



***Faecalibaculum rodentium* Remodels Retinoic Acid Signaling to Govern Eosinophil-Dependent Intestinal Epithelial Homeostasis**

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SUMMARY

The intestinal epithelium plays critical roles in sensing and integrating dietary and microbial signals. How microbiota and intestinal epithelial cell (IEC) interactions regulate host physiology in the proximal small intestine, particularly the duodenum, is unclear. Using single-cell RNA sequencing of duodenal IECs under germ-free (GF) and different conventional microbiota compositions, we show that specific microbiota members alter epithelial homeostasis by increasing epithelial turnover rate, crypt proliferation, and major histocompatibility complex class II (MHCII) expression. Microbiome profiling identified *Faecalibaculum rodentium* as a key species involved in this regulation. *F. rodentium* decreases enterocyte expression of retinoic acid-25 producing enzymes *Adh1*, *Aldh1a1*, and *Rdh7*, reducing retinoic acid signaling required to maintain certain intestinal eosinophil populations. Eosinophils suppress intraepithelial lymphocyte-mediated production of interferon- γ that regulates epithelial cell function. Thus,

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AUTHOR CONTRIBUTIONS

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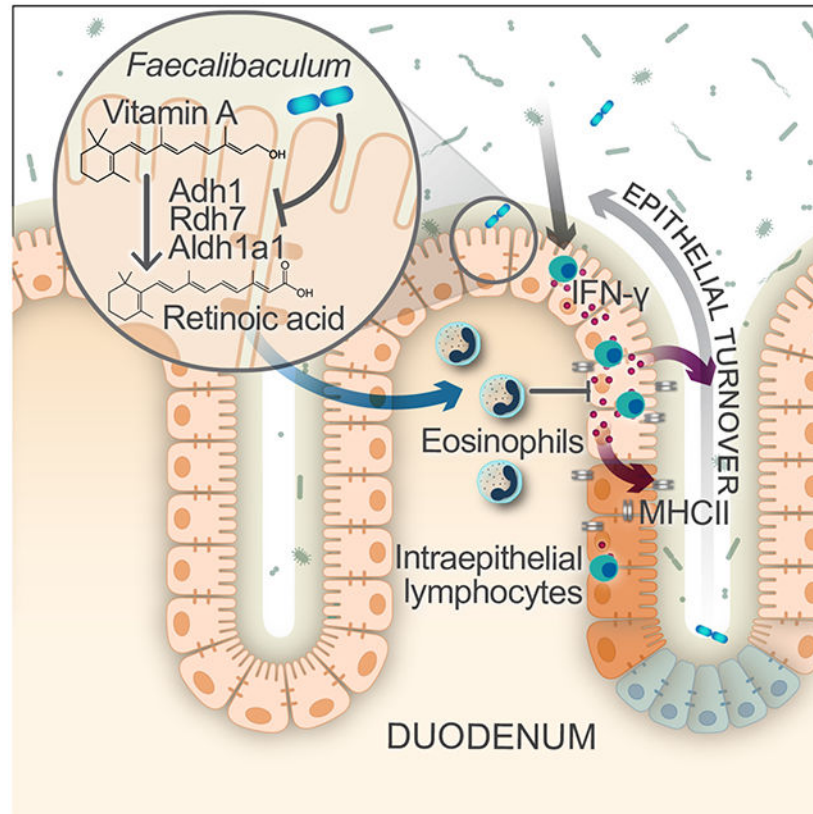
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DECLARATION OF INTERESTS

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we identify a retinoic acid-eosinophil-interferon- γ -dependent circuit by which the microbiota modulates duodenal epithelial homeostasis.

Graphical Abstract



eTOC blurb

The intestinal epithelium is a central node for communication between the microbiota and immune system. Cao *et al.* show that *Faecalibaculum rodentium* can promote epithelial proliferation and turnover by dampening retinoic acid production that supports survival of intestinal eosinophils, which in turn suppress pro-proliferative IFN- γ production.

INTRODUCTION

The intestinal epithelium consists of specialized cell types, which function in nutrient uptake, barrier integrity, and host defense (Allaire et al., 2018; Hooper, 2015; Solis et al., 2020). These cells sense dietary and microbial signals, such as short chain fatty acids, secondary bile acids, and tryptophan metabolites, and relay them to immune cells (Larsen et al., 2020; Soderholm and Pedicord, 2019). The microbiota influences intestinal epithelial cell (IEC) function by inducing the expression of antigen-presenting molecules (Tuganbaev et al., 2020), regulating the frequency of epithelial subsets, including tuft and goblet cells (Howitt et al., 2016; Nadjisombati et al., 2018; Schneider et al., 2018), and increasing the rate of epithelial turnover (Khoury et al., 1969). The microbiota also modulates the production of

Initially, we focused our investigation on intestinal stem cells (ISCs, cluster 2), which give rise to all differentiated mature IECs, as a representation of the progenitor populations which shared similar significantly differentially expressed genes (DEGs) (Table S1). IH ISCs differed more from Jax/GF ISCs overall, with 240 DEGs between IH and Jax/GF ISCs, but only 44 DEGs between Jax and GF ISCs. Gene ontology (GO) analysis of DEGs from GF and Jax vs. IH ISCs identified an enrichment in metabolic processes (including fatty acid and metal ion metabolism) in GF/Jax ISCs, while enriched categories in IH ISCs mostly related to immune responses (Fig. 1B). Most of the top DEGs, as ranked by significance, were related to major histocompatibility complex class II (MHCII) and MHCI expression in IH ISCs (Fig. 1C). This finding is in line with the “response to interferon (IFN)- γ ” category in IH ISCs (Fig. 1B), as IFN- γ can induce both MHCII and MHCII expression (Skoskiewicz et al., 1985; Zhou, 2009). These differences in immune response genes were specific to IH ISCs, not simply the presence of a microbiota, as DEGs in Jax versus GF ISCs were also mainly related to lipid and metal ion metabolism (Fig. S2A-B). Furthermore, comparing Jax vs IH ISCs showed an enrichment in immune response and MHCII signatures similar to that seen in the IH vs GF/Jax comparison. These analyses support that most of the transcriptional differences attributable to the presence of a microbiota are driven by the IH microbiota (Fig. S2C-D). To study the ISC compartment *in vivo*, we derived *Lgr5-GFP-creERT2* mice, which express GFP in *Lgr5*⁺ ISCs, onto a Jax or IH microbiota background. We did not observe a significant difference in the proportion of ISCs in Jax and IH mice by flow cytometry or immunofluorescence (Fig. S2E-G) but confirmed increased MHCII expression in IH ISCs (Fig. 1D). We also examined gene signatures for ISC subsets identified by Biton et al., 2018 under the different microbiota conditions (Fig. S2H). We found that all microbiota conditions had more ISC-I and ISC-II compared to ISC-III and that the expression of all 3 signatures was highest in IH mice, suggesting that these ISCs are most similar to the ISCs profiled in Biton *et al.* and may have overall higher expression of stem-related genes. *Gpx2*, a gene with both pro- and anti-proliferative effects in stem cells and cancer (Florian et al., 2010; Jiao et al., 2017; Li et al., 2020), was also highly upregulated in IH ISCs, which we confirmed by qPCR (Figs. 1C, S2I), prompting us to investigate the proliferation of ISCs and epithelial turnover rate under the different microbiota conditions. We measured crypt Ki67 expression, which marks all non-G0 phase cells, and performed 48 hours of continuous bromodeoxyuridine (BrdU) labeling, which is incorporated during DNA replication, to measure epithelial turnover. IH mice had increased numbers of Ki67⁺ cells in the crypts as well as a higher epithelial turnover rate (the length of crypt-villus axis labeled by BrdU divided by the total axis length)_compared to GF and Jax mice (Fig. 1E-H). We also confirmed by flow cytometry that IH mice have a higher frequency of Ki67⁺ *Lgr5*-GFP ISCs than Jax mice (Fig. 1I). Cell cycle scoring of ISCs from the scRNAseq dataset also showed that IH ISCs had a slightly higher score for the S and G2/M cell cycle phases compared to GF and Jax mice (Fig. S2J). Overall, we found that microbiota composition, not just presence, is critical for regulation of epithelial phenotypes. In particular, the IH but not Jax microbiota is able to induce MHCII expression, crypt proliferation, and increased epithelial turnover rate.

The microbiota modulates intestinal immune populations via enterocyte retinoic acid production

We next examined the distinct mature IEC subsets that arise from these ISCs, focusing on absorptive enterocytes, where we observed the most pronounced differences compared to other differentiated cell types (Table S1). Unexpectedly, even though Jax and IH both possess SPF microbiota, their enterocytes had strikingly different gene expression profiles, with Jax enterocytes closely resembling those from GF mice, resulting in their categorization into a separate cluster from IH enterocytes (Fig. 1A). GO analysis of DEGs in GF/Jax vs IH enterocytes revealed a similar pattern as seen in ISCs, with GF/Jax enterocytes enriched in fatty acid metabolism and IH enterocytes enriched in immune response pathways (Fig. 2A). The top DEGs enriched in IH enterocytes as ranked by significance included MHCII-related genes and defense response genes such as the anti-microbial peptide *Reg3g*, the mucin *Muc13*, and the microbiota-epithelial cell segregation-promoting *Lypd8* (Fig. 2B). IH enterocyte MHCII expression was similar or higher than ISC (Fig. S3E). MHCI genes were also upregulated in IH enterocytes (Fig. S3F). As in ISCs, the differences in immune response-related genes were specific to IH enterocytes. Comparison of Jax vs. GF enterocytes from cluster 1 did not identify activation of immune pathways (Fig. S3A-B), while comparison of IH vs Jax enterocytes revealed similar enriched genes as when comparing IH to GF and Jax (Fig. S3C-D). We confirmed high MHCII expression in total IECs from IH mice and low expression in GF and Jax IECs by flow cytometry (Fig. 2C). Among genes highly expressed in GF and Jax enterocytes, three are related to RA production (Fig. 2B): *Aldh1a1*, an aldehyde dehydrogenase that converts retinaldehyde to RA, as well as the alcohol dehydrogenase *Adh1* and retinol dehydrogenase *Rdh7*, which can catalyze the upstream conversion of retinol to retinaldehyde (Kumar et al., 2012). Overall, IH enterocytes exhibited an immune activation phenotype, whereas GF and Jax enterocytes were enriched in expected metabolic processes and RA production, demonstrating that the specific composition of the microbiota can shape IEC function.

Dietary retinol (vitamin A) is absorbed by epithelial cells primarily in the proximal small intestine and then processed to RA (Villablanca et al., 2011), which affects intestinal immunity via signaling through the RAR and RXR receptors (Czarnewski et al., 2017; Hall et al., 2011a). Some of these effects include: recruiting T, B, and innate lymphoid cells (ILCs), controlling the balance between different T helper cell and ILC subsets, and supporting the survival of CD11b⁺CD103⁺ dendritic cells (DCs) (Iwata et al., 2004; Kim et al., 2015; Larange and Cheroutre, 2016; Mora et al., 2006). We confirmed decreased mRNA expression of the RA-producing enzymes *Adh1*, *Aldh1a1*, and *Rdh7* in IH mice by qPCR (Fig. 2D). Next, to assess RA production, we measured Aldh activity using the Aldefluor assay. We found that GF IECs have the highest Aldh activity, followed by Jax IECs, and IH IECs had the lowest activity (“Test” samples) (Fig. 2E). All IECs lacked Aldh activity in the presence of the Aldh inhibitor DEAB (“Ctrl” samples). The other major source of RA in the gut is DCs in the intestinal lamina propria (Villablanca et al., 2011). Duodenal GF, Jax, and IH DCs produced similar levels of RA (Fig. 2F), suggesting that any differential levels of RA production are likely due to epithelial-derived RA. Additionally, we found that while epithelial Aldh activity was also lower in the jejunum and ileum of IH mice than Jax mice (Fig. S3G-H), overall Aldh activity was highest in duodenal epithelial cells, consistent

with previous reports (Villablanca et al., 2011). Duodenal RA levels measured by liquid chromatography-mass spectrometry (LC-MS) were slightly elevated in GF and Jax mice compared to IH mice (Fig. S3I). Jax intestinal eosinophils also had higher expression of the RAR target gene *Tgm2* (Moore et al., 1984) than IH eosinophils (Fig. S3J). Furthermore, we found higher expression of an RA target gene signature in GF and Jax mice compared to IH mice in many epithelial cell types, particularly enterocytes, with cluster 1 (GF/Jax enterocytes) having higher expression compared to cluster 7 (IH enterocytes) (Fig. S3K). Together, these results suggest that GF and Jax mice have higher RA production and signaling than IH mice.

