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Interleukin-17B Antagonizes Interleukin-25-Mediated Mucosal Inflammation

Highlights

- IL-25 expression is pathogenic for mucosal inflammation
- IL-17B expression is protective against colitis and asthma
- IL-17B inhibits the pro-inflammatory function of IL-25

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In Brief

Among the IL-17 cytokines, IL-17B has long been a functional mystery. Dong et al. demonstrate that IL-17B is expressed by colon epithelial cells and that IL-17B is able to antagonize the pro-inflammatory function of IL-25 by binding the same target receptor. Furthermore, IL-17B is protective whereas IL-25 is pathogenic in colitis.

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Interleukin-17B Antagonizes Interleukin-25-Mediated Mucosal Inflammation

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SUMMARY

The interleukin-17 (IL-17) family of cytokines has emerged as a critical player in inflammatory diseases. Among them, IL-25 has been shown to be important in allergic inflammation and protection against parasitic infection. Here we have demonstrated that IL-17B, a poorly understood cytokine, functions to inhibit IL-25-driven inflammation. IL-17B and IL-25, both binding to the interleukin-17 receptor B (IL-17RB), were upregulated in their expression after acute colonic inflammation. Individual inhibition of these cytokines revealed opposing functions in colon inflammation: IL-25 was pathogenic but IL-17B was protective. Similarly opposing phenotypes were observed in *Citrobacter rodentium* infection and allergic asthma. Moreover, IL-25 was found to promote IL-6 production from colon epithelial cells, which was inhibited by IL-17B. Therefore, our data demonstrate that IL-17B is an anti-inflammatory cytokine in the IL-17 family.

INTRODUCTION

Members of the interleukin-17 (IL-17) family of cytokines have recently emerged as critical players in inflammation. IL-17A and IL-17F are predominately expressed by CD4$^+$ T helper 17 (Th17) cells, but can be produced by other lymphocytes as well (Dong, 2008; Cupedo et al., 2009; Takatori et al., 2009; Reynolds et al., 2010; Sawa et al., 2010). Recently, IL-17C and its receptor IL-17 receptor E (IL-17RE) have been described to regulate Th17 cell responses and epithelial cell-dependent colon immunity (Chang et al., 2011; Ramirez-Carrozzi et al., 2011; Song et al., 2011; Reynolds et al., 2012). IL-25 (IL-17E) is unique in that it induces T helper 2 (Th2) cell-mediated inflammation (Fort et al., 2001; Angkasekwinai et al., 2007; Zaph et al., 2008). IL-25 thus is protective against parasitic infection and is produced by multiple cell types (Fallon et al., 2006; Owyang et al., 2006; Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010). IL-25 signals through a heterodimeric receptor comprised of IL-17 receptor A (IL-17RA) and IL-17 receptor B (IL-17RB), both of which are necessary for IL-25 to promote cytokine production from target cells (Rickel et al., 2008; Angkasekwinai et al., 2010). Outside of parasitic infection, much less is known about the roles of IL-25 in the colon. Injection of IL-25 throughout the course of DSS-induced colitis results in a protective response; mice exhibit less inflammation and increased survival (Mchenga et al., 2008). Conversely, in a model of oxazolone-induced colitis, neutralizing IL-25 or IL-17RB can decrease colonic inflammation (Camelo et al., 2012). Finally, commensal flora drives the expression of IL-25 in the colon, which might serve to limit the numbers of Th17 cells and IL-22-expressing RORγt$^+$ group 3 innate lymphoid cells (Zaph et al., 2008; Sawa et al., 2011).

Little is known about IL-17B. The initial cloning and characterization reveals that human IL-17B can induce tumor necrosis factor α (TNF-α) and IL-1β production by THP-1 cells (Li et al., 2000). Moreover, IL-17B is substantially expressed in the paws of arthritic mice and polyclonal anti-IL-17B treatment ameliorates collagen-induced arthritis (Yamaguchi et al., 2007). Indeed, mice injected with IL-17B exhibit a neutrophilia phenotype similar to those injected with IL-17A (Schwarzenberger et al., 1998; Shi et al., 2000). However, IL-17B and IL-25 share a common receptor, IL-17RB (Shi et al., 2000; Huang et al., 2014), suggesting that there might be unexplored and unique functions of IL-17B, although the affinity of IL-17B for IL-17RB is weaker compared to IL-25 (Lee et al., 2001). Non-immune functions have been attributed to IL-17B as well, including roles in development (You et al., 2005), fracture (Kokubu et al., 2008), and cancer (Sanders et al., 2010; Huang et al., 2014).

Given that IL-17RB is expressed by mucosal epithelial cells (Shi et al., 2000; Lee et al., 2001; Zhao et al., 2010), we have investigated the potential role of IL-17B and IL-25 in the regulation of mucosal inflammation. Opposing disease phenotypes by IL-17B- and IL-25-deficient animals were observed in models of

