Microbiota-dependent expression of CTLA-4 by innate lymphoid cells restrains IFNγ dependent colitis

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Abstract

The maintenance of intestinal homeostasis is a fundamental process critical for organismal integrity. Sitting at the interface of the gut microbiome and mucosal immunity, adaptive and innate lymphoid populations regulate the balance between commensal micro-organisms and pathogens. Checkpoint inhibitors (CPI), particularly those targeting the CTLA-4 pathway, disrupt this fine balance and can lead to inflammatory bowel disease (IBD) and immune checkpoint colitis (CPI-C). Here, we show that CTLA-4 is expressed by innate lymphoid cells (ILC) and that its expression is regulated by ILC subset-specific cytokine cues in a microbiota-dependent manner. Genetic deletion or antibody blockade of CTLA-4 demonstrates that this pathway plays a key role in intestinal homeostasis and is conserved in human IBD and CPI-induced colitis (CPI-C). We propose that this population of CTLA-4-positive ILC may serve as an important target for the treatment of idiopathic and iatrogenic intestinal inflammation.

Introduction

Cytotoxic T lymphocyte–associated antigen 4 (CTLA-4), or CD152, is regarded as one of the key inhibitory molecules expressed by conventional CD4+ and CD8+ T cells1, 2, 3, 4, 5, 6. CTLA-4 is homologous to CD28, a costimulatory molecule expressed on the surface of naïve CD4+ and CD8+ T cells that promotes T cell activation and proliferation7, 8. Both CD28 and CTLA-4 bind to the ligands CD80/B7.1 and CD86/B7.2, which are usually found on the surface of antigen presenting cells (APC)9, 10, 11. CTLA-4 has a 10-100-fold greater affinity for both CD80 and CD86 and outcompetes CD28 binding and can also remove the ligands from cells by a process of transendocytosis12, 13. Regulatory T cells (Treg) suppress aberrant and excessive immune responses via constitutive expression of CTLA-414, 15, 16. The importance of this is highlighted in Ctla4 knockout mice, which die prematurely from multi-organ failure due to excessive immune activation and lymphoproliferation17, 18. Furthermore, in recent years, cancer patients undergoing treatment with anti-CTLA-4 and other immune checkpoint inhibitors (CPI) that block inhibitory molecules and enhance immune activation against tumours, have been found to develop autoimmune and immune-mediated inflammatory diseases19, 20, 21, 22, 23, 24, 25, 26, 27.

The discovery of innate lymphoid cells (ILCs) has been important for the understanding of immune responses, especially at barrier surfaces28, 29, 30, 31, 32, 33, 34, 35. ILC1 can be activated by stimulation with the cytokines IL-12 and IL-18, express the transcription factor T-bet, and are potent producers of interferon-γ (IFNγ)28, 36. ILC2 cells can be activated by alarmin cytokines, including IL-25 and IL-33, highly express the transcription factor GATA3 and are strong producers of interleukin (IL)-5 and IL-1337, 38, 39. ILC3 cells are activated by the cytokines IL-23, IL-1β, IL-6, IL-27 and TGF-β and express the transcription factor RORγt but can be further subdivided according to whether they express natural cytotoxicity receptors (NCRs). NCR+ ILC3 are potent producers of IL-2240, 41 and NCR- ILC3 produce IL-17A42, 43. Furthermore, previously CD4+ lymphoid tissue inducer (LTi) cells were considered as a subtype of ILC3 cells due to their expression of RORγt, however, they have now been considered their own ILC subtype. In adults, LTi cells are predominately described as RORγt+ CCR6+ NCR– CD4+ cells and are high producers of
IL-22. ILCs can also transdifferentiate into other ILC lineages, with parallels to T<sub>H</sub> cell plasticity<sup>30,44,45,46,47,48,49,50,51</sup>. CTLA-4 and another inhibitory receptor, PD-1, are expressed by ILC2 during <i>Nippostrongylus brasiliensis</i> infection, or stimulation with recombinant IL-25. Transcripts encoding CTLA-4 are increased in intestinal inflammation in both bulk RNA-sequencing and single-cell RNA-sequencing (scRNA-seq) analyses<sup>52,53,54,55,56</sup>.

Despite recent discoveries and investigations into the immunopathology and biology of ILCs, there are still key limitations in the understanding of these immune cells and how they are regulated. Here in this study, we aim to further investigate and understand the role of surface checkpoint molecules on ILCs and how their expression maintains homeostasis. We show that the checkpoint molecules CTLA-4 and PD-1 are expressed in intestinal lamina propria (LP) ILCs. Furthermore, our data also highlight the presence of CTLA-4 on ILC subsets, and its importance in regulating immunological responsiveness in the colon and its regulation by microbial and inflammatory cues.

**Results**

**Immune checkpoint molecules are expressed across ILC populations in the colon**

We have previously described the importance of ILCs in mediating colitis<sup>30,34,35,50</sup>. Moreover, we have found that ILC1 are a significant driver for the development of DSS-induced colitis, and described how early IFNγ production is likely to be key in the induction of colitis by ILC1<sup>51</sup>. We therefore performed a comprehensive analysis of the expression of well-characterised checkpoint molecules on murine ILCs at single-cell resolution. First, we utilised two publicly available scRNA-seq datasets of FACS-sorted ILCs from the small intestine lamina propria (SI LP) of wild-type (WT) mice<sup>56,57</sup>. The first dataset identified ex-ILC3/ILC1, ILC2, NCR<sup>+</sup> ILC3s, LTi-ILC3 and two undetermined ILC clusters (Fig. 1A). We found that <i>Ctla4</i> expression was predominantly detected in ex-ILC3/ILC1, and to a lesser extent in NCR<sup>+</sup> ILC3 (Figs. 1B and C). PD-1 (encoded by <i>Pdcd1</i>) was not highly expressed in NCR<sup>+</sup> ILC3s and ex-ILC3/ILC1 subsets but was detected in ILC2 and LTi-like ILC3 (Figs. 1B and C). PD-L1 and PD-L2 (encoded by <i>Cd274</i> and <i>Pdcd1lg2</i>, respectively) were detected more widely across all four clusters, but with ILC2 exhibiting the highest expression of PD-L2 (Figs. 1B and C). Interestingly, <i>Lag3</i> and VISTA (encoded by <i>Vsir</i>) expression was also apparent in LTi-like ILC3, NCR<sup>+</sup> ILC3 and ex-ILC3/ILC1 subsets, but less so in ILC2 (Figs. 1B and C). Expression of <i>Havcr2</i>, which encodes for Tim-3, could not be detected in this dataset. These data indicate that ILCs express inhibitory immune checkpoint genes. To confirm <i>Ctla4</i> expression in NCR<sup>+</sup> ILC subsets, we probed a second dataset, which utilised sorted NKp46<sup>+</sup> ILC and could, therefore, be used to investigate gene expression by NK cells, as well as ILC1 and NKp46<sup>+</sup> ILC3 (Fig. 1D). In agreement with our initial findings, we again detected transcripts encoding <i>Ctla4</i> in NKp46<sup>+</sup> ILC3 and ILC1 subsets (Figs. 1E and F). <i>Pdcd1</i> was not highly expressed in ILC1 and NKp46<sup>+</sup> ILC3, but PD-L1 and PD-L2 expression was found across ILC1, NKp46<sup>+</sup> ILC3 and NK cells (Figs. 1E and F). The distribution of <i>Lag3</i> and <i>Vsir</i> expression was consistent with the first dataset, with high expression across NCR<sup>+</sup> ILC3 and ILC1 subsets (Figs. 1E and
F). In this dataset, expression of Havcr2 could only be detected at very low levels across all three ILC subsets (Supplementary Fig. 1A and B).

