ARTICLE

Genetic and commensal induction of IL-18 drive intestinal epithelial MHCII via IFNγ

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Major histocompatibility complex class II (MHCII) is dynamically expressed on intestinal epithelial cells (IECs) throughout the intestine, but its regulation remains poorly understood. We observed that spontaneous upregulation of IEC MHCII in locally bred Rag1−/− mice correlated with serum Interleukin (IL)-18, was transferrable via co-housing to commercially bred immunodeficient mice and could be inhibited by both IL-12 and IL-18 blockade. Overproduction of intestinal IL-18 due to an activating Nlrpc mutation upregulated IEC MHCII via classical inflammasome machinery independently of immunodeficiency or dysbiosis. Immunodeficiency or dysbiosis increased IL-18 transcription, which synergized with Nlrc4 inflammasome activity to drive elevations in serum IL-18. This IL-18-MHCII axis was confirmed in several other models of intestinal and systemic inflammation. Elevated IL-18 reliably preceded MHCII upregulation, suggesting an indirect effect on IECs, and mice with IL-18 overproduction showed activation or expansion of type 1 lymphocytes. Interferon gamma (IFNg) was uniquely able to upregulate IEC MHCII in enteroid cultures and was required for MHCII upregulation in several in vivo systems. Thus, we have linked intestinal dysbiosis, systemic inflammation, and inflammasome activity to IEC MHCII upregulation via an intestinal IL-18-IFNg axis. Understanding this process may be crucial for determining the contribution of IEC MHCII to intestinal homeostasis, host defense, and tolerance.

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INTRODUCTION

The mammalian intestinal epithelium is a heterogeneous single-cell barrier that separates host tissues from a complex microbial ecosystem. It must shepherd vital nutrients systemically from the lumen, exclude commensal microbes, repair itself, and mobilize appropriate immune responses to intestinal pathogens. Among the greatest challenges this barrier faces is negotiating the balance between host defense and immune tolerance. Notably, antigen presentation is critical to both outcomes.

Though the expression of major histocompatibility complex class II (MHCII) is canonically reserved for “professional” antigen presenting cells, like B- and dendritic cells (DCs), intestinal epithelial cells (IECs) are also capable of processing peptides and expressing them on surface MHCII. IEC MHCII appears to be expressed on both luminal and basolateral surfaces, and on a wide variety of IECs from the duodenum through the colon. Both murine and human studies concur that elevated colonic MHCII expression is observed in gut inflammatory disorders including colitis and graft-versus-host disease (GVHD), where local production of interferon gamma (IFNg) by intestinal T cells appears sufficient to upregulate IEC MHCII.

Despite broad appreciation for the phenomenon, little is known about its regulation or contributions to barrier integrity, host defense, and tolerance. The conventional view of intestinal antigen presentation suggests that DCs acquire antigen and migrate to Peyer’s Patches and mesenteric lymph nodes to initiate immune responses to food-, commensal-, and pathogen-derived antigens. Recent studies using conditional deletion of MHCII have largely corroborated this view. For example, intestinal CD11c+ DCs, and not IECs, were found to be required for Th17 responses to Segmented Filamentous Bacteria (SFB) colonization, as well as bacterial driven-T cell colitis.

However, other studies suggest exceptions to the “professional” APC paradigm in the intestine. MHCII expression by Rorc expressing cells was required to prevent the development of spontaneous IBD in a microbiota-dependent manner. The specific role for MHCII expression by IECs remains controversial. Biton et al. showed that IEC MHCII expression was important for epithelial remodeling post infection. Koyama et al. recently identified that ileal IEC MHCII expression promoted intestinal graft-versus-host responses to cytotoxic alloantigens via the microbiota, IL-12, and IFNg. Consistent with a role for IEC MHCII in gut T-cell activation, mice selectively lacking IEC MHCII developed both more severe infectious (C. rodentium) colitis as well as worsened T-cell transfer or IL-10 receptor blockade colitis (the latter requiring H. hepaticus).

MHCII expression on traditional APCs can be stimulated by various cytokines and chemokines. Other than its association with IFNg, little is known about the regulation of IEC MHCII. Specifically, the conditions, chronology, and upstream events leading to IEC MHCII remain largely unknown. Herein, we show that Rag1 deficiency is associated with a transferable dysbiosis
that drives MHCII expression on both small and large IECs. IL-18 and IL-12 are necessary drivers for this IEC MHCII expression. Various genetic models of IL-18 overproduction show that constitutive IL-18 can itself (independently of dysbiosis or immunodeficiency) drive MHCII and supports a role for inflamma-
some machinery in the regulation of both serum IL-18 and SI IEC MHCII. Specifically, our results support a collaborative model in which microbial induction of IL-18 cooperates with inflammasome-mediated proL-18 maturation to drive changes in serum IL-18, expansion of innate and adaptive type 1 lymphocytes, and IFNг-
mediated IEC MHCII. These models, as well as models of systemic inflammation and commensal pro-tost colonisation, elucidate a novel mechanism of IEC MHCII regulation via an intestinal IL-18-IL-
12/IFNg axis.

