HOIL1 regulates group 2 innate lymphoid cell numbers and type 2 inflammation in the small intestine

Matthew J Wood  
*University of Illinois at Chicago*

Jeffrey N Marshall  
*University of Illinois at Chicago*

Victoria L Hartley  
*University of Illinois at Chicago*

Ta-Chiang Liu  
*Washington University School of Medicine in St. Louis*

Kazuhiro Iwai  
*Kyoto University*

See next page for additional authors

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HOIL1 regulates group 2 innate lymphoid cell numbers and type 2 inflammation in the small intestine

Matthew J. Wood1,4, Jeffrey N. Marshall1, Victoria L. Hartley1, Ta-Chiang Liu2, Kazuhiro Iwai3, Thaddeus S. Stappenbeck2,5 and Donna A. MacDuff1,2

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INTRODUCTION

Inflammatory bowel disease (IBD) affects around 1% of the US population and prevalence continues to increase in developed countries1. IBD is a complex disease influenced by both genetic and environmental factors, and specific treatments are therefore only effective for a subset of patients. Patients with mutations in heme-oxidized IRP2 ubiquitin ligase 1 (HOIL1; official gene name RBCK1), experience a complex immune disorder involving autoinflammation and inflammatory bowel disease-like symptoms, increased susceptibility to bacterial infections, progressive muscular amylopectinosis and myopathy2. Gastrointestinal symptoms in HOIL1-deficient mice include abdominal pain, bloody and mucous stools, colonic lesions and eosinophilic infiltration3. HOIL1, HOIL1-interacting protein (HOIP; official name RNF31) and SHANK-associated RH domain-interacting protein (SHARPIN) form an E3 ubiquitin ligase complex called the linear ubiquitin chain assembly complex (LUBAC). Patients with mutations in HOIP display similar clinical and cellular phenotypes to HOIL1 deficient patients2.

LUBAC is the only enzyme known to generate linear (methionine-1-linked) polyubiquitin chains due to the unique E3 ubiquitin ligase activity of HOIP, and has been shown to regulate NFκB activation and programmed cell death downstream of many innate immune receptors, including TNFR1, IL1R1, IL-17R and toll-like receptors (TLRs)4–6. LUBAC also regulates CD40, B and T cell receptor, inflammasome and RIG-I-like receptor signaling pathways. Accordingly, HOIL1 and LUBAC are important for the efficient induction of type 1 inflammatory cytokines and interferons, and to control bacterial and viral infections7–9.

In mice, complete loss of HOIP or HOIL1 expression results in embryonic lethality due to essential roles in hematopoiesis and in limiting TNFα-induced cell death10,11. SHARPIN-deficient mice are viable, but exhibit defects in immune development as well as severe systemic inflammation within the first two months of life12. To study the physiological consequences of HOIL1-deficiency, we have employed a HOIL1-mutant mouse model (Hoil1−/− herein) that expresses the N-terminal domain of HOIL1 at approximately ten percent of wild-type levels, enabling partial stabilization of LUBAC and viability of homozygous mice8,13,14. Expression of both HOIL1 and HOIP is reduced, and LUBAC function is impaired in ileum of mice by 18 months of age, similar to those observed in mice8,13,14. We previously demonstrated that these mice are a relevant model of human HOIL1-deficiency, since they exhibit immunodeficiency or hyperinflammatory responses, depending on the pathogenic challenge8. Macroscopically, naïve Hoil1−/− mice are indistinguishable from their wild-type (Hoil1+/+) littermates, but glycogen-like deposits are observed in the cardiac tissue of mice by 18 months of age, similar to those observed in humans with mutations in HOIL18.

Patients with mutations in HOIL1 experience a complex immune disorder including intestinal inflammation. To investigate the role of HOIL1 in regulating intestinal inflammation, we employed a mouse model of partial HOIL1 deficiency. The ileum of HOIL1-deficient mice displayed features of type 2 inflammation including tuft cell and goblet cell hyperplasia, and elevated expression of Il13, Il5 and Il25 mRNA. Inflammation persisted in the absence of T and B cells, and bone marrow chimeric mice revealed a requirement for HOIL1 expression in radiation-resistant cells to regulate inflammation. Although disruption of IL-4 receptor alpha (IL4Rα) signaling on intestinal epithelial cells ameliorated tuft and goblet cell hyperplasia, expression of Il5 and Il13 mRNA remained elevated. KLRG110, CD9010 group 2 innate lymphoid cells were increased independent of IL4Rα signaling, tuft cell hyperplasia and IL-25 induction. Antibiotic treatment dampened intestinal inflammation indicating commensal microbes as a contributing factor. We have identified a key role for HOIL1, a component of the Linear Ubiquitin Chain Assembly Complex, in regulating type 2 inflammation in the small intestine. Understanding the mechanism by which HOIL1 regulates type 2 inflammation will advance our understanding of intestinal homeostasis and inflammatory disorders and may lead to the identification of new targets for treatment.