Blockade of CTLA-4 and PD-1 are linked to checkpoint inhibitor induced colitis in cancer patients\(^{58,59,60}\), and genetic variants in Ctxla4 are linked to predisposition to IBD\(^ {61,62}\). Using our recently reported pre-clinical model of immune checkpoint colitis\(^{63}\), we identified NCR\(^+\) ILC1/3 and ILC2 clusters using scRNA-seq from sorted CD45\(^+\) colonic lamina propria cells of healthy wild-type mice. Here, we found similar expression of immune checkpoints on ILC subsets as described above. Ctxla4, Lag3, Pdcd1lg2 and Vsir exhibited higher expression in NCR\(^+\) ILC1/3 than ILC2, whereas Cd274 expression was higher in ILC2 (Supplementary Fig. 1C). Thus, results from all three scRNA-seq datasets indicate that CTLA-4, as well as other immune checkpoint molecules, are expressed by certain subsets of ILC.

**CTLA4 is expressed on ILC2, NKp46\(^-\) ILC and NKp46\(^+\) ILC subsets**

To validate the scRNA-seq data at the protein level, we used ow cytometry to measure CTLA-4 levels in Rag2\(^-/-\) mice (lacking T cells) and, as a negative control, in Rag2\(^-/-\) x Ctxla4\(^-/-\) double knockout mice. CTLA-4 staining on ILC subsets was specific and we were able to detect CTLA-4 expression by ILC1, ILC2, NKp46\(^+\) ILC3 and NKp46\(^-\) ILC3 from Rag2\(^-/-\) mice (Figs. 2A-B and Supplementary Fig. 2A).

In PMA and ionomycin stimulated colonic lamina propria (cLP) CD127\(^+\) ILCs from BALB/c wildtype mice, there was minimal CTLA-4 expression by ILC containing KLRG1 (a specific marker of ILC2) (Fig. 2C). However, in KLRG1\(^-\) ILCs, we observed CTLA-4 in subsets of both NKp46\(^+\) and NKp46\(^-\) ILCs (Fig. 2C). Utilising a C57BL/6 ROR\(^\gamma\)t-eGFP reporter mouse line, we observed co-expression of CTLA-4 in both ROR\(^\gamma\)t-eGFP\(^+\) and ROR\(^\gamma\)t-eGFP\(^-\) ILCs (Fig. 2C), further suggesting that CTLA-4 expression is frequent among NKp46\(^+\) ILC3 and NKp46\(^-\) CCR6\(^-\) ILC3. CTLA-4 staining was significantly increased upon stimulation with PMA and ionomycin compared to unstimulated ILC subsets isolated from both Rag2\(^-/-\) mice and WT animals (Fig. 2D-F). These data highlight that non-soecific activation activation of ILC can increase the expression of CTLA-4.

To further understand if CTLA-4 could be induced in a subset specific manner, we FACS purified KLRG1\(^+\) and KLRG1\(^-\) cLP ILC to determine which cytokines could induce CTLA-4 expression in CD90\(^+\) ILC2 (KLRG1\(^+\) sorted) and CD90\(^+\) NKp46\(^+\) (KLRG1\(^-\) sorted) ILC in vitro (Supplementary Fig. 2B). Interestingly, we found that a combination of rIL-25 and rIL-33 was a potent inducer of CTLA-4 expression in ILC2 (Fig. 2G). In contrast, CTLA-4 expression by NKp46\(^+\) ILC was most strongly induced by rIL-12 + rIL-18 (Fig. 2H). CTLA-4 expression in NKp46\(^+\) ILCs and ILC2s was not further enhanced when a cocktail of rIL-1\(\beta\), rIL-6, rIL-27 and rTGF-\(\beta\) was added to the medium (Fig. 2G-H). These in vitro data indicate that the canonical cytokines used to stimulate the respective ILC2 and NKp46\(^+\) ILC subsets can induce the expression of CTLA-4.

An IL-10-producing regulatory ILC subset in the intestine has been previously reported\(^ {61}\), although more recent reports have challenged the validity of this finding\(^ {64}\). Typically, CTLA-4 expressing Foxp3\(^+\) CD4\(^+\)
T\textsubscript{Reg} cells produce the anti-inflammatory cytokine IL-10\textsuperscript{65}. Therefore, we sought evidence for a population of CD127\textsuperscript{+} ILC co-expressing IL-10 and CTLA-4 in the cLP and small intestine lamina propria of BALB/c. We detected very few IL-10-producing ILCs upon stimulation with PMA and ionomycin (Supplementary Fig. 3). In contrast, we found a substantial CTLA4\textsuperscript{+} ILC population among those stimulated ILCs, but IL-10 and CTLA-4 co-expression was not observed, indicating that CTLA-4 expression is separable from IL-10 production.

