Article

Immunity

Interleukin-23-Independent IL-17 Production Regulates Intestinal Epithelial Permeability

Highlights

- Neutralizing IL-17 results in increased gut permeability in a DSS injury model
- γδ T cells are the major source of gut-protective IL-17 and are IL-23 independent
- IL-17 regulates the cellular localization of the tight junction protein occludin
- The data explain IBD exacerbation with anti-IL-17 but amelioration with anti-IL-23

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In Brief

Neutralizing IL-17 has efficacy in psoriasis but is ineffective for the treatment of Crohn's disease. Cua and colleagues demonstrate that IL-23-independent γδ T cell-derived IL-17 regulates occludin tight junction protein and limits gut permeability. Anti-IL-23 therapy preserves protective IL-17 while limiting inflammation during acute gut injury.

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Interleukin-23-Independent IL-17 Production Regulates Intestinal Epithelial Permeability

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SUMMARY

Whether interleukin-17A (IL-17A) has pathogenic and/or protective roles in the gut mucosa is controversial and few studies have analyzed specific cell populations for protective functions within the inflamed colonic tissue. Here we have provided evidence for IL-17A-dependent regulation of the tight junction protein occludin during epithelial injury that limits excessive permeability and maintains barrier integrity. Analysis of epithelial cells showed that in the absence of signaling via the IL-17 receptor adaptor protein Act-1, the protective effect of IL-17A was abrogated and inflammation was enhanced. We have demonstrated that after acute intestinal injury, IL-23R$^+$ γδ T cells in the colonic lamina propria were the primary producers of early, gut-protective IL-17A, and this production of IL-17A was IL-23 independent, leaving protective IL-17 intact in the absence of IL-23. These results suggest that IL-17-producing γδ T cells are important for the maintenance and protection of epithelial barriers in the intestinal mucosa.

INTRODUCTION

Interleukin-17A (IL-17A) is the hallmark cytokine of the T helper (Th17) cell subset of CD4$^+$ T cells, which have been implicated as the primary pathogenic population in a range of autoimmune disorders (Langrish et al., 2005; Louten et al., 2009; Sallusto and Lanzavecchia, 2009). IL-23 has been shown to promote the terminal differentiation and expansion of Th17 effector cells and is thought to orchestrate chronicity and severity in disease models such as experimental autoimmune encephomyelitis (EAE) and inflammatory bowel disease (IBD) (McGeachy et al., 2009; Park et al., 2005; Yen et al., 2006). In Crohn's disease (CD), SNPs in the *IL23R* locus are associated with disease susceptibility (Duerr et al., 2006), and patients with active CD present with increased numbers of IL-23- and IL-17-expressing cells in the gut lamina propria (Hölttä et al., 2008).

However, increasing evidence suggests opposing effects of the cytokines IL-23 and IL-17 in their contribution to intestinal immunopathology. Murine models of innate (Buonocore et al., 2010) and T-cell-driven (Ahern et al., 2010; Yen et al., 2006) colitis demonstrate an inflammatory role for IL-23, and neutralizing IL-23 had a protective effect. The efficacy of neutralizing IL-23 clinically has also shown some promise. Administration of ustekinumab, a monoclonal antibody against the p40 shared subunit of IL-12 and IL-23, has shown positive responses in CD patients resistant to anti-tumor necrosis factor alpha (TNF-α) treatment (Sandborn et al., 2012), and recent data from a Phase 2a study for MEDI2070—a monoclonal antibody against the p19 subunit of IL-23—has shown great promise in treating TNF-α-therapy-resistant CD patients (Sands et al., 2015, J. Crohn's Colitis, abstract). 42.4% of the patients on MEDI2070 experienced a clinical response and reduced inflammatory scores compared to 10% in the placebo group and treatment has not increased adverse events, suggesting a more favorable benefit-risk profile compared with the inhibition of both IL-12 and IL-23 through sequestration of p40. In contrast to IL-23, various murine models of colitis suggest a protective role for IL-17A. IL-17A neutralization increases tissue damage in a dextran sodium sulfate (DSS) model of IBD (Ogawa et al., 2004), and IL-17A- or IL-17 receptor alpha (RA)-deficient T cells results in exacerbated colitis when transferred into RAG-1-deficient recipients (O'Connor et al., 2009). Importantly, phase II clinical trials with secukinumab, targeting IL-17A (Hueber et al., 2012), or brodalumab, targeting IL-17RA (Targan et al., 2012), is ineffective in treating CD and results in either higher rates of adverse events or worsening of CD, respectively. Although the data clearly do not show any efficacy for neutralizing IL-17A or IL-17RA in CD, the current understanding of the mechanism of IL-17-mediated protective effects in both mouse and man is lacking.

Given the suggested role of IL-17A in maintaining barrier function of epithelial tissues (Kinugasa et al., 2000), we were interested in addressing the protective effects of IL-17A on preserving epithelial integrity in a DSS model of acute colonic injury. We demonstrate that IL-17A promoted epithelial barrier function by regulating the cellular localization of the tight junction protein occludin during DSS-mediated injury and protected the mice from excessive gut permeability. In addition, our data

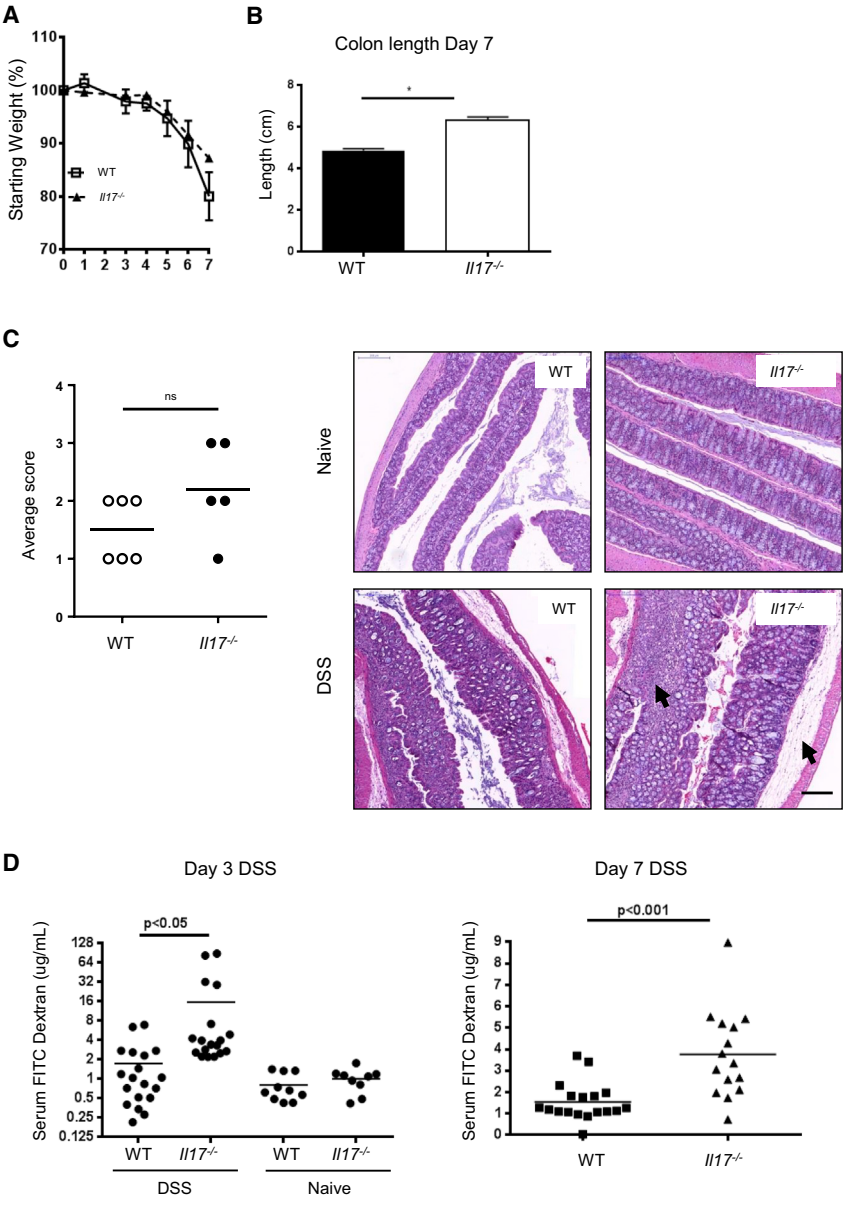

Figure 1. *Il17*$^{-/-}$ Mice Suffer Worse Epithelial Injury and Enhanced Gut Permeability after DSS Administration

(A) Weight loss of WT (squares) and *Il17*$^{-/-}$ (triangles) mice over time during DSS treatment, representative data from three independent experiments, n = 5–7/group, mean ± SEM.