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**Fig. 1**  **Hoil**⁻/⁻ **mice exhibit type 2 inflammation in the distal ileum.** a Representative images of H&E stained sections of ileum from **Hoil**⁺/⁺ and **Hoil**⁻/⁻ mice. Scale bars represent 50 μm (left panel) and 20 μm (right panels). b Representative images of PAS/Alcian Blue stained sections. c Goblet cells per villus in **Hoil**⁺/⁺ and **Hoil**⁻/⁻ ileum. d-h Relative **Il4**, **Il5**, **Il13** (d), **Tnf**, **Ifng** (f), **Il25**, **Il33**, **Il18** and **Tslp** (g) mRNA levels, and **Il4**, **Il5** (e), **Il-25**, **Il-33** and **Tslp** (h) protein levels in ileum of **Hoil**⁺/⁺ and **Hoil**⁻/⁻ mice. i **Il18**, **Il25**, **Il33** and **Tslp** mRNA levels in **Hoil**⁺/⁺ and **Hoil**⁻/⁻ IEC and LP fractions relative to **Hoil**⁺/⁺ IEC median. j DCLK1 (red) and DAPI (blue) stained sections of ileum from **Hoil**⁺/⁺ (left) and **Hoil**⁻/⁻ (right) mice (scale = 50 μm). k Enumeration of tuft cells in villi and crypts of **Hoil**⁺/⁺ and **Hoil**⁻/⁻ ileum. Each symbol represents a sample from an individual mouse and colored bars represent the median. mRNA levels are expressed as relative to the median level for **Hoil**⁺/⁺. Histological enumerations and measurements represent the mean from >10 villi per mouse. All mice were aged between 6 and 9 weeks. H&E Hematoxylin and Eosin. IEC Intestinal epithelial cell fraction, LP Lamina propria fraction. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.001 by Mann-Whitney (c-h, k) or ordinary 2-way ANOVA (l).
Here, we show that expression of HOIL1 in a radiation-resistant cell type is required to limit type 2 inflammation in the small intestine. Excessive expression of type 2 inflammatory cytokines in HOIL1-deficient tissue did not require T cells or B cells, IL4Ra-dependent tuft cell hyperplasia or induction of IL-25. Global gene expression and flow cytometric analyses revealed that group 2 innate lymphoid cell (ILC2) numbers were increased in the absence of HOIL1 and independent of IL-25 induction. Antibiotics
treatment alleviated the inflammation, indicating a role for microbial sensing. Our data reveal a novel role for HOIL1 in regulating type 2 inflammation in the intestine, contributing to a broader understanding of the mechanisms of intestinal homeostasis and disease.

RESULTS
HOIL1-deficient mice exhibit type 2 intestinal inflammation in the distal ileum
To investigate whether HOIL1 deficiency causes intestinal inflammation in mice, we examined the distal ileum from specific pathogen-free (SPF) Hoil1−/− and Hoil1+/+ co-housed littermates. Histological analysis revealed goblet cell hyperplasia in the distal ileum of Hoil1−/− mice (Fig. 1a–c), relative to tissues from Hoil1+/+ littermates. This histological change is characteristic of type 2 inflammation observed after intestinal helminth infection.15,16 Consistently, mRNAs for type 2 inflammatory cytokines Il4, Il5 and Il13 were elevated in Hoil1−/− compared to Hoil1+/+ ileum (Fig. 1d). An increase in Il-4 and Il-5 protein was also detected in the homogenized tissue (Fig. 1e). However, we did not detect changes in type 1 inflammatory cytokine mRNAs, Ifng and Tnf (Fig. 1f), indicating that Hoil1−/− mice do not experience a generalized, non-specific inflammation, or a shift from type 1 to type 2 cytokine production. Type 2 inflammation can be caused by infection with a common intestinal protozoan, Tritrichomonas muriis, present in some SPF mouse colonies. However, Tritrichomonas muriis was not detected in fecal samples from our mice (not shown).

