

Regulation of human intestinal T-cell responses by Type 1 Interferon-STAT1 signalling is disrupted in inflammatory bowel disease

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Conflict of Interest

No authors had any conflict of interest in the preparation of this manuscript.

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Author Contribution

EG designed, performed and analysed the experiments and wrote the manuscript. AS designed experiments and wrote the manuscript. TS performed experiments and wrote the manuscript. NM performed experiments and wrote the manuscript. MP and JL performed and analysed experiments. JOL and TTM designed experiments and assisted in the manuscript preparation.

Keywords

Interferon, T cell, Inflammatory Bowel Disease, STAT1, human

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ABSTRACT

Type 1 Interferon (IFN-1) promotes regulatory T-cell function to suppress inflammation in the mouse intestine, but little is known about IFN-1 in the human gut. We therefore assessed the influence of IFN-1 on CD4⁺ T-cells isolated from human colon tissue obtained from healthy controls or patients with inflammatory bowel disease (IBD). Immunofluorescent imaging revealed constitutive expression of IFN β in human intestinal tissue, and colonic T-cells were responsive to exogenous IFN-1 as assessed by phosphorylation of Signal Transduction and Activator of Transcription 1 (pSTAT1) and induction of Interferon Stimulated Genes (ISGs). Unlike their blood counterparts, intestinal T-cells from non-inflamed regions of IBD colon displayed enhanced responsiveness to IFN-1, increased frequency of pSTAT1⁺ cells, and greater induction of ISGs upon IFN-1 exposure *in vitro*.

In healthy tissue, antibody neutralisation of IFN β selectively reduced T-cell production of the pro-regulatory cytokine IL-10 and increased IFN γ synthesis. In contrast, neutralisation of IFN β in IBD tissue cultures increased the frequency of T-cells producing inflammatory cytokines but did not alter IL-10 expression. These data support a role for endogenous IFN-1 as a context-dependent modulator of T-cell function that promotes regulatory activity in healthy human intestine, but indicate the IFN-1/STAT1 pathway is dysregulated in inflammatory bowel disease.

INTRODUCTION

In the healthy intestine, regulatory mechanisms limit T-cell responsiveness to the commensal microbiota and environmental antigens in order to maintain homeostasis,

but dysregulation of these mechanisms can lead to pathological inflammation and development of inflammatory bowel disease (IBD). Accordingly, intestinal T-cells remain one of the main targets for IBD therapy¹, but the endogenous factors that regulate T-cell activity in the human gut are not fully understood.

The balance of T-cell regulatory and effector functions are determined partly during priming in gut-associated lymphoid tissue or mesenteric lymph nodes. Signals derived from the activating dendritic cell (DC) and local environment can influence the differentiation of naive CD4⁺ T-cells towards a variety of effector or regulatory fates. However, it is now clear that T-cell properties are not fixed during priming but instead these cells retain functional plasticity and their function can be modified by signals encountered later upon homing to the intestinal mucosa.^{2, 3, 4}

Type 1 Interferon (IFN-1), predominantly IFN α and IFN β , has emerged as a potent context/timing-dependent modifier of T-cell function.⁵ IFN-1 binds to the IFN-1 receptor (IFNAR) and can differentially activate members of the Signal Transduction and Transcription (STAT) family to determine downstream cytokine production.⁶ For example, in murine T-cells IFN-1 signalling via STAT1 inhibits IFN γ production, whereas signalling via STAT4 instead promotes IFN γ .⁷

IFN-1 limits viral replication in acute infection, but can also contribute to viral persistence in chronic infection models via the induction of IL-10 producing CD4⁺ T-cells. This suggests a potential role for IFN-1 in chronic T-cell-driven inflammatory disorders.^{8, 9, 10} In mice, constitutive low-level production of IFN β occurs in several tissues even in the absence of viral infection.¹¹ This suggests that, at least in mice, endogenous IFN-1 may regulate T-cell expression of STAT proteins and control responsiveness to subsequent stimuli.⁶ In murine models, IFNAR

deficiency increases susceptibility to Dextran Sodium Sulphate (DSS) colitis¹², whereas administration of IFN-1 can ameliorate DSS colitis via a mechanism that depends on TLR9/bacterial DNA^{13, 14}. However, reports that local delivery of IFN-1 by a transgenic *Lactobacillus acidophilus* led to increased severity of DSS colitis¹², underscore the complex role of IFN-1 in maintaining mucosal integrity.

In a T-cell transfer model of colitis, IFN-1 signalling induced by poly(I:C) can ameliorate gut inflammation via a mechanism thought to require both FoxP3+ regulatory T-cells^{15, 16}, and IFNAR expression by the grafted colitogenic T-cells¹⁷. In human multiple sclerosis, therapeutic administration of IFN-1 promotes T-reg IL-10 expression while restricting IL-17-driven inflammation¹⁸. While genome-wide association studies have detected *IFNAR* variants associated with increased risk for developing IBD¹⁹, IFN-1 therapy displayed only limited efficacy in human IBD²⁰. Given that constitutive expression and function of IFN-1 in human intestinal immunity is not known, we aimed to determine how IFN-1 influences signalling pathways at single-cell resolution in defined populations of intestinal T-cells in health and IBD.

RESULTS

IFN-1 is constitutively expressed in human colon and modulates the function of intestinal T-cells

To assess whether IFN-1 influences T-cell function in the human intestine, we first sought evidence that T cells are responsive to IFN-1 in human colon. To do this, we assessed mRNA expression levels of interferon stimulated genes (ISGs; myxovirus resistance A - *MxA* and 2'-5' oligoadenylate synthetase - 2'5'OAS) in cells migrated out of human colonic biopsy tissue during overnight incubation in the presence or absence of IFN-1. The intestinal T-cells were then isolated by FACS sorting and

subjected to qRT-PCR. Alternatively, cells were obtained without IFN-1, but then exposed or not to brief (15 minute) IFN-1 stimulation, and then permeabilized for assessment of pSTAT1 expression. Both approaches revealed that IFN-1 treatment of either healthy colon or IBD tissue increased intestinal T-cell expression of *MxA* (median 30-fold, figure 1A) and 2'5'OAS (median 50-fold, figure 1B), and induced a ~10-fold increase in the frequency of CD4⁺ T-cells expressing pSTAT1 (Figure 1D, representative FACS plots figure 1C).

We next sought to identify direct evidence of IFN-1 in the human colon, focusing on the IFN β isoform thought to co-ordinate many key effects of IFN-1⁶. Technical challenges have hampered previous attempts to directly detect IFN-1 in human tissues, but a recent publication successfully observed IFN β expression in human skin using IHC analysis of freshly frozen clinical samples²¹. We therefore used a similar IHC approach to visualize endogenous IFN β in colonic tissue. IFN β was detected, distributed in the epithelium and also throughout the lamina propria in close association with intestinal T-cells, in both controls and IBD patients (Figure 1 E-F and supplementary Figure 1).

We next sought evidence that human intestinal T-cells can be influenced by IFN-1 exposure *in situ*. We detected pSTAT1 in both naïve and memory subsets of CD4⁺ T-cells both before and after IFN-1 stimulation, but the frequency of pSTAT1⁺ cells was significantly higher in the stimulated memory cell population (supplementary Figure 2). Accordingly, expression of the T-cell activation marker CD69 was detected only among CD45RA⁻ memory cells, and the CD69⁺ population included a higher frequency of pSTAT1⁺ cells, suggesting increased responsiveness to IFN-1 specifically in the activated memory T-cell compartment (supplementary Figure 3).

Since these cells exhibited low-level 'constitutive' phosphorylation of STAT1 and expression of ISGs even in the absence of IFN-1 supplementation (Figure 1 A-D), we next assessed whether exposure to endogenous IFN-1 during the cell isolation process was sufficient to induce STAT1 signalling. To do this, we established walk-out cultures in the presence or absence of neutralising anti-IFN β antibody and assessed T-cell expression of total STAT1 and pSTAT1. As phosphorylation is a brief process and the neutralisation was conducted over the 24 hour walk-out period, it was felt more likely to detect an effect in total STAT1 expression, which is known to be influenced by IFN-1 signalling ⁶. Neutralisation of IFN β significantly reduced the frequency of U-STAT1+ CD4+ T-cells detected (Figure 2 A and B), suggesting that constitutively produced IFN β was biologically active in these cultures. Additionally, these cells had reduced responsiveness to further IFN-1 stimulation with decreased frequency of pSTAT1+ cells (Figure 2 C and D). This is consistent with endogenous IFN β priming CD4+ T cells in their responsiveness to IFN-1. In contrast, addition of neutralising anti-IFN γ did not significantly alter STAT1 phosphorylation or total STAT1 levels (data not shown). Collectively these data support a role for IFN β produced constitutively within human intestinal tissue as a potential modulator of gut T-cell function, signalling at least in part via STAT1 activation.