(B) Colon length at day 7, combined data from three experiments, mean ± SEM. *p < 0.05.

(C) Pathology scores of disease severity, means indicated; ns, not significant. Combined data from two experiments. H&E of colons reveal enhanced edema and lymphocytic infiltrate in *Il17*$^{-/-}$ colons (arrows) after DSS, 20× magnification shown. Scale bar in lower right panel represents 200 μm. Combined data from two experiments.

(D) Detection of FITC-dextran in plasma showing increased colon permeability in *Il17*$^{-/-}$ over WT after 3 and 7 days of DSS, representative data from three experiments, or combined data from three experiments (day 7), means indicated, *p < 0.01.

All statistics generated by one-way ANOVA, with Tukey's multiple comparisons. See also Figure S1.

RESULTS

Gut Epithelial Integrity Is Compromised in the Absence of IL-17 during Injury

In order to investigate the role of IL-17 in disease progression after gut mucosal injury, we administered DSS in the drinking water to WT or *Il17*$^{-/-}$ mice for 7 days. Mice were monitored and scored according to weight loss, consistency of stool, rectal bleeding, and colon length. Overall weight loss and colon length were comparable between WT and *Il17*$^{-/-}$ mice after 7 days of DSS treatment (Figures 1A and 1B). Upon gross physical and histological examination, disease pathology was comparable between *Il17*$^{-/-}$ colons and WT colons (Figure 1C). Although both groups of mice exhibited epithelial disruption and the presence of abscesses, the absence of IL-17 was further characterized by enhanced bleeding into the mucosal lumen as well as increased edema and cellular infiltrate into the submucosal layer (Figures 1C). To further characterize the damage to the epithelial layer, permeability was quantified by orally administering FITC-dextran to mice on day 3 and day 7 of DSS treatment and measuring the amount present in the serum (Figure 1D). Although baseline permeability in naive mice was similar between WT and *Il17*$^{-/-}$ mice, the diffusion of FITC-dextran across the epithelium after DSS treatment was significantly increased in colons from *Il17*$^{-/-}$ mice compared to those from WT mice at both time points, suggesting that IL-17 deficiency reduced the ability to maintain appropriate barrier function during DSS-induced injury. To test whether the increased permeability from *Il17*$^{-/-}$ mice observed in DSS-induced injury was specific

show that IL-17A could mediate these protective effects by signaling through the IL-17R adaptor protein Act-1 on epithelial cells. We examined the production of IL-17 by multiple adaptive and innate cell populations after intestinal insult and found that the initial source of tissue-protective IL-17A was primarily a tissue-resident IL-23R^{+} γδ T cell as opposed to a CD4^{+} αβ T cell or an innate lymphoid cell (ILC). The colonic γδ T cells did not require IL-23 signaling for the production of IL-17A, and mice deficient for IL-23R were protected from the increased barrier dysfunction after DSS treatment that is seen in *Il17*$^{-/-}$ mice. Importantly, neutralizing IL-23 dampened adaptive and innate cell activation while maintaining the production of protective IL-17A during epithelial injury. Taken together, these data provide a mechanism by which IL-17A-producing γδ T cells help protect the epithelial barrier against excessive permeability after injury.

to the model, we neutralized IL-17 in another mouse model of colitis, the T cell transfer model. Anti-IL-17A antibodies did not have any impact on weight loss throughout the course of the disease as compared to controls, but the mice exhibited increased FITC dextran permeabilization between 3 and 4 weeks after T cell transfer (Figures S1A and S1B). Neutralization of IL-23R had a protective effect on the mice, clearly differentiating IL-17 from IL-23 in colitis.

Sub-cellular Localization of the Tight Junction Protein Occludin Is Altered in the Absence of IL-17A after Intestinal Injury

An important component of maintaining intestinal barrier function is the formation and maintenance of tight junctions between epithelial cells. The tight junction complex, composed of proteins including claudins, occludins, and zonula occludens 1 (ZO-1), is a critical structure in regulating intestinal permeability and the epithelial paracellular pathway (Turner, 2009). Because IL-17A-deficient mice exhibited increased intestinal permeability after gut injury, we hypothesized that the epithelial tight junctions would be compromised in these animals. Transcripts of *Cldn7*, *Tjp1*, and *Ocln* did not show any differences between WT and *Il17*$^{-/-}$ mice at baseline, and whereas expression was modulated at day 3 after DSS, IL-17 deficiency did not impact *Tjp1* or *Ocln* (Figure 2A) expression. Although transcripts of *Ocln* were similar between WT and *Il17*$^{-/-}$ colon tissue samples, altered intra-cellular localization could explain the increase in permeability and loss of barrier function. Tight junction disruption, including internalization of occludin from the tight junction, has previously been demonstrated to contribute to increased intestinal permeability (Clayburgh et al., 2005). Cross sectional images of the colonic crypts of epithelial cells in naive mice showed clear staining of occludin colocalized with F-actin on the apical surface of the cell (Figures S2 and 2B). In contrast to naive or control Ig-treated mice, in animals treated with anti-IL-17A, the colon epithelial cells showed diffuse occludin staining that appeared to extend into cytoplasm as well as a loss of co-localization with F-actin. These results demonstrate that whereas occludin message and protein expression do not seem to be altered with IL-17A neutralization, its cellular localization and ultimately tight junction function is impacted. To determine whether IL-17 had a direct impact on occludin subcellular localization, we turned to an in vitro system using the human epithelial colon adenocarcinoma line, Caco-2. Caco-2 cells express transcripts for *IL17RA* and *IL17RC* and respond to recombinant IL-17 (Figure S3). TNF-α concentration is markedly increased in mouse models of IBD as well as CD patients, and TNF-α has been shown to disrupt tight junctions and increase epithelial barrier permeability in Caco-2 cells (Ma et al., 2004). Indeed, the addition of TNF-α into Caco-2 monolayers resulted in altered cellular localization for occludin, leading to a disorganized staining pattern that extended into the cytoplasm (Figure 2C). This TNF-α-mediated disruption of occludin localization was dramatically decreased in the presence of IL-17A. These results show that IL-17A can directly regulate TNF-α-mediated disruption of tight junction proteins in Caco-2 cells, and the data complement the effects of neutralizing IL-17A observed in vivo.

