

Interleukin-37 regulates innate immune signaling in human and mouse colonic organoids

Joannie M Allaire¹, Anita Poon¹, Shauna M Crowley¹, Xiao Han¹, Navjit Moore¹, Martin Stahl^{1,2}, Brian Bressler³, Kevan Jacobson¹, Xiaoxia Li⁴, Bruce A Vallance^{1*}.

¹Department of Pediatrics, BC Children's Hospital, University of British Columbia, Vancouver, British Columbia, Canada.

²STEMCELL Technologies, Vancouver, British Columbia, Canada.

³Division of Gastroenterology, Department of Medicine, University of British Columbia, Vancouver, BC, Canada.

⁴Department of Immunology, Cleveland Clinic Foundation, Cleveland, Ohio, USA.

*Corresponding author: Bruce A. Vallance (BAV) bvallance@cw.bc.ca

ABSTRACT

Intestinal epithelial cells (IEC) reside in close proximity to the gut microbiota and are hypo-responsive to bacterial products, likely to prevent maladaptive inflammatory responses. This is in part due to their strong expression of Single Ig IL-1 related receptor (SIGIRR), a negative regulator of interleukin (IL)-1 and toll-like receptor signaling. IL-37, an anti-inflammatory cytokine that inhibits innate signaling in diverse cells by signaling through SIGIRR. Despite the strong expression of SIGIRR by IEC, few studies have examined whether IL-37 can suppress their innate immune signaling. We characterized innate immune responses of human and murine colonoids to bacteria (FliC, LPS) and host (IL-1 β) products and the role of IL-37/SIGIRR in regulating these responses. We demonstrated that human colonoids responded only to FliC, but not to LPS or IL-1 β . While colonoids derived from different donors displayed significant inter-individual variability in the magnitude of their innate responses to FliC stimulation, all colonoids released a variety of chemokines. Interestingly, IL-37 attenuated these responses through inhibition of p38 and NF κ B signaling pathways. We determined that this suppression by IL-37 was SIGIRR dependent, in murine organoids. Along with species-specific differences in IEC innate responses, we show that IL-37 can promote IEC hypo-responsiveness by suppressing inflammatory signaling.

Introduction

Intestinal epithelial cells (IEC) line the luminal surface of the mammalian gastrointestinal (GI) tract, and are uniquely positioned in that they are in constant and direct contact with both the gut microbiota as well as the underlying immune system. In this location, IEC play a central role in coordinating mucosal immunity, including promoting host defense by being responsive to luminal threats such as bacterial pathogens¹. IEC accomplish this by expressing various pattern recognition receptors such as toll-like-receptors (TLR)²⁻⁴. These TLR enable IEC to sense those microbial pathogens that are able to escape the gut lumen and reach them, triggering IEC intrinsic pro-inflammatory and anti-microbial response, as well as signaling the presence of the invading microbes to underlying immune cells⁴⁻⁶. However, due to their frequent exposure to bacterial products released by commensal bacteria in the gut lumen under baseline conditions, IEC are less responsive to bacterial products than professional immune cells. This IEC hypo-responsiveness is likely aimed at preventing maladaptive inflammatory responses against the resident gut microbiota. This dichotomy suggests IEC undergo some form of dynamic innate immune regulation. Recent findings support this paradox by showing that human primary IEC are responsive to host (IL-1 α , TNF α) and bacterial (flagellin) products, while being poorly responsive to other specific bacterial products such as LTA and LPS⁷. Another study showed that even though human IEC express the innate receptor for LPS (TLR4), they lack expression of the adaptor proteins necessary for proper activation of this innate receptor².

Another way that IEC can regulate their own innate immune signaling is through the expression of negative regulators. We and others have shown that IEC strongly express Single Ig IL-1 related receptor (SIGIRR), a negative regulator of interleukin (IL)-1 and TLR signaling^{8,9}. Originally considered an orphan receptor, SIGIRR suppresses innate signaling by sequestering adaptor proteins downstream of MyD88, inhibiting intracellular signaling that would otherwise activate p38 MAP kinase or the transcription factor NF κ B and their target genes^{10,11}. Previous studies using transformed IEC lines have shown that SIGIRR regulates NF κ B-mediated chemokine (ie. IL-8) responses to IL-1 β as well as TLR ligands (flagellin)⁹. Correspondingly, *Sigirr* deficient (-/-) mice have proven highly susceptible to intestinal inflammation and enteric infections, primarily due to the loss of *Sigirr* expression in their gut epithelium, leading to exaggerated inflammatory and antimicrobial responses^{12,13}. These studies have highlighted the

importance of SIGIRR in controlling mucosal inflammation in the gut, yet its role as an orphan receptor has limited its potential to function as a therapeutic target.

This concept has changed since the discovery of IL-37, a newly discovered anti-inflammatory cytokine belonging to the IL-1 family¹⁴. In a variety of human diseases, elevated IL-37 levels have been linked with increased inflammation levels¹⁵⁻¹⁷. Specifically, IL-37 appears to play a key role in suppressing innate immunity, directly inhibiting inflammatory signaling in various cell types¹⁸⁻²⁰. Interestingly, IL-37 is a human-specific cytokine and has not been discovered in mice. When human IL-37 is transgenically expressed in mice, it exhibits a wide range of anti-inflammatory features, acting through several mechanisms depending on the cellular and microenvironmental context^{14,21,22}. For example, IL-37 can act intracellularly binding to SMAD3, translocating to the nucleus and directly suppressing the transcription of various cytokine and chemokine genes^{22,23}. In humans, IL-37 can also be produced and secreted extracellularly by immune cells²⁴. Once in the extracellular environment, IL-37 binds to cells using a heterodimeric receptor comprised of both SIGIRR and the IL-18 receptor to inhibit innate immune signaling via specific inhibition of the NF κ B and p38 MAPK pathways^{21,25}.

Despite IEC expressing higher levels of SIGIRR than other cells in the gut, thus far, no study has examined the ability of IL-37 and SIGIRR to regulate innate signaling within primary-derived IECs (ie. enteroids or colonoids). In this study, we evaluated the responsiveness of primary colonic IEC (colonoids), derived from healthy donors, to the pro-inflammatory cytokine IL-1 β and to the microbial products flagellin (FliC) and LPS. Only the bacterial product FliC induced a strong inflammatory response in human colonoids, whereas these cells showed a very limited response to IL-1 β as compared to transformed cell lines (Caco-2) or mouse colonoids. Although we observed significant inter-individual variability upon FliC stimulation, all colonoids responded by releasing several chemokines (IL-8, CCL2 and CCL20). In contrast, mouse colonoids were found to be responsive to both FliC and IL-1 β but only secreted a limited number of chemokines (Cxcl1 and Ccl20). For the first time, we show that the anti-inflammatory cytokine IL-37 attenuates the innate immune responses of human and mouse colonoids and this attenuation, at least in murine cells was Sigirr dependent. Taken together, our results show

exogenous IL-37 can suppress innate immune responses of primary IEC and that important inter-species differences in IEC responses to specific inflammatory stimuli occur.

Results

Pediatric and adult colonoids transcribe similar levels of innate immune genes

IL-37 expression and function have been intensively studied in immune cells such macrophages, neutrophils, and T cells, but little attention has been paid to epithelial cells. To better understand the innate immune response of primary intestinal epithelial cells (IEC) and whether it is impacted by IL-37, human-derived organoids were grown from the sigmoidal biopsies of pediatric or adult healthy donors according to well described methods^{26,27}. These colonoids were exposed to the cytokine IL-1 β or bacterial products (LPS or flagellin (FliC)) for 4 hours and then assessed for their immune response by qPCR, ELISA and Western Blot analysis. After being cultured for 10 to 12 days, both adult and pediatric colonoids showed similar macroscopic and histological features, including budding and IEC thickening (Supp 1A). Since IL-37 typically acts under inflammatory conditions, expression of various innate receptors was evaluated in human colonoids to determine their capacity to respond to pro-inflammatory stimuli. Recently published studies have demonstrated site specific innate receptor expression as well as human specific innate responses by IEC to host or microbial products^{2,3,28}. By real-time qPCR analysis, both pediatric and adult colonoids were confirmed to transcribe mRNA for several innate immune receptors including *TLR4*, *TLR5*, *IL-1R* and *IL-18R1* (Supp 1B). Gene transcription of *SIGIRR* and the novel anti-inflammatory cytokine, *IL-37* were also confirmed by qPCR analysis, showing similar levels between pediatric and adult samples (Supp 1C). Human colonoids showed high expression of *SIGIRR* mRNA (Ct values around 25-27) but very low levels of *IL-37* (Ct values around 31-33) (Supp 1D). IL37 protein secretion was not detected in IEC under all stimulation conditions (Supp 1E).

Human colonoids are highly responsive to *Salmonella* flagellin

To analyze the inflammatory response and functionality of naïve, primary IEC, mature 3D colonoids in Matrigel were treated with *Salmonella* flagellin (FliC 100 ng/ml) for 4 hours. Colonoids were also treated with IL-1 β (20 ng/ml) or the bacterial product LPS (1 μ g/ml). FliC stimulation of colonoids elicited strong upregulated transcription of various cytokine and chemokine genes at the mRNA level, with *CCL20* induced 120-fold, and *IL-8* and *TNF α* induced 40-fold compared to untreated conditions (Fig 1A). In contrast, IL-1 β stimulation induced only a 4 to 6-fold increase in mRNA levels of these cytokines (Fig 1A). To determine if these changes

led to protein secretion, we collected supernatant of stimulated 3D colonoids in Matrigel, to look at basolateral secretion, and performed ELISA. Consistent with mRNA, FliC, among the 3 stimuli used, was the strongest inducer of IEC innate responses with a strong CCL20 and IL-8 secretion, whereas IL-1 β induced little or no secretion of these chemokines (Fig 1B). The low or lack of innate response of human colonoids to IL-1 β stimulation is not caused by the ineffectiveness of IL-1 β , as IL-1 β , even at a concentration of 5 times less, was able to induce higher levels of IL-8 and CCL20 secretion by Caco2 cells (Supp 1F). LPS also failed to induce the secretion of IL-8 and CCL20 by human colonoids, similar to a recent finding³ (Fig 1B).

