 3C). *L. reuteri* also improved villus-to-crypt ratios in mice fed a low-tryptophan diet ($P = 0.02$; Fig. 3D). Together, these results suggest that even in the context of a low-tryptophan diet, supplementation with *L. reuteri* was sufficient to produce AhR ligands that then modulated gluten immunopathology.

Because *L. reuteri* may have anti-inflammatory effects independent of AhR ligands, we further investigated the therapeutic potential of intestinal AhR activation by treatment with an AhR agonist, 6-formylindolo (3,2-b) carbazole (Ficz), compared to vehicle control (Fig. 4A). After gluten treatment, NOD/DQ8 mice developed higher intraepithelial lymphocyte counts, a lower villus-to-crypt ratio, increased *Il15* expression, and mucosal barrier dysfunction (Fig. 4, B to G) compared to mice not exposed to gluten. Compared with basal conditions (day 0), gluten-treated mice had higher intestinal expression of *Ahr* and AhR target genes such as *Cyp1a1*, *Il22*, and *Il17*, suggesting an overall state of immune activation (Fig. 4D). However, the degree of increase in *Ahr* and AhR target gene expression in gluten-treated NOD/DQ8 mice remained below that observed in control C67Bl/6 mice fed a normal gluten-containing diet, particularly for *Ahr* and *Il22* expression (fig. S5). Moreover, higher expression of these AhR target genes was observed in NOD/DQ8 mice treated with Ficz compared with mice receiving vehicle, confirming activation of AhR by the agonist (Fig. 4D). Ficz did not affect intestinal *Il15* expression (Fig. 4D), but it improved intraepithelial lymphocyte counts, villus-to-crypt ratios, paracellular permeability, ion transport, and other markers of overall gut inflammation (Fig. 4, B to G). Together, these results demonstrate that pharmacological modulation of the AhR pathway could ameliorate gluten immunopathology in NOD/DQ8 mice challenged with gluten.

Impaired AhR activation and gut microbiota metabolites in patients with celiac disease

To study the relevance of gut microbiota–derived AhR ligands in celiac disease pathogenesis, we analyzed the concentrations of specific gut microbiota metabolites known to activate AhR in stool from patients with active celiac disease, patients with celiac disease on a gluten-free diet for 2 years, and nonceliac control individuals. Patients with active celiac disease had lower fecal concentrations of AhR agonists including tryptamine, indole-3-aldehyde, and indole-3-lactic acid ($P = 0.02$; Fig. 5A and fig. S6, A to C). A higher fecal concentration of tryptophan was also observed in nonceliac controls compared to patients with active celiac disease ($P = 0.02$; Fig. 5B). In contrast, xanthurenic acid and kynurenic acid, products of tryptophan metabolism through the kynurenine pathway, were higher in stool of patients with celiac disease compared with nonceliac controls ($P = 0.004$, Fig. 5C and fig. S6, D and E). In patients with celiac disease treated with a gluten-free diet, no difference in fecal concentrations of the AhR agonists tryptamine, indole-3-aldehyde, and indole-3-lactic acid were observed compared with patients with active celiac disease (Fig. 5A and fig. S6, A to C). However, patients with celiac disease treated with a gluten-free diet had higher concentrations of indole-3-acetic acid, known to be an AhR agonist ($P = 0.04$; Fig. 5D) (*13*–*15*, *24*).

To investigate the functional relevance of these findings, we studied the capacity of the fecal microbiota to activate AhR in patients with active celiac disease. Consistent with the impaired production of AhR ligands, fecal samples