**ILC1 upregulate CTLA-4 in a microbiota-dependent manner**

The composition of the intestinal microbiota has been shown to influence colitis susceptibility in both the general population and cancer patients treated with CPI\textsuperscript{66,67,68,69}. Therefore, we investigated whether altering the intestinal microbiota would affect CTLA-4 expression in ILCs in the lamina propria of mice. To investigate this, WT mice were given a cocktail of antibiotics (ampicillin, neomycin, vancomycin and metronidazole) in drinking water over a period of two weeks. After this period, CTLA-4 expression on ILC subsets was analysed using flow cytometry. NKp46\textsuperscript{+} ILC3s and ILC1s had the highest expression of CTLA-4 after stimulation *in vitro* (Fig. 2E and F). Therefore, we looked at NKp46\textsuperscript{+} ILCs and interestingly, we found that cLP NKp46\textsuperscript{+} IFN\textsubscript{γ}\textsuperscript{+} ILC from the antibiotic-treated mice had a lower MFI for CTLA-4, upon restimulation with PMA and ionomycin, in comparison to control wild-type mice (Figs. 3A and B). These data illustrate that the microbiota may play a role in regulating CTLA-4 expression in NKp46\textsuperscript{+} IFN\textsubscript{γ}\textsuperscript{+} ILCs.

To investigate further if the microbiota was important for regulating the expression of CTLA-4 in ILCs, we used germ-free (GF) wild-type mice and compared them to control specific pathogen-free (SPF) wild-type mice. CTLA-4 expression was significantly reduced in all colonic ILC subsets of GF mice when compared to SPF mice, even after PMA and ionomycin stimulation (Figs. 3C and D). We conclude that CTLA-4 is upregulated on ILCs in response to the microbiota.

**CTLA-4 restrains inflammation in the colon**

We next investigated whether inflammation is a driver of immune checkpoint expression on ILCs. We analysed the expression of checkpoint genes using RNA-seq from colonic tissue segments from six different pre-clinical models of colitis, including T cell-mediated models (*Il10\textsuperscript{−/−}* mice and T cell transfer colitis), chemically induced models (Dinitrobenzene sulfonic acid (DNBS) induced colitis and dextran sulphate sodium (DSS) induced colitis) and two models of ILC-dependent colitis (*Tbx21\textsuperscript{−/−} x Rag2\textsuperscript{−/−}*) Ulcerative Colitis (TRUC) and anti-CD40 colitis in *Rag\textsuperscript{−/−}* mice). *Ctla4* was the most highly upregulated checkpoint gene in all the colitis models, with less pronounced changes in the expression of *Pdcd1*, *Lag3*, *Vsir* and *Havcr2* (Fig. 4A). We observed induction of *Ctla4* in the colon in both innate immune mediated models of colitis (Fig. 4A). The level of *Ctla4* upregulation in the colon of TRUC mice (>18-fold, FDR < 0.005), was equivalent to the level observed in T cell mediated models of disease. TRUC mice completely lack all adaptive lymphocytes, and disease is dependent on the intestinal microbiota and mucosal ILCs\textsuperscript{70},
further supporting the idea that CTLA-4 expressing ILC regulate immunological responsiveness in the colon in a microbiome-dependent manner.

To further investigate the functional importance of CTLA-4 in the innate immune compartment of the colon, we again utilised the $\text{Rag2}^{-/-} \times \text{Ctla4}^{-/-}$ model. Unlike $\text{Ctla4}^{-/-}$ mice, which develop a spontaneous and fatal phenotype\textsuperscript{17,18}, $\text{Rag2}^{-/-} \times \text{Ctla4}^{-/-}$ mice did not exhibit greater spleen and colon weights compared to $\text{Rag2}^{-/-}$ mice (Supplementary Fig. 4A). To determine whether loss of $\text{Ctla4}$ resulted in molecular perturbations in the colonic innate immune system, we analysed gene expression changes in the colon of $\text{Rag2}^{-/-} \times \text{Ctla4}^{-/-}$ compared to $\text{Rag2}^{-/-}$ mice under homeostatic specific pathogen free (SPF) conditions using RNA-seq. There were 344 differentially expressed genes (DEGs; FDR < 0.05) in the colon of $\text{Rag2}^{-/-} \times \text{Ctla4}^{-/-}$ in comparison with $\text{Rag2}^{-/-}$ mice, including 272 upregulated genes and 72 downregulated genes. Notably, many of the most upregulated genes were associated with immune activation ($\text{Ccl7, Cxcl10, Il18bp, Stat2, Stat1}$) and interferon stimulation ($\text{Gbp3, Gbp7, Irf7, Irf9, Ifit1}$) (Fig. 4B-C).

Biological pathway analysis demonstrated significant activation of multiple immune pathways, including interferon signalling ($P < 4 \times 10^{-10}$), dendritic cell maturation ($P < 0.0006$) and TREM1 signalling ($P < 0.007$) (Fig. 4D). The most strongly predicted upstream activator of the gene expression changes observed in the colon of $\text{Rag2}^{-/-} \times \text{Ctla4}^{-/-}$ mice was $\text{Ifng}$ (Z-score = 8.5, $P < 1.1 \times 10^{-50}$) (Fig. 4E and Supplementary Fig. 5A). Other upstream regulators predicted to be activated included $\text{Stat1}$, the canonical signalling pathway triggered by $\text{Ifng}$ (Z-score = 7.5, $P < 6.9 \times 10^{-66}$), $\text{Tnf}$ (Z-score = 5.5, $P < 2 \times 10^{-22}$) and type 1 interferon pathway components, including $\text{Ifna, Ifnar}$ and $\text{Irf7}$ (Fig. 4E and Supplementary Fig. 5A). Together, these data indicate that when CTLA-4 is deficient from ILCs, there is a transcriptional drive towards a more pro-inflammatory IFN$\gamma$-mediated transcriptional profile.

We next investigated whether this pro-inflammatory gene expression in the innate immune compartment following loss of CTLA-4 was associated with increased susceptibility to colitis. Administration of 5% DSS in the drinking water resulted in accelerated weight loss and increased colon mass in $\text{Rag2}^{-/-} \times \text{Ctla4}^{-/-}$ mice in comparison with $\text{Rag2}^{-/-}$ mice, consistent with more severe colitis in the absence of CTLA-4 (Fig. 4F). Flow cytometry analysis of cytokines showed an increase in IFN$\gamma$-producing ILCs in $\text{Rag2}^{-/-} \times \text{Ctla4}^{-/-}$ mice in comparison to $\text{Rag2}^{-/-}$ mice after treatment with DSS but no change in IL-13/IL-5 producing ILC2 cells (Figs. 4G and H and Supplementary Figs. 6A-B).