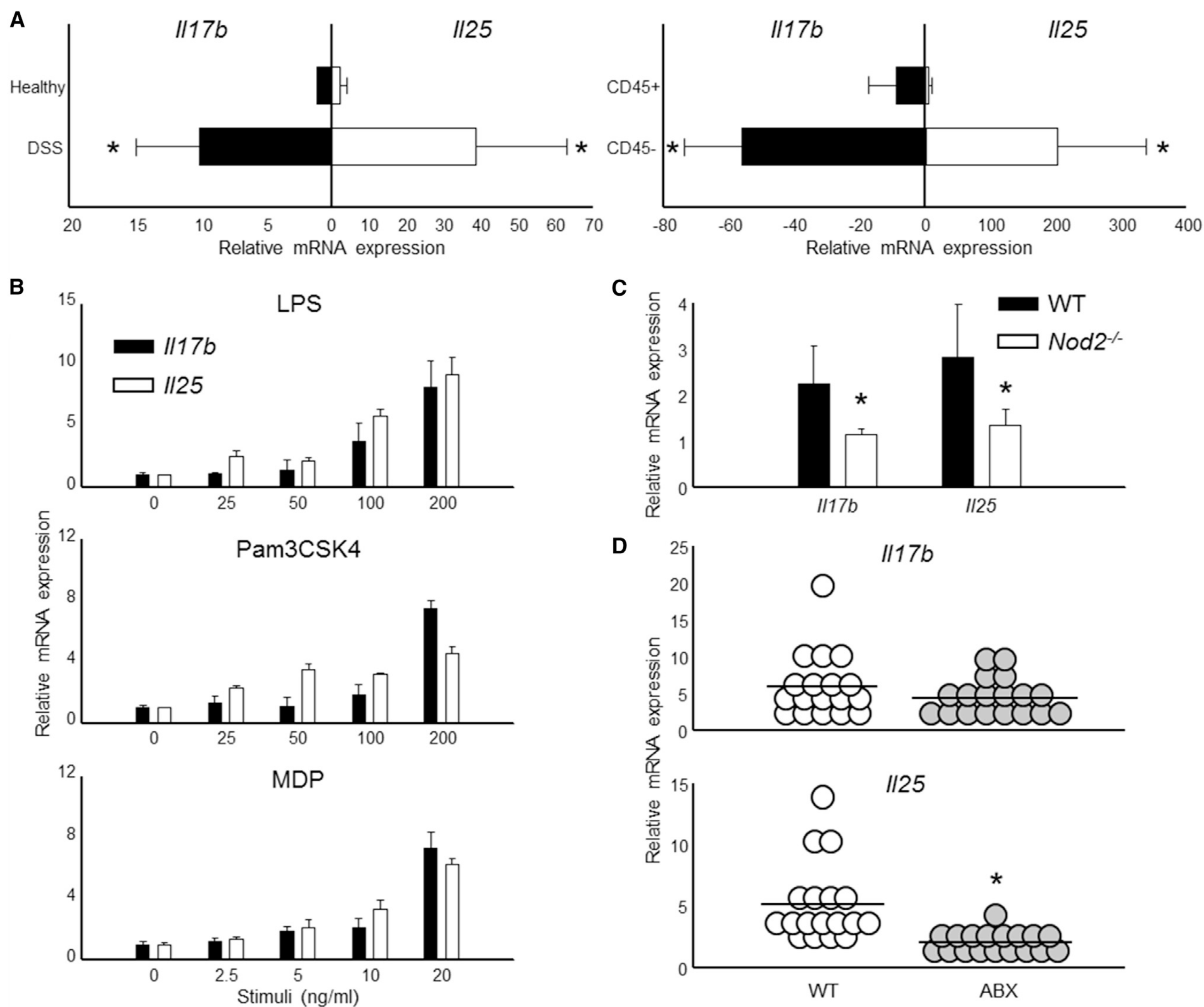

Figure 1. Colon Epithelial Cells Produce IL-17B and IL-25 in Acute Colitis

(A) Colon samples were derived from healthy controls or from animals after 8 day DSS-induced colitis (left). DSS samples were further processed and separated into leukocyte ($CD45^+$) and epithelial cell ($CD45^-$) fractions (right). The relative expression of *Il17b* and *Il25* was then measured by qPCR. n = 5 mice per group.

(B) Primary colon epithelial cells derived from healthy mice were activated through TLR (LPS, Pam3CSK4) and NOD2 (MDP) pathways for 6 hr. *Il17b* and *Il25* mRNA expression was assessed by real-time RT-PCR.

(C) Total colon tissue was isolated from healthy WT and NOD2-deficient animals and then assessed for *Il17b* and *Il25* expression by qPCR. n = 5 mice per group.

(D) C57BL/6 animals were left untreated (WT) or administered an antibiotic regiment (ABX) for 4 weeks to deplete microbiota. Total colon tissue was then obtained and the expression of *Il17b* and *Il25* was measured by qPCR. n = 18 mice per group.

All gene values were normalized to the expression of the housekeeping gene *Actb*. Data are presented as mean + SD error bars and are representative of at least three independent experiments. Student's t test, *$p < 0.05$. See also Figure S1.

acute colitis, airway inflammation, and *Citrobacter rodentium* infection. Overall, our studies have identified a critical inhibitory function for IL-17B in IL-25-mediated mucosal inflammation.

RESULTS

IL-17B and IL-25 Are Expressed by Colon Epithelial Cells

To determine the function of IL-17B and IL-25 in colon inflammation, we first examined mRNA expression of *Il17b* and *Il25* in total colon tissues isolated from healthy C57BL/6 mice (WT) as well as WT mice administered DSS for 8 days (Figure 1A). We found that there was basal expression of both cytokines in the colon under steady-state conditions. However, induction of acute colitis by DSS led to a substantial increase in the expression of *Il17b* (~10-fold) and *Il25* (~35-fold) mRNA (Figure 1A) as well as protein (Figure S1). Given that multiple tissues have been shown to express both cytokines (Li et al., 2000; Shi et al., 2000; Lee et al., 2001; You et al., 2005; Sanders et al., 2010), we next examined the contribution of hematopoietic and non-hematopoietic cells in the production of each. We first isolated colons

from DSS-induced WT animals and then separated cells on the basis of CD45 and CD16/32 expression. We found that the majority of *Il17b* and *Il25* mRNA expression was clearly in the colon epithelial cell (CEC) ($CD45^{-}CD16/32^{-}$) fraction (Figure 1A), which is consistent with our previous report that epithelial cells are a major source of IL-25 (Angkasekwinai et al., 2007).

The increased expression of IL-17B and IL-25 during colitis led us to investigate which signal(s) regulated expression. In vitro stimulation of CECs with toll-like receptor-4 (TLR4) agonist (LPS) and TLR1 and 2 agonist (Pam3CSK4) led to a dose-dependent mRNA increase of both cytokine genes (Figure 1B). We also stimulated CECs with muramyl dipeptide (MDP) and saw a similar enhancement of *Il17b* and *Il25* expression (Figure 1B), suggesting that the nucleotide oligomerization domain-2 (NOD2) pathway is important for the expression of both by CECs as well. Indeed, deficiency in NOD2 led to reduced basal *Il17b* and *Il25* expression in healthy colons compared to WT controls (Figure 1C).

Because IL-17B and IL-25 were both expressed in colons from healthy animals, we thought that expression might be regulated by the microbiota. To investigate this hypothesis, we depleted the gut microbiota via a broad antibiotic regiment (Ivanov et al., 2008). Similar to a previous report (Sawa et al., 2011), we found that antibiotic depletion of WT animals led to a substantial decrease of *Il25* expression in the colon (Figure 1D). Surprisingly, antibiotic depletion only moderately reduced *Il17b*, suggesting that the signals governing IL-17B production are more restricted.

Deficiency in IL-25 Confers Protection against DSS-Induced Colitis

We next tested WT and IL-25-deficient (*Il25*$^{-/-}$) mice (Angkasekwinai et al., 2010) for the development of colon inflammation 8 days after DSS administration. Both groups started to lose weight ~5 days after treatment, but *Il25*$^{-/-}$ mice exhibited considerable protection from the wasting disease manifesting in WT controls (Figure 2A). Further analysis after euthanasia revealed that the moderate weight loss observed in *Il25*$^{-/-}$ animals was directly attributable to decreased colonic inflammation; characteristic inflammatory parameters such as colon length and H&E histology revealed that *Il25*$^{-/-}$ mice were protected from inflammatory destruction, tissue remodeling, and leukocyte infiltration typical of this mouse model (Figures 2B–2D).

After establishing the pathogenic function of IL-25 in colonic inflammation, we next hypothesized that this phenotype is driven by the action of CECs (Figure 1A). Therefore, we generated a mouse strain with epithelial-specific deletion of IL-25 by crossing mice with floxed *Il25* to mice with a cre recombinase under control of the villin 1 promoter (*Vil1-cre*). The resulting epithelial cell-specific conditional *Il25*$^{-/-}$ mice (*Il25*$^{fl/fl}$ × *Vil1-cre*) were then subjected to DSS-induced colitis. As expected, *Il25*$^{fl/fl}$ × *Vil1-cre* animals exhibited resistance to DSS-induced weight loss and colon shortening compared to WT controls (Figures 2E and 2F). Importantly, the expression of *Il25* in the colons derived from *Il25*$^{fl/fl}$ × *Vil1-cre* animals after DSS treatment was ~4% of the expression found in co-housed WT littermates, further demonstrating the importance of IL-25 expression by CECs (Figure 2G). Similar to *Il25*$^{-/-}$ animals, colon tissue was less inflamed in *Il25*$^{fl/fl}$ × *Vil1-cre* mice compared to WT (Figures 2H and 2I).

We next asked whether IL-25 regulates inflammation through the production of pro-inflammatory mediators after the induction of colitis. Indeed, colons isolated from *Il25*$^{-/-}$ mice after DSS administration were markedly reduced in mRNA and protein expression of the IL-1β, IL-6, and TNF-α cytokines and the CCL2 chemokine (Figure 2J). Thus, our results indicate that IL-25, whose expression in epithelial cells is enhanced in DSS colitis, plays a pathogenic role by regulating pro-inflammatory mediators.

