Bacteroidales recruit IL-6-producing intraepithelial lymphocytes in the colon to promote barrier integrity

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Interactions between the microbiota and distal gut are important for the maintenance of a healthy intestinal barrier; dysbiosis of intestinal microbial communities has emerged as a likely contributor to diseases that arise at the level of the mucosa. Intraepithelial lymphocytes (IELs) are positioned within the epithelial barrier, and in the small intestine they function to maintain epithelial homeostasis. We hypothesized that colon IELs promote epithelial barrier function through the expression of cytokines in response to interactions with commensal bacteria. Profiling of bacterial 16S ribosomal RNA revealed that candidate bacteria in the order Bacteroidales are sufficient to promote IEL presence in the colon that in turn produce interleukin-6 (IL-6) in a MyD88 (myeloid differentiation primary response 88)-dependent manner. IEL-derived IL-6 is functionally important in the maintenance of the epithelial barrier as *IL-6*^{-/-} mice were noted to have increased paracellular permeability, decreased claudin-1 expression, and a thinner mucus gel layer, all of which were reversed by transfer of *IL-6*^{+/+} IELs, leading to protection of mice in response to *Citrobacter rodentium* infection. Therefore, we conclude that microbiota provide a homeostatic role for epithelial barrier function through regulation of IEL-derived IL-6.

INTRODUCTION

The mammalian gastrointestinal tract supports the existence of trillions of bacteria. A critical mutualism exists within the intestinal mucosa, where microbes can not only promote health but also promote multiple mucosal diseases.¹ Tissue barrier function is a critical determinant of health, and epithelial cell homeostasis is crucial for maintenance of effective barrier function. Although epithelial homeostasis depends upon interactions with microbes, innate immune cells, and stroma,² the precise roles of these factors have yet to be elucidated.

Intraepithelial lymphocytes (IELs) are a unique population of antigen-experienced T cells anatomically associated with epithelial cells that function to protect the host from microbial invasion and maintain epithelial homeostasis.³ Our understanding of IELs is derived from murine studies in the small intestine (SI) where a majority of IELs are TCR $\gamma\delta$ ⁺ CD8 α ⁺.

One hallmark of SI IELs is the expression of CD103 (α E integrin) that allows them to home to the SI. CD103 interaction with E-cadherin on epithelial cells maintains the localization of SI IELs.⁴ Microbial sensing through NOD2 (nucleotide-binding oligomerization domain-containing protein 2) and the MyD88 (myeloid differentiation primary response 88) pathway result in epithelial cell production of interleukin-15 (IL-15) that stimulates IEL proliferation and effector functions,^{5,6} including maintenance of barrier function through several mechanisms. For example, $\gamma\delta$ IELs of the SI stimulate epithelial cells to secrete the antimicrobial peptide RegIII γ ,⁷ and IEL secretion of transforming growth factor- β , interferon- γ , and keratinocyte growth factor protects and repairs epithelial cells after injury.³ However, many of these features and functions are unique to $\gamma\delta$ IELs of the SI, whereas this represents a minor subpopulation of IELs in the colon. Our

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understanding of how *colon* IELs contribute to barrier function is still unclear.

Previous work has demonstrated that $\text{TCR}\beta^+ \text{CD4}^- \text{CD8}^-$ IELs in the murine and human colon produce IL-6 early after mechanical and inflammatory injury to the epithelium.⁸ IL-6 is known to signal through two mechanisms: *cis*-signaling in which soluble IL-6 binds its heterodimer receptor of IL-6R α and gp130 on the cell surface; or *trans*-signaling in which soluble IL-6 and secreted IL-6R α form a complex and then bind membrane-bound gp130.⁹ Generally, IL-6R α is found on lymphocytes and hepatocytes whereas the co-receptor gp130 is ubiquitously present;⁹ the presence of IL-6R on epithelia and how IL-6 signals in these cells remains unknown.

Upon ligation of the IL-6R α and gp130, one of two pathways is activated: the signal transducer and activator of transcription 3 (STAT3) or Src-homology tyrosine phosphatase (SHP2)/Ras/extracellular signal-regulated kinase (ERK). Colonic epithelial cell homeostasis and wound healing appears to require the STAT3 pathway.¹⁰ Some studies suggest that IL-6 signaling stimulates epithelial stem cell proliferation,⁸ whereas others have suggested that IL-6 protects intestinal epithelial cells from apoptosis during dextran sodium sulfate-induced colitis and *Citrobacter rodentium* infection in mice.^{11–13} Nevertheless, the source of, and stimulus for, IL-6 during intestinal epithelial homeostasis have not been established.

In the present studies, we hypothesized that colon IELs promote epithelial barrier function through the secretion of cytokines, particularly IL-6, stimulated by interactions with commensal microbes. Our results demonstrate that bacterial members in the order Bacteroidales aid in establishing IL-6-producing IELs in the colon, and MyD88-dependent signals are required for IEL production of IL-6. Importantly, the loss of IL-6-producing IELs impairs the integrity of the epithelial barrier through reductions in both tight junction expression and mucus thickness that are important in the host's defense against *C. rodentium* colitis. These studies suggest a novel pathway of microbiota stimulation of cytokine production in intestinal epithelial barrier homeostasis.

