Epithelial IL-23R Signaling Licenses Protective IL-22 Responses in Intestinal Inflammation

Highlights

- IL-23R transduces signals into the intestinal epithelium
- Il23r<sup>AIEC</sup> mice are susceptible to DSS colitis and have a disturbed gut microflora
- Epithelial IL-23R is required for optimal secretion of the c-type lectin Reg3b
- The c-type lectin Reg3b promotes recruitment of IL-22-producing cells as an alarmin
- Systemic substitution of Reg3b rescues the gut barrier defect of Il23r<sup>AIEC</sup> mice

In Brief

Aden et al. show that epithelial IL-23R signaling initiates a Reg3b-dependent chemoattraction of IL-22-producing neutrophil granulocytes into the intestinal lamina propria, limiting flagellated bacteria content and intestinal inflammation.
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SUMMARY

A plethora of functional and genetic studies have suggested a key role for the IL-23 pathway in chronic intestinal inflammation. Currently, pathogenic actions of IL-23 have been ascribed to specific effects on immune cells. Herein, we unveil a protective role of IL-23R signaling in intestinal epithelial cells. Mice deficient in IL-23 expression in intestinal epithelial cells (Il23R<sup>IEC</sup>) have reduced Reg3β expression, show a disturbed colonic microflora with an expansion of flagellated bacteria, and succumb to DSS colitis. Surprisingly, Il23R<sup>IEC</sup> mice show impaired mucosal IL-22 induction in response to IL-23. αThy-1 treatment significantly deteriorates colitis in Il23R<sup>IEC</sup> animals, which can be rescued by IL-22 application. Importantly, exogenous Reg3β administration rescues DSS-treated Il23R<sup>IEC</sup> mice by recruiting neutrophils as IL-22-producing cells, thereby restoring mucosal IL-22 levels. The study identifies a critical barrier-protective immune pathway that originates from, and is orchestrated by, IL-23R signaling in intestinal epithelial cells.

INTRODUCTION

Interleukin-23 (IL-23) is a heterodimeric cytokine of the IL-12 superfamilly, consisting of two subunits, IL-23p19 and IL-12p40, and exerts important functions on T cell-driven immune responses in several organ systems (Parham et al., 2002). Overwhelming genetic and functional evidence has pointed to a pro-inflammatory role of the IL-23/IL-23 receptor (IL-23R) signaling pathway in inflammatory bowel disease (IBD) and other immune diseases (Croxford et al., 2012). Inhibition of IL-23 signaling has been shown to ameliorate several murine models of intestinal inflammation (Eison et al., 2007). So far, the pro-inflammatory effects of IL-23 have been ascribed to perpetuating pathogenic Th17 responses, with increased production of the inflammatory cytokines IL-17A and IL-17F (Aggarwal et al., 2003; Leppkes et al., 2009). Several genome-wide association studies have identified a number of genetic variants in the genes encoding Il23R, Il12A (IL-12p40), JAK2, and STAT3 as independent risk (or protective) factors for both ulcerative colitis (UC) and Crohn's disease (CD) (Barrett et al., 2008). So far, a decreased responsiveness of T cell populations to IL-23 is conferred by the presence of a single rare coding variant (R381Q, rs11209026) in the Il23R gene that is associated with decreased susceptibility for IBD (Sarin et al., 2011). Although the link between disease protection and a loss-of-function variant in IL-23 signaling is compelling, it is worth noting that the exact functional role of IL-23R signaling in intestinal homeostasis has not been defined yet, and several conundrums remain.

First, IL-23R activation in mucosal immune cells is a pivotal inducer of the cytokine IL-22, a member of the IL-10 family. IL-22 has been shown to exert protection against intestinal inflammation in either adoptive transfer, chemically induced, or infectious models of colitis (Sugimoto et al., 2008). Therefore, therapeutic inhibition of pro-inflammatory IL-23R signaling may also lead to downregulation of IL-22-dependent signals with unclear consequences.
Second, high susceptibility to experimental colitis was demonstrated in a mouse model with genetic ablation of IL23p19 (Becker et al., 2006). The paradoxical finding has been attributed to a direct cross-regulation of IL12p35/p40. Importantly, all findings reported so far have concentrated on the role of direct IL-23 signaling in leukocytes, although recent evidence points to a role of IL-23 signaling in epithelial cells types (Hasnain et al., 2014).

In a previous study, we found IL-23R to be expressed in the intestinal epithelium of normal subjects and IBD patients (Raelson et al., 2007). Here we report that intestinal epithelium-specific deletion of the Il23r gene renders mice susceptible to experimental colitis by impairing IL-22-driven protective mucosal immunity against flagellated commensals.