Production of IL-4, IL-5 and IL-13 can be induced by IL-25, IL-33, TSLP and IL-18.15,16,19 Il25 and Il13 mRNAs and IL-25 protein were slightly elevated in Hoil1−/− ileum relative to Hoil1+/+ ileum (Fig. 1g, h). However, no differences in Il18 and Tsp5 mRNA, or IL-33 and TSLP total protein, were observed. In order to increase the sensitivity of mRNA detection, we separated the epithelial cell and lamina propria (LP) cell fractions. Expression of Il13, Tsp5 and Il18 mRNA was similar for Hoil1−/− and Hoil1+/+ mice within each cell fraction (Fig. 1i). However, Il25 mRNA was significantly higher in the Hoil1−/− IEC fraction. Tuft cells are the primary producers of IL-25 in the small intestine, undergo hyperplasia in response to IL-13 during helminth infection, and can be identified by their unique expression of DCLK1.17,20-22 Accordingly, DCLK1+ cells were significantly increased in the distal ileum of Hoil1−/− mice (Fig. 1j, k). Taken together, these data show that HOIL1 deficiency in mice results in a type 2-like inflammation in the distal ileum associated with excessive expression of Il4, Il5, Il13 and Il25 mRNAs and histological changes.

Symbiotic microbes promote type 2 inflammation in the absence of HOIL1
We next examined the post-natal development of type 2 inflammation in the ileum of Hoil1−/− mice. No significant differences in Il5, Il13 or Ifng mRNA expression were measured in the intestine from newborn Hoil1−/− and Hoil1+/+ mice (Fig. 2a). At 3 weeks of age, both Il5 and Il13 mRNAs were slightly, but not significantly, elevated in the ileum of Hoil1−/− mice (Fig. 2b). These data suggest that Hoil1−/− mice develop intestinal inflammation with age, possibly due to increasing microbial exposure and diversity. To test whether intestinal microbes drive intestinal inflammation in Hoil1−/− mice, we treated 6 to 8 week-old mice with a broad-spectrum cocktail of antibiotics for two weeks by daily oral gavage. At 7 and 14 days after starting antibiotics treatment, bacterial 16S DNA levels in stool were below the limit of detection (Fig. 2c). Following 14 days of antibiotic treatment, expression of Il4, Il5 and Il13 mRNA in distal ileum of Hoil1−/− mice was reduced to the level measured in Hoil1+/+ mice treated with water (Fig. 2d, e). These mRNAs were also reduced in the ileum of Hoil1+/+ mice by antibiotics treatment, but to a lesser extent. No differences in Il18, Tsp5, Il25, Ifng or Tnf expression were measured between Hoil1−/− and Hoil1+/+ mice, although expression was reduced by antibiotics treatment (Fig. 2f, h). Il25 remained slightly elevated in tissue from antibiotics-treated Hoil1−/− mice despite Il13 and Il4 being reduced to water-treated Hoil1+/+ levels or below. The number of goblet and tuft cells in antibiotics-treated Hoil1−/− mice was reduced almost to Hoil1+/+ frequencies, which may be a direct effect of loss of microbial exposure to the IECs, or an indirect effect via a reduction in IL-13 expression (Fig. 2g–h). These data indicate that microbial exposure contributes to aberrant type 2 inflammation in the absence of HOIL1.