Phosphorylation of STAT1 and IFN β responsiveness are enhanced in intestinal CD4+ T-cells from IBD patients.

In order to assess whether IFN-1:STAT1 signalling is altered in human IBD, we next compared CD4+ T-cell responsiveness to IFN β and pSTAT1 expression between IBD patients and control donors. To do this, we focused our analyses on cells isolated from macroscopically and histologically non-inflamed areas of colon in order to exclude

signalling signatures associated with active inflammation. Even in unstimulated tissue cultures, we observed marked expression of pSTAT1 among CD4⁺ T-cells isolated from the intestinal mucosa (Figures 1 C and D), although the total frequency of pSTAT1⁺ cells was significantly higher in colonic tissue from IBD patients (Figure 1D). Upon supplementation with exogenous IFN-1, both IBD colon samples and control tissues exhibited ~10-fold increases in the proportion of derivative cells containing pSTAT1 (Figure 1D). Similar data were obtained in parallel analysis of samples of inflamed mucosal tissue from IBD patients, although the total proportion of pSTAT1⁺ cells detected was lower (Supplementary Figure 4). These data indicated that under both resting and inflammatory conditions, CD4⁺ T-cells in IBD colon exhibit increased STAT1 activation compared with their counterparts in control tissue samples. We therefore proceeded to investigate whether the increased levels of pSTAT1 detected in CD4⁺ T-cells in IBD tissue could indicate enhanced Th1 polarization, as described in an earlier report²². However, our analyses revealed that pSTAT1 was expressed at comparable levels in both Tbet⁺ (Th1 cells) and Tbet⁻ subsets of gut CD4⁺ T-cells (Figure 3A-C), indicating that pSTAT1 was not a surrogate marker of Th1 cells in this setting.

Collectively these data demonstrated that STAT1 phosphorylation and putative IFN-1 signalling were modulated in T-cells that migrated out of IBD colon, irrespective of whether the tissue was overtly inflamed. Conceptually, these effects could reflect intrinsic T-cell properties, exposure to soluble factors released by the cultured tissue, or a combination of these effects. To test for the presence of IBD-associated factors that might initiate STAT1 phosphorylation, we assessed the ability of IBD tissue culture supernatants to induce pSTAT1 in CD4⁺ T-cells derived from control colon tissue. Despite extensive

investigation, we found no consistent evidence that soluble factors released from IBD tissue could up-regulate pSTAT1 in healthy colonic CD4+ T-cells (data not shown). These findings indicated that IBD-associated changes in STAT1 phosphorylation and responsiveness to IFN-1 were at least in part due to intrinsic properties of the T-cells. Accordingly, we observed that gut T-cells from IBD patients exhibited greater IFN-1-induced ISG expression (Figure 3D-E). Furthermore, we detected a strong trend towards reduced expression of the STAT1 regulator *SOCS1* in intestinal T-cells freshly isolated from IBD patients (Figure 3F). Taken together, these data indicate that altered IFN-1 responsiveness and STAT1 phosphorylation in IBD are at least in part due to changes in gut T-cell-intrinsic properties.

IFN β :STAT1 signalling exerts differential effects on T-cell function in healthy tissue and IBD colon

When we assessed the ability of IFN-1 to activate alternative STAT family members in intestinal CD4+ T-cells, we observed marked phosphorylation of both STAT3 (2-fold increase) and STAT5 (4-fold increase) in response to IFN-1 exposure, but we did not detect any differences between patients and controls either at baseline or in the cytokine-treated cultures (Figure 4A). These data suggested that differential effects of IFN β on STAT activation in T-cells from IBD patients were specific to STAT1. We therefore proceeded to investigate whether the increased IFN-1 responsiveness and STAT1 phosphorylation observed in IBD patient T-cells was restricted to the gut or reflected system-wide changes in T-cell function. To do this, we analysed CD4+ T-cells in peripheral blood and observed that the trace numbers of pSTAT1+ cells were significantly increased by IFN-1 stimulation, particularly within the CD45RA- memory/effector compartment

(32.1%±22 pSTAT1+ post stimulation) in CD45RA- cells versus 18.2%±15.2 in CD45RA+ cells, $p < 0.05$ ANOVA). However, we observed no significant difference in IFN β responsiveness between IBD patients and controls even when comparing gut-homing CD4+ T-cells ($\beta 7$ integrin+) with CD4+ T-cells targeted to other sites ($\beta 7$ -negative; Figure 4B). These data indicated that IBD-associated changes in IFN-1 responsiveness and pSTAT1 expression of intestinal CD4+ T-cells are local effects that do not reflect systemic alterations in these patients.

There is increasing evidence from murine models that constitutive IFN β expression can support regulatory T-cell responses^{15, 16}, but the function of this cytokine in the human intestine is currently unknown. To address this question, we obtained T-cells from colonic biopsy tissue cultured in the presence or absence of neutralising anti-IFN β antibody and measured cytokine production and expression of master regulator transcription factors upon re-stimulation with anti-CD3 and anti-CD28. Addition of anti-IFN β to the biopsy cultures did not alter T-cell expression of the transcription factors T-bet or FoxP3 as assessed by intracellular staining (Figure 5A, B), and ROR γ t expression was very low throughout (data not shown). Nonetheless, intracellular cytokine staining of cells isolated from healthy tissue revealed that anti-IFN β treatment induced a significant decrease in the frequency of CD4+ T-cells producing IL-10 (Figure 5C), with a trend towards an increased frequency of IFN γ -producing cells (Figure 5D), accompanied by a significant increase in supernatant concentration of IFN γ detected by ELISA (Figure 6B). TNF α and IL-17A production were also observed, although IFN β neutralisation failed to alter the amount detected either by intracellular staining (Figures 5E and 5F) or ELISA measurement (Figures 6C and 6D). These findings suggest a potentially important role for endogenous IFN β in modulating the

balance of IFN γ and IL-10 production by local T-cells in healthy tissues to favour the expression of anti-inflammatory cytokines. This effect is likely due to functional modulation by environmental signals rather than altered T-cell differentiation fates, since the transcription factor profiles observed were not altered by IFN-1 exposure.

These 'pro-regulatory' effects of IFN β treatment in control tissues were absent when we repeated these experiments using colon samples from IBD patients, even though the tissues analysed were obtained from non-inflamed regions of the intestine (macroscopically and histologically normal; Figures 5 and 6). In IBD biopsy organ culture, treatment with neutralising anti-IFN β induced an increase in the proportion of intestinal T-cells producing IFN γ , TNF α and IL-17A, and trend towards increased production of IL-10 (Figure 5A), accompanied by a significant increase in supernatant IL-10 levels as assessed by ELISA (Figure 6A). Consistent with previous reports, T-cells expressing T-bet or Foxp3 were more frequent in IBD tissue than control samples (Supplemental Figure 5)^{23, 24}, but as observed in healthy tissue expression of these transcriptional regulators was not altered by neutralisation of IFN β (figures 5A and 5B). Therefore, endogenous IFN β has a pro-regulatory effect in healthy tissue, where this cytokine promotes IL-10 production, whereas in IBD our data indicate that IFN β instead restricts a number of effector T-cells producing cytokines in the intestine.

DISCUSSION

This study reports that endogenous IFN β promotes STAT1 phosphorylation in human intestinal T-cells to exert pro-regulatory effects in the healthy gut. We also present evidence that this axis is disrupted in IBD, where endogenous IFN β instead restricts effector T-cell cytokine responses, even when assessing macroscopically and histologically non-inflamed areas of the gut.

We observed that T-cells isolated from human intestinal tissue were responsive to IFN-1 *in vitro*, as demonstrated by phosphorylation of multiple STAT signalling proteins and marked induction of ISG expression. The induction of ISG expression was on total (CD3+) T cells and the differential contribution of CD8 and CD4 populations cannot be determined. We also detected that a proportion of T-cells migrating out of cultured biopsy tissue expressed the phosphorylated form of STAT1, which is the primary family member involved in IFN-1 signalling. This finding suggested that gut T-cells may be exposed to IFN β cytokine *in situ*, and accordingly we observed for the first time that IFN β is present in human colonic tissue. It is unclear what the source of the IFN-1 is although the epithelium appears to be important and this would be an interesting area for further research.

There is increasing evidence from murine models that constitutive production of IFN-1, and IFN β in particular, can exert immunoregulatory effects^{15, 16, 25}. Consistent with this concept, neutralisation of IFN β in biopsy cultures from healthy donors significantly reduced the generation of IL-10+ T-cells, whereas the frequency of IFN γ + T-cells and quantity of IFN γ detected in the culture supernatants were increased.