RESULTS
Rag1 deficiency is associated with a microbiota-dependent increase in small and large intestinal epithelial MHCII

Inducible expression of MHCII has been reported on murine small and large IECs in a variety of circumstances\(^4,5,9\). We observed consistently high MHCII expression on Epcam\(^+\) colonic and small intestinal (SI) epithelial cells in Rag1\(^{-/-}\) mice relative to wild-type (WT) controls (Fig. 1a and Supplementary Fig. 1). SI IEC MHCII was sometimes observed to be elevated in unstimulated WT mice, suggesting greater sensitivity of SI IECs vs. colonic IECs. Treatment of Rag1\(^{-/-}\) mice with broad spectrum antibiotics normalized colonic MHCII expression (Fig. 1b). Given this microbial associa-
tion, we compared IEC MHCII in Rag1\(^{-/-}\)/mice bred in our colony (Pitt) vs. mice imported directly from a commercial source (Jackson Laboratory). Importantly, Rag1\(^{-/-}\)/ mice showed neither colonic nor SI IEC MHCII expression, suggesting a commensal inducer of IEC MHCII present uniquely in our colony (Fig. 1c).

To better understand the mechanisms driving MHCII expression in Rag1\(^{-/-}\)/mice, we directly tested this link by assessing MHCII expression in mice specifi-
cally lacking the IL-18 gene in Vil1-expressing IECs. Loss of intestinal Il-18 diminishes IEC MHCII upregulation, supporting a role for in

Genetic models of IL-18 overproduction cause SI MHCII upregulation independently of the microbiome or immunodeficiency

We have previously shown that mice bearing an activating mutation in the NLRC4 inflammasome (Nlrc4\(^{TS/TS}\)) exhibited increased serum IL-18 derived from IECs\(^16\). SI IECs from these mice also showed increased proliferation and transcription of genes related to MHCII antigen presentation. We readily detected spontaneous IEC MHCII upregulation on Epcam\(^+\) SI IECs (and to a lesser extent colonic IECs) from Nlrc4\(^{TS/TS}\) mice (Fig. 4a). Contrasting with Rag1\(^{-/-}\)/mice, Nlrc4\(^{TS/TS}\) mice treated with broad spectrum antibiotics (MANV) showed neither

diminished IEC MHCII (data not shown)\(^16\).

Elevated IEC MHCII is transferable to immunodeficient, but not immunocompetent hosts

To determine the nature of microbiota-driven IEC MHCII expres-
sion in Pitt Rag1\(^{-/-}\)/mice, we co-housed them for 5 weeks with imported Jackson Rag1\(^{-/-}\)/mice. Co-housing resulted in a significant increase in IEC MHCII (Fig. 2a). To identify unique microbial factors driving changes in MHCII, we performed 16S

Microbial dysbiosis drives MHCII expression in Rag1\(^{-/-}\)/mice

Since IL-18 in Rag1\(^{-/-}\)/mice is intestinally derived\(^16\), we directly tested this link by assessing MHCII expression in mice specifically lacking the IL-18 gene in Vil1-expressing IECs. Loss of intestinal IL-18 normalized both serum IL-18\(^16\) and MHCII (Fig. 4c). As in Rag1\(^{-/-}\)/mice, antibody-based blockade of the IL-18R or IL-12 failed to affect SI IEC MHCII expression in Nlrc4\(^{TS}\) mice (Supplementary Fig. 3c, d).

We hypothesized that other models associated with excess IL-18 would also increase IEC MHCII. We found that mice with transgenic expression of IL-18\(^18\), mice lacking IL-18/’s endogenous inhibitor IL-
18 binding protein (Il18bp\(^+\)/ mice)\(^9\), and mice with genetic hyperactivity of the Pyrin inflammasome (Mefv\(^{W736X/W736X}\)) all developed elevation of SI MHCII expression, again solidifying the link between IL-18 overproduction and elevated epithelial MHCII (Fig. 4d-f).

Capase-1 and gasdermin-D are required for basal serum IL-18 and Nlrc4-inflammasome induced elevations of serum IL-18 and IEC MHCII

Elevated serum IL-18 in Nlrc4\(^{TS}\) mice is derived from the intestinal epithelium\(^16\). To better understand the mechanisms driving epithelial release of IL-18 and subsequent IEC MHCII induction,
we evaluated several strains lacking either intestinal IL-18 or key components of the inflammasome pathway. Notably, mice with WT Nlrc4 specifically lacking IL-18 in intestinal epithelia (Il18<sup>fl/fl</sup>ΔIEC) showed lower IL-18 levels than Cre-negative “WT” controls, suggesting that basal serum IL-18 is intestinally derived (Fig. 5a).