Given RA's importance in regulating intestinal immunity and its higher abundance in the proximal small intestine, we profiled duodenal lamina propria immune cells to determine the effect of epithelial-derived RA in Jax and IH mice (Fig. 3, Fig. S4, Fig. S5A-M). To examine which immune cell differences are specific to RA, we administered the retinoic acid receptor (RAR) inhibitor BMS493 (Germain et al., 2009). Compared to Jax mice, IH mice (with lower RA levels) had a lower proportion of CD11b⁺CD103⁺ DCs, whose expansion is dependent on RA (Klebanoff et al., 2013) (Fig. 3A). As expected, inhibiting RAR signaling also decreased CD11b⁺CD103⁺ DCs in both Jax and IH mice. We also noted a potential effect of vehicle (DMSO) treatment alone on this DC population, indicating the importance of comparing inhibitor-treated to vehicle-treated populations. Intestinal Tregs and Th17 cells are also regulated by either high or low levels of RA (Cha et al., 2010; Hall et al., 2011b; Mucida et al., 2007; Wang et al., 2010; Xiao et al., 2008). IH mice had a higher proportion of total $\alpha\beta$ T cells and Th17 cells but lower Tregs than Jax mice (Fig. 3B-D). However, these differences do not seem to mainly depend on RA, as BMS493 treatment did not cause Jax T cells to resemble IH proportions. Other cell types, including Th1, Th2, and ILCs, also differed between Jax and IH mice, but again, these differences were not directly regulated by RA as BMS493 administration did not replicate the effect on their proportions (Fig. S5A-M).

Eosinophils, which are regulated by RA *in vitro* when isolated from human peripheral blood (Ueki et al., 2008), were also significantly higher in Jax than IH mice (Fig. 3E). RAR signaling inhibition decreased the eosinophils in Jax mice to IH proportions but had no effect on IH eosinophils. Among immune cells profiled, only eosinophils exhibited this pattern of being decreased in Jax but not IH mice after treatment. We did not observe differences in the lamina propria expression of *Il5*, an important cytokine for eosinophil accumulation and survival (Rothenberg and Hogan, 2006), between Jax and IH mice (Fig. S5N), indicating IL-5 was not responsible for the increased eosinophils observed in Jax mice. To confirm that RAR signaling occurs in eosinophils and is blocked by BMS493, we sorted proximal small intestinal eosinophils and measured the expression of select RARs and the RAR target gene *Tgm2* (Moore et al., 1984). Intestinal eosinophils express *Rara* and *Rarb*, and inhibiting RAR signaling decreased *Tgm2* expression (Fig. S5O-P). Using an *in vitro* system with isolated bone marrow eosinophils, we directly tested the effect of RA on eosinophil survival. Incubation with RA increased eosinophil survival, though less so than interleukin (IL)-5 (Fig. 3F). These results suggest that higher RA production in Jax mice increases eosinophil levels, potentially via enhanced survival, and is the main driver behind differential eosinophil frequencies in Jax versus IH mice. Following inhibition of

RAR signaling, some eosinophils remain in both groups. These data suggest a role for RA in regulating certain intestinal eosinophils and raise the possibility of different eosinophil cell states or subsets with varying dependence on RA.

Eosinophils regulate epithelial turnover and MHCII expression by suppressing IFN- γ -producing intraepithelial lymphocyte (IEL) subsets in a microbiota-dependent manner

The proximal small intestine, where we found that RA regulates eosinophils, contains the highest levels of eosinophils in the gut (Chu et al., 2014). Eosinophils in other tissues promote proliferation during the tissue repair process after injury or infection (Goh et al., 2013; Heredia et al., 2013; Vicetti Miguel et al., 2017), and the intestinal epithelium is highly proliferative at steady state. Thus, we wondered if eosinophils may be responsible for the distinct IEC proliferation phenotypes in IH and Jax mice. To test this hypothesis, we used eosinophil-deficient PHIL mice derived onto either a Jax or IH microbiota background. As we initially observed, IH wild-type (WT) mice had a higher epithelial turnover rate and more proliferating Ki67⁺ cells than Jax WT mice (Figs. 1E-H, 4A-B). On a Jax microbiota background, eosinophils did not affect turnover or Ki67⁺ cells (Fig. 4A-B). However, on an IH microbiota background, PHIL mice had increased epithelial turnover and Ki67⁺ cells compared to WT mice. A similar pattern was observed in MHCII expression, with very low expression in both Jax WT and PHIL mice, higher expression in IH WT mice, and highest expression in IH PHIL mice (Fig. 4C). These results suggest that eosinophils have no regulatory effect when there is little proliferative signal, as in Jax mice. However, when an increased proliferative signal is present, eosinophils act as a negative regulator to dampen excess proliferation. To determine if eosinophils also regulate the response to injury, we employed a model of small intestinal injury by injecting an anti-CD3 antibody that results in T cell-mediated epithelial damage (Merger et al., 2002). Similar to the patterns in turnover, IH (especially IH PHIL) mice had more extensive intestinal damage that was characterized by villous atrophy and crypt injury, as evaluated in histological injury scores (Fig. 4D-F). Thus, eosinophil regulation of MHCII expression, IEC proliferation, and/or IFN- γ -related signals may affect sensitivity to inflammatory injury.

IFN- γ can regulate IEC proliferation (Nava et al., 2010) and induce MHCII expression in epithelial cells (Skoskiewicz et al., 1985). Eosinophils suppress IFN- γ production by lamina propria T cells in the colon and stomach (Arnold et al., 2018) and response to IFN- γ was enriched in IH ISCs (Fig. 1B). Therefore, we measured *Ifng* mRNA levels in the epithelial and lamina propria fractions of Jax and IH WT and PHIL mice. In the epithelial fraction, which contains both epithelial and immune cells, IH PHIL had the highest *Ifng* expression (Fig. 5A), mirroring the patterns in epithelial turnover and MHCII expression. No differences were observed in the lamina propria (Fig. S6B). This epithelial *Ifng* likely comes from IELs, which are the majority of immune cells in the epithelium and known IFN- γ producers (McDonald et al., 2018). Epithelial cells do not produce IFN- γ under homeostatic conditions (Haber et al., 2017), and we did not detect *Ifng* transcripts in our epithelial scRNA-seq dataset. Thus, we next characterized IEL subsets and their IFN- γ production. IELs can be divided into TCR $\alpha\beta$ ⁺ IELs, which develop from naïve T cells encountering foreign antigen in the periphery, and TCR $\gamma\delta$ ⁺ IELs, which are more innate-like and develop after recognition of self-ligands (McDonald et al., 2018). TCR $\alpha\beta$ ⁺

IELs can be further sub-divided into CD8 $\alpha\beta^+$ IELs; CD4 $^+$ IELs, which depend on MHCII expression by IECs (Bilate et al., 2020; Tuganbaev et al., 2020); and CD8 $\alpha\alpha^+$ IELs, which are similar to TCR $\gamma\delta^+$ IELs that share CD8 $\alpha\alpha$ expression. A majority of IELs in IH WT mice were TCR $\alpha\beta^+$, whereas Jax WT mice had a majority of TCR $\gamma\delta^+$ IELs (Figs. 5B, S6A). Jax PHIL mice had a similar IEL profile as Jax WT mice, but IH PHIL mice had even more TCR $\alpha\beta^+$ and fewer TCR $\gamma\delta^+$ IELs than IH WT mice. Within the TCR $\alpha\beta^+$ IEL subsets, IH mice had fewer CD8 $\alpha\alpha^+$ IELs, more CD8 $\alpha\beta^+$ IELs, and more CD4 $^+$ IELs than Jax WT and PHIL mice (Fig. 5C). IH PHIL mice were similar to IH WT mice, with an additional increase in CD4 $^+$ IELs, correlated with highest MHCII expression in IH PHIL mice. Overall, these findings reflect a shift from innate-like IELs towards more conventional, peripherally educated IELs in IH and especially IH PHIL mice compared to Jax mice. Most IEL subsets in IH PHIL mice produced more IFN- γ than in Jax WT and PHIL mice (Fig. 5D). Additionally, certain subsets, including total TCR $\alpha\beta^+$, CD8 $\alpha\beta^+$, and CD4 $^+$ IELs, produced more IFN- γ across all conditions compared to TCR $\gamma\delta^+$ and CD8 $\alpha\alpha^+$ IELs. Therefore, increased IFN- γ production in IELs in IH PHIL mice is due to both a per cell increase in IFN- γ production as well as a shift in IEL populations to higher IFN- γ -producing subsets. Overall, we concluded that IH microbiota increases IFN- γ production in IELs and that eosinophils suppress this microbiota-induced increase.

To determine if the increased IFN- γ production in IH and IH PHIL mice was a driver of differences in epithelial phenotype, we used an IFN- γ neutralizing antibody (Ab). There was no effect in Jax WT and Jax PHIL mice injected with the Ab, as expected based on the low IFN- γ production in Jax mice (Figs. 5E-F, S6C). However, IFN- γ blockade abrogated the difference in IEC turnover between IH WT and IH PHIL mice without affecting Ki67 $^+$ crypt cells and also decreased MHCII expression. This difference in cell turnover versus Ki67 $^+$ cells could be because Ki67 marks all non-G0 phase cells. ISCs often remain in a quiescent G1 phase (Carroll et al., 2018), so a change in IFN- γ -dependent proliferation could occur without changes in Ki67. As a complementary method to track proliferation, we measured BrdU $^+$ cells in the crypt 2 hours after BrdU injection. We found that anti-IFN- γ Ab reduced BrdU $^+$ cells in IH WT and PHIL mice, indicating reduced proliferation (Fig. 5G-H). Additionally, we observed very few TUNEL $^+$ apoptotic IECs under any condition (Fig. S6D), suggesting that IFN- γ -mediated effects are not due to changes in apoptosis. Overall, these findings demonstrate that eosinophil restraint of epithelial turnover depends on suppression of IFN- γ .

***Faecalibaculum rodentium* regulates RA-eosinophil-epithelial cell circuit**

After observing that decreased RA production by IECs in IH mice results in fewer eosinophils, increased IFN- γ production, and increased IEC turnover rates and MHCII expression, we investigated if these differences could be modulated by microbiota transfer. Because IH IECs differed more from both Jax and GF IECs, we chose to transfer IH microbiota to Jax mice. A one-time gavage of IH stool or cecal contents in Jax mice (without any antibiotic pre-treatment of Jax mice) resulted in reduced IEC *Adh1*, *Aldh1a1*, and *Rdh7* expression, decreased eosinophil proportion, and shifts in IEL composition toward more TCR $\alpha\beta^+$, CD8 $\alpha\beta^+$, and CD4 $^+$ IELs and increased IFN- γ production (Fig. 6A-D). As a result, MHCII expression and Ki67 $^+$ crypt cells also increased after transfer,

with a greater increase in Jax PHIL mice receiving IH contents compared to Jax WT recipients (Fig. 6E-G). The turnover rate was only slightly different between WT and PHIL post-transfer, possibly because the newly-introduced IH microbiota induced a very high turnover rate reminiscent of baseline IH PHIL mice in both groups. Pre-treatment of IH mice with broad-spectrum antibiotics prior to transfer of their microbiota to Jax mice eliminated the regulatory activity of the transfer (Fig. S6E). Co-housing Jax and IH mice in the same cage also resulted in Jax mice taking on an IH phenotype (Fig. S6F). Collectively, these results indicate that antibiotic-sensitive member(s) of the IH microbiota responsible for RA-eosinophil-epithelial regulation can be transferred to Jax mice and confer immunomodulatory effects.