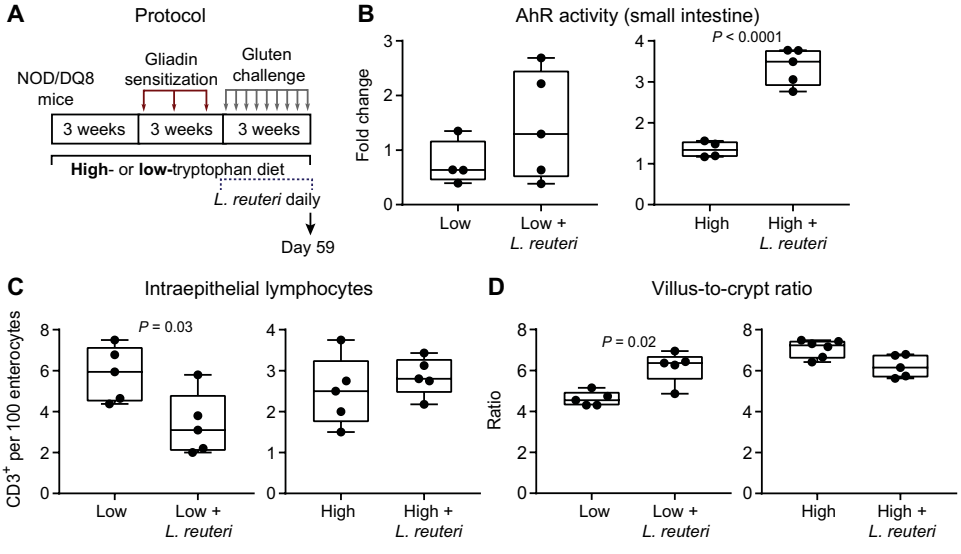

Fig. 3. *L. reuteri* treatment increases AhR activation and attenuates gluten immunopathology in NOD/DQ8 mice. (**A**) Shown is the protocol for *L. reuteri* treatment of NOD/DQ8 mice fed a high- or low-tryptophan diet and exposed to gluten after sensitization with gliadin. (**B**) Shown is AhR activity in the small intestinal contents of gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 4$ to 5 per group) or high-tryptophan ($n = 4$ to 5 per group) diet after *L. reuteri* treatment. (**C**) $CD3^+$ intraepithelial lymphocytes ($CD3^+$ per 100 enterocytes) were counted in the small intestine of gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 5$ per group) or high-tryptophan ($n = 5$ per group) diet after *L. reuteri* treatment. (**D**) Shown are villus-to-crypt ratios in gluten-treated NOD/DQ8 mice fed a low-tryptophan ($n = 5$ per group) or high-tryptophan ($n = 5$ to 6 per group) diet after *L. reuteri* treatment. Each dot represents an individual mouse. Data from one representative experiment of two independent experiments are presented as median with interquartile range and whiskers extending from minimum to maximum. Statistical significance was determined by Student's two-tailed *t* test (B and C) or Mann-Whitney test (D).