One striking observation from the FliC stimulation experiments was the significant variability among the 6 colonoid lines (each derived from a different patient) in the magnitude of their innate immune response (Fig 1B). CCL20 secretion by the different colonoid lines ranged from 200 to 1200 pg/ml after 4h of FliC stimulation despite ensuring equal colonoid density, size and protein quantity. To explore the expression/secretion of other cytokines and chemokines the colonoid innate immune response to FliC and how this differed among individuals, we used a Milliplex Luminex assay detecting 9 cytokines and chemokines (IL-6, IL-10, IL-18, IL-8, CCL2, CCL3, CCL4, CCL5 TNF α) (Fig 1C). Notably, FliC-stimulated colonoids produced CCL2 (MCP-1) at high levels similar to the levels detected for IL-8, followed by CCL5 and TNF α at more modest levels (Fig 1D). ELISA also confirmed the high levels of IL-8 secreted (Fig 1B). Furthermore, it is important to mention that under these specific conditions, IL-10, IL-6, IL-18, CCL3 and CCL4 secretion were not detected (Fig 1C).

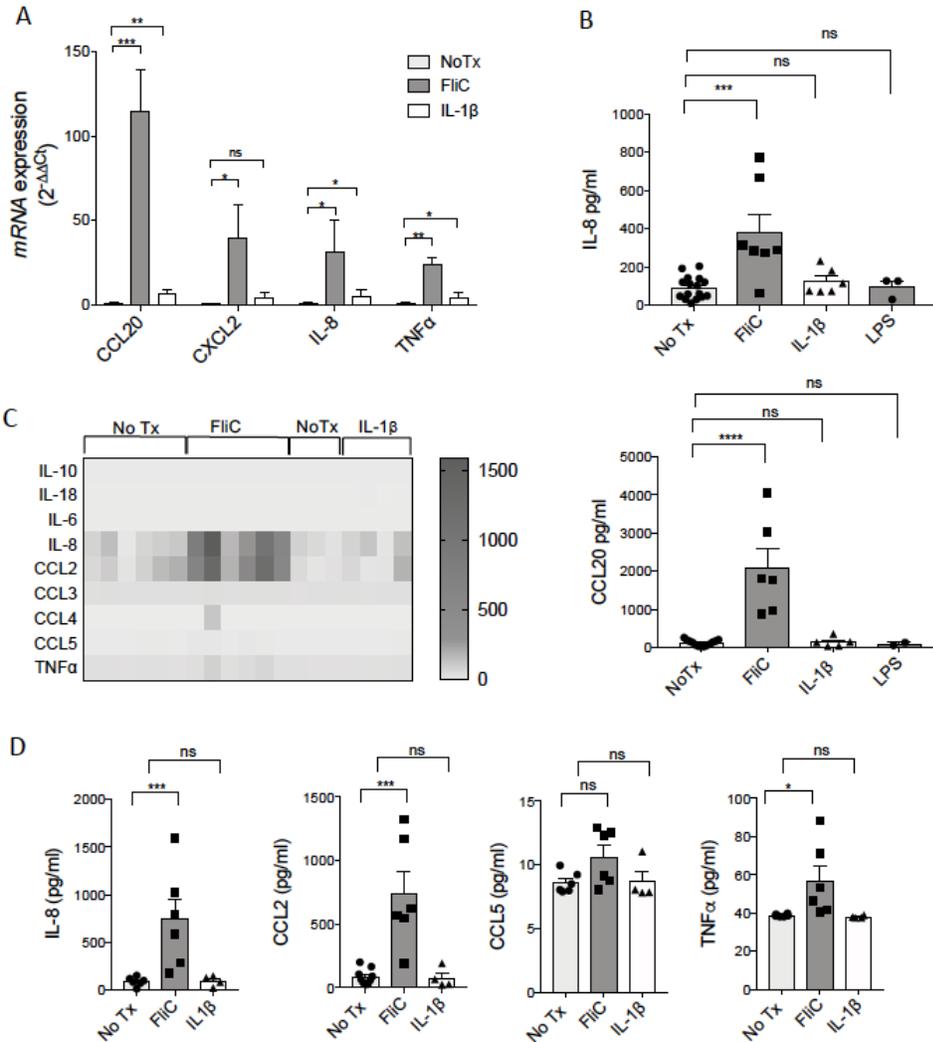


Figure 1: Human IEC responses to host (IL-1β) and bacterial stimuli (FliC, LPS)

A) qPCR analysis of inflammatory gene transcription after FliC or IL-1β stimulation for 4h, expressed as fold change over untreated colonoids. B) IL-8 and CCL20 protein levels secreted basolaterally by human colonoids after 4h of stimulation with FliC or IL-1β. C) Heat map representation of chemokine concentrations in the supernatants of colonoids after 4h of stimulation with FliC or IL-1β. D) Concentration of chemokines detected by Multiplex Luminex Assay in the supernatants of colonoids after 4h of stimulation with FliC or IL-1β. Mean and SEM are indicated. All data shown were repeated in 2 independent experiments, n= 6 donors (4 adult and 2 pediatric). *, P = 0.01 to 0.05; **, P = 0.001 to 0.01; ***, P = 0.0001 to 0.001. ns= not significant.

IL-37 suppresses innate immune responses of human colonoids to different stimuli

We next determined if IL-37 would inhibit IEC immune responses. A dose response curve for IL-37 stimulation revealed that, much like peripheral blood mononuclear cells (PBMC)²¹, picogram levels of IL-37 (1000 pg/ml to 100 pg/ml) were sufficient to suppress FliC-induced inflammatory responses in colonoids (Supp 2A). Therefore, 100 pg/ml of IL-37 was selected to evaluate its effect on innate immune responses of FliC stimulated colonoids. The mRNA levels of various cytokines and chemokines such as *CCL20*, *IL-8*, *CXCL2* and *TNF α* were significantly reduced in the presence of IL-37 (Fig. 2A). These mRNA changes were found to largely correlate with protein levels as demonstrated through ELISA (Fig 2B) or Milliplex Luminex analysis (Fig 2C). However, the majority of colonoids lines (4 of 6) showed a significant reduction of CCL20 and IL-8 secretion, while the colonoids from 2 individuals were unresponsive to IL-37 treatment (Supp 2B-2C). To further investigate the effects of IL-37, we pursued our analysis only on the IEC/colonoids derived from responsive donors. Among these responsive colonoids, a significant reduction was observed at the protein levels for IL-8 secretion (34 % reduction) and CCL20 at 25% (Fig. 2B) as well as significant decrease in CCL2 and TNF α levels in response to IL-37 treatment (Fig 2C).