Excess production of Il13 and Il5 occurs independently of goblet and tuft cell hyperplasia and IL-25 induction in Hoil1−/− ileum
During helminth infection, IL-13 stimulation of IECs drives epithelial cell changes, including goblet and tuft cell hyperplasia similar to that observed in the Hoil1−/− mice. Through a feed-forward mechanism, increased production of IL-25 by tuft cells promotes further production of IL-13, IL-5 and IL-4.16,18,19 To determine whether the elevated levels of IL-13 were responsible for the epithelial abnormalities observed, we examined the role of IL-13/IL-4 signaling specifically in IECs by crossing Hoil1−/− mice to I4rafl/fl mice and VillinCre (ΔIEC) transgenic mice.23,24 Histological analysis revealed that deletion of I4ra on IECs largely rescued the epithelial cell abnormalities in Hoil1−/− mice (Fig. 3a–d). Consistently, Il25 and Il13 mRNAs were reduced in Hoil1−/− I4rafl/flIEC tissue to levels comparable to Hoil1−/− I4rafl/fl and Hoil1+/+ I4rafl/flIEC tissue (Fig. 3f). Surprisingly, Il5 and Il13 mRNAs remained elevated in Hoil1−/− I4rafl/flIEC tissue, despite Il25 and Il13 mRNA being reduced to Hoil1−/− I4rafl/flIEC levels (Fig. 3g). Il5 mRNA was slightly reduced in the absence of IL-4Rα expression, indicating partial dependence. IL-25, IL-33, TSLP, IL-13, J. S. and IL-13 protein levels were highly variable in whole tissue samples and, although they appeared to be slightly lower in Hoil1−/− I4rafl/flIEC tissue compared to Hoil1−/− I4rafl/gf tissue, these differences were not significant (Fig. 3h, i). Il13 and Il5 mRNAs were also elevated in other regions of the gastrointestinal tract such as the jejunum and, to a lesser extent, the mesenteric lymph nodes (MLN) and colon (Fig. 3j–l). Differences in Il4 mRNA expression were not detectable in the MLN, suggesting that IL-4 may not be a driving component of this pathway. Together, these data show that increased IL-13/IL-4 signaling in IECs via I4ra triggers goblet and tuft cell hyperplasia and the induction of IL-25 in Hoil1-deficient
ileum, but that an IL4Ra-dependent increase in IL25, IL33 or Tslp is not required to drive the excessive IL13 and IL5 mRNA expression.

**Aberrant type 2 inflammation in Hoil1−/- ileum is not dependent on T cells**

Type 2 CD4+ helper T cells (Th2) and group 2 innate lymphoid cells (ILC2) are considered to be the primary producers of IL-13 and IL-4, and ILC2 are almost exclusive producers of IL-25. We sought to determine that the type 2 phenotype. However, IL4 and IL5 mRNA levels in jejune (f) from Hoil1−/- mice, including ILCs, NK cells, dendritic cells, and mast cells. The percentage of CD3+ T cells was significantly reduced in Hoil1−/- mice, indicating that these cells may also be dysregulated in the absence of Hoil1 (Fig. 4b, c).

To determine whether T cells are required for the type 2 inflammation in the absence of Hoil1, we examined Hoil1−/- mice. Goblet and tuft cell numbers were increased in the ileum of Hoil1−/- mice compared to Hoil1−/- mice (Fig. 4d–f). IL5, IL13 and IL25 mRNAs were elevated in Hoil1−/- mice (Fig. 4g, h), indicating that T cells are not required to trigger the inflammatory phenotype. However, IL4 expression was not elevated in Hoil1−/- mice compared to Hoil1−/- ileum (Fig. 4g), indicating that T cells are the major producers of IL-4, as expected. Together, these data show that T cells are not required for type 2 inflammation in Hoil1-deficient ileum, and suggest that another cell type, such as ILC2, is required.

**Expression of Hoil1 is required in radiation-resistant cells to regulate intestinal type 2 inflammation**

We next asked whether Hoil1 is required in cells derived from the bone marrow to limit type 2 inflammation, and generated reciprocal bone marrow chimeras after lethal irradiation of Hoil1−/- (WT) and Hoil1−/- (KO) mice. Chimerism was confirmed by measuring expression of Hoil1 (Rbck1) mRNA in the ileum and relative amounts of Hoil1−/- and Hoil1−/- genomic DNA in blood (Fig. 5a, b). Histological and gene expression analyses performed after 16 weeks revealed that transfer of KO bone marrow into WT mice was not sufficient to trigger goblet cell hyperplasia or excessive IL13 expression (Fig. 5c–e). Furthermore, transfer of WT bone marrow into KO mice was not sufficient to suppress goblet cell hyperplasia or IL13 induction. These data indicate that expression of Hoil1 in a radiation-resistant, non-bone marrow-derived cell type is required to prevent aberrant type 2 inflammation.

**HOIL1 limits ILC2 numbers in the small intestine**

We considered that HOIL1 may control the production of a factor that regulates type 2 cytokine expression. We previously determined that changes in expression of Il18, Tslp, Il25 or Il33 were unlikely to be responsible (Fig. 3). To assess a broader range of potential regulators, we measured the mRNA expression of a number of factors that have been shown either to suppress or to promote the production of type 2 cytokines31,32. We examined Hoil1−/- mRNA levels in jejune (f) from Hoil1−/- mice, including ILCs, NK cells, dendritic cells, and mast cells. The percentage of CD3+ T cells was significantly reduced in Hoil1−/- mice, indicating that these cells may also be dysregulated in the absence of Hoil1 (Fig. 4b, c).