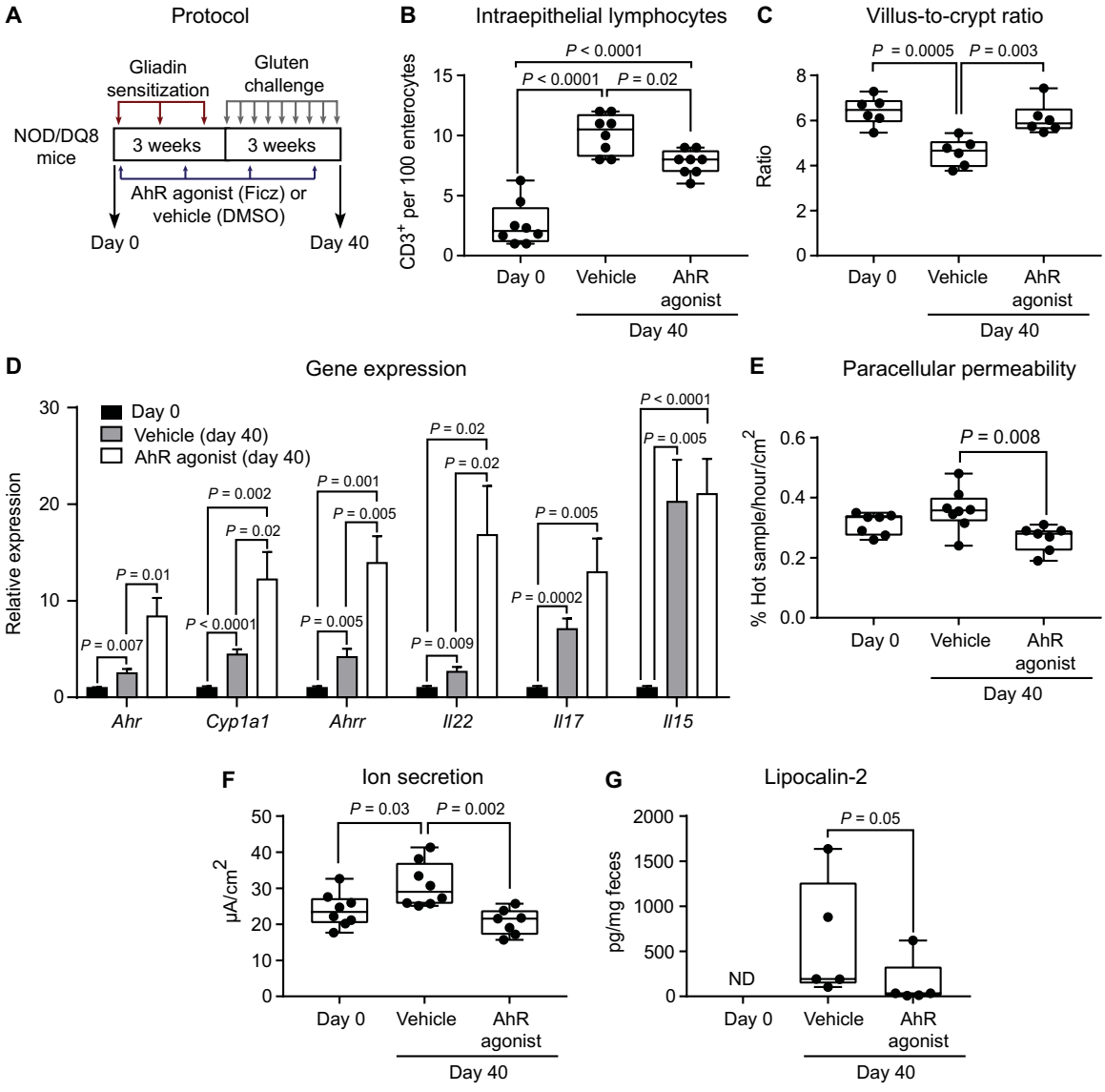

Fig. 4. Treatment with an AhR agonist reduces gluten immunopathology in NOD/DQ8 mice. (**A**) Shown is the protocol for pharmacological treatment with the AhR agonist Ficz [6-formylindolo (3,2-b) carbazole] of gliadin-sensitized and gluten-challenged (gluten-exposed) NOD/DQ8 mice. DMSO, dimethyl sulfoxide. (**B**) $CD3^+$ intraepithelial lymphocytes ($CD3^+$ per 100 enterocytes) were counted in the small intestine of naïve NOD/DQ8 mice at day 0 ($n = 8$) and of gluten-exposed NOD/DQ8 mice after treatment with Ficz ($n = 8$) or vehicle control ($n = 8$) at day 40. (**C**) Villus-to-crypt ratios were measured in the small intestine of naïve NOD/DQ8 mice at day 0 ($n = 6$) and of gluten-exposed NOD/DQ8 mice after treatment with Ficz ($n = 6$) or vehicle control ($n = 6$) at day 40. (**D**) Shown is expression of AhR pathway genes in the proximal small intestine of naïve NOD/DQ8 mice at day 0 ($n = 6$ to 8) and of gluten-exposed NOD/DQ8 mice after treatment with Ficz ($n = 7$ to 8) or vehicle control ($n = 7$ to 8) at day 40. (**E** and **F**) Small intestinal barrier function was assessed at day 40 by (E) paracellular permeability to ^{51}Cr-EDTA flux (% hot sample per hour per square centimeter) and (F) ion secretion (microamperes per square centimeter) in gluten-exposed NOD/DQ8 mice receiving Ficz treatment ($n = 7$) or vehicle control ($n = 8$). Small intestinal barrier function was compared to that for naïve mice at day 0 ($n = 7$ to 8). (**G**) Fecal lipocalin-2 was quantified in naïve NOD/DQ8 mice at day 0 and in gluten-exposed NOD/DQ8 mice receiving Ficz treatment ($n = 5$) or vehicle control ($n = 5$) at day 40 (ND, not detected). Each dot represents an individual mouse. Data from one independent experiment are presented as median with interquartile range and whiskers extending from minimum to maximum or as mean ± SEM. Statistical significance was determined by one-way ANOVA with Tukey post hoc test (B, C, and F), Kruskal-Wallis followed by a Dunn's post hoc test (E), Student's two-tailed *t* test (D), or Mann-Whitney test (G).

from patients with active celiac disease had a lower capacity to activate AhR ($P = 0.02$; Fig. 5E). The capacity of the fecal microbiota to activate AhR was rescued in patients with celiac disease treated with a gluten-free diet ($P = 0.04$; Fig. 5E). Duodenal expression of *Ahr* and AhR pathway genes such as *Cyp1a1*, *Il22*, and *Il17* was lower, whereas that of *Il15*, a key cytokine in celiac disease, was higher in patients with active celiac disease compared with nonceliac controls (Fig. 5F). Consistent with the increased production of the AhR ligand indole-3-acetic acid by the gut microbiota of patients with celiac disease fed a gluten-free diet, expression of AhR pathway genes, such as *Cyp1a1* and *Il22*, was also increased.

DISCUSSION

The capacity of the gut microbiota to produce tryptophan-based AhR ligands influences the balance between intestinal homeostasis