IL-17 Signals through an Act-1 Pathway in Epithelial Cells to Maintain Barrier Integrity

Act-1 is an essential adaptor protein used by IL-17R to mediate downstream cytokine signaling during an inflammatory response (Qian et al., 2002, 2007). We would therefore hypothesize that if IL-17 is maintaining epithelial integrity during DSS colitis, we would expect that in the absence of Act1 signaling, gut epithelium would exhibit enhanced pathology after treatment with DSS. Act-1 was originally cloned as an NF-κB activator, but it has also been shown to negatively regulate B cell function and humoral responses (Qian et al., 2004). In order to test the specific contribution of IL-17R signaling on epithelial cells, we utilized a mouse with a conditional deletion of *Act1* driven by the expression of an epithelial-specific protein K18, leaving Act-1 signaling intact in B cells. We induced DSS colitis in control mice (*K18creAct1*$^{fl/+}$) and epithelial-specific Act-1-deficient mice (*K18creAct1*$^{fl/-}$) and examined changes in the overall inflammatory response in the absence of IL-17-mediated Act-1 signaling in epithelial cells (Figure 3A). The expression of many inflammatory markers was upregulated in the *K18creAct1*$^{fl/-}$ mice compared to control mice. These data suggest that the Act-1 signaling pathway plays a role in IL-17-dependent maintenance of epithelial barriers by regulating the extent of inflammation during DSS-induced colitis. In addition, we found that mice with the epithelial-specific deficiency in Act-1 exhibited significantly increased gut permeability on day 3 after DSS-induced colitis, as measured by the presence of FITC-dextran in the serum (Figure 3B). Together, these data provide one possible mechanism by which IL-17 signaling directly on epithelial cells maintains mucosal integrity and functional epithelial tight junctions.

Early IL-17 in the Intestinal Lamina Propria Is Produced by a RAG- and RORγt-Dependent Population

In order to elucidate changes in proinflammatory cytokine expression in the absence of IL-17, we analyzed colon samples from WT and *Il17*$^{-/-}$ mice 3 days after DSS administration by RT-PCR. Expression of *Ifng*, *Il22*, *Il23a*, and *Tnf* was comparable between the two groups (Figure S4). To determine the specific contribution of cytokines from immune cells, colonic lamina propria lymphocytes (cLPLs) were isolated and stimulated overnight with either αCD3 or IL-23, and supernatants were analyzed for cytokine protein. WT cells produced IL-17A, IL-17F, IL-22, and IFN-γ in response to both stimuli (Figure 4A). Supernatants from cells deficient in IL-17A had no inherent defect in overall cytokine production; all protein concentrations were similar to WT mice, with the exception of IL-17A. Taken together, these data support the conclusion that the production of other cytokines by cLPLs is independent of the ability to produce IL-17A and suggest that the increased pathology in the colon of *Il17*$^{-/-}$ mice is due primarily to the absence of IL-17A.

We next sought to identify the primary source of IL-17 by comparing WT and *Rag1*$^{-/-}$ mice responses to DSS administration. We found that compared to WT cLPL cultures, RAG1-deficient cLPLs produced less IL-17A and IFN-γ in response to IL-23 or αCD3 stimulation (Figure 4B), suggesting that both of these cytokines are mainly secreted by a RAG1-dependent population. In contrast, IL-23, but not αCD3, induced

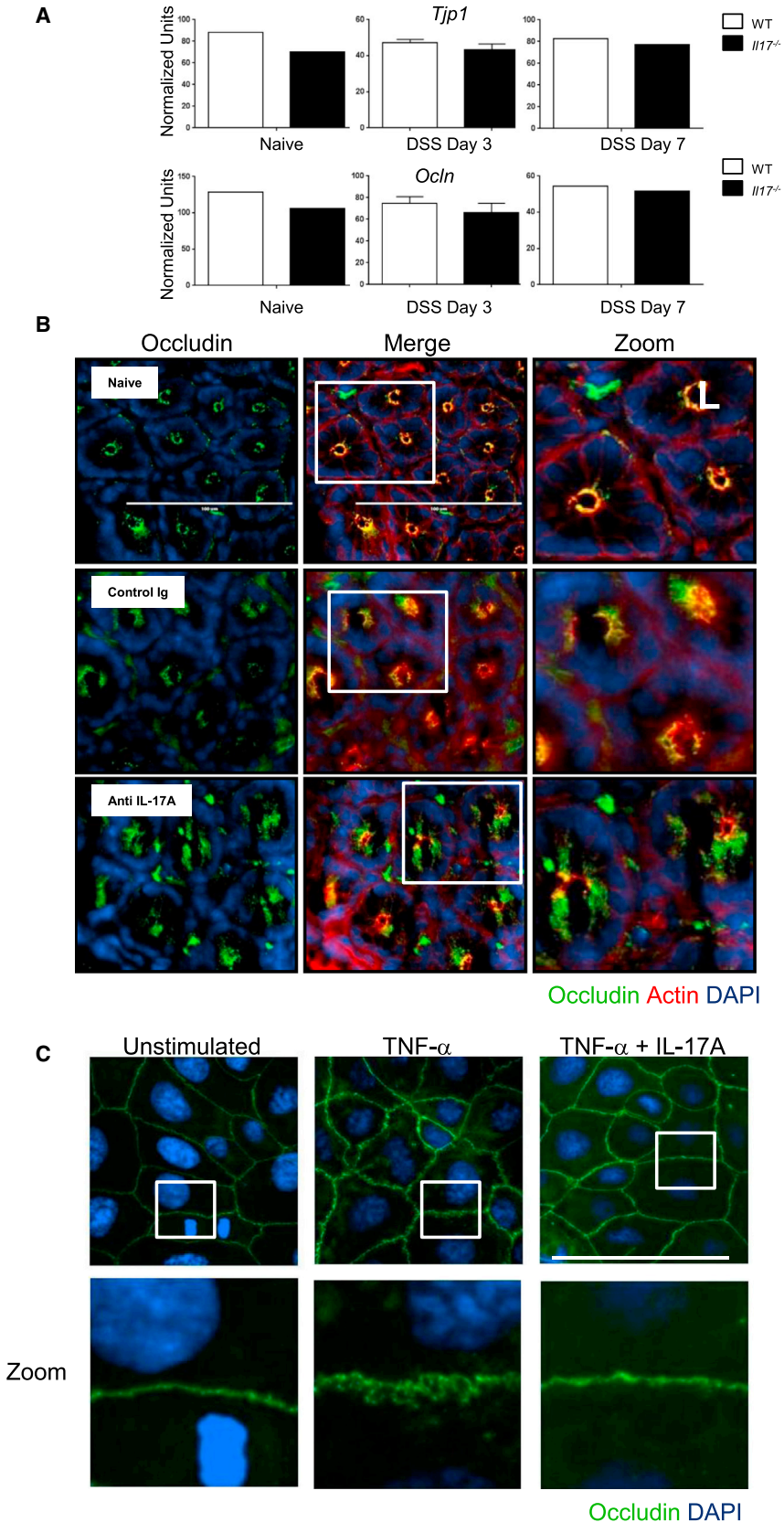

Figure 2. Dysregulation of Occludin Cellular Localization in the Absence of IL-17A after DSS-Induced Injury

(A) Colonic tissues were analyzed by RT-PCR for *Tjp1* and *Ocln* transcripts at day 3 and day 7 after DSS treatment. Representative data from two experiments. Error bars indicate SEM.

(B) C57BL/6 mice administered IgG or anti-IL-17A (20 mg/kg) 2 days before DSS treatment. Immunofluorescence images of occludin (green), F-actin (red), and DNA (blue) of distal colon segments 3 days after DSS. The third column represents a magnified image from the white box in the second column. Scale bars represent 100 μm. Images representative of three experiments. See also Figure S2.

(C) Caco-2 cells plated on poly-L-lysine-coated coverslips, treated with recombinant human TNF-α (10 ng/ml) or TNF-α (10 ng/ml) + recombinant human IL-17A (10 ng/ml) and cultured for 24 hr. Immunofluorescence images of occludin (green) and DNA (blue). The bottom row represents a magnified image from the corresponding white box in the first row. Scale bar represents 100 μm. Images are representative of three experiments. See also Figure S3.