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DISCUSSION
In this study, we have identified a critical role for HOIL1 in regulating type 2 inflammation in the small intestine of mice. HOIL1-mutant mice exhibited characteristic goblet and tuft cell hyperplasia that was associated with increased expression of IL-4, IL-5, IL-13 and IL-25. Goblet and tuft cell hyperplasia and \textit{Il25} induction were dependent on signaling through IL4Rα on IECs. However, \textit{Il13} and \textit{Il5} mRNA and ILC2 numbers remained
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Fig. 4  T cells are not required to drive type 2 inflammation in Hoil1−/− ileum. a IL4, IL5 and IL13 mRNA levels in Hoil1+/+ and Hoil1−/− IEC and LP cell fractions relative to Hoil1+/+ IEC. b Representative flow plots gated on live, CD45+ LP cells from Hoil1+/+ and Hoil1−/− ileum showing the gating strategy and intracellular IL-13 expression in CD3+ and in CD11b+ CD3− CD19− cell populations. c Quantification of IL-13+ cells (left panel) and percentage (of total CD45+ cells, right panel) for the indicated cell populations from Hoil1+/+ and Hoil1−/− ileum. d, e H&E (d) and DCLK1 and DAPI (e) stained sections of ileum from Hoil1+/+ Rag1−/− and Hoil1−/− Rag1−/− mice (scale = 50 μm). f Enumeration of goblet cells per villus in ileum from Hoil1+/+ Rag1−/− and Hoil1−/− Rag1−/− mice. g, h IL4, IL5, IL13 (g), IL25 and IL33 (h), mRNA levels in Hoil1+/+ Rag1−/− and Hoil1−/− Rag1−/− distal ileum relative to Hoil1+/+ Rag1−/−. Each symbol represents a sample from an individual mouse and bars represent the median. All mice were aged between 6–9 weeks. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by 2-way ANOVA with Tukey’s multiple comparisons test (a) or Mann-Whitney test (c, f-h).

Fig. 5  Expression of HOIL1 in non-hematopoietic cells is required to suppress type 2 inflammation in the ileum. a Rbck1 mRNA levels (Hoil1, exons 3–4) in ileum from bone marrow chimeric mice relative to WT + WT. WT + WT: Hoil1+/+ mice with Hoil1+/+ bone marrow; WT + KO: Hoil1+/+ mice with Hoil1−/− bone marrow; KO + WT: Hoil1−/− mice with Hoil1+/+ bone marrow; and KO + KO: Hoil1−/− mice with Hoil1−/− bone marrow. b Percentage of WT (Rbck1 intron 7, left panel) or KO (neomycin-resistance cassette, right panel) gDNA in blood from bone marrow chimeric mice relative to WT + WT or KO + KO controls. c Il13 mRNA levels in ileum from bone marrow chimeric mice relative to WT + WT. d Representative H&E stained sections of ileum from bone marrow chimeric mice (scale = 50 μm). e Enumeration of goblet cells per villus in ileum from bone marrow chimeric mice. Each symbol represents a sample from an individual mouse and colored bars represent the median. Histological enumerations and measurements represent the mean from >10 villi per mouse. Chimeric mice were analyzed 16 weeks after reconstitution. H&E Hematoxylin and Eosin. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by Brown-Forsythe and Welch one-way ANOVA with Dunnett’s T3 multiple comparisons test (a-c), or ordinary one-way ANOVA with Tukey’s multiple comparisons test (e).

significantly elevated when tuft cell and IL-25 induction were blocked by deletion of IL4Ra on IECs, demonstrating that HOIL1 functions upstream of IL4Ra in the feed-forward cycle to regulate ILC2.

Although Th2 cells, ILC2, eosinophils and mast cells can express type 2 inflammatory cytokines, analysis of Hoil1−/− Rag1−/− mice demonstrated that T cells are not required for inflammation. These findings were consistent with an increase in intracellular IL-13 observed in a CD11b+CD3+CD19− population, but not in the CD3+ or CD11b+ cell populations from the Hoil1-deficient small intestine. Furthermore, RNA-Seq analysis of CD45+ cells identified an increase in mRNA expression of six ILC2-associated genes, two of which (Nmur1 and Il5) are specific for ILC2. Subsequent flow cytometric analysis revealed a four to five-fold increase in LinKLRG1−CD90+ ILC2 in Hoil1-deficient tissue, which was independent of IL4Ra signaling on IECs, tuft cell expansion and further induction of IL-25. These KLRG1−CD90+ ILC2 may be similar to the inflammatory ILC2 that have been reported to proliferate in the small intestine, then migrate to the lung and other tissues in response to helminth infection or IL-25 treatment34,35,36.