Defective IL-17B Expression Exacerbates the Development of Acute Colitis

To examine the role of IL-17B in colon inflammation, we generated a mouse strain containing a genetrap in the upstream promoter of *Il17b* (IL-17B-deficient). This strain develops normally and exhibits substantially reduced *Il17b* mRNA and protein expression in mucosal tissues (Figure S2). Next we performed DSS-induced colitis in these animals. Surprisingly, defective IL-17B led to an opposing phenotype compared to *Il25* deletion (Figure 2). IL-17B-deficient mice began to lose weight earlier than their WT counterparts (Figure 3A) and colons were consistently shorter (Figure 3B). Moreover, H&E analysis revealed greater leukocyte infiltration, thickening, and goblet cell destruction as a result of IL-17B deficiency (Figures 3C and 3D). To determine the basis for these observations, we next isolated mRNA and protein from colons of WT and IL-17B-deficient mice (Figure 3E). In contrast to our observations in *Il25*$^{-/-}$ animals (Figure 2), we observed that inflammatory mRNA and protein (IL-1β, IL-6, TNF-α, and CCL2) was markedly increased in animals lacking IL-17B. Overall, these results suggest that although both IL-25 and IL-17B bind to IL-17RB, they differentially regulate the development of colonic inflammation.

IL-17B Inhibits IL-25-Induced IL-6 Production

To further investigate the mechanisms underlying the opposing phenotypes in these animals, we first examined the contribution of IL-17RB signaling. Bone marrow chimeras were generated with IL-17RB present and/or absent (Watarai et al., 2012) in the hematopoietic and non-hematopoietic compartments. Mice deficient in IL-17RB in both compartments exhibited resistance to DSS, similar to *Il25*$^{-/-}$ mice (Figure 4A). Conversely, mice with IL-17RB deficiency only in leukocytes exhibited weight loss consistent with WT. Finally, animals with deletion of IL-17RB in the non-immune cells exhibited protection from DSS colitis (Figure 4A). Thus, CECs are not just the primary source of IL-25 (Figure 2), but are probably the primary cells responding to IL-25 in this model.

To investigate how IL-17RB promotes colonic inflammation, we next examined the effect of IL-25 on the production of IL-6 by CECs. We focused on IL-6 production because of the correlation of expression with disease severity (Figures 2J and 3E) and its importance in colon inflammation and cancer (Atreya et al., 2000; Gay et al., 2006; Yen et al., 2006; Tajima et al., 2008; Grivennikov et al., 2009). When treating either the young adult mouse colon (YAMC) cell line or primary CECs with IL-25, we observed a dose-dependent effect of inducing IL-6 production (Figure 4B). In contrast, IL-17B had no effect in promoting IL-6 or other mRNAs from YAMCs or CECs (Figure S3A). Because

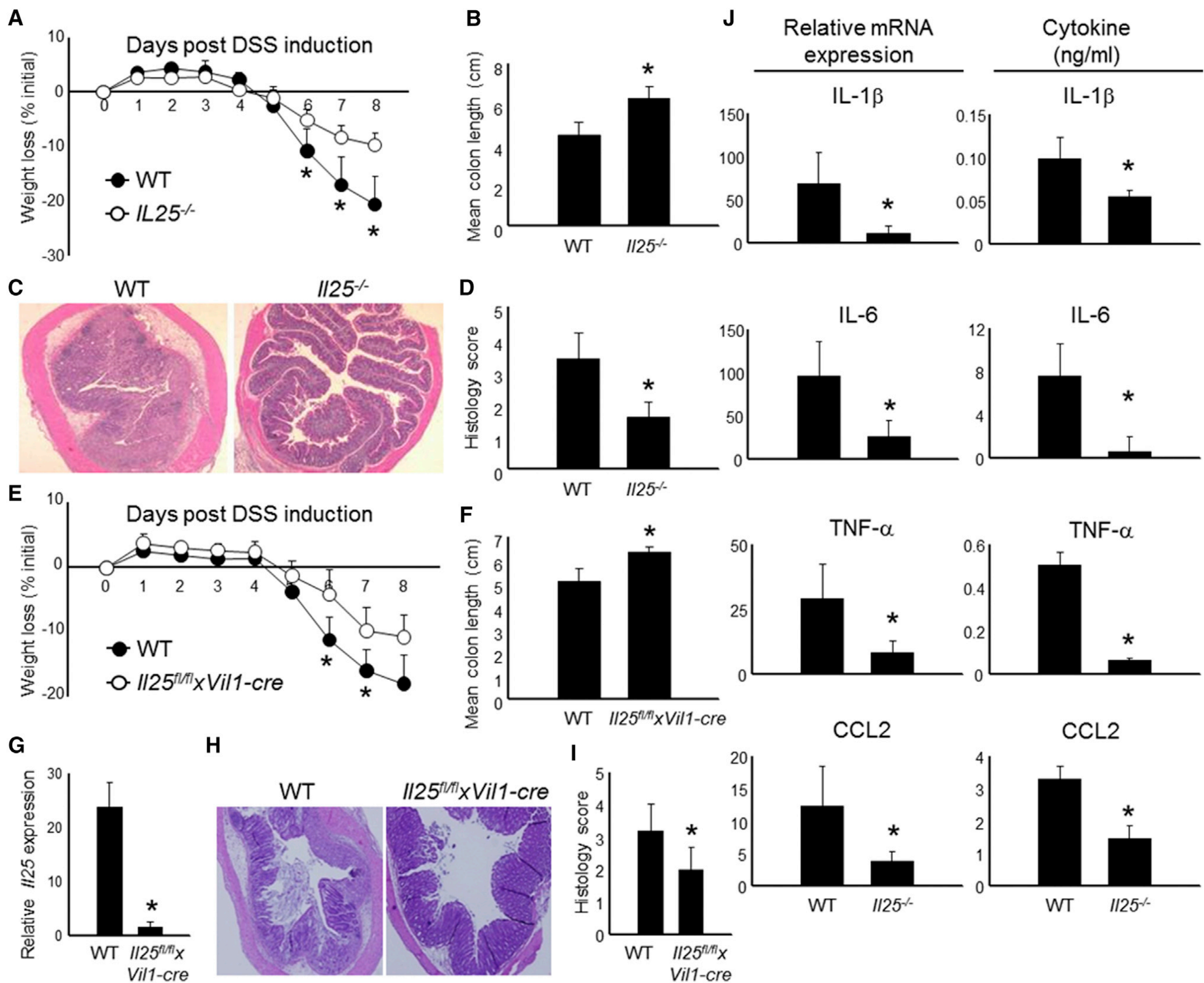

Figure 2. IL-25-Deficient Mice Are Protected from DSS-Induced Colitis

(A) Acute colitis was induced in WT and *Il25*$^{-/-}$ mice by the addition of DSS in drinking water (n = 5–6 per group). Weight loss during colitis progression is shown.

(B) Mice with colitis were euthanized on day 8, colon length was measured from individual mice, and data were combined.

(C) Representative H&E histology from intermediate colon sections derived from the mice in (A).

(D) Combined histological scores of all mice and all sections (proximal, intermediate, and distal) in (A).

(E) Weight loss of WT and *Il25*$^{fl/fl}$ × *Vil1-cre* mice throughout the course of DSS-induced colitis. n = 5–6 mice per group.

(F) Combined individual colon lengths from the mice in (E).

(G) Combined expression of total colon *Il25* mRNA from each mouse in (E).

(H) Representative H&E histology from middle colon sections from mice in (E).

(I) Combined histological scores from all colon sections of DSS mice presented in (E).

(J) Total colon tissue was isolated from mice 8 days after the induction of DSS colitis and equivalent biopsy sections were taken for inflammatory mRNA analysis (qPCR) or were cultured in media overnight for cytokine analysis in supernatants (ELISA).

The data from individual mice were combined for each group. All gene values were normalized to the expression of *Actb*. Data from individual mice are presented as mean + SD error bars and are representative of at least three independent experiments. Student's t test, *$p < 0.05$ for comparisons between WT control and *Il25*$^{-/-}$ groups.

