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

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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 is 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-1β) 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-1β. 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 NFκB 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.
LPS stimulated peripheral blood mononuclear cells (PBMC) that secreted IL-37 (see Fig. S1 online) and IL-37 levels. IL-37 protein secretion was not detected in IEC under all stimulation conditions as opposed to very low SIGIRR mRNA IL-37 were also confirmed by qPCR analysis, showing similar levels between pediatric inflammatory cytokine, including IL-1β, as IL-1β, even at a concentration of 5 times less, was able to induce higher levels of IL-8 and CCL20. 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-1β 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-1β 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-1β but only secreted a limited number of chemokines (Cxc1 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 extensively 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. These colonoids were exposed to the cytokine IL-1β or bacterial products (LPS or flagellin (FliC)) for 4 h 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 (see Fig. S1 online). 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. 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 (see Fig. S1 online). 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 (see Fig. S1 online). Human colonoids showed high expression of SIGIRR mRNA but very low levels of IL-37. IL37 protein secretion was not detected in IEC under all stimulation conditions as opposed to LPS stimulated peripheral blood mononuclear cells (PBMC) that secreted IL-37 (see Fig. S1 online). 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 h. Colonoids were also treated with IL-1β (10 ng/ml) or the bacterial product LPS (1 μg/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 TNFa induced 40-fold compared to untreated conditions (Fig. 1a). In contrast, IL-1β stimulation induced only a 4 to sixfold 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 basal secretional 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-1β induced little or no secretion of these chemokines (Fig. 1b). The low or lack of innate response of human colonoids to IL-1β stimulation is not caused by the ineffectiveness of IL-1β, as IL-1β, even at a concentration of 5 times less, was able to induce higher levels of IL-8 and CCL20 secretion by Caco2 cells (see Fig. S1 online). LPS also failed to induce the secretion of IL-8 and CCL20 by human colonoids, similar to a recent finding7 (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 4 h 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, TNFα) (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 TNFα 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).

**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 (see Fig. S2 online). 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 TNFα 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
Figure 2. IL-37 effect on human IEC FliC responses. (a) qPCR analysis of inflammatory genes after FliC and IL-37 exposure for 4 h 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 4 h of stimulation with FliC and IL-37. (c) CCL2, CCL5 and TNFα protein levels secreted basolaterally by colonoids after 4 h 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 min 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 min of stimulation with FliC with or without IL-37 (left). Equal loading confirmed with β-Actin as well as total protein stain of membrane (see Fig. S4 online). Densities relative to total protein or β-Actin are shown (right). Mean and SEM are indicated from n = 4 donors (2 adult and 2 pediatric). All data shown are representative of at least 3 independent experiments. Statistical significance calculated using unpaired Student’s t-test *, 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.
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 (see Fig. S2 online). 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 TNFα levels in response to IL-37 treatment (Fig. 2c).

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. To assess whether the effect of IL-37 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 confluence was well established, monolayers were treated with FliC (100 ng/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 only modestly increased after FliC stimulation and IL-37 did not reduce its activation level (Fig. 2e).

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 signaling. 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. IL-37 treatment (Fig. 2c).

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 MAPK. 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 appeared more cystic and less mature when grown 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, even though a trend for increase cytokine production was observed (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 enabled IL-37 to decrease CCL20 secretion by 46% (Fig. 3d) as 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 (Figs. 2b, 3d). Thus, aside from showing that the presence of SB202190 limits IL-37 activation in colonoids, we also confirm that IL-37 inhibits both p38 and NFκB activation and downstream signaling pathways, by showing a reduction in the phosphorylation of NFκB and p38, but with no modulation of total protein levels (Fig. 3e).