The differences between the intracellular staining of the intestinal T cells compared to the ELISA data may be due to the contribution of other cell types in the supernatants

and their differential response to IFN β . It is likely that other IFN-1 subtypes are also present in the environment and it is not possible to conclude about the functional importance of all IFN-1 in this system. However, the overall findings are consistent with data from the murine T-cell transfer model of colitis, in which IFN-1 was found to be critical for maintaining regulatory T-cell function^{15, 16}. However, in contrast with the experimental models, our data did not demonstrate any effect of IFN-1 on expression of FoxP3, which is less tightly linked to regulatory function in the human system²⁶.

It has been suggested that the IFN-1 effects on T-cell function are context dependent²⁷. Consistent with this concept, our data demonstrate that the effects of IFN β differ in healthy and IBD mucosa. Our findings are best explained by plasticity of intestinal T cells and their responsiveness to local environmental signals. Our analysis provided no evidence for altered differentiation to major T cell fates as defined by expression of master transcriptional regulators.

Phosphorylation of STAT1 in gut T-cells was demonstrated within 15min of exposure to IFN-1, suggesting a direct effect of IFN-1 on T cells, but it is not possible to exclude a contribution from other cell types in these cultures to many of the effects measured. The varied results obtained by ELISA analysis of supernatants from both control and IBD tissue cultures (Figures 6 and 8) do suggest a differential role of IFN-1 on non-T-cells, although the small tissue samples available precluded further investigation of this issue. Indeed, an early study on the role of interferon on LPMCs suggested that, in regards to cytotoxicity, LPMCs from IBD patients can be relatively *hyporesponsive* to exogenous interferon²⁸.

Several of our observations indicate altered IFN-1 responsiveness and STAT1 phosphorylation of gut T-cells in IBD. For this analysis, we focused primarily on tissue sampled from macroscopically and histologically non-inflamed areas of intestine in order to identify underlying immunoregulatory changes that might otherwise be masked by ongoing inflammatory processes. The frequency of pSTAT1+ CD4+ T-cells detected was higher in non-inflamed IBD colon than in control tissue both in unstimulated cultures and after exposure to IFN-1 *in vitro*. This difference was specific for STAT1 in the intestinal mucosa, since we were unable to identify any modulation of alternative IFN-1-sensitive STATs or detect any changes in T-cells derived from the blood. Indeed, we were also unable to induce phosphorylation of STAT1 in control T-cells upon incubation with the culture supernatant of IBD biopsies, suggesting that enhanced pSTAT1 expression by IBD T-cells is an intrinsic property of these cells rather than being conferred by a soluble factor.

Previous studies have typically focused on analysing STAT expression in whole samples of inflamed IBD tissue (containing numerous different cell types). Instead, we have for the first time used a novel phosflow-based method to measure the phosphorylated forms of STAT proteins at the single cell level in distinct subsets of human leukocytes in the absence of overt mucosal inflammation. Using this approach, we observed an increased frequency of pSTAT1+ intestinal CD4+ T-cells in tissue from IBD patients, consistent with a previously shown trend for increased pSTAT1 levels in whole tissue homogenates, and increases in total STAT1 protein identified in CD4+ lamina propria cells²⁹. However, we did not observe the previously reported increases in STAT3 expression and phosphorylation in IBD^{29, 30}, which may reflect

cell-type specific differences or could perhaps be attributed to our approach of analysing non-inflamed tissue.

In addition to increased expression of pSTAT1, intestinal CD4⁺ T-cells from non-inflamed IBD intestinal tissue were significantly more responsive to IFN-1 stimulation as assessed by induction of ISG expression. Given the direct relationship between exposure to IFN-1 and expression of this gene set, this observation again suggests direct effects of IFN-1 on the T-cell population. Suppressor of Cytokine Signalling 1 (SOCS1) is a major regulator of STAT1 signalling in T-cells, and conditional KO of *Socs1* in murine T-cells has been shown to exacerbate colitis³¹. SOCS1 is induced by IFN-1 and acts via numerous mechanisms as part of a negative feedback loop in IFN-1-STAT1 signalling³². In our study, there was a strong trend towards reduced SOCS1 expression in intestinal T-cells freshly isolated from non-inflamed IBD tissue; we observed a corresponding increase in total STAT1 levels in these cells. It is therefore likely that reduced SOCS1 control of STAT1 signalling contributes to increased IFN-1 sensitivity of gut T-cells in IBD. Consistent with this altered responsiveness to IFN-1, we further observed that neutralization of IFN β during IBD biopsy culture increased the frequency of derivative T-cells producing IFN γ , TNF α or IL-17A. These data suggest that the non-inflamed IBD mucosa represents a modified signalling environment in which endogenous IFN β acts to restrain production of pro-inflammatory cytokines rather than promoting IL-10 expression, perhaps to prevent the development of overt inflammation. We also detected a strong trend towards reduced ISG expression by gut T-cells when measured directly after isolation from IBD tissues compared with control tissue. Based on these observations, we propose that in IBD tissue the constituent T-cells exhibit altered responsiveness to endogenous

IFN β and other subsequent environmental signals, which could in part explain the seemingly conflicting early data on the role these cytokines play in IBD.

While the efficacy of IFN-1 treatment in IBD has been largely disappointing²⁰, there have been promising early results from a phase 1 trial of UC patients treated with the IFN-1-inducing TLR9 agonist DIMS0150³³. More recently, it has been demonstrated that patients with either UC or Crohn's disease have a distinct 'virobiota' compared to household controls³⁴, suggesting that IFN-1 may be an important factor in the complex interactions between viruses, bacteria and the host immune system during the pathogenesis of IBD.

In summary, this study demonstrates that human colon constitutively expresses IFN β , which has a selective STAT1-dependent pro-regulatory effect on cytokine production by intestinal T-cells. This finding is consistent with mouse models in which IFN-1 signalling via STAT1 inhibits IFN γ production³⁵. Our observations are also in agreement with the concept that the balance of STAT proteins and their activation determines the outcome of subsequent exposure to IFN-1 and other local triggers. Specifically, in this view IFN-1 maintains a basal repertoire STAT expression that forms a platform for specific T-cell responses⁶.

This IFN-1-shaped level of T-cell responsiveness would be an important determinant of local immune responses against viruses and other pathogens in the human intestine while preventing an excessive inflammatory response.

METHODS

Patient samples and cell isolation

Intestinal tissues were collected from patients with IBD during endoscopy or surgery, and control tissues were collected from subjects who were undergoing follow-up because of rectal bleeding, altered bowel habits, or cancer. The diagnosis for each patient was made using clinical parameters, radiographic studies, and histological criteria. All tissue and blood donors gave informed written consent, and the study was approved by the local research ethics committee. Patient information is presented in supplementary information.

Cells were extracted from intestinal tissue by enzymatic digestion or “walk-out” culture as previously described^{36, 37, 38}. Briefly, the tissue was treated with 1mmol/L dithiothreitol (Sigma-Aldrich St. Louis, MO, USA) to remove faeces and mucus, followed by 1mmol/L EDTA (Sigma-Aldrich) in Hank's balanced salt solution (Sigma-Aldrich) to remove epithelium. Digestion was by 1 mg/mL collagenase D (Roche Applied Science Penzberg, Upper Bavaria, Germany) in HEPES-buffered RPMI 1640 medium (Sigma-Aldrich) containing 2% foetal calf serum (PAA Little Chalfont, UK) and 20 µg/mL deoxyribonuclease I (Roche Applied Science). A lamina propria (LP) cell suspension was passed through a 40µm cell strainer. Alternatively, biopsies were cultured for “walk-out” following EDTA processing. The denuded tissue was placed in 0.5mL complete medium (RPMI1640 Dutch Modification supplemented with 2mM L-glutamine, 100U/mL penicillin, 100µg/mL streptomycin, 100µg/mL gentamicin (all Sigma-Aldrich) and 10% heat-treated (56 °C for 30 minutes) foetal calf serum (FCS; PAA). Cultures were established in 24 well plates (Becton Dickinson) at one biopsy in

0.5 mL of medium per well and placed in a humidified incubator at 37 °C with 5% CO₂ in air. In some experiments the medium was supplemented with IFN β (Peprotech) at 1000IU/ml or with an equal volume of the diluent Phosphate Buffer Saline (PBS) as a control. Alternatively, human anti-IFN β antibody or an isotype-matched mouse IgG1 control antibody (both from Biolegend) were added at 1 μ g/ mL (Biolegend) The biopsies were then discarded after 48hrs culture and egressed cells collected from the remaining medium by centrifugation at 400 x g for 5 minutes. Typically, 80% of viable cells isolated from non-inflamed or control samples were CD3+, and 75% of these cells were CD4+. Viability was assessed using Live/Dead™ staining.