To pinpoint which component of the IH microbiota is responsible for the microbiota's regulatory capacity, we performed 16S rRNA gene amplicon sequencing of stool from Jax and IH mice under the previously tested experimental conditions. Since both cohousing and direct microbiota transfer recapitulated the IH-like RA-eosinophil-epithelial regulatory capacity in Jax mice, we focused on bacterial species absent in Jax mice and Jax mice receiving antibiotic-treated IH microbiota but present in the other groups. Microbiome communities clustered into two groups, with one group comprising samples from high RA/high eosinophil/low epithelial turnover mice (Jax and Jax with Abx-treated IH transfer) and the second comprising low RA/low eosinophil/high epithelial turnover mice (IH, IH cohoused, Jax cohoused, and Jax with IH transfer) (Fig. S7A). Differentially abundant bacterial genera that distinguish these two groups were identified using linear discriminant analysis effect size (LEfSe) analysis (Segata et al., 2011) (Fig. 7A). In particular, *Bifidobacterium*, *Faecalibaculum*, and *Lachnospiraceae A2* genera were enriched in IH mice and Jax mice with IH cohousing/transfer compared to Jax, which was confirmed by qPCR on stool samples (Fig. 7B). *Bifidobacterium* and *Faecalibaculum* were also detected in the duodenal luminal contents of IH mice (Fig. 7B), raising the possibility of a direct local effect on the epithelial-immune interplay we observed. To further investigate this interaction, we performed fluorescence *in situ* hybridization (FISH) using fluorochrome-tagged 16S rRNA gene probes specific for *F. rodentium*. We detected *F. rodentium* in the duodenal crypts (Fig. 7C). In contrast, when we examined *F. rodentium* localization in the colon, we observed it principally in the colonic contents (Fig. S7B). Although there are relatively few bacteria in the duodenum, as expected given the overall lower bacterial density, this observation suggests that *F. rodentium* could adhere to or directly interact with duodenal IECs.

To investigate if any of these identified bacteria can regulate epithelial cell phenotypes, we monocolonized GF mice with strains of these genera that were isolated from IH mice. *B. pseudolongum* and *F. rodentium* but not *Lachnospiraceae A2* successfully monocolonized GF mice (Fig. S7C). *F. rodentium* alone reduced *Aldh1a1* expression and eosinophil proportions compared to GF mice, thereby increasing epithelial turnover and Ki67⁺ crypt cells (Fig. 7D-G). *B. pseudolongum* reduced *Adh1* expression but did not affect eosinophils or epithelial turnover, perhaps because other enzymes can compensate for *Adh1* activity, whereas only *Aldh1a1* in IECs can convert retinaldehyde to RA. However, we did not observe an effect of *F. rodentium* or *B. pseudolongum* on MHCII or IFN- γ production (Fig. S7D-F). Thus, we posit that a different bacterial component(s) in the IH microbiota acts

as a separate signal for IFN- γ induction in IELs. *F. rodentium* suppresses RA to regulate eosinophils, which then modulate IFN- γ production induced by this second source. The observation that *F. rodentium* still increases proliferation and turnover, despite not inducing IFN- γ , also indicates that both IFN- γ -dependent and independent mechanisms are involved in controlling this process. *F. rodentium* also only slightly increased anti-CD3 induced injury (Fig. S7G), suggesting that while heightened proliferation may increase susceptibility, other factors likely including IFN- γ signaling also regulate the response to injury or initial damage severity. Thus, *F. rodentium* is one IH microbiota member sufficient to recapitulate its RA-eosinophil-epithelial regulation, though other members also play a role in a full SPF microbiota, particularly in inducing IFN- γ .

DISCUSSION

We have uncovered a retinoic acid-eosinophil-IFN- γ circuit by which the microbiota can regulate duodenal IEC turnover and MHCII expression. Specific microbiota members including *F. rodentium* can activate this circuit, thereby altering both intestinal epithelial and immune homeostasis. Thus, we identify multiple points of regulation for epithelial turnover rate, modulation of which is critical for maintaining barrier function and allowing repair after injury or infection. Furthermore, this finding emphasizes the interconnected nature of microbiota-epithelial-immune interactions, with IECs as both sensors and readouts of changes in microbiota composition and function.

Our work highlights the impact of different SPF microbiota and the importance of certain microbiota members. Although both Jax and IH mice possess complex microbiota with many bacterial species, in many aspects of epithelial and immune cell function, we found that Jax mice more closely resemble GF mice than IH mice. GF mice have long been known to have a slower small intestinal epithelial turnover rate compared to SPF mice (Khoury et al., 1969). However, we further demonstrate that a specific species present in many mouse microbiotas, *F. rodentium* (Chang et al., 2015; Cox et al., 2017; Zagato et al., 2020), can accelerate epithelial turnover. In terms of RA regulation, IECs from SPF mice produce less RA than GF mice due to suppression of *Rdh7* expression (Grizotte-Lake et al., 2018). In our study, we extend this work to show that only IH microbiota, and specifically *F. rodentium*, was able to suppress IEC RA production. In addition to *Rdh7*, we also found that *Adh1* and *Aldh1a1* expression was reduced in IH IECs, perhaps because of different microbiota compositions. Recent work has also shown that certain microbiota members, such as segmented filamentous bacteria (SFB, called *Candidatus savagella*), can produce RA (Woo et al., 2021). Although our mice lack SFB (data not shown), there may be RA-producing species that could represent another RA source.

Additionally, our observations reveal an immunomodulatory role of *F. rodentium*, an understudied microbiota member. *F. rodentium* is a Gram-positive, anaerobic member of the family *Erysipelotrichaceae* (Chang et al., 2015; Cox et al., 2017). It is found predominantly in the murine gut, though a closely related species, *Holdemanella bififormis*, is present in humans (Zagato et al., 2020). *F. rodentium* in the ileum (where it is found in the mucus layer) and colon reduces tumor growth in mice through production of short-chain fatty acids (Zagato et al., 2020). We also found that *F. rodentium* can colonize duodenal crypts.

Further work is needed to determine if *F. rodentium* regulates IEC RA production through metabolites or direct contact with IECs.

Our work also provides insight into regulation of MHCII expression in ISCs by both the microbiota and eosinophils. The IH microbiota can induce epithelial MHCII expression, which has also been attributed to SFB and their attachment properties (Tuganbaev et al., 2020). Our IH mice lack SFB, and future work is needed to identify IFN- γ -inducing bacteria that regulate MHCII expression in our system. This higher MHCII expression is associated with lower levels of RA, whereas RA has previously been shown to promote MHCI expression on colon tumor cells (Bhattacharya et al., 2016). This apparently divergent function of RA could be due to RA signaling through eosinophils to regulate MHCII in our system compared to the direct action on tumor cells, as well as the difference between normal and cancerous cells, as RA did not promote MHCI expression on normal epithelium (Bhattacharya et al., 2016). Functionally, ISC MHCII can regulate CD4+ T helper cell subsets that in turn modulate ISC fates, and ablation of MHCII on epithelial cells can increase Lgr5⁺ ISC numbers (Biton et al., 2018). We did not find a difference in Jax and IH ISC frequency, perhaps due to the difference in genetic ablation used in that study versus the lack of inducing signal we observed. Suppression of ISC MHCII by high-fat diet can promote intestinal tumorigenesis, and ISC stemness also influences antigen-presentation machinery expression and the immune environment in colorectal cancer (Beyaz et al., 2021; Chen et al., 2021). Thus, our work demonstrating the role of microbiota, eosinophils, and IEL-derived IFN- γ in regulating MHCII expression could aid in mechanistic understanding of how MHCII is controlled in relevant disease settings.

We also uncovered a pivotal role for the microbiota and RA in regulating intestinal eosinophils. Jax mice with high RA had more intestinal eosinophils than IH mice with low RA, and inhibiting RAR signaling reduced Jax eosinophils to IH levels. This finding is in accordance with observations that SPF mice have fewer intestinal eosinophils than GF mice (Jiménez-Saiz et al., 2020) and provides a mechanism for that regulation. Another recent study found that eosinophils regulate the host response to microbial colonization, but the microbiota did not control eosinophil abundance (Ignacio et al., 2022). Our finding that only specific bacteria regulate eosinophil abundance provides an explanation for the discrepancies in these previous findings. Furthermore, the eosinophils remaining in Jax mice after inhibiting RAR signaling and the lack of an effect in IH mice suggest that there are different eosinophil functional states or subsets in the duodenum with distinct requirements for RA. Consistent with prior observations in human eosinophils (Ueki et al., 2008), we found that eosinophils express RAR and RXR receptors and that RAR signaling is active in intestinal eosinophils. Future work will also determine if RA-dependent and RA-independent eosinophils have different functional properties and if this contributes to differences in Jax and IH eosinophil function.

Additionally, we demonstrated that intestinal eosinophils suppress production of IFN- γ by IELs to restrict excessive epithelial turnover. This anti-proliferative role contrasts with the pro-proliferative function of eosinophils in injury and repair contexts (Goh et al., 2013; Heredia et al., 2013), which may be due to the homeostatic nature of the intestinal interaction and presence of resident eosinophils, whereas pro-proliferative eosinophils are

rapidly recruited after an insult. Gastric and colonic eosinophils can modulate lamina propria IFN- γ production by T cells via a PD-L1-dependent pathway (Arnold et al., 2018). It remains to be determined if intestinal eosinophils directly interact with IELs to regulate IFN- γ production and if a similar pathway mediates that regulation. We found that neutralization of IFN- γ abrogates increased epithelial turnover in IH WT and PHIL mice. Both type I (IFN- α/β) and type II (IFN- γ) IFNs can promote epithelial proliferation (Eriguchi et al., 2018; Sun et al., 2015). We further show that specific SPF microbiota can alter IFN- γ production by IELs, similar to what is known for lamina propria CD8⁺ T cells (Tanoue et al., 2019). This second microbiota signal in addition to suppression of RA production and eosinophil levels is required to increase IEC turnover, as the absence of eosinophils in Jax PHIL mice does not itself result in faster turnover rates as seen in IH PHIL mice. These findings point to eosinophils as an critical regulator of intestinal homeostasis by fine-tuning microbiota inputs, adding to growing work showing that eosinophils have homeostatic functions beyond allergic and parasitic responses (Ignacio et al., 2022; Shah et al., 2020).

In summary, we identified a mechanism by which microbiota members including *F. rodentium* can promote epithelial proliferation through a RA-eosinophil-IFN- γ -dependent pathway. This role for the microbiota in controlling duodenal IECs illustrates the importance of deeper exploration of microbiota-host interactions in the proximal small intestine, which could open up therapeutic avenues for upper gastrointestinal tract diseases like celiac disease, eosinophilic esophagitis, and environmental enteric dysfunction. Further study of how eosinophils contribute to intestinal epithelial homeostasis could also identify targets for modulating epithelial proliferation and preventing tumor initiation.

STAR METHODS

Resource Availability

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wendy S. Garrett (wgarrett@hsph.harvard.edu).

Materials Availability—Bacterial strains isolated from our facility mice are available upon request.

Data and Code Availability

- Single-cell RNA-seq data and 16S rRNA amplicon sequencing data have been deposited at SRA as BioProject PRJNA763366.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental Model and Subject Details

Mice—Wild-type C57BL/6J mice (called in-house, IH) were bred and housed in microisolator cages in the specific-pathogen-free (SPF) barrier facility at the Harvard T.H.