**CTLA-4$^+$ ILC are present in IBD patients**

We sought to determine whether our findings in mice translated across to patients with IBD. We first looked to see if $\text{CTLA4}$ expression was upregulated under inflammatory conditions in the human colon. We sampled colonic biopsies from patients with active ulcerative colitis (UC) and healthy control subjects without intestinal inflammation. RNA-seq data analysis showed that $\text{CTLA4}$, and to a lesser extent $\text{PDCD1}$ and $\text{LAG3}$, were all upregulated in the colon of UC patients as compared to healthy control subjects (Fig. 5A). To determine whether CTLA-4 was expressed by human colonic ILCs, we extracted lamina
proportion mononuclear cells of patients undergoing colonoscopy and performed multiparameter flow cytometry. We found that CTLA-4 was present in human ILC1 and NCR− ILC3 cells (Fig. 5B and C and Supplementary Fig. 7A). CTLA-4 MFI levels on ILC1 were also increased in the context of intestinal inflammation in IBD patients compared to healthy controls (Fig. 5D). These data indicate that CTLA-4 is upregulated on ILC1 in IBD.

**ILC1 are expanded and mediate CPI-induced colitis**

In recent years, blockade of immune checkpoints, in particular CTLA-4 and PD-1, has been found to cause a new form of IBD, CPI-induced colitis (CPI-C). ILC clusters were identified in our pre-clinical model of CPI-C, but the role of immune checkpoint blockade in relation to ILCs was not well described in patients with CPI-induced colitis. Since expression of immune checkpoint proteins was observed in ILC populations in wild-type mice (Figs. 1 and 2 and Supplementary Fig. 1), and we know from previous work that ILCs are important cells for the pathogenesis of IBD, we hypothesised that there could be a role for ILCs in CPI-induced colitis.

Analysis of the transcriptional changes observed in wild-type mice treated with combination CPI therapy revealed an increase in the expression of genes associated with interferon signalling, such as *Stat1, Stat4, Gbp5* and *Gbp6*, as well as an increase in the expression of *Ifng* itself, in the cluster identified as NCR+ ILC1/3 in mice with CPI-colitis versus control healthy mice (Figs. 6A-B), when looking at the scRNA-seq dataset from our pre-clinical model of CPI-C treated mice. Furthermore, there was an increase in the expression of genes encoding cytotoxic molecules, such as *Gzmb, Gzma* and *Prf1* (Figs. 6A-B). Further analysis at the pathway level using the GSEA Hallmark database showed an increase in the expression of genes associated with interferon alpha and interferon gamma pathways in the ILC clusters, indicating an IFNγ related phenotype (Fig. 6C). These pathway data were validated using Qiagen's Ingenuity Pathway Analysis (IPA) software to compare control and CPI-induced colitis models. Predicted upstream regulators of ILCs in CPI-induced colitis included *Tbx21*, encoding the transcription factor T-bet, and genes encoding the cytokines *Il2, Il18* and *Il21*, suggesting that ILC1 may contribute to colitis in this model (Fig. 6D). The retinoic acid receptor (RAR)γ- agonist CD437 and the synthetic retinoid St1926 were also predicted to play a role in the gene expression changes observed in colonic ILC in CPI-induced colitis (Fig. 6D).

To validate this transcriptomic data at the protein level, we measured IFNγ and IL-17A production from IL-7R+ CD90+ ILCs in mice treated with CPI-induced colitis compared to healthy control mice (Supplementary Fig. 8A). No differences were seen in cytokine production from ILCs in wild-type mice (Figs. 6E and F). However, in wild-type mice, disease is likely mostly driven by T cells. To scrutinise the role of ILCs in the absence of T cells, we induced CPI-colitis in *Rag2−/−* mice. Notably, even in the absence of an adaptive immune system, we still observed induction of disease in *Rag2−/−* mice, including significantly increased colonic and splenic mass and an increase in the recruitment of neutrophils to the colon (Figs. 6G-I). Overall, the number of IFNγ producing ILCs was similar in CPI-colitis as compared to wild-type mice. However, we did observe a significant increase in polyfunctional IFNγ/IL-17A producing cells in the colon of mice with CPI-colitis (Figs. 6J and K), consistent with a pathogenic role for these cells. 

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To determine if this phenotype could be observed in humans, we compared ILC subsets in the peripheral blood from patients with CPI-induced colitis, healthy control subjects and patients with IBD. The proportional abundance of ILCs was significantly dysregulated in patients with CPI-induced colitis in comparison with healthy control subjects or patients with IBD, with a proportional expansion of ILC1s and relative loss of ILC2 (Fig. 6L and Supplemental Fig. 8B). These data are consistent with ILC1 contributing to intestinal inflammation during CTLA-4 blockade in patients with CPI-induced colitis.

**Discussion**

This study provides insight into the regulation of CTLA-4 in ILCs, and how blockade of this crucial regulatory molecule leads to exacerbated disease in mouse models of colitis and in patients with conventional IBD and CPI-induced IBD. The barrier surfaces, and the colon in particular, are challenged with maintaining immunological restraint against a multitude of different commensal bacteria that vary across individuals and time\(^ {72} \), whilst remaining poised to repel invading pathogens. In humans and in mice, we identified CTLA-4 expression in colonic ILCs, a population of tissue-resident innate lymphocytes that have been implicated as important early effector cells in host defence against pathogens and restitution of the mucosal barrier, including in conventional inflammatory bowel diseases\(^ {73, 74, 75, 76} \). Our findings suggest that CTLA-4 is an important controller of the host response to the microbiome in the colon. These data are consistent with clinical data from \( \text{CTLA4} \) heterozygote patients who develop life-threatening autoimmunity, including severe intestinal inflammation\(^ {77, 78} \).

CTLA-4 expression being shown previously by ILC2 cells during parasite infection, or stimulation with recombinant IL-25 and IL-33 and in response to alarmins\(^ {52, 53, 54, 55, 56, 79} \). In our experiments, CTLA-4 expression was low in ILC2s in both the scRNA-seq data and in PMA and ionomycin stimulated ILC2s. However, there was a much greater expression of CTLA-4 from ILC2s stimulated \textit{in vitro} with IL-25 and IL-33, which supports the previous findings. When CTLA-4 is inhibited on ILCs, type 1 immunity becomes activated, leading to further gut inflammation. We show that \( \text{Ctla4} \) is predominantly expressed by ILC1, NCR\(^ - \) ILC3 and NCR\(^ + \) ILC3 subsets. Notably, \( \text{Ctla4} \) expression was increased in \( \text{Rag2}^-/- \) mice lacking adaptive immune cells and was upregulated following cell stimulation. For the first time, we show that CTLA-4 plays an important role in restraining innate immune activation in the colon. Genetic deletion of \( \text{Ctla4} \) in the innate immune compartment triggered a pro-inflammatory transcriptional programme in the colon and predisposed to more severe innate-immune mediated colitis, confirming that CTLA-4 curtail ILC-mediated mucosal inflammation.