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors or patients by centrifugation over Ficoll-Paque PLUS (GE Healthcare Little Chalfont, UK) at 650g for 20min.

Antibody Labelling and Flow Cytometry

Cells were incubated with monoclonal antibodies on ice for 15 minutes in 100 μ L fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline with 2%v/v FCS mmol/L EDTA and 0.2% sodium azide), washed by centrifugation, and fixed in 1% (wt/vol) paraformaldehyde. Cells were acquired on a FACSCanto II (BD Biosciences), using CellQuest software (BD Biosciences) and analyzed using WinList 6.0 software (Verity Topsham, ME). Positively stained cells were identified relative to isotype-matched control antibody staining. A full list of the monoclonal antibodies used is provided in Supplementary Materials.

Analysis of phosphorylated antigens by flow cytometry

A technique for measuring intracellular phosphorylated antigens in single cells was developed and optimized as part of this study. LPMCs isolated by walk-out were re-suspended in 100 μ L complete medium with gentamicin in 5mL FACS tubes and either left unstimulated or stimulated with 40,000 IU/mL IFN α 2A (Peprotech), for 15min in a 37°C water bath. The cells were then fixed by addition of an equal volume of 4 % w/v paraformaldehyde (PFA) for 10min in a 37°C water bath.

The fixed cells were washed by centrifugation in PBS and then incubated in 1mL of 70% cold methanol (VWR, USA) on ice for 30min for permeabilisation. The cells were washed twice in FACS buffer and then re-suspended in 100 μ L FACS buffer for simultaneous cell surface and intracellular antibody labelling. Cells were labelled as appropriate and incubated for 30-60min at room temperature. After one further wash with FACS buffer the cells were re-suspended in 300 μ L of FACS buffer and analysed by flow cytometry as described.

Stimulation of T-cells for cytokine analysis

LPMCs were cultured in 200 μ L complete medium in 96 well plates (Beckton Dickinson), and Low Endotoxin Azide Free (LEAF) anti-CD3 and anti-CD28 antibodies (Biolegend) were added each at a final concentration of 1 μ g/mL. The plates were put in a humidified incubator at 37°C with 5% CO₂ in air for 20h. After 20 hours the cells were collected into 5mL FACS tubes, and the Golgi-blocking agent monensin (VWR) was added at 3 μ M final concentration and the tube contents mixed thoroughly. The FACS tubes were replaced in the incubator for 4 hours. Cells were then collected and

processed by flow cytometry after centrifugation and supernatants were stored at -80°C.

Isolation of intestinal or peripheral T-cells by flow sorting

LPMCs, collected by walk-out technique, were washed by addition of excess FACS buffer, centrifugation at 400 x g for 5 min and discarding of supernatant. The cells were then resuspended into 100 µL filter sterilised (0.2 micron) modified FACS buffer (lacking sodium azide) and incubated with relevant monoclonal antibodies for 15 minutes on ice protected from light. Cell populations within LPCs were isolated by flow-sorting using a FACS Aria cytometer (Becton Dickinson). In order to sort only viable T-cells, the agent 7-amino-actinomycin D (7-AAD; Biolegend) was used in combination with CD3⁺ labelling.

Quantitative Real-Time Polymerase Chain Reaction

RNA extraction and reverse transcription were performed using the RNeasy Micro Kit and QuantiTect Reverse Transcription Kit, respectively. Quantitative real-time polymerase chain reaction (PCR) was performed using the QuantiFast SYBR Green PCR Kit (all from Qiagen Venlo, Limburg, Netherlands) on a 7500 Real-Time PCR System (Applied Biosystems Foster City, CA). Full primers are listed in Supplementary Materials. Expression data were normalized to GAPDH using the $2^{-\Delta Ct}$ method.

Multiplex ELISA

Measurement of TNF α , IL-10, IFN γ , and IL-17A in culture supernatants was performed using the FlowCytomix™ Multiplex Kit (eBioscience) according to the manufacturer's instructions. Data was acquired on a Canto II Flow Cytometer (BD Bioscience), set up

as recommended by the kit manufacturer and data analysed using FlowCytomixPro™ (eBioscience) software.

Immunohistochemistry

Colonic biopsies were snap frozen in liquid nitrogen. Slides were prepared by the pathology service at the Royal London Hospital (Barts Health, UK). Slides were fixed with addition of 1 mL of PFA 4%. 1 mL blocking serum (5% BSA, 0.1% Tween in PBS) was added followed by addition of primary antibodies. The antibodies used were as shown in supplementary table XXX. Secondary antibodies were applied: AF555 goat anti-mouse IgG (Invitrogen, USA), and/or AF488 donkey anti-rabbit IgG (Invitrogen, USA). Mounting medium was with DAPI (Vectorshield, Vector Laboratories, USA).

Analysis was performed with a Research Systems Microscope BX61 (Olympus), with digital image capture at 100x (standard) or 200x (high power) using the software system SmartCapture v3.0 (Digital Scientific, UK).

Statistics

Statistical analyses were performed using SigmaStat 3.5 (Systat Software San Jose, CA). Comparisons between 2 groups of paired, normally and non-normally distributed data were analyzed using Paired t test, Student t test and Signed rank test, respectively. Data sets of more than 2 groups were compared by analysis of variance (ANOVA) with pairwise comparisons by Holm–Sidak test. $P < 0.05$ was regarded as statistically significant.

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Figure Legends

Figure 1. Human Intestinal T cells are responsive to IFN-1 in health and IBD. A-B Intestinal samples were isolated from controls (n=8) or IBD patients (n=10) in the presence of IFN-1 for 24 hours or Unstimulated (US) and cells were sorted for CD3+ viable cells. RNA was extracted and expression of ISGs MxA (A) and 2'5'OAS (B) were measured by qRT-PCR. **C** Representative flow cytometry plots of cells isolated by walk out. Cells were stimulated (lower panel) or not (upper panel) IFN-1 for 15 minutes. **D** Combined results of controls (n=16) and IBD patients (n=24) showing frequency of pSTAT1+ CD4+ T cells with IFN-1 stimulation or not (US). White boxes indicate 1st to 3rd quartiles with middle line indicating median value. **E-F** Fresh frozen sections prepared from control colon tissue for immunohistochemistry. CD3 as green (AF488). **E** isotype staining mIgG1 (AF555 - red). **F** IFN β (AF555 - red). Representative of 5 independent experiments.

Figure 2 Endogenous IFN-1 supports U-STAT1 expression and IFN-1:pSTAT1 responsiveness in human intestinal CD4+ T cells. A-D LPMCs were walk-out in the presence of anti-IFN β antibody or isotype control. Cells were analysed by flow cytometry gated on scatter and CD4+. **A** Expression (total) (U-)STAT1 in CD4+ intestinal T cells (n=20). **B** As per A, showing paired values from control samples only (n=8). **C** pSTAT1 expression in CD4+ intestinal t cells (n=20). **D** As per C, showing paired values from control samples only (n=8).

Figure 3 Intestinal T cells are more responsive to IFN-1 in IBD, not simply due to an increase Th1 phenotype. LPMCs were isolated by walk-out and processed for phosflow (A-C) or intestinal T cells were flow sorted and mRNA processed for qRT-PCR (D-F). **A** Representative flow cytometry plot of pSTAT1 expression in T-bet+ (black line) and T-bet (grey line) gated on CD4+ T cells (dotted line mIgG2A isotype). **B** 2D representative FACS plot of CD4+ T cells showing co-expression of T-bet and pSTAT1. **C** Proportion of all T-Bet+CD4+ T cells that are also pSTAT1+ (n=23). Grey line indicates median. **D-E** Expression of ISGs MxA (D) and 2'5' OAS (E) after 24 hours walk-out cell isolation in the presence of IFN-1, comparing the samples with IFN-1 to those without in controls (n=8) and IBD (n=10) **F** Intestinal T cells were isolated immediately by collagenase digestion before RNA extraction. Expression of SOCS1 from controls (n=8) and IBD (n=10). Bars indicate median expression with lines indicating standard deviation.

Figure 4. IBD-associated alteration in STAT phosphorylation are specific for STAT1 and are not seen in the peripheral blood. A Walk out cells were isolated from controls (n=16) or IBD patients (n=24), and either stimulated with IFN-1 for 15 minutes or not (US). The percentage of pSTAT3+ (left) or pSTAT5+ (right) CD4+ T cells are shown. **B.** PBMCs were isolated from IBD patients (n=18) and age matched controls (8). Cells were analysed for pSTAT1 signal after 15 minute stimulation with IFN-1, depending on T cells subset, CD3+CD4+CD45RA β 7 γ (memory β 7 γ) CD3+CD4+CD45RA β 7 γ (memory β 7 γ) or CD3+CD4+CD45RA+ (naive).