Chan School of Public Health. These mice are distinct from other mice in our facility that we have previously described in Howitt et al., 2016, in that the IH mice in this paper are maintained free of the protozoan *Tritrichomonas muris*. C57BL/6 WT (called Jax) and *Lgr5-GFP-creERT2* mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. PHIL mice (Lee et al., 2004) were obtained from Dr. Elizabeth Jacobsen at the Mayo Clinic (Scottsdale, Arizona). All genetically modified mouse lines were bred on a C57BL/6J background and were bred as heterozygotes to generate littermate controls. Both male and female mice were used. Mice were used experimentally between 6-12 weeks of age.

Germ-free WT C57BL/6J mice were bred and maintained in semi-rigid gnotobiotic isolators in the Harvard T. H. Chan Gnotobiotic Center for Mechanistic Microbiome Studies.

To rederive genetically modified lines onto a Jax microbiota background, pups were taken on the day of birth or one day after and raised by a dam of the desired microbiota background. These Jax mice were maintained in SPF facility caging but all SPF cages were changed by the investigators to prevent mixing of microbiota. Microbiota status was monitored by qPCR and Jax background mice were regularly rederived onto freshly ordered Jax mice.

Animal studies and experiments were approved and carried out in accordance with Harvard Medical School's Standing Committee on Animals and the National Institutes of Health guidelines for animal use and care.

Bacterial strains and gnotobiotic colonization—*Bifidobacterium pseudolongum* was isolated from BIH mice from our facility by plating stool on selective *Bifidobacterium* iodoacetate medium agar plates (Sasajima et al., 2010). *Lachnospiraceae A2* was isolated from BIH mice by plating stool on brain-heart infusion (BHI) plates supplemented with 1 g/L inulin. 48 colonies of each condition were screened by colony polymerase chain reaction (PCR) using *Bifidobacterium* genus or *Lachnospiraceae A2* primers. *Faecalibaculum rodentium* was obtained from the DSMZ collection (#103405) and was also isolated from our IH mice by plating on Eggerth-Gagnon plates (ATCC medium 2840) and screening by colony PCR with *F. rodentium* primers. All of these strains were cultivated under anaerobic conditions at 37°C.

For gnotobiotic experiments, *B. pseudolongum* was grown in MRS medium (BD) supplemented with 0.5 g/L L-cysteine for 24 hours, *F. rodentium* in DSMZ medium 104 (modified PYG medium) for 24 hours, and *Lachnospiraceae A2* in BHI (BD) supplemented with 5 g/L yeast extract, L-cysteine, and hemin (Sigma) for 48 hours. Cultures were observed to be turbid and concentrated 10x for gavage in GF mice. Monocolonization was confirmed by Sanger sequencing of stool DNA, and germ-free status was confirmed by qPCR with universal bacterial 16S primers.

Method Details

scRNAseq

Cell isolation and sorting: Epithelial cells for sequencing were isolated similarly to as described in Biton et al., 2018. The duodenum was excised, opened longitudinally, rinsed

in PBS, and sliced into fragments about 2-5mm in length. The tissue was incubated in 20 mM EDTA (VWR) in PBS on ice for 45 min with occasional inversion, then shaken vigorously. The tissue was then transferred to subsequent EDTA-PBS solutions for a total of 4 times, for 5 minutes each, and then shaken. Fractions were examined under a light microscope and crypt-enriched fractions were collected and combined. This crypt fraction was passed through a 70 μ M filter, centrifuged, dissociated with pre-warmed TrypLE express (Invitrogen) for 1 min at 37 °C, centrifuged, and resuspended in 1% BSA (Roche), 5 mM HEPES (Corning), 1 mM EDTA. Cells from each condition were stained with a distinct barcoded antibody (Cell-Hashing antibody, TotalSeq-A, Biolegend). Epithelial (live EpCAM⁺CD45⁻Ter119⁻CD31⁻) cells were sorted on a FACSAria (BD).

Sequencing and analysis: Single cell RNA-seq experiments were performed by the Brigham and Women's Hospital Single Cell Genomics Core. ~7,500 cells from each condition were resuspended in 0.4% BSA in PBS at a concentration of 2,000 cells per μ l, pooled together, then loaded onto a single lane (Chromium Next GEM Chip G, 10X Genomics) followed by encapsulation in a lipid droplet (Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1, 10X Genomics) followed by cDNA and library generation according to the manufacturer's protocol.

mRNA libraries were sequenced to an average of 30,000 reads per cell and HTO (Cell Hashing antibodies) libraries sequenced to an average of 5,000 reads per cell on an Illumina Novaseq. scRNA-seq reads were processed with Cell Ranger v3.1, which demultiplexed cells from different samples and quantified transcript counts per putative cell. Quantification was performed using the STAR aligner against the mm10 transcriptome.

Further processing and visualization was performed using the Seurat v3 R package (Stuart et al., 2019). Cells with either less than 200 or more than 6,000 detected genes or >0.12 mitochondrial fraction were excluded from further analysis. Cell expression was normalized followed by selection of highly variable features, data scaling and cell clustering. We identified genes that are differentially expressed (had an adjusted P value lower than 0.05 and fold change >1.5 or <0.5) using the MAST test (Finak et al., 2015) implemented in Seurat v3.

Clusters were manually annotated using gene signatures described in Haber et al., 2017 and Biton et al., 2018. Genes used for cluster identification and annotation are included in Fig. S1A-B. In particular, cluster 3 was defined as immature enterocytes based on high expression of *Ccl25* and *Nfe2l2*. Cluster 9 was identified as goblet and Paneth because the majority of cells expressed goblet cell markers *Agr2* and *Fcgbp*, while a small number highly expressed Paneth cell markers *Lyz1* and *Defa26*. Cluster 10 was identified as stem (Aqp1) because of the expression of *Aqp1*, *Pdgfa*, and *Shhg8*, as well as low *Olfm4* expression. Cluster 12 was identified as tuft because of *Trpm5*, *Dclk1*, and *Il25* expression.

ISC subset analysis was performed using gene signatures from Biton et al., 2018. RA gene signature (*Tgm2*, *Isx*, *Rara*, *Rarb*, *Rarg*, *Rbp1*, *Rbp2*, *Cd38*, *Egr1*, *Cdx1*, *Oat*) was chosen from previously described RA target genes (Balmer and Blomhoff, 2002; Dekaney et al., 2008; Nakshatri and Chambon, 1994). Cell cycle analysis was performed using the Seurat

CellCycleScoring function and built-in gene lists. Enrichment of differentially expressed genes in gene ontology categories was determined using the topGO package (Alexa et al., 2006). P values were calculated using Fisher's exact test.

Intestinal lamina propria and epithelial cell isolation—The small intestine and/or colon were removed and flushed with ice-cold sterile PBS using a 19-gauge feeding needle. The duodenum (defined as the first 7 cm following the stomach), jejunum (the 10 cm following the duodenum), and ileum (the last 10 cm of the small intestine, immediately proximal to the cecum) were excised and Peyer's patches were removed. Intestines were then opened longitudinally and gently agitated at 4°C in PBS, 2% FBS (Gibco), 5mM HEPES (Corning), 1mM DTT (Sigma) for 10 min. The tissue was then transferred into prewarmed PBS, 2% FBS, 5mM HEPES, 5mM EDTA and rotated at 37°C for 15 minutes followed by vigorous shaking to remove epithelial cells. This was repeated and epithelial cells from both fractions were combined and washed with PBS prior to epithelial digestion. The remaining non-epithelial tissue was used for lamina propria cell isolation. The tissue was rinsed twice in PBS, placed into an Eppendorf tube with RPMI, minced with scissors, and digested in RPMI containing 5% FBS, 5mM HEPES, 1% penicillin/streptomycin (Corning), 0.5 units/ml Dispase II (StemCell Technologies), 50 µg/ml DNase (Roche), and 0.25 mg/mL collagenase A (Roche) for 45 minutes at 37°C. For epithelial cell isolation, the epithelial fraction was digested in RPMI containing 5% FBS, 5mM HEPES, 1% penicillin/streptomycin, 0.5 units/ml Dispase II, and 50 µg/ml DNase for 10 minutes at 37°C. Both the epithelial and lamina propria fraction were then passed through 40 µm filters and washed with PBS, 2% FBS, 1mM EDTA.

Flow cytometry—Single cell suspensions were initially Fc blocked with anti-CD16/CD32 (clone 93, Biolegend) and then stained with surface antibodies and viability dye (20 min on ice). For transcription factor staining, after surface staining, cells were fixed for 45 min with eBioscience Transcription Factor Staining Set and then stained intracellularly for 45 min at room temperature. For Ki67⁺ staining of Lgr5-GFP cells, after surface staining, cells were fixed for 20 min with BD Cytotfix, followed by fixation for 45 min with eBioscience Transcription Factor Staining Set and then stained intracellularly for 45 min at room temperature. For cytokine (IFN-γ) staining, 1 million isolated epithelial fraction cells were stimulated with eBioscience Cell Stimulation Cocktail (with protein transport inhibitors) for 4h at 37 ° C in RPMI with 10% fetal bovine serum and penicillin/streptomycin. Cells were then washed, blocked, surface stained, and fixed in BD Cytotfix/Cytoperm for 20 minutes. Intracellular staining was performed for 45 min on ice. Samples were acquired on an LSRII or FACS Symphony (BD). Antibodies used are listed in the Key Resources Table.

Histology and fluorescence microscopy—The duodenum was opened longitudinally and rolled before overnight fixation in 4% paraformaldehyde (Sigma). The tissue was then embedded in paraffin and cut into 5µm thick sections at the Harvard Medical School Rodent Histopathology Core. For immunofluorescence staining, sections were initially deparaffinized and rehydrated. Heat-mediated antigen retrieval was performed in Target Retrieval Solution, Citrate pH 6.1 (Agilent) for 20 minutes. Afterwards, the slides were washed in PBS and blocked in PBS containing 3% BSA (Roche), 3% donkey serum

(Jackson Immunoresearch), 0.1% Triton X-100 (Sigma), 0.1% saponin (Sigma) for 1 hour at room temperature. Primary antibodies were incubated overnight at 4°C and secondary antibodies were applied for 1.5 hours at room temperature. Primary antibodies included: rat anti-BrdU (1:200 dilution, ab6326, Abcam), rat anti-Ki-67 (1:300 dilution, 14-5698-82, ThermoFisher Scientific), mouse anti-E-Cadherin (1:400 dilution, 36/E-Cadherin, BD Biosciences). DNA was labeled with DAPI (0.5 µg/ml). TUNEL staining was performed using the Click-iT™ Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor™ 594 dye (ThermoFisher) per the manufacturer's instructions.

For Lgr5-GFP visualization, the duodenum was opened longitudinally and rolled, fixed overnight in 4% paraformaldehyde, placed in 20% sucrose for 6h, and then placed in 30% sucrose overnight until the tissue sank. The tissue was then embedded in OCT and frozen at -80°C. 8µm frozen sections were cut and labeled with goat anti-GFP (Abcam ab6673) and rat anti-EpCAM (Invitrogen 14-5791-81).

For 16S rRNA gene FISH, intestinal tissue was fixed overnight in methanol-Carnoy's fixative followed by routine paraffin embedding and sectioning. After deparaffinization, fluorescence *in situ* hybridization was performed at 50°C for 90 min in 5% formamide-0.1% SDS-TBS buffer with 2.5 ng/µl of each *F. rodentium*-specific probes 5'-Cy3- GCCAACCAACTAATGCACCG-3' and 5'-Cy3- CCGGGAATACGCTCTGGAAA-3' (Zagato et al., 2020), and 5 ng/µl of a eubacterial 16S RNA sequence specific probe EUB338 (5'-AF488-GCTGCCTCCCGTAGGAGT-3'). Slides were then washed in pre-warmed washing buffer at 50°C and stained with DAPI to visualize nuclei.

Image analysis was performed in Fiji (a version of ImageJ). Epithelial turnover was quantified by dividing the length of the crypt-villus axis labeled by BrdU after 48h of treatment by the total crypt-villus length labeled by DAPI. Ki67⁺ and BrdU⁺ cells after 2h BrdU pulse were counted in the crypts. For both epithelial turnover and Ki67⁺ cell quantification, 15-20 villi/crypts were usually counted per mouse and averaged for a final value.