Multiple inflammasomes, caspases, and cell death pathways are present in IECs<sup>13</sup> and activation of the NLR4 inflammasome in IECs can trigger caspase-1 and caspase-8, both of which can cleave inflammasome substrates<sup>14</sup>. Serum IL-18 in Casp<sup>−/−</sup> mice, even in the presence of the NLR4<sup>−/−</sup> mutation, was below WT levels, and such mice did not upregulate IEC MHCII (Fig. 5b). This suggests there is little/no contribution of caspase-8 (or any other protease) to either basal or Nlrc4<sup>−/−</sup>-enhanced IL-18 production or subsequent IEC MHCII induction. Identical results were obtained in mice lacking the pore-forming inflammasome substrate gasdermin-D<sup>−/−</sup> (Fig. 5c). Thus, IEC IL-18 production, basal and Nlrc4<sup>−/−</sup>-driven serum IL-18, and spontaneous IL-18 dependent MHCII upregulation require the classical inflammasome components caspase-1 and gasdermin-D.

Rag1 deficiency synergizes with Nlrc4 hyperactivity in the elevation of serum IL-18

Paradoxically, IEC IL-18 transcription is slightly decreased in Nlrc4<sup>−/−</sup> mice<sup>16</sup>. As such, their elevated IL-18 arises due to increased inflammasome-mediated proll-18 cleavage and export. In locally bred Rag1<sup>−/−</sup> mice, we suspected that microbial dysbiosis drove elevated IL-18 transcription. Indeed, re-analysis of ileal transcription from Mao et al.<sup>24</sup> showed that when SFB-colonized WT, TCR<sub>α</sub>α<sup>−/−</sup>, and Rag1<sup>−/−</sup> mice were co-housed, only immunodeficient mice upregulated IL-18; separately housed (SFB-naive) TCR<sub>α</sub>α<sup>−/−</sup> mice did not upregulate IL-18 (Fig. 5d). We reasoned that if increased serum IL-18 in Rag1<sup>−/−</sup> mice is the product of increased IL-18 transcription, then combining this mechanism with Nlrc4<sup>−/−</sup>-mediated proll-18 cleavage should result in (at least) additive IL-18 overproduction. Indeed, Nlrc4<sup>−/−</sup>/WT; Rag1<sup>−/−</sup> mice had approximately tenfold higher serum IL-18 than either parental strain, supporting the complementary nature of these mechanisms (Fig. 5e).

IL-18 dependent upregulation of IEC MHCII occurs broadly in response to systemic inflammation and <i>Tritrichomonas</i> colonization

We postulated that other stimuli associated with changes in local intestinal and systemic IL-18 would also influence IEC MHCII expression. Repeated systemic stimulation through toll-like receptor (TLR) nine results in an inflammatory phenotype reminiscent of human macrophage activation syndrome, which increases serum IL-18 and is dependent on an IL-12-IFNg axis<sup>16,25</sup>. Repeated CpG administration induced high expression of MHCII on colonic and SI IECs (Fig. 6a).

Colonization with the gut-specific commensal protist <i>Tritrichomonas</i> has been associated with intestinal inflammasome activation and lamina propria T-cell IFNg production<sup>16,29</sup> as well as Tuft cell activation and induction of type 2 cytokines<sup>30</sup>. We gavaged WT mice with <i>Tritrichomonas</i> enriched from the cecal contents of a colonized mouse and noted both extensive establishment of <i>Tritrichomonas</i> colonization in recipient animals and robust upregulation of intestinal (particularly colonic...
MHCII (Fig. 6a). Interestingly, Tritichomonas colonization did not appreciably elevate serum IL-18 (Fig. 6b), supporting a remarkably gut-specific immune response. Specific deletion of IL-18 in IECs also resulted in decreased IEC MHCII upregulation with either TLR9 stimulation or Tritrichomonas colonization (Fig. 6c, d), particularly in the colon.

Elevated IL-18 precedes induction of IEC MHCII
Interestingly, elevated IL-18 occurred around weaning in Rag1−/− mice (~3–4 weeks of age), preceding MHCII induction (6 weeks of age) (Supplementary Fig. 4a, b). Despite Nlrc4+/- mice elevating serum IL-18 as early as 2 days of age, they too did not upregulate MHCII until 3–4 weeks of age, suggesting an important role for time/weaning in IL-18-induced MHCII expression (Supplementary Fig. 4c). This was largely replicated in Il18bp+/− mice (Supplementary Fig. 4d), although they took slightly longer to achieve stable and high MHCII elevation. In addition, 10-day-old WT pups treated with CpG showed elevated serum IL-18, but not IEC MHCII (Supplementary Fig. 4e).