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 4 h, 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-1β stimulation was combined with IL-37 treatment. IL-37 reduced mRNA levels of Cxcl1 (reduced 33%) and Ccl20 (reduced 43%) in Sigirr+/- colonoids, but to a lesser degree Tnfα, 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 Nfκb activation in human derived colonoids and Nfκb is downstream of Sigirr signaling pathways, we explored if IL37 exerts its inhibitory effect on Nfκb through its use of Sigirr. We again utilized our 2D colonoid-derived monolayer system and assessed Nfκb nuclear translocation and activation through immunostaining. Monolayers derived from WT and Sigirr-/- mouse colonoids were treated with inflammatory stimuli (IL-1β (10 ng/ml) or FliC (100 ng/ml)) for 30 min to induce Nfκb translocation to the nucleus (Fig. 5e for IL-1β) (See Fig. S3 online for FliC). When the monolayers of either genotype were stimulated with IL-1β, the majority of cells showed nuclear co-localisation of Nfκb (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 Nfκb cells in the Sigirr+/- colonoid monolayers that was not observed in the Sigirr-/- monolayers (Fig. 5e). A similar reduction in nuclear Nfκb staining was observed with Sigirr+/- colonoids, but not with Sigirr-/- colonoids, when both were treated with FliC plus IL-37 (see Fig. S3 online). Similar to our findings with human organoids, Western blot analysis on mouse colonoids confirmed that IL-37 reduces both Nfκb activation and p38 after IL-1β stimulation (Fig. 5f). The same trend was also observed for FliC-treated colonoids in the presence of IL-37 (data not shown). These results suggest that extracellular IL-37 acts on IEC through a Sigirr dependent pathway to inhibit FliC or IL-1β induced Nfκb and p38 activation.

**Figure 4.** Mouse IEC responses to host (IL-1β) and bacteria stimuli (FliC). (a) qPCR analysis of innate receptor and negative regulator gene transcription by mouse colonoids expressed as relative expression over reference genes. (b) qPCR analysis of chemokines and cytokines after 4 h of stimulation with FliC and IL-1β expressed as fold change over untreated WT (Sigirr+/+). (c) Cxcl1 and Ccl20 protein levels secreted basolaterally by colonoids derived from WT and Sigirr-/- mice after 4 h of stimulation with FliC or IL-1β. (d) Heat map representation of various chemokine concentration in the supernatant of colonoids after 4 h of stimulation with FliC or IL-1β. Mean and SEM are indicated. Mean and SEM are indicated from n = 4 mouse organoid lines derived from each genotype. All data shown are representative of at least 2–3 independent experiments. Statistical significance calculated using one-way ANOVA *, 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 4 h 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 4 h of stimulation with FliC and IL-37. (c) qPCR analysis of key inflammatory genes after with IL-1β stimulation, in concert with IL-37 for 4 h on Sigirr+/+ (WT) and Sigirr−/− mouse colonoids expressed as fold change over untreated colonoids. (d) Cxcl1 (left) and Ccl20 (right) protein levels secreted basolaterally by WT and Sigirr−/− mouse colonoids after 4 h of stimulation with IL-1β and IL-37. (e) Immunostaining against Nfkb (red), Actin Phalloidin (green) and DAPI (blue) of 2D monolayer after 30 min of stimulation with IL-1β with or without IL-37 (left). Counts of Nfkb positive nuclei from immunostaining (Right). (f) Western blot analysis of phospho and total NFkB, phospho and total p38 in mouse colonoids after 30 min of stimulation with IL-1β with or without IL-37 (left). Equal loading was confirmed with β-Actin as well as total protein stain of membrane (see Fig. S6 online). Densities relative to total protein or β-Actin are shown (right). Mean and SEM are indicated from n = 4 mouse organoid lines derived from each genotype. All data shown are representative of at least 3 independent experiments. Statistical significance calculated using one-way ANOVA *, 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. 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 (murine colonoids). Specifically, IL-37 reduced NFκB and p38 activation and chemokine secretion typically seen after stimulation of colonoids with bacterial FltC, as well as the cytokine IL-1β. 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-1β 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-1β stimulus, emphasizing previous findings that species differences can greatly affect IEC behavior and their innate immune responses.

