INTESTINAL EPITHELIAL CELLS FROM INFLAMMATORY BOWEL DISEASE PATIENTS PREFERENTIALLY STIMULATE CD4+ T CELLS TO PROLIFERATE AND SECRETE INTERFERON-\(\gamma\)

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Running title: IBD IECs stimulate CD4+ T cells

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Abstract

Previous studies have suggested that intestinal epithelial cells (IECs) have the capacity to function as non-professional antigen presenting cells that in the normal state preferentially activate CD8+ T cells. However, under pathological conditions, such as those found in inflammatory bowel disease (IBD), persistent activation of CD4+ T cells is seen. The aim of this study was to determine whether the IBD-IECs contribute to CD4+ T cell activation. Freshly isolated human IECs were obtained from surgical specimens of patients with or without IBD and co-cultured with autologous or allogeneic peripheral blood T lymphocytes. Co-cultures of normal T cells and IECs derived from IBD patients resulted in the preferential activation of CD4+ T cell proliferation that was associated with significant IFN-γ, but not IL-2, secretion. Cytokine secretion and CD4+ T cell proliferation was inhibited by pre-treatment of the IBD IECs with the anti-DR mAb L243. In contrast, normal IECs stimulated the proliferation and cytokine secretion by CD4+ T cells to a significantly lesser degree than IBD IECs. Furthermore, blockade of HLA-DR had a lesser effect in the normal IEC-CD4+ T cell co-cultures. We conclude that IECs can contribute to the ongoing CD4+ T cell activation seen in IBD. We suggest that the apparent differences between the secreted levels of IFN-γ indicate that it may play a dual role in intestinal homeostasis, in which low levels contribute to physiologic inflammation while higher levels are associated with an uncontrolled inflammatory state.

Key words: Mucosal inflammation, Crohn’s disease, ulcerative colitis, Interferon-γ, cytokines.
**Introduction**

Inflammatory bowel disease (IBD) is a chronic inflammatory intestinal disorder of unknown etiology comprised of two phenotypes: Crohn’s disease (CD) and ulcerative colitis (UC). While the precise cause of inflammation is as yet undefined, it is believed that an antigen driven response against non-pathogenic bowel flora is one of the initial events [1, 2]. This has been clearly demonstrated in animal models of IBD [3-5] and has been suggested in human disease [6-11]. Unlike the normal state, where an immunologic response towards normal bowel flora and food antigens has been proposed to mediate controlled, “physiologic”, inflammation, in IBD, inflammation is uncontrolled. The inflammatory cells and mediators responsible for such loss of control are as yet unknown.

Murine models of IBD demonstrate that CD4+ T cell differentiation plays a pivotal role in determining the type of immune response generated in the gut and that distinct cytokine profiles characterize each CD4+ T cell subset (Th1, Th2, Th3/Tr1) [12-16]. In humans, however, these distinctions are not as clear and extrapolation from animal models is not straightforward [17]. In Crohn’s disease, a Th1 CD4+ T cell differentiation pathway is responsible for macrophage activation, granuloma formation and increased tissue levels of TNF-α [18-21]. For interferon-γ (IFN-γ) and IL-2, however, conflicting data exist, as both increased and decreased levels of these cytokines have been reported under various conditions [22-24]. In UC, the cytokine profile is even less defined. Attempts to demonstrate a clear Th2 cytokine profile have failed. The only cytokines reported to be consistently increased are IL-5, IFN-γ and, recently, IL-13 [22, 25, 26]. The role of regulatory Th3/Tr1 cells [27, 28], generally associated with tolerance, have not yet been defined in IBD and both increased [29], and decreased [30] IL-10 levels have been reported.