IFNg is a unique and potent driver of MHCII expression on IECs in vitro
As IECs have little/no expression of the IL-18 or Il-12 receptors (Supplementary Fig. 5a) and elevated IL-18 in many of our models precedes the upregulation of MHCII, we hypothesized that IL-18 did not act directly on epithelial cells to upregulate MHCII. Stimulation of WT SI organoids with high-dose IL-18 (50 ng/mL) failed to upregulate MHCII, whereas IFNg stimulation did, even at doses as low as 0.1 ng/mL (Supplementary Fig. 5b and data not shown). To determine whether any other stimuli could induce IEC organoid MHCII, we stimulated with various cytokines and pathogen-associated molecules chosen based on their receptor expression in WT IECs or their presence within the intestinal microenvironment. Strikingly, only IFNg was able to upregulate

Fig. 2 Elevated MHCII in Rag1−/− mice is driven by a transferable microbial dysbiosis. Locally (Pitt) and commercially bred (Jackson) Rag1−/− mice were kept isolated or co-housed for 5 weeks. At sacrifice, mice were analyzed for epithelial MHCII expression by flow cytometry (a). Each dot represents a single mouse and is representative of two independent experiments with 2–3 mice per condition. Cecal stool was collected and its DNA analyzed by 16S analysis. Jaccard principal coordinate analysis plots of cecal fecal diversity (b) and family diversity (c), for each mouse and is representative of a single experiment. gMFI geometric mean fluorescent intensity, PC principal coordinate.
MHCI expression on IECs (Supplementary Fig. 5b). Similar stimulation of human fetal small intestine organoids showed upregulation of MHCI with IFNg, but not IL-18, LPS, or CpG stimulation (Supplementary Fig. 5c) supporting IFNg as a primary driver of MHCI expression in both murine and human IECs.

IEC MHCI expression requires IFNg signaling in vivo. In vivo, T-cell transfer colitis and irradiation1,5 require IFNg to upregulate IEC MHCI. We sought to determine whether IFNg was an absolute requirement for IEC MHCI induction in the variety of IL-18-dependent systems described above. Using Ifng−/− bone marrow chimeras and Ifngr1−/− mice, we found that induction of either SI or colonic IEC MHCI in Rag1−/− (Fig. 7a), Nlrc4−/− (Fig. 7b), TLR9-stimulated (Fig. 7d), and Trichromonas (Fig. 7d) colonized mice required IFNg or the IFNg receptor. Though not comprehensive, these data suggest IFNg is likely to be required for IEC MHCI upregulation regardless of whether the stimulus is acute or chronic, local or systemic.

Since IEC MHCI induction requires IFNg but is not observed prior to weaning, we first confirmed that neonatal IECs express the IFNg receptor (Supplementary Fig. 4f). As murine intestines are not fully populated with lymphocytes until after weaning29,32, we hypothesized that IL-18 acted on IFNg-producing intestinal lymphocytes to increase IEC MHCI. Neither T- nor B-cells are absolutely required for IEC MHCI upregulation, given the robust responses we observed in Rag1−/− mice. Investigation of colonic lamina propria innate lymphoid cells (ILCs) in Rag1−/− mice did not show an increase in the proportion of colonic NK or ILC1 cells, but rather a shift in their expression of KLRG1 consistent with an activated phenotype (Fig. 8a–c). Ex vivo stimulation with PMA/ionomycin resulted in dramatic loss of ILC/NK cells, preventing direct confirmation of their IFNg production. By contrast, examination of SI lamina propria of Nlrc4−/− mice showed no change in NK/ILC1 populations, but a dramatic increase in both CD4 and CD8 T-cells (Fig. 8d–g). These cells showed a non-significant trend towards more type I differentiation/activation. These data suggest that chronic IL-18 is associated with activation and/or expansion of diverse, and likely redundant, IFNg-competent intestinal lymphocyte populations.

DISCUSSION

Intestinal epithelial expression of MHCI was described nearly 50 years ago, and has since then largely existed as a phenomenon associated with specific microbial or inflammatory triggers, or its analysis restricted to specific portions of the intestine32. More recent selective deletion and single-cell technologies have ushered a resurgence of interest in the phenomenon. Though many studies implicate DCs as the main sources of productive intestinal antigen presentation8,9,34, others nevertheless highlight intestinal lymphocyte populations.

Ex vivo stimulation with PMA/ionomycin resulted in dramatic loss of ILC/NK cells, preventing direct confirmation of their IFNg production. By contrast, examination of SI lamina propria of Nlrc4−/− mice showed no change in NK/ILC1 populations, but a dramatic increase in both CD4 and CD8 T-cells (Fig. 8d–g). These cells showed a non-significant trend towards more type I differentiation/activation. These data suggest that chronic IL-18 is associated with activation and/or expansion of diverse, and likely redundant, IFNg-competent intestinal lymphocyte populations.