RESULTS

Bacteria of the order Bacteroidales are sufficient to maintain the colon IEL population

Studies utilizing germ-free and antibiotic-treated mice have demonstrated that the presence of IELs in the SI is dependent on microbial colonization.^{6,14,15} Microbial stimulation of NOD2 and the MyD88 pathway in the SI epithelium lead to epithelial IL-15 secretion and the recruitment of IELs.^{5,6} We verified that microbial depletion through administration of broad-spectrum antibiotics (1 mg ml⁻¹ each of ampicillin, metronidazole, and neomycin, and 0.5 mg ml⁻¹ vancomycin in drinking water) for 1 week significantly reduced IELs in the colon as assessed by immunofluorescence staining for epithelial CD3⁺ cells (Figure 1a,b) and flow cytometry (Supplementary Figure S1 online); this loss of IELs was reversible as recolonization by cohousing with control littermates resulted in an increase in IEL numbers. Furthermore, we confirmed the

requirement for microbial stimulation of Toll-like receptors and/or IL-1 family pathways in the colon as *MyD88*^{-/-} mice were found to have significantly reduced colon IELs (Figure 1c). In an attempt to identify bacteria necessary to maintain the colon IEL population, mice were treated with individual antibiotics or in combination for 1 week followed by analysis of colon IELs by flow cytometry. All antibiotic treatments decreased the total number of colon IELs (Figure 1d). As expected, treatment of mice with individual antibiotic classes had differing impacts on the microbial populations (Supplementary Figure S2a). It is notable that all antibiotics had a significant influence on reducing members of the class Bacteroidia. Based on these initial findings, we performed 16S ribosomal RNA sequencing of the fecal microbiome from five untreated and five combination antibiotic-treated mice. When comparing relative abundance of operational taxonomic units (OTUs) at the order level, the Wilcoxon rank test demonstrated that Bacteroidales were most significantly reduced in antibiotic-treated mice (Figure 1e and Supplementary Figure S2b), and within Bacteroidales, a Wilcoxon rank test identified the genus *Alistipes* were the most significantly reduced by antibiotic treatment (Supplementary Figure S2c). We measured by quantitative PCR (qPCR) the relative abundance of this genus in our single-antibiotic treated mice compared with untreated mice. Although *Alistipes* bacterial DNA was readily detected by qPCR of its 16S ribosomal RNA in untreated mice, it did not amplify in the samples from antibiotic-treated mice (Supplementary Figure S2d), confirming that *Alistipes* were depleted by each antibiotic treatment. Recolonized mice, which have an increased IEL population compared with antibiotic-treated mice (Figure 1a,b and Supplementary Figure S1), demonstrate increased *Alistipes* compared with the antibiotic-treated mice (Supplementary Figure S2d). These data suggest that Bacteroidales, including *Alistipes*, are important for the presence of IELs in the colon.

To test our hypothesis that Bacteroidales are sufficient for the presence of colon IELs, we monocolonized germ-free mice with *Alistipes onderdonkii* as well as closely related and well-characterized *Bacteroides fragilis* and *Bacteroides thetaiotamicron*.^{16,17} We confirmed the *B. thetaiotamicron* and *B. fragilis* were also depleted in abundance in our antibiotic-treated mice (Supplementary Figure 2e). As controls, germ-free mice were gavaged with an unrelated species *Escherichia coli* or phosphate-buffered saline (PBS). Colonization of mice with each species was not significantly different as confirmed by culture of cecal contents (Supplementary Figure S3). Monocolonization with *A. onderdonkii*, *B. fragilis*, and *B. thetaiotamicron* significantly increased the number of IELs in the colon compared with *E. coli* and PBS-gavaged mice (Figure 1f), confirming that at least some species within the order Bacteroidales, but not *E. coli*, are sufficient to maintain IELs in the colonic epithelium.

IL-6 secretion by IELs requires bacterial signals

Colon IELs previously have been shown to produce IL-6,⁸ and this we verified by intracellular cytokine staining and flow

Figure 1 Bacteria in the class *Bacteroides* maintain the colon intraepithelial lymphocyte (IEL) population. **(a–d)** Groups of 3–9 C57Bl/6 male and female mice aged 8–12 weeks were untreated, treated with antibiotics for 1 week, or treated with antibiotics followed by recolonization for 1 week each. Data are from two independent experiments. **(a)** Immunofluorescence of methacarn-fixed, paraffin-embedded colon tissue from mice was performed and representative images shown at $\times 400$. Bar = 20 μm . Dotted lines outline crypts and arrows point to IELs as identified as CD3⁺ (green) cells in the epithelial layer (β -catenin, red). Nuclei were stained with bis-benzimide (blue). **(b)** CD3⁺ epithelial cells were counted in four well-oriented high-powered fields from immunofluorescence staining performed in 6 untreated, 7 antibiotic-treated, 6 recolonized, and 3 germ-free mice and shown as the mean number of cells per crypt $\pm$ s.e.m. $^{**}P < 0.01$ and $^{***}P < 0.0001$ as determined by Kruskal–Wallis analysis with Dunn's post test. **(c,d)** The absolute number of epithelial CD3⁺ cells harvested from the colons of mice was determined by flow cytometry. Each dot represents an individual mouse and bars are the mean $\pm$ s.e.m. Statistical analysis for **c** was performed using a two-tailed Student's *t*-test; $^{**}P < 0.01$. In **d**, statistical analysis was performed using a one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test; $^{***}P < 0.001$ and $^{****}P < 0.0001$. **(e)** The bacterial 16S ribosomal RNA (rRNA) sequencing from fecal DNA extracted from 5 untreated and 5 antibiotic-treated (Abx) mice was performed. Order-level differences in relative abundances $\pm$ s.e.m. are shown with Wilcoxon rank test performed for statistical analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$. **(f)** Germ-free male and female mice aged 8–12 weeks were gavaged with phosphate-buffered saline (PBS), *Alistipes onderdonkii*, *Bacteroides fragilis*, or *Bacteroides thetaiotamicron* and allowed to colonize for 2 weeks. Epithelial cells were harvested and CD3⁺ cells evaluated by flow cytometry. Each dot represents an individual mouse and bars are the mean $\pm$ s.e.m. $^{*}P < 0.05$ as determined by one-way ANOVA with Dunnett's post test.

cytometry analysis of IELs from untreated C57Bl/6 mice (**Supplementary Figure S4**). However, the stimulus for IL-6 production is unclear. Therefore, we evaluated the production of IL-6 in response to resident bacteria. Again, mice were treated with broad-spectrum antibiotics for 1 week $\pm$ recolonization by cohousing with untreated littermates for 1 week. IELs were magnetically sorted and mitogen stimulated *ex vivo*. Purity of our EDTA liberation and magnetic sorting of IELs was confirmed by flow cytometry (**Supplementary Figure S5**). As shown in **Figure 2a**, IL-6 tracked with the

presence of microbial-derived signals under conditions of antibiotic treatment and recolonization. IL-6 was not produced by potential contaminating lamina propria T cells because CD3⁺ cells isolated from collagenase digests of colon tissue following EDTA liberation of IELs did not produce detectable IL-6 after mitogen stimulation (**Figure 2a**). Toll-like receptor and/or IL-1 family signals through MyD88 were also required for IEL secretion of IL-6, as IELs from *MyD88*^{−/−} mice did not secrete detectable levels of IL-6 (**Figure 2a**). As antibiotic treatment resulted in decreased numbers of IELs in the SI⁶ and