Figure 5 IFN β neutralization decreases IL-10 production from intestinal CD4+ T cells in healthy controls, but increases multiple cytokines in samples from

non-inflamed IBD samples. LPMCs were isolated in the presence of anti-IFN β or isotype control (mIgG1). LPMCs were then collected and stimulated with CD3/CD28 antibodies for 24 hours. Intracellular cytokines or transcription factors were measured by flow cytometry as follows: A T-bet, B FoxP3 C IL-10, D IFN γ E TNF α and F IL-17. Cells were gated on scatter and CD4 $^+$

Figure 6 IFN β neutralisation increases IFN γ concentrations in supernatants from control mucosa, but increased IL-10 concentrations from non-IBD mucosa. Cells were isolated in the presence of neutralising anti-IFN β antibody or isotype control (mIgG1). The supernatant was collected and IL-10 (A), IFN γ (B), TNF α (C), IL-17 (D), and IFN α and were measured by multiplex ELISA (n=11 controls, n=11 IBD)

Supplementary Figures

Supplemental figure 1. IFN β was detectable in the lamina propria of control colon. Fresh frozen sections prepared from control colon tissue for immunohistochemistry. CD3 as green (AF488). **A** isotype staining mIgG1 (AF555 - red) in control sample. **B** IFN β (AF555 - red). **C** isotype in control with lymphoid follicle seen. **D** IFN in lymphoid follicle (same as C) Representative of 5 independent experiments

Supplemental figure 2. Most Walk-out cells are memory and these are the responsive cells. **A** CD4 $^+$ T cells are shown demonstrated CD45RA staining. **B** CD45RA $^+$ (naive) or **C** CD45RA $^-$ (memory) CD4 $^+$ T cells after IFN-1 stimulation. **D** pSTAT1 $^+$ expression in gated CD4 $^+$ intestinal T cells based on CD45RA expression. Representative of 7 experiments. Statistical analysis by paired t test.

Supplemental figure 3. T cell activation marker CD69 is only seen in memory cells and correlates with pSTAT1 expression. **A** Representative FACS plot of gated CD4 $^+$ walk-out intestinal T cells showing CD45RA and CD69 co-expression. **B** CD45RA $^-$ CD69 $^+$ and **C** CD45RA $^+$ CD69 $^+$ showing pSTAT1 expression after IFN-1 stimulation. Representative of 7 experiments. **D** Combined data comparing pSTAT1 $^+$ frequency in gated CD4 $^+$ intestinal T cells between CD69 $^+$ and CD69 $^-$ cells. Statistical analysis by paired t test.

Supplementary figure 4. Expression of pSTAT1 is more frequent in Intestinal T cells from non-inflamed areas in IBD patients. Intestinal biopsies of IBD patients (normal macro/microscopically – Non-Inflamed or Inflamed), were processed by the walk out method. **A,B** Percentage of pSTAT1 $^+$ CD4 $^+$ T cells in IBD (n=24) patients in both unstimulated (**A**) and after 15 minute stimulation with IFN-1 (**B**). **C** Total STAT1 expression (phosphorylated and non-phosphorylated; U-STAT1 $^+$) as a percentage of intestinal CD4 $^+$ in unstimulated samples and **D** U-STAT1 Mean Fluorescence Intensity (MFI) levels from the same samples. Boxes indicate 1 st and 3 rd quartiles, with the middle line the median. Statistical analysis by paired t test

Supplemental figure 5. pSTAT1 expression is not solely representative of a TH1 phenotype. Expression of transcription factors T-Bet (A) and FoxP3 (B) as a percentage of all walk-out CD4⁺ T cells from control (n=10) or Non-inflamed (n=12) or inflamed (n=12) IBD samples.

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Author Contribution

EG designed, performed and analysed the experiments and wrote the manuscript. AS designed experiments and wrote the manuscript. TS performed experiments and wrote the

manuscript. NM performed experiments and wrote the manuscript. MP and JL performed and analysed experiments. JOL and TTM designed experiments and assisted in the manuscript preparation.

Figure 1

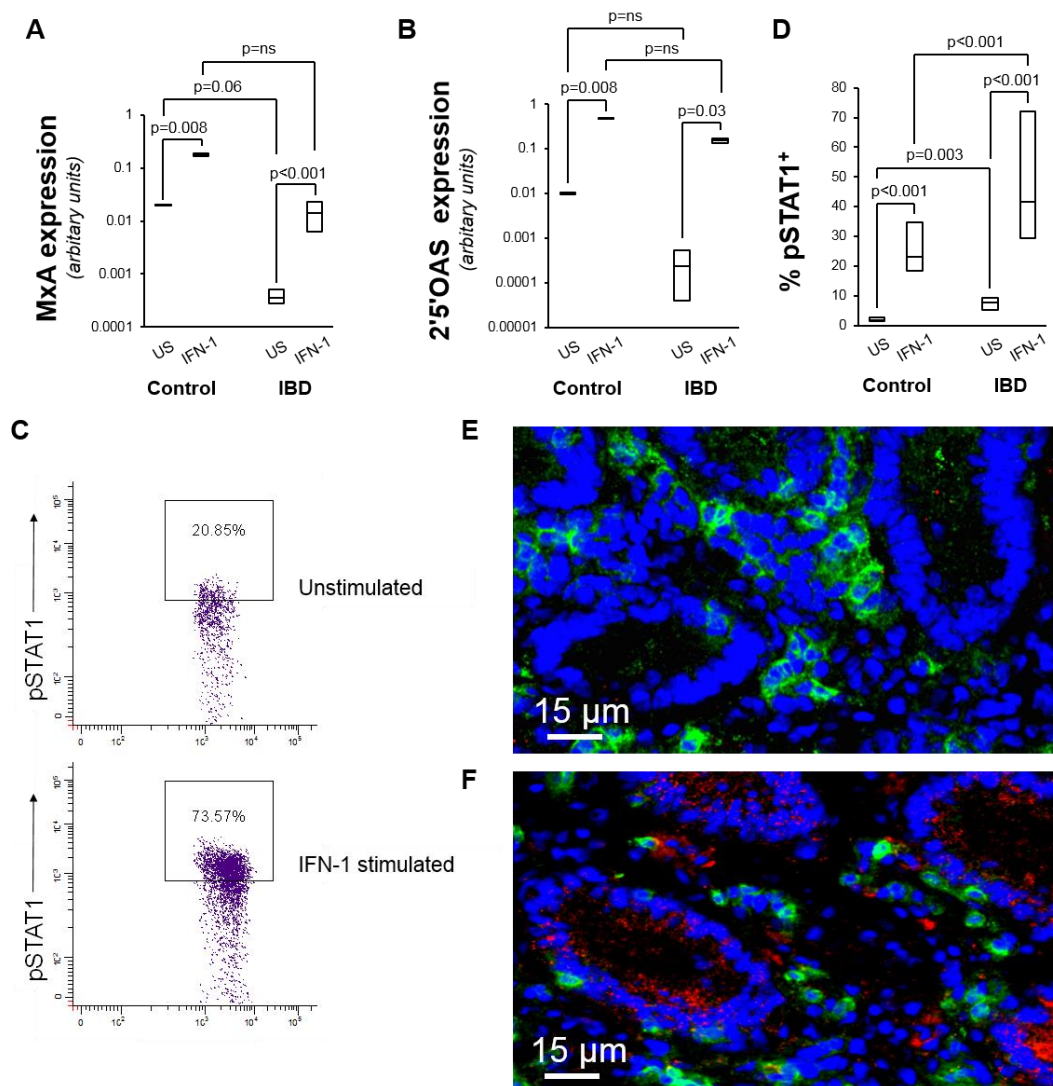
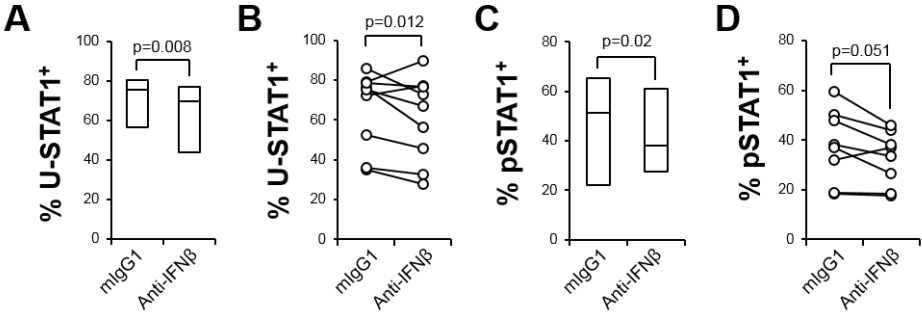
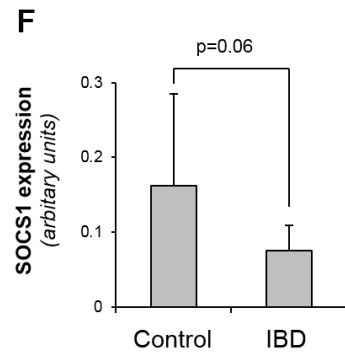
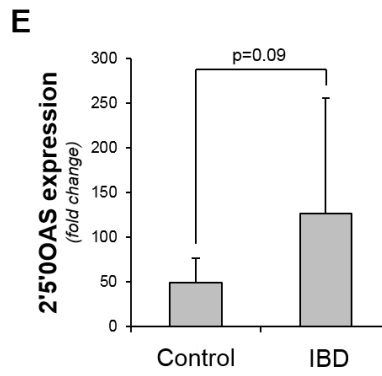
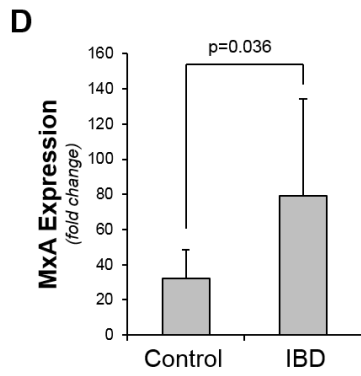
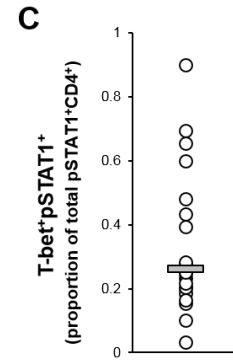
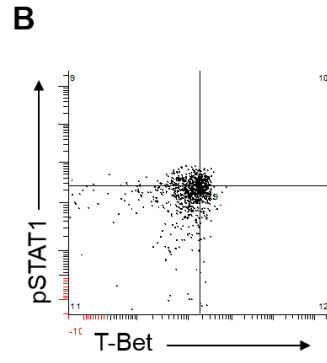
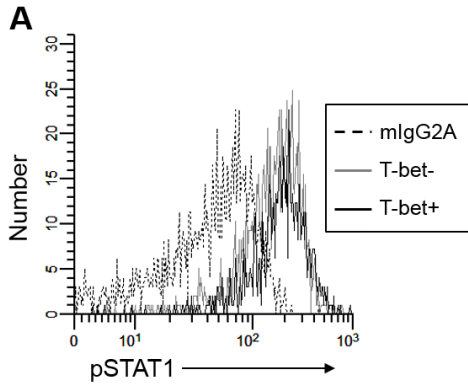