We found that Rag1−/− mice in our colony had elevated serum IL-18 and colonic and SI MHCI expression. IL-18 and IEC MHCI were antibiotic-sensitive in Rag1−/− mice, suggesting the activity of specific commensal mediators. Co-housing commercially sourced Rag1−/− mice (low for IL-18 and MHCII) with those locally bred resulted in a pronounced fecal microbial shift characterized by the increased abundance of Helicobacter, Prevotella, and Mucispirillum (Deferribacteracea) genera. Similar co-housing of WT mice did not induce serum IL-18 or IEC MHCI despite a similar microbial shift, suggesting a role for immunodeficiency in microbial-driven MHCI induction. Though the resolution of this analysis did not permit the identification of specific bacterial species, Helicobacter, Prevotella, and Mucispirillum are all...
organisms bound strongly by IgA\textsuperscript{14,15}. The interaction of these bacteria and host immunodeficiency may have enabled them to interact more directly with IECs and driven IL-18 transcription. A similar induction of IL-18 was observed in dysbiotic TCR\textsuperscript{a−/−} mice (Fig. 5d), suggesting T-cell functions, such as production of barrier-maintaining cytokines, T-cell dependent antibody responses, or limiting the outgrowth of ILCs could prevent the dysbiosis and IEC MHCII induction observed in Rag1\textsuperscript{−/−} mice.

Strengthening the link between elevated IL-18 and IEC MHCII, we found that NLRC4 inflammasome hyperactivity drove IEC-dependent IL-18 overproduction and upregulated MHCII and related genes\textsuperscript{16}. We also observed robust IEC MHCII expression on SI, but not colonic cells in other known IL-18 overproducing mouse strains (IL18\textsuperscript{tg+/−/wt}, IL18bp\textsuperscript{−/−} and MEFV\textsuperscript{VT264A/V726A}), suggesting differential regulation of SI and colon IECs, and confirming a strong link between IL-18 and IEC MHCII. IEC MHCII was IL-18

Fig. 4 NLRC4 inflammasome hyperactivity, IL-18bp deficiency, and transgenic IL-18 expression increase SI MHCII expression. Epithelial MHCII including representative histograms in Nlrc4\textsuperscript{TS/TS} mice at baseline (a), following treatment with broad spectrum antibiotics (b), and with or without deletion of intestinal epithelial IL-18 (c). SI MHCII in Il18\textsuperscript{+/+} mice (d), Il18bp\textsuperscript{−/−} mice (e), and MEFV\textsuperscript{VT264A/V726A} mice (including serum IL-18) (f). MEFV\textsuperscript{VT264A/V726A} mice were assessed at 4 weeks of age due to spontaneous systemic inflammation arising in adulthood. Samples are SI unless otherwise indicated. Each symbol represents a single mouse. Representative of \(n \geq 3\) experiments (a, c), combined results of \(n = 2\) experiments (b, d–f) and representative of a single experiment (h). Statistical significance is shown on each graph and is determined by unpaired \(t\) test \((n = 2\) groups). MANV antibiotics (Metronidazole, ampicillin, neomycin, vancomycin), gMFI geometric mean fluorescent intensity, Unt untreated (no antibiotics).
dependent, as blocking IL-18 signaling or deleting IL-18 from IECs significantly decreased MHCII expression in these models.

In NLRC4 hyperactive mice, both elevated serum IL-18 and epithelial MHCII including representative histograms in adult mice of the indicated genotypes (a–c). Terminal ileum IL-18 read counts in co-housed, segmented filamentous bacteria (SFB)-colonized WT, Rag1−/− and TCRα−/− mice compared to separately housed TCRα−/− mice. Data are derived from Mao et al.24 and accessed from GSE86780 (d). Serum IL-18 in mice bearing combined Nlrc4 hyperactivating and Rag1 mutations (e). Combined results of n = 4 (a) or n = 2 (e) independent experiments with n > 2 mice per condition. b, c Representative of n = 2 independent experiments with n = 4–6 mice per genotype. Statistical significance is shown on each graph and is determined by unpaired t test (n = 2 groups) or one-way ANOVA (n = 3 groups). gMFI geometric mean fluorescent intensity, Δ IEC Villin-Cre+. The dotted lines represent the average gMFI of Gasdermin-D or Caspase-1 heterozygous littermate controls.