Figure 2 Intraepithelial lymphocytes (IELs) utilize bacterial signals for stimulation of interleukin-6 (IL-6) secretion. IELs were harvested from colons of mice and mitogen stimulated *ex vivo*. IL-6 secretion into the supernatant was measured by enzyme-linked immunosorbent assay (ELISA), and IL-6 from unstimulated IELs was subtracted from that of the mitogen-stimulated IELs from the same mouse. Data are from groups of 3–9 male and female mice aged 8–12 weeks in two independent experiments and shown as the mean $\pm$ s.e.m. Statistical significance was determined by one-way analysis of variance (ANOVA) with Dunnett's test. **(a)** IL-6 secretion from IELs and LPLs isolated from untreated C57Bl/6 mice as well as antibiotic-treated (Abx), recolonized, and *MyD88*^{-/-} mice. * $P < 0.05$ and **** $P < 0.0001$. ND, not detected; NT, not tested. **(b)** IEL secretion of IL-6 from germ-free mice gavaged with phosphate-buffered saline (PBS) or monocolonized with bacteria. * $P < 0.05$.

colon (**Figure 1** and **Supplementary Figure S1b**), we queried whether the production of IL-6 may be because of the reduced number of IELs. When normalized to the average number of CD4⁺ CD8⁺ IELs, secretion of IL-6 continued to track with the presence of the microbiota (**Supplementary Figure S6b**). Because *A. onderdonkii*, *B. fragilis*, and *B. theta* maintain the IEL population in gnotobiotic mice, we tested whether these strains could modulate IELs to produce IL-6 relative to PBS and *E. coli*. Colonization with *A. onderdonkii*, *B. fragilis*, or *B. theta* led to variable IL-6 production by IELs that was substantially higher than that produced by IELs isolated from *E. coli* or PBS-gavaged germ-free mice (**Figure 2b** and **Supplementary Figure S6c**).

IL-6 promotes barrier function and induces epithelial claudin-1 and mucin-2 expression

Although epithelial damage is limited by IL-6 signaling,^{8,11–13} surprisingly little is known about IL-6 and epithelial barrier function. To understand how IL-6 affects the epithelial barrier, we utilized T84 cells, a well-characterized human colon epithelial cell line widely used to study barrier regulation.¹⁸ IL-6R α protein was observed in lysates from both T84 cells and murine primary epithelial cells, indicating that these cells could respond to IL-6 *cis*-signaling (**Figure 3a**). Addition of IL-6 strongly induced STAT3 phosphorylation and SOCS3 expression in T84 cells, as determined by western blot and qPCR, respectively, verifying that IL-6 is able to signal through epithelial-expressed surface IL-6 receptors (**Figure 3b,c**). To define functional responses to IL-6, T84 cells were plated on membrane-permeable supports in either the absence or presence of IL-6 and monitored during the formation of barrier as measured by transepithelial resistance and permeability to fluorescein isothiocyanate (FITC)-dextran. As shown in **Figure 3d,e**, by day 4 of cell culture, IL-6 induced a prominent increase in the rate of barrier formation by transepithelial resistance ($P < 0.01$) and concomitant

decrease in paracellular permeability ($P < 0.0001$), revealing that activation of IL-6 receptor on intestinal epithelia promotes barrier formation.

Because paracellular permeability of FITC-dextran was affected by IL-6, we assessed the effect of IL-6 on epithelial tight junction proteins. T84 cells were treated with IL-6, and expression of a panel of tight junction proteins was profiled by qPCR using previously described methods.¹⁹ This approach identified a significant increase in the expression of CLDN1. A so-called “tight claudin,” CLDN1 plays an integral role in determining barrier function in IECs.²⁰ As shown in **Figure 3f**, exposure of epithelia to IL-6 induced CLDN1 mRNA and was confirmed at the protein level (**Figure 3g**). Such observations strongly implicate a role for IEL-derived IL-6 in the induction of epithelial barrier integrity via claudin-1.

Another important component of the epithelial barrier is the mucus layer. Adherent to the epithelium is a tight matrix of mucin-2 glycoprotein (MUC2) followed by a loosely associated matrix at the luminal edge. The inner layer is sterile whereas commensal bacteria colonize the loose outer layer.²¹ Because studies have demonstrated higher epithelial susceptibility to *C. rodentium* in *IL-6*^{-/-} mice,¹² and STAT3 can induce MUC2 gene expression,^{22,23} we reasoned that IL-6 may also have an effect on the mucus layer during homeostasis. T84 cells treated with IL-6 were noted to have increased MUC2 expression (**Figure 3h**), suggesting that IL-6 may also influence mucus production as part of its role in the maintenance of barrier function.

IL-6 maintains the epithelial barrier *in vivo*

Next, we aimed to verify that IEL-produced IL-6 was necessary to maintain the epithelial barrier through the same mechanisms *in vivo*. First, we assessed the epithelial permeability of FITC-dextran in *IL-6*^{-/-} mice compared with controls and antibiotic-treated mice. Compared with untreated mice, mice administered antibiotics as well as *IL-6*^{-/-} mice