What initiates the aberrant activation of CD4+ T cells in IBD? Aberrant antigen presentation by professional antigen presenting cells (APC) such as monocytes and dendritic
cells has been suggested [31-35]. However, recent work from our laboratory and others has pointed to a possible role of the intestinal epithelial cell in the activation of CD4+ T cells in IBD. The constitutive expression of MHC class II molecules on IECs and their increased expression in IBD [36] make the direct interaction of IECs with CD4+ T cells a realistic possibility. Moreover, the demonstration of CD58 and CD86 on IECs in normal [37] and in IBD (UC) patients [38], respectively, and of B7H on normal as well as IBD-IECs [39, 40] suggests that these cells are able to deliver co-stimulatory signals, thereby initiating active immunity. In the microenvironment of the IECs, however, co-stimulation may be less critical, as the vast majority of lamina propria lymphocytes (LPL) are memory cells [41, 42]. The demonstration, by Hershberg’s group [37, 43], of direct activation of CD4+ T cell clones by DR+ IEC lines and our own work demonstrating activation of CD4+ associated p56-\textit{lek} by IBD-IECs [44], strongly support the IEC’s potential as a direct activator of CD4+ T cells.

When normal IECs are used as APCs, activation of CD8+ T cells is seen [44-46]. This occurs through the IEC surface molecules gp180 (CD8 ligand) and CD1d, which associate and form a MHC class I-like complex [46] that stimulates CD8+ T cell activation, which is reflected in T cell proliferation [47-49]. In IBD, gp180’s expression is decreased [44], so stimulation of CD8+ suppressor T cells does not occur [50], and the delicate balance between CD8+ T cell suppression and CD4+ T cell active immunity could be skewed towards the latter. As data suggesting a possible direct interaction between IECs and CD4+ T cells have accumulated, we hypothesized that such direct interactions may occur in the normal gut but would be elevated in IBD, thus contributing to CD4+ T cell over-activity. In order to address this hypothesis, we compared the proliferation and cytokine secretion profiles of CD4+ T cells that were co-cultured with fresh human IECs from normal and IBD tissues. In this study we demonstrate that IBD-IECs preferentially stimulate CD4+ T cells \textit{in vitro} resulting in their
proliferation and in IFN-γ secretion. We also show that MHC class II molecules, whose expression is increased on IBD IECs, are involved in the mediation of this interaction.

**Methods**

**Isolation of Intestinal Epithelial Cells (IECs):** Surgical specimens were obtained from colonic resections performed at the Mount Sinai Medical Center. Forty five IEC: PBT cell co-cultures were generated. IECs were obtained from 18 normal mucosal samples (at least 10 cm distal to colonic malignancies) 13 CD, 10 UC and 4 diverticulitis (non-IBD inflammatory control) patients.

IECs were isolated by a method described previously [51]. Briefly, specimens were washed with PBS, the mucosa was stripped from the submucosa and placed in 1 mM dithiothreitol (Sigma, St. Louis, MO) for 10 minutes. The pieces were then washed with PBS and incubated twice in dispase (Boehringer Mannheim, Indianapolis, IN), 3mg/ml in RPMI 1640 medium (Mediatech Cellgro, Herndon, VA), for 30 minutes in a 37 °C water bath. The cell suspension was collected and centrifuged on a percoll density gradient. IECs are located at the 0-30% layer interface. Cells were washed x 3 with RPMI, and re-suspended in serum free medium (AIM-V, 50u/ml penicillin, 50 µg/ml streptomycin, 2mM glutamine all from GIBCO BRL, Grand Island, NY) for co-culture experiments. Cells were >95% viable by trypan-blue exclusion, and the purity of IECs in the suspension was > 95%, as determined by staining with FITC-conjugated anti-epithelial cell specific antigen (ESA, Biomed a Corp. CA) and flow cytometric analysis using Cell Quest software. IEC preparations were irradiated (3000 rads) prior to co-culture with T cells.

**Isolation of peripheral blood T cell subpopulations:** Peripheral blood CD3+, CD4+, CD8+, or whole T cells were negatively selected from normal donor blood using the RosetteSep™ procedure (StemCell Technologies Inc, Vancouver, Canada) according to the manufacturer’s instructions. In brief, tetrameric antibody complexes directed against glycophorin A on RBC
and against cell surface markers on human hematopoietic cells (anti-CD16, CD19, CD36, CD56, and anti-CD4 or anti-CD8) were added to whole blood (50µl/ml) and incubated at room temperature. After 20 minutes, samples were diluted with PBS/2% FBS, layered over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 20 minutes (the unwanted cells pellet along with the RBC). The interface was collected, washed twice with PBS/2% FBS and re-suspended in serum-free medium (Aim-V). The purity of CD4+ and CD8+ T cell suspensions was >98% with <1% CD8+ contamination in CD4+ T cell suspension and vice versa, as determined by flow cytometric analysis.

