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

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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 auto-inflammation 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 patients3.

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)4,5. 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 HOIL1−/− 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 challenge15. 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 HOIL116.
Fig. 1  *Holl1*<sup>+/−</sup> mice exhibit type 2 inflammation in the distal ileum.  

**a** Representative images of H&E stained sections of ileum from *Holl1*<sup>+/−</sup> and *Holl1*<sup>−/−</sup> 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 *Holl1*<sup>+/−</sup> and *Holl1*<sup>−/−</sup> ileum.

**d–h** Relative *Il4*, *Il5*, *Il13* (d), *Tnf*, *Ifng* (f), *Il25*, *Il33*, *Il18* and *Tslp* (g) mRNA levels, and *Il4*, *Il5* (e), *Il25*, *Il33* and *Tslp* mRNA levels in ileum of *Holl1*<sup>+/−</sup> and *Holl1*<sup>−/−</sup> mice. *Il18*, *Il25*, *Il33* and *Tslp* mRNA levels in *Holl1*<sup>+/−</sup> and *Holl1*<sup>−/−</sup> IEC and LP fractions relative to *Holl1*<sup>+/−</sup> IEC median.  

**i**–**j** DCLK1 (red) and DAPI (blue) stained sections of ileum from *Holl1*<sup>+/−</sup> (left) and *Holl1*<sup>−/−</sup> (right) mice (scale = 50 μm).

**k** Enumeration of tuft cells in villi and crypts of *Holl1*<sup>+/−</sup> and *Holl1*<sup>−/−</sup> 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 *Holl1*<sup>+/−</sup>. 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 (i).
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, IL4Rα-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+/+ littersmates. This histological change is characteristic of type 2 inflammation observed after intestinal helminth infection.

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, Il12 and Tnf (Fig. 1f), indicating that Hoil1−/− mice do not experience a generalized, non-specific inflammatory response, or a shift from type 1 to type 2 cytokine production. Type 2 inflammation can be caused by infection with a common intestinal protozoan, Trichomonas muri, present in some SPF mouse colonies. However, Trichomonas muri 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, IL25 and IL33 mRNA levels and IL-25 protein were slightly elevated in Hoil1−/− ileum relative to Hoil1+/+ ileum (Fig. 1g, h). However, no differences in Il12 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 layer from the lamina propria (LP) cell fractions. Expression of Il13, Tsp5 and Il18 mRNA was similar for Hoil1−/− and Hoil1+/+ ileum 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. 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 mRNA 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 Ilng 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 mRNA levels were also reduced in the ileum of Hoil1+/+ mice by antibiotics, treatment, but to a lesser extent. No differences in Il12, Tsp5, Il33, Ilng or Tnf expression were measured between Hoil1−/− and Hoil1+/+ mice, although expression was reduced by antibiotics treatment (Fig. 2e, f). 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–j). 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. 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 Il4raf/flox mice and VillinCre (ΔIEC) transgenic mice. Histological analysis revealed that deletion of Il4raf on IECs largely rescued the epithelial cell abnormalities in Hoil1−/− mice (Fig. 3a–e). Consistently, Il25 and Il33 mRNAs were reduced in Hoil1−/−/Il4raf/flox tissue to levels comparable to Hoil1+/−/Il4raf+/+ and Hoil1−/−/Il4raf−/− tissue (Fig. 3f). Surprisingly, Il5 and Il13 mRNAs remained elevated in Hoil1−/−/Il4raf−/− tissue, despite Il25 and Il33 mRNA being reduced to Hoil1−/−/Il4raf−/− 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, IL-12 and IL-13 protein levels were highly variable in whole tissue samples and, although they appeared to be slightly lower in Hoil1−/−/Il4raf−/− tissue compared to Hoil1−/−/Il4raf+/+ 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 IL4Ra triggers goblet and tuft cell hyperplasia and the induction of IL-25 in Hoil1-deficient
ilium, but that an IL4Ra-dependent increase in Il12, Il13 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-5. We sought to identify the major producers of IL-13 in the ileum of Hoil1−/− mice. First, we determined that Il4, Il5 and Il13 mRNAs were expressed primarily in the LP cell fraction (Fig. 4a). Using flow cytometry, we observed that small percentages of CD3+ cells (T cells) and of CD11b+ (myeloid) cells expressed IL-13 upon stimulation, and were similar in Hoil1−/− and Hoil1+/+ LP (Fig. 4b, c). However, 10–15% of CD3+CD11b+CD19− cells expressed IL-13, and a higher percentage of these cells were producing IL-13 in the Hoil1−/− LP. This cell fraction includes ILCs, NK cells, dendritic cells, and mast cells. The percentage of CD3+ T cells was significantly reduced in Hoil1−/− LP, 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−/−Rag1−/− mice. Goblet and tuft cell numbers were increased in the ileum of Hoil1−/−Rag1−/− mice compared to Hoil1+/−Rag1−/− mice (Fig. 4d–f). Il4, Il5, Il13 and Il25 mRNAs were elevated in Hoil1−/−Rag1−/− compared to Hoil1+/−Rag1−/− ileum (Fig. 4g, h), indicating that T cells are not required to trigger the inflammatory phenotype. However, Il4 expression was not elevated in Hoil1−/−Rag1−/− ileum compared to Hoil1+/−Rag1−/− (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.