Interestingly, our results also revealed that even picomolar concentrations (1000 pg/ml to 100 pg/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 defined.

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 line. 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.

In this study, IL-37 suppressed chemokine secretion levels from colonoids by 20 to 45%. We believe this would be biologically significant, and in keeping with previous reports, that IL-37 does not fully inhibit innate immune response but rather serves to balance excessive inflammation. It would be interesting to verify if this chemokine inhibition would translate into more physiological effects such as reduced chemotaxis of immune cells, normally induced by these chemokines. Unfortunately, using the current organoid system would be impractical since the chemokine levels are too low to induce migration of neutrophils or other immune cells. A scale-up of the organoid cultures (30–50 fold) or a co-culture system could help to answer these questions in the future.

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. 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. 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 during disease states, IEC may produce greater levels of IL-37, since some studies have shown high immunohistochemical staining for IL-37 in the inflamed gut epithelium of IBD patients. In our system, we detected low levels of IL-37 mRNA without seeing any increase in protein expression (see Fig. S1 online). 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 yet. 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 it 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 cells. 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. 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 FltC stimulation, mouse IEC produce stronger innate immune responses following IL-1β stimulation than they do when exposed to FltC. 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 NFκB 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 as well as blood from 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, H07-02861). 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 previously. 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. 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-acetylcysteine (Sigma-Aldrich), nicotinamide (Sigma-Aldrich), mEGF (Invitrogen), A83-01 (Tocris), SB 202,190 (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. 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. 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 2×5 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-1β (10 ng/ml; Sigma) or murine IL-1β (10 ng/ml; PeproTech) or LPS O55:B5 (1000 ng/ml; InvivoGen) or murine IL-1β (10 ng/ml; InvivoGen) or mIL-1β (10 ng/ml; InvivoGen) with or without recombinant IL-37 (100 to 0.1 ng/ml; R&D) or corresponding volume of base media for 4 h.

**Colonoid-derived monolayer seeding and stimulation.** Monolayers derived from colonoids (human or mice) were generated as previously described with modifications. 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 2×5 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 24 h after seeding. 72 h after seeding, confluent monolayers were stimulated with colonoid media supplemented with FliC (100 ng/ml; InvivoGen) or murine IL-1β (10 ng/ml; InvivoGen) with or without recombinant IL-37 (100 ng/ml; R&D) or corresponding volume of base media for 30 min.

**Monolayer immunostaining.** For colonoid monolayer immunostaining, after 30 min of stimulation, coverslips were fixed in 4% PFA for 15 min at room temperature. Then the cells were rinsed in PBS 1X twice, and...
pelleted and the supernatant was collected for IL-37 analysis by ELISA.

bottomed 96 well-plate and stimulated with LPS (100 ng/ml; InvivoGen). After 24 h of stimulation, cells were

Western blot images were taken using a ChemiDoc imaging system (Bio-Rad) and

α-rabbit IgG:HRP (1:2000; Cell Signaling Technologies #7076) or horse α-mouse IgG:HRP (1:2000; Cell Signal-

ing with rabbit monoclonal phospho-p38 (1:2000; Cell Signaling Technologies #9211), total p38 (1:2000; Cell

Bis–Tris Gel (Life Technologies), transferred to PVDF membrane (Life Technologies), followed by immunoblot-

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 immunoblot-

sections were viewed on a Zeiss AxioImager microscope and images taken using an AxioCam HRm camera operating through Zen software.

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 (R&D systems), human IL-8 ELISA MAX Deluxe Set (BioLegend), murine Ccl20 and Cxcl1 (R&D systems)).