IL-25 induced IL-6, we next asked whether this effect was dependent on the IL-17RA and IL-17RB heterodimer as well as the Act1 adaptor, all of which are known to be required for IL-17RB signaling in other cells (Chang et al., 2006; Qian et al., 2007; Rickel et al., 2008; Claudio et al., 2009; Angkasekwinai et al., 2010). Stimulation of IL-17RA- (data not shown), IL-17RB- (Figure 4C), or Act1 (*Traf3ip2*)-deficient (Figure 4D) CECs with IL-25 resulted in little to no IL-6, indicating that IL-25 signaling in CECs is comparable to other cell types.

Although we could not observe a discernible effect of IL-17B, we investigated whether IL-17B influences IL-25-mediated IL-6 expression, considering the opposing colitis phenotypes (Figures 2 and 3). We isolated primary CECs and stimulated them with a fixed concentration of IL-25 (200 ng/ml) accompanied

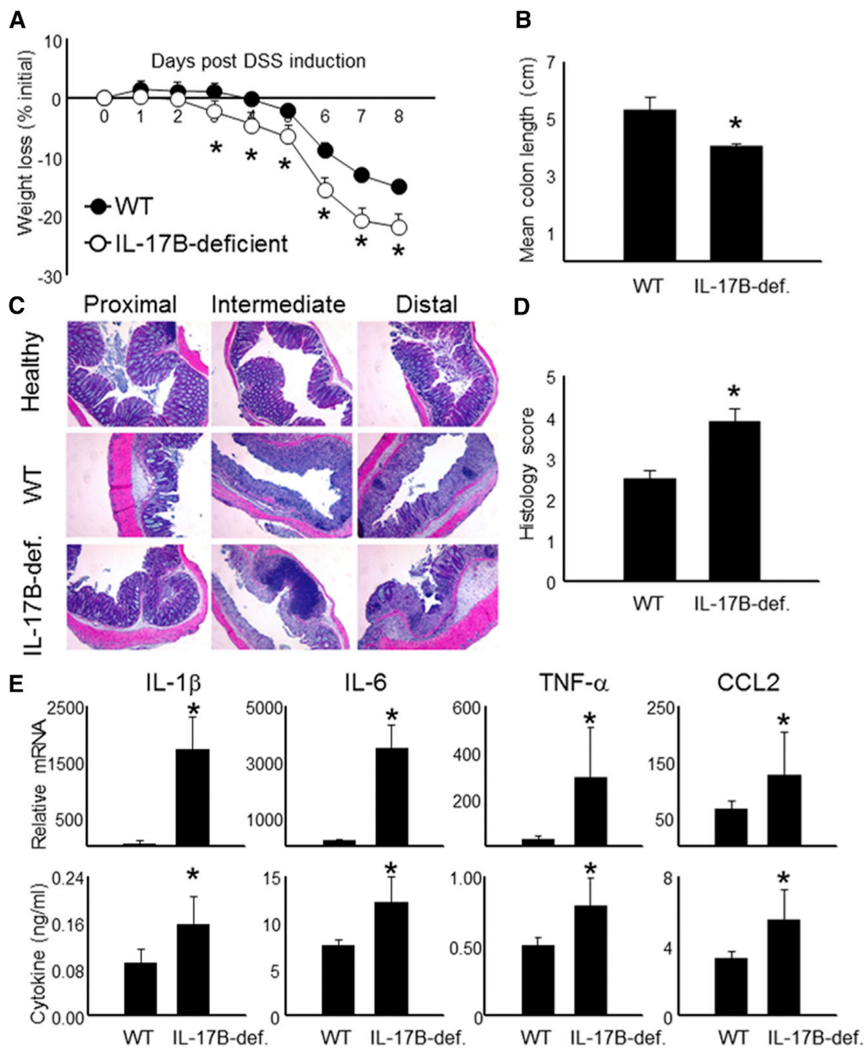

Figure 3. IL-17B-Deficient Mice Exhibit Exacerbated Colitis

(A) Weight loss (percent of starting weight) was assessed daily in WT and IL-17B-deficient mice after DSS administration. n = 5–6 mice per group.

(B) Colons were isolated from individual mice after sacrifice at day 8 and then measured and combined for each group.

(C and D) Colon tissue derived from the mice in (A) were sectioned and then stained with H&E (C) to measure infiltration and inflammation (D) in individual mice after DSS onset at day 8.

(E) Total colon tissue was divided into similar sections and then combined for each mouse. mRNA was then isolated and gene values were measured by qPCR with the expression of *Actb* as a reference. Supernatants from the colon were also assayed for cytokine expression by ELISA.

Data are presented as mean + SD error bars and are representative of at least three independent experiments. Student's t test; $^*p < 0.05$ in comparison of IL-17B-deficient to WT controls. See also Figure S2.

by simultaneous treatment of increasing IL-17B. We found that IL-17B treatment resulted in a reduction of IL-6 (Figure 4E). High-dose IL-17B almost completely abolished IL-6 mRNA and protein, suggesting that IL-17B might restrict IL-25 signaling. Conversely, IL-17B could not inhibit IL-6 expression induced by IL-17 (Figure S3B). These results suggest that IL-17B inhibits IL-25 binding to IL-17RB as a means to dampen IL-25 signaling. Therefore, we analyzed the binding of IL-25-hIg to IL-17RB or IL-17RB and IL-17RA in the presence of increasing IL-17B (Figures 4F and S3C). Low-dose IL-17B was unable to inhibit IL-25 binding to HEK293 cells expressing IL-17RB alone. However, high doses of IL-17B substantially inhibited binding. Moreover, when cells expressed both IL-17RB and IL-17RA, IL-17B could inhibit IL-25 binding at lower concentrations. These data suggest that IL-25 binds to IL-17RB regardless of IL-17RA, though dimerization of IL-17RB to IL-17RA is critical for the transmission of signal (Figures 4B–4D). IL-17B preferentially inhibited IL-25 binding to the heterodimeric form of the receptor (Figure 4F). However, IL-17A had no effect on IL-25 binding to IL-17RB or the IL-17RB and IL-17RA complex.

Because IL-17B deficiency led to greater colitis severity, we asked whether treatment of WT or IL-17B-deficient mice with IL-17B could improve disease severity. We found that treatment of mice by i.p. IL-17B injection at the start of DSS was sufficient to improve weight loss (Figure S3D) in WT animals. Furthermore, IL-17B treatment in IL-17B-deficient animals led to a partial rescue from wasting disease.

To test whether the severe DSS phenotype in IL-17B-deficient mice exaggerated IL-25 signaling, we crossed IL-25- and IL-17B-deficient mice to create double-deficient mice (IL-17B-deficient, *Il25*$^{-/-}$). We treated these mice with DSS and found that additional IL-25 deletion greatly improved wasting in IL-17B-deficient animals (Figure 4G). Moreover, colonic inflammation was identical in the WT and IL-17B-deficient, *Il25*$^{-/-}$ animals (Figure 4H) as was *Il6* mRNA expression in the colon (Figure 4I). Thus, the deficiency of both IL-17B and IL-25 reverts a single-deficient animal back to the WT phenotype.