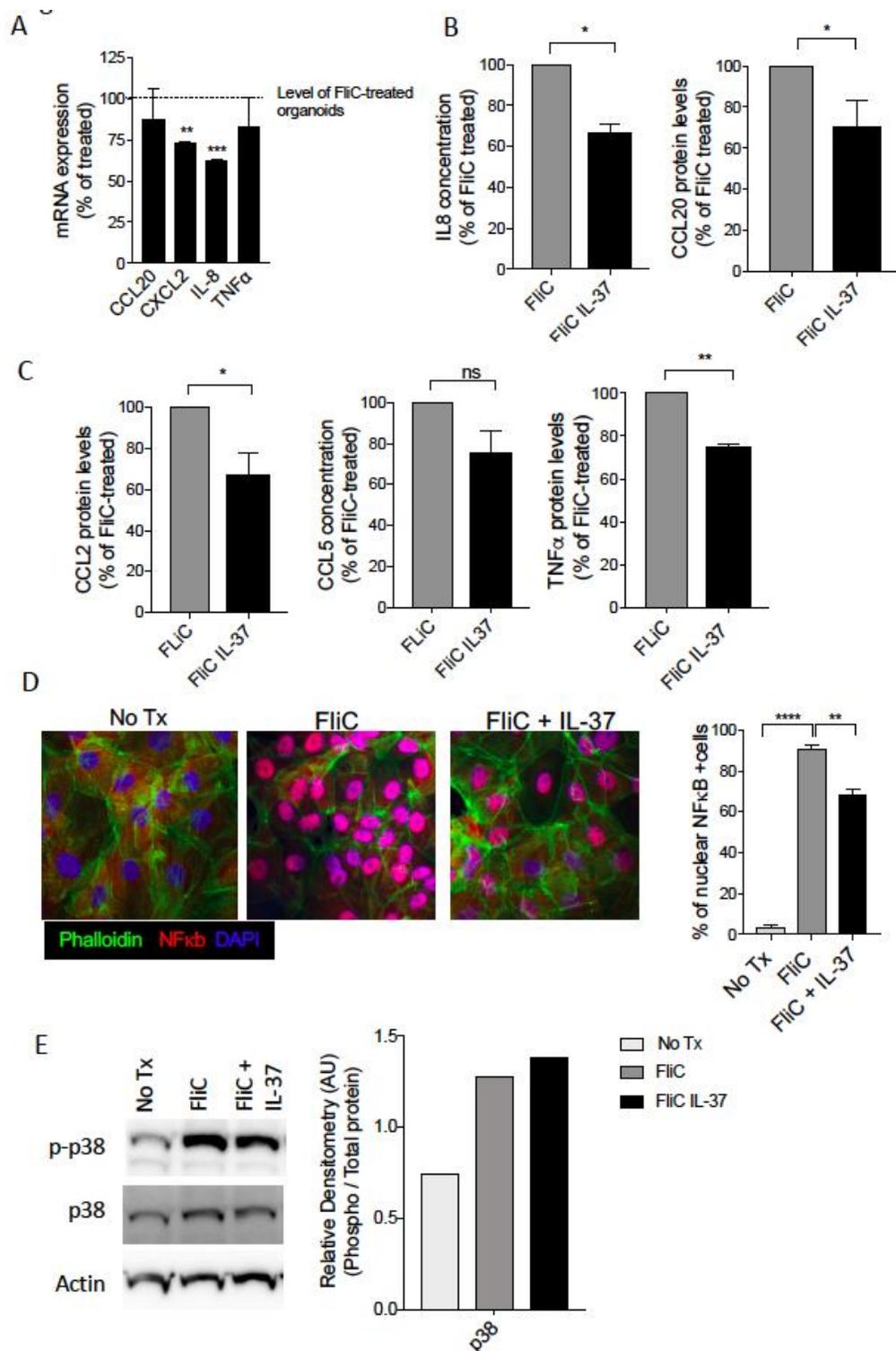


Figure 2: IL-37 effect on human IEC FliC responses. A) qPCR analysis of inflammatory genes after FliC and IL-37 exposure for 4h expressed as percentage of change over FliC treated colonoids. B) IL-8 and CCL20 protein levels (detected by ELISA) secreted basolaterally by human colonoids after 4h of stimulation with FliC and IL-37. C) CCL2, CCL5 and TNF α protein levels secreted basolaterally by colonoids after 4h of stimulation with FliC and IL-37 (detected

by Milliplex Luminex assay). D) Immunostaining against NFκB (red), actin (Phalloidin - green) and DAPI (blue) of 2D monolayer after 30 minutes of stimulation with FliC with or without IL-37 (left). Counts of NFκB positive nuclei from immunostaining (right). E) Western blot analysis of phospho and total p38, after 30 minutes of stimulation with FliC with or without IL-37. Equal loading confirmed with Actin as well as total protein stain of membrane (see Fig. S4). Mean and SEM are indicated. All data shown were repeated in 2 independent experiments, n= 4 donors (3 adult and 1 pediatric). *, P = 0.01 to 0.05; **, P =0.001 to 0.01; ***, P= 0.0001 to 0.001; ****, P= 0.00001 to 0.0001; ns= not significant.

IL-37 dramatically suppresses FliC-induced activation of NFκB in human colonoids

In immune cells, the downstream immunosuppressive effects of IL-37 via SIGIRR are hypothesized to occur through intracellular inhibition of the NFκB or p38 MAPK signaling cascades, leading to reduced inflammatory signals including attenuated cytokine and chemokine secretion^{21,25}. To assess whether the effect of IL37 observed in the IEC/colonoids was due to its suppression on p38 or NFκB activation, we took advantage of a 2D monolayer system derived from human colonoids to assess NFκB activation through immunostaining. This involved the dissociation of 3D colonoids and plating single cells onto an ECM-coated coverslip to form a monolayer. After 5 days of culture, when confluency was well established, monolayers were treated with FliC (100ng/ml) for 30 min to provide sufficient time for NFκB translocation to the nucleus, indicative of its activation (Fig. 2D). As expected, exposure to FliC led to the majority of NFκB migrating to the nuclei of the IEC. When the monolayers were also exposed to IL-37, significant reductions in both the number of cells with nuclear NFκB signal, and the intensity of the nuclear staining were observed (Fig. 2D). Surprisingly, western blot analysis showed that the activation and phosphorylation of p38 was low after FliC stimulation and IL-37 did not reduced its activation level (Fig 2E).

IL-37 moderately suppresses FliC-induced p38 activation in human colonoids

SB202190 is an important media component necessary for the growth and proliferation of organoids, however it is also a potent inhibitor of p38 MAPK²⁷. Even though the colonoids were treated in a serum-growth factor free medium (base medium; Advanced DMEM/F12 supplemented with GlutaMAX and HEPES), they were grown in the presence of SB202190 until their stimulation, which could impact their ability to respond to IL-37 and FliC. To address this, we grew colonoids in an organoid media lacking SB202190. There were alterations in the appearance and growth rate of the colonoids in the absence of SB202190 (Fig 3A-B). The

colonoids were more cystic and less differentiated in media lacking SB202190 as compared to regular organoid media (Fig 3A). Despite these findings, the absence of SB202190 did not significantly alter the secretion of chemokines by colonoids in response to FliC treatment (Fig 3C). We further assessed the effect of IL-37 on FliC stimulated colonoids grown without SB202190. Here we observed that SB202190 suppressed the full biological activity of IL-37 since its removal decreased CCL20 secretion to 46% in the presence of IL-37 (Fig 3D) compared to the 25% reduction displayed with colonoids grown in regular organoid media (with SB202190) (fig 2B). Surprisingly, removal of SB202190 did not further decrease IL-37 mediated suppression of IL-8 release (Fig 2B and Fig 3D). We thus confirm that IL-37 inhibits both p38 and NFkB activation and downstream signalling pathways, by showing a reduction in the phosphorylation of NFkB and p38 with no modulation in total protein levels (Fig 3E).

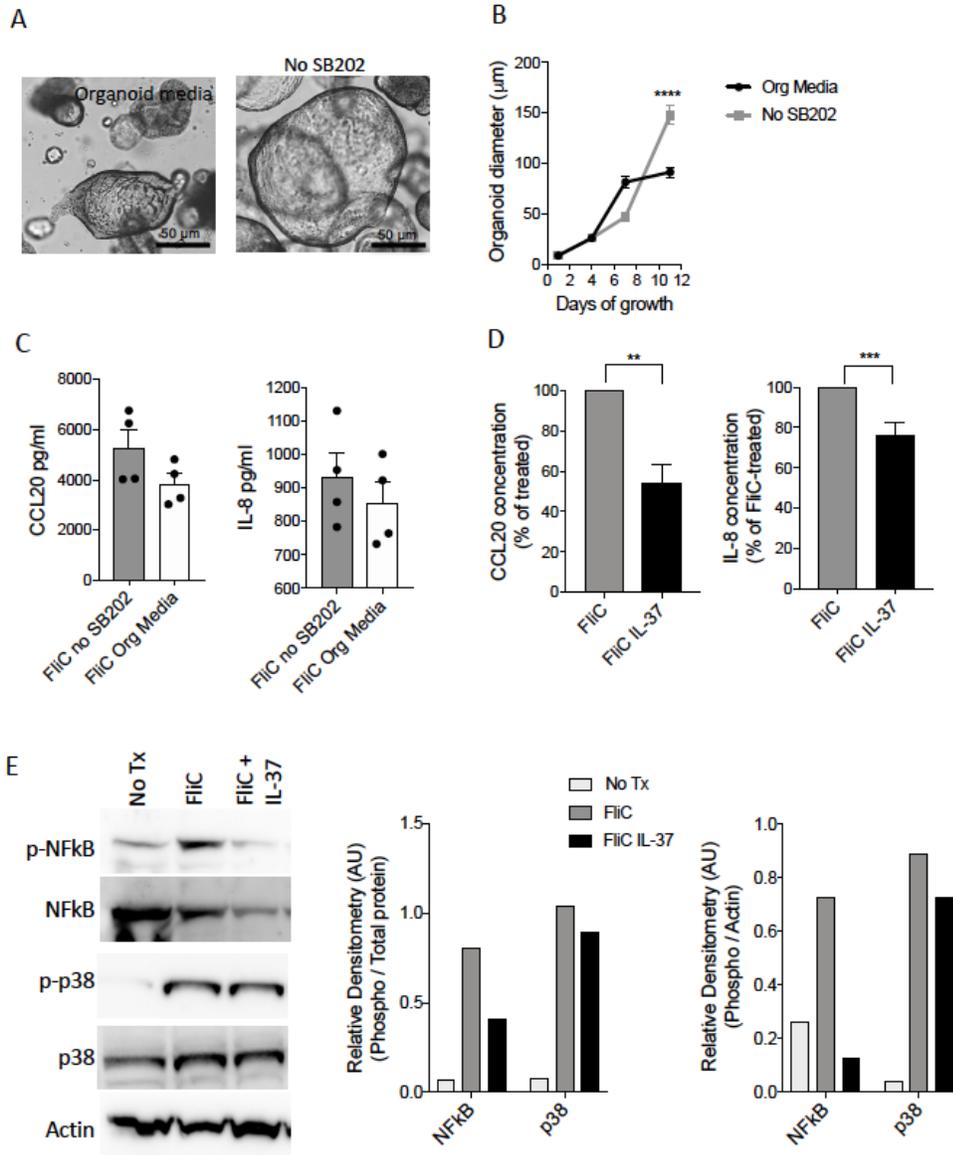


Figure 3: SB202190 effect on IL37 immunosuppressive responses A) Brightfield images of a representative human colonoids grown in standard organoid Media (left) or in No SB202190 media (right). B) Growth curve of colonoids grown in standard organoid media or in No SB202190 media for 14 days. C) IL-8 and CCL20 protein levels secreted basolaterally after 4h of stimulation with FliC of human colonoids grown in standard organoids media or in No SB202190 media. D) IL-8 and CCL20 protein levels secreted basolaterally by human colonoids grown in No SB202190 media after 4h of stimulation simultaneously with FliC and IL-37. E) Western blot analysis of phospho and total NFkB, phospho and total p38 in human colonoids grown in No SB202 media after 30 minutes of stimulation with FliC with or without IL-37. Equal loading confirmed with Actin as well as total protein stain of membrane (see Fig. S5). Mean and SEM are indicated. All data shown are repeated in 2 independent experiments, n= 4 (2 adult and 2 pediatric) donors. *, P = 0.01 to 0.05; **, P = 0.001 to 0.01; ***, P = 0.0001 to 0.001; ****, P = 0.00001 to 0.0001. Comparison lacking annotations are not significant.