Eosinophil isolation and sorting—For intestinal eosinophils, the lamina propria was processed as described for flow cytometry and cells were sorted with a two-step MACS process according to the manufacturer's instructions, first by labeling with MHCII-PE antibody (Biolegend) and using Anti-PE Microbeads (Miltenyi Biotech) to deplete MHCII⁺ cells, and then sorting SiglecF⁺ cells with Anti-Siglec-F MicroBeads (mouse) (Miltenyi Biotech). The purity of SiglecF⁺MHCII⁻ eosinophils was usually >95%. For bone marrow eosinophils, the tibia and femur were harvested, cleaned of muscle, one end of the bone cap was cut off, and bone marrow was collected by centrifuging at 12,000 rpm for 2 minutes. Red blood cells were removed with Ammonium Chloride solution (STEMCELL Technologies). SiglecF⁺ cells were sorted from the remaining bone marrow cells Anti-Siglec-F MicroBeads (mouse) (Miltenyi Biotech). The purity of SiglecF⁺ eosinophils was usually >75%.

In vitro eosinophil culture—Sorted bone marrow eosinophils were cultured in RPMI with Glutamax, 10% FBS, penicillin/streptomycin, sodium pyruvate, and 50 µM beta-

mercaptoethanol for 24h. In some conditions, 10 ng/mL IL-5 (Biolegend) and/or 100 nM all-trans retinoic acid (Sigma) was included in the culture. After 24h, cell viability was measured by flow cytometry.

RNA/DNA isolation and RTq-PCR—For RNA isolation from total intestinal tissue, tissue was snap frozen and lysed in Qiazol (Qiagen) for RNA extraction following manufacturer's instructions. For RNA isolation from eosinophils, sorted cells were lysed in Qiazol and processed with the Direct-zol RNA Miniprep Kit (Zymo Research). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Bacterial DNA isolation from stool or intestinal contents was performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen). qPCR was performed using the Kapa SybrFast mastermix. Primers used are listed in Table S2.

BrdU—Mice were injected intraperitoneally (i.p.) with 100 μ l of a 3mg/mL BrdU (Sigma) solution in PBS and simultaneously started on drinking water containing 0.8 mg/mL BrdU and 0.5% glucose. Drinking water was continued for 48 hours to measure epithelial turnover.

For short term BrdU incorporation, mice were injected i.p. with 100 μ l of a 3mg/mL BrdU and sacrificed after 2 hours.

Aldefluor assay—Epithelial and lamina propria cells were isolated as described. The assay was performed using the ALDEFLUOR Kit (StemCell Technologies) according to the manufacturer's instructions, at a concentration of 500,000 cells/mL with 30 min incubation time.

Retinoic acid quantification—RA in intestinal tissue was measured by liquid chromatography mass spectrometry via a method adapted from (Kane and Napoli, 2010) and (Grizotte-Lake et al., 2018). Briefly, RA was extracted from intestine under yellow light to prevent retinoid isomerization and degradation using a two-step liquid-liquid extraction method. Glass tubes and pipettes were used to minimize RA adherence to plastic. 60-120 mg tissue was snap frozen in liquid nitrogen and homogenized with 1000 μ L of 0.9% saline on ice using a dounce homogenizer and 50 μ L of internal standard (RA-d5 at a concentration of 200pg/ μ L) in ethanol was added. To extract retinoids 2 mL of ethanol containing 0.025 M KOH was added, the sample was briefly vortexed and 10 mL hexane was added. After centrifugation at 800 x g for 2 min the hexane layer was removed. To the remaining aqueous layer, 120 μ L of 4 M HCl was added, the sample vortexed, and 10 mL hexane was added to the acidified sample to extract atRA. After centrifugation at 800 x g for 2 min, the top hexane layer containing RA was collected and evaporated to dryness at 25-30°C under a gentle stream of nitrogen. The atRA containing residue was resuspended in 60 μ L DMSO. The resuspended extracts were transferred to amber glass vials with glass inserts.

LC-MS analysis was performed by the Harvard Center for Mass Spectrometry. RA was measured by LC-MS on an Agilent 6460 LCMS triple-quad system. 5 μ l of sample was injected and separated on a Phenomenex Luna C18 (150 x 2mm) column. Mobile phase A consisted of 10mM ammonium acetate and mobile phase B was methanol. A linear gradient was generated over 15 min with a flow rate of 0.3 mLs/min: 0 min, 70% B; 10 min, 95% B;

12 min 95% B; 12.1 min, 70% B; 15 min 70% B. The MS was operated in multiple reaction monitoring mode with negative ionization. The gas temperature was 350°C, gas flow rate was 12 liters/min, nebulizer pressure was 35 psi, sheath gas temperature was 400°C, and sheath gas flow rate was 12 liters/min.

Retinoic acid receptor inhibitor—Mice were injected i.p. with 220 µg of the pan-retinoic acid receptor inverse agonist BMS493 (Tocris) or vehicle control DMSO (Corning) daily for 8 days, in a volume of 25 µl.

Anti-CD3 injury—Mice were injected i.p. with 50 µg anti-CD3ε or Armenian hamster IgG isotype control (Biolegend). On day 3 after injection, the duodenum was excised, opened longitudinally, and prepared for paraffin embedding as described above in the histology section. Hematoxylin and eosin stained slides were evaluated by a blinded pathologist and histological injury was scored on a scale of 0-4 for the parameters of villus to crypt ratio, crypt injury/loss, and monocyte and polymorphonuclear cell infiltration. The summed score was multiplied by a conversion factor of 1-4 based on the percent of intestine length involved by injury/abnormality.

IFN-γ neutralization—Mice were injected i.p. with 200 µg of anti-IFN-γ (BioXCell clone XMG1.2) neutralizing antibody or PBS every other day for 8 days. BrdU treatment started on day 6, 48h before the endpoint.

Transfer of intestinal contents—Stool or cecal contents were collected into 1 mL of sterile PBS. Usually 2 stool pellets (approximately 10-20% of total liquid volume) were used. Contents were homogenized by mashing with a pestle and vortexing, and 100 µl of liquid was orally gavaged into recipient mice.

Antibiotic treatment—IH mice were provided with 0.5g/L vancomycin (Alfa Aesar), 1 g/L metronidazole (Sigma), 1 g/L neomycin (Alfa Aesar), 1 g/L ampicillin (Corning), and 0.2 g/L amphotericin B (Alfa Aesar) in their drinking water *ad libitum* with water changes every 3 days. For experiments with antibiotic pre-treatment of donor mice prior to cecal content transfer, antibiotic treatment was for 2 weeks.

Cohousing experiments—In general, unless otherwise noted, mice were cohoused with littermates at weaning and all mice on a Jax background were maintained in cages separate from in-house mice. For experiments in which Jax and IH mice were cohoused, equal numbers of Jax and IH mice were placed in the same cage for 2 weeks.

16S rRNA sequencing—DNA was isolated from stool pellets by phenol-chloroform extraction after bead beating as previously described (Lobel et al., 2020). The 16S rRNA gene sequencing protocol was adapted from the Earth Microbiome Project (Thompson et al., 2017). The 16S rRNA V4 region was amplified from the extracted DNA by PCR and sequenced by using the 2 150–base pair paired-end reading on a MiSeq instrument (Illumina, San Diego, CA). Analysis of 16S rRNA sequence data was performed using Microbiome Helper scripts and QIIME v1.9 (Caporaso et al., 2010; Comeau et al., 2017). Sequences were clustered into operational taxonomic units (OTUs) at a similarity threshold

of 97% by using the sortmerna-sumac method of open-reference OTU picking. OTUs were subsequently mapped to a subset of the SILVA 132 database containing only sequences from the V4 region of the 16S rRNA gene to determine taxonomies (Quast et al., 2013). To account for variations in sequencing depth, OTU tables were rarefied to the lowest sequence depth among samples. Data were visualized in R using the phyloseq package (McMurdie and Holmes, 2013). Differentially abundant OTUs between groups at the genus level were identified using LefSE (Segata et al., 2011).

Quantification and Statistical Analysis

Data were analyzed with GraphPad Prism (version 9.0). Data are shown as mean with individual data points as noted. For comparison between two independent experimental groups, a two-tailed Mann-Whitney U test was used. For comparison between more than two groups, one-way ANOVA followed by Holm-Sidak's test was performed. For comparison in experiments with two independent variables, two-way ANOVA followed by Holm-Sidak's test was performed. Details of statistical analysis and sample size are provided in the figure legends. No samples were excluded from any experiments performed in this study unless in the case of technical failure. Experimenters were not blinded to experimental conditions. Differences of $P < 0.05$ were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Microbiota promotes epithelial turnover and suppresses retinoic acid production
- Retinoic acid supports intestinal eosinophil survival
- Eosinophils suppress IFN- γ production by intra-epithelial lymphocytes
- IFN- γ increases epithelial turnover and MHCII expression

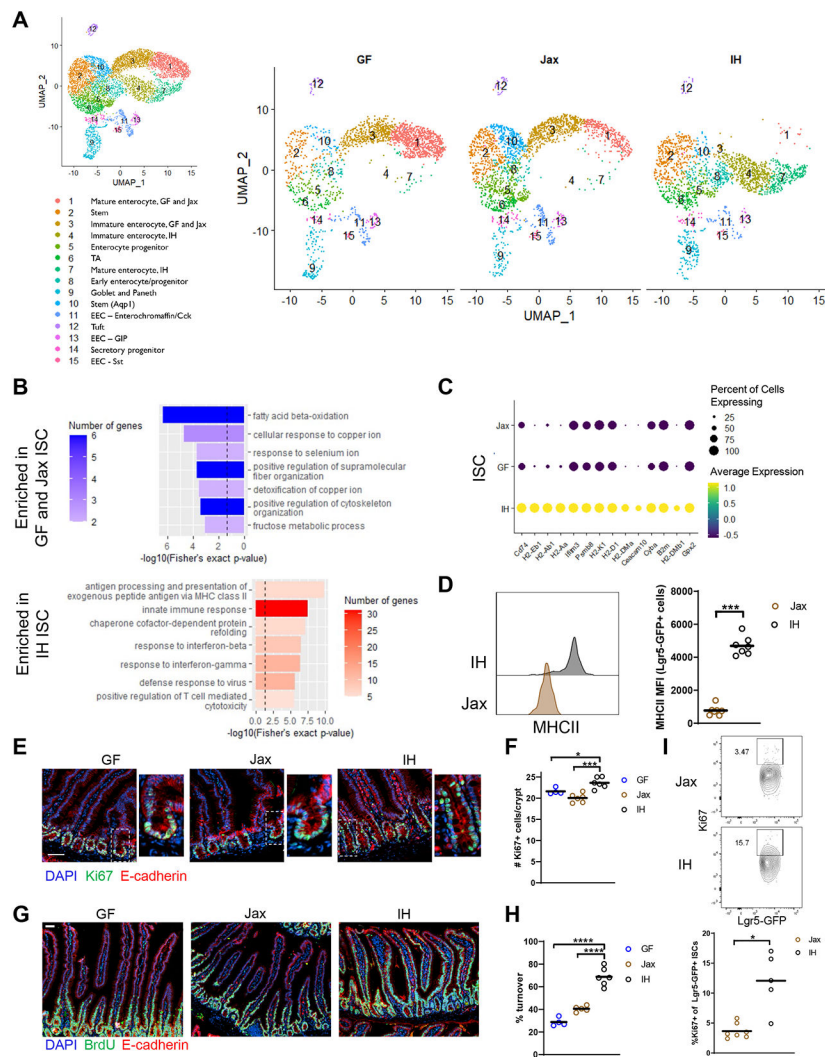


Figure 1. Certain microbiota features regulate intestinal stem cell proliferation and epithelial turnover

(A) UMAP representation of scRNAseq of sorted live EpCAM⁺CD45⁻Ter119⁻CD31⁻ IECs. n=1. TA, transit amplifying; EEC, enteroendocrine. (B) GO categories for DEGs enriched in IH vs GF and Jax ISCs (dashed line indicates p value = 0.05). (C) Top DEGs in IH vs GF and Jax ISCs ranked by significance. (D) MHCII expression in ISCs. (E) Representative images (higher magnification on right) (scale bar 50 μ m) and (F) quantification of Ki67⁺ cells. (G) Representative images (scale bar 50 μ m) and (H) quantification of epithelial turnover after 48h continuous BrdU labeling. (I) Representative plots and quantification of Ki67 expression in Lgr5-GFP ISCs. Each symbol (D, F, H, I) represents data from an individual mouse. Data are pooled from 2-3 experiments (D, F, H, I). Data are shown as mean with individual data points. *p < 0.05, **p < 0.01, ***p < 0.001, Mann-Whitney U test (D, I), one-way ANOVA with Holm-Sidak's post-test (F, H). See also Figures S1, S2, Table S1.