Opposing Roles of IL-17B and IL-25 in Response to *C. rodentium*

To determine whether IL-17B and IL-25 functions are specific to acute colitis or further manifest in general inflammatory responses, we orally infected WT, IL-17B-deficient, and *Il25*$^{-/-}$ mice with *Citrobacter rodentium*, a murine-specific, Gram-negative model for human *E. coli* infection (Luperchio and Schauer, 2001). All experiments were performed on mice co-housed or on a rotating cage system to minimize differential microbiota effects (Elinav et al., 2011). IL-17B-deficient mice consistently exhibited a mild, but significant, weight loss compared to WT animals (Figure 5A). This weight loss correlated with an extensive bacterial burden in the feces, indicating a failure to properly control the pathogen (Figure 5B). The bacterial load in the liver and spleen, an indicator of dissemination, was also higher with

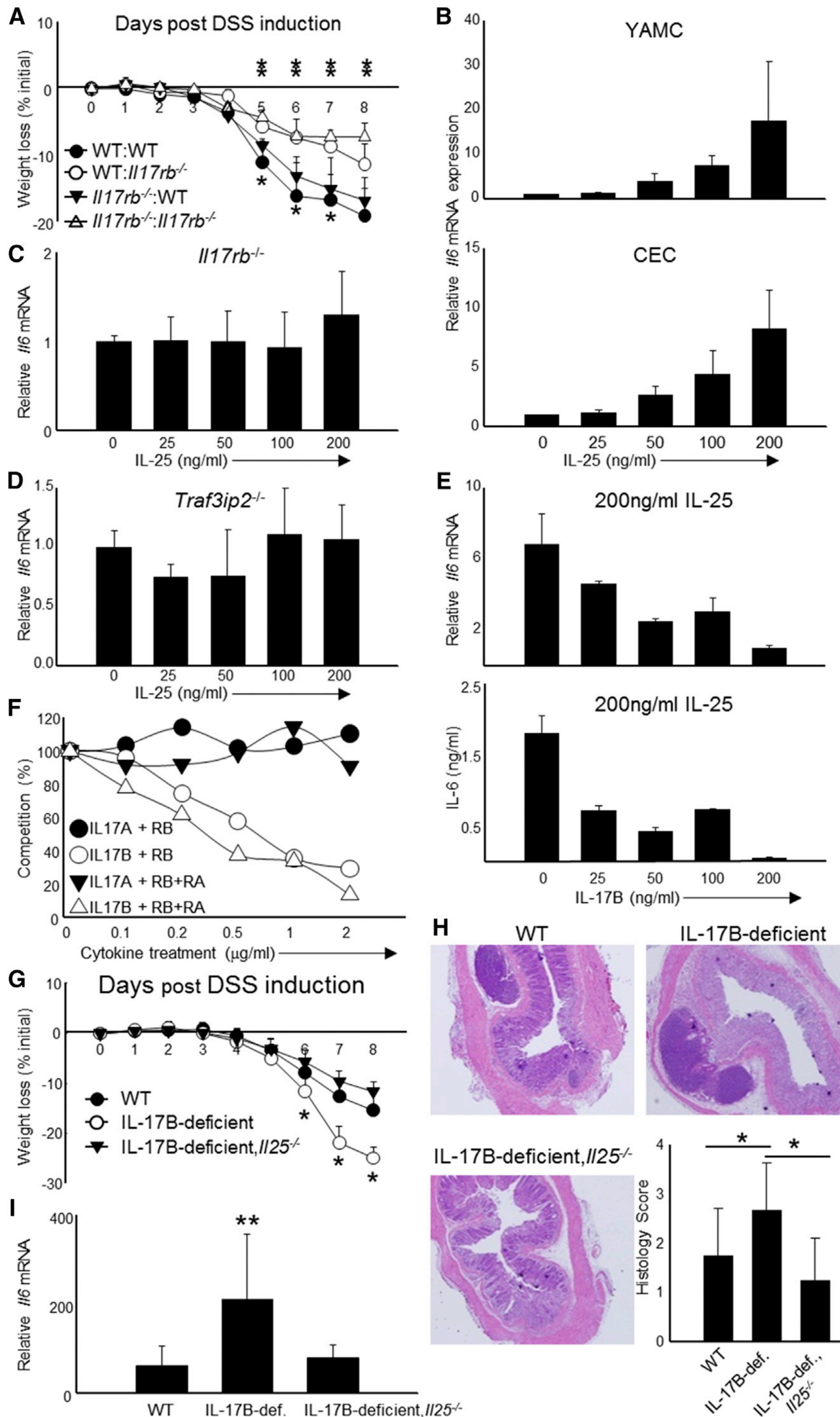

Figure 4. IL-17B Inhibits IL-25-Mediated IL-6 Production in Colon Epithelial Cells

(A) Bone marrow chimeric mice were generated with WT and *Il17rb*$^{-/-}$ animals as donors and recipients. The first genotype designation in the legend identifies hematopoietic cell origin (WT: or *Il17rb*$^{-/-}$:) and the second represents non-hematopoietic cell origin (:WT or :*Il17rb*$^{-/-}$).

(B) IL-6 expression was measured by qPCR after 6 hr stimulation with recombinant IL-25 in a colon epithelial cell line (YAMC) and primary colon epithelial cells (CECs).

(C and D) IL-6 mRNA expression was similarly measured from CECs derived from IL-17RB-deficient (C) and Act1 (*Traf3ip2*$^{-/-}$)-deficient (D) mice.

(E) IL-6 was examined in CECs after fixed IL-25 stimulation (200 ng/ml) followed by simultaneous treatment of increasing IL-17B concentrations. IL-6 was measured by qPCR (6 hr stimulation, top) and ELISA (24 hr stimulation, bottom).

(F) Summary of competition experiments measuring the ability of increasing concentrations of IL-17B or IL-17A to displace IL-25-hIg bound to murine IL-17RB (RB) or IL-17RB and IL-17RA (RB+RA). Competition (%) is the percentage of IL-25 displacement at each IL-17B or IL-17A concentration compared those treated with only IL-25.

(G) Combined weight loss data from WT, IL-17B-deficient, and IL-17B and IL-17E double-deficient (IL-17B-deficient, *Il25*$^{-/-}$) mice after DSS administration. n = 7–8 mice per group.

(H) Representative H&E histology and combined histological scores of all colon sections from the mice shown in (G).

(I) Combined IL-6 mRNA expression from total colon samples derived from the mice in (G).

Data are presented as mean + SD error bars and are representative of two independent experiments (A) and at least three independent experiments in (B)–(I). mRNA data were obtained by examining relative gene expression to *Actb*. Student's t test; *p < 0.05 for daily comparisons of WT:WT mice to *Il17rb*$^{-/-}$:*Il17rb*$^{-/-}$ mice and **p < 0.05 for daily comparisons of WT:*Il17rb*$^{-/-}$ mice to *Il17rb*$^{-/-}$:*Il17rb*$^{-/-}$ mice (A); *p < 0.05 for daily comparisons of IL-17B-deficient to IL-17B-deficient, *Il25*$^{-/-}$ *mice* (G); **p < 0.05 for comparison of IL-17B-deficient to WT and IL-17B-deficient, *Il25*$^{-/-}$ *mice* (I). See also Figure S3.

IL-17B deficiency; however, only a modest count was obtained from these organs, possibly due to the relatively low CFU for infection (Figure S4A). Thus, IL-17B deficiency is pathogenic for *Citrobacter rodentium* infection, similar to DSS-induced colitis, although the mechanism probably is different due to the importance of IL-6 in combating *C. rodentium* (Dann et al., 2008).

We next performed similar experiments with *Il25*$^{-/-}$ mice. For reasons unknown, in these experiments WT animals had a moderate weight loss (Figure 5C). Nonetheless, *Il25*$^{-/-}$ mice did exhibit a slight, but significant, improvement in weight compared to WT mice (Figure 5C), indicating that IL-25 is important for *C. rodentium* defense. Further analysis revealed a similar trend where fecal bacteria were significantly reduced in *Il25*$^{-/-}$ mice (Figure 5D). The protective effect observed in *Il25*$^{-/-}$ mice, however, did not reach the threshold for statistical significance for spleen and liver comparisons (Figure S4A).