Figure 2





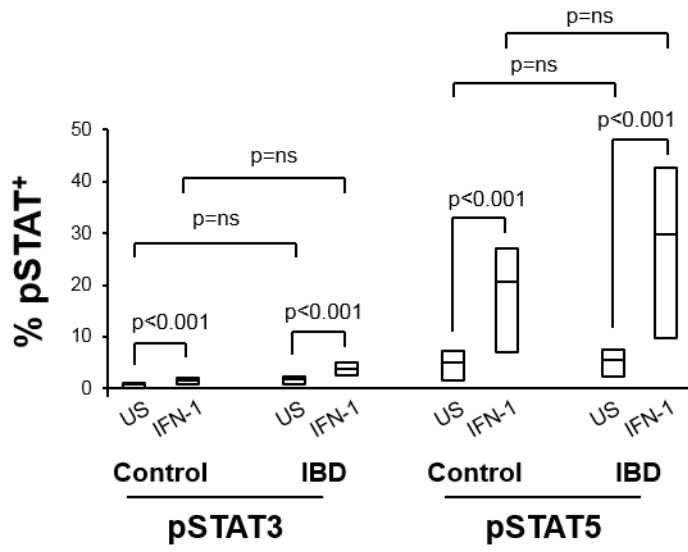
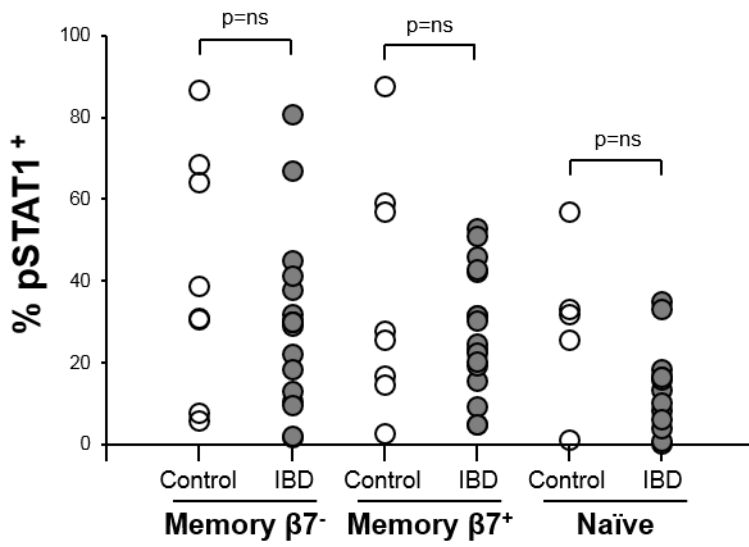
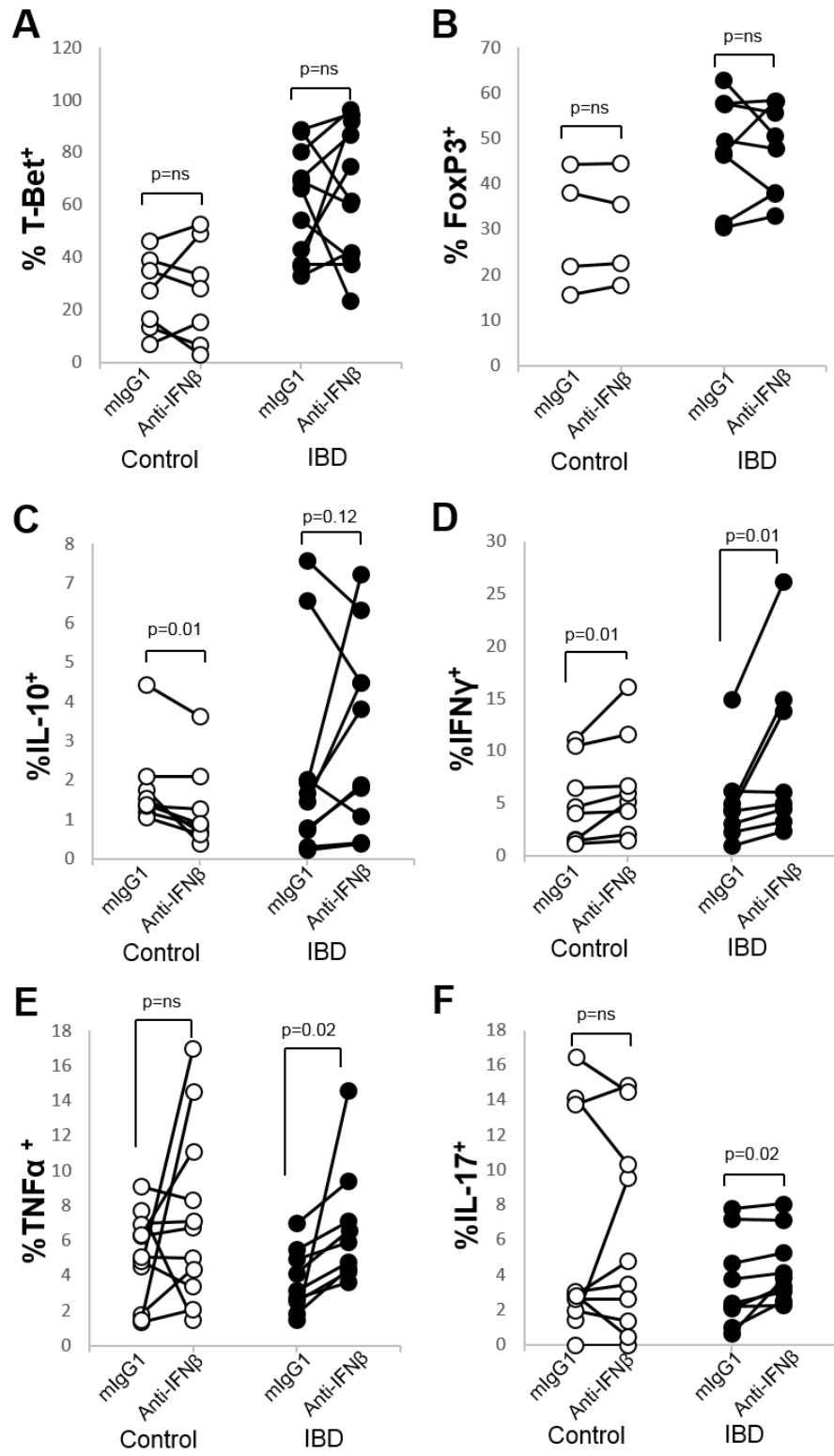
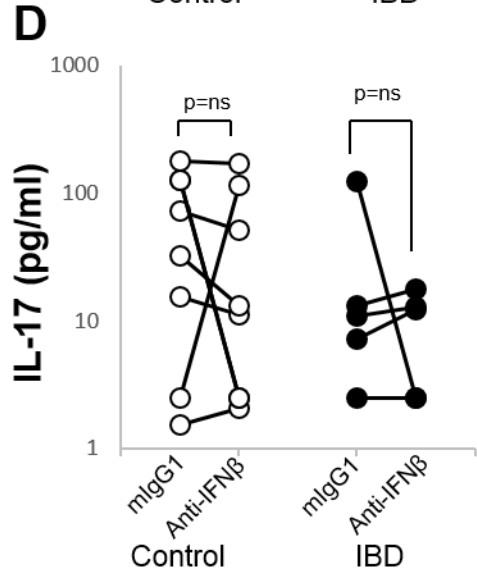
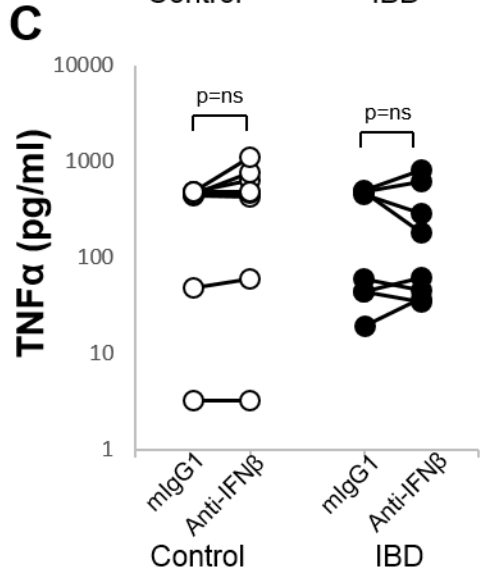
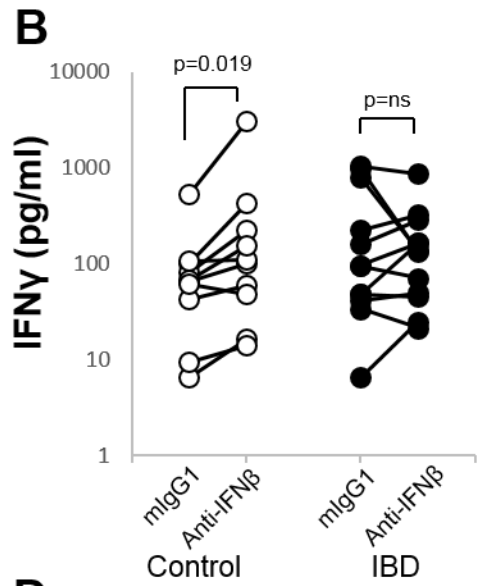
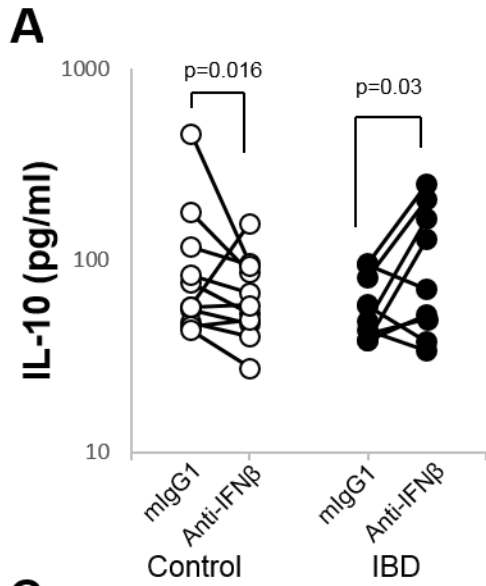
A**B**