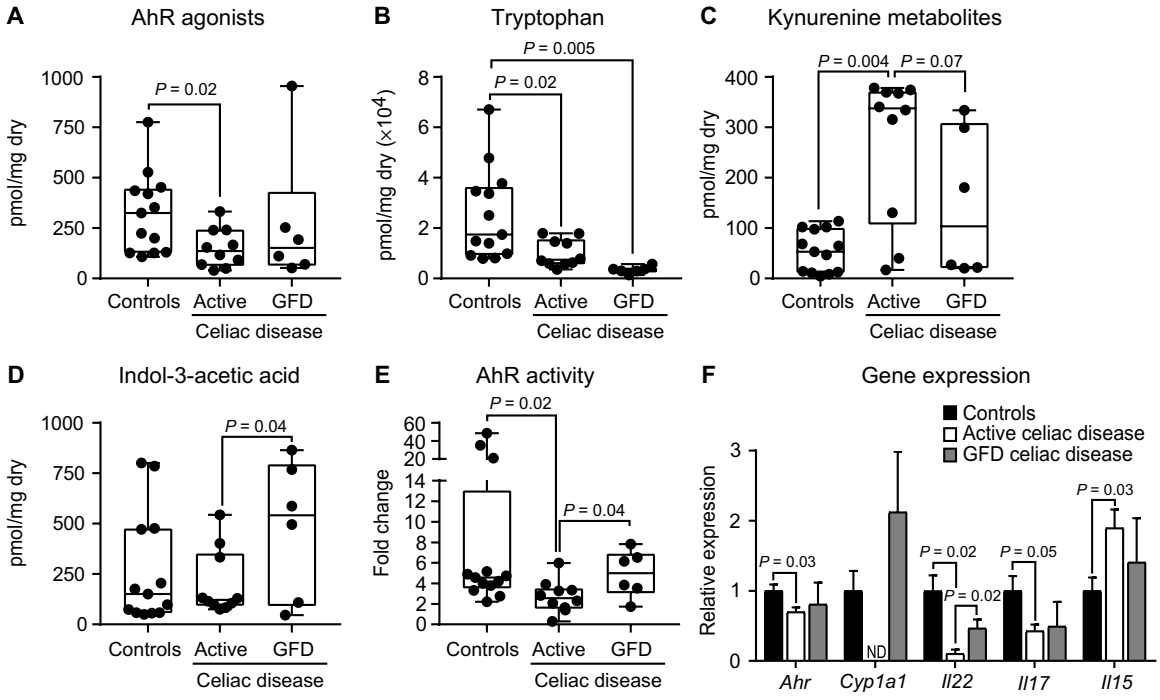

Fig. 5. Patients with celiac disease show decreased AhR activation and AhR ligand concentrations in stool compared to nonceliac controls. (**A** to **D**) Shown are fecal concentrations of total AhR agonists (tryptamine, indole-3-aldehyde, and indole-3-lactic acid) (A), tryptophan (B), total kynurenine metabolites (xanthurenic and kynurenic acid) (C), and indole-3-acetic acid (D) in stool from patients with active celiac disease ($n = 10$), patients with celiac disease on a gluten-free diet (GFD; $n = 6$), or controls without celiac disease ($n = 13$). (**E**) Shown is AhR activity in fecal samples from patients with active celiac disease ($n = 10$), patients with celiac disease on a gluten-free diet ($n = 6$), or controls without celiac disease ($n = 13$). (**F**) Shown is expression of AhR pathway genes in duodenal biopsies from patients with active celiac disease ($n = 4$ to 6), patients with celiac disease on a gluten-free diet ($n = 3$ to 5), or controls without celiac disease ($n = 5$). Each dot represents an individual human participant. Data are presented as median with interquartile range and whiskers extending from minimum to maximum or mean ± SEM. Statistical significance for AhR agonists and indole-3-acetic acid was determined by Mann-Whitney test or Student's two-tailed *t* test, respectively. Statistical significance for other parameters was determined by one-way ANOVA with Tukey post hoc test or Kruskal-Wallis test, followed by a Dunn's post hoc test or Mann-Whitney test.

(*13*, *14*, *17*, *30*) and chronic inflammation, notably through impairment of IL-22–mediated anti-inflammatory mechanisms (*24*, *25*). Shifts in intestinal microbial composition have been described in patients with active celiac disease (*21*–*23*), but the link between altered gut microbiota composition and AhR pathway activation is unclear. Using a NOD/DQ8 mouse model that develops moderate inflammation upon sensitization to gliadin and challenge with gluten, we showed that a diet enriched in tryptophan shifted the gut microbiota toward a higher abundance of *Lactobacillus* and *R. gnavus*, which are known AhR ligand producers (*15*, *17*, *24*, *29*). Metabolomic and transcriptomic analysis indicated that this shift was accompanied by an increased production of AhR ligands and activation of the AhR pathway, which promoted gut homeostasis and ameliorated gluten immunopathology in NOD mice expressing HLA-DQ8, a risk gene for celiac disease. Experimental modulation of the AhR pathway with either a combination of AhR ligand producing bacterial strains *L. reuteri* CNCM-I5022 and CNCM-I5429 (*15*, *24*, *25*) or pharmacological activation of AhR using Ficz reduced gluten immunopathology. Last, relevance to human disease was demonstrated by the reduced amounts of specific gut microbial metabolites known to activate AhR in fecal samples from patients with active celiac disease compared to nonceliac controls.

An AhR pathway role in celiac disease is indirectly supported by altered IL-17 and IL-22 production by gluten-specific T cells in a macaque model of celiac disease (*31*) and in biopsies from patients with celiac disease (*32*). We found lower expression of key genes mediating the physiological activation of the AhR pathway (*Cyp1a1*, *Il22*, and *Il17*) in the duodenum of patients with celiac disease compared to nonceliac controls. Additional support comes from studies with *Ahr*$^{-/-}$ mice, which exhibit changes in T cell receptor αβ–positive (TCRαβ$^+$) CD4$^+$ and CD8$^+$ intraepithelial lymphocytes in the colon, a subset of T cells that increases in active celiac disease and that responds to a gluten-free diet (*33*). In NOD/DQ8 mice previously described to have a genetic impairment in natural killer cells (*34*), we observed an increase in CD3$^+$ intraepithelial lymphocytes with a low-tryptophan diet, suggesting that a lower AhR-activating capacity of the gut microbiota in the absence of dietary substrate may affect this key celiac disease–associated T cell subset. Last, a recent study has shown lower AhR expression in CD4$^+$, CD8$^+$ T cells, and CD56$^+$ natural killer cells isolated from both the intestinal lamina propria and the intraepithelial compartment of patients with active celiac disease (*20*). However, the drivers of these changes remain unknown. We hypothesized that previously described shifts in the gut microbiota in active celiac disease (*21*–*23*) could influence the AhR pathway through impaired production of AhR ligands.