RESULTS

II23R\textsuperscript{-/+EC} Mice Show Exacerbated Morbidity and Associated Mortality in Experimental Colitis

To initially test the ability of IL-23 to elicit a signal in intestinal epithelial cells in vitro, MdeK cells were stimulated with recombinant IL-23 (100 ng/ml), and STAT3 phosphorylation and target gene induction were assessed by western blot and real-time PCR, respectively. The experiments revealed a moderate increase in phosphorylation of STAT3 after 30 min as well as a slight (compared with the effect of IL-22, which was tested in parallel) but significant induction of the S100a9 mRNA after 6 hr. Under baseline conditions, Il23r mRNA is present at very low levels in purified murine intestinal epithelial cells, which were upregulated ~5-fold by induction of colonic inflammation (2% dextran sodium sulfate [DSS] for 3 days; Figure S1D). Using a lacZ knockout (KO) reporter mouse, which expresses β-galactosidase contained within the deletion cassette under the endogenous promoter, we further demonstrated that the Il23r promoter is active throughout the colonic epithelium (Figure S1E).

We generated a conditional allele of the Il23r gene (Il23r\textsuperscript{flox/flox} mice) (Figure S2A) and crossed Il23r\textsuperscript{flox/flox} mice to C57Bl/6 VillinCre mice to obtain a specific deletion of Il23r in the intestinal epithelium (termed Il23r\textsuperscript{flox/flox}Il23r\textsuperscript{DIEC} hereafter) and corresponding IL23r\textsuperscript{flox/flox} (Il23r\textsuperscript{DIEC}) littermates, as depicted in the breeding scheme (Figure S2C). Continuous genotyping revealed no evidence for aberrant germline deletion (Figure S2B). Genotypes were confirmed using tail, tissue, and fecal DNA genotyping (Figure S2D). Although Il23r mRNA in purified intestinal epithelial cell fractions from Il23r\textsuperscript{DIEC} mice was below the detection threshold (Figure S2E), it was present in Il23r\textsuperscript{DIEC} animals at low levels (~30-fold lower compared with lamina propria mononuclear cells). Furthermore, to test the impaired signaling downstream of epithelial IL-23R in vivo, we injected 1.5 μg of IL-23 (or PBS as sham) intraperitoneally (i.p.) and assessed epithelial STAT3 phosphorylation 1 hr later. Only in IL-23-injected colonic epithelial cells in Il23r\textsuperscript{DIEC}, but not in Il23r\textsuperscript{DIEC}, mice could a significant increase of nuclear pSTAT3 immunoreactivity be observed, thereby providing further evidence for direct epithelial IL-23R activity, which is inactivated in Il23r\textsuperscript{DIEC} mice (Figures S1F and S1G).

Epithelial II123R-23 Signaling Controls the Abundance of Flagellated Intestinal Bacteria

To determine the effect of epithelial II123R signaling on gut microbiome composition, we performed 16S rDNA profiling of II23R\textsuperscript{-/+EC} and II23R\textsuperscript{DIEC} feces before and after DSS treatment. Principle coordinate analysis (PCoA) on Bray Curtis distances revealed four distinct clusters (Figure 2A) based on genotype and treatment. At higher taxonomical levels, the major bacterial phylum Firmicutes (Figure S5) showed a significantly increased abundance in naïve (p = 0.010) and DSS-treated (p = 0.041) II23R\textsuperscript{DIEC} mice compared with control littermates. Interestingly, overall mostly flagellated bacterial groups (e.g., Lachnospiraceae, Helicobacter, Escherichia/Shigella, Clostridium groups) were more prevalent in II23R\textsuperscript{DIEC} mice with further expansion upon DSS treatment (Figure 2B). Functional metagenomic prediction using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) algorithm (Langille et al., 2013) inferred a significant increase of genes assigned to flagellar assembly pathways in II23R\textsuperscript{DIEC} mice compared with II23R\textsuperscript{DIEC} mice at baseline and after DSS treatment (Figure 2C). Using a HEK-Blue Toll-like receptor (TLR)-5 reporter assay (Cullender et al., 2013), we indeed confirmed an increased abundance of biologically active flagellin in II23R\textsuperscript{DIEC} compared with II23R\textsuperscript{DIEC} feces in response to DSS treatment (Figure 2D). Importantly, co-housing II23R\textsuperscript{DIEC} mice with an excess of II23R\textsuperscript{DIEC} littermates could correct disease activity and flagellar expansion, indicating that altered microbiota significantly contributes to the increased disease risk of II23R\textsuperscript{DIEC} mice (Figures S5B and S5C).