The proliferation and activation of ILC2 can be induced by IL-25, TSLP, and IL-33, along with additional signals such as cysteinyl leukotrienes, NMU, or Notch ligands32–34,37,39. We were unable to detect differences in TSLP or IL-33 mRNA or protein expression and, although IL-25 was elevated in the Hoil1-deficient ileum, the
IL-4Rα-dependent increase in IL-25 was not required. Global mRNA analysis of CD45+ and CD45- cells did not reveal candidates except genes associated with ILC2. One possibility is that HOIL1 plays a cell-intrinsic role in regulating ILC2, and this would be consistent with a requirement for HOIL1 in radiation-resistant, non-bone marrow-derived cells, since some ILC2 are thought to self-renew in tissues. A recent study identified LUBAC as a component of the IL17RA/IL17RC receptor signaling complex (RSC) required for efficient signal transduction and NFκB activation. The same study identified a negative feedback loop for the IL-17RSC, although LUBAC did not appear to be involved. Since IL-25 signals through IL17RB, which is highly expressed on gut ILC2, it is plausible that HOIL1 and LUBAC regulate tonic IL-25/IL17RB signaling and therefore ILC2 numbers and activation state (Fig. 8).

Other mechanisms are possible including ILC2-intrinsic regulation of IL17RB expression or signaling through other receptors, or ILC2-extrinsic roles for IL-27, interferons, neuropeptides such as NMU, or lipid mediators such as prostaglandins and leukotrienes. Further studies, such as cell type-specific deletion of Hoil1, will be required to distinguish these possibilities.

Although IL-25 is a well-established activator of intestinal ILC2, examination of antibiotics-treated mice indicated that a signal other than IL-25 is involved. Antibiotics treatment reduced Il4, Il5 and Il13 mRNA levels in Hoil1+/+ tissue to levels similar to Hoil1−/− tissue from water-treated mice. Il25 mRNA, however, was only partially reduced by antibiotics, and reduction of Il25 mRNA (by blocking IL4Ra signaling) was not sufficient to reduce Il5 and Il13 mRNA. Others have shown that resting ILC2 numbers and Il5 expression are largely unaffected by the absence of microbes in wild-type mice. However, the additional ILC2 we observed in the absence of HOIL1 may be activated ILC2 and subject to additional modes of regulation. Future studies will need to determine whether loss of microbial exposure reduces ILC2 numbers or activation state, and to determine whether HOIL1 regulates this response to microbes in an ILC2-intrinsic or extrinsic manner (Fig. 8).

Methods

Mice

All mice used in this study were on a C57BL/6J background. Mice were housed in accordance with Federal and University guidelines and protocols were approved by the University of Illinois Chicago Animal Care Committee and the Animal Studies Committee of Washington University.
Fig. 7 HOIL1 regulates ILC2 numbers in the small intestine. a Counts per million reads (CPM) for the indicated genes identified as differentially expressed in CD45+ cells from Hoil1f/fI4ra− and Hoil1f/fI4ra+Ieto ileum relative to Hoil1f/fI4ra− and Hoil1f/fI4ra+Ieto by RNA-Seq analysis, q ≤ 0.05 for both comparisons. b Heat map/dot plot representation of the indicated genes in cell types identified in LP from untreated and OVA-treated mice by scRNAseq8. c Heat map/dot plot representation of Rbck1 expression in intestinal epithelial cell subsets identified by scRNAseq8, d e mRNA levels for the indicated genes in whole ileum (d) or MLN (e) measured by qRT-PCR and expressed as relative to Hoil1f/fI4ra−. f Representative flow plots gated on live, CD45+ Lin (CD3, CD4, CD5, CD11b, CD11c, CD19, NK1.1) cells showing expression of CD90.2 and KLRG1. g Quantification of panel F for Lin− CD90.2 KLRG1− and Lin+ CD90.2+ KLRG1+ cells (percentage of total CD45+ cells). Each symbol represents a sample from an individual mouse and colored bars represent the median. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by 2-way ANOVA with Tukey’s multiple comparisons test.