Western blotting. Colonoids were resuspended in RIPA buffer with protease inhibitors and phosphatase inhibitor, sonicated, then centrifuged at 16,000g 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 (1:2000; Cell Signaling Technologies #9211), total p38 (1:2000; Cell Signaling Technologies #9212), phospho-NFkB (1:2000; Cell Signaling Technologies #3033), total NFkB (1:2000; Cell Signaling Technologies #8242) or mouse monoclonal anti-β-actin (1:2000; ABM # G043), then with horse α-rabbit IgG:HRP (1:2000; Cell Signaling Technologies #7076) or horse α-mouse IgG:HRP (1:2000; Cell Signaling Technologies #7076). Western blot images were taken using a ChemiDoc imaging system (Bio-Rad) and densitometry analysis of the obtained images were done using ImageJ 1.45S software (Wayne Rasband, NIH).

Milliplex luminexx assay. Human colonoid supernatant (25 µl) was used to measure cytokine concentra-
tions using a custom-designed multi-analyte Cytokine Human Magnetic Panel bead array, (MilliporeSigma) 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 availability
All data and reagents are available upon request to the corresponding author. No data sets were generated during the current study.

Received: 11 December 2020; Accepted: 25 March 2021
Published online: 15 April 2021

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Table 1. Primer list for qPCR analysis.

| Target gene | Primer FWD | Primer REV |
|-------------|------------|------------|
| CCL20       | GCT ACT CCA CCT CTF GGG CG | ACC TCC AAC CCC AGC AAG GAG CTT CT |
| CXCL2       | GAAAGCCTGCTCAACCCCAG | TGGTCACTGATTGCCATTT |
| IL1R        | ATGAAATTTGATCGTCCGCTTGT | ACCAGGCAATGTAAGTTCCTGT |
| IL1R8       | AAGAACCCGAGTTTGAGATG | GAGCGATTTAGCTTTAGTTT |
| IL7         | AGTGACCATCACCCTGACG | CCCACCTGAGCCCTATAAAA |
| IL8 (CXCL8) | TGT GTG AAG GTG CAG TTT TGC | GCA CCC AGT TTT CTT TGC GGG |
| SIGIRR      | GCTGACTGCAAGGCAGAGAGA | ACTGTGGAGGCGTGTAAGGG |
| TLR4        | AAGGCCAAAAGGATGATTGTG | CTGGAGCAGGCTTCCTCAC |
| TLR5        | TGC ATC CAG ATG CCT TTT AG | TGC TGA TGG CAT TGC TAA AG |
| TNFα        | TTCCGACAAGATGACCTTGAC | TCAGCCCTCTTTCTTCTT |
| GAPDH (reference) | ATG ACC TGG CCA CAG CC | CCG TGG TCC GTG CTT CTC T |
| TBP (reference) | GCC CGA AAC GCC GAA TAT | TCC TGG TGC CAG CAG CAG |

| Mouse primers |
|--------------|
| Cxcl1        | TGC ACC CAA ACC GAA GTC AT | TGG TCA GAA GGC AGC GCT CAA |
| Cxcl2        | CCT GCC AAG GGT TGA CTT CA | TCC TGT CGG GCA GTG |
| Ccl20        | GCC TCT CTT ACA TGA AGC CGC | CCA GGT TGG CTT TGG ATC AGC |
| Il1r1        | AAGAATGCTGCTCTGCGCTT | AGACATTCATATTTCATCTCT |
| Tlr4         | TGGCGTGTATCATACATCCTCG | TGGCACATGAAAGCTGGTCTTA |
| Tlr5         | TGGAGGCGAGTGGAAATCAG | GTGACGATCTGGGGGTGGT |
| Sigirr       | CCC TGC TCT ATG TTA AGT GTC G | TCA GGT TCA CCA AAA GGT CG |
| Tnfα         | CATCTTCCAAAATTAGTCTGACAA | TGG GAG TAG ACA AGG TAC AAC CC |
| Rplpol (reference) | AGA TGC GGA AGA TGC TGT TGG C | TGG CCT AGA CCA GGT TTC |
| Gapdh (reference) | ATG ACC TTT CCA CAG CC | CCC ATC ACC ATC TTC CAG |