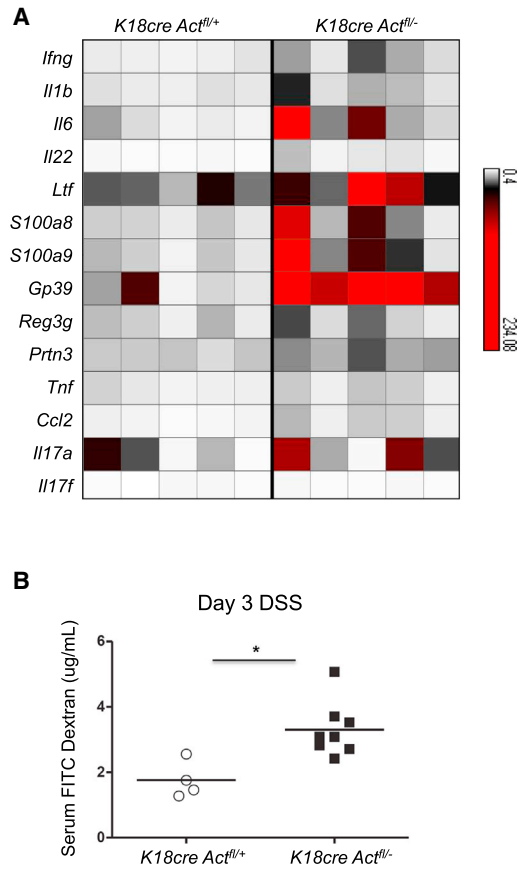

Figure 3. Enhanced Inflammatory Signature in the Absence of IL-17-Induced Act-1 Signaling in Epithelial Cells

(A) Epithelial cells from the distal colons of control or *K18creAct1*$^{fl/-}$ DSS-treated mice were analyzed by RT-PCR. Transcripts of inflammatory genes represented in a heat map as fold change over the amounts in naive mice. Each column represents an individual mouse; n = 5/group.

(B) Detection of FITC-dextran in plasma after 3 days of DSS. One of two experiments is shown; *p value = 0.01; means indicated.

IL-17F and IL-22 production in *Rag1*$^{-/-}$ mice similar to the concentrations observed in WT mice (Figure 4B). These data indicate that the induction of IL-17F and IL-22 is RAG-1 independent.

We have previously shown that production of IL-17A by CD4$^+$ T cells is dependent on the transcription factor RORγt (Ivanov et al., 2006). We sought to determine whether production of IL-17A by CD3$^+$ lymphocytes was dependent on RORγt by treating *Rorc*$^{-/-}$ mice with DSS for 3 days and examining the cytokine production in culture supernatants from isolated cLPLs. The absence of RORγt not only abrogated IL-17A and IL-17F production in response to αCD3 as expected but also significantly reduced IL-22 production after TCR ligation (Figure S5). Together these data provide evidence that RORγt is required for the trans-activation of IL-23 signaling and optimal production of IL-17A, IL-17F, and IL-22. Importantly, the results provided here implicate "innate" IL-17- and IL-22-secreting populations in the gut that require RORγt for their effector function and contribute to gut protection after injury.

γδ T Cells Are the Primary Source of Early IL-17A Production

Innate intestinal populations including ILC3s and tissue-resident γδ T cells are early sources of both IL-17 and IL-22 (Martin et al., 2009; Spits et al., 2013). In order to identify the specific contribution of the various RORγt-expressing population to IL-17 and IL-22 production, we isolated cLPLs from *Rorc-Gfp*^{TG} mice after DSS administration and used surface marker expression to phenotype the different populations. Using GFP expression to detect RORγt-expressing cells, we identified three major RORγt$^+$ populations in the colon after DSS treatment: CD3$^+$ γδ T cells, CD4$^+$ αβ T cells, and CD3$^-$ ILC3s (Figure 5A). Intracellular staining for IL-17A and IL-22 revealed that γδ T cells were the major source of IL-17A in the lamina propria after DSS-induced injury, with more than 30% of cells expressing the cytokine (Figures 5A and 5B) and very few IL-22-producing γδ T cells. In contrast to γδ T cells, ILC3s were the predominant source of IL-22 with approximately 50% of the population staining positive for this cytokine, and very few cells were positive for IL-17. CD4$^+$ T cells only had a minor contribution to IL-17 and IL-22 at this early time point. These data suggest that IL-17A and IL-22 are initially produced by distinct innate cell populations in the gut lamina propria in response to injury. In addition, we demonstrated that γδ T cells are the primary IL-17A-producing population. Thus, given their ability to rapidly respond to proteins and inflammatory cytokines (Cua and Tato, 2010), γδ T cells, as opposed to ILC3s, are important in maintaining epithelial integrity in our model through the secretion of IL-17.

In general, distinct subsets of γδ T cells exist and have unique function and homing capabilities, including the ability to produce either IL-17A or IFN-γ in response to a variety of stimuli (Martin et al., 2009; Ribot et al., 2009). We next determined the capacity of γδ T cells in the mucosal lamina propria to also produce IFN-γ after injury induction. When naive cLPLs were isolated and stimulated ex vivo with either PMA and ionomycin or IL-23, we found that whereas CD4$^+$ T cells were the largest IFN-γ-producing population, it was γδ T cells that again were the major IL-17A producers (Figure 5C). In addition, after DSS treatment, the γδ T cells were still the main source of IL-17A protein (Figures 5C and 5D). Although γδ T cells were the major producers of IL-17A in the colon lamina propria, a minor population of CD4$^+$ T cells was also positive for this cytokine ex vivo, particularly after PMA and ionomycin stimulation. To test the specific contribution of γδ T cells in barrier function during acute DSS injury, we utilized *Tcrd*$^{-/-}$ mice for our studies. We did not find any γδ T cells in the colon lamina propria of *Tcrd*$^{-/-}$ mice (Figure 5E) and did not observe any compensatory increase in the frequency of IL-17-producing CD4$^+$ T cells or CD4$^-$CD8$^-$ double-negative T cells (Figure 5F). Consistent with our hypothesis of a protective role for γδ T cells during DSS injury, *Tcrd*$^{-/-}$ mice exhibited increased FITC dextran permeability (Figure 5G). Finally, we did not observe a protective role for CD4$^+$ T cells, as their depletion in vivo did not impact gut permeability (Figure 5G). These data provide evidence that γδ T cell populations in the gut lamina propria are readily able to produce IL-17A protein upon activation and that these innate T lymphocytes are the early source of tissue-protective IL-17A during gut epithelial injury.

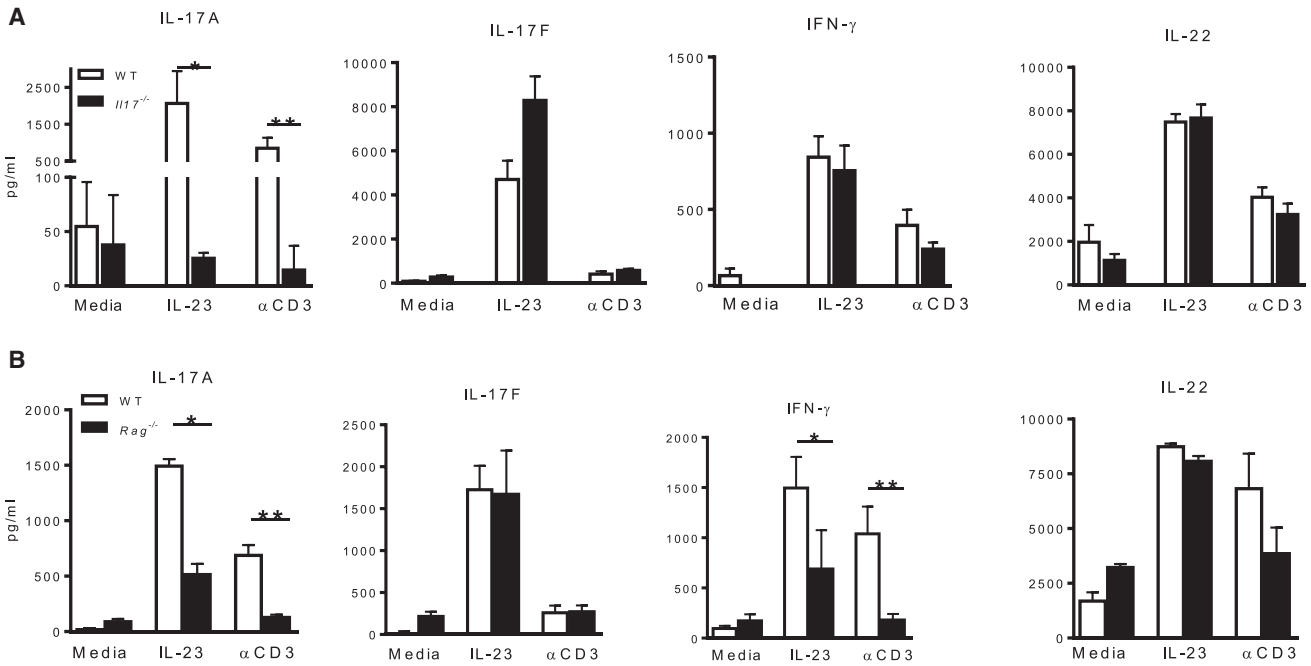

Figure 4. Cytokine Production by Colonic Lamina Propria Cells after DSS-Induced Injury