**Hoi1 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 cytokines. We examined Hoil1+/−Il4ra−/− mice since the epithelial changes and IL-25 induction are blocked, but Il13 and Il5 mRNA overexpression persists in these mice (Fig. 3). However, Il10, Tgfβ1, Il12p40, Tnfsf15 (TL1A), Tnfsf17 (GITRL), Il6, Csf2 (GM-CSF) or Il1b mRNAs were not consistently dysregulated in tissue from both Hoil1+/−Il4ra−/− and Hoil1+/−Il4ra−/− mice relative to their HOIL1-sufficient littermates (Fig. 6a). Il27 mRNA was significantly reduced, and IL-27 has been shown to regulate ILC and CD4+ T cell responses.

To take a more comprehensive approach to identifying transcriptional differences, we sorted CD45+ and CD45− cells from the ileum of Hoil1+/−Il4ra−/− mice and performed RNA sequencing (Supplementary Table 1). Unexpectedly, in the CD45 fraction, Rbck1 (Hoil1) was the only gene identified as differentially expressed (DE) in the Hoil1+/−Il4ra−/− and Hoil1+/−Il4ra−/− ileum relative to the Hoil1+/+Il4ra−/− and Hoil1+/−Il4ra−/− mice. However, in the CD45− fraction, Nmur1, Il17, 1700601F12Rik, Il13, Il17b, Epas1, in addition to Rbck1, were identified as DE in Hoil1+/−Il4ra−/− tissue, but unaffected by IL4Ra signaling on IECs (Fig. 7a, Supplementary Table 1).

To determine which specific cell types express these DE genes in the small intestine during a type 2 inflammatory response, we queried the Immunological Genome Project (ImmGen) database and published single-cell RNA sequencing datasets. Krg1 can be expressed by ILC2, NK cells and Th2 cells, and Il17b, 1700601F12Rik and Epas1 can be induced in activated ILC2 and Th2 cells. However, Il5 and Nmur1 mRNAs are relatively specific to ILC2 among CD45− cells within the small intestine (Fig. 7b). Rbck1 expression was variable among immune cell and epithelial cell populations, with notable expression in several dendritic cell subtypes, ys T cells and germinal center B cells, as well as in tuft cells, goblet cells, transit amplifying cells and enterocyte progenitors (Fig. 7b, c).

Using qRT-PCR, we confirmed that Nmur1 was more highly expressed in distal ileum from Hoil1+/−Il4ra−/− and Hoil1+/−Il4ra−/− mice (Fig. 7d). Krg1 mRNA was also slightly elevated, although this was not significant in the Hoil1+/+Il4ra−/− tissue. Epas1 mRNA expression was not detectably different in whole tissue. Il17b and Siglecf were elevated in tissue from Hoil1+/−Il4ra−/− but not Hoil1+/−Il4ra−/− mice, indicating that changes in their expression are dependent on IL4Ra signaling in IECs, which is consistent with expression of both markers on tuft cells. Furthermore, Nmur1 was more highly expressed in the MLN from Hoil1+/−/Il4ra−/− and Hoil1+/−Il4ra−/− mice, but Siglecf and Il17b were not (Fig. 7e). Nmur1 is a neuropeptide receptor that has recently been shown to be preferentially expressed on ILC2, and to induce ILC2 activation and proliferation in response to neurenomin (NMU) produced by mucosal neurons. These findings suggested that ILC2 numbers or activation state are dysregulated in the small intestine of Hoil1-deficient mice. Flow cytometry revealed that the frequency of Lin−Klrk1+ CD90.2+ ILC2 was approximately fivefold higher in the ileum of Hoil1+/− mice, and remained elevated even in the absence of IL4Ra signaling on IECs (Fig. 7f, g), indicating that Hoil1 limits number of ILC2 in the small intestine.
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 $\text{Il}25$ induction were dependent on signaling through IL4R$\alpha$ on IECs. However, $\text{Il}13$ and $\text{Il}5$ mRNA and ILC2 numbers remained
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) in ileum 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 (left panel) and tuft cells (right panel) per villus in ileum from Hoil1+/+Rag1−/− and Hoil1−/−Rag1−/− mice. g–i 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+ cell 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 11S) 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 IEC, 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 treatment35,36.