Hool1f/f (Rbck1tm1Kiwia) and Hoil1f/f-Rag1f/f mice have been described previously8,13. Co-housed Hoil1f/f and littermates were used as wild-type controls. I4rafox/flox mice were a gift from Ajit Chacko, and bred to Hoil1f/f mice. VillinCre (B6.Cg-Tg(Vil1-cre)997Gum/J) mice were purchased from The Jackson Laboratory and bred to Hoil1f/fI4rafox/flox mice. Male and female mice were included in all analyses. Mice from at least two litters were used to generate each data set. No mice were excluded from the analyses.

IEC and LP cell separation
Ileum and jejunum were flushed with PBS, Peyer’s patches removed, opened longitudinally, and cut into 1 cm pieces. Two washes with HBSS supplemented with 10% bovine calf serum, 15 mM HEPES, 5 mM EDTA and 1.25 mM DTT were performed at room temperature for 20 min under continuous rotation followed by 20 s of vortexing in PBS pH 7.4. IECs were collected and resuspended in TRI-reagent (Sigma). Remaining tissue containing the LP fraction was homogenized in TRI-reagent.

Flow cytometry
Ileum and jejunum were flushed as described above for LP separation. LP pieces were transferred to 15 ml RPMI 1640 supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine with 0.5 mg/ml Collagenase VIII (Sigma). Samples were shaken vigorously by hand and placed in a shaking incubator at 220 rpm and 37 °C for 15 min with additional manual shaking at 10 min. After 15 min, 35 ml ice-cold complete media was added. Samples were washed twice with FACS buffer (PBS pH 7.4, 1% FBS, 2 mM EDTA) and filtered with 100 μm and 70 μm strainers. Single cell suspensions were incubated with CD16/CD32, normal mouse, rat and hamster serum, then stained with fluorophore-conjugated antibodies against: CD45 (30-F11), CD11b (M1/70), CD19 (6D5), Siglec-F (S17007L), CD3 (17A2), IL7R (A7R34). Viable cells were identified using a PE-anti-IL-13 antibody (eBio13A). Flow cytometry was performed using a BD Fortessa X20 or CytoFLEX S (Beckman Coulter), and data were analyzed using FlowJo10.5 (TreeStar Inc.). Flow cytometry gating strategy was based on fluorescence minus one (FMO), unstained, and isotype controls.

Fluorescence-activated cell sorting and RNA-Seq analysis
IEC and LP cell fractions were prepared as described above, and 50% of the IEC combined with the LP cells. Cells were stained with Zombie NIR fixable viability dye (BioLegend). For identification of ILC populations, positive cells were excluded by staining for CD3 (17A2), CD4 (GK1.5), CD5 (S3-7.3), CD11b (M1/70), CD11c (N418), CD19 (6D5) and NK1.1 (PK136). CD45 (30-F11), CD90.2 (30-H12), and KLRG1 (2F1) were used for positive identification. For intracellular staining, cells were re-stimulated for 4 h with PMA/Ionomycin and Brefeldin A (Biolegend), according to manufacturer’s recommendations, then fixed and permeabilized using a Foxp3/Transcription Factor buffer set (eBioscience), according to manufacturer’s instructions. Cells were stained with a PE-anti-IL-13 antibody (eBio13A). Flow cytometry was performed using a BD Fortessa X20 or CytoFLEX S (Beckman Coulter), and data were analyzed using FlowJo10.5 (TreeStar Inc.). Flow cytometry gating strategy was based on fluorescence minus one (FMO), unstained, and isotype controls.

Analysis of scRNA-seq datasets
scRNA-seq datasets of small intestine LP (GEO: GSE124880) and epithelium (GEO: GSE92233)10 were probed for expression of genes of interest through the Broad Institute’s Single Cell Portal.

Antibiotic treatment
Mice were treated by daily oral gavage with either sterile dH2O or 100 mg/kg ampicillin, 100 mg/kg neomycin, 50 mg/kg vancomycin, and 100 mg/kg metronidazole dissolved in sterile dH2O16. Stool pellets collected on days 0, 4, 7, and 14. Randomization of animals into treatment groups was not explicitly performed, but determined by cage assignment at weaning prior to genotyping.

Fecal DNA isolation
DNA was isolated from homogenized fecal pellets by double phenol: chloroform:isooamyl alcohol extraction and isopropanol precipitation19.

Tritrichomonas mursi testing
Fecal pellets were collected from at least two breeding cages from each mouse strain: Hoil1f/f, Hoil1f/f-Rag1f/f, Hoil1f/fRag1f/fIrα−Irβ− (2-3 pellets per cage), and shipped to IDEXX BioAnalytics for testing for Tritrichomonas mursi. All samples were negative.