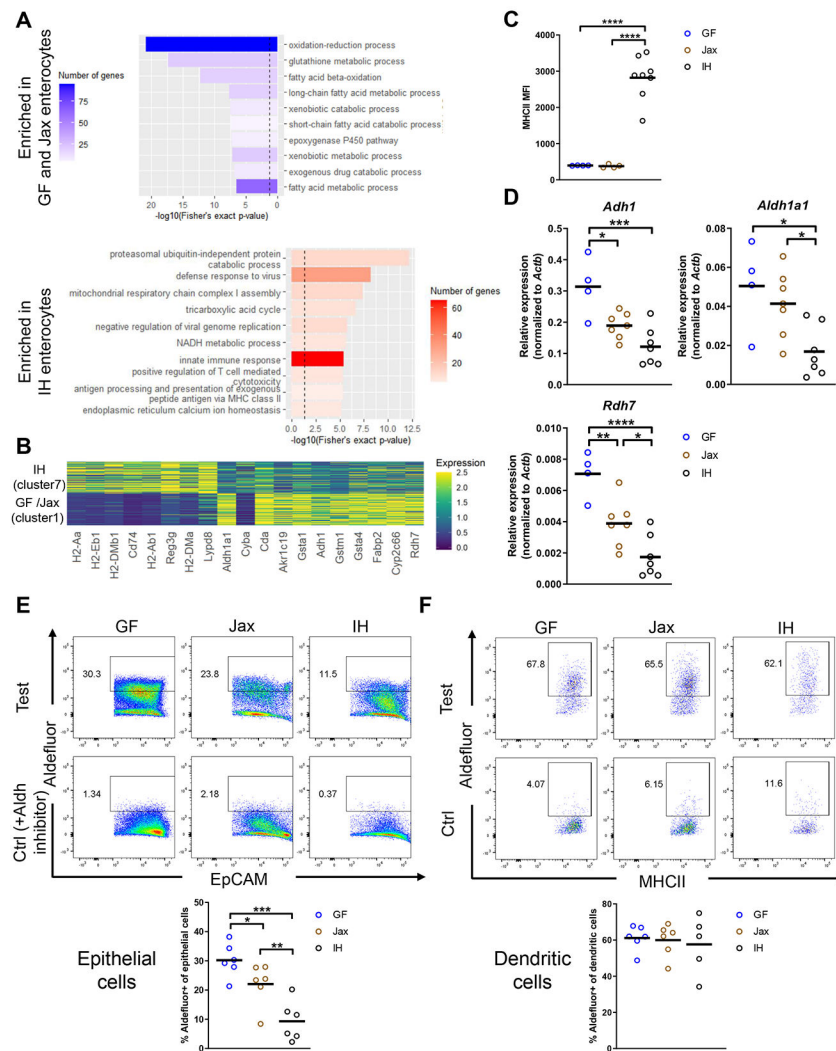


Figure 2. The microbiota modulates enterocyte phenotypes and retinoic acid production (A) GO categories of DEGs in GF/Jax and IH enterocytes (clusters 1 and 7, dashed line indicates p value = 0.05). (B) Top DEGs in GF/Jax and IH enterocytes ranked by significance. (C) MHCII MFI in total IECs. (D) mRNA expression in epithelial fraction. (E-F) Measurement of Aldh enzyme activity via Aldefluor assay in duodenal IECs (EpCAM⁺CD45⁻) or dendritic cells (CD45⁺CD11c⁺MHCII⁺CD64⁻). Control samples included an Aldh inhibitor (DEAB). Gating strategy as in Fig. S2E and Fig. S4A. Each symbol (C-F) represents data from an individual mouse. Data reflect at least 2 independent experiments (C-F). Data are shown as mean with individual data points. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, one-way ANOVA with Holm-Sidak's post-test (C-F). See also Figure S3.

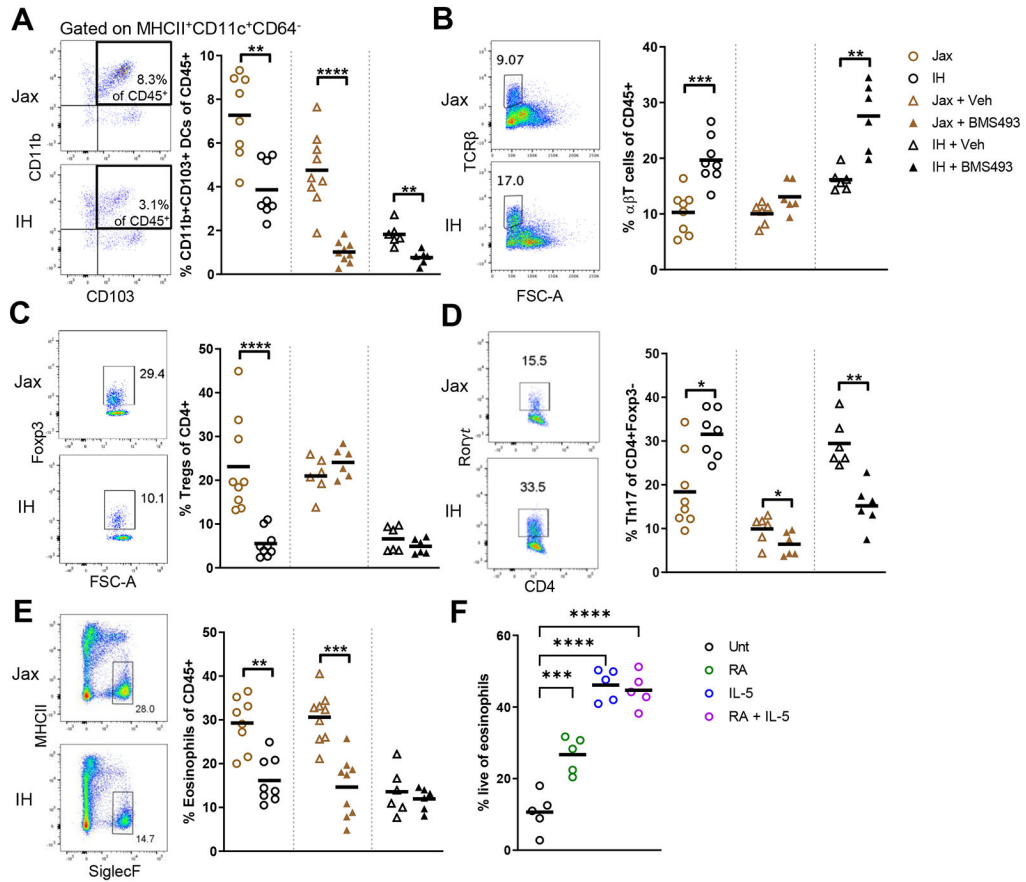


Figure 3. Differential epithelial cell production of retinoic acid in Jax and IH mice regulates intestinal immune populations

(A-E) Flow cytometric profiling of lamina propria immune populations at steady state, or after treatment with 220μg RAR inhibitor BMS493 or vehicle (DMSO) for 8 days. (F) Live eosinophils measured by flow cytometry after 24h culture of sorted bone marrow eosinophils with 10ng/mL IL-5, 100nM RA, or both.

Each symbol represents data from an individual mouse. Data reflect at least 2 independent experiments. Data are shown as mean with individual data points. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Mann-Whitney U test (A-E), one-way ANOVA with Holm-Sidak's post-test (F). See also Figures S4, S5.

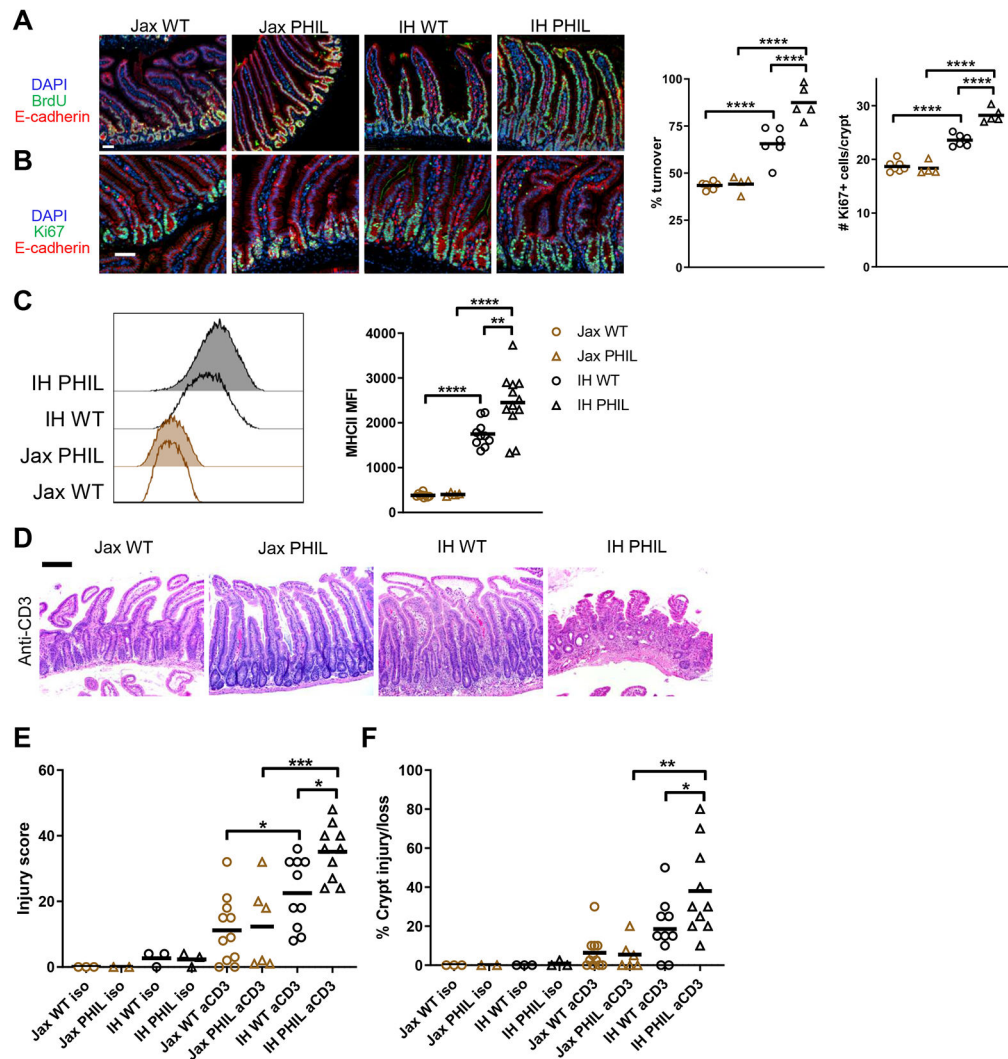


Figure 4. Eosinophils regulate epithelial cell turnover, MHCII expression, and response to injury
Representative images (scale bar 50 μ m) and quantification of (A) Epithelial turnover after 48h continuous BrdU labeling and (B) Ki67⁺ cells. (C) MHCII expression on epithelial cells. (D) Representative hematoxylin and eosin staining (scale bar 200 μ m), (E) Histological injury score, and (F) Percent crypt injury/loss in duodenum on day 3 after injection of anti-CD3.