Lower *Lactobacillus* abundance including strains with known capacity to produce AhR ligands (*15*, *17*, *24*, *25*) has been described in the small intestine and feces of patients with active celiac disease (*21*–*23*). Proteobacteria have been reported to be overrepresented in several intestinal and extraintestinal diseases with an inflammatory

phenotype (*26*, *27*). Our previous work showed that Proteobacteria members in duodenal contents of patients with celiac disease, such as *Pseudomonas aeruginosa*, degrade gluten differently than do bacteria from healthy participants, resulting in production of peptides that stimulate gluten-specific T cells (*9*). In the current study, an enriched tryptophan diet shifted the gut microbiota of gluten-naïve NOD/DQ8 mice toward a higher abundance of *Lactobacillus* and decreased Proteobacteria, leading to higher production of AhR ligands that promoted intestinal homeostasis. Gluten sensitization and challenge was a major independent driver of this gut microbiota shift, but the capacity of the gut microbiota to produce AhR ligands was preserved in mice that had received the enriched tryptophan diet. On the other hand, the concentration of kynurenine, a tryptophan metabolite produced by host cells through IDO1 enzyme activity and implicated in chronic inflammatory diseases (*28*, *35*), was increased in the feces of mice fed the low-tryptophan diet and in fecal samples from patients with celiac disease (*36*). Although kynurenine is an AhR agonist when used at high concentrations in vitro, its low concentrations in different animal tissues and fluids make it unlikely to be a major AhR agonist in vivo, especially when compared to the indole derivatives (*13*, *14*, *37*). The ability of the gut microbiota or host cells to metabolize tryptophan into AhR ligands or kynurenine, respectively, depends on the availability of tryptophan in the gut (*13*–*15*, *24*, *25*). Together, this suggests that both impaired tryptophan metabolism by the gut microbiota and higher tryptophan metabolism by host cells can lead to defective AhR signaling and increased production of kynurenine metabolites that are associated with an inflammatory state. On the basis of previous studies, it is also possible that the gut microbiota changes induced by the enriched tryptophan diet has other beneficial effects related to gut microbial metabolism of immunogenic wheat proteins (*9*, *10*, *38*).

To directly investigate bacterial modulation of the AhR pathway in our NOD/DQ8 mouse model, we used a combination of two *L. reuteri* strains with known AhR ligand–producing capacity (*15*, *24*, *25*) in the context of low-tryptophan and enriched tryptophan diets. We found that *L. reuteri* supplementation increased the capacity of the small intestinal microbiota to activate AhR, particularly in mice fed the enriched tryptophan diet, indicating a synergism between the substrate and its metabolizer. The slight increase in AhR activity in mice fed a low-tryptophan diet and *L. reuteri* was sufficient to improve villus-to-crypt ratios and decrease intraepithelial lymphocyte counts. On the other hand, *L. reuteri* supplementation in mice fed the enriched tryptophan diet did not improve further the beneficial effects of the enriched tryptophan diet. This suggested that the increased gut microbiota–derived endogenous production of AhR agonists induced by tryptophan supplementation was enough to reduce gluten immunopathology and that increasing activation of the AhR pathway with *L. reuteri* did not reduce disease severity further. Together, these results demonstrate that gluten immunopathology is ameliorated by dietary tryptophan and *L. reuteri* and suggest both independent and likely shared pathways for these interventions. Our results agree with previous studies in colitis and metabolic syndrome showing that *Lactobacillus* strains capable of metabolizing tryptophan into AhR ligands improve disease outcomes (*24*, *25*). It has recently been shown that *L. reuteri* is able to reprogram gut $CD4^+$ intraepithelial lymphocytes into immunoregulatory T cells in an AhR-dependent manner (*39*), suggesting a possible mechanism for improvement of intestinal barrier integrity in the context of celiac disease. However, we cannot rule out additional beneficial effects of *L. reuteri*, such as mucosal barrier enhancement or wheat immunogenic peptide detoxification, as previously demonstrated for other *Lactobacillus* strains including *L. reuteri* (*9*, *38*, *40*).

To further investigate the role of AhR activation in the improvement of gluten immunopathology, we pharmacologically modulated the AhR pathway using Ficz. Our results show that Ficz treatment improved intestinal permeability and inflammation in NOD/DQ8 mice treated with gluten. Studies have shown that intestinal AhR activation in mice by Ficz or diet-derived AhR ligands decreased development of colon cancer (*41*), experimental colitis (*24*, *42*), and *Citrobacter rodentium* colitis severity (*43*, *44*) through IL-22 production (*14*). Production of AhR ligands by the gut microbiota is required for IL-22 production (*24*), which is involved in mucosal wound healing (*45*) and production of antimicrobial proteins by intestinal epithelial cells (*46*, *47*). Several sources of IL-22 have been identified in the gut, including production by T helper 22 cells, TCRγδ intraepithelial lymphocytes, and innate lymphoid cells (*46*, *48*). Here, we observed increased duodenal *Il22* expression after AhR signaling induced by Ficz treatment or treatment with an enriched tryptophan diet in NOD/DQ8 mice, suggesting that the gut microbiota/AhR/IL-22 axis could play a role in celiac disease pathogenesis.

Our results are relevant to humans because impaired gut microbial production of AhR ligands was detected in a cohort of patients with active celiac disease. This decreased production of AhR ligands was associated with reduced gut microbiota AhR agonist activity and reduced duodenal expression of *Ahr* and AhR pathway genes such as *Il22* and *Il17*. Our results agree with a previous study showing lower AhR expression in inflamed mucosa of patients with celiac disease (*20*) and identify a potential defect in gut microbiota–specific AhR activation in celiac disease. These results suggest the hypothesis that inflammation in active celiac disease promotes a shift in gut microbiota composition resulting in impaired AhR agonist production that affects IL-22 production. Likewise, a diet low in factors that serve as substrates for gut microbial–derived AhR ligands could contribute to celiac disease. This hypothesis is supported by the results in this study showing that a gluten-free diet in patients with celiac disease increased the production of AhR ligands by the gut microbiota, improved activation of the AhR pathway in the intestine, and increased expression of *Il22*, a cytokine known to participate in intestinal recovery in mice and humans (*14*, *24*, *25*, *45*, *46*). Evidence of higher tryptophan metabolism through the kynurenine pathway was observed in patients with celiac disease on a gluten-free diet, suggesting that in these patients, tryptophan was consumed by both the gut microbiota and host cells to produce AhR ligands and kynurenine metabolites, respectively. The consumption of tryptophan by these two pathways could be the reason for the decreased fecal concentrations of tryptophan observed in patients with celiac disease on a gluten-free diet compared to nonceliac controls.