Epithelial II123R-23 Signaling Licenses Mucosal II22 Production

The intestinal epithelium interacts with immune cells that critically contribute to tissue regeneration by producing II-22 (Sanos et al., 2011). We thus hypothesized that altered mucosal II-22 levels may contribute to the disease risk in II23R\textsuperscript{DIEC} mice. Indeed, ex vivo stimulation with IL-23 led to strong II-22 mRNA upregulation (Figure 3A) in crude small intestinal crypts prepared from II23R\textsuperscript{DIEC} animals, which was significantly attenuated in crypts from II23R\textsuperscript{DIEC} mice. Known II-22-expressing cells in the gut consist of a heterogeneous cell population comprising...
Th17 cells, innate-type immune cells (e.g., natural killer [NK] cells, innate lymphoid cells) (Buonocore et al., 2010; Cox et al., 2012) and neutrophilic granulocytes (Zindl et al., 2013). Because isolated splenocytes showed indistinguishable IL-22 induction between Il23Rfl and Il23RDIEC animals (Figure 3C), we asked whether extravasation of IL-22-producing cells may contribute to different IL-22 levels in Il23Rfl and IL23RDIEC mice. Because treatment with αThy-1 antibody inhibits the extravasation of leukocytes (including T cells, innate lymphoid cells [ILCs], and neutrophils) into the lamina propria (Wetzel et al., 2006; Cox et al., 2012), Il23Rfl and Il23RDIEC mice were treated for 2 consecutive days with 100 or 200 μg of a Thy-1 antibody (clone T24/31), and a significant reduction of CD3+ lymphocytes (Figure 3D) as well as neutrophils (Gr1+, data not shown) was observable. IL-23-induced IL-22 expression was blunted in preparations derived from animals that underwent αThy-1 administration at a high dose, regardless of their genotype (Figure 3E), demonstrating that epithelial cells are not a direct source of IL-22 in response to IL-23.

Subsequently, we analyzed the effect of αThy-1 treatment as an in vivo model to reduce the mucosal IL-22 level in DSS-induced colitis (Cox et al., 2012). Il23Rfl and Il23RDIEC mice were treated prior to induction of DSS colitis (2%) and then every 3 days with 200 μg αThy-1 (Il23Rfl + αThy1; Il23RDIEC + αThy1) or an immunoglobulin G (IgG) (Il23Rfl, Il23RDIEC) control (clone LTF-2) (Figure 3H). Il23RDIEC animals treated with αThy1 antibody became moribund shortly after induction with the 2% DSS regimen, as determined by a dramatic weight loss over the first 10 days, compared with αThy1-treated Il23Rfl mice (Figure 3F). This was accompanied by impaired intestinal proliferation.

Figure 1. Increased Lethality and Disease Activity in Il23RDIEC Mice in Chronic Intestinal Inflammation
Colitis was induced by cyclic administration of 2% DSS (n = 10 male animals/group).
(A and B) Weight loss (A) and overall survival (B) were monitored every other day until day 30.
(C) Representative histologies (H&E) of DSS-treated Il23RDIEC and Il23Rfl mice on day 30.
(D) The histological score displays the combined score of inflammatory cell infiltration and tissue damage.
(E) Colonic sections from Il23RDIEC and Il23Rfl mice were immunostained with antibodies for BrdU.
(F) Positively stained cells were counted per high-power field (HPF, 400×).
(G) The columns represent the median of the colon length obtained from 10 animals/group postmortem on day 30.
(H) Overall disease activity index on day 30.
(I) Colon transcript levels of STAT3-dependent proliferation-associated Reg3b and Reg3g mRNA in Il23RDIEC (n = 10 males) and IL23Rfl (n = 10 males) mice (qRT-PCR).
Significance was determined using two-tailed Student’s t test and is expressed as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
because the length of the BrdU+ epithelial lining (Figures 3I and 3J) and the number of BrdU+ cells per crypt (Figures 3K and 3L) were both reduced in Il23RΔIEC mice, which was further deterio-
rated by a Thy-1 treatment. In line with previous reports on IL-22-
deficient mice (Kreymborg et al., 2007; Zheng et al., 2008), a Thy-1 treatment in Il23RΔIEC mice increased the abundance of flagellated (54.38% in Il23RΔIEC and only 39.41% in Il23Rfl mice) bacterial groups (Figure 3G).