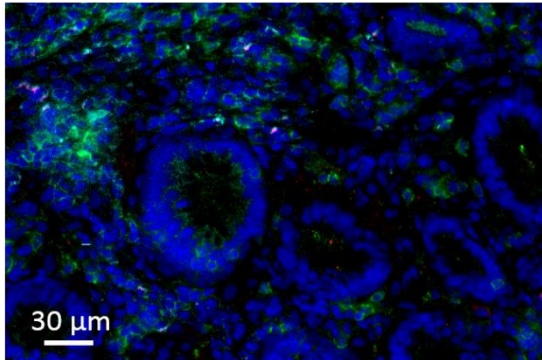
Figure 5



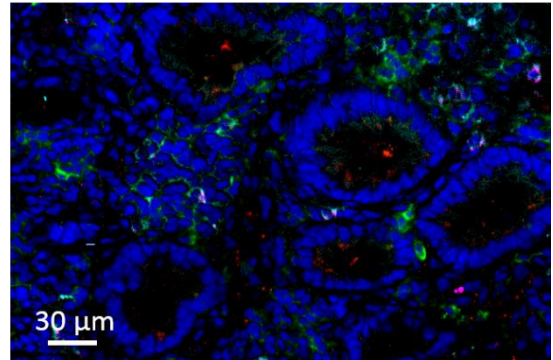


Supplementary Figure 1

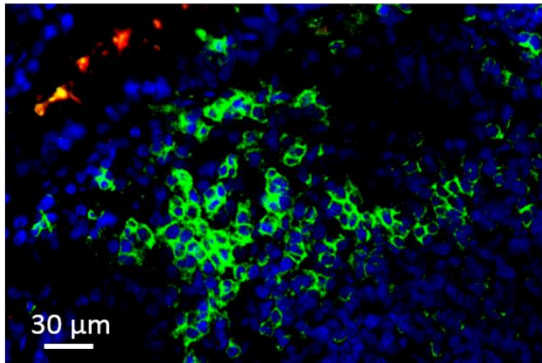
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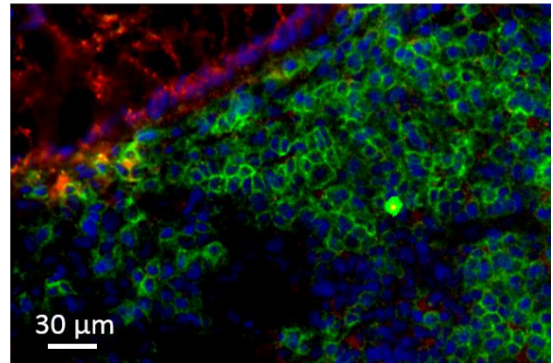
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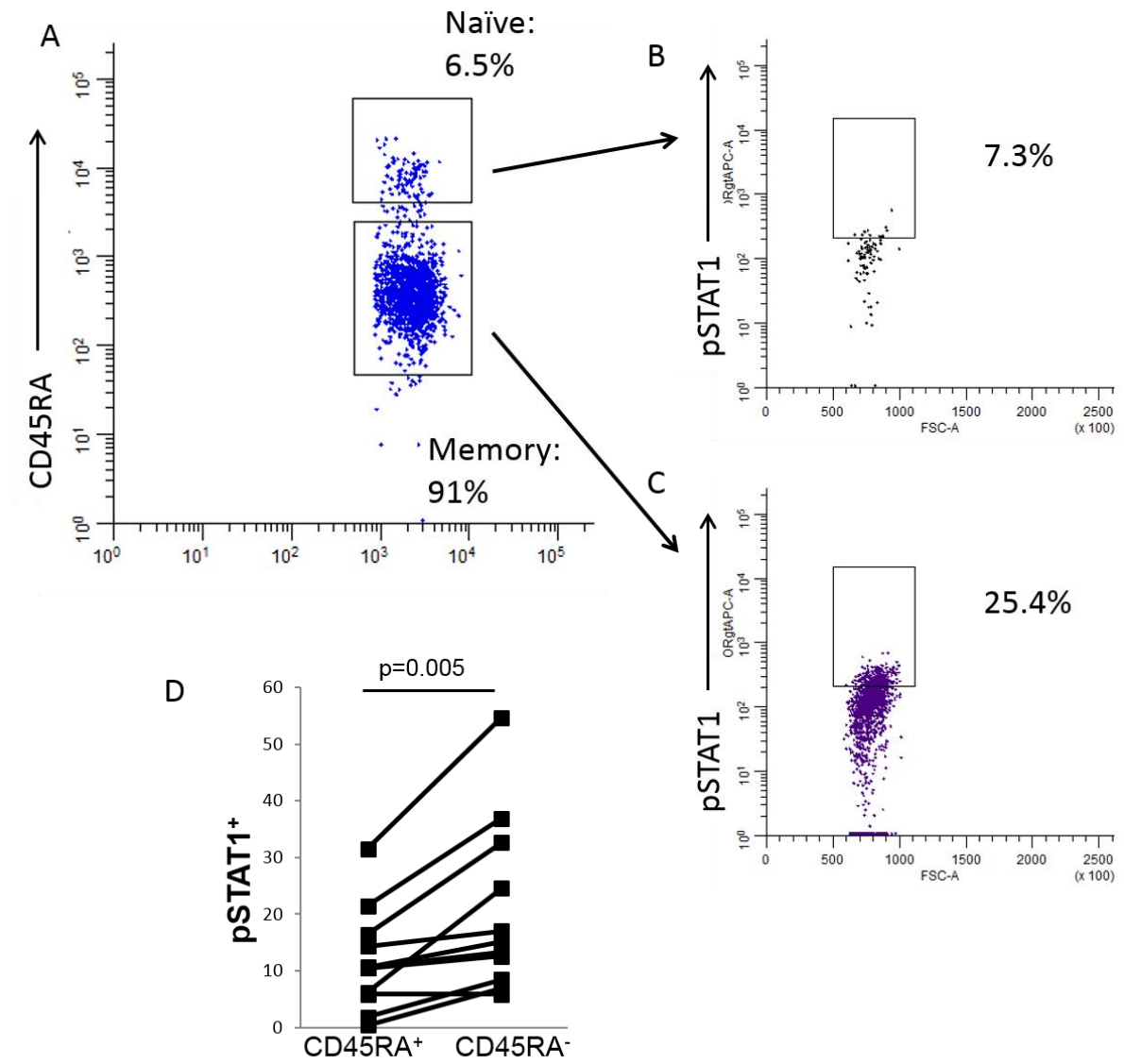