We also found that \( \text{Ctla4} \) expression was enhanced by ILC activation and regulated by microbial and inflammatory cytokines. Our data support a microbially-driven regulatory circuit of inducible \( \text{Ctla4} \) expression in ILCs. We propose that gut colonisation with pro-inflammatory bacteria triggers upregulation of \( \text{Ctla4} \) by ILC, leading in turn to suppression of microbially-driven immune activation. However, when the CTLA-4-driven regulatory circuit is lost, unopposed mucosal immune activation leads to colitis development. This is consistent with the shifts in the composition of the intestinal microbiota observed in...
immunotherapy-treated cancer patients who go on to develop CPI-induced colitis\textsuperscript{67, 68, 69}. Moreover, manipulation of the intestinal microbiota looks to be a promising therapeutic option in the management of CPI-induced colitis\textsuperscript{80, 81}.

CTLA-4-expressing ILCs were found in both patients with IBD and patients with CPI-induced colitis, potentially identifying a novel therapeutically tractable cellular target. Based on the gene expression changes observed in colonic ILCs in CPI-induced colitis, upstream analysis predicted significant activation of proximal mediators, such as cytokines that induce IFN\textgamma{} production (\textit{Il18, Il21, Il27} and \textit{Il2}), transcription factors (\textit{Tbx21}), and MAPK kinases (\textit{Map2k1/2}), flagging them as potentially druggable targets. The list of identified drug targets also includes the selective retinoic acid receptor (RAR)\gamma{} agonist CD437, and the retinoid ST1926, which were predicted regulators of colonic ILC transcriptomes in CPI-induced colitis.

In conclusion, this study provides new insights into the pattern of CTLA-4/Ctla-4 expression on both human and mouse ILC, and its regulation by microbial and specific inflammatory cues. Moreover, we show that CTLA-4 plays a key role in restraining innate immune activation in the colon.

**Methods**

**Animal husbandry**

C57BL/6 and BALB/c wild-type mice (both Charles River) and BALB/c \textit{Rag2}\textsuperscript{−/−} mice (Taconic) were sourced commercially. A colony of colitis-free \textit{Rag2}\textsuperscript{−/−} x \textit{Tbx21}\textsuperscript{−/−} (TRnUC) mice was generated as described previously\textsuperscript{35}. BALB/c \textit{Ctla4}\textsuperscript{−/−} mice were kindly provided by A. Sharpe (Harvard, Boston) and used to generate \textit{Rag2}\textsuperscript{−/−} x \textit{Ctla4}\textsuperscript{−/−} mice. \textit{Rorc}\textsuperscript{GFP} mice were a kind gift of Dr Gérard Eberl. All these mice were housed in specific pathogen–free facilities at King’s College London Biological Services Unit, Imperial College London Central Biomedical Services, University College London Biological Services Unit or Charles River Laboratories. C57BL/6 germ-free mice were housed in germ-free facilities at St. George’s University London and were kindly provided by Professor Mike Curtis.

**Cell isolation**

\textit{cLP} and Peyer’s Patch-free SI LP leukocytes were isolated using a published method\textsuperscript{82, 83}. Briefly, the epithelium was removed by incubation in HBSS lacking Mg\textsuperscript{2+} or Ca\textsuperscript{2+} (Invitrogen) supplemented with EDTA and HEPES. The tissue was further digested in HBSS supplemented with 2% foetal calf serum (FCS Gold, PAA Laboratories), 0.5 mg/ml collagenase D, 10 \mu{}g/ml DNase I and 1.5 mg/ml dispase II (all Roche). The LP lymphocyte-enriched population was harvested from a 40%-80% Percoll (GE Healthcare) gradient interface. Leukocytes from human gut and blood were isolated using a published protocol\textsuperscript{84}.

**Flow cytometry**
Flow cytometry was performed as previously described\textsuperscript{50,83,85}. LIVE/DEAD\textsuperscript{TM} stain (ThermoFisher Scientific Inc.) was used to determine cell viability. Lineage cocktails used included antibodies against CD3, CD45R, CD19, CD11b, TER-119, Gr-1, CD5 and FcεRI for murine and human ILC. The FoxP3 staining kit was used for intracellular staining of CTLa-4, transcription factors and cytokines. In case of cytokine analysis, cells were pre-stimulated with 100ng/ml PMA and 2µM ionomycin in the presence of 6µM monensin for 4 hours prior to FACS analysis as indicated. Samples were acquired using an LSRFortessa\textsuperscript{TM} cell analyser (Becton Dickinson, USA) and data were analysed using FlowJo software (Tree Star, USA).

**ILC sorting and in vitro culture**

Single-cell suspensions from colonic lamina propria were stained with fluorescently labelled antibodies as described and analysed and sorted using a BD FACSARia III cell sorter (BD Biosciences). Antibodies against CD45, lineage markers and IL-7Rα were used to separate CD45\textsuperscript{+} Lin\textsuperscript{−} CD127\textsuperscript{+} cells. ILC were cultured in DMEM supplemented with 10% FCS, 1 x GlutaMax (Gibco), 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM HEPES, 1x non-essential amino acids (Gibco), 1 mM sodium pyruvate and 50 µM β-mercaptoethanol (Gibco). 20,000 sorted ILCs were plated per well of a flat-bottom 96-well plate pre-coated with 4,000 OP9-DL1 following an established method. The medium was further supplemented with rmIL-7 and rhIL-2 (both at 10 µg/ml) and further recombinant mouse cytokines as indicated. All cytokines were used at a final concentration of 10 µg/ ml unless indicated otherwise. Cells were harvested and analysed by flow cytometry after 48 hours in culture. In some conditions, the cells were stimulated with 100 ng/ml PMA and 2 µM ionomycin in the presence of 6 µM monensin for 4 hours prior to cell harvest.