To demonstrate the direct role of IL-22 in the setting, mice were pretreated with a Thy-1 prior to DSS administration as described above. a Thy-1-treated Il23RΔIEC mice were then either supplemented with PBS (+PBS) or recombinant (rec.) IL-
22 (+IL-22) every other day and sacrificed on day 10. Notably, exogenous IL-22 completely rescued DSS-induced weight loss (Figure 4A), increased colon length (Figure 4D), reduced the abundance of flagellated bacteria (data not shown), and improved wound healing (as measured by BrdU staining and statistical assessment of the BrdU+ area) (Figures 4B and 4C), indicating that lack of IL-22 is indeed a decisive element for the observed phenotype in Il23RΔIEC mice. The findings indicate that coordination of both the epithelial renewal and the compo-
sition of the microbiota relies on the presence of functional IL-
23R in intestinal epithelial cells, which influences optimal mucosal IL-22 secretion.
Intestinal Epithelial IL-23R Activation Induces Reg3b Expression

We next addressed the potential mechanism that mediates the influence of intestinal IL-23R deficiency on IL-22 production by leukocytes. From other studies, which showed direct activation effects on myelomonocytic cells (Löhrner et al., 2015), we postulated that the C-type lectin Reg3b, a known STAT3 target gene in intestinal epithelial cells could be a decisive epithelium-derived co-factor to modulate IL-22 expression.

Reg3b expression was already significantly lower in small intestinal and colonic crypts from Il23rfl mice compared with Il23rfl animals at baseline (Figure 5A) and was further induced in epithelial cells upon DSS colitis in an IL-23-dependent manner in Il23rfl but not in Il23rfl mice (Figure 5B). Reg3b could be directly induced in epithelial cells upon i.p. injection with recombinant IL-23 in Il23rfl but not Il23rfl mice (Figure 5C), and induction was mediated by epithelial STAT3, because STAT3D mice failed to upregulate Reg3b in the colon epithelium compared with their STAT3fl littermates (Figure 5D). As an endogenous strong inducer of IL-23 (Kinnebrew et al., 2012), we further employed i.p. injection of the TLR5 ligand flagellin, which also led to upregulation of epithelial Reg3b in Il23rfl animals (Figures S6A and S6C) but not in Il23rfl mice. Notably, this regulation pattern induced by the endogenous flagellin/IL-23 model also coincided with differential IL22 mRNA and protein induction (Figures S6A and S6B). The failure to upregulate IL22

Figure 3. Epithelial Signaling Licenses IL-22 Production

(A–C) Crude epithelial crypt preparations from the small intestine (A), colon (B), and primary splenocytes (C) from untreated mice were isolated and stimulated for 3 hr with IL-23 (50 ng/ml), and IL-22 mRNA levels were investigated by real-time PCR. Data are representative of a minimum of 3 animals/genotype and experiment.

(D) Depletion of leukocytes using anti Thy-1 antibody treatment of 100 or 200 μg (T24/31) per mouse 2 days before crypt isolation. Note that such preparations contain associated leukocytes. The effect of the Thy-1 antibody was confirmed by FACS staining of small intestine lamina propria CD3+ cells.

(E) Isolated cell fractions were stimulated for 3 hr with IL-23 (50 ng/ml), and gene expression of IL-22 was assessed.

(F) Weight loss was monitored from day 3 every day until day 10.

(G) Increased abundance of flagellated bacteria upon aThy-1 administration in Il23rfl mice.

(H) Colitis was induced by administration of 2% DSS in the following groups: Il23rfl (n = 6; 3 male [m], 3 female [f]), Il23rfl (n = 7; 4 m, 3 f), Il23rfl + aThy1 (n = 5; 3 m, 2 f), and Il23rfl + aThy1 (n = 5; 4 m, 1 f). Anti-Thy-1 administration occurred 48 hr prior to DSS induction, on day 0, and then every third day by injection (i.p.) of 200 μg aThy1 or IgG control.

(I and K) Colon Swiss rolls from Il23rfl, Il23rfl, Il23rfl + aThy1, and Il23rfl + aThy1 mice were immunostained with antibodies for BrdU. Representative pictures are shown with red lines indicating the area of BrdU absent cells in the colon mucosa (I) or the number of BrdU+ cells per crypt (M) in colon Swiss rolls reveals a dramatic reduction of proliferation in Il23rfl mice and Il23rfl + aThy1 mice

Significance was determined using two-tailed Student’s t test and is expressed as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
upon flagellin stimulation was not observable in mixed splenocyte cultures from Il23R^DIEC mice, thus rendering a systemic TLR5 signaling defect unlikely (data not shown).