IL-37 suppresses chemokine production by murine colonoids

Currently, a murine homologue of IL-37 has not been identified, however previous studies have shown that human IL-37 can efficiently act on murine monocytes and lymphocytes to suppress innate immune signaling¹. We examined whether IL-37 could also suppress immune signaling within murine colonoids, and whether such actions would depend on Sigirr expression. Colonic colonoids were generated from *Sigirr* deficient (*Sigirr*^{-/-}) mice, and their littermate controls (*Sigirr*^{+/+}), and were grown in 3D culture as previously described^{26,29}. We first determined the capacity of murine colonoids to respond to pro-inflammatory stimuli by evaluating the expression of various innate receptors. As shown in Fig 4A, both *Sigirr*^{+/+} (WT) and *Sigirr*^{-/-} (KO) colonoids expressed higher levels of *Tlr4*, *Tlr5*, *Il1r* and *Il18r1* (Fig. 4A). Moreover, WT colonoids showed *Sigirr* gene expression whereas its total absence was observed in KO colonoids (Fig 4A).

The murine colonoids were then stimulated with either *Salmonella* flagellin (FliC) (100 ng/ml), or murine Il-1 β (10 ng/ml) to match the inflammatory stimuli used on human colonoids. Key murine inflammatory genes were then assessed including the chemokines *Ccl20*, *Cxcl2*, *Cxcl1* (murine homologue of IL-8) and the cytokine *Tnfa*. All genes showing significantly increased transcription upon stimulation, with Il-1 β inducing the greatest increases (30 to 100-fold change) (Fig 4B). ELISA analysis also showed significant amounts of Cxcl1 and Ccl20 secreted after FliC and Il-1 β stimulation (Fig 4C). To further characterize the innate response of these murine colonoids, we used a Milliplex Luminex assay covering 9 cytokines and chemokines (Il-6, Il-10, Cxcl1, Cxcl2, Ccl2, Ccl3, Ccl4, Ccl5, Tnfa). Surprisingly, the only secreted molecule detected on our panels was Cxcl1, whereas the other molecules (Il-6, Il-10, Cxcl2, Ccl2, Ccl3, Ccl4, Ccl5, Tnfa) were not significantly induced (Fig 4D). These levels of Cxcl1 were similar to those detected by ELISA, confirming that this chemokine is strongly induced after FliC stimulation and was induced even more strongly following Il-1 β stimulation (Fig 4C).

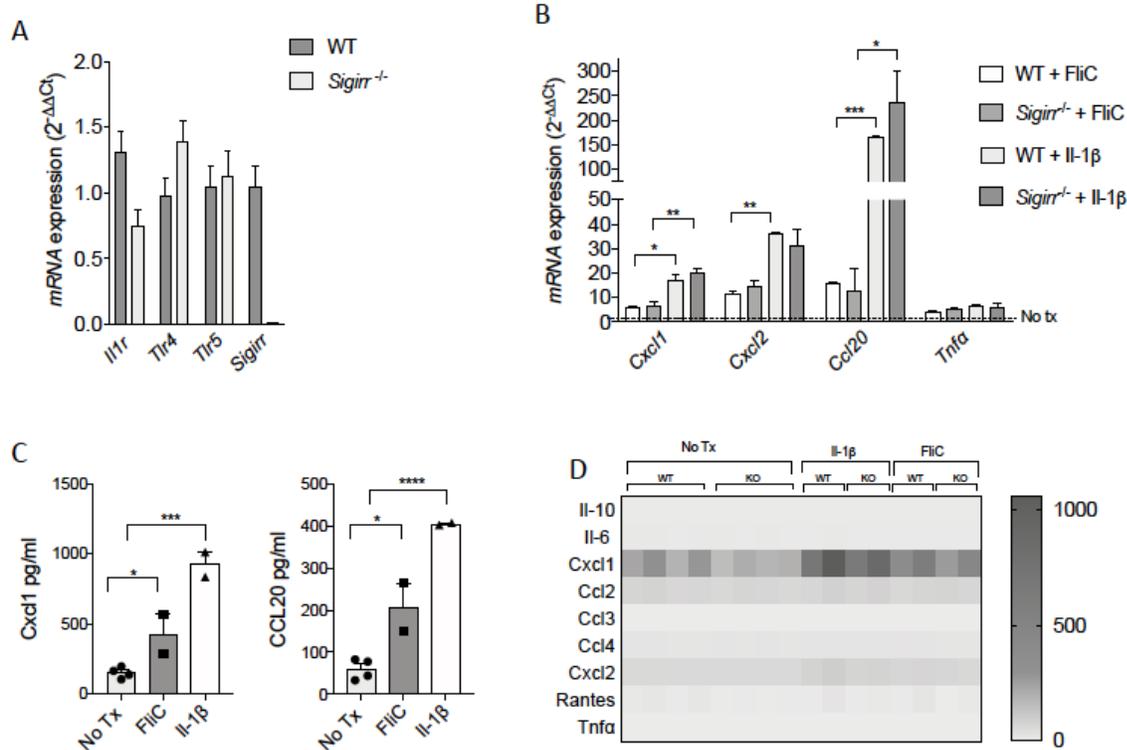


Figure 4: Mouse IEC responses to host (IL-1 β) and bacteria stimuli (FliC)

A) qPCR analysis of innate receptor and negative regulator gene transcription by mouse colonoids expressed as fold change over WT (*Sigirr*^{+/+}) colonoid expression. B) qPCR analysis of chemokines and cytokines after 4h of stimulation with FliC and IL-1 β . C) Cxcl1 (left) and Ccl20 (right) protein levels secreted basolaterally by colonoids derived from WT and *Sigirr*^{-/-} mice after 4h of stimulation with IL-1 β and IL-37. D) Heat map representation of various chemokine concentration in the supernatant of colonoids after 4h of stimulation with FliC or IL-1 β . Mean and SEM are indicated. All data shown repeated in 2 independent experiments, n= 2 mice of each genotype for each experiment. *, P = 0.01 to 0.05; **, P = 0.001 to 0.01; ***, P = 0.0001 to 0.001; ****, P = 0.00001 to 0.0001. Comparison lacking annotations are not significant.

Sigirr mediates the effect of IL-37 on murine colonoids

We next sought to clarify whether IL-37 would suppress this innate response of murine colonoids, as well as determine whether its actions depended on the expression of Sigirr. Upon addition of IL-37 at the same time as FliC, and measured after 4h, IL-37 was found to successfully reduce inflammatory responses in *Sigirr*^{+/+} colonoids. IL-37 significantly reduced mRNA levels of *Cxcl1* (reduced 25%), *Cxcl2* (reduced 25%) and *Ccl20* (33%) as compared to colonoids not receiving IL-37, however this suppression was not observed with *Sigirr*^{-/-} colonoids (Fig 5A). To clarify whether these IL-37 effects were seen at the protein level, we

analyzed chemokine/cytokine secretion by ELISA. The same trends were observed at the protein level, with *Cxcl1* and *Ccl20* showing the greatest reductions in levels when IL-37 was added to stimulated *Sigirr*^{+/+} colonoids and this suppression was not observed with *Sigirr*^{-/-} colonoids (Fig 5B).

Similar effects were observed when Il-1 β stimulation was combined with IL-37 treatment. IL-37 reduced mRNA levels of *Cxcl1* (reduced 33%), *Ccl20* (reduced 43%) and to a lesser degree *Tnfa*, in *Sigirr*^{+/+} colonoids. This suppression was not observed in *Sigirr*^{-/-} colonoids suggesting that the suppressive effects of exogenous IL-37 on murine IEC is *Sigirr* dependent (Fig. 5C). These IL-37 effects were also observed at the protein level, with *Cxcl1* and *Ccl20* secretion significantly reduced when IL-37 was added to stimulated *Sigirr*^{+/+} colonoids (Fig. 5D). This suppression of innate signaling was not observed in *Sigirr*^{-/-} colonoids following treatment with IL-37 (Fig. 5D). These data suggest that the ability of IL-37 to suppress inflammatory signaling within murine IEC is dependent on the expression of *Sigirr*.

Due to our earlier results that demonstrate IL37 inhibits *Nfkb* activation in human derived colonoids and *Nfkb* is downstream of *Sigirr* signaling pathways³⁰⁻³², we explored if IL37 exerts its inhibitory effect on *Nfkb* through its use of *Sigirr*. We again utilized our 2D colonoid-derived monolayer system and assessed *Nfkb* nuclear translocation and activation through immunostaining. Monolayers derived from *Sigirr*^{+/+} and *Sigirr*^{-/-} colonoids were treated with inflammatory stimuli (Il-1 β (10ng/ml) or *FliC* (100ng/ml)) for 30 min to induce *Nfkb* translocation to the nucleus (Fig. 5E for Il-1 β) (Supp 3 for *FliC*). When the monolayers of either genotype were stimulated with Il-1 β , the majority of cells showed nuclear co-localisation of *Nfkb* (red) and DAPI (blue) (Fig. 5E). When the stimulated monolayers were treated with IL-37, there was a significant reduction in the number of positive nuclear *Nfkb* cells in the *Sigirr*^{+/+} colonoid monolayers that was not observed in the *Sigirr*^{-/-} monolayers (Fig. 5E). A similar reduction in nuclear *Nfkb* staining was observed with *Sigirr*^{+/+} colonoids, but not with *Sigirr*^{-/-} colonoids, when both were treated with *FliC* plus IL-37 (Supp 3). These results demonstrate that extracellular IL-37 acts on IEC through a *Sigirr* dependent pathway to inhibit *FliC* or Il-1 β induced *Nfkb* activation.