Figure 3 Interleukin-6 (IL-6) signals in colon epithelia and enhances epithelial barrier function via induction of claudin-1 and mucin-2. **(a)** IL-6R α protein in T84 and primary murine epithelial cells was determined by western blot. **(b)** T84 colon epithelial cells were cultured to confluence in the absence or presence of 50 ng ml⁻¹ recombinant human IL-6. Protein was harvested after 0, 10, and 30 min of IL-6 exposure. Western blot confirmed phosphorylation of signal transducer and activator of transcription 3 (STAT3) after IL-6 exposure. **(c)** After 24 h of IL-6 exposure, RNA from T84 cells was harvested and evaluated by quantitative PCR (qPCR) for SOCS3 expression and normalized to actin. Data are the mean $\pm$ s.e.m. fold induction of SOCS3 in IL-6 treated cells compared with untreated cells. An unpaired two-tailed Student's *t*-test was used to determine statistical significance. **P* < 0.05. **(d)** T84 cells were cultured on membrane permeable supports in the absence or presence of IL-6. Transepithelial resistance (TER) was recorded daily and shown as the mean $\pm$ s.e.m. An unpaired two-tailed Student's *t*-test was performed at each time point to determine statistical significance. ***P* < 0.01. **(e)** T84 transwells were evaluated for paracellular flux of fluorescein isothiocyanate (FITC)-dextran. The rate of flux is shown as the mean $\pm$ s.e.m. An unpaired two-tailed Student's *t*-test demonstrated significance. ****P* < 0.0001. **(f)** After 24 h of IL-6 exposure, RNA from T84 cells was extracted evaluated for CLDN1 expression by qPCR. Data are the mean fold induction of CLDN1 $\pm$ s.e.m. in IL-6-treated cells relative to untreated cells. Statistical analysis using an unpaired two-tailed Student's *t*-test revealed significance. **P* < 0.05. **(g)** Cellular lysates from unexposed and IL-6-exposed T84 cells at 24 h were evaluated for claudin-1 protein by western blot. **(h)** RNA from T84 cells with and without IL-6 treatment was evaluated for MUC2 expression by qPCR. Data are the mean fold induction of MUC2 $\pm$ s.e.m. in treated cells compared with untreated cells. Statistical analysis using an unpaired two-tailed Student's *t*-test revealed significance. **P* < 0.05.

showed a greater than threefold increase in serum levels of FITC-dextran (*P* = 0.001). This permeability defect was reversible, as recolonization of antibiotic-treated mice led to permeability normalization, indifferent from untreated mice (Figure 4a). Similar to T84-cultured cells treated with IL-6, we found claudin-1 protein staining by immunohistochemistry significantly increased in the colonic epithelium of IL-6^{+/+} mice compared with IL-6^{-/-} mice (Figure 4b), suggesting that IL-6-stimulated claudin-1 expression may be one mechanism that promotes barrier integrity. In addition, we confirmed a mucus layer defect in the colons of IL-6^{-/-} mice relative to wild-type mice by immunofluorescence. Mice deficient for IL-6 demonstrated a significantly reduced mucus layer thickness (Figure 4c,d). Thus, we conclude

that IL-6 is critical in maintaining the intestinal barrier via multiple mechanisms, including stimulation of tight junction formation and mucus production.

IEL-derived IL-6 decreases epithelial permeability and protects from *C. rodentium* colitis

To demonstrate the IEL source of IL-6 was necessary, we isolated colon IELs from donor IL-6^{+/+} or IL-6^{-/-} mice and transferred them into IL-6^{+/+} or IL-6^{-/-} recipients. After 1 week, barrier permeability was assessed by the presence of serum FITC-dextran after oral gavage. Unlike transferring IL-6^{-/-} IELs into IL-6^{-/-} mice, transfer of IL-6^{+/+} IELs into recipient IL-6^{-/-} mice resulted in restoration of barrier permeability similar to transfer of IL-6^{+/+} IELs into IL-6^{+/+}

Figure 4 Epithelial barrier integrity is impaired in the absence of interleukin-6 (IL-6). Male and female 8–12-week-old C57Bl/6 and *IL-6*^{-/-} mice were treated as previously described. Experiments were performed using littermates in groups of three mice and repeated twice. **(a)** Untreated, antibiotic-treated (Abx), recolonized, and *IL-6*^{-/-} mice were gavaged with 0.6 mg kg⁻¹ body weight 4 kDa fluorescein isothiocyanate (FITC)–dextran. After 4 h, sera were collected from the mice, the fluorescence at 492 nm measured, and the amount of dextran calculated. Data are the mean concentration of dextran ± s.e.m. Statistical analyses were performed by one-way analysis of variance (ANOVA) with Dunnett's test. ****P* < 0.001. **(b)** Claudin-1 (brown) *in vivo* was evaluated by immunohistochemistry. Representative photos from 1 of 5 *IL-6*^{+/+} and 1 of 4 *IL-6*^{-/-} mice are shown at × 400. Bar = 20 μm. **(c)** Fluorescent *in situ* hybridization using a universal bacterial probe (red) was performed on *IL-6*^{+/+} and *IL-6*^{-/-} mice. Nuclei were labeled with bis-benzimide (blue) and the mucus layer labeled with wheat germ agglutinin (green). Representative images are shown at × 400. Bar = 20 μm. **(d)** Measurement of the mucus layer was performed in 3 areas of each of 4–6 high-powered fields (× 400) per mouse (6 *IL-6*^{+/+} and 6 *IL-6*^{-/-} mice). Data are the mean ± s.e.m. mucus thickness. An unpaired two-tailed Student's *t*-test demonstrated significance. ****P* < 0.001.

mice (**Figure 5a**). In a separate experiment, we confirmed that IELs will track to the colon when transferred in this manner using IELs isolated from CD45.1 congenic C57Bl/6 that were injected into wild-type, CD45.2 recipients (**Supplementary Figure S7**). These data demonstrate that IEL-derived IL-6 is sufficient to maintain an effective epithelial barrier.

Previous studies have shown *IL-6*^{-/-} mice have decreased survival and worse histopathology when infected with *C. rodentium*.¹² Thus, to demonstrate that IEL-produced IL-6 would restore barrier protection during disease, we orally infected mice with this pathogen following transfer of donor *IL-6*^{+/+} or *IL-6*^{-/-} IELs into *IL-6*^{+/+} or *IL-6*^{-/-} recipients. *IL-6*^{-/-} mice that received *IL-6*^{-/-} IELs had significantly more weight loss and increased intestinal histopathology after 12 days of infection compared with transfer of *IL-6*^{+/+} IELs into recipient *IL-6*^{-/-} mice and *IL-6*^{+/+} IELs into *IL-6*^{+/+} mice (**Figure 5b–e**). Therefore, we conclude that IL-6 expression by IELs is sufficient to restore protection against *C. rodentium* infection.