To rule out an indirect role of autocrine IL-22 as a Reg3b inducer, freshly isolated epithelial cells from wild-type (WT) animals were stimulated with IL-23 or IL-22 in the presence or absence of a neutralizing aIL-22 antibody for 3 hr. IL-23 and IL-22 both induced expression of Reg3b in intestinal epithelial cells, whereas only IL-22- but not IL-23-induced Reg3b expression was blocked by the neutralizing aIL-22 antibody (Figure 5E).

Last, intestinal organoids (Figure 5F) from Il23R^DIEC and Il23R^fl (Sato et al., 2009) mice were stimulated with IL-23 for 24 hr, and mRNA transcript levels of Reg3b showed significant epithelial Reg3b expression in organoids derived from Il23R^fl but not Il23R^DIEC cultures (Figure 5G). IL-22 was not detectable, again excluding autocrine IL-22 effects on Reg3b expression (Figure 5H). These data demonstrate that IL-23 induced Reg3b in intestinal epithelial cells via epithelial cell-expressed IL-23R.

Systemic Reg3b Administration Protects Il23R^DIEC Mice from DSS-Induced Colitis by Restoring IL-22 Production and Recruitment of Neutrophils

We hypothesized that, if indeed Reg3b derived by epithelial cells is important for the observed phenotype in Il23R^DIEC mice, then systemic administration of Reg3b should protect Il23R^DIEC mice from colitis and influence IL-22 production within the inflamed mucosa. Therefore, colitis was induced by 2% DSS, and Il23R^DIEC mice received either PBS or rec. Reg3b intraperitoneally every other day for 10 days at a dose far below the concentrations that have been shown to have a direct effect on microbiota when applied luminally in earlier studies (Zheng et al., 2008). Systemic Reg3b treatment significantly improved DSS-induced body weight loss (Figure 6A), reconstituted epithelial proliferation (Figures 6B and 6C), and increased local production of IL-22 in Reg3b-treated colon explant cultures (Figure 6F). Given that mucosal IL-22 secretion negatively regulates the expansion of flagellated β-proteobacteria, we next analyzed the bacterial composition of the fecal microbiota using 16S rRNA sequencing. A Bray Curtis PcoA plot showed significant (nonparametric multivariate ANOVA [NPMANOVA], p = 0.0089) differentiation between untreated and Reg3b-treated Il23R^DIEC mice (Figure 6G), and systemic Reg3b treatment resulted in a significant decrease of flagellated bacteria in the colonic feces of Il23R^DIEC mice (Figure 6H).

Because we had excluded the possibility of a direct synergistic effect of Reg3b stimulation on IL-23-induced IL22 expression by co-stimulation experiments in crude crypt preparations (data not shown), we next tested the hypothesis whether endogenous Reg3b could serve as a chemoattractant principle for IL-22-producing cells also in the intestine because expression of Reg3b was recently described to control the accumulation of activated immune cells in myocardial ischemia (Lörchner et al., 2015). Indeed, Il23R^DIEC mice exhibited reduced numbers of Thy-1^+ CD3^+, and CD4^+ cells in the small intestinal lamina propria compared with their Il23R^fl littermates under baseline conditions (Figures S7A–S7C). We specifically observed diminished numbers of neutrophilic granulocytes in the colonic lamina propria, as indicated in fluorescence-activated cell sorting (FACS)
staining (Gr-1, Figure S7D) and colon histology of untreated IL23RIEC compared with Il23Rfl mice (Figures S7 E and S7F).

Neutrophil numbers were notably increased in the lamina propria of Reg3b-treated Il23RIEC animals despite the clear anti-inflammatory effect of Reg3b treatment (Figures 6 D and 6E).

To further test the chemotactic role of Reg3b for immune cells, WT mice were injected i.p. with PBS or recombinant endotoxin-free Reg3b, and peritoneal cells were collected. Cytospin and FACS analysis of peritoneal cell composition showed a significant influx of neutrophils 6 hr after Reg3b administration, whereas the level of macrophages, eosinophils, and basophils remained unchanged (Figures 6J–6O and data not shown). In line with a positive influence of Reg3b on chemotaxis, Cxcl1 production was also increased in peritoneal fluids (Figure 6P) and sera (Figure 6Q) after Reg3b treatment. Finally, we could show that Reg3b KO mice produce less IL-22 in response to flagellin compared with similarly treated WT littermate controls (Figure 6 I).