Together, the results suggest that patients with active celiac disease have impaired tryptophan metabolism by the gut microbiota and defective AhR activation, as well as higher tryptophan metabolism by host immune cells leading to proinflammatory kynurenine metabolites (fig. S7) (*28*, *35*). In patients with celiac disease, the gluten-free diet partly corrected impaired tryptophan metabolism by reducing inflammation-driven kynurenine production and increasing gut microbiota–dependent AhR ligand production leading to AhR activation and *Il22* expression, which may have aided in intestinal recovery (fig. S7).

Our study has several limitations. The cohort of patients with celiac disease was small, and all of the patients with celiac disease in the gluten-free diet group were in clinical remission. Future studies should confirm the results in a larger cohort of patients with celiac disease and should also include nonresponders to the gluten-free diet to investigate persistent AhR pathway abnormalities, because this would be a population of interest to target therapeutically. Also, our results in the NOD/DQ8 mice should be confirmed in other mouse models expressing HLA-DQ2 (*49*), which is expressed by most patients with celiac disease and in transgenic mouse models overexpressing IL-15 or other factors associated with celiac disease (*50*).

In summary, our study identifies a potential pathogenic mechanism related to the impaired production of AhR ligands by the intestinal microbiota in celiac disease. In NOD/DQ8 mice, we show that gluten-induced immunopathology can be reversed through a combination of an enriched tryptophan diet and *L. reuteri* supplementation that resulted in production of AhR ligands. Our study suggests that tryptophan catabolites derived from the metabolic activity of the intestinal microbiota could be used as markers of gut dysbiosis. Currently, the only treatment for celiac disease is a strict, life-long adherence to a gluten-free diet. Many patients with celiac disease suffer from persistent symptoms despite following a gluten-free diet. Our results should prompt interventional studies in this patient group to evaluate tryptophan supplementation in combination with probiotic organisms, such as *L. reuteri* strains, that produce AhR ligands from the dietary substrate. This therapeutic approach could also be useful as a preventative strategy in at-risk populations.

MATERIALS AND METHODS

Study design

The objectives of the study were to investigate whether AhR ligand production by the gut microbiota influenced immunopathology induced by gluten. Several approaches were used to address this question. Transgenic NOD mice expressing the DQ8 celiac disease susceptibility gene were maintained under specific pathogen–free conditions. All animal experiments were carried out in accordance with the McMaster University animal utilization protocols and with approval from the McMaster University Animal Care Committee and McMaster Animal Research Ethics Board. Mice were subjected to interventions aimed at enhancing the intestinal AhR pathway as follows: (i) Treatment with a low- or high-tryptophan diet before and after gluten exposure, (ii) oral supplementation with AhR ligand–producing *Lactobacillus* during gluten treatment, or (iii) treatment with either an AhR agonist or vehicle during gluten treatment. Gluten treatment consisted of a previously described method of sensitization and challenge. Fecal microbiota composition, AhR activation by intestinal contents, and fecal AhR ligand production were investigated in NOD/DQ8 mice fed a low- or high-tryptophan diet and before or after gluten treatment. Signs of intestinal inflammation, AhR activation, and gluten immunopathology were examined after exposure to a high- or low-tryptophan diet and after gluten treatment, *Lactobacillus* supplementation, or treatment with an AhR agonist. Studies were planned with the minimum number of animals per group ($n = 4$) to observe significant differences reproducibly. Mice were randomly assigned to groups. Statistical outliers or samples where technical issues were encountered were removed from analysis. The specific number of mice used in each experimental group is included in the figure legends.

To provide more clinical relevance, we also analyzed the concentrations of specific fecal microbiota metabolites known to activate AhR in stool from patients with active celiac disease, patients with celiac disease on a gluten-free diet, and controls without celiac disease. Participants with inflammatory bowel disease, peptic ulcer, or reflux disease and those taking immunosuppressants, glucocorticosteroids, antibiotics, or probiotics were excluded. All of the participants provided signed written informed consent. The study was approved by the Hamilton Integrated Research Ethics Board (REB# 12-599-T). Analysis of data was performed in a blinded fashion when possible.