**Fig. 5** Classical inflammasome components are required and synergize with immunodeficiency to modulate Nlrc4-inflammasome induced elevations of serum IL-18 and IEC MHCII. Serum IL-18 and epithelial MHCII including representative histograms in adult mice of the indicated genotypes (a–c). Terminal ileum IL-18 read counts in co-housed, segmented filamentous bacteria (SFB)-colonized WT, Rag1−/− and TCRα−/− mice compared to separately housed TCRα−/− mice. Data are derived from Mao et al.24 and accessed from GSE86780 (d). Serum IL-18 in mice bearing combined Nlrc4 hyperactivating and Rag1 mutations (e). Combined results of n = 4 (a) or n = 2 (e) independent experiments with n > 2 mice per condition. b, c Representative of n = 2 independent experiments with n = 4–6 mice per genotype. Statistical significance is shown on each graph and is determined by unpaired t test (n = 2 groups) or one-way ANOVA (n = 3 groups). gMFI geometric mean fluorescent intensity, Δ IEC Villin-Cre+.

synergy between factors driving IEC IL-18 transcription (e.g., dysbiosis and immunodeficiency) and factors promoting IEC inflammasome activation.

We consistently observed differences in the regulation of colonic vs. SI IEC MHCII such that genetically-mediated excess of IL-18 or NLRC4 inflammasome activation predominantly affected SI IECs, whereas Tritrichomonas colonization, TLR9 stimulation, and Rag1 deficiency/dysbiosis altered both colonic and SI MHCII. We also observed occasional spontaneous increases in WT SI, but not colonic, IEC MHCII expression. SI MHCII may be more sensitive to transient stimuli (e.g., SFB colonization8,13,36) and also more durable, as blockade of IL-18 or IL-12 via systemic antibody injection was able to alter colonic but not SI IEC expression (Fig. 3 and Supplementary Fig. 3). Likewise, genetic ablation of IEC IL-18 had a more profound effect on colonic vs. SI IEC MHCII expression (particularly in the TLR9 and Tritrichomonas systems). These
Regional differences in IEC MHCII regulation could be for several reasons: (1) greater feedback inhibition by microbial products in the colon leading to less sensitivity/durability of MHCII expression, (2) a greater abundance of IL-18 responsive/IFNg-producing lymphocytes in the SI, or (3) other mechanisms.

In various systems, we found that IEC MHCII induction did not occur until about the time of weaning, typically days to weeks after elevation of systemic IL-18. Weaning is a dynamic time in the murine intestine, witnessing an increase in exposure to food antigens, a window where waning luminal IgA from ingested breast milk is not yet replaced by endogenous IgA, a temporary flash of intestinal STAT3 signals, and an influx of intestinal lymphocytes. Food and microbial signals may not be necessary for IEC upregulation of MHCII, as lymphocytes enter the human intestine in utero and human IECs can upregulate MHCII around 18 weeks gestation. We speculate that the

Fig. 6  *Trichomonas* colonization and systemic TLR9 activation induce IL-18 dependent upregulation of colonic and SI IEC MHCII. Serum IL-18 and gMFI of epithelial MHCII, including representative histograms in CpG or *Trichomonas*-enriched stool. Two weeks post treatment initiation, mice were sacrificed and analyzed for colonic and small intestinal epithelial MHCII expression by flow cytometry (a) and serum IL-18 by cytometric bead array (b). MHCII expression on small and large intestinal epithelial cells in littermates with or without intestinal epithelial IL-18 treated with two CpG injections (c) or colonized with *Trichomonas* for 2 weeks (d). Dotted lines represent unstimulated MHCII in *I18* controls. Each figure is a representative of two independent experiments and each individual mouse is denoted by a datapoint with 3–5 mice per treatment/genotype. Statistical significance is shown on each graph and was determined by unpaired t test (n = 2 groups) or one-way ANOVA (n = 3 groups). TLR toll-like receptor, gMFI geometric mean fluorescent intensity, Unt untreated, *Tu* Tritrichomonas colonized, ΔIEC Villin-Cre+.
influx and effector differentiation of lymphocytes competent to produce IFNγ may be crucial for driving IEC MHCII in response to local stimuli, particularly IL-18. Obviously, IFNγ production by T-cells is not absolutely required given robust IEC MHCII upregulation in dysbiotic Rag1−/− mice. Indeed, we observed activation of colonic NK1.1-expressing ILCs in locally bred, but not Jackson, Rag-deficient mice. Our analysis was not able to distinguish NK from ILC1 cells, but both express the IL-18 receptor and are competent to produce IFNγ. In NLRC4Tg mice, the most likely producers of IFNγ appeared to be the dramatically expanded population of (largely) Tbet+ CD4 and CD8 T-cells in the lamina propria. Thus, both T-cells and ILCs may be sufficient IFNγ producers for the induction of IEC MHCII.