Each symbol represents data from an individual mouse. Data reflect at least 3 independent experiments. Data are shown as mean with individual data points. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Holm-Sidak's post-test.

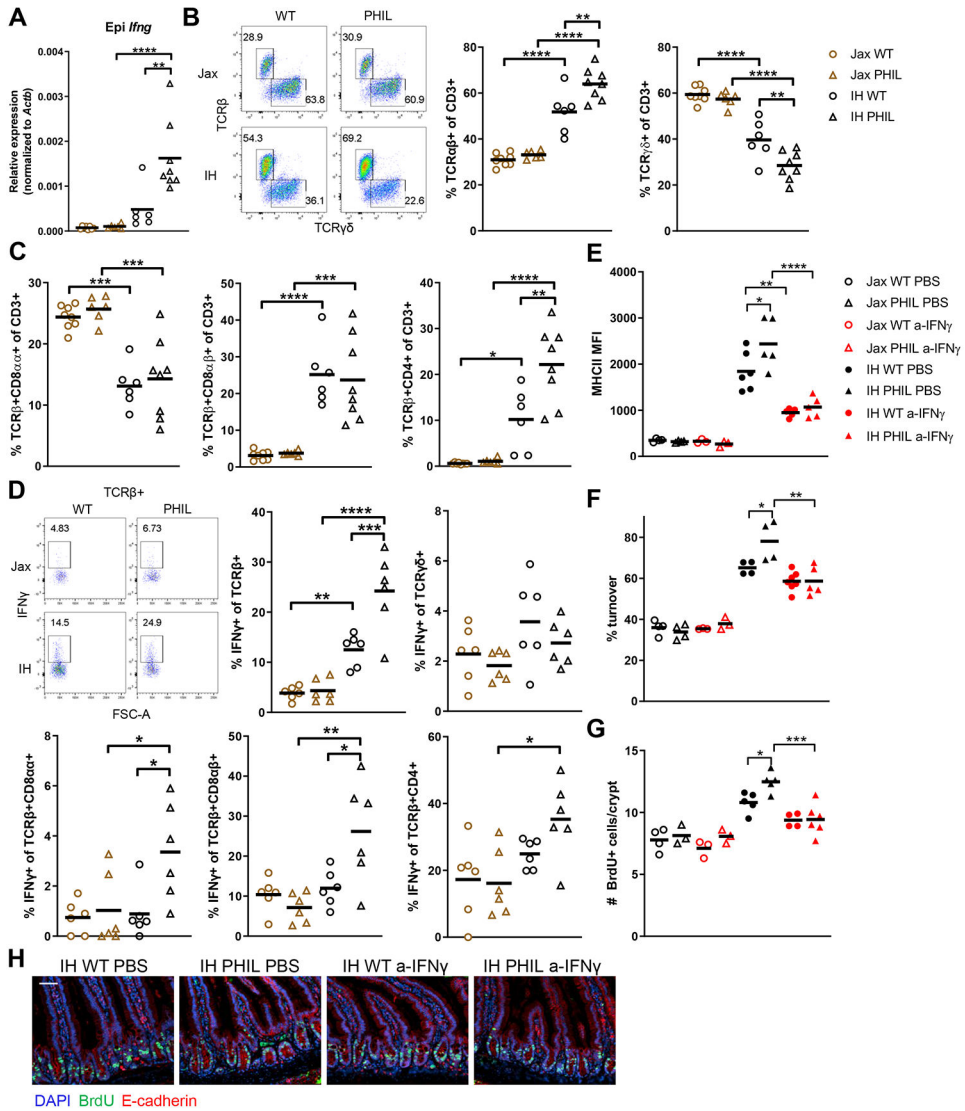


Figure 5. Eosinophils suppress IFN- γ -producing IEL subsets to regulate epithelial cell turnover and MHCII expression in a microbiota-dependent manner

(A) *Ifng* mRNA expression in epithelial fraction. (B-C) Flow cytometric profiling of IEL populations. (D) IFN- γ production by IEL populations. (E) IEC MHCII expression, (F) Epithelial turnover after 48h continuous BrdU labeling, and (G-H) BrdU⁺ cells after 2h short-term labeling in mice treated every other day for 8 days with 200 μ g anti-IFN- γ neutralizing Ab or PBS.

Each symbol represents data from an individual mouse. Data reflect at least 2 independent experiments. Data are shown as mean with individual data points. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Holm-Sidak's post-test. See also Figure S6.

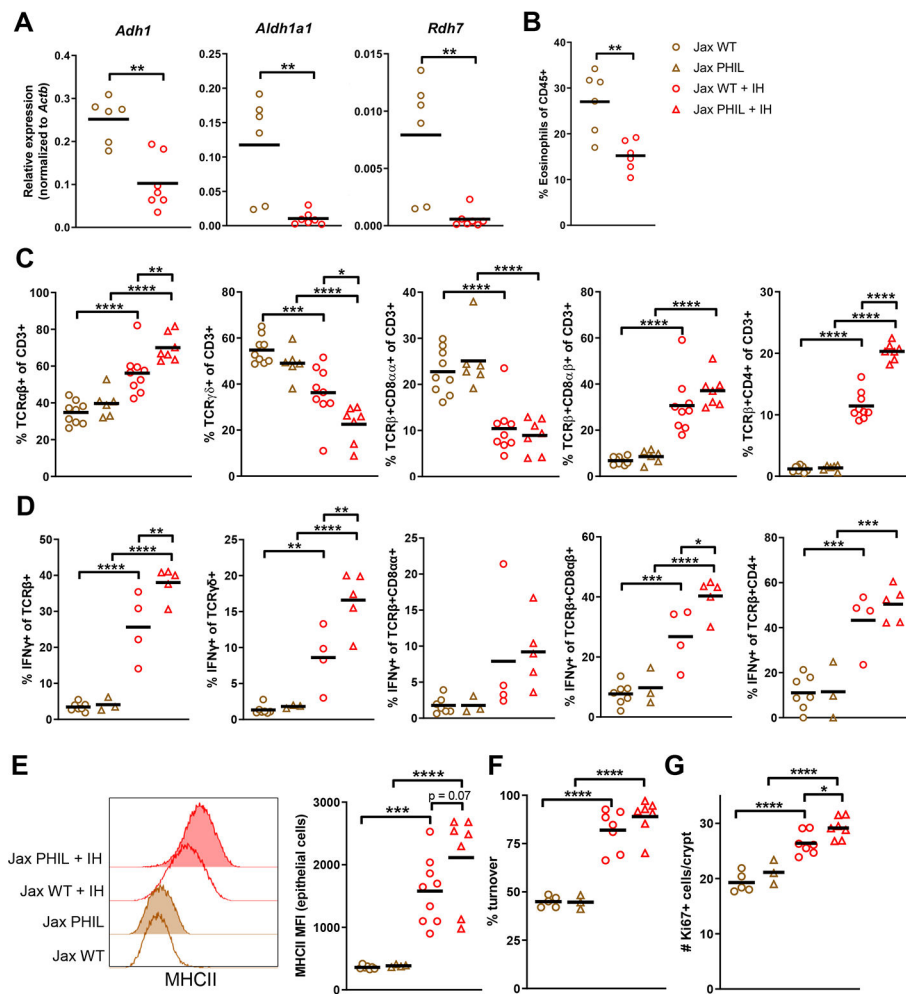


Figure 6. IH microbiota regulates epithelial-eosinophil crosstalk

Jax mice received one transfer of IH microbiota contents via gavage. (A) mRNA expression in epithelial fraction 3 wks post-transfer. (B) Eosinophil levels 2 wks post-transfer. (C) IEL subsets and (D) IEL IFN- γ production 3 wks post-transfer. (E) MHCII expression on IECs 3 wks post-transfer. (F) Epithelial turnover after 48h continuous BrdU labeling and (G) Ki67⁺ cells 4 wks post-transfer.

Each symbol represents data from an individual mouse. Data reflect at least 2 independent experiments. Data are shown as mean with individual data points. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Mann-Whitney U test (A-B), two-way ANOVA with Holm-Sidak's post-test (C-G). See also Figure S6.

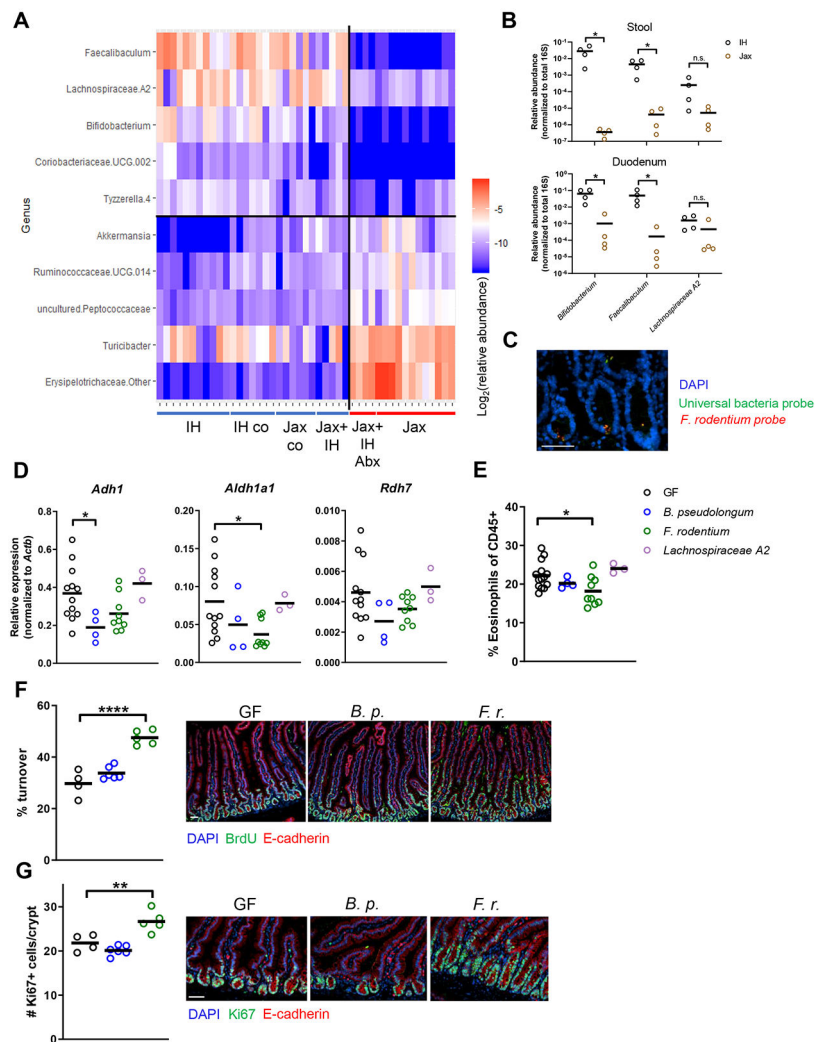


Figure 7. 16S rRNA gene amplicon survey analysis identifies *Faecalibaculum* as a regulator of a retinoic acid-eosinophil-epithelial circuit

(A) Heatmap of differentially abundant genera identified by LefSE analysis of 16S rRNA gene amplicon sequencing of stool from different microbiota conditions. (B) Colonization levels in gut regions determined by qPCR. (C) 16S FISH for *F. rodentium* in IH duodenum. (D-G) GF mice were mono-colonized for 2 wks. (D) mRNA expression in epithelial fraction. (E) Eosinophil levels measured by flow cytometry. (F) Epithelial turnover after 48h continuous BrdU labeling. (G) Ki67⁺ cells. Each symbol (B, D-G) represents data from an individual mouse. Data reflect at least 2 independent experiments. Data are shown as mean with individual data points. *p < 0.05, Mann-Whitney U test (B), one-way ANOVA with Holm-Sidak's post-test (D-G). See also Figure S7.