Colonic LPLs restimulated with media, IL-23, or αCD3 overnight and supernatant analyzed by ELISA or luminex to determine concentrations of IL-17A, IL-17F, IFN-γ, and IL-22.

(A) Cytokine protein concentrations detected in supernatants of cLPLs isolated from either WT (open bars) and *Il17*$^{-/-}$ (closed bars) mice, *$p < 0.016$; **$p = 0.007$. Data are representative of two independent experiments. Error bars indicate SEM. See also Figure S4.

(B) WT and *Rag*$^{-/-}$ mice: IL-17A, *$p = 0.0001$, **$p < 0.005$; IFN-γ, *$p < 0.048$, **$p < 0.006$. Data are representative of two independent experiments. Error bars indicate SEM. See also Figure S5.

IL-17 Production by γδ T Cells Is Independent of IL-23 Signaling

Having shown that the tissue-resident RORγt$^+$ γδ T cells are potent producers of IL-17 in response to IL-23, we wanted to determine whether IL-23 signaling was required for the early IL-17 production during injury. To do this, we made use of the *Il23r-Gfp* homozygous genetically targeted mice that allowed us to compare IL-17 production in the presence or absence of IL-23R expression. GFP$^+$ cells isolated from *Il23r-Gfp*$^{+/-}$ and *Il23r-Gfp*$^{+/+}$ littermates after DSS administration were restimulated ex vivo and compared for IL-17 production. Significantly less IL-17 production was observed in the GFP$^+$ cLPLs from *Il23r-Gfp*$^{+/+}$ than from *Il23r-Gfp*$^{+/-}$ mice (Figure 6A), indicating that IL-23 does contribute to a portion of the overall IL-17 production early after injury. Although there was a significant reduction in the number of γδ T cells in the colon in the absence of IL-23R signaling, the proportion of IL-17$^+$ cells was comparable between strains (Figure 6B). In contrast, no difference was found in either the proportion of CD4$^+$ cells present in the colon or in the percentage of IFN-γ$^+$ cells whether or not IL-23R was present (Figures S6A and S6B). We hypothesized that the IL-23-independent γδ T cells would still be able to produce protective IL-17 and confer barrier function in the absence of IL-23R signaling during DSS injury. Although both WT and *Il23r*$^{-/-}$ mice rapidly lost weight (Figure 6C) and exhibited epithelial disruption and the presence of abscesses, *Il23r*$^{-/-}$ mice did not exhibit a decrease in barrier function after administration of DSS at both day 3 and day 7 (Figure 6D). Serum FITC dextran concentration was lower in *Il23r*$^{-/-}$ mice than in WT animals at day 3. In support of the maintenance of barrier function, *Il23r*$^{-/-}$ mouse colon epithelial cells did not exhibit cytoplasmic localization of occludin but had a staining pattern similar to WT epithelial cells in the crypts (Figure 6E). These data suggest that the early dysregulation of barrier function during DSS is dependent on the absence of IL-17, but not IL-23. Barrier function in IL-23R-deficient mice was comparable to WT mice, if not enhanced at early time points, and we show that in contrast to neutralizing IL-17A alone, targeting IL-23 preserves protective IL-17A produced by IL-23R-independent colonic γδ T cells, which promotes maintenance of epithelial barrier function.

DISCUSSION

IL-17 promotes the expression of proteins in mucosal tissues that are involved with barrier function during an infection with pathogenic organisms (Kao et al., 2004; Kinugasa et al., 2000; Ogawa et al., 2004). Some of these proteins, such as cellular tight-junction protein, ZO-1, become de-localized early after DSS treatment and this is thought to be a marker of epithelial injury (Poritz et al., 2007). In addition to these studies, our data have shown that the absence of IL-17A results in increased epithelial injury and compromised barrier function after DSS treatment. We have shown that IL-17A regulated the cellular localization of the tight junction protein occludin within the crypts

of colon epithelial cells during injury, and IL-17R-dependent activation of Act-1 in epithelial cells prevented excess inflammation after DSS. Mice with a conditional deletion of Act-1 in epithelial cells showed an increase in gut permeability after DSS, reinforcing the protective role for IL-17 in barrier function. TNF-α-mediated barrier disruption in Caco-2 monolayers was reversed with the addition of recombinant IL-17A, suggesting a direct role for IL-17 and IL-17R signaling on epithelial cells in the regulation of occludin. IL-17 is a key regulator of antimicrobial responses at mucosal surfaces, and in cooperation with IL-22 induces the expression of antimicrobial proteins including beta defensins, calgranulin, and lipocalin (Kolls et al., 2008). IL-17 contributes to defense against the gram-negative pathogens *Klebsiella pneumonia* and *Citrobacter rodentium* as well as having an essential role in mucocutaneous immunity against *Candida albicans* (Puel et al., 2011). Given the role of IL-17 in antimicrobial responses, there is the possibility of an altered microbiota in IL-17-deficient mice contributing to increased gut permeability in the DSS injury model. Although these concepts need to be further investigated, the data presented here demonstrate one potential mechanism by which IL-17A signaling through Act-1 supports barrier function: by maintaining occludin localization at tight junctions during DSS injury.

IL-17 has generally been classified as a pathogenic cytokine in a variety of inflammatory diseases. Although IL-17 and IL-17R signaling was shown to contribute to pathogenicity in a DSS model of colitis (Qian et al., 2007), it is important to note that the data we present suggest a protective role for IL-17A during an acute model of gut injury. In fact, in the clinical trial with sekukinumab for Crohn's disease (Hueber et al., 2012), it was specifically the patients with elevated CRP concentrations that experienced a treatment-associated worsening of disease, suggesting an important protective role for IL-17 during an acute and active inflammatory response, consistent with our findings in the acute DSS injury model. Chronic and long-term production of IL-17 mediates inflammatory effects and neutrophil recruitment into the gut, and a more subtle, low-dose chronic DSS injury model could allow us to address how long-term increases in gut permeability and IL-17 can influence IBD disease course. We have identified an important function of IL-17 modulating TNF-α-driven disruptions in epithelial tight junction proteins, and whether this applies in the clinic needs to be tested.