IL-22 is critical cytokine for combating *Citrobacter* infection (Zheng et al., 2008). Therefore, we analyzed *Il22* mRNA and protein in infected colons (Figure S4B). We found that IL-22

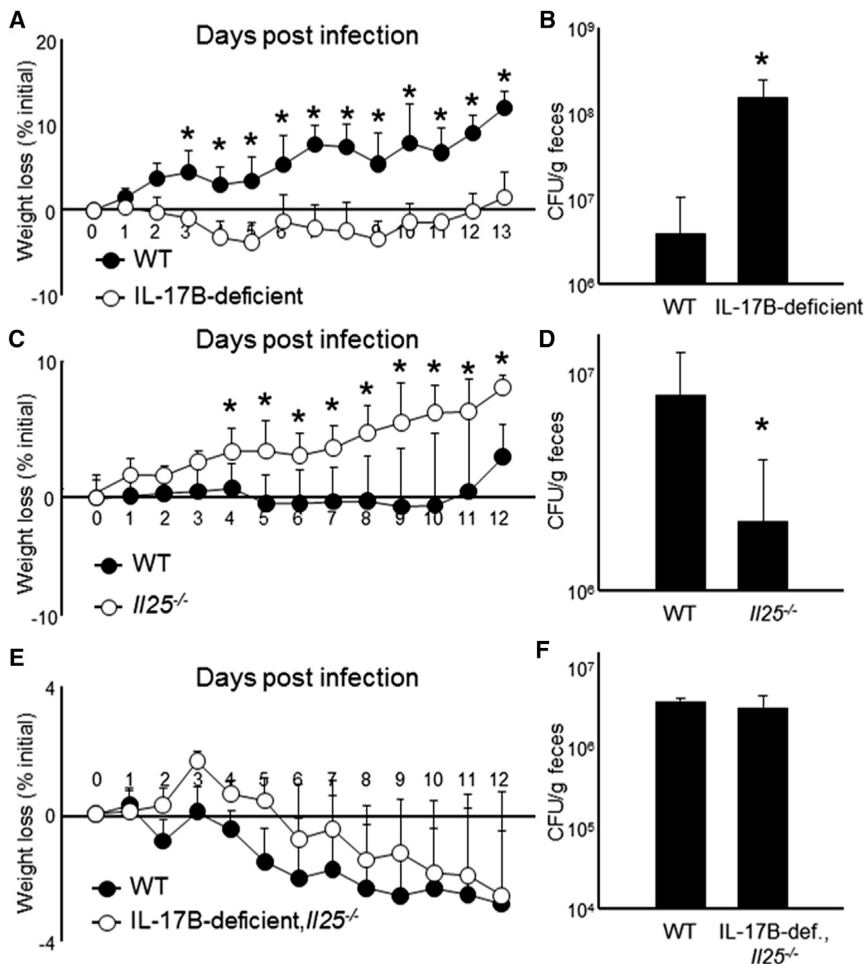

Figure 5. IL-17B Is Protective against *Citrobacter rodentium* Infection

(A, C, E) WT and IL-17B-deficient (A), WT and *Il25*$^{-/-}$ (C), or WT and IL-17B-deficient, *Il25*$^{-/-}$ (E) mice were orally infected with 2×10^9 CFUs of *Citrobacter rodentium* and weighed daily for 12–13 days. n = 7–18 mice per group.

(B, D, F) After harvest, feces were collected, homogenized, and then examined for *Citrobacter rodentium* growth on MacConkey agar after overnight incubation.

Data are presented as mean + SD error bars and are representative of at least three independent experiments. Student's t test; $^*p < 0.05$. See also Figure S4.

expression was decreased in *Il25*$^{-/-}$ mice and increased in IL-17B-deficient mice, which was somewhat surprising considering that IL-17B-deficient mice were susceptible and *Il25*$^{-/-}$ mice were protected against *Citrobacter* infection. Thus, the functions of IL-25 and IL-17B in the innate response to pathogens are complex and likely to involve other factors outside of IL-6 and IL-22. When analyzing IL-17B-deficient, *Il25*$^{-/-}$ animals, we found that deletion of both cytokines during infection resulted in a phenotype equivalent to WT animals, including weight loss, bacteria load, and IL-22 production (Figures 5E, 5F, S4A, and S4B), similar to that observed in DSS experiments (Figures 4G–4I).

Opposing Functions of IL-17B and IL-25 in Airway Inflammation

Previous work has demonstrated that IL-25 mediates allergic inflammation by promoting Th2 cell responses and facilitating eosinophil recruitment (Fort et al., 2001; Angkasekwinai et al., 2007, 2010). Consequently, animals lacking IL-25 are protected against the development of experimental asthma. In allergic asthma induced by OVA and alum, IL-17B is also induced in the lung tissue (Figure S5A). Although lack of IL-25 led to a reduction of eosinophils in this model, the bronchoalveolar lavage fluid (BALF) of IL-17B-deficient mice was marked by substantial eosinophil influx (Figure 6A). When we examined various Th2 cell mediators, we expectedly found that the IL-4, IL-5, and IL-13 cytokines were reduced in *Il25*$^{-/-}$ and exacerbated in IL-17B-deficient mice (Figure 6B). A similar profile was obtained when analyzing the total lung mRNA (Figure 6C). IL-9, regulated by IL-25 (Angkasekwinai et al., 2010), was increased in the lungs of IL-17B-deficient animals as well (Figure 6C). Similarly, the BALF of *Il25*$^{-/-}$ mice exhibited a loss of the Th2 cell compartment upon OVA restimulation whereas IL-17B-deficient BALF contained increased numbers of Th2 cells (Figure 6D). To further examine Th2-cell-mediated responses, we restimulated total splenic and mediastinal lymph node cells with OVA for 3 days and analyzed cytokine production. We found a similar exacerbation of Th2 cell responses in the cells isolated from IL-17B-deficient mice (Figure 6E). Thus, IL-25 strongly promotes allergic airway inflammation through enhanced Th2 cell responses whereas IL-17B conversely restrains Th2 cell responses in the airway.

To determine the outcome of deleting both cytokines in this model, we performed a similar set of experiments in IL-17B-deficient, *Il25*$^{-/-}$ mice (Figures S5B–S5D). We found that respiratory infiltration as well as Th2 cell cytokine production was equivalent between WT and IL-17B-deficient, *Il25*$^{-/-}$ animals. These results indicated that removal of both cytokines counteracts the phenotypes observed in single-deficient mice, similar to what was observed for DSS (Figure 4) and *Citrobacter rodentium* infection (Figure 5).