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 ligands32,34–36. 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. Future 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 IL4Rα 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/6 J background. Mice were housed in accordance with Federal and University guidelines and protocols approved by the University of Illinois Chicago Animal Care Committee and the Animal Studies Committee of Washington University.
Flow cytometry
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.

Analysis of scRNA-seq datasets
scRNA-seq datasets of small intestine LP (GEO: GSE124880) and epithelium (GEO: GSE9233A) 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 dH2O. 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 precipitation.

Trichomonas muri testing
Fecal pellets were collected from at least two breeding cages from each mouse strain: 1) Hoil1+/−, Il4ra−/− and Hoil1−/−Il4ra−/− in 2) Hoil1−/−Il4ra−/− and Hoil1+/−Il4ra−/− mutants. scRNA-seq libraries were prepared for both conditions and sequenced on the Illumina HiSeq platform. Libraries were generated and sequenced in two independent experiments by the Broad Institute Single Cell Portal.

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. Tissues 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), Il13 (Mm.PT.58.31366752), Il9 (Mm.PT.58.41769240), Tnf (Mm.PT.58.12575861), Il18 (Mm.PT.58.42776691), Il25 (Mm.PT.58.28942186), Il33 (Mm.PT.58.12022572), Iiled (Mm.PT.58.41321689), Rbck1 (Mm.PT.58.30767649), Il6 (Mm.PT.58.10005566), Il10 (Mm.PT.58.13531087), Il12p40 (Mm.PT.58.12009997), Tgfb (Mm.PT.58.11254750), Tnfsf15 (Mm.PT.58.43939933), Tnfsf18 (Mm.PT.56a.8500128), CsF (Mm.PT.58.91861111), Il1b (Mm.PT.58.41616450), Il17 (Mm.PT.58.11487953), Nmur1 (Mm.PT.58.32232111), Rps29 (Mm.PT.58.30803964), Ppia (Mm.PT.58.13819524), Il17rb (Mm.PT.58.12616779), Siglecf (Mm.PT.58.6685529) (Integrated DNA Technologies). 16 s qPCR was performed using PowerSYBR Green assay (Invitrogen) and primers: 515 F (5'-CAGGGTATCTAATCC-3') and 805 R (5'-GTGCCAGCMGCCGCGGTAA-3'). Transcript levels were quantified using the relative standard curve method, with Rps29 as the reference gene.

**Histology**
Distal ileum (last 6 cm up to the cecum) was flushed with PBS followed by 10% buffered formalin and opened longitudinally, flattened and pinned in 10% buffered formalin for 24 h followed by washing with 70% ethanol. Strips of tissue were embedded in 2% agar prior to paraffin embedding. Blocks were sectioned and stained with Hematoxylin and Eosin. For immunofluorescent staining, antigen retrieval was performed by boiling in 10 mM Tri-sodium citrate (dihydrate) with 0.05% Tween 20, pH 6.0 for 20 min. Sections were blocked with PBS containing 5% FBS and 0.1% TRITON X-100 for 3 h, and then incubated with rabbit anti-mouse DCLK1 (ab31704, Abcam) or rabbit anti-mouse Lysozyme (ab108508, Abcam) in PBS + 5% FBS at 4°C overnight. Sections were washed with PBS + 0.3% Tween 20 and incubated with Alexa Fluor 555 donkey anti-rabbit (A31572, Invitrogen) for 1 h at 4°C. Sections were washed with PBS + 0.3% Tween 20, and counterstained and mounted with Prolong Gold antifade reagent with DAPI (Invitrogen). Imaging was performed on a BX-TH10 microscope (Keyence). Tuft and goblet cell quantification was based on an average of at least 10 villi and crypts per mouse. Blinding was performed 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.
DATA AVAILABILITY
RNA-Seq data are available at NCBI Gene Expression Omnibus (GEO) through accession number GSE196550.

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AUTHOR CONTRIBUTIONS
M.J.W., T.C.L., K.I., T.S.S., and D.A.M. designed the study; M.J.W., J.N.M., V.L.H., and D.A.M. performed experiments; M.J.W., J.N.M., V.L.H., and D.A.M. analyzed the data and performed statistical analyses; M.J.W., V.L.H., T.C.L., T.S.S., and D.A.M. interpreted the data; M.J.W. and D.A.M. drafted the paper; all authors were involved in discussing the data and provided feedback on the paper.
COMPETING INTERESTS
The authors declare no competing interests.

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