Although the protective role of IL-17A has been discussed in the literature, few studies have analyzed specific IL-17A-producing cell populations for their functional significance within the inflamed tissue. Our results involving the gut mucosa concur with previously published work with lung and skin models in which subsets of γδ T cells are important for barrier protection (D'Souza et al., 1997; Jameson et al., 2002; Nakasone et al., 2007). In addition to their protective role in the lung and skin, we have provided evidence in this study that γδ T-cell-derived IL-17A in the colonic lamina propria also provided protection within the colon during DSS injury. We have shown that colonic γδ T cells were the primary producers of gut-protective IL-17A and were dependent on RORγt but not IL-23 for their function. RORγt$^+$ T helper 17 (Th17) cells are also characterized by the production of IL-17. However, in our animal housing conditions, we found that CD4$^+$ Th17 cells made up a very minor subset of IL-17-producing cells at 3 days after DSS treatment. In contrast to the subset of gut γδT cells that produced constitutive IL-17, CD4$^+$ T cells required PMA and ionomycin stimulation ex vivo to detect IL-17, which might not be representative of their production in vivo. Further confirming the minor contribution of CD4$^+$ T-cell-derived IL-17, depleting CD4$^+$ T cells did not have an impact on FITC dextran permeability, whereas *Tcrd*$^{-/-}$ mice showed a clear increase in gut leakiness. The *Tcrd*$^{-/-}$ study combined with the CD4$^+$ T cell depletion experiment demonstrate the critical function of IL-17-producing γδ T cells in protecting barrier surfaces. Our analysis of colonic tissue after early injury confirms the existence of a protective role for γδ T-cell-derived IL-17A in the colon that works to limit permeability of the epithelial barrier in order to reduce common complications of mucosal injury, such as excessive edema and anemia.

Our data show that IL-22 production, often associated with IL-17-secreting cells, is independent of the ability to produce IL-17A (Liang et al., 2006; Veldhoen et al., 2008). Colonic LPLs isolated from *Il17*$^{-/-}$ mice and re-stimulated with IL-23 after injury led to normal IL-22 production despite the absence of IL-17. It is tempting to speculate whether the balance between IL-17 and IL-22 can mediate their protective or pathogenic effects on the epithelium. This interplay between IL-17 and IL-22 has previously been demonstrated in lung epithelial cells (Sonnenberg et al., 2010). However, further work will need to be done to determine whether the pathogenic or protective effect of IL-22 in the gut epithelium can be regulated depending on the presence or absence of IL-17A. Although IL-22 is generally thought to be protective at mucosal surfaces, in certain circumstances it is known to promote inflammation. IL-22 has been shown to stimulate epithelial cell production of chemokines that recruit inflammatory myeloid cells including CXCL1, CXCL2, and CXCL5 (Eken et al., 2013), as well as the proinflammatory cytokine IL-18 (Muñoz et al., 2015). Thus, uncontrolled production of IL-22 in concert with other cytokines might contribute to inflammation. We have demonstrated that neutralizing IL-23R signaling in a T cell transfer model of colitis was efficacious in limiting disease, even when treated at later time points, whereas neutralizing IL-17 recapitulated many of the similar phenotypes we observed in the DSS model of colitis, including increased gut permeability and weight loss. In contrast to IL-23, these two different mouse models of gut damage and human clinical trials consistently demonstrate a protective role for IL-17.

Although IL-23 is an important cytokine for driving IL-17 responses, we have shown that the early protective IL-17A is independent of IL-23 and that the absence of IL-23 signaling does not lead to excessive barrier dysfunction during DSS injury. These findings provide a potential explanation of the dramatic differences between neutralizing IL-23 and IL-17A in DSS injury and epithelial permeability. Although there was a significant decrease in the frequency of IL-17-producing cells in IL-23R-deficient animals, IL-23-independent production of protective IL-17 was still intact in the γδ population found in the colonic tissue. We propose that neutralizing IL-23 minimizes tissue inflammation and T cell and myeloid cell activation while leaving the protective function of IL-17 from γδ T cells intact. This early IL-17 production by γδ T cells in our model establishes this population as a major player in limiting inflammation-induced damage in gut epithelium after an injury through an Act1-dependent mechanism of protection. It is notable that γδ T intra-epithelial

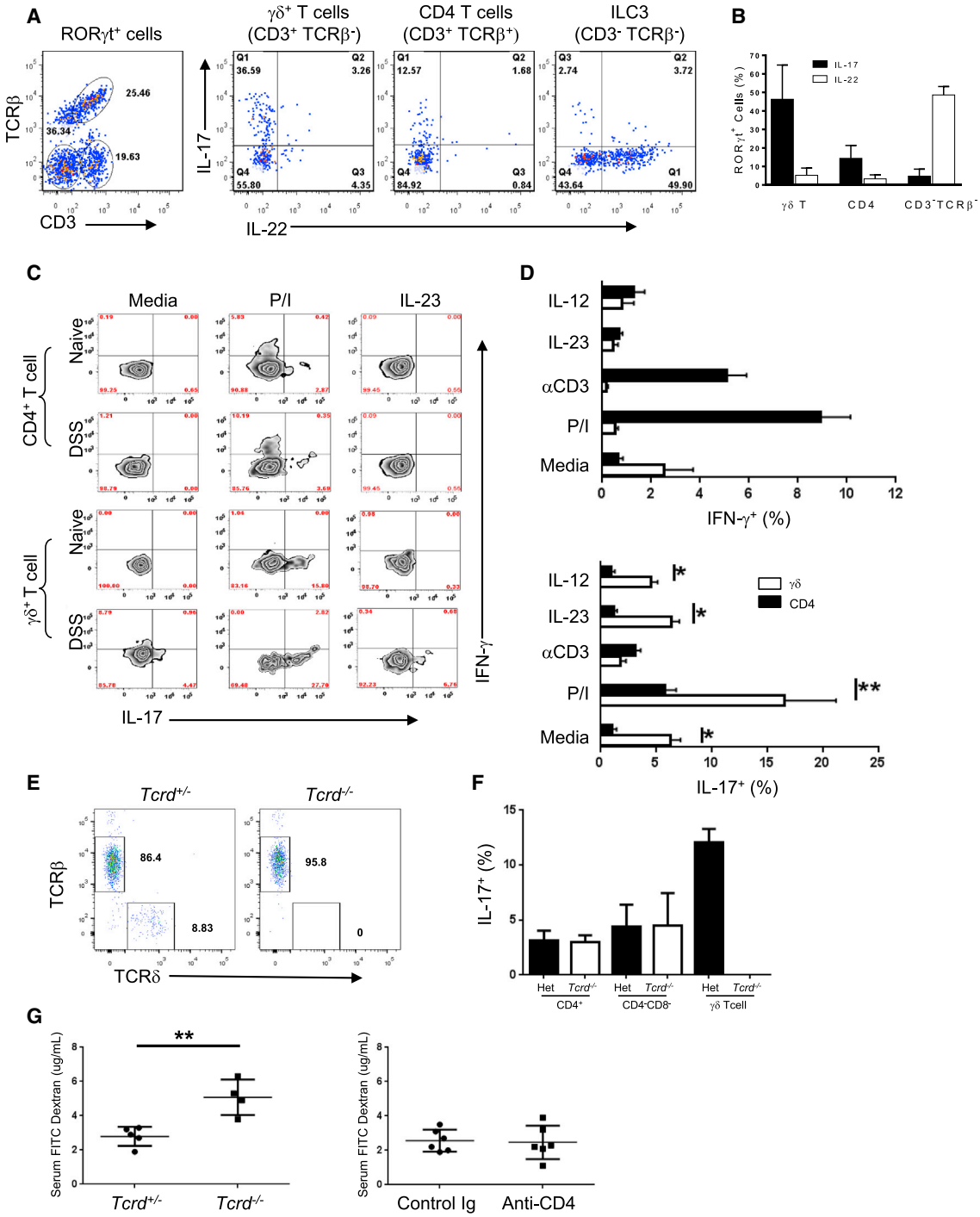

Figure 5. Different Subsets of cLPLs Produce either IL-17 or IL-22 after Restimulation

(A) Dot plots of the three major RORγt-expressing populations from cLPLs isolated after 3 days of DSS. Intracellular cytokine analysis of cLPLs showing IL-17 versus IL-22 production for each population after 3 hr of restimulation in vitro. Representative dot plots from three independent experiments are shown.

(B) Percentage of RORγt-GFP$^+$ cells expressing either IL-17 or IL-22 by population, combined data from two experiments are shown, means ± SD.