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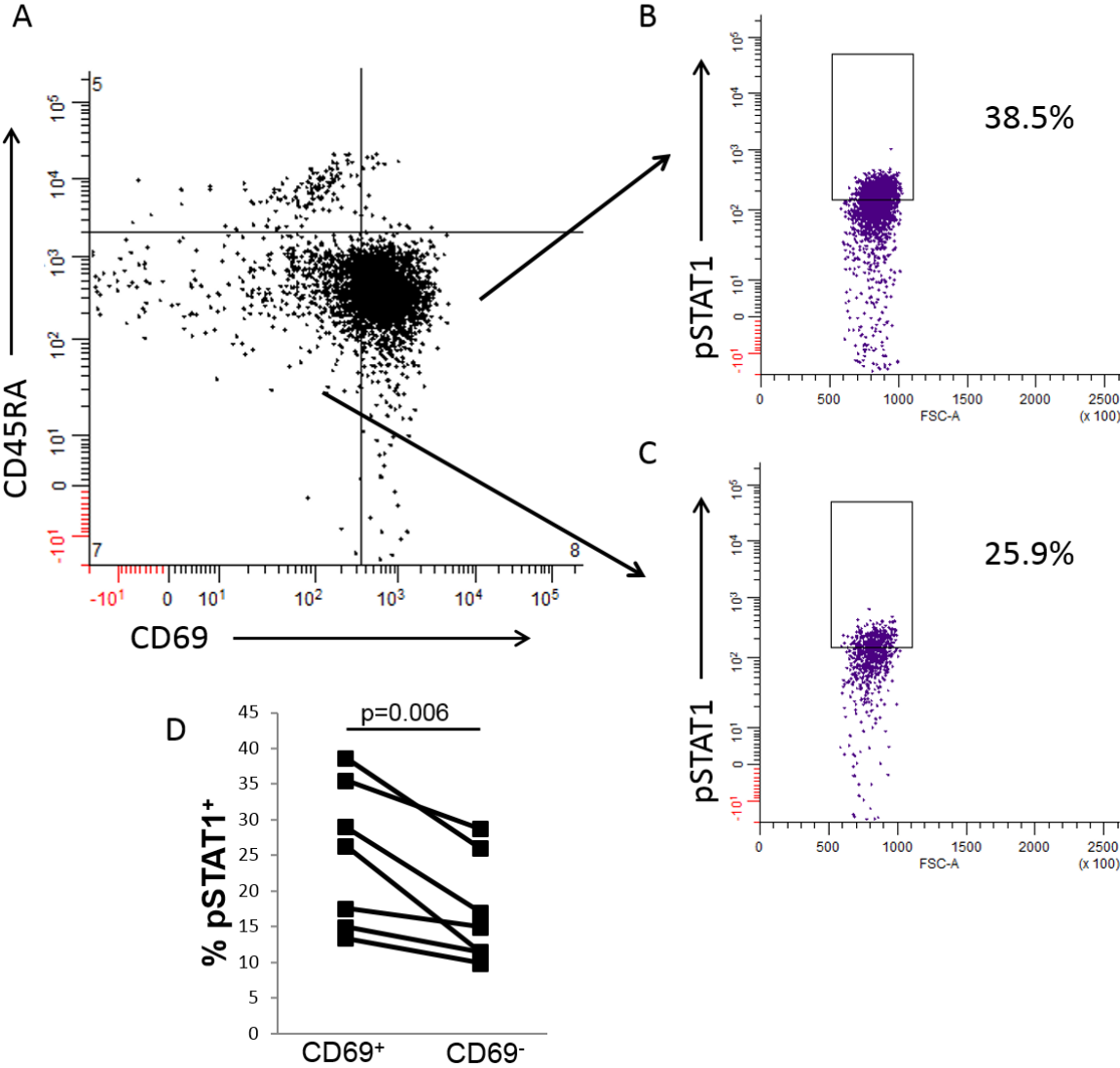
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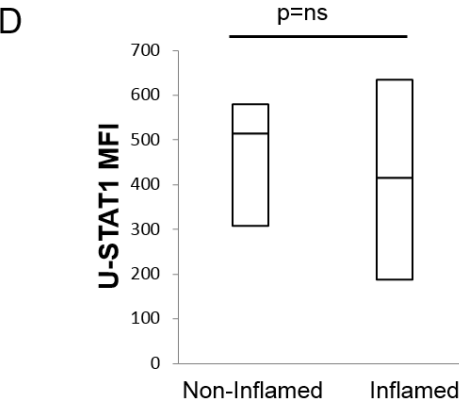
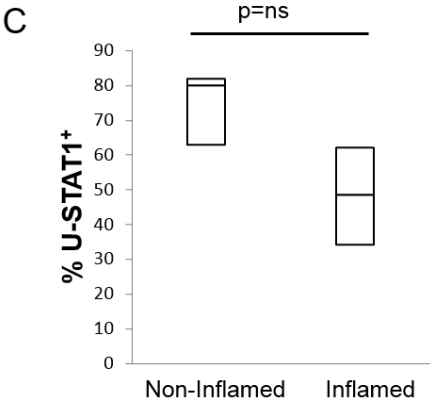
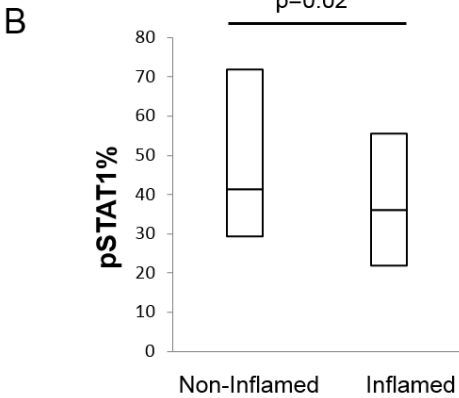
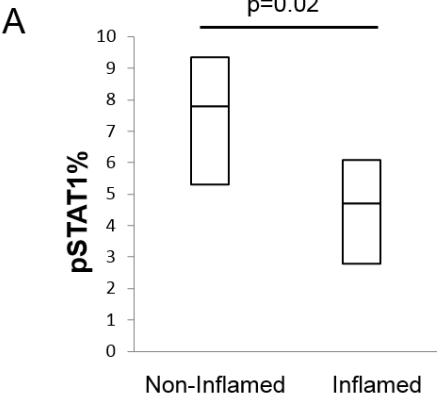
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5