IL-6 produced by IELs restores claudin-1 expression and mucus thickness in *IL-6*^{-/-} mice during *C. rodentium* infection

Analyses of tissue sections from mice treated with IEL transfers followed by *C. rodentium* infection for 12 days demonstrated that transfer of *IL-6*^{+/+} IELs into *IL-6*^{-/-} mice repaired the defective epithelial barrier of *IL-6*^{-/-} mice. Transfer of *IL-6*^{+/+} IELs into *IL-6*^{-/-} mice resulted in significantly reduced bacterial translocation and increased mucus thickness compared with *IL-6*^{-/-} IELs into *IL-6*^{-/-} mice (**Figure 6a–c**). Claudin-1 expression increased with *IL-6*^{+/+} IELs given to *IL-6*^{-/-} mice similar to *IL-6*^{+/+} IELs to *IL-6*^{+/+} but nearly absent in *IL-6*^{-/-} IELs to *IL-6*^{-/-} mice (**Figure 6d,e**). Together, these results indicate that IL-6 produced by IELs can improve some functions of the epithelial barrier that are impaired in *IL-6*^{-/-} mice.

DISCUSSION

IL-6 is an important cytokine for intestinal epithelial repair after injury. However, details regarding its role during intestinal

Figure 5 Intraepithelial lymphocyte (IEL)-produced interleukin-6 (IL-6) repairs the epithelial barrier and protects from *Citrobacter rodentium* colitis. (a) IELs from *IL-6*^{+/+} or *IL-6*^{-/-} mice were harvested, magnetically sorted, and transferred into recipient *IL-6*^{+/+} or *IL-6*^{-/-} mice. After 1 week, intestinal barrier permeability was evaluated by fluorescein isothiocyanate (FITC)-dextran flux. Two experiments of 2–3 male and female mice per group were performed. Data are the mean serum concentration of dextran $\pm$ s.e.m. **** P < 0.0001 by one-way analysis of variance (ANOVA) with Tukey's test. (b) At 2 days following IEL transfer, 4–7 mice per group were infected with *C. rodentium* by oral gavage and monitored by daily weights. Mice were euthanized 12 days after infection. Data are the mean percentage of starting weight $\pm$ s.e.m. A two-way repeated-measures ANOVA with Dunnett's test determined statistical significance. * P < 0.05 and ** P < 0.01. (c) Methacarn-fixed, paraffin-embedded colon tissue from 12-day infected mice in (b) were stained by hematoxylin and eosin (H&E) and evaluated in a blinded manner for histologic damage as assessed by the number of organized inflammatory aggregates and ulcers along the entire colon. These are shown as the mean $\pm$ s.e.m. for each treatment group. * P < 0.05 by Kruskal–Wallis test with Dunn's multiple comparisons test. (d) Five well-oriented high-powered fields (HPFs) per mouse were viewed at $\times 200$ and number of crypts counted in each section. Data are the mean crypts/HPF $\pm$ s.e.m. ** P < 0.01 by one-way ANOVA with Tukey's test. (e) Representative histology is shown at $\times 200$. Bar = 50 μ m.

homeostasis, stimulus for secretion, cellular source, and mechanistic function were unknown. In this study, we hypothesized that IEL-derived IL-6 was critical for intestinal homeostasis. Our results demonstrate that a subset of Bacteroidales is sufficient to maintain IELs in the colon where they produce IL-6 dependent upon MyD88 signals. Moreover, IEL-derived IL-6 is functional and promotes barrier function via claudin-1 protein expression and increased mucus thickness that are protective against *C. rodentium* infection.

Despite our findings, several questions remain regarding how IELs are recruited to the colon and stimulated to produce IL-6. Multiple prior studies have shown microbiota to be regulators of the IEL population through NOD2 and MyD88 signaling in epithelial cells.^{6,7} Our studies are in agreement and further demonstrate that specific microorganisms, namely members of the bacterial order Bacteroidales, are sufficient to maintain the colon IEL population, although the mechanism by which the species within Bacteroidales maintain colon IELs

is not immediately clear. In the SI, MyD88 signaling in epithelial cells results in IL-15 production that retains a portion of the IEL population there.⁵ However, these data do not explain why certain bacterial strains within Bacteroidales were able to maintain the IEL population in our studies. More recent studies have identified cell surface short-chain fatty acid receptors on populations of mucosal T cells²⁴ and past work has suggested that one of the short-chain fatty acids, butyrate, may provide a proliferative or homing signal for some CD8⁺ populations of IEL in the rat colon.²⁵

Although our studies focus upon the role of IEL-derived IL-6, there are additional sources of IL-6 within the intestinal mucosa including epithelial cells, monocytes, and other T cells. Yet, transfer of *IL-6*^{+/+} IELs into *IL-6*-deficient mice was sufficient to reverse the intestinal permeability defect apparent in *IL-6*^{-/-} mice, indicating that IELs are a dominant source of IL-6 for mucosal barrier function. Although the purity of our transferred cells is robust, reducing the possibility of