**In vivo murine antibody and faecal microbiota transplant treatment**

Faecal content extracted from the caecum of TRUC mice was pelleted by centrifugation and reconstituted in sterile PBS with 25% Glycerol and then frozen down at -80\textdegree C. At the beginning of experiments, aliquots were thawed and 200 µl of the faecal solution was orally gavaged into mice. Mice were intraperitoneally administered with anti-CTLA4 (clone 9H10, BioXCell) (200 µg) and anti-PD-1 (clone RMP1-14, BioXCell) (250 µg) once per week for three weeks, as per the method used by Wei et al.\textsuperscript{58}.

**Antibiotic treatment**

Cages of mice were treated 2 weeks, prior to anti-CD40 treatment, with an Abx cocktail [1 g/L ampicillin (A), 0.35 g/L vancomycin (V), 1 g/L metronidazole (M), 1 g/L neomycin (N), Sigma] in the drinking water. Mice continued the Abx cocktail during the anti-CD40 treatment. Water bottles were changed once per week.

**Single-cell RNA-seq**

Colonic LPMCs from mice were initially sorted using a FACS Aria machine (BD Biosciences) based on live CD45\textsuperscript{+} gates and taken immediately to the 10X Chromium. Cells were suspended at 1x10\textsuperscript{6}/mL in PBS and 10,000 cells were loaded onto the Chromium\textsuperscript{TM} Controller instrument within 15 min after completion
of the cell suspension preparation using GemCode Gel Bead and Chip, all from 10x Genomics (Pleasanton, CA), and following the manufacturer's recommendations. Briefly, cells were partitioned into Gel Beads in Emulsion in the Chromium™ Controller instrument where cell lysis and barcoded reverse transcription of RNA occurred. Libraries were prepared using 10x Genomics Library Kits (3’ end V3 kit) and sequenced on an Illumina HiSeq2500 according to the manufacturer's recommendations. Read depth of more than 200 million reads per library, or an approximate average of 20,000 reads per cell was obtained with a recovery of 5000 cells.

The raw 10X Genomics sequencing libraries were processed using the Cell Ranger suite v.3.0.1 to demultiplex base call files, generate single cell feature counts for each library, and finally combine these data into one feature by barcode matrix. Read alignment and gene expression quantification made use of the CellRanger pre-built mouse (mm10 v. 3.0.0) reference data. The individual UMI count matrices were normalised to the same effective sequencing depth before they were aggregated. The merged UMI count matrix was imported in R v.3.6.1 and quality checks were carried out to mitigate the effects of technical artefacts on downstream analyses. Filtering steps were taken to remove genes detected in less than 3 cells (barcodes), and barcodes with: 1) more than 12,400 UMIs (determined as three median absolute deviations above the median barcode library size); or 2) less than 198 detected genes (determined as three median absolute deviations below the median number of genes for all barcodes after log2 transformation); or 3) expression of the epithelial cell adhesion molecule (Epcam) or collagen alpha-1(I) chain (Col1a1) that would suggest contamination by epithelial cells or fibroblasts, respectively; or 4) co-expression of genes encoding chains of the Cd3 complex (Cd3d, Cd3e and Cd3g) and those of the B cell antigen receptor (Cd79a, Cd79b) to limit further the impact of multiplets on downstream analyses. The filtered dataset was imported into Seurat v3.1.5, and the anchor-based integration workflow was followed to account for biological and technical batch differences between mice and sequencing libraries. For each sample, the UMI counts were normalised using the LogNormalize method and a scale factor equal to 10000, and the top 10000 most highly variable genes were identified using the vst method. Up to 10000 integration anchor cells were identified for each pair of count matrices after dimensionality reduction to 20 coordinates via Canonical Component Analysis. These anchor sets guided the iterative process integrating the individual sample data into one shared space with all genes passing initial quality checks. The expression data were scaled after regressing out the following sources of biological and technical variation: mouse id, sequencing library preparation and sequencing batches, number of UMIs detected for each cell and percent of UMIs for mitochondrial genes in each cell. After running Principal Component Analysis of the integrated expression data and inspecting the scree plot, 30 coordinates were used to represent the whole dataset into two dimensions using tSNE.

To cluster the integrated dataset, the k = 20 nearest neighbours of each cell were determined based the Euclidean distance of their expression profiles projected onto the first 30 principal components previously identified. A shared nearest neighbour graph was then built from these data to represent the neighbourhood overlap between pairs of cells using the Jaccard similarity index. From this graph, 24 cell clusters (identified with integers from 0 to 23) were computed by the Louvain algorithm for modularity.
optimisation with a resolution parameter equal to 0.8. Markers for each cluster were identified by
differential expression analysis using MAST\textsuperscript{87}. Only genes expressed in at least 5% of the cells in the
cluster under consideration or the rest of the cell population were tested. Genes were considered
differentially expressed if the absolute value of the natural log fold change was greater than 0.25 and the
Bonferroni-adjusted P value was less than 0.001.

Clusters were annotated to broad cell types using the SingleR (v.1.0.6) package\textsuperscript{88} and the Immunologic
Genome Project (ImmGen) transcriptomic datasets for sorted populations of mouse immune cells\textsuperscript{89}. To
this end, SingleR was used to calculate cluster-level gene expression profiles from the individual cell’s
data, and then to classify them using its correlation-based iterative algorithm with default parameter
settings. The classification process yielded confident assignments based on the pruned scores for all but
two sub-populations.

**Bulk RNA-seq analysis**

For bulk RNAseq analysis, RNA was extracted from distal colon segments and purified cells with Qiazol reagent (Invitrogen). In this method, distilled solutions of RNA were purified using chloroform and isopropanol and eluted with ethanol before being resuspended in RNAse-free water. RNA was cleaned up using the Qiagen RNeasy Microkit and performing the DNA clean-up protocol as listed in the manufacturer’s guidelines. RNA samples were then checked for quality, contamination and concentration using a NanoDrop, Qubit spectrophotometer and Bioanalyzer. RNA with only a RIN score of above 7 were used for RNA sequencing. RNA was then stored at -80°C prior to further analysis.

The quality of the raw library files was inspected with fastQC. Raw reads were trimmed and filtered to
remove adaptor contamination and poor-quality bases using trimmomatic. Depending on the source
organism, the resulting read files were mapped to the GRCm38 (mouse) genome assembly using Hisat2
with default parameters. The number of reads mapping to the genomic features annotated in Ensembl
with a MAPQ score higher than or equal to 10 was calculated for all samples using htseq-count with
default parameters.