Gluten sensitization and challenge

Female and male 8- to 12-week-old NOD AB° DQ8 (NOD/DQ8) mice were bred in a conventional specific pathogen–free facility at McMaster University and maintained on a gluten-free diet (Envigo, TD.05620) unless otherwise stated. All mice had unlimited access to food and water. NOD/DQ8 mice were sensitized with 500 μg of pepsin-trypsin digest of gliadin and 25 μg of cholera toxin (Sigma-Aldrich, St. Louis, MO) by oral gavage once a week for 3 weeks, to break oral tolerance to gliadin (*3*, *51*). Mice were then challenged by oral gavage with 10 mg of gluten (Sigma-Aldrich) dissolved in acetic acid three times a week for 3 weeks ("gluten treatment") (*3*, *51*). Mice were euthanized 18 to 24 hours after the final gluten challenge. In additional experiments, male and female 8- to 12-week-old specific pathogen–free C57Bl/6 mice maintained on a normal gluten-containing chow (Envigo 2918) were included.

Probiotic and pharmacological treatments

NOD/DQ8 mice were injected intraperitoneally with Ficz (Enzo Life Sciences, 1 μg per mouse) or vehicle (dimethyl sulfoxide, Sigma-Aldrich) at days 1, 10, 20, and 30 during gluten sensitization and challenge. For the treatments with bacteria known to produce AhR ligands (*24*, *25*), mice were gavaged six times a week for 3 weeks with 10^9 colony-forming units of a combination of *L. reuteri* CNCM-I5022 and *L. reuteri* CNCM-I5429. Bacteria were grown in de Man, Rogosa and Sharpe broth supplemented with cysteine (0.5 mg/ml) and Tween 80 (1 mg/ml) for 18 to 20 hours. Oral gavage with de Man, Rogosa and Sharpe medium was performed in control mice.

Custom tryptophan rodent diet

NOD/DQ8 mice were fed a customized version of Envigo TD.01084 in which tryptophan concentration was adjusted at 0.1% (low-tryptophan diet) or 1% (high-tryptophan diet) (table S1). Three weeks after the beginning of the diets, mice were gliadin-sensitized and gluten-challenged.

Measurement of AhR activity

The AhR activity of human and animal stool samples was measured using a luciferase reporter assay method, as described previously (*24*, *25*). Briefly, H1L1.1c2 cells, which contained the dioxin response element–driven firefly luciferase reporter plasmid pGudLuc1.1, were seeded into a 96-well plate and stimulated with human or animal stool suspensions or small intestinal contents for 24 hours. Luciferase activity was measured using a luminometer, and the results are reported as fold changes based on the negative luciferase activity of the control. All values were normalized on the basis of the

cytotoxicity of the samples using the Lactate Dehydrogenase Activity Assay (Promega, Madison, USA).

Metabolite measurements

The metabolite concentrations in stool and serum samples were determined by a specific method using high-perfomance liquid chromatography–coupled to high-resolution mass spectrometry as previously described (*52*–*54*). IDO activity was assessed by measurement of kynurenine-to-tryptophan ratio (*25*, *55*).

16*S* ribosomal RNA gene sequencing

Total DNA was extracted from mouse fecal samples, and data were sequenced as previously described (*10*) and analyzed as previously described (*56*). Briefly, the V3 region of the 16*S* ribosomal RNA gene was amplified and sequenced on the Illumina MiSeq sequencing system. Sequences obtained were aligned to each other using the Paired-End Read (PEAR) merger, and clustering of reads into operational taxonomic units was performed using the Quantitative Insights into Microbial Ecology (QIIME) version 1.8.0 (*57*), and data were loaded into R using the phyloseq package for downstream processing. A total of 4,012,804 reads were obtained with an average of 74,311.19 per sample and ranged from 11,512 to 169,626 per sample.

Measurement of gut barrier function

Barrier function was assessed by Ussing chamber technique as previously described (*51*). Briefly, sections of jejunum from each mouse were mounted in an Ussing chamber with an opening of 0.6 cm^2. Net active transport across the epithelium was measured via a short-circuit current response (I_{sc}, microamperes) injected through the tissue under voltage-clamp conditions. Baseline I_{sc} (microamperes per square centimeter) was recorded at equilibrium 20 min after mounting jejunum sections. Mucosal-to-serosal transport of macromolecules was assessed by adding ^{51}Cr-EDTA in the luminal side of the chamber. ^{51}Cr-EDTA fluxes were calculated by measuring the proportion of radioactive ^{51}Cr-EDTA detected in the serosal side of the chamber after 2 hours compared to the radioactive ^{51}Cr-EDTA placed in the luminal side at the beginning of experiment.

Gene expression analysis using quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from human or animal duodenum samples using RNeasy Mini Kit (Qiagen, Hilden, Germany) with the deoxyribonuclease treatment, according to the manufacturer's instructions. RNA concentration was determined using a NanoDrop spectrophotometer (Qiagen). Quantitative reverse transcription polymerase chain reaction (PCR) was performed using iScript Reverse Transcriptase (Bio-Rad) and then a SsoFast EvaGreen Supermix (Bio-Rad) in a Mastercycler ep realplex apparatus (Eppendorf) with specific human or mouse oligonucleotides. Additional information on the primers used is available in table S2. Quantitative PCR (qPCR) data were analyzed using the $2^{-\Delta\Delta Ct}$ quantification method with human or mouse *Gapdh* as the endogenous control.

Histology and immunohistochemistry

Cross sections of the proximal small intestine were fixed in 10% formalin and embedded in paraffin. Enteropathy was determined by measuring villus-to-crypt ratios in a blinded fashion, as previously described (*51*). The presence of intraepithelial lymphocytes in the sections was performed by immunostaining for $CD3^+$ cells as described previously (*3*, *51*) Briefly, sections were stained with polyclonal rabbit anti-mouse CD3 (Dako) and in each section, intraepithelial lymphocytosis was determined by counting $CD3^+$ intraepithelial lymphocytes per 20 enterocytes in five randomly chosen villus tips.