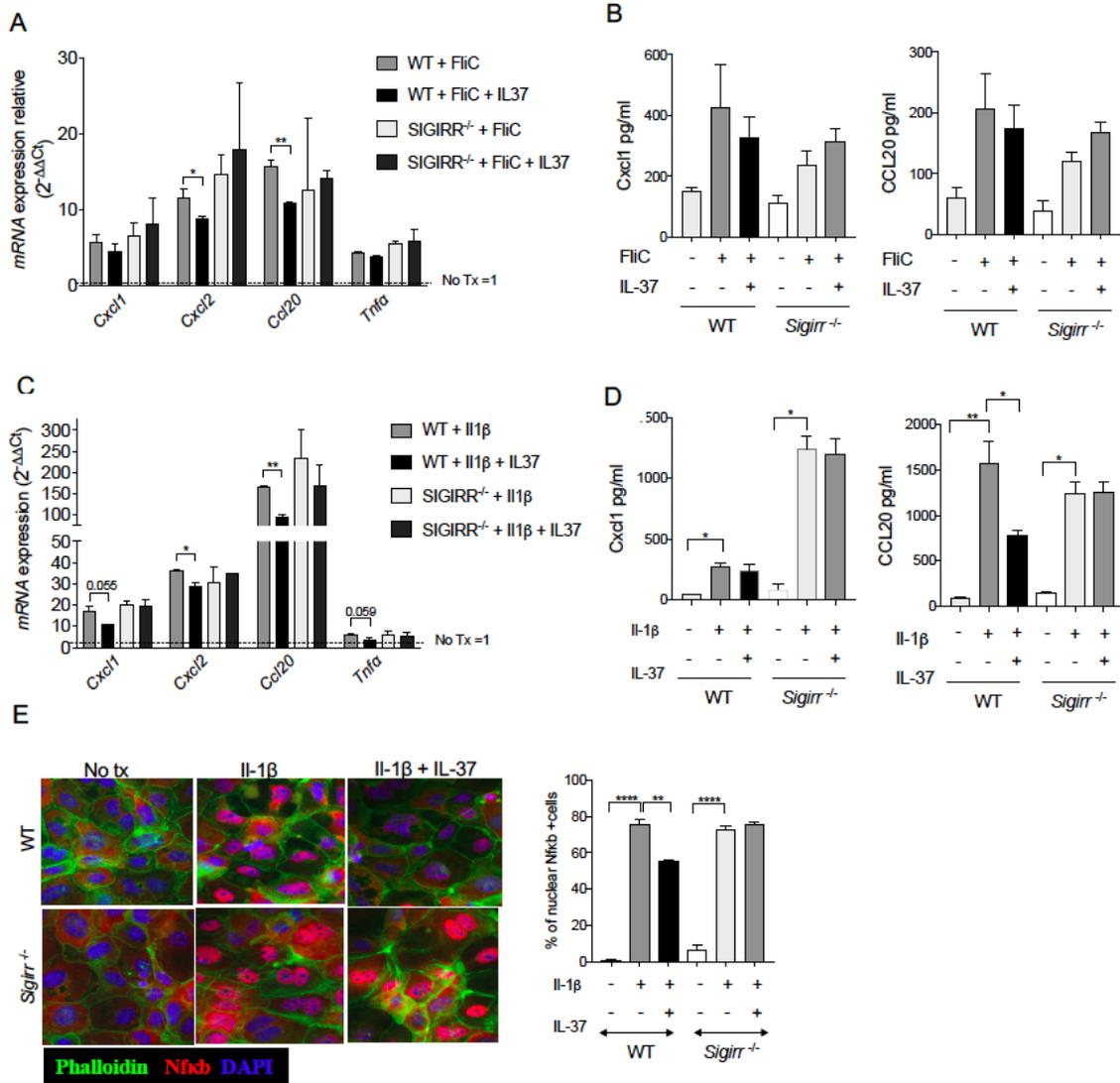


Figure 5: IL-37's suppression of innate responses in mouse organoids is Sigirr dependent.
 A) qPCR analysis of main inflammatory genes after FliC stimulation with IL-37 for 4h on *Sigirr*^{+/+} (WT) and *Sigirr*^{-/-} mouse colonoids expressed as fold change over untreated colonoids.
 B) Cxcl1 (left) and Ccl20 (right) protein levels secreted basolaterally by WT and *Sigirr*^{-/-} mouse colonoids after 4h of stimulation with FliC and IL-37. D) Immunostaining against Nfkb (red), Actin Phalloidin (green) and DAPI (blue) of 2D monolayer after 30 minutes of stimulation with Il-1b with or without IL-37 (left). Counts of Nfkb positive nuclei from immunostaining (Right). Mean and SEM are indicated. All data shown repeated in 2 independent experiments, n= 2 mice of each genotype for each experiment. *, P = 0.01 to 0.05; **, P = 0.001 to 0.01; ***, P = 0.0001 to 0.001; ****, P = 0.00001 to 0.0001. Comparison lacking annotations are not significant.

Discussion

IL-37 is a novel anti-inflammatory cytokine that plays an important role in regulating innate immunity. The majority of studies on IL-37 have focused on its ability to regulate the function of immune cells in various tissues and diseases, whereas its potential effects on the gut epithelium have been largely overlooked^{19,20}. By using human and murine colonoids and colonoid-derived monolayers, we show for the first time that extracellular IL-37 can effectively suppress IEC inflammatory responses in a manner dependent on Sigirr (in murine colonoids). Specifically, IL-37 reduced NF κ B and p38 activation and chemokine secretion typically seen after stimulation of colonoids with bacterial FliC, as well as the cytokine Il-1 β . This is the first study showing that IL-37 can suppress immune signaling in human and murine colonoids and that the effect is Sigirr dependent in mice.

Through the use of human colonoids, we investigated their innate immune responses and the effects of IL-37 on primary tissue-derived IEC, that unlike cell lines, have not undergone transformation or cell culture adaptation. Organoids provide several advantages over traditional cell culture methods, since they recapitulate the physiological and biological properties of the tissue of origin. Our results highlight the inter-individual variability that can occur when studying primary tissues and organoids derived from patients. Each colonoid line likely behaves in ways influenced by the specific genetic, epigenetic and environmental factors that influenced the original donor. Our results also surprisingly reveal that human colonoids are unresponsive to IL-1 β stimulation despite expressing *IL1R*, as compared to the transformed Caco-2 cell line. In contrast, murine derived colonoids were found to respond very actively to the Il-1 β stimulus, emphasizing previous findings that species differences can greatly affect IEC behavior and their innate immune responses²⁰.

Interestingly, our results also revealed that even picomolar concentrations (1000pg/ml to 100pg/ml) of IL-37 are sufficient to significantly inhibit murine and human IEC innate immune responses with micromolar concentration being less effective. The finding that higher doses of IL-37 can be less effective has been previously described in human macrophages, yet the mechanisms have yet to be clearly defined²¹. Our results also support previous findings that IL-37 can suppress the secretion of several chemokines such as CCL2, CCL5 and CXCL8 by the

transformed T84 IEC line³³. While this earlier study explored the ability of T84 cells to express IL-37, our results suggest that non-transformed colonic IEC do not produce significant levels of IL-37. Our experimental design instead used recombinant IL-37, enabling our assessment of extracellular IL-37 actions in its secreted form. This approach also gives us the advantage of discriminating the extracellular effect of IL-37 on IEC over the effect it could potentially have when produced intracellularly, as demonstrated with the use of CRISPR/Cas9 to delete IL37 in T84 cells.

In cells that produce IL-37, IL-37 can act intracellularly by binding to SMAD3 and then translocate to the nucleus to repress transcription of specific target genes^{14,22,34}. IL-37 can also be secreted into the extracellular milieu where it will bind to specific heteroreceptors (IL-18 and SIGIRR) to inhibit innate immune signaling within its target cells^{21,25}. By using recombinant IL-37, we focused our efforts on defining the role of extracellular IL-37 on IECs. While we cannot rule out the possibility that some effects seen in human colonoids could reflect the actions of intrinsic IL-37 production, we found that human colonoids in fact produce very low levels of IL-37, as assessed by both qPCR and ELISA. Nevertheless, it is possible that *in vivo*, and particularly during disease states, IEC may produce greater levels of IL-37, since some studies have shown strong immunohistochemical staining for IL-37 in the inflamed gut epithelium of IBD patients²². In our system, we detected low levels of IL-37 mRNA without seeing any increase in protein expression (Supp 1). This suggests that colonic IEC from healthy individuals might be missing key elements (transcription factor, cytokine stimulation, differentiation state) required to produce functional levels of IL-37. Further investigations should focus on IL-37 expression by IECs and under which conditions, and by which IEC subtypes it may be expressed.

IL-37 does not appear to be expressed by mice or its human orthologue has not been discovered yet³⁵. However previous studies have shown that human IL-37 can act on murine cells in a similar fashion to its actions on human cells. By using murine colonoids, we confirmed that human recombinant IL-37 is effective on murine IECs, providing a new system to study IL-37 and its signaling pathways and downstream targets. In this study, colonoids derived from *Sigirr*^{+/+} and *Sigirr*^{-/-} mice were used to prove that extracellular IL-37 requires Sigirr expression to suppress inflammatory signaling. This data suggests that IL-37 acts on mouse IEC in a similar

manner to what has been previously published with immune cells²¹. SIGIRR's ability to regulate gut inflammation and innate immune responses by IEC have been previously described in different *in vitro* and *in vivo* models^{4,7,23}. Here, our data support the concept that Sigirr, along with the IL-18R, functions as a hetero-receptor that mediates the regulator actions of IL-37. While the exact mechanisms remain to be determined, it is tempting to speculate that during clinical forms of intestinal inflammation, IL-37 could be secreted in the environment to act on SIGIRR expressing IEC to repress innate immune signaling, limiting the development of excessive inflammation. These speculations certainly require further clarification. However, our study is the first study to support the hypothesis that IL37 can act on primary IEC and further studies are needed to better understand how IL-37 and SIGIRR interact to balance inflammatory responses in the gut.