(C) Detection of intracellular IL-17 (x axis) and IFN-γ (y axis) in cLPLs from either naive or day 3-DSS-treated mice after 3–4 hr restimulation ex vivo. Representative zebra plots from two independent experiments show either a CD4$^+$ T cell gate (top two rows) or a γδ$^+$ T cell gate (bottom two rows).

(D) Bar graph showing percentage of either CD4$^+$ or γδ T cells from DSS-treated mice that are positive for IFN-γ (top) and IL-17 (bottom), combined data from two experiments, with means ± SEM (*$p < 0.0001$; **$p < 0.04$). Two-tailed, Student's t test performed.

(E) Colon LPLs from *Tcrd*$^{+/-}$ and *Tcrd*$^{-/-}$ mice gated on live, CD3$^+$Thy1$^+$ cells. Representative of two independent experiments.

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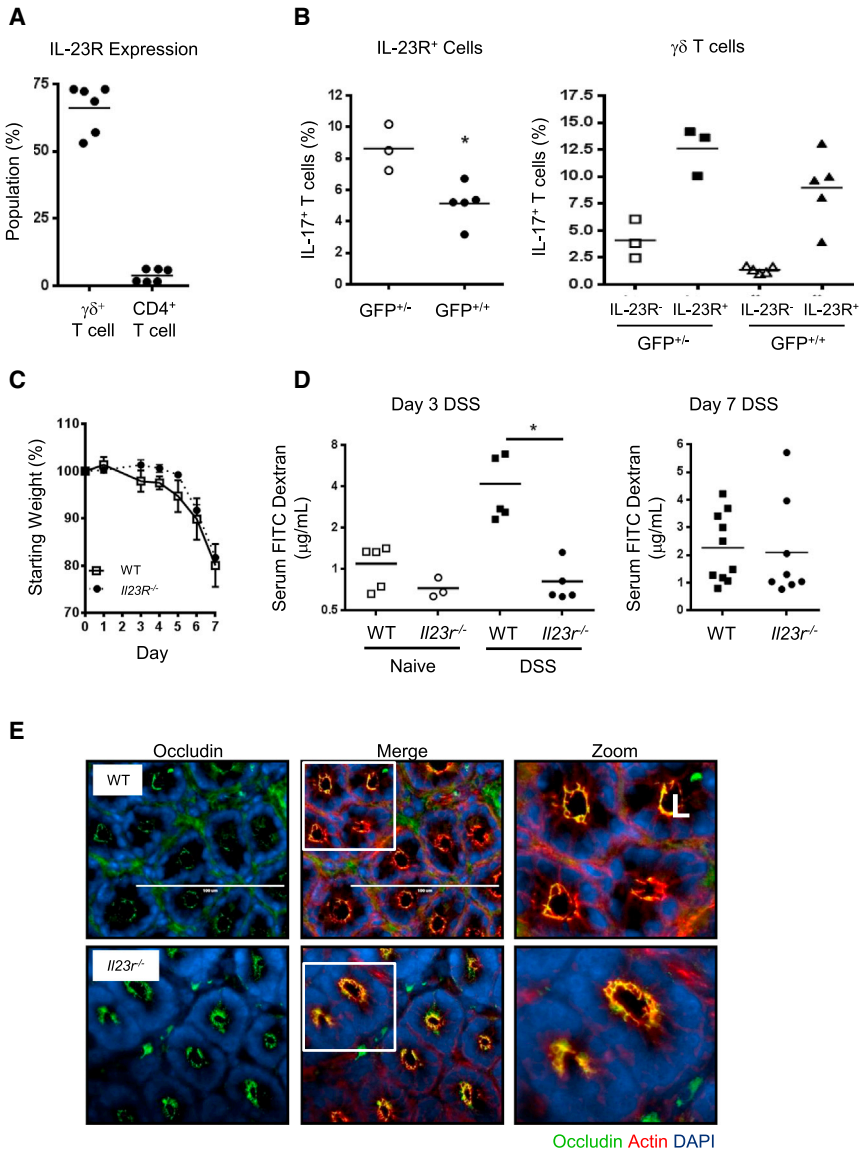

Figure 6. IL-23R-Deficient Mice Do Not Exhibit Increased Gut Permeability after DSS Treatment

(A) IL-23R expression in different lamina propria lymphocyte populations as determined by *Il23r-Gfp* reporter using *Il23r-Gfp*$^{+/-}$ mice, combined data from two experiments; means indicated.

(B) IL-17 production is decreased overall in the absence of IL-23R signaling as shown by comparison of *Il23r-Gfp*$^{+/-}$ (GFP$^{+/-}$) (IL-23R present) and *Il23r-Gfp*$^{+/+}$ (GFP$^{+/+}$) (IL-23R absent) mice for all GFP^{+} cLPLs (left) or γδ T cells only (right). Data from two experiments; means indicated. See also Figure S6.

(C) Weight loss of WT (squares) and *Il23r*$^{-/-}$ (circles) mice over time during DSS treatment, representative data from three independent experiments, n = 5–7/group, mean ± SEM.

(D) Detection of FITC-dextran in serum after 3 and 7 days of DSS. Representative data from two experiments, or combined data from two experiments (day 7), means indicated, *p < 0.01. All statistics generated by one-way ANOVA, with Tukey's multiple comparisons.

(E) Immunofluorescence images of occludin (green), f-actin (red), and DNA (blue) of distal colon segments from WT or *Il23r*$^{-/-}$ mice 3 days after DSS. The third column represents a magnified image from the white box in the second column. Representative of three independent experiments.

lymphocytes (IELs) can also stimulate mucosal healing after 6 days of DSS treatment and recruit macrophages to injured areas to prevent commensal penetration after damage (Ismail et al., 2009; Pull et al., 2005). Therefore, it is clear from these studies that there exist multiple mechanisms by which γδ T cells and their secretion of IL-17A are important for the maintenance, protection, and repair of epithelial barriers in intestinal mucosa.

Recently, a clinical study tested the efficacy of an anti-IL-17A monoclonal antibody in treatment of Crohn's disease. Blockade of IL-17A in these patients exacerbated their disease and was characterized by an increase in inflammatory markers over baseline (Hueber et al., 2012). Furthermore, for a subset of patients taking the drug, 44% developed additional infections, whereas none in the placebo group had an adverse event related to infection (Hueber et al., 2012). Similar results were obtained in a separate study targeting IL-17RA (Targan et al., 2012), further confirming the deleterious effects of neutralizing IL-17A or IL-17 signaling for the treatment of CD. Although elimination of IL-17A has been efficacious in other inflammatory disorders such as psoriasis, its failure in the treatment of CD is yet another cautionary tale of the pleiotropic effects of cytokines used as therapeutics (Shen and Durum, 2010). These clinical studies are a strong endorsement of the protective effects of IL-17A in human intestinal mucosa and provide further support for the data presented within this paper.

EXPERIMENTAL PROCEDURES

Mice

Il17$^{-/-}$, *Rorc*$^{-/-}$, *Rorc(γt)-Gfp*^{TG}, *Il23r-Gfp*, and *Tcrd*$^{-/-}$ mice were derived as previously described and bred and housed within micro isolator caging units

(F) Colon LPLs as in (E), stimulated with PMA and ionomycin and gated on the populations indicated. Representative of two independent experiments. Error bars indicate SEM.