Figure 6 Transfer of IL-6⁺ intraepithelial lymphocytes (IELs) into IL-6^{-/-} mice restores the mucus layer and claudin-1 expression. **(a)** Fluorescent *in situ* hybridization (FISH) was performed on tissues from **Figure 5** and the number of bacteria located within intestinal tissue in each of 20 high-powered fields (HPF, at $\times 400$) were counted and shown as the mean bacteria/HPF $\pm$ s.e.m. $^*P < 0.05$ and $^{***}P < 0.001$ by Kruskal–Wallis test with Dunn’s multiple comparisons test. **(b)** Mucus thickness in 3 areas of each of 4 HPFs per mouse was measured in sections from **(a)** and displayed as the mean mucus thickness $\pm$ s.e.m. Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey’s post test. $^{***}P < 0.001$ and $^{****}P < 0.0001$. **(c)** Representative FISH is shown at $\times 400$. Bar = 20 μ m. A universal bacterial probe (red) was used to mark bacteria; nuclei were labeled with bis-benzimide (blue); and the mucus layer labeled with wheat germ agglutinin (green). Dashed white lines outline the epithelial and luminal borders of the inner mucus layer. Arrowheads point to areas of bacterial translocation. **(d)** Claudin-1 protein expression from the experiment in **Figure 5** was evaluated by immunohistochemistry and assessed a numeric score for each mouse based on the level of staining: 0 = no staining, 1 = faint, 2 = mild; 3 = moderate; 4 = intense. The mean staining intensity per group $\pm$ s.e.m. is shown and statistical significance was assessed using the Kruskal–Wallis test with Dunn’s multiple comparisons test. $^*P < 0.05$. **(e)** Representative claudin-1 immunohistochemistry (brown) is shown at $\times 400$. Bar = 20 μ m.

inadvertently transferring other cellular sources of IL-6, some IELs trafficked to the SI and a small percentage to the spleen. It remains unclear how this pattern of trafficking might influence intestinal permeability. Nonetheless, given that most cells return to the intestine suggests that the IEL activities remain mostly local.

Our data reveal an important function for IEL-derived IL-6 in maintaining epithelial homeostasis and protection during barrier impairment. One mechanism is via IL-6 signaling through STAT3 to induce expression of claudin-1.

Interestingly, per our review of the CLDN1 promoter sequence, there are four predicted STAT3-binding sites, supporting our findings. It is notable that recent work implicates IL-6 as a cytokine in the disruption of epithelial barrier function *in vitro* through increased expression of the “leaky” tight junction claudin-2.^{26,27} These studies, though, demonstrated the pathway to occur through IL-6 activation of the Akt and ERK pathways that we did not find in our studies. Such a discrepancy between cytokines functioning as both barrier disruptors and barrier promoters has been described before. For example,

interferon- γ has long been known to disrupt epithelial barrier function,²⁸ whereas more recent studies have shown an important role for interferon- γ in the restitution of epithelial barrier through a mechanism involving induction of IL-10 receptors on intestinal epithelial cells.²⁹ Notably, *in vivo* studies have supported a protective role for IL-6 by promoting intestinal epithelial proliferation and wound healing.⁸ Placed in context, such results indicate a dynamic process wherein IL-6 produced by differing cell types may modulate barrier function differently during inflammation, tissue repair, and homeostasis.

Goblet cell function including mucus production (MUC2 expression) and secretion is known to be modulated by cytokines, particularly T helper type 2 cell response-driven cytokines (STAT6 signaling) and IL-22 and IL-10 (via STAT3).^{22,23,30} Because IL-6 also acts via the STAT3 pathway in our studies, we reasoned that it would also modulate MUC2 expression. We found increased MUC2 expression *in vitro* in T84 cells with IL-6 treatment, similar to others' observations in LS180 colon adenocarcinoma cell line,³¹ and increased mucus thickness in the presence of IEL-derived IL-6 *in vivo*. However, *in vivo*, our data do not address whether IL-6 may be acting directly on MUC2 expression in goblet cells or indirectly through other cells and mediators that stimulate goblet cell function. In addition, our evaluation of the mucus layer *in vivo* was limited to wheat germ agglutinin staining and thickness of the layer. It does not depict the quality of or glycan content in the layer.

Although we interpret our data as IL-6⁺ IELs stimulate enterocyte claudin-1 and mucin-2 to protect mice during *C. rodentium* infection, the barrier integrity may only be an indirect consequence of reduced inflammation. IL-6 produced by IELs may lead to protection of epithelial cell apoptosis or stimulation of epithelial proliferation as previously suggested by others,^{8,11–13} resulting in a more intact barrier with increased claudin-1 and mucin-2 presence. In support of this, others have shown the tight junction claudin-3 to be disrupted only when *C. rodentium* attaches to enterocytes,³² suggesting that tight junctions are influenced by pathogen attachment and not host signals. Thus, an alternate interpretation of our data would be that disease severity is decreased in the presence of IL-6⁺ IELs causing reduced *C. rodentium* access to the epithelium and less disruption of claudin-1. Nevertheless, this interpretation still supports the conclusion that IL-6⁺ IELs are protective.

Although our data indicate that IEL-produced IL-6 acts directly on epithelial cells to promote barrier integrity, it remains unclear whether the protective influences of IL-6 during *C. rodentium* infection are directly mediated by our proposed mechanism. Numerous cells, cytokines, and signaling pathways are involved in the murine response to *C. rodentium*.³³ Where IL-6 intersects with these pathways remains unknown, although one study using IL-6^{-/-} mice suggested its role was upstream of IL-17 and IL-22.³⁴ In that report, IL-17A and IL-17F signaling through IL-17RC were dispensable but IL-22 was required for survival during infection. However, using alternate approaches for genetic deletion of

IL-17 homologs and their receptors, others have found this pathway to be important for protection against *C. rodentium*, with IL-17C acting in synergy with IL-22.^{35,36} Thus, it is reasonable to query whether IL-6⁺ IELs transferred in our studies function to stimulate IL-17 and IL-22 pathways that protect against *C. rodentium* infection in addition to our proposed mechanism.

Taken together, this work highlights the importance of a finely balanced mutualism between the host and microbiota within the mucosa. A clearer understanding of the molecular basis of such crosstalk between gut-derived signals and the IELs within the mucosa are likely to shed important light on potential therapeutic targets for mucosal disease.

METHODS

Animals. C57Bl/6, CD45.1 congenic (B6.SJL-Ptprca Pepcb/BoyJ), IL-6^{-/-} and MyD88^{-/-} breeding pairs on the C57Bl/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). Strains were bred and maintained in the University of Colorado Anschutz Medical Campus animal facility in standard specific pathogen-free conditions as well as in germ-free isolators. All use was approved by the institutional animal care and use committee. Both male and female mice were used at the age of 8–12 weeks. To control for variations in microbiota, littermates and cohousing were utilized. Treatment of mice with antibiotics was done by placing 0.5 mg ml⁻¹ vancomycin and 1 mg ml⁻¹ each of ampicillin, metronidazole, and neomycin (Sigma, St Louis, MO) as a combination or individually into drinking water supplemented with 20 mg ml⁻¹ grape-flavored sugar-sweetened Kool-Aid (Kraft Foods, Deerfield, IL). Untreated mice were provided drinking water with Kool-Aid alone. After 1 week, mice were either euthanized for studies or recolonized by cohousing antibiotic-treated mice with unmanipulated littermates for 1 week.