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD45 APC-Cy7 (30-F11)	BioLegend	Cat#:103116; RRID:AB_312981
Anti-mouse CD45 Pacific Blue (30-F11)	BioLegend	Cat#:103126; RRID:AB_493535
Anti-mouse CD45 APC (30-F11)	BioLegend	Cat#:103112; RRID:AB_312977
Anti-mouse CD45 BUV395 (30-F11)	BD Biosciences	Cat#:564279; RRID:AB_2651134
Anti-mouse CD90.2 (53-2.1) PE/Cy7	BioLegend	Cat#:140310; RRID:AB_10643586
Anti-mouse CD90.2 (30-H12) BUV805	BD Biosciences	Cat#:741909; RRID:AB_2871223
Anti-mouse Lineage markers Pacific Blue (17A2/RB6-8C5/RA3-6B2/Ter-119/M1/70)	BioLegend	Cat#:133310; RRID:AB_11150779
Anti-mouse Lineage markers FITC (145-2c11/RB6-8C5/RA3-6B2/Ter-119/M1/70)	BioLegend	Cat#:133302; RRID:AB_10697030
Anti-mouse CD64 (X54-5/7.1) PE/Cy7	BioLegend	Cat#:139314; RRID:AB_2563904
Anti-mouse SiglecF (E50-2440) AlexaFluor 647	BD Biosciences	Cat#:562680; RRID:AB_2687570
Anti-mouse SiglecF (E50-2440) PE	BD Biosciences	Cat#:552126; RRID:AB_394341
Anti-mouse/human CD11b (M1/70) FITC	BioLegend	Cat#:101206; RRID:AB_312789
Anti-mouse CD11c (N418) Pacific Blue	BioLegend	Cat#:117322; RRID:AB_755988
Anti-mouse CD103 (2E7) PE	eBioscience	Cat#:12-1031-81; RRID:AB_465798
Anti-mouse I-A/I-E (MHCII) (M5/114.15.2) PerCP	BioLegend	Cat#:107624; RRID:AB_2191073
Anti-mouse EpCAM APC (G8.8)	BioLegend	Cat#:118214; RRID:AB_1134102
Anti-mouse EpCAM PE/Cy7 (G8.8)	BioLegend	Cat#:118216; RRID:AB_1236471
Anti-mouse CD3 (17A2) FITC	BioLegend	Cat#:100204; RRID:AB_312661
Anti-mouse CD4 (GK1.5) BUV496	BD Biosciences	Cat#:612952; RRID:AB_2813886
Anti-mouse CD4 (GK1.5) PE/Cy7	BioLegend	Cat#:100422; RRID:AB_312707
Anti-mouse CD4 (RM4-5) AlexaFluor 488	BioLegend	Cat#:100529; RRID:AB_389303
Anti-mouse CD4 (RM4-5) PerCP-Cy5.5	BioLegend	Cat#:100540; RRID:AB_893326
Anti-mouse CD8a (53-6.7) APC	BioLegend	Cat#:100712; RRID:AB_312751
Anti-mouse CD8a (53-6.7) APC/Cy7	BioLegend	Cat#:100714; RRID:AB_312753
Anti-mouse CD8b (YTS156.7.7) PE	BioLegend	Cat#:126607; RRID:AB_961300
Anti-mouse TCR gamma delta (GL3) APC/Fire 750	BioLegend	Cat#:118136; RRID:AB_2650828
Anti-mouse TCR beta (H57-597) Pacific Blue	BioLegend	Cat#:109226; RRID:AB_1027649
Anti-mouse TCR beta (H57-597) PerCP-Cy5.5	BioLegend	Cat#:109228; RRID:AB_1575173
Anti-mouse/rat RORgt (B2D) APC	eBioscience	Cat#:17-6981-82; RRID:AB_2573254
Anti-mouse/rat RORgt (B2D) PE	eBioscience	Cat#:12-6981-82; RRID:AB_10807092
Anti-mouse GATA3 (L50-823) Alexa Fluor 488	BD Biosciences	Cat#:560077; RRID:AB_1645303
Anti-mouse GATA3 (L50-823) BV711	BD Biosciences	Cat#:565449; RRID:AB_2739242
Anti-mouse T-bet (4B10) PE/Cy7	BioLegend	Cat#:644824; RRID:AB_2561761
Anti-mouse/rat/human Foxp3 (150D) PE	BioLegend	Cat#:320008; RRID:AB_492980
Anti-mouse Foxp3 (MF-14) BV421	BioLegend	Cat#:126419; RRID:AB_2565933

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-mouse IFN gamma (XMG1.2) PE/Cy7	BioLegend	Cat#:505826; RRID:AB_2295770
Anti-mouse I-A/I-E (MHCI) (M5/114.15.2) PE	BioLegend	Cat#:107608; RRID:AB_313323
Anti-mouse Ki-67 (SolA15) PE/Cy7	Thermo Fisher Scientific	25-5698-82; RRID:AB_11220070
TotalSeq-A0309 anti-mouse Hashtag 9 Antibody (M1/42; 30-F11)	BioLegend	Cat#:155817; RRID:AB_2750042
TotalSeq-A0310 anti-mouse Hashtag 10 Antibody (M1/42; 30-F11)	BioLegend	Cat#:155819; RRID:AB_2750043
TotalSeq-A0311 anti-mouse Hashtag 11 Antibody (M1/42; 30-F11)	BioLegend	Cat#:155821; RRID:AB_2750136
TotalSeq-A0312 anti-mouse Hashtag 12 Antibody (M1/42; 30-F11)	BioLegend	Cat#:155823; RRID:AB_2750137
InVivoMab anti-mouse IFN gamma (XMG1.2)	Bio X Cell	Cat#:BE0055; RRID:AB_1107694
Mouse monoclonal anti-E-cadherin primary antibody (36/E-Cadherin)	BD Biosciences	Cat#:610181; RRID:AB_397580
Rat monoclonal anti-Ki-67 primary antibody (SolA15)	eBioscience	Cat#:14-5698-82; RRID:AB_10854564
Rat monoclonal anti-BrdU primary antibody (BU1/75 (ICR1))	Abcam	Cat#:ab6326; RRID:AB_305426
Goat polyclonal anti-GFP primary antibody	Abcam	Cat#:ab6673; RRID:AB_305643
Rat monoclonal anti-EpCAM primary antibody (G8.8)	Invitrogen	Cat#:14-5791-81; RRID:AB_953624
Donkey anti-rat IgG AlexaFluor 647 secondary antibody	Invitrogen	Cat#: A78947; RRID:AB_2910635
Donkey anti-mouse IgG AlexaFluor 647 secondary antibody	Abcam	Cat#:ab150111; RRID:AB_2890625
Donkey anti-rat IgG AlexaFluor 594 secondary antibody	Jackson Immunoresearch	Cat#:712-585-153; RRID:AB_2340689
Anti-mouse CD3e (145-2C11)	BioLegend	Cat#:100340; RRID:AB_11149115
Armenian Hamster IgG Isotype Ctrl Antibody	BioLegend	Cat#:400940; RRID:AB_11203529
Bacterial and Virus Strains		
<i>Faecalibaculum rodentium</i>	DSMZ	#103405
<i>Lachnospiraceae A2</i>	This paper	N/A
<i>Bifidobacterium pseudolongum</i>	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
BMS493	Tocris	3509
TrypLE Express	Invitrogen	12-604-013
HEPES	Corning	25-060-CI
Fetal bovine serum	Gibco	10438-026
Sodium pyruvate	Corning	25-000-CI
2-Mercaptoethanol	Sigma	M3148
Dithiothreitol	Sigma	00-5523-00
Penicillin/streptomycin	Corning	30-002-CI
Collagenase D	Roche	11088882001
Collagenase A	Roche	10103586001
DNase I	Roche	10104159001
Dispase	StemCell Technologies	07913
Ammonium Chloride Solution	StemCell Technologies	07850
Cell Stimulation Cocktail (plus protein transport inhibitors)	eBioscience	00-4975-03
Paraformaldehyde	Sigma	441244

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bromodeoxyuridine	Sigma	B5002
QIAzol	Qiagen	79306
Bovine serum albumin fraction V	Roche	03116956001
Normal donkey serum	Jackson ImmunoResearch	017-000-121
Triton-X 100	Sigma	T8787
Saponin	Sigma	S4521
Aqua-Poly/Mount	Polysciences	18606-20
Target Retrieval Solution, pH 6.1	Agilent	S169984-2
RPMI + GlutaMAX	Gibco	61870-036
EDTA	VWR	E522
Phenol – chloroform – isoamyl alcohol mixture	Sigma	77617
UltraPure Buffer-Saturated Phenol	Thermo Fisher Scientific	15513039
DMSO	Corning	25-950-CQC
Glucose	Sigma	G7021
Brain Heart Infusion	BD Biosciences	237500
MRS Broth	BD Biosciences	288130
Hemin	Sigma	H9039
Yeast extract	BD Biosciences	212750
Vancomycin hydrochloride	Alfa Aesar	J62790-06
Metronidazole	Sigma	M1547
Neomycin sulfate	Alfa Aesar	J61499-14
Amphotericin B	Alfa Aesar	J61491-03
Ampicillin sodium salt	Corning	61-238-RM
Retinoic acid	Sigma	R2625
Retinoic acid-d5	Toronto Research Chemicals	R250202
Hexane	Sigma	32293
Hydrochloric acid	Sigma	258148
Recombinant mouse IL-5 (carrier free)	BioLegend	581502
Critical Commercial Assays		
Live/Dead Fixable yellow dead cell stain kit	Invitrogen	L34959
Live/Dead Fixable Near-IR dead cell stain kit	Invitrogen	L10119
ALDEFLUOR Kit	STEMCELL Technologies	01700
BD Cytotfix/Cytoperm Fixation Buffer	BD Bioscience	554714
Foxp3 / Transcription Factor Staining Buffer Set Kit	eBioscience	00-5523-00
iScript Reverse Transcription Supermix	Bio-Rad	1708891
SYBR FAST Universal qPCR Master Mix	KAPA Biosystems	KK4619
DNA-free DNA Removal Kit	Invitrogen	AM1906
Direct-zol RNA Miniprep Kit	Zymo Research	R0251
Anti-PE Microbeads	Miltenyi Biotec	130-048-801

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-Siglec-F Microbeads	Miltenyi Biotec	130-118-513
Click-iT™ Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor™ 594 dye	Thermo Fisher Scientific	C10618
Deposited Data		
scRNA-seq	This paper	SRA BioProject PRJNA763366
16S rRNA amplicon sequencing	This paper	SRA BioProject PRJNA763366
Experimental Models: Organisms/Strains		
Mouse: Lgr5-EGFP-IRES-creERT2	Jackson Laboratory	008875
Mouse: PHIL	The Mayo Clinic, Dr. Elizabeth Jacobsen	N/A
Mouse: C57BL/6J	Jackson Laboratory or bred in own facility	000664, from Jackson room MP15
Oligonucleotides		
See STAR methods for list of quantitative RT-PCR primers	This paper	N/A
Software and Algorithms		
BD FACSDiva v6.2	BD Biosciences	N/A
Prism v9	GraphPad	N/A
STAR aligner version 2.7	(Dobin et al., 2013)	https://github.com/alexdobin/STAR
phyloseq	(McMurdie & Holmes, 2013)	https://github.com/joey711/phyloseq
QIIME v1.9	(Caporaso et al., 2010)	http://qiime.org/
LefSE	(Segata et al., 2011)	https://huttenhower.sph.harvard.edu/lefse/
Seurat v3	(Stuart et al., 2019)	https://satijalab.org/seurat/
topGO	(Alexa et al., 2006)	https://bioconductor.org/packages/release/bioc/html/topGO.html
NSI-Element Basic Research software	Nikon	N/A
ImageJ v1.53c	ImageJ	https://imagej.nih.gov/ij/
FlowJo v10.7.1	TreeStar	N/A