**Co-culture assays:** 1 x 10^6 IECs from normal or IBD patients were co-cultured with 1 x 10^6 allogeneic whole T cells, or CD4+ or CD8+ T cells in AIM-V medium, in a total volume of 1 ml/well in 24 well flat bottom plates (Falcon™, BD Biosciences, San Jose, CA). Plates were incubated in a 37 °C, 5% CO2 humidified incubator for 5 days. In each experiment, IECs, whole T, CD4+ and CD8+ T cells were incubated alone (negative control) or with conventional APCs, (non-T cells, positive control). Cell free supernatants were harvested 36 and 120 hours after incubation and frozen at -70 °C until ELISA assays were performed. In several experiments, IECs from normal and IBD patients were co-cultured with allogeneic normal peripheral blood T cells generated from the same donor. In yet another series of experiments, normal and IBD IECs were incubated with autologous or allogeneic peripheral blood T cells (including cross over studies, where IECs from a normal donor were co-cultured with IBD peripheral blood T cells).

**Proliferation assessment:**

**Membrane labeling of T cells with CFSE:** Prior to the co-culture assays, CD4+, CD8+ and whole T cells were re-suspended in PBS at 5x10^6/ml and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR). Cells were incubated
for 10 minutes at 37 °C with 1mM (final concentration) CFSE, washed twice with RPMI and used in the co-culture experiments.

**Flow cytometric analysis**: Labeling of T cells with CFSE was performed before co-cultures. Five days after incubation, T cells were re-suspended in PBS and incubated for 30 minutes with antibodies. The following antibodies were used: anti-CD3, anti-CD4 and anti-CD8 (conjugated with PE, PerCP or APC) and relevant isotype controls (all from Becton Dickinson, San Jose, CA). T cell proliferation was assessed by flow cytometry and four-color analysis of CD3+ lymphocytes using Cell Quest software (Beckton Dickinson). Non-proliferating cells remain CFSE-high. In contrast, CFSE expression of proliferating cells decreases in proportion to the number of cell divisions (CFSE-low). After gating on CD3+ cells, the percentages of CD4+ and CD4- as well as those of CFSE-high and CFSE-low cells were determined. The data obtained using this approach were confirmed using standard \(^{3}\text{H}\) Thymidine incorporation assays.

**\(^{3}\text{H}\)-Thymidine incorporation assays**: Five days after incubation, cells were resuspended and placed in 100ul triplicate microwell cultures for pulsing. Cells were pulsed with \(^{3}\text{H}\)-thymidine \(1\mu\text{Ci}\) for 18 hours, harvested (PHD Cambridge harvester Cambridge Technologies, Cambridge, MA, USA) and read by a \(\beta\) counter (Beckman 3801, Fullerton, CA, USA).

**Enzyme–linked Immunosorbent Assays**: Interferon \(\gamma\), IL-2 and IL-10 production by co-cultured T cells was determined by cytokine ELISA sets purchased from BD-Pharmingen (San Diego, CA). ELISA protocols were used according to the manufacturers’ instructions.

**RT-PCR**: A total of 1 \(\mu\)g of RNA, extracted with Trizol reagent 24-36 hours after stimulation of T cells with IECs, was used from each well for cDNA synthesis. Reverse transcription was performed with Moloney Murine Leukemia Virus reverse transcriptase at 42 °C for 1 h in a total volume of 30 \(\mu\)l. The reaction was stopped by heat inactivation and the cDNA was diluted to 100 \(\mu\)l. For each PCR reaction, 2.5\(\mu\)l cDNA, 10 pM of forward and
reverse primer, platinum Taq 0.2u with the supplied buffer, and 2mM MgCl₂ and 200 µM
dNTPs were used in a 25 µl final volume. The PCR cycle was 4 minutes at 95 °C to activate
the enzyme, then 94 °C for 45 seconds, 60 °C for 45 seconds, and 72 °C for 1 minute for 30
cycles followed by 10-minute extensions at 72 °C.
The following primers were used [52]:

**Interferon-γ:**
5’ primer: 5’-ATGAAATATACAAGTTATATCTTGGCTTT-3’
3’ primer: 5’-GATGCTCTTTCGACCTCGAAACAGCAT-3’

**β-actin:**
5’ primer: 5’-TGACGGGGTCACCCACACTGTGCCCATCTA-3’
3’ primer: 5’-CTAGAAGCATTGCGGTGGACGATGGAGGG-3’

**Blocking assays:** In some experiments, prior to the co-culture, IECs were incubated with a
purified blocking anti-DR monoclonal antibody (L243, ATCC, Rockville, MD) at 35 µg/ml
for 1 hour at 4 °C, or an isotype control (monoclonal antibody 204-IgG2b anti-B cell
differentiation factor (BCDF), which is not expressed by IECs). The cells were then washed
twice with PBS and re-suspended in AIM-V for co-culture experiments. An aliquot of these
IECs was stained by PE-conjugated L243 (BD-Pharmingen) to assess HLA-DR expression
on fresh IECs. These cells were counterstained with FITC-conjugated anti-epithelial cell
specific antigen (ESA, Biomed Corp, CA) to confirm that HLA-DR expression was
restricted to IECs. Non-viable IECs were excluded by propidium-iodide (PI) staining. HLA-
DR expression was determined by flow-cytometry using Cell Quest software (Becton
Dickinson).

**Statistical analysis:** We used two-sample paired t tests of the means for comparisons
between cytokine concentrations originating from the same specimen. For all other
comparisons, we used the Mann-Whitney test. P values of <0.05 were considered to represent statistical significance.

Results

IECs from IBD patients preferentially stimulate CD4+ T cell proliferation

First we analyzed the effect of IECs from IBD or normal control patients on the proliferative responses of T cells. Non-stimulated T cells (incubated in medium only) had <1% spontaneous proliferation (Fig. 1a). Normal IECs promoted higher CD4- (CD8+) T cell proliferation; the percentages of CD4- and CD4+ CFSE-low (proliferating) T cells were 5.3±1.2% and 2.4±0.4%, n=7, respectively (p=0.04). In contrast, IBD IECs promoted significantly higher CD4+ vs. CD4- T cell proliferation; in CD co-cultures, the percentages of CD4+ and CD4- CFSE-low (proliferating) T cells were 10.4±2.2% and 3.5±0.9%, respectively (n=6, p=0.009), and in UC co-cultures the percentages of CD4+ and CD4- CFSE-low (proliferating) T cells were 10.2±3.6% and 7.6±3.4%, respectively (n=5, p=0.02). When comparing CD4+ T cell proliferation between IBD and normal-IEC-stimulated co-cultures, there was a higher proliferation of CD4+ T cells in IBD-IECs T-cell co-cultures (CD vs. normal p=0.002, UC vs. normal p=0.04) (Fig. 2).

We then determined whether IECs would affect T cell proliferation when normal or IBD IECs were co-cultured with pure CD4+ T cells. CD4+ T cells were negatively selected from whole blood of healthy volunteers. These were >98% CD3+ CD4+ and did not proliferate when incubated alone (Fig. 3). As seen in Figs. 3 and 4, normal IECs had the potential to directly interact with CD4+ T cells and to stimulate CD4+ T cell proliferation. This was more pronounced in IBD-IECs stimulated co-cultures. The percentage of CD4+ CFSE-low (proliferaing) T cells in CD IEC:CD4+ T cell co-cultures was 13.4±1.9 (n=7, p=0.001 vs. normal) and 19.5±7.2 in UC co-cultures (n=4, p=0.02 vs. normal) vs. 3.3±1.3 in normal IEC:CD4+ T cell co-cultures (n=6). Non T cells induced even higher CD4+ T cell
proliferation (21.7±3.2%, n=9, p=0.03). In order to validate the CFSE data, as well as quantitate the magnitude of the proliferative response, an alternate approach to measuring cell proliferation, i.e., ³H-thymidine incorporation was used. In normal IEC stimulated co-cultures, mean CD4+ T cell proliferation was 5864±1980 cpm (n=22). This increased to 7837±1966 cpm (n=18) when CD IECs were the stimulator cells (p=0.003 vs normal), and to 17450±6147 cpm (n=11) when UC-IECs were the stimulator cells (p<0.00008 vs. normal, Fig. 5). This suggests that immunologically significant proliferative responses of CD4+ T cells may be evoked by IECs, and that these responses are significantly increased when IBD IECs are the stimulator cells.