RNA isolation
Whole 1 cm tissue samples of distal ileum (1 cm from the cecum), jejenum (10–11 cm from the stomach), distal colon, or mesenteric lymph nodes were snap-frozen and stored at −80 °C. Samples were homogenized in TRI-Reagent (Sigma) using zirconia/silica beads and a Mini-Beadbeater 24 (BioSpec). RNA was isolated according to the manufacturer’s instructions. RNA samples were treated with Turbo DNA-free DNase (Invitrogen) and 1
**Fig. 8** Model of role of HOIL1 in regulating type 2 inflammatory signaling. HOIL1 acts in a radiation-resistant cell type to suppress ILC2 proliferation and the production of IL-4, IL-5, and IL-13 in the presence of commensal microbes. HOIL1 may function to suppress the production of a positive regulatory factor (orange circles) upstream of IL-4, IL-5 and IL-13, or may be required for the negative regulation of ILC2 through a cell-extrinsic (pink circles) or cell-intrinsic mechanism, such as inhibition of IL17RB (IL25R) signaling. HOIL1 functions outside of the IL-13–tuft cell – IL-25 feed-forward loop. In the absence of HOIL1, excessive IL-4, IL-5 and IL-13 can trigger chronic type 2 inflammation including goblet and tuft cell hyperplasia.

μg of RNA used as a template for cDNA synthesis with random primers and ImProm-II reverse transcriptase (Promega).

**Quantitative PCR**
Quantitative PCR (qPCR) was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems) using predesigned probe-based assays for: Il4 (Mm.PT.58.32703659), Il5 (Mm.PT.58.41498972), Il6 (Mm.PT.58.31366752), Ifng (Mm.PT.58.41769240), Tnf (Mm.PT.58.12575861), Il18 (Mm.PT.58.42776691), Il25 (Mm.PT.58.28942186), Il13 (Mm.PT.58.12022572), Tgfb (Mm.PT.58.41321689), Rbck1 (Mm.PT.58.30767649), Il6 (Mm.PT.58.10005566), Il10 (Mm.PT.58.13531087), Il12 (Mm.PT.58.12049997), Tgfβ (Mm.PT.58.11254750), Tnfsf15 (Mm.PT.58.43933933), Tnf (Mm.PT.56.8500128), Csf2 (Mm.PT.89.1861111), Il1b (Mm.PT.58.41616450), Il27 (Mm.PT.88.11487953), Nmur1 (Mm.PT.58.32232111), Rgs1 (Mm.PT.30.803964), Eps8 (Mm.PT.58.13819524), Il17rb (Mm.PT.81.2616779), Siglec6 (Mm.PT.86.685529) (Integrated DNA Technologies). 16 s qPCR was performed using PowerSYBR Green assay (Invitrogen) and primers: 5′-GACTACCCAGTATCTAATCC-3′ and 5′-GTGCCAGCMGCCGCTAA-3′. Transcript levels were quantified by assigning slides a mouse tag number, and matching to genotype post-quantification.

**Bone marrow chimeric mice**
Recipient mice were exposed to 1200 rad of whole body irradiation and injected intravenously with 10 million whole bone marrow cells from donor mice. Mice were allowed to reconstitute for 16 weeks before sacrifice for analysis of intestinal tissue. Mice were bled at 12 to 14 weeks post-irradiation to determine percent chimerism. Genomic DNA was isolated from blood and analyzed by qPCR for the presence of Rbck1/Hoil1 intron 7 (Hoil1+/-) or the neomycin-resistance cassette (Hoil1+/+), with Rag2 as a normalization control.

**Total protein isolation and ELISAs**
Whole 1 cm tissue samples of distal ileum (1 cm from the cecum) were homogenized in PBS with Halt phosphatase and protease inhibitors (Thermo scientific) using sterile zirconia/silica beads and a Mini-Beadbeater 24 (BioSpec). Supernatant was reserved for further analysis and total protein quantified using DC Protein assay (Bio-Rad). Cytokine production was determined in distal ileum by ELISA using R&D DuoSet for IL-33 and IL-13, and Biolegend ELISA MAX for TSLP, IL-4, IL-5, and IL-25 following the manufacturers’ instructions and analyzed with a microplate reader (BioTek Synergy 2).

**Statistical analyses**
Data were analyzed with Prism 9 software (GraphPad Software, San Diego, CA). Statistical significance was determined by tests as indicated in the figure legends.
COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
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Correspondence and requests for materials should be addressed to Donna A. MacDuff.

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