In summary, our study has demonstrated important differences in the innate immune response of human and murine primary IEC. While human IEC are highly reactive to FliC stimulation, mouse IEC produce stronger innate immune responses following Il-1 β stimulation than they do when exposed to FliC. Moreover, these species differences are reflected in the panel of chemokines induced, with human IEC secreting a greater array of immune molecules (CCL2, CCL5, CCL20, IL8). By taking advantage of the colonoid system, we show for the first time, the anti-inflammatory role of IL-37 on primary human IEC by reducing NF κ B and p38 signalling while we corroborated that its extracellular role depends on Sigirr expression in mouse IEC. Taken together, these data show that both human and mouse colonoids respond (albeit differently) to bacterial products, and their responses can be suppressed by IL-37, through its ability to signal through SIGIRR.

Methods:

Ethics statement

De-identified intestinal biopsies from paediatric and adult patients were collected using experimental methods and protocols approved by the Clinical Research Ethics Boards of the University of British Columbia, the British Columbia Children's and Women's Research Review Committee, and from the Providence Health and Clinical Research Ethics Board (H14-0391, H15-01977, H20-02483). Written informed consent was obtained from all participants (or legal guardians) prior to inclusion in this study. All following investigations and methods were performed in accordance with relevant guidelines and regulations of the Tri-council Policy Statement: Ethical Conduct for Research Involving Humans (Government of Canada) and followed the principles of the Declaration of Helsinki.

All mouse experiments were performed according to protocols approved by the University of British Columbia's Animal Care Committee and in direct accordance with the Canadian Council on Animal Care (CCAC). Authors complied to ARRIVE guidelines for reporting animal research.

Mouse strain

Sigirr^{-/-} mice have been described previously¹⁰. *Sigirr*^{-/-} and *Sigirr*^{+/+} mice were bred under specific pathogen-free conditions at the BC Children's Hospital Research Institute, with male mice (4 mice total from each genotype) being used for organoids isolation at 8–12 weeks of age.

Generation of mouse colonoids and maintenance.

Murine colonoids were isolated as previously described^{28,29,36}. In brief, mouse colonic crypts were isolated and washed before being diluted 1:1 in Matrigel (Corning). After the Matrigel solidified, organoid media (base media (Advanced DMEM/F12, supplemented with Pen Strep GlutaMAX and HEPES) with 50% WRN supplemented with N2 (Invitrogen), B27 (Invitrogen), N-acetylcystine (Sigma-Aldrich), nicotinamide (Sigma), mEGF (Invitrogen), A83-01 (Tocris), SB 202190 (Sigma-Aldrich), Y-27632 (Abmole)) was added to the well and incubated at 37°C with 5% CO₂. Media was changed every three days and the colonoids were passaged every five to seven days.

Generation of human colonic colonoids from biopsies and maintenance.

Intestinal crypts were isolated from de-identified pediatric or adult sigmoid colon biopsies and grown as colonoid cultures using well described methods^{27,29,37}. The solution containing the colonic crypts was centrifuged and washed twice with base media then diluted 1:1 in Matrigel (Corning). This was pipetted into multiple domes in a 24-well plate and incubated at 37°C with 5% CO₂. After the Matrigel solidified, organoid media (same as mouse organoids) was added to the well and incubated at 37°C with 5% CO₂. Media was changed every three days and the colonoids were passaged every seven to ten days.

Stimulation of human and mice colonoids

For experiments, colonoids were passaged using TrypLE techniques as already described but with modifications^{3,28,36}. First, Matrigel domes were disrupted by adding ice-cold PBS containing 5 mM EDTA and incubating on ice for 15 min. Colonoids were washed twice with base media, resuspended in TrypLE express (Gibco) and incubated at 37°C with 5 % CO₂ for 2 x 5 min. The colonoids were then rapidly disrupted into single cell suspensions with repeated pipetting through a p1000 tip, and an equal volume of organoid media was added. Cells were centrifuged, then resuspended in base media. The cells were counted using an automated cell counter and the appropriate number of cells (15,000 cells/well for human; 10,000 cells/well for mouse) were seeded using Matrigel as described in the colonoid isolation section in a 24-well plate. Colonoids were stimulated (days 5-7 after seeding for mice, day 10-14 for human) with base media with FliC (100 ng/ml; InvivoGen) or human IL-1 β (10ng/mL; Sigma) or murine IL-1 β (10ng/mL; Peprotech) or LPS O55:B5 (1000 ng/ml; InvivoGen) with or without recombinant IL-37 (100 to 0,1 ng/ml; R&D) or corresponding volume of base media for 4h.

Colonoid-derived monolayer seeding and stimulation.

Monolayers derived from colonoids were generated as previously described with modifications^{28,29,36}. First, the growth media was removed, then Matrigel domes were disrupted and colonoids were resuspended in TrypLE express (Gibco) and incubated at 37°C with 5 % CO₂ for 2 x 5 min. The colonoids were then rapidly disrupted into single cell suspensions with gentle pipetting through a p1000 tip, and an equal volume of monolayer media (base media

supplemented with 50% WRN, N2 (Invitrogen), B27 (Invitrogen), mEGF (Invitrogen) and Y-27632 (Abmole) was added. Cells were centrifuged, then resuspended in monolayer media and added dropwise to Geltrex (Gibco) coated coverslips in 24-well plates. Monolayers were incubated at 37°C with 5% CO₂ and media changed 24h after seeding. 72h after seeding, confluent monolayers were stimulated with monolayer media supplemented with FliC (100 ng/ml; InvivoGen) or *mIL-1β* (10 ng/mL; Invitrogen) with or without recombinant IL-37 (100 ng/ml; R&D) or a corresponding volume of base media for 30 minutes.

Monolayer immunostaining

For colonoid monolayer immunostaining, after 30 min of stimulation, coverslips were fixed in 4% PFA for 15 minutes at room temperature. Then the cells were rinsed in PBS 1X twice, and treated with PBS, 0.1% Triton X-100 and 0.05% Tween 20 for 15 mins, then blocked with 2% donkey serum in PBS, 0.01% Triton X-100 and 0.05% Tween 20 overnight. Coverslips were then stained with anti-NFκB antibody (1:100; Cell Signaling #8242) overnight at 4°C followed by secondary antibody staining with anti-rabbit Alex Fluor-4588 and Alexa Fluor 468-phalloidin for 60 min, washed and mounted using ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) for DNA staining. Sections were viewed on a Zeiss AxioImager microscope and images taken using an AxioCam HRm camera operating through Zen software.

RNA extraction for colonoids

For mRNA extraction after simulation, Matrigel was disrupted as described in previous sections. Harvested colonoids were resuspended in 300 ul of Trizol and lysed at room temperature for 15 min. RNA was extracted using Direct-zol RNA micro-Prep (Zymo research) following the manufacturer's protocol.

Real time qPCR analysis

Total RNA from colonoids quantified using a NanoDrop Spectrophotometer (ND1000). 500ng of RNA was reverse-transcribed using 5X All-In-One RT (ABM), followed by dilution of the cDNA at 1:5 in RNase/DNasefree H₂O. The qPCR was carried out using a Bio-Rad CFX connect Real-time PCR detection system, with the specificity for each of the PCR reactions confirmed by

melting point analysis. Quantitation was performed using CFX Maestro™ software (Bio-Rad). The expression of genes was normalized to that of *Rplp0* and *GAPDH*. Primer sequences for all genes analyzed are summarized in Table 1.

Human primers		
Target gene	Primer FWD	Primer REV
<i>CCL20</i>	GCT ACT CCA CCT CTG CGG CG	ACC TCC AAC CCC AGC AAG GTT CT
<i>CXCL2</i>	GAAAGCTTGTCTCAACCCCG	TGGTCAGTTGGATTTGCCATTTT
<i>IL1R</i>	ATGAAATTGATGTTTCGTCCCTGT	ACCACGCAATAGTAATGTCCTG
<i>IL18R1</i>	AAGAACGCCGAGTTTGAAGAT	GAGCAGTTGAGCCTTACGTTT
<i>IL37</i>	AAGTCATCCATCCCTTCAGC	CCCACCTGAGCCCTATAAAA
<i>IL8 (CXCL8)</i>	TGT GTG AAG GTG CAG TTT TGC	GCA CCC AGT TTT CCT TGG GG
<i>SIGIRR</i>	GCTGACTGCAAGGACAGAGA	ACTCGTGGAGGCTGTAGTGG
<i>TLR4</i>	AAGCCGAAAGGTGATTGTTG	CTGAGCAGGGTCTTCTCCAC
<i>TLR5</i>	TGC ATC CAG ATG CTT TTC AG	TGC TGA TGG CAT TGC TAA AG
<i>TNFa</i>	TTCCAGAAGATGATCTGATGC	TCAGCCTCTTCTCCTTCT
Mouse primers		
<i>Cxcl1</i>	TGC ACC CAA ACC GAA GTC AT	TTG TCA GAA GCC AGC GTT CAA
<i>Cxcl2</i>	CCT GCC AAG GGT TGA CTT CA	TTC TGT CTG GGC GCA GTG
<i>Ccl20</i>	GCC TCT CGT ACA TAC AGA CGC	CCA GTT CTG CTT TGG ATC AGC
<i>Il1r1</i>	AAGTAATGCTGTCCTGGGCT	AGCACTTTCATATTCTCCATTTGT
<i>Tlr4</i>	TGGCTGGTTTACACATCCATCGGT	TGGCACCATTGAAGCTGAGGTCTA
<i>Tlr5</i>	TGGAGCCGAGTGAGAAATCAG	GTGACGATCCTGGGGTGT
<i>Sigirr</i>	CCC TGC TCT ATG TTA AGT GTC G	TCA GGT TCA CCA AAA GGT CG
<i>Tnfa</i>	CATCTTCTCAAATTCGAGTGACAA	TGG GAG TAG ACA AGG TAC AAC CC

Table 1: Primer list for qPCR analysis

Enzyme-linked immunosorbent assay (ELISA)

After 4 h stimulation of colonoids in Matrigel dome, the media was collected and centrifuged at 1000 g at 4 °C for 10 min, then the supernatant was collected and stored at -80 °C. 100 uL of the supernatant (or dilution) was used per well in duplicate and the ELISAs were performed according to manufacturer's instructions (human CCL20, CXCL1 and CXCL2 R&D, human IL-8 ELISA MAX™ Deluxe Set BioLegend, murine Ccl20 and Cxcl1 R&D).