Taken together, these data provide evidence for a mechanism involving IL-23R-dependent epithelial Reg3b expression, which is needed for adequate attraction of IL-22 secreting cells, most notably neutrophils. In addition to a direct regenerative effect, this mechanism may help to control colonic microbiota composition.

**DISCUSSION**

Intestinal homeostasis requires a balanced interplay of a plethora of cell types. A growing body of evidence highlights the key role of transducing tonic signals from commensal bacteria into cytokine-mediated epithelial regenerative programs (Veldhoen et al., 2008; Martin et al., 2009; Mielke et al., 2013; Leung et al., 2014). So far, the epithelium has been mainly thought to “passively” receive cytokine signals and transduce them into an antimicrobial and regenerative response that is known to be dependent on STAT3 (Pickert et al., 2009).

Here we demonstrate that IL-23-dependent activation of the intestinal epithelium serves as a crucial modulatory factor toward an orchestrated immune response from epithelial cells that subsequently control antimicrobial and regenerative processes in the inflamed mucosa.

It must be noted that the expression levels of IL-23R in intestinal epithelial cells are low compared with immune cells. Although we provide evidence for direct signaling and IL-23R-dependent target gene expression in IECs and Il23r mRNA upregulation during intestinal inflammation, the different expression patterns between the epithelial compartment and immune cells warrant an adequate discussion of the potential pitfalls of the model. We have controlled for the precision of the Cre excision by the villin-Cre line in Il23rflEC mice both by demonstrating the genomic deletion, which is only present in IECs but not in other cellular compartments, as well as by showing that mixed immune cells (splenocytes) from Il23rflEC mice respond functionally normal to IL-23 stimulation. In an independent cross for which we used the lysM-Cre line, deletion of the Il23r gene in the myeloid compartment caused a clear impairment of IL-23-dependent target gene expression in CD11c+ cells (unpublished data), demonstrating that
cre-mediated excision is principally also able to impair IL-23R-dependent signals in another genetic model. The employed villin-cre line has been characterized extensively in many studies, making an inadequate activation of the promoter in specific immune cells extremely unlikely as the underlying cause of the phenotype. We thus think that, despite the low level of expression, it is justified to assume that the observed protective effects are directly linked to an activation of IL-23R on intestinal epithelial cells.

We report that Il23r−/−IEC mice rapidly succumb to chronic DSS-induced colitis because of reduced wound healing and impaired IL-22-mediated protective immune responses. Il23r−/−IEC animals significantly differ in their composition of the gut microbiota with expansion of flagellated bacterial genera. Interestingly, the expansion of flagellated bacterial taxa became even more pronounced upon DSS challenge or inhibition of leukocyte extravasation and correlated with the severity of colitis. These observations agree with findings that the abundance of

Figure 6. Systemic Reg3b Substitution Rescues Il23r−/−IEC Mice by Increasing Local IL-22 Production

DSS colitis was induced in Il23r−/−IEC + Reg3b (n = 5; 3 m, 2 f) and Il23r−/−IEC + (n = 9; 5 m, 4 f) mice as described above. Mice received 2 μg rec. Reg3b or an equivalent volume of PBS i.p. on day 1 and every second day.

(A) Weight loss was monitored from day 3 every day until day 10.

(B) Colon Swiss rolls from Il23r−/−IEC and Il23r−/−IEC + Reg3b mice were immunostained with antibodies for BrdU. Representative pictures are shown, with red lines indicating the BrdU-negative area.

(C) Representative pictures of colonic sections stained with anti-BrdU antibody.

(D and E) Representative H&E pictures of colon showing increased neutrophil influx in Reg3b-treated mice (D) and statistical evaluation of counted neutrophils per HPF (E).

(F) IL-22 ELISA of colon explants from Il23r−/−IEC + Reg3b versus Il23r−/−IEC mice.

(G) Principle coordinate analysis (Gray Curtis distance matrices) of fecal flora obtained by 16S rDNA V4 sequencing from Il23r−/−IEC + Reg3b versus Il23r−/−IEC animals after DSS treatment (see the Supplemental Experimental Procedures for details).

(H and I) Abundances of dominant bacterial groups (genus level) after DSS treatment in the feces of Il23r−/−IEC + Reg3b versus Il23r−/−IEC mice according to the flagellatin status (flagellated, non-flagellated, or unknown) in response to DSS (H), IL-22 ELISA from WT or Reg3b KO explants after stimulation with 1 μg ultrapure flagellin for 2 h i.p. (I).