For monocolonization experiments, germ-free C57Bl/6 mice (obtained from the National Gnotobiotic Rodent Resource Center, University of North Carolina, Chapel Hill, NC, and bred and maintained locally in sterile vinyl isolators) were gavaged with 200 μ l liquid bacterial culture of the following obtained from American Type Culture Collection (ATCC, Manassas, VA): *Alistipes onderdonkii* WAL 8169, *Bacteroides thetaiotamicron* VPI-5482, *Bacteroides fragilis* NCTC 9343, and *Escherichia coli* K12. At the time of animal euthanasia, cecal contents were serially diluted in PBS and cultured on 10% sheep red blood cell agar plates to determine bacteria colonization levels.

Permeability of the colonic epithelial barrier was evaluated by absorption of FITC-dextran. Mice were given 0.6 mg kg⁻¹ body weight of 4 kDa FITC-dextran (Sigma) by oral gavage, and sera were collected after 4 h. The amount of fluorescence was measured with a fluorimeter (Promega, Madison, WI) at 485/530 nm. A standard curve was generated to calculate the amount of dextran that was present in the sera.

C. rodentium infection was performed utilizing the protocol by Bhinder *et al.*³⁷ Briefly, 200 μ l of a fresh overnight culture of *C. rodentium* (ATCC, strain DBS100) was orally gavaged into each mouse to induce disease. Body weight was monitored daily throughout the study that was terminated 12 days after infection at which time mice were euthanized and tissues collected for analyses.

Isolation, analysis, and transfer of IELs and primary epithelial cells.

After euthanasia of mice, the colon with cecum was removed, flushed with PBS without magnesium or calcium, and placed in PBS with 1 mM EDTA. The tissues were vortex-agitated for 10 min at room temperature, placed through 70 μ m nylon mesh, and pelleted by centrifugation. Cells were resuspended in PBS with 5% fetal calf serum, counted, and used for further analyses.

For flow cytometry, 0.5 ng ml^{-1} fluorescently labeled antibodies (eBioscience, San Diego, CA; BioLegend, San Diego, CA; and Tonbo Biosciences, San Diego, CA) were added to the cells: CD3 ϵ (17A2), CD4 (GK1.5), CD8 α (53-6.7), CD8 β (H35-17.2), TCR β (H57-597), TCR $\gamma\delta$ (GL3), CD45 (30-F11), CD45.1 (A20), and Ghost Dye Violet 510 (Tonbo). Intracellular staining was performed using commercial intracellular staining fixation and permeabilization buffers (Tonbo) and anti-IL-6 (MP5-20F3) or Rat IgG1 isotype control (Tonbo). Cells were run on a LSR II flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (V.10.0.8, Ashland, OR).

Purification of IELs was performed by magnetic separation using the EasySep Mouse T Cell Isolation Cocktail (Stem Cell Technologies, Vancouver, BC, Canada) and biotinylated anti-EpCAM (G8.8). Isolated cells were then resuspended in RPMI-1640 media with 10% fetal calf serum and divided into two wells of a 96-well cell culture plate. Then, 10 ng ml^{-1} phorbol 12-myristate 13-acetate and $1 \mu\text{g ml}^{-1}$ ionomycin were added to one well. Cells were incubated at 37°C for 2 h with protein transport inhibitor cocktail (eBioscience; for intracellular cytokine staining) or for 5 h without a protein transport inhibitor (for ELISA). Cell culture supernatants were harvested for cytokine analysis by ELISA (Meso Scale Discovery, Rockville, MD).

For experiments in which IELs were transferred into recipient mice, magnetically sorted IELs from C57Bl/6 mice were labeled with CellVue Jade Cell Labeling Kit (eBioscience), or IELs from CD45.1 congenic mice were counted and suspended in PBS at a concentration of $1\text{--}2 \times 10^6$ cells per ml. Then, $100 \mu\text{l}$ of cells were then injected intravenously via the tail vein into recipient mice.

Assessment of microbiota. DNA from feces was isolated using a commercial kit (UltraPure Fecal DNA, Mol Bio, Carlsbad, CA). The concentration of DNA was determined by spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA) and normalized to fecal pellet weight. qPCR for *Bacteroidia* class, *Clostridiales* order, *Enterobacteriaceae* family, *Enterococcus* family, *Lactobacillaceae* family, *Alistipes* genus, and *Bacteroides thetaiotamicron*- and *Bacteroides fragilis*-specific 16S DNA was performed and normalized to a universal bacterial 16S primer set.^{38,39} **Supplementary Table 1** lists primer sequences used and their references.

For microbiome analysis, harvested DNA from fecal pellets was PCR amplified with broad-range bacterial primers targeting the 16S ribosomal RNA gene V1V2 region and pooled amplicons subjected to Illumina MiSeq sequencing, as previously described.^{40–42} Assembled sequences were aligned and classified with SINA (1.3.0-r23838)⁴³ using the 418,497 bacterial sequences in Silva 115NR99 (ref. 44) as reference configured to yield the Silva taxonomy. OTUs were produced by clustering sequences with identical taxonomic assignments. Analyses of OTU relative abundance and biodiversity were conducted using Explicite software.⁴⁵ All samples had a Good's coverage index $> 99\%$, indicating excellent depth of sequencing coverage.

When analyzing the microbiome data to determine differences between C57Bl/6 untreated and antibiotic-treated mice ($N = 5$ each group), the relative abundance of OTUs at the order level was compared using the Wilcoxon rank test to identify significant differences between groups. A P value of < 0.05 was considered significant. Within the most significant order *Bacteroidiales*, the family-level OTUs were compared with the Wilcoxon rank test.