Differential expression analysis between sample groups was performed in R using the Wald test as
implemented in the DESeq2 package. P-values were adjusted for multiple testing according to the
Benjamini and Hochberg procedure. The P- and corresponding FDR values were re-estimated empirically
with fdrtool, when the histograms of the initial P-value distributions showed that the assumptions of the
test were not met.

**Pre-clinical colitis models**

Dextran sulphate sodium (DSS) (36–50 KDa, MP Biomedicals, Ontario, USA) colitis was induced in mice
by adding 3% DSS for wild-type model and 5% for $\text{Rag2}^{-/-}$ mice to the drinking water for a period of 5
days, after which DSS was removed. Mice were culled after 5 days of being on normal water. Anti-CD40
induced colitis in Rag2−/− mice following i.p. injection of 150 µg of rat anti-mouse FGK4.5 (Bio X Cell, West Lebanon, USA). T cell transfer colitis was induced by injecting (i.p.) FACs sorted 0.5x10^6 live naïve CD4+CD25−CD62L+CD44− splenic T cells into Rag2−/− mice. DNBS colitis was induced using DNBS (Sigma-Aldrich, France) resuspended in 50 µl of 30% ethanol (EtOH) in PBS. The DNBS solution was administered on day 1 by injection with a tuberculin syringe (Terumo, France) and a flexible plastic probe inserted 3.5 cm into the colon. TRUC colitis has been described elsewhere.[35,70]

**Human samples**

Studies in human tissues received ethical approval from Guy’s and St Thomas’s Trust (REC number: 15/LO/1998). Colonic lamina propria mononuclear cells (cLPMCs) were isolated using either grids or by the digestion protocol described above from endoscopically acquired biopsy specimens. Typically, 12–16 biopsies were taken from each patient. Peripheral blood samples taken from patients and healthy controls and PBMCs were isolated using a standard ficoll density gradient centrifuge method. Isolated cLPMCs or blood PBMCs were stimulated with 100 ng/ml PMA and 2 µM ionomycin in the presence of 6 µM monensin for 4 hours and then stained for flow cytometry as described above.

**Statistics**

Results are expressed as mean. Data were analysed using Student’s t-test or Mann-Whitney U test or Kruskal-Wallis Test for grouped data, as appropriate, using GraphPad Prism 9.0 (GraphPad Inc., USA). ns: non-significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

**Declarations**

**Acknowledgements**

We thank the BRC FlowCore at King’s College London for technical help and Dr Gérard Eberl (Institut Pasteur, Paris) for providing Rorc^GFP^ mice. We also would like to thank Professor Mike Curtis (King’s College London), and Rene Ocho, Emma Mustafa and Robert Bond (all St. George’s University London) for generating germ-free mice.

**Author Contributions**

Study concept and design (GML, NP), acquisition of data (JWL, JHS, GB, LBR, DC, OSO), data analysis and interpretation (JWL, JHS, GB, LBR, OSO, DC, RGJ), technical support (EMR, FH, GMJ, ER, RM, TK), patient recruitment and consenting (HI, OSO, AC, SP), obtained funding (GML, LSKW, NP, JFN, RGJ, TK), drafting of manuscript (JWL, JHS), edits to manuscript (LSKW, RGJ), study supervision (GML, NP, LSKW, RGJ).

**Financial support**
This study was supported by grants awarded by the Wellcome Trust (NP, WT101159, LSKW, 220772/Z/20/Z) and the UKRI Medical Research Council (GL and RGJ, MR/M003493/1 and MR/R001413/1; GL, MR/K002996/1; LSKW, MR/S0091401/1). JFN acknowledges an RCUK/UKRI Rutherford Fund fellowship (MR/R024812/1), and GMJ (203757/Z/16/A) and ER (215027/Z/18/Z) were funded by a PhD fellowship from the Wellcome Trust. The research was also supported by the National Institute for Health Research (NIHR) Biomedical Research Centre at Guy’s and St Thomas and King’s College London (GL). The work of TK was supported by the UKRI BBSRC Gut Microbes and Health Institute Strategic Programme (BB/R012490/1 and its constituent projects BBS/E/F/000PR10353 and BBS/E/F/000PR10355) as well as a BBSRC Core Strategic Programme Grant for Genomes to Food Security (BB/CSP1720/1 and its constituent work packages, BBS/E/T/000PR9819 and BBS/E/T/000PR9817). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health. Work at the CRUK City of London Centre Single Cell Genomics Facility and Cancer Institute Genomics Translational Technology Platform was supported by the CRUK City of London Centre Award [C7893/A26233].

The authors have no conflict of interest to declare.

References


**Figures**
Figure 1

The immune checkpoint transcriptional landscape across intestinal ILC clusters

(A) UMAP plot showing the six ILC populations previously identified from CD45⁺ Lin⁻ CD127⁺ sorted cells by Fiancette et al. (B) UMAP plots and (C) violin plots showing the expression of canonical immune checkpoint inhibitory molecules across the six identified ILC subsets (median shown by the yellow
Figure 2

(D) UMAP plot showing the three populations of Nkp46+ sorted cells previously identified by Krzywinska et al. (E) UMAP plots and (F) violin plots showing the expression of canonical immune checkpoint inhibitory molecules across the three identified ILC populations (median shown by the yellow triangle).
CTLA-4 is present on ILC2s and NCR⁺ ILCs

(A) Representative flow cytometry plots and (B) summary dot plots from $Rag^{2/-}$ and $Rag^{2/-} \times Ctl\alpha^{4/-}$ mice showing the proportion of CTLA-4-expressing ILC1, ILC2, NKp46⁺ ILC3s and NKp46⁻ ILC3 cells. (C) Flow cytometry plot showing CTLA-4 in the lamina propria of the colon of WT mice. LPMC were stimulated with PMA, ionomycin and monensin for 4 hours. (D) Representative flow cytometry plots and (E) summary dot plots from wild-type (WT) mice and (F) $Rag^{2/-}$ mice showing the proportion of CTLA-4-positive KLRG1 and NKp46 ILC upon stimulation with PMA and ionomycin. (G) KLRG1⁺ and (H) NKp46⁺ KLRG1⁻ cLP ILC were FACS sorted and cultured for 48 hrs in the presence of rIL7, rIL2 and rIL-15 (unstim), with the addition of either PMA and ionomycin, rIL-12 and rIL-18, rIL-1β and rIL-23, rIL-25 and rIL-33 or rIL-12, rIL-18, rIL-1β, rIL-6, rIL-27 and rTGF-β (cocktail). The proportion of CTLA-4-positive KLRG1⁺ CD90.2⁺ and KLRG1⁻ NKp46⁺ CD90.2⁺ cLP ILC was measured upon seeding KLRG1⁺ and KLRG1⁻ cLP ILC, respectively. * P<0.05 ** P<0.01 **** P<0.0001 for Mann Whitney U Test for B and 2-sided Kruskal-Wallis Test for E-H.
Figure 3