(J–Q) Peritoneal exudates of five wild-type mice were harvested 6 h after intraperitoneal injection of either sterile saline solution (K) or 100 μg/kg of purified, endotoxin-free Reg3b (N), and relative numbers of neutrophils or macrophages were assessed by cytospin (J and M), or Gr-1 and F4/80 staining was quantified by FACS analysis (K, L, N, and O). Representative flow cytometric analyses (K and N) of peritoneal cell populations and statistical analysis (L and O) show that Reg3b triggers the influx of neutrophils. Also shown is an ELISA on the indicated cytokines on peritoneal fluid (F) and sera (Q) after Reg3b stimulation as described above. Significance was determined using two-tailed Student’s t test and is expressed as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
flagellated intestinal bacteria is a critical factor for the severity of experimental colitis (Rhee et al., 2005; Vijay-Kumar et al., 2007; Ivison et al., 2010). One may therefore presume that the expansion of flagellated taxa in IL23R<sup>−/−</sup> mice may result from the lack of flagellin-induced IL-22 secretion, which is known to require endogenous IL-23 from dendritic cells (Kinnebrew et al., 2012). Recent studies in IL23<sup>−/−</sup> mice (Zenewicz et al., 2013; Behsens et al., 2014) and our own data reconstituting IL23R<sup>−/−</sup> mice with exogenous IL-22 corroborate this hypothesis. As in many other DSS-driven models, the altered microbiota augments the inflammatory phenotype observed in IL23R<sup>−/−</sup> mice, whereas co-housing with an excess of floxed littermates rescues IL23R<sup>−/−</sup> mice from disease risk.

Our data indicate that epithelial IL-23R signaling exerts pivotal control over mucosal IL-22 secretion through a newly described mechanism. Interestingly, our screening for potential chemokines secreted by the epithelium revealed that IL-23-dependent secretion of the c-type lectin Reg3b is likely to act as a paracrine factor amplifying IL-22 production in the lamina propria. The few studies on the effect of Reg3 proteins on intestinal mucosal immunity describe mainly a direct antimicrobial activity because mice deficient in Reg3g display a different mucus architecture (Vaishnava et al., 2011), and luminal application of high doses of recombinant Reg3g seems to reduce colonization with *Salmonella* (van Ampting et al., 2012). However, the exclusive direct antimicrobial effect is much less clear for Reg3b. The fact that genetic ablation of Reg3b in mice leads to prolonged colonization with *Y. pseudotuberculosis* in ileal Peyer’s patches without affecting luminal bacterial levels indicates a potential effect of Reg3b beyond its presence in the intestinal mucus (Dessein et al., 2009; van Ampting et al., 2012). A very recent report in the ischemic heart points toward a role of Reg3b in mediating macrophage trafficking to control protective immune cell infiltration into the affected heart tissue (Lorcher et al., 2013). Furthermore, Reg3b is skewing macrophage polarization through an unknown mechanism (Gironella et al., 2013).

Although we could not demonstrate a direct synergistic effect of Reg3b on IL-23-mediated induction of IL-22 expression in vitro, we show by intraperitoneal injection that the protein acts as a chemoattractant for neutrophils. Interestingly, neutrophils have recently been reported to serve as a source for mucosal IL-22 in DSS-induced inflammation (Zindl et al., 2013). In line with the idea that epithelium-derived Reg3b could control mucosal levels of IL22-producing neutrophils, intraperitoneal Reg3b administration attenuated the increased clinical susceptibility to DSS-induced inflammation in IL23R<sup>−/−</sup> mice. Under such conditions, Reg3b injection clearly upregulated neutrophil numbers in the lamina propria and restored IL-22-secretion in IL23R<sup>−/−</sup> animals.

We thereby demonstrate that IL-23R-dependent Reg3b triggers an influx of IL-22-producing immune cells. It remains to be determined whether the alarmin Reg3b may dictate the recruitment of only one or also other IL-22-producing cell types; however, our data clearly point to neutrophils as a plausible candidate. It also remains to be determined whether Reg3b is actively secreted on the basolateral side in the physiological setting or whether it may only enter the lamina propria via epithelial barrier defects. Taken together, we could show that the epithelial IL-23R licenses mucosal IL-22 production and serves as a critical factor orchestrating protective mucosal immune responses in the context of microbiota-fueled inflammation. These findings have important implications for future therapeutic developments in chronic inflammatory diseases because the rationale of IL-23 neutralization is the dampening of TH17 cell activity. However, in clinical settings like IBD, where IL-22 secretion might be an important protective factor, proregenerative functions of IL-23 on epithelial cells should be kept in mind.