Western blotting

Colonoids were resuspended in RIPA buffer with protease inhibitors and phosphatase inhibitor, sonicated, then centrifuged at 16,000xg for 20 min at 4°C. Total protein was estimated and 10 µg of whole cell lysate prepared according to manufacturer's instructions in 1X Bolt LDS Sample Buffer with 1X Bolt Reducing Agent (Life Technologies) and heated at 70.C for 10 min. Proteins were separated by Bolt 12% Bis-Tris Gel (Life Technologies), transferred to PVDF membrane (Life Technologies), followed by immunoblotting with rabbit monoclonal phospho-p38 (4631; 1:2000; Cell Signaling Technologies), total p38 (4631; 1:2000; Cell Signaling Technologies), phospho-NFκB (3033; 1:2000; Cell Signaling Technologies), total NFκB (8242; 1:2000; Cell Signaling Technologies) or mouse monoclonal anti-β-actin (G043; 1:2000; ABM), then with horse α-rabbit IgG:HRP (7076; 1:2000; Cell Signaling Technologies) or horse α-mouse IgG:HRP (7076; 1:2000; Cell Signaling Technologies).

Milliplex Luminex Assay

Human colonoid supernatant (25 µl) was used to measure cytokine concentrations using a custom-designed multi-analyte Cytokine Human Magnetic Panel bead array, (Millipore, Sigma) consisting of CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (Rantes), IL-10, IL-8, IL-18, IL-6, and TNFα, according to the manufacturer's protocol. Mouse colonoid supernatant (25 µl) was used on a custom-designed multi-analyte Cytokine Mouse Magnetic Panel bead array, (Millipore, Sigma) consisting of Ccl2 (Mcp-1), Ccl3 (Mip-1α), Ccl4 (Mip-1β) Ccl5 (Rantes), Il-10, Il-8, Il-18, Il-6, and Tnfα, according to the manufacturer's protocol. Results were obtained with a Flexmap 3D system with Luminex xPONENT software version 4.2 (both from Luminex Corp.; Austin, TX, USA). Cytokine concentrations were determined using Milliplex Analyst software (version 3.5.5.0, Millipore).

Statistical analysis.

All results presented in this study are expressed as the mean values ± standard errors (SEM). Mann-Whitney U-test, student *t*-test and one-way ANOVA were performed using GraphPad Prism software, version 7.0c for MAC OS X. A p-value of 0.05 or less was considered significant, with asterisks denoting significance in the figures.

DATA AVAILAIBILITY

All data and reagents are available upon request to the corresponding author. No data sets were generated during the current study.

Figure legends

Figure 1: Human IEC responses to host (IL-1 β) and bacterial stimuli (FliC, LPS)

A) qPCR analysis of inflammatory gene transcription after FliC or IL-1 β stimulation for 4h, expressed as fold change over untreated colonoids. B) IL-8 and CCL20 protein levels secreted basolaterally by human colonoids after 4h of stimulation with FliC or IL-1 β . C) Heat map representation of chemokine concentrations in the supernatants of colonoids after 4h of stimulation with FliC or IL-1 β . D) Concentration of chemokines detected by Multiplex Luminex Assay in the supernatants of colonoids after 4h of stimulation with FliC or IL-1 β . Mean and SEM are indicated. All data shown were repeated in 2 independent experiments, n= 6 donors (4 adult and 2 pediatric). *, P = 0.01 to 0.05; **, P =0.001 to 0.01; ***, P= 0.0001 to 0.001. ns= not significant.

Figure 2: IL-37 effect on human IEC FliC responses. A) qPCR analysis of inflammatory genes after FliC and IL-37 exposure for 4h expressed as percentage of change over FliC treated colonoids. B) IL-8 and CCL20 protein levels (detected by ELISA) secreted basolaterally by human colonoids after 4h of stimulation with FliC and IL-37. C) CCL2, CCL5 and TNFa protein levels secreted basolaterally by colonoids after 4h of stimulation with FliC and IL-37 (detected by Milliplex Luminex assay). D) Immunostaining against NFkB (red), actin (Phalloidin - green) and DAPI (blue) of 2D monolayer after 30 minutes of stimulation with FliC with or without IL-37 (left). Counts of NFkB positive nuclei from immunostaining (right). E) Western blot analysis of phospho and total p38, after 30 minutes of stimulation with FliC with or without IL-37. Equal loading confirmed with Actin as well as total protein stain of membrane (see Fig. S4). Mean and SEM are indicated. All data shown were repeated in 2 independent experiments, n= 4 donors (3 adult and 1 pediatric). *, P = 0.01 to 0.05; **, P =0.001 to 0.01; ***, P= 0.0001 to 0.001; ****, P= 0.00001 to 0.0001; ns= not significant.

Figure 3: SB202190 effect on IL37 immunosuppressive responses A) Brightfield images of a representative human colonoids grown in standard organoid Media (left) or in No SB202190 media (right). B) Growth curve of colonoids grown in standard organoid media or in No SB202190 media for 14 days. C) IL-8 and CCL20 protein levels secreted basolaterally after 4h of stimulation with FliC of human colonoids grown in standard organoids media or in No

SB202190 media. D) IL-8 and CCL20 protein levels secreted basolaterally by human colonoids grown in No SB202190 media after 4h of stimulation simultaneously with FliC and IL-37. E) Western blot analysis of phospho and total NFkB, phospho and total p38 in human colonoids grown in No SB202 media after 30 minutes of stimulation with FliC with or without IL-37. Equal loading confirmed with Actin as well as total protein stain of membrane (see Fig. S5). Mean and SEM are indicated. All data shown are repeated in 2 independent experiments, n= 4 donors (2 adult and 2 pediatric). *, P = 0.01 to 0.05; **, P =0.001 to 0.01; ***, P= 0.0001 to 0.001; ****, P= 0.00001 to 0.0001. Comparison lacking annotations are not significant.

Figure 4: Mouse IEC responses to host (Il-1 β) and bacteria stimuli (FliC)

A) qPCR analysis of innate receptor and negative regulator gene transcription by mouse colonoids expressed as fold change over WT (*Sigirr*^{+/+}) colonoid expression. B) qPCR analysis of chemokines and cytokines after 4h of stimulation with FliC and Il-1 β . C) Cxcl1 (left) and Ccl20 (right) protein levels secreted basolaterally by colonoids derived from WT and *Sigirr*^{-/-} mice after 4h of stimulation with Il-1 β and IL-37. D) Heat map representation of various chemokine concentration in the supernatant of colonoids after 4h of stimulation with FliC or Il-1 β . Mean and SEM are indicated. All data shown repeated in 2 independent experiments, n= 2 mice of each genotype for each experiment. *, P = 0.01 to 0.05; **, P =0.001 to 0.01; ***, P= 0.0001 to 0.001; ****, P= 0.00001 to 0.0001. Comparison lacking annotations are not significant.

Figure 5: IL-37's suppression of innate responses in mouse organoids is Sigirr dependent.

A) qPCR analysis of main inflammatory genes after FliC stimulation with IL-37 for 4h on *Sigirr*^{+/+} (WT) and *Sigirr*^{-/-} mouse colonoids expressed as fold change over untreated colonoids. B) Cxcl1 (left) and Ccl20 (right) protein levels secreted basolaterally by WT and *Sigirr*^{-/-} mouse colonoids after 4h of stimulation with FliC and IL-37. D) Immunostaining against Nfkb (red), Actin Phalloidin (green) and DAPI (blue) of 2D monolayer after 30 minutes of stimulation with Il-1 β with or without IL-37 (left). Counts of Nfkb positive nuclei from immunostaining (Right). Mean and SEM are indicated. All data shown repeated in 2 independent experiments, n= 2 mice of each genotype for each experiment. *, P = 0.01 to 0.05; **, P =0.001 to 0.01; ***, P= 0.0001 to 0.001; ****, P= 0.00001 to 0.0001. Comparison lacking annotations are not significant.

Figure S1: Innate receptor expression and IL-1 β responses of human IEC

A) Brightfield images of representative colonoids derived from sigmoid colon biopsies taken from pediatric (left) and adult (right) donors. B) qPCR analysis of innate receptor expression by human colonoids as fold change over adult colonoid expression. C) qPCR analysis of *SIGIRR* and *IL-37* by human colonoids expressed as fold change over adult colonoid expression. D) IL-8 and CCL20 protein levels secreted by human colonoids versus Caco-2 cell lines after 4h of stimulation with IL-1 β . All data shown was repeated in 2 independent experiments, n= 6 (4 adult, 2 pediatric) donors.

Figure S2: Dose response of IL-37 on human IEC responses to FliC stimulation.