Since we have previously shown that the CD8+ T cells proliferating in normal IECs: peripheral blood T cell co-cultures are restricted by a complex of CEA and CD1d [9], one would predict that T cell responses to allogeneic vs. autologous IECs would be roughly comparable. CD1d is non-polymorphic with a limited repertoire of antigens that are capable of binding into the antigen binding groove (lipids or glycolipids). As seen in Fig. 6 and 7, T cell proliferation in response to autologous vs. allogeneic IECs were comparable (p=ns). This was true when normal as well as IBD IECs were the stimulating cells (Fig. 6, normal, 3 different experiments, Fig. 7 CD, representing 3 different experiments), and was demonstrated both by ³H-thymidine as well as CFSE assessments of proliferation. This proliferation, however, was still lower than the response to allogeneic monocytes/DCs. The question that remains is whether there is an intrinsic defect in PB T cells from IBD patients to respond to normal IECs (i.e. is the defect at the level of the T cell or the IEC). To address this directly we established crossover co-cultures of normal IECs with CFSE labeled T cells from normal and IBD patients. As seen in Fig. 7, T cells, whether from normal or IBD patients, proliferated similarly in all co-cultures. Moreover, supporting the data presented thus far, normal IECs evoked a moderate proliferative response in allogeneic CD PB T cells
that was comparable to normal autologous PB T cells proliferation. In the crossover
experiment CD IECs elicited a higher proliferative response in normal PB T cells, this being
comparable to the proliferative response of autologous CD PB T cells. These data suggest
that IBD T cells are intrinsically similar to normal control PB T cells in their response to
normal IECs.

**CD4+ T cells that are stimulated by IBD-IECs secrete more interferon-γ than normal**

**IEC-stimulated CD4+ T cells**

We then determined whether the preferential stimulation of CD4+ T cell proliferation by
IBD IECs was accompanied by a difference in cytokine production. In preliminary kinetic
studies we observed an increase in interferon-γ secretion, above background, starting at 24-48
hours after incubation and reaching a plateau at days 5-6. Thus, interferon-γ concentrations
were determined at two points in time (36 and 120 hours). IECs and non-stimulated CD4+ or
CD8+ T cells incubated alone secreted <40 pg/ml interferon-γ throughout the incubation
period. There was no significant difference between interferon-γ secretion seen in normal and
IBD IECs (control for contaminating IEL) or by CD4+ or CD8+ T cells alone used in the
different co-cultures. At 36 hours, interferon-γ secretion by CD4+ T cells co-cultured with
normal IECs was similar to background levels. However at 120 hours an increase to a mean
concentration of 205.6±54.9 pg/ml was observed (p=0.008 vs. 36 hours). In contrast, IBD
IECs stimulated significantly higher interferon-γ secretion by CD4+ T cells. At 36 hours, the
interferon-γ concentration in CD, UC and NL IEC:CD4+ T cell co-cultures was 249±80.7
(n=10), 216.4±60.5 (n=8), and 28.8 pg/ml (n=12), respectively (p=0.01 for CD vs. NL and
0.002 for UC vs. NL). This increased secretion persisted in both CD and UC vs. normal IEC-
stimulated co-cultures over a 5-day period (Fig. 8a). At 120 hours, the interferon-γ
concentration in CD, UC and NL IEC:CD4+ T cell co-cultures was 1014±238, 1139±263.4
and 205.6±54.9 pg/ml, respectively (p=0.001 for CD vs. NL and 0.0005 for UC vs. NL).

Interestingly, this secretion was ~80% of the maximal interferon-\(\gamma\) secretion stimulated by professional APCs (Non T cells). Interferon-\(\gamma\) secretion by CD4+ T cells stimulated by IECs from four patients with diverticulitis was <150 pg/ml at 120 h (data not shown). The ELISA results were supported by interferon-\(\gamma\) mRNA expression, assessed by RT-PCR (see Fig. 12). These results suggest that the difference between normal and IBD co-cultures is not merely kinetic, but that the signal for interferon-\(\gamma\) secretion induced by IBD IECs is stronger than the one induced by normal IECs.