**EXPERIMENTAL PROCEDURES**

**Mice**

Weight- and gender-matched mice (genetic background C57BL/6JxSv129, backcrossed for at least six generations) were used at an age of 8–12 weeks for all experiments. For DSS experiments, the indicated numbers (minimum n = 5 per genotype) of animals were employed. IL23R<sup>+/+</sup> mice were generated by targeted insertion of LoxP sites flanking exon 4 of the IL23R gene (Geno-way). Villin-Cre animals (strain B6.SJL-Tg(Vil-cre)997Gum/J) were purchased from Jackson Laboratory. Conditional knockout of IL23R in the intestinal epithelium was established by crossing Villin-Cre with IL23R<sup>−/−</sup> mice, resulting in IL23R<sup>−/−</sup> or IL23R<sup>+/+</sup>. It has been well established that this knockout, apart from some expression of the transgene in renal tubuli, is specific for the intestinal epithelium and does not involve immune cells in the intestine (El Marjou et al., 2004). STAT3<sup>fl/fl</sup> and STAT4<sup>fl/fl</sup> mice were provided by Christine Watson (Cambridge University). Reg3b WT and Reg3b KO mice were kindly provided by Stephen P. Hunt (University College London). The ß-galactosidase reporter mouse (IL23R knockin/knockout mouse [Il23rtm1a (EUCOMM)Wtsi mouse with lacZ cassette]) was kindly provided by the EUCOMM Consortium. Mice were maintained in a 12-hr light-dark cycle under standard conditions and were provided with food and water ad libitum. Procedures involving animal care were conducted according to national and international laws and policies and with appropriate permission. Appropriate permission was given by the local animal safety review board of the federal ministry of Schleswig Holstein.

**Antibodies and Reagents**

Murine αThy-1 (clone T24/31) was purchased from BioXcell. Flagellin (FLA-ST ultrapure) was purchased from InvivoGen. Recombinant murine Reg3b was purchased from R&D Systems. Recombinant murine IL-22 was purchased from PeproTech, and recombinant murine IL-23 was purchased from BioLegend.

**FACS**

Flow cytometry was performed on a FACSCalibur (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences) using standard parameters and antibodies (details can be found in the Supplemental Experimental Procedures).

**Isolation of Primary Cells and Cultivation of Intestinal Organoids**

Murine intestinal epithelial cells and lamina propria cells were isolated using the Hank’s balanced salt solution (HBSS)-EDTA method followed by enzymatic digestion. Mouse intestinal organoids were cultivated as described before (Sato et al., 2009). For details, see the Supplemental Experimental Procedures.

**Induction of Collitis, Determination of Clinical Scores, and Histology**

For chronic colitis induction, mice were supplied with 2% DSS (molecular mass, 40 kDa; TdB Consultancy) dissolved in drinking water for 5 days, followed by 5 days of regular drinking water. The disease activity index (DAI) was obtained as described previously (Siegmund et al., 2001). Consumption of drinking water was measured daily. A high-resolution mouse video endoscopic system was used on day 30 of the experiment (Karl Storz AIDA VET), and endoscopic scores were obtained as described previously (Wirtz et al., 2007). For induction of short colitis, mice were supplied with 2% DSS dissolved in drinking water for 3 days and sacrificed immediately for intestinal
epithelial cell isolation as described elsewhere (Pickert et al., 2009). Histopathological analyses were performed according to standard methods (see the Supplemental Experimental Procedures for details).

**Colon Culture**

Colon tissues were harvested from C57BL/6 mice and used for the induction of colitis. Colon tissues were cultured in 24-well flat-bottom culture plates in RPMI 1640 medium (Gibco) for 24 hr at 37°C. Supernatants were analyzed for IL-22 concentration using an IL-22 ELISA kit according to the manufacturer’s protocol (PeproTech).

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism (version 4.0) for Windows software (GraphPad). Statistical significance was evaluated by Mann-Whitney U test for nonparametric data or Student’s t test for parametric data unless indicated otherwise. p values less than 0.05 were considered statistically significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.07.054.

**AUTHOR CONTRIBUTIONS**

K.A. and P.R. conceived and designed the study; acquired, analyzed, and interpreted the data; performed statistical analysis; and wrote the manuscript. A.R., M.F.P., J.K., F.T., S.P., A.B., M.J., R.H., R.S.T., S.B.B., O.W., and S.L. contributed to the interpretation of data and writing of the manuscript. A.R., M.F.P., and T.S. contributed equally.

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