DISCUSSION

The pro-inflammatory functions of IL-17 have been well documented (Dong, 2008). Here, we have demonstrated further functional complexity among the IL-17 family of cytokines. Adding to a well-established role in type 2 immunity, we found that IL-25 was pathogenic in acute colitis, whereas IL-17B was conversely protective. These opposing effects were similar in *Citrobacter rodentium* infection and allergic asthma. Notably, we have found

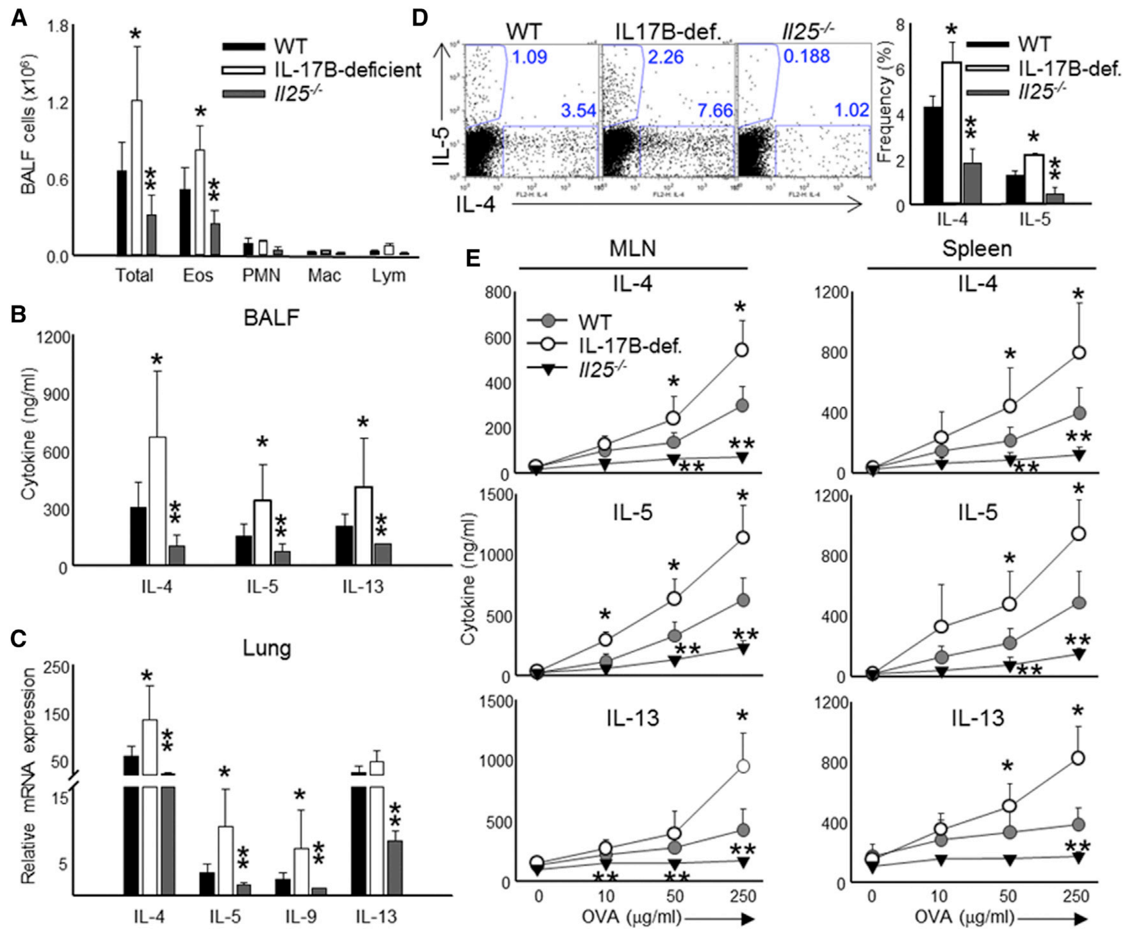

Figure 6. IL-17B and IL-25 Have Opposing Roles during Allergic Asthma

(A) Total cell counts in the BAL fluid of WT, IL-17B-deficient, and IL-25-deficient animals sensitized with OVA in alum at day 0 and 14, followed by intranasal challenge with OVA at days 14, 25, and 26.

(B) BALF derived from the animals in (A) was assayed for the presence of IL-4, IL-5, and IL-13 by ELISA.

(C) Combined mRNA analysis of Th2 and Th9 cell-associated genes from total lung samples derived from the mice in (A).

(D) Representative (left) and combined analysis (right) of Th2 cell frequencies in the BALF of OVA and alum sensitized animals. T cells isolated from the BALF were restimulated with 50 μg/ml OVA prior to intracellular cytokine staining for IL-4 and IL-5.

(E) Analysis of splenic and mediastinal lymph node Th2 cell compartments after 3 days restimulation with increasing doses of OVA. IL-4, IL-5, and IL-13 protein was measured from the supernatants by ELISA.

Data are presented as mean of data obtained from individual mice + SD error bars and are representative of at least three independent experiments. Student's t test; *p < 0.05 for comparisons of WT to IL-17B-deficient and **p < 0.05 for comparisons of WT to *Il25*$^{-/-}$. See also Figure S5.

a unique role for IL-17B in inhibiting IL-25-mediated IL-6 production by CECs, making IL-17B an antagonistic and anti-inflammatory cytokine in this family.

IL-25 is generally thought of as a Th2-cell-derived factor, promoting allergic asthma responses but also protecting against parasitic infection (Reynolds et al., 2010). Here, we have shown that IL-25 in acute colitis promoted IL-6 production from CECs, which is consistent with a report of IL-25 promoting the release of IL-6 from eosinophils (Wong et al., 2005). Throughout the course of this study, IL-25-dependent IL-6 production was the most consistently inhibited by IL-17B treatment. However, at this time we cannot rule out the influence of other mediators in promoting the protective and pathogenic phenotypes observed in IL-25- and IL-17B-deficient mice, respectively. Most likely, IL-17B antagonism of IL-25 is influencing multiple facets of the mucosal immune response, which is the subject of our current investigations.

Our finding that IL-25 deletion protects against DSS differs from another showing that injecting IL-25 protects against the development of DSS colitis (Mchenga et al., 2008). Though we cannot fully explain the discrepancy between reports, IL-25 stimulation of primary CECs in vitro promoted the production of IL-6, consistent with decreased inflammation observed in *Il25*$^{-/-}$ mice. Indeed, when we injected WT mice directly with IL-25 (not shown) or IL-17B, we observed phenotypes opposite to those found in cytokine-deficient mice. Thus, different housing conditions or microbiota might influence results obtained from recombinant protein injection.

IL-17B has long been a mysterious member of the IL-17 cytokine family. Previous work has demonstrated that IL-17RB might

be oncogenic whereas IL-17B might contribute to tumor cell growth (Tian et al., 2000; Furuta et al., 2011; Huang et al., 2014). The original report characterizing IL-17B demonstrates that this cytokine stimulates the release of TNF-α and IL-1β from THP-1 cells. However, we did not observe production of pro-inflammatory mediators after IL-17B stimulation, consistent with IL-17B being unable to induce IL-6 from fibroblasts (Li et al., 2000). Instead, the primary effect we observed for IL-17B treatment in CECs was direct inhibition of IL-25-mediated IL-6 production. This of course begs the question of how exactly IL-17B can inhibit IL-25-mediated IL-6 production in CECs. One possibility is that IL-17B competes with IL-25 for binding IL-17RB. Indeed, we found that IL-17B inhibited IL-25 binding to IL-17RB or IL-17RA and IL-17RB complexes. Because previous work has demonstrated that IL-17A and IL-17F form heterodimers, we investigated this possibility as well. However, exhaustive attempts to identify IL-17B and IL-25 heterodimers were unsuccessful (not shown), suggesting that competition for IL-17RB binding causes functional antagonism. IL-17B had higher activity in blocking IL-25 binding to the IL-17RA and IL-17RB heterodimer compared to IL-17RB alone. Conversely, IL-25 was capable of binding IL-17RB in the absence of IL-17RA, suggesting that IL-17RA was more important for signaling. These binding assays have limitations considering that we cannot rule out the contribution of endogenous IL-17 receptors that might be expressed in the human HEK cells, because we do not know whether the murine IL-17RB could form heterodimers with endogenous human IL-17RA. Another caveat is that only mouse receptors were co-expressed in these cells and more direct studies need to be performed in the future on both mouse and human IL-17RA and IL-17RB similar to studies on IL-17 and IL-17F (Toy et al., 2006; Kuestner et al., 2007). Finally, we need to determine whether IL-17RB is internalized after binding of IL-17B, IL-25, or both. Differential IL-17RB internalization could represent another mechanism governing IL-17B regulation of IL-25.