Our mouse and human enteroid studies not only supported an indirect role for IL-18 in driving IEC MHCII, they expanded upon previous findings3,5,8 to suggest that IFNγ was uniquely capable of inducing IEC MHCII. Why IFNγ would drive IEC MHCII even under homeostatic conditions, and why upregulation of IEC MHCII is the unique domain of IFNγ, remain mysterious. The latter may relate to a greater diversity of cytokine- and pattern recognition-receptors on classical APCs vs. IECs, or to the differential usage of promoters driving the MHC Class II Transactivator (CIITA)5,8. All

**Fig. 7** Diverse in vivo triggers of MHCII expression all require IFNγ. Epithelial cell gMFI and representative histograms in lethally irradiated Rag1−/− mice reconstituted with either WT or Ifng−/− (gKO) bone marrow (a), NLRC4Tg mice with or without Ifngr1 (b), and Ifngr1−/− and Ifngr1−/− littermates treated with CpG (c), or gavaged with Tritrichomonas-enriched stool (d). qPCR showing equal colonization with Tritrichomonas (d, inset). Data show combined results for two independent experiments, except for b, which shows a representative experiment from n = 2, with 3–5 mice per condition. Statistical significance is shown on each graph and was determined by unpaired t test.

BMCs bone marrow chimeras, gMFI geometric mean fluorescent intensity, gKO Ifng−/−, TMu Tritrichomonas, Unt untreated (no irradiation or reconstitution).
Fig. 8 Elevated IL-18 alters lamina propria immune cell composition in Rag1−/− and NLRC4TS/TS mice. Quantification of lamina propria lymphocytes by flow cytometry. Total live single CD45+ (a) and ILC (b) cell populations in Jackson WT vs. Jackson and Pitt Rag1−/− mice. Representative flow plots and quantification showing KLRG1 NK1.1 double-positive cells (c). T cell and NK cell composition (d, e) including representative flow plots and Tbet+ cells (f) in in-house WT vs. NLRC4TS/TS. Isolated ILCs were stimulated with Brefeldin, PMA and ionomycin for 2.5 h and IFNg expression quantified (g). Summary diagram (h) created with BioRender.com. a–c Representative on n = 2 experiments with three mice per experimental group. d–g Combined results from n = 3 experiments with n = 1–2 mice per group. Statistical significance is shown on each graph and was determined by unpaired t test. Jax Jackson Rag1−/− mice.
CIITA promoters are IFNγ-responsive, but promoter IV is more commonly used in non-hematopoietic cells and specifically regulates IFNγ-inducible CIITA expression. Overall, these data link the diverse mechanisms contributing to intestinal IL-18 to local production of IFNγ and geographically diverse upregulation of IEC MHCII (Fig. 8h). They provide a uniquely broad picture of the regulation of this energetically costly process, but questions pertaining to the significance of IEC MHCII in barrier integrity, host defense, and tolerance induction remain open. As these functions gain greater clarity, understanding IEC MHCII regulation will be critical for identifying and correcting pathology related to its dysregulation and exploiting this process to treat intestinal disease.

MATERIALS AND METHODS

Mice
All mice were bred and manipulated under specific pathogen-free conditions in 12-h light-dark cycles with food and water available ad libitum. All procedures were approved by the University of Pittsburgh animal care and use committee. All mice are on a C57Bl/6 background and originated from Jackson Laboratories, except the IL-18R−/−/− (Dr. Tomoaki Hoshino, Kurume University), MEFVβ2G/Aβ2G/A mice (Drs. Daniel Kartner and Jae Jin Chae, NHGRI), IL18bp−/− (Knockout Mouse Project), IL18 (Dr. Richard Flavell, Yale University) and the NLRC4−/−/− (Dr. Tomoaki Hoshino, Kurume University) and the Nlrc4TS/TS/Knockout Mouse Project, −/− mice were lethally irradiated with 1100 cGy (split dose, 2 h apart) in an XRAD320 (Precision Xray Inc) irradiator. The following day mice were reconstituted with 4–6 million WT or IFNγ−/− bone marrow cells. Intestinal scaps were collected 4–6 weeks post treatment initiation.

Supplementary Fig. 6 shows the workflow strategy for the colonic epithelium. Lamina propria cells were isolated according to described protocols and stimulated for 2.5 h with PMA/ionomycin and Brefeldin A to study cytokine secretion. Filtered single cell suspensions were stained with anti-mouse antibodies against CD3(OTK3), CD4(RM4-5), CD8b(YTS 156.7.7), CD45.2(104), CD90.3(S3-2.1), B220(RA3-6B2), Epac2(G8.8), Fixable Live Dead Stain (Thermo Fisher, Waltham, MA), IFNγ(XMG1.2), IFNγ(R2F1), NK1.1(2F1), TCRδ(G5-1.2) (eBioscience, San Diego, CA) or Biolegend (San Diego, CA). To study cytokine secretion. Filtered single cell suspensions were stained with anti-mouse antibodies against CD3(OTK3), CD4(RM4-5), CD8b(YTS 156.7.7), CD45.2(104), CD90.3(S3-2.1), B220(RA3-6B2), Epac2(G8.8), Fixable Live Dead Stain (Thermo Fisher, Waltham, MA), IFNγ(XMG1.2), IFNγ(R2F1), NK1.1(2F1), TCRδ(G5-1.2) (eBioscience, San Diego, CA) or Biolegend (San Diego, CA).