Clinical cohort

Eleven patients (7 females, mean age of 36.8 years) with positive anti–tissue transglutaminase (TG2) immunoglobulin A (IgA) test at diagnosis who attended the Adult GI Diseases Clinic at McMaster University were recruited after confirming active celiac disease by the presence of duodenal atrophy (>Marsh IIIa). Participant demographics are shown in table S3. Patients with concurrent or past history of inflammatory bowel disease (Crohn's disease, ulcerative colitis, or undetermined colitis), as determined by laboratory, endoscopic, or bowel imaging were excluded. Eighteen other patients (10 females, mean age of 51.7 years) undergoing endoscopy in which organic disease including peptic ulcer, reflux disease, inflammatory bowel disease, and celiac disease were ruled out were recruited as nonceliac controls. Six patients (three with active celiac disease and three nonceliac controls) were taking a proton pump inhibitor in the month before enrollment. An additional six patients with celiac disease on a gluten-free diet for >2 years (four females, mean age of 35.3 years) who were in clinical remission (with a negative anti-TG2 IgA test) and who attended the Adult GI Disease Clinic at McMaster University were included in the study. Patients taking immunosuppressants, glucocorticosteroids, antibiotics, or probiotics were excluded. When possible, genetic analysis was performed for celiac disease risk genes. Fecal samples were collected and kept frozen and stored until analysis. Not all patients were used for all determinations because of sample availability. The specific number of patients is included in the figure legends.

Statistical analysis

GraphPad Prism version 6.0 (San Diego, CA, USA) was used for all analyses and preparation of graphs. The data from one or two independent experiments are presented as median (interquartile range) or mean ± SEM. Normal distribution was determined by D'Agustino-Pearson omnibus normality test, Shapiro-Wilk test, and Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie correction. For datasets that failed normality tests, nonparametric tests were used to analyze significant differences. Multiple comparisons were evaluated statistically by one-way analysis of variance (ANOVA) and post hoc Tukey test or nonparametric Kruskal-Wallis test followed by a post hoc Dunn's test. For comparisons between two groups, significance was determined using the two-tailed Student's *t* test or nonparametric Mann-Whitney test. In all determinations, statistical outliers (using the ROUT method) or samples where technical issues were encountered, such as poor RNA quality, poor tissue quality for Ussing chambers, or poor histological orientation, were removed from analysis. Exact numbers and tests used are provided in each figure legend. Differences corresponding to $P < 0.05$ were considered significant.

For gut microbiota analysis, R Statistics, with the stats and vegan packages, was used to perform the statistical analysis. Data transformation was used when required and when possible to achieve a normal distribution (logarithmic, square root, inversion, and inverted logarithm). Differences between whole bacterial communities were tested by permutational multivariate ANOVA (PERMANOVA) calculated

using an unweighted UniFrac distance. Multiple comparisons were evaluated statistically by one-way ANOVA or Kruskall-Wallis test. Statistically significant differences were then evaluated by two-tailed Student's *t* test or Wilcoxon rank-sum test, and multiple testing was corrected via false discovery rate (FDR) estimation.

SUPPLEMENTARY MATERIALS

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Fig. S1. Higher fecal and serum AhR ligands in NOD/DQ8 mice fed a high-tryptophan diet are associated with greater *Lactobacillus* abundance.

Fig. S2. Gluten treatment shifts the gut microbiota composition of NOD/DQ8 mice regardless of diet intervention.

Fig. S3. Fecal tryptophan and AhR ligand concentrations before and after gluten treatment in NOD/DQ8 mice.

Fig. S4. Tryptophan supplementation increases AhR ligand concentrations in gluten-treated NOD/DQ8 mouse serum.

Fig. S5. Expression of AhR pathway genes in C57Bl/6 and NOD/DQ8 mice.

Fig. S6. Patients with active celiac disease have reduced AhR ligands in stool and increased kynurenine metabolites.

Fig. S7. Proposed scheme for tryptophan metabolism in the gut of patients with celiac disease.

Table S1. Composition of low- and high-tryptophan diets.

Table S2. qPCR primers used for gene expression analysis.

Table S3. Demographics of the clinical cohort.

Data file S1. Individual-level data for all figures.

View/request a protocol for this paper from *Bio-protocol*.

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Aryl hydrocarbon receptor ligand production by the gut microbiota is decreased in celiac disease leading to intestinal inflammation

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The advantage of a high-tryptophan diet

Although 40% of the worldwide population express celiac disease susceptibility genes, only 1% will develop the disorder, suggesting a role for environmental factors, including the gut microbiota. Lamas *et al.* show that celiac disease is associated with an impaired capacity of the gut microbiota to metabolize tryptophan into aryl hydrocarbon receptor (AhR) ligands in patients with celiac disease. Gluten-induced immunopathology in mice expressing a celiac disease susceptibility gene was ameliorated after AhR pathway activation by a high-tryptophan diet, or by treatment with a pharmacological AhR agonist or bacteria producing AhR ligands. AhR pathway modulation by the gut microbiota may have potential as a therapeutic strategy for treating celiac disease.