(G) Detection of serum FITC dextran after 3 days of DSS. Representative of two independent experiments. Error bars indicate SEM. **p < 0.004. Two-tailed, unpaired t test.

at MRL (Awasthi et al., 2009; Ivanov et al., 2006; Lochner et al., 2008; Nakae et al., 2002). *Rag1*$^{-/-}$ and WT C57BL/6 or littermate controls were used as age- and sex-matched controls where indicated (Jackson Laboratories). *K18creAct1*$^{fl/-}$ mice were provided by Xiaoxia Li and have been described previously (Qian et al., 2007; Swaidani et al., 2009) All animal procedures were approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

DSS and FITC-Dextran Administration

Mice were given 3.5% DSS in drinking water for 3 days before lamina propria cells were isolated from the colons for in vitro culture or a 1 cm length of the distal colon was snap frozen for taqman analysis. Alternatively, DSS drinking water was administered for 7 days and mice were monitored for weight loss and signs of disease. RNA isolation and real-time PCR was performed as previously described (Fehniger et al., 1999; McGeachy et al., 2009). Mice were gavaged with FITC-Dextran (4 kDa, Sigma Aldrich) as previously described (Dawson et al., 2009) 3 hr prior to fluorometric analysis of FITC fluorescence in plasma.

T-Cell-Driven Colitis

Spleen cells from BL/6 mice were processed through 100 μm nylon filters and purified for CD4 using magnetic bead separation (Myltenyi). $CD3^+CD4^+CD25^-CD45RB^{hi}$ T cells were sorted with FACS Aria (BD). The cells (3×10^5) were injected intravenously, and mice were monitored and weighed for 5 weeks after injection. At the study endpoint, mice were sacrificed, and serum and intestines were collected for analysis. Anti-IL-23R (Merck, mouse IgG1) and anti-IL-17A (Merck, mouse IgG1) and isotype control (Merck, mouse IgG1) were administered s.c. at day 14 after transfer at a dose of 50 mpk, and once weekly until 42 days after transfer.

Histology

Dissected colons were fixed in 10% NBF before embedding in paraffin. Hematoxylin and eosin staining was performed on sections of tissue for grading of pathology. All images were acquired with the Zeiss Mirax Midi scanner with a 20× objective.

Immunofluorescence Microscopy

Distal colons were flushed with PBS, embedded in Tissue-Tek O.C.T. compound (SAKURA Finetechnical Company) in cryomolds, and snap frozen in liquid nitrogen for cryosectioning. Cryosections were prepared on a Leica Cryostat (Leica Microsystems) at −21°C in 5 μm thickness. Sections were mounted on glass slides and fixed in 100% ethanol at 4°C for 30 min followed by 3 min of −20°C acetone fixation at room temperature. The slides were washed in PBS and blocked in FBS and rabbit serum. The tissue sections were stained with a monoclonal occludin antibody OC-3F10 (Life Technologies) at 4°C overnight. After washing in PBS, the sections were stained with a rabbit anti-mouse IgG Alexa Fluor 488 conjugated secondary antibody (Life Technologies) and Alexa Fluor 546 Phalloidin (Life Technologies) for 60 min at room temperature. The tissue sections were treated with ProLong Mountant with DAPI (Life Technologies) and covered with a coverslip. Fluorescence was visualized on the EVOS FL Cell Imaging System (Life Technologies) at 40× magnification.

Colonic Lamina Propria Cell Isolation

Colons were removed from naive and DSS-treated mice and epithelial cells were stripped by incubating in a 37°C water bath in cell dissociation solution made with HBSS (BioWhittaker), 5 mM EDTA (Invitrogen), and 10 mM HEPES (Life Technologies). Supernatant with IEL and epithelial cells was discarded and colonic tissue was then incubated in a digestion cocktail containing HBSS, 10% FCS (Hyclone Laboratories), 1 mg/ml collagenase type IV, 0.5 mg/ml DNaseI, and 0.5 mg/ml dispase (all from Sigma-Aldrich) in a 37°C water bath. Digested tissue was processed through a 70 μm filter and washed before lymphocytes were separated using a percoll gradient (GEHealthcare) and resuspended in complete RPMI (Mediatech) supplemented with 10% FCS (Hyclone), 1% HEPES, 50 μM 2-mercaptoethanol (Invitrogen), 1% sodium pyruvate, and penicillin and streptomycin (both from Mediatech).

Cell Culture

Colonic lamina propria cells were isolated as above and put into single-cell suspensions at 1 or 2×10^6 cells/ml. Cells were plated for a final volume of 200 μl and restimulated with media alone, 40 ng/ml of IL-23 (DNAX), 10 ng/ml of IL-12 (DNAX), or 4 μg/ml of αCD3 (BioXcell) for 20 hr or with PMA and ionomycin for 3 hr. Caco-2 [Caco2] (ATCC HTB-37) were cultured in Eagle's Minimum Essential Medium in 20% FBS according to ATCC instructions.

Flow Cytometry

For intracellular cytokine staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (both Sigma-Aldrich) in the presence of Golgi-plug (BD Biosciences) for 4 hr in complete medium. Surface staining was then performed in the presence of Fc-blocking antibodies (2.4G2, BD) and using αCD3 (500A2, BD), αCD4 (RMU-5, BD), αTCRβ (H57-597, BD), α-γδTCR (UC7-13D5, eBioscience; or GL3, BD), and α-NKp46 (29A1.4, eBioscience). Cells were then fixed and permeabilized with cytofix-cytoperm kit (BD) as directed before intracellular staining using antibodies against IFN-γ, IL-22, and IL-17A (all from BD). All samples were collected with Canto II (BD) and data were analyzed with FlowJo software (Tree Star).

RNA Isolation

For gene expression analysis, RNA was isolated by homogenizing colons into RNA STAT-60 (Tel-Test) using a polytron homogenizer, then extracting total RNA according to the manufacturer's instructions. After isopropanol precipitation, total RNA was re-extracted with phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich) using phase-lock light tubes (5 Prime, Thermo Fisher Scientific). For DSS-treated samples, total RNA was isolated using RNeasy Midi method (QIAGEN), where tissue was first homogenized into RLT Buffer plus beta-Mercaptoethanol using a polytron homogenizer, and according to manufacturer's instructions. Total RNA was then re-cleaned over Rneasy Mini columns (QIAGEN) according to manufacturer's protocol.

Real-Time Quantitative PCR

DNase-treated total RNA was reverse-transcribed with QuantiTect Reverse Transcription (QIAGEN) according to manufacturer's instructions. Primers were designed with Primer Express (Applied Biosystems, Life Technologies) or obtained commercially from Applied Biosystems. Gene-specific preamplification was done on 10 ng cDNA per Fluidigm Biomark manufacturer's instructions (Fluidigm). Real-time quantitative PCR was then done on the Fluidigm Biomark using one of two different chemistries. For the first chemistry, two gene-specific unlabelled primers were utilized at 400 nM with Taqman Gene Expression Master Mix plus EvaGreen. For the second chemistry, two unlabelled primers at 900 nM each were used with 250 nM of FAM-labeled probe (Applied Biosystems, Life Technologies) with Taqman Universal PCR Master Mix with UNG. Samples and primers were run on 96.96 Array(s) per manufacturer's instructions (Fluidigm). The absence of genomic DNA contamination was confirmed with primers that recognize genomic region of the CD4 promoter. Ubiquitin transcripts was measured in a separate reaction and used to normalize the data by the Δ-Δ Ct method. (Using the mean cycle threshold value for ubiquitin and the gene of interest for each sample, the Equation 1.8 ^ (Ct ubiquitin minus Ct gene of interest) $\times 10^4$ was used to obtain the normalized values.)

Luminex

Plasma cytokine concentrations were measured with Luminex system (Millipore Corporation). Plasma samples were collected and spun at 6,000 rpm for 15 min at 4°C and was collected and stored at −80°C analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.09.003.

AUTHOR CONTRIBUTIONS

Conceptualization, C.M.T. and D.J.C.; Methodology, J.S.L., C.M.T., and D.J.C.; Investigation, J.S.L., C.M.T., B.J.-S., F.G., C.C., Y.C., W.M.B., M.J.,

G.A., and T.K.M.; Writing – Original Draft, J.S.L., C.M.T., and D.J.C.; Writing – Review & Editing, J.S.L. and D.J.C.; Resources, X.L.; Supervision, D.J.C.

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