ILC express CTLA-4 in a microbiota-dependent manner

(A) Representative flow plots and (B) summary dot plot showing the percentage of CTLA-4-positive NKp46+ IFNγ+ ILCs and CTLA-4 MFI in these cells, after PMA and ionomycin stimulation, in control untreated compared to antibiotic-treated mice * P< 0.05 Mann Whitney U Test. (C) Representative flow plots and (D) summary statistics showing the percentage of different ILC subsets (ILC1s, ILC2s, NCR+ proteins...
ILC3s and NCR: ILC3s), after PMA and ionomycin stimulation, that are positive for CTLA-4 in specific pathogen free (SPF) and germ-free (GF) conditions. ** P<0.01 *** P<0.001 Mann Whitney U Test.

Figure 4

CTLA-4 restrains innate immune activation in colitis
(A) Heatmap of the changes in immune checkpoint expression in distal colon segments taken from different models of colitis ($Il10^{-/-}$ (n=3), DSS (n=4), T cell transfer (n=4), DNBS (n=4), TRUC (n=4) and anti-CD40 (n=4)) compared to control mice (controls were: WT mice (n=4) for $Il10^{-/-}$, DSS and DNBS models; $Rag2^{-/-}$ mice (n=4) for T cell transfer, TRUC and anti-CD40 models. (B) Volcano plot showing the gene expression profile of distal colon segments taken from $Rag2^{-/-} \times Ctl4^{-/-}$ mice (n=4) compared to $Rag2^{-/-}$ mice (n=4). Positive log2 fold-changes indicate upregulation in $Rag2^{-/-} \times Ctl4^{-/-}$ mice, while negative log2 fold-changes indicate upregulation in $Rag2^{-/-}$ mice. (C) The most significantly upregulated differentially expressed genes involved in immune activation and interferon-stimulated genes from colon segments taken from $Rag2^{-/-} \times Ctl4^{-/-}$ mice (n=4) compared to $Rag2^{-/-}$ mice (n=4). (D) Canonical pathways (Ingenuity Pathway Analysis) activated in colon segments from $Rag2^{-/-} \times Ctl4^{-/-}$ mice (n=4) compared to $Rag2^{-/-}$ mice (n=4). (E) Upstream regulators predicted to control the gene expression changes observed in $Rag2^{-/-} \times Ctl4^{-/-}$ mice (n=4) compared to $Rag2^{-/-}$ mice (n=4). (F) Colon mass and weight change of $Rag2^{-/-} \times Ctl4^{-/-}$ mice (n=8) and $Rag2^{-/-}$ mice (n=8) treated with 5% DSS. * P< 0.05 Mann Whitney U test. (G) Representative flow cytometry plots and (H) summary dot plot showing IFN$\gamma$ and IL-17A cytokine production from ILCs from the cLP in $Rag2^{-/-} \times Ctl4^{-/-}$ mice (n=8) and $Rag2^{-/-}$ mice treated with 5% DSS. * P<0.05 Mann Whitney U test.
CTLA-4⁺ ILC1 are present in IBD patients

(A) Heatmap showing immune checkpoint molecule expression (RNA-seq) in the sigmoid colon of healthy patients (HC) (n=6) and patients with ulcerative colitis (n=15). (B) Representative flow plots and (C) summary statistics showing the percentage of different ILC subsets (ILC1s, NCR⁺ ILC3s and NCR⁻ ILC3s)
from patients with IBD (n=5) and healthy controls (n=5) that were positive for CTLA-4 after treatment with PMA, ionomycin and monensin for 3 hours. * P< 0.05 Multiple Mann Whitney U test. (D) Bar graph showing the CTLA-4 MFI in different ILC subsets (ILC1, NCR+ ILC3 and NCR- ILC3) in patients with IBD (n=5) compared with healthy controls (n=5) * P<0.05 Multiple Mann Whitney U Test.
**NCR^+ ILC are expanded in CPI-colitis**

(A) Chair plots showing differentially expressed transcripts ranked by decreasing log2 fold change and coloured by estimated false discovery rate (FDR) across the scRNA-seq in our pre-clinical model from both ILC clusters (ILC2s and NCR^+ ILC1/3s) in mice with CPI-induced colitis (n=3) vs control wild-type BALB/c mice (n=3). (B) Violin plots showing the expression levels of cytokines across the two ILC clusters in wild-type BALB/c control mice (n=3) and mice with CPI-induced colitis (n=3). (C) Pathways, identified using GSEA Hallmark, upregulated in the two ILC clusters in mice with CPI-induced colitis (n=3) vs control mice (n=3). (D) Upstream regulators predicted to mediate the gene expression changes in CPI-induced colitis vs control samples from both ILC clusters. (E) Representative flow cytometry plots and (F) summary dot plot showing IFNγ and IL-17A cytokine production from Lin^- IL-7R^+ ILCs in wild-type mice treated with CPI-colitis. (G) Colon and (H) spleen mass in untreated *Rag2^-/-* mice and *Rag2^-/-* mice given FMT only or FMT+CPI. * P<0.05 ** P<0.01 **** P<0.0001 Kruskal-Wallis Test. (I) Gr-1^+ neutrophil infiltration between untreated *Rag2^-/-* mice and *Rag2^-/-* mice given FMT only or FMT+CPI. * P<0.05 Kruskal-Wallis Test. (J) Representative flow cytometry plots and (K) summary dot plot showing IFNγ and IL-17A cytokine production from Lin^- IL-7R^+ ILCs in *Rag2^-/-* mice treated with CPI-colitis. (L) Summary statistics showing the percentage of PMA, ionomycin-treated ILC subsets (ILC1, ILC2 and ILC3) in peripheral blood from healthy controls (n=40), patients with IBD (n=31) and patients with CPI-induced colitis (n=7) *P< 0.05 **P<0.01.

**Supplementary Files**

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- Supplementaryfigures.pdf