CD3 and CD4 expression was assessed by flow cytometry on the BD LSR II, and analyzed using FCAP Array (BD Biosciences, v. 3.0, San Jose, CA). Organoid cultures
Murine. WT organoid cultures were generated from SI samples according to the manufacturer’s protocol (Stem Cell Technologies, Vancouver, Canada) in IntestiCult™ Organoid Growth Medium. Organoids were routinely passaged and maintained in Matrigel domes every 7–10 days. For TLR and cytokine stimulation, mature organoids were seeded at a density of ~300 organoids/well in a 12 well plate. Following organoid budding (3–5 days post passage), the media was removed and replaced with TLR/kitokine supplemented media and the samples incubated at 37°C for 3.5 days. Organoids were removed, stained, and analyzed as described above for the IEC samples. All murine cytokines were purchased from Peprotech (Rockyhill, NJ) and used at a concentration of 10 ng/mL, apart from IL-18 (MBL International, –/− mice were lethally irradiated with 1100 cGy (split dose, 2 h apart) in an XRAD320 (Precision Xray Inc) irradiator. The following day mice were reconstituted with 4–6 million WT or IFNγ−/− bone marrow cells. Intestinal scaps were collected 4–6 weeks post treatment initiation.

BMCS. Rag1−/− mice were lethally irradiated with 1100 cGy (split dose, 2 h apart) in an XRAD320 (Precision Xray Inc) irradiator. The following day mice were reconstituted with 4–6 million WT or IFNγ−/− bone marrow cells. Intestinal scaps were collected 4–6 weeks post treatment initiation.

Human. SI enteroids were isolated from surgically resected fetal (<24 weeks) SI tissue according to described protocols. Samples were procured from the Health Science Tissue Bank of UPMC Magee-Womens Hospital in accordance with the guidelines set forth by the University of Pittsburgh Ethics Committee. Briefly, 100 crypts were plated in Matrigel in 48 well plates containing a 50:50 vol/vol ratio of human intestinal stem cells media (plus 50 mM EGF) and WNR-conditioned media. Three days post plating the tissue was removed and replaced with TLR/Cytokine supplemented media (same concentrations as the murine organoids). Organoids were harvested 3.5 days post stimulation according to the methods described in ref. and stained and analyzed for IEC MHCII surface expression.

Co-housing and 16S sequence analysis
Three- to five-week-old Rag1−/− mice bred locally or purchased commercially (Jackson labs) and/or WT mice were co-housed for 5 weeks prior to sacrifice. Cecal stool samples were collected at the time of death and frozen until processing. Fecal DNA was extracted using the QiaAMP Stool DNA Mini Kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions and sequenced by Microbiome Insights (University of British Columbia, Canada). Microbiome informatics were performed by AHPB using QIIME 2020.2.0. Raw sequences were quality-filtered and denoised with DADA2. Amplicon sequence.
variants (ASVs) were aligned with mafft and used to construct a phylogeny with fasttree21,22. Alpha diversity metrics (observed OTUs), beta diversity metrics (Jaccard Similarity) and Principal Coordinate Analysis were estimated after samples were rarefied to 63,000 (subsampled without replacement) sequences per sample. Taxonomy was assigned to ASVs using naive Bayes taxonomy classifier against the Greengenes 18_5 99% OTUs reference sequences23,24. All plots were made with publicly available R packages. Full sequence data are available upon request.

Analysis of publicly available data sets
Publicly available data sets were obtained from the Gene Expression Omnibus. Briefly, terminal ileum IL-18 read counts in co-housed, SFB-colonized WT, Rag²−/− and TCRα−/− mice were obtained from Mao et al. and accessed from GSE8678024. Normalized read counts from bulk RNA-seq of FACS-sorted IECs from neonatal intestine derived from mice 4 h after subcutaneous injection of saline.25 t-SNE plot (perplexity = 25) colored by inferred cell type or gene expression of select murine cytokine receptors from Haber et al.31 visualized in Single-Cell Expression Atlas.

Statistics
Unpaired Student’s t tests or one-way ANOVAs with Tukey post correction were performed in GraphPad Prism (Version 8.3.1) as identified in the figure legends. Results were considered significant if p < 0.05.

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AUTHOR CONTRIBUTIONS
S.C. and L.V.D.K. planned and analyzed the experiments, which were conducted by L.V.D.K., C.S., V.D., E.W., J.V., L.Y., and S.C. A.H.P.B. performed the 16S analysis and is supervised by T.H. The manuscript was written by L.V.D.K. and S.C. and all authors reviewed and approved the manuscript prior to submission.

ADDITIONAL INFORMATION
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