Our current model is that colon inflammation enhances IL-25 pro-inflammatory signaling while IL-17B is concurrently induced to restrain IL-25. IL-25 has a higher affinity for IL-17RB alone compared to IL-17B. However, treatment with IL-17B throughout the course of DSS colitis led to protection in WT animals, indicating that a shift in the IL-25 to IL-17B ratio can influence the course of inflammation. Previous work has demonstrated that IL-17F in some situations antagonizes IL-17 signaling, including forming heterodimeric complexes with IL-17 that have weaker activity compared to IL-17 alone (Wright et al., 2008). We believe that the antagonism exerted on IL-25 signaling by IL-17B, however, is unique to this family because IL-17F by itself can activate signaling pathways important for inflammation (Yang et al., 2008) but we have yet to find a stand-alone function for IL-17B. Moreover, IL-17B could not antagonize IL-6 production induced by IL-17 in CECs, demonstrating the specificity of IL-17B action. Therefore, further study is needed to determine whether IL-17B alone can transmit a functional signal to any cell type and to determine the precise mechanism for how IL-17B is inhibiting IL-25 binding to IL-17RB.

The prospect of modulating IL-17RB signaling for the treatment of colitis at this time seems promising. Our findings that recombinant IL-17B injections throughout the course of DSS administration can improve disease outcome in WT animals further supports this theory. Whether IL-17B is as important in human IBD is currently under investigation. The production of IL-25 has been linked to human IBD even though polymorphism is not believed to be a risk factor (Büning et al., 2003). Two recent reports have shown that inflammatory bowel disease (IBD) patients have decreases in IL-25 (Caruso et al., 2009; Su et al., 2013). These results might suggest that IL-25 is a protective factor in human IBD; however, IL-17B expression in these patients has yet to be determined. Decreased IL-25 expression in human IBD might also be representative of a failure of IL-17B to control inflammation. Thus, further investigation is necessary to determine the role of IL-17B in human IBD as well as determining whether IL-17RB represents a viable therapeutic target.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from NCI, and *Nod2*$^{-/-}$ and *Vil1-cre* mice were purchased from The Jackson Laboratory. *Il25*$^{fl/fl}$ and IL-25-deficient mice have been previously described (Angkasekwinai et al., 2010). *Il25*$^{fl/fl}$ mice were crossed with *Vil1-cre* mice on the B6 background to generate *Il25*$^{fl/fl}$ × *Vil1-cre* mice. IL-17B-deficient mice were generated by gene trapping in the R1 ES cell line (clone 371C12; Toronto Centre for Phenogenomics, Centre for Modeling Human Disease). Both IL-25- and IL-17B-deficient mice have been backcrossed to B6 background for >8 generations. *Il17rb*$^{-/-}$ and *Traf3ip2*$^{-/-}$ (Act1) mice have previously been described (Chang et al., 2011; Watarai et al., 2012). All experiments were conducted on 6- to 12-week-old animals with protocols approved by the Rosalind Franklin University of Medicine and Science and MD Anderson Cancer Center Institutional Animal Care and Use Committees. Additional information on experimental mice can be found in the Supplemental Experimental Procedures.

Antibiotic Depletion of Commensal Flora

Commensal flora was depleted with a previously described regiment (Ivanov et al., 2008). In brief, mice were administered water containing a cocktail of ampicillin (Sigma-Aldrich), neomycin (Fisher Scientific), vancomycin (Fisher), and metronidazole (Fisher) for 4 weeks.

DSS-Induced Colitis

All experiments were performed with 6- to 12-week-old mice on the C57BL/6 background that were fed DSS in drinking water at a concentration of 2%–3.5% (w/v) for a total period of 5 days. Starting weights were recorded and then mice were weighed and monitored daily until the experimental endpoint (day 8). For analysis, total colon tissue was harvested from animals and colon lengths were recorded. Colon sections were then divided into proximal, intermediate, and distal sections for H&E histological analysis. Sections from each region were also combined in plated in media overnight for supernatant analysis of cytokines by ELISA. Sections were also combined and lysed in TRIzol (Life Technologies) for analysis of mRNA expression by qPCR. Additional analysis methods, including mRNA, histology, and protein analysis, can be found in the Supplemental Experimental Procedures.

Cell Lines and CECs

YAMC cells were a generous gift from Dr. Dingzhi Wang at the University of Texas MD Anderson Cancer Center. The isolation of CECs has been described previously (Reynolds et al., 2012). Primary CECs were plated and rested in 96-well dishes prior to stimulation with LPS (Sigma-Aldrich), Pam3CSK4 (Invivogen), MDP (Invivogen), or the indicated concentration of mouse IL-17B and/or IL-25 (R&D Systems) for 6 hr (mRNA) or 24 hr (ELISA). Additional experimental details can be found in the Supplemental Experimental Procedures.

Cytokine Binding

To analyze IL-25 binding to IL-17RB, a recombinant IL-25 tagged with human Ig (IL-25-hIg) was raised in *Drosophila* S2 cells as described previously

(Angkasekwinai et al., 2007). In brief, HEK293T cells were retrovirally transfected with constructs encoding murine *Il17ra* or *Il17rb*. After transfection, the indicated concentrations of IL-17A, IL-17B (R&D Systems), or IL-25-hIg were added simultaneously for 30 min followed by the determination of IL-25-hIg surface binding by flow cytometery using anti-hIg conjugated to APC. Additional details can be found in the Supplemental Experimental Procedures.

Generation of Bone Marrow Chimeras

Recipient WT or $Il17rb^{-/-}$ mice (6–8 weeks) were irradiated with 750 rad and injected intravenously with 5×10^6 donor cells as indicated. After 6 weeks, reconstituted mice were administered 3% DSS in drinking water as described above.

Citrobacter rodentium Infection

C. rodentium (ATCC 51459) infection was performed with 2×10^9 CFU as previously described (Hu et al., 2013). Mice were weighed for 13–14 days after infection and colon mRNA and cytokine production was analyzed as described above for DSS colitis. Bacterial load was assessed by plating fecal, liver, or splenic homogenates overnight on MacConkey agar. Additional details for this model can be found in the Supplemental Experimental Procedures.

OVA-Induced Asthma

Mice were immunized i.p. with 50 μg of grade VII OVA (Sigma) emulsified in alum (Thermo) at days 0 and 14 followed by i.n. administration of 50 μg OVA at days 14, 25, and 26 as described previously (Angkasekwinai et al., 2010). In brief, at day 28, BALF was collected for IL-4, IL-5, and IL-13 determinations by ELISA (BD Biosciences). BALF was also analyzed for leukocyte infiltration via flow cytometry and Dif-Quick (Fisher) histological staining. Finally, lymphocytes isolated from the BALF were restimulated with OVA to determine Th2 cell generation by staining with antibodies specific for IL-4 and IL-5 (eBioscience). Lung tissue was collected and homogenized in TRIzol for the determination of cytokine gene expression by qPCR. Mediastinal lymph nodes and spleens were collected from each mouse, homogenized, and then stimulated with the indicated concentrations of OVA for 3 days. IL-4, IL-5, and IL-13 expression was then measured in the supernatants by ELISA.

Statistical Analysis

Statistical comparisons were performed by two-tail paired Student's t test. p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.03.008.

AUTHOR CONTRIBUTIONS

C.D., J.M.R., and Y.-H.L. conceived the project, designed the experiments, and wrote the manuscript. J.M.R. and Y.-H.L. performed most of the experiments. Y.S., X.W., P.A., K.C.N., S.F., and S.H.C. contributed to specific experiments. H.W. provided mice and intellectual input.

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