A) qPCR analysis of inflammatory gene transcription after exposure to FliC for 4h. B) IL-8 and CCL20 protein levels secreted basolaterally by human colonoids after 4h of stimulation simultaneously with various IL-37 doses, showing all data (right) and responsive donors only (left). C) IL-8 protein levels secreted basolaterally by human colonoids after 4h of simultaneous stimulation with FliC and different IL-37 doses, showing all data (right) and only responsive donors (left). All data shown was repeated in 2 independent experiments, n= 6 (4 adult, 2 pediatric) donors for all data, n= 4 (3 adult, 1 pediatric) donors for responsive.

Figure S3: IL-37 reduce FliC induced Nfkb activation in mouse organoids.

Immunostaining against Nfkb (red) Phalloidin (green) and DAPI (blue) of 2D monolayer after 30 minutes of stimulation with FliC with or without IL-37 (left). Counts of Nfkb positive nuclei from immunostaining (Right). All data shown repeated in 2 independent experiments, n= 2 mice of each genotype for each experiment.

REFERENCES

1. Allaire, J. M. *et al.* The Intestinal Epithelium: Central Coordinator of Mucosal Immunity. *Trends Immunol.* **39**, 677–696 (2018).
2. Price, A. E. *et al.* A Map of Toll-like Receptor Expression in the Intestinal Epithelium Reveals Distinct Spatial, Cell Type-Specific, and Temporal Patterns. *Immunity* **49**, 560–575.e6 (2018).
3. Kayisoglu, O. *et al.* Location-specific cell identity rather than exposure to GI microbiota defines many innate immune signalling cascades in the gut epithelium. *Gut* gutjnl-2019-319919 (2020) doi:10.1136/gutjnl-2019-319919.
4. Abreu, M. T. Toll-like receptor signalling in the intestinal epithelium: How bacterial recognition shapes intestinal function. *Nat. Rev. Immunol.* **10**, 131–143 (2010).
5. Frantz, A. L. *et al.* Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with downregulation of polymeric immunoglobulin receptor, mucin-2, and antibacterial peptides. *Mucosal Immunol.* **5**, 501–512 (2012).
6. Gibson, D. L. *et al.* MyD88 signalling plays a critical role in host defence by controlling pathogen burden and promoting epithelial cell homeostasis during *Citrobacter rodentium*-induced colitis. *Cell. Microbiol.* **10**, 618–631 (2008).
7. Ruan, W. *et al.* Enhancing responsiveness of human jejunal enteroids to host and microbial stimuli. *J. Physiol.* **598**, 3085–3105 (2020).
8. Kadota, C. *et al.* Down-regulation of single immunoglobulin interleukin-1R-related molecule (SIGIRR)/TIR8 expression in intestinal epithelial cells during inflammation. *Clin. Exp. Immunol.* **162**, 348–361 (2010).
9. Khan, M. A. *et al.* The Single IgG IL-1–Related Receptor Controls TLR Responses in Differentiated Human Intestinal Epithelial Cells. *J. Immunol.* **184**, 2305–2313 (2010).
10. Wald, D. *et al.* SIGIRR, a negative regulator of Toll-like receptor - Interleukin 1 receptor signaling. *Nat. Immunol.* **4**, 920–927 (2003).
11. Guo, J., Zhan, X., Xu, G., Mao, C. & Wei, R. Transcriptomic analysis reveals that IL-1R8/Sigirr is a novel macrophage migration regulator and suppresses macrophage proliferation through p38 MAPK signaling pathway. *Biomed. Pharmacother.* **124**, (2020).
12. Sham, H. P. *et al.* SIGIRR, a Negative Regulator of TLR/IL-1R Signalling Promotes Microbiota Dependent Resistance to Colonization by Enteric Bacterial Pathogens. *PLoS Pathog.* **9**, (2013).
13. Xiao, H. *et al.* The Toll-Interleukin-1 Receptor Member SIGIRR Regulates Colonic Epithelial Homeostasis, Inflammation, and Tumorigenesis. *Immunity* **26**, 461–475 (2007).
14. Nold, M. F. *et al.* IL-37 is a fundamental inhibitor of innate immunity. *Nat. Immunol.* **11**, 1014–1022 (2010).
15. Huang, N. *et al.* Interleukin-37 alleviates airway inflammation and remodeling in asthma via inhibiting the activation of NF- κ B and STAT3 signalings. *Int. Immunopharmacol.* **55**, 198–204 (2018).
16. Imaeda, H. *et al.* Epithelial expression of interleukin-37b in inflammatory bowel disease. *Clin. Exp. Immunol.* **172**, 410–416 (2013).
17. Wang, Y. C., Weng, G. P., Liu, J. P., Li, L. & Cheng, Q. H. Elevated serum IL-37 concentrations in patients with sepsis. *Medicine (Baltimore)*. **98**, e14756 (2019).
18. Jiang, M. *et al.* IL-37 inhibits invasion and metastasis in non-small cell lung cancer by suppressing the IL-6/STAT3 signaling pathway. *Thorac. Cancer* **9**, 621–629 (2018).

19. Theoharides, T. C., Tsilioni, I. & Conti, P. Mast cells may regulate the anti-inflammatory activity of IL-37. *Int. J. Mol. Sci.* **20**, (2019).
20. Wang, D. W. *et al.* Interleukin-37 Enhances the Suppressive Activity of Naturally Occurring CD4 + CD25 + Regulatory T Cells. *Sci. Rep.* **6**, 1–11 (2016).
21. Li, S. *et al.* Extracellular forms of IL-37 inhibit innate inflammation in vitro and in vivo but require the IL-1 family decoy receptor IL-1R8. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 2497–2502 (2015).
22. Bulau, A. M. *et al.* Role of caspase-1 in nuclear translocation of IL-37, release of the cytokine, and IL-37 inhibition of innate immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 2650–2655 (2014).
23. Nold, M. F. *et al.* IL-37 is a fundamental inhibitor of innate immunity. *Nat. Immunol.* **11**, 1014–1022 (2010).
24. Rudloff, I. *et al.* Monocytes and dendritic cells are the primary sources of interleukin 37 in human immune cells. *J. Leukoc. Biol.* **101**, 901–911 (2017).
25. Nold-Petry, C. A. *et al.* IL-37 requires the receptors IL-18R α and IL-1R8 (SIGIRR) to carry out its multifaceted anti-inflammatory program upon innate signal transduction. *Nat. Immunol.* **16**, 354–365 (2015).
26. Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262–265 (2009).
27. Sato, T. *et al.* Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett’s epithelium. *Gastroenterology* **141**, 1762–1772 (2011).
28. Holly, M. K. *et al.* Salmonella enterica infection of murine and human enteroid-derived monolayers elicits differential activation of epithelium-intrinsic inflammasomes. *Infect. Immun.* **88**, (2020).
29. Fernando, E. H. *et al.* A simple, cost-effective method for generating murine colonic 3D enteroids and 2D monolayers for studies of primary epithelial cell function. *Am. J. Physiol. - Gastrointest. Liver Physiol.* **313**, G467–G475 (2017).
30. Riva, F. *et al.* TIR8/SIGIRR is an interleukin-1 receptor/toll like receptor Family member with regulatory functions in inflammation and immunity. *Front. Immunol.* **3**, (2012).
31. Garlanda, C. *et al.* Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 3522–3526 (2004).
32. Garlanda, C., Riva, F., Bonavita, E., Gentile, S. & Mantovani, A. Decoys and regulatory ‘receptors’ of the il-1/toll-like receptor superfamily. *Front. Immunol.* **4**, 1–13 (2013).
33. Günaltay, S., Ghiboub, M., Hultgren, O. & Hörnquist, E. H. Reduced IL-37 Production Increases Spontaneous Chemokine Expressions in Colon Epithelial Cells. *Dig. Dis. Sci.* **62**, 1204–1215 (2017).
34. Sharma, S. *et al.* The IL-1 Family Member 7b Translocates to the Nucleus and Down-Regulates Proinflammatory Cytokines. *J. Immunol.* **180**, 5477–5482 (2008).
35. Cavalli, G. & Dinarello, C. A. Suppression of inflammation and acquired immunity by IL-37. *Immunol. Rev.* **281**, 179–190 (2018).
36. Crowley, S. M. *et al.* Intestinal restriction of Salmonella Typhimurium requires caspase-1 and caspase-11 epithelial intrinsic inflammasomes. *PLoS Pathog.* **16**, e1008498 (2020).
37. Rees, W. D. *et al.* Enteroids Derived From Inflammatory Bowel Disease Patients Display Dysregulated Endoplasmic Reticulum Stress Pathways, Leading to Differential Inflammatory Responses and Dendritic Cell Maturation. *J. Crohns. Colitis* **14**, 948–961 (2020).

ACKNOWLEDGMENTS

We thank C. Ma and M. Diaz Gomez for their assistance with animal handling and cell culture procedures respectively. We thank the Vallance lab for feedback and valuable discussions. We thank B. Cai from the Kollman Lab for her assistance with the Multiplex Luminex assay. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and from Crohn's and Colitis Canada (CCC) to B.A.V. J.M.A was supported by 3 post-doctoral fellowships from Canadian Institutes of Health Research (CIHR), Michael Smith Foundation for Health Research (MSFHR) and Fonds de recherche du Québec – Santé (FRQS). S.M.C. was supported by Four-Year Doctoral Fellowships from the University of British Columbia. A.P. was supported by a summer studentship from Natural Sciences and Engineering Research Council of Canada (NSERC).

AUTHOR CONTRIBUTIONS

J.M.A. designed the study, performed experiments, analyzed and interpreted the data, and wrote the manuscript with B.A.V. | K.J. and B.B. recruited human subjects, obtained informed consent, and provided biopsies and clinical details. | X.L. provided mouse strains. | A.P., S.M.C., H.H. and M.S. provided help with organoids experiments. A.P. also performed ELISA and qPCR experiments. | N.M. helped with qPCR experiments | H.H. and S.M.C. provided input on experimental design and helped interpret the data. | B.A.V. acquired funding, interpreted data and supervised the study. | All authors edit and review the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.