Histology, fluorescent *in situ* hybridization, and immunohistochemistry. Colon tissues were dissected from mice and placed whole and unflushed in methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid) fixative overnight. Tissues were then washed two times in 100% methanol for 30 min each and then two times in xylenes for 20 min each followed by embedding into paraffin. For histological examination $5 \mu\text{m}$ sections were cut, stained with hematoxylin and eosin, and coverslipped.

For fluorescent *in situ* hybridization FISH, $5 \mu\text{m}$ sections of methacarn-fixed, paraffin-embedded mouse colon were

deparaffinized by heating at 72°C for 30 min followed by passage through xylenes and then alcohols. Slides were crosslinked with ultraviolet subtype-C for 20 min in PBS and then air dried. A eubacterial fluorescent *in situ* hybridization probe (EUB-338; 5'-GCTGCCTCCCGTAGGAGT-3'),⁴⁶ 5' labeled with AlexaFluor568 (Molecular Probes, Thermo Fisher Scientific, Waltham, MA), was reconstituted with sterile water and diluted to a working concentration of $10 \text{ ng } \mu\text{l}^{-1}$ with hybridization buffer (20 mM Tris-HCl, 0.1% sodium dodecyl sulfate, 0.9 M NaCl, pH 7.2, 5% formamide). The sections were hybridized at 50°C overnight. Slides were washed in wash buffer (hybridization buffer without SDS or formamide) at 50°C for 30 min. Slides were then counterstained with AlexaFluor 488 wheat germ agglutinin (Molecular Probes) and 4',6-diamidino-2-phenylindole, mounted with ProLong antifade (Molecular Probes), and evaluated by fluorescent microscopy (AxioCam MR c5 attached to an AxioImager A1 microscope, Zeiss, Oberkochen, Germany).

Immunohistochemistry was performed on $5 \mu\text{m}$ sections of methacarn-fixed, paraffin-embedded mouse colon. Following deparaffinization and rehydration, tissue was treated with 3% H_2O_2 in methanol for 15 min at room temperature and then washed with deionized water followed by PBS. Primary antibody claudin-1 (rabbit polyclonal, Invitrogen, Thermo Fisher Scientific, Waltham, MA) diluted 1:200 with 3% bovine serum albumin in PBS was applied to tissue overnight at 4°C . Secondary antibody horseradish peroxidase-conjugated anti-rabbit (Bio-Rad, Hercules, CA) diluted 1:3,000 with 3% bovine serum albumin in PBS was applied for 1 h at room temperature. Following washing, slides were developed with DAB using a commercial kit (Vector Laboratories, Burlingame, CA), counterstained with hematoxylin, and coverslipped. Slides were evaluated with light microscopy.

Cell culture and western blot. T84 cells (ATCC, Manassas, VA) cultured in Dulbecco's modified Eagle's medium/F12 (Thermo Fisher Scientific) with 10% fetal bovine serum were grown to confluence in 24-well plates. Media were replaced with and without 50 ng ml^{-1} recombinant human IL-6 (Tonbo Biosciences). After 24 h, cells were lysed in either Trizol for RNA extraction or Tris-lysis buffer containing protease inhibitors (Pierce, Thermo Fisher Scientific, Waltham, MA). Protein concentration was determined by BCA (Pierce). Protein was separated by electrophoresis on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with antibodies to actin (polyclonal, Abcam, Cambridge, MA), STAT3 (79D7, rabbit monoclonal antibody, Cell Signaling Technology, Danvers, MA), pSTAT3 (3E2, mouse monoclonal antibody, Cell Signaling Technology), IL-6R α (polyclonal, Santa Cruz, Dallas, TX), and claudin-1 (rabbit polyclonal, Invitrogen).

T84 cells were then grown to confluence on 0.33 cm^2 , $0.4 \mu\text{m}$ permeable polyester inserts (Corning, Corning, NY) in the absence or presence of 50 ng ml^{-1} IL-6. Transepithelial resistance was measured using the EVOM2 voltammeter (World Precision Instruments, Sarasota, FL) and expressed as Ohms $\times \text{cm}^2$. Paracellular permeability was assayed using FITC-Flux assay: confluent T84 monolayers on 0.33 cm^2 , $0.4 \mu\text{m}$ permeable polyester inserts were washed and equilibrated in Hanks' balanced salt solution. Then, 1 mg ml^{-1} FITC-dextran, 3 kDa (Sigma) was added to the apical compartment and samples were taken from the basolateral compartment every 30 min for 2 h. Fluorescence was determined using a Glomax Multi fluorescent plate reader (Promega) and represented as the change in fluorescence over time.

RNA isolation and qPCR. RNA from primary cells or T84 cells was extracted using Trizol. RNA concentration was determined by spectrophotometry (Nanodrop), and then converted to complementary DNA (cDNA) using iScript (Bio-Rad). qPCR of cDNA for actin, SOCS3, claudin-1, -2 and -4, and mucin-2 was performed.^{19,29} **Supplementary Table 1** lists the primer sequences used. Reactions consisted of $1 \mu\text{l}$ cDNA, $0.6 \mu\text{M}$ each of forward and reverse primers, $1 \times$ SYBR Green PCR Master Mix (Applied Biosystems, Foster City,

CA), and water for a total volume of 17 μ l. Samples were denatured at 95 °C for 2 min, cycled 40 times through 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s, and then denaturation curves determined from 58 °C through 95 °C. All qPCR assays were conducted in a Applied Biosystems 7500 real time PCR system. Specificity of amplicon was verified by agarose gel electrophoresis.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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AUTHOR CONTRIBUTIONS

K.A.K. and S.P.C. designed the study, performed experiments, analyzed and interpreted data, and wrote the paper. H.M.S., E.H.R., E.L.S., G.M., A.K.W., N.O. and E.L.C. helped perform experiments and analyze data. J.D.H., D.I., C.E.R. and D.N.F. processed samples, sequenced, and analyzed microbiome data. All authors contributed to reviewing and revising the manuscript and approved the final draft for submission.

DISCLOSURE

The authors declared no conflict of interest.

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