We next determined whether CD8+ T cells stimulated by normal or IBD IECs also contribute to the overall production of interferon-\(\gamma\). As seen in Fig. 8b, in contrast to IEC-stimulated CD4+ T cells (Fig. 8a), CD8+ T cells secreted low concentrations of interferon-\(\gamma\) (<200 pg/ml) whether stimulated by normal, CD or UC IECs. When CD4+ and CD8+ T cells from the same healthy donor were stimulated by the same IEC preparation, interferon-\(\gamma\) secretion by the stimulated CD4+ T cells after 120 hours was 2.4 higher than the stimulated CD8+ T cells in the normal co-cultures, 5 times higher in UC co-cultures (p=0.002 vs. NL) and 15 times higher in CD co-cultures (p=0.008 vs. NL), suggesting a greater contribution to interferon-\(\gamma\) secretion by CD8+ T cells in the normal IEC-stimulated co-cultures (Fig. 8c).

The different PBT cell preparations that are used in the co-cultures may have different responses in allogeneic co-cultures. To directly compare the effect of IECs from normal and IBD patients, CD4+ T cells from the same healthy donor were incubated simultaneously with IECs from normal or IBD patients. As demonstrated in Fig. 9, CD4+ T cells proliferate more and expressed and secreted more interferon-\(\gamma\) when IBD-IECs (compared with normal IECs) were the stimulator cells.

**Interleukin-2 secretion**
To determine whether the increase in interferon-γ production by IBD-IECs stimulated CD4+ T cells paralleled a more classic Th1 cytokine profile, IL-2 secretion was measured in the same co-culture supernatants. IL-2 levels at 120 hours in NL, CD and UC co-cultures were 34±10.2, 66.4±39 and 185.3±77 pg/ml, respectively (Table 1) (p=NS). When CD4+ T cells were stimulated by conventional APCs (Non T cells), maximal stimulation resulted in IL-2 levels of 864±199 pg/ml at 120 hours (p=0.02 vs. normal IEC-induced IL-2 secretion by CD4+ T cells).

Interferon-γ concentrations that were secreted in the same NL, CD and UC co-cultures were 205.6±54.9, 1014±238, and 1139±263.4 pg/ml, respectively, and 1556±90 in Non T-stimulated CD4+ T cell co-cultures. Thus, IEC-stimulated interferon-γ secretion by CD4+ T cells is relatively higher than the corresponding value for IL-2.

Interleukin 10 secretion

IL-10 is an anti-inflammatory/inhibitory cytokine secreted by regulatory T cells, IECs and macrophages [53]. Its presence or absence in IEC:T cell co-cultures could help to explain the observed differences in cytokine secretion profiles. Low (<35 pg/ml) IL-10 levels were demonstrated in all co-cultures (Table 1), compared with concentrations of 340±170 pg/ml at 120h, observed when CD4+ T cells were maximally stimulated by conventional APCs (Non-T cells), (p=0.03 vs. normal IEC-induced IL-10 secretion by CD4+ T cells).

Increased stimulation of CD4+ T cells by IBD IECs is mediated by HLA-DR

The data described suggest that direct interactions between IECs and CD4+ T cells do occur and induce CD4+ T cell proliferation and interferon-γ secretion. In previous studies, increased HLA-DR expression on IBD IECs and a low constitutive HLA-DR expression on normal IECs was demonstrated [36]. Therefore, we hypothesized that HLA-DR molecules, which are a prerequisite for the interaction of antigen presenting cells and CD4+ T cells, could mediate the interaction of IECs with CD4+ T cells. HLA-DR expression was
determined by staining freshly isolated human IECs from normal and IBD tissues with an anti-HLA-DR mAb (L243). Two and a half-5.5% (MFI=32±11) of normal IECs expressed HLA-DR. In contrast, 45-97% of CD (MFI=1052±406) and 34-62% (MFI=385±114) of UC IECs expressed HLA-DR. Not only was the percentage of stained cells significantly higher in IBD, but the density of expression was also significantly higher (Table 2). The HLA-DR expression on IECs from non-IBD inflammatory controls was similar to that seen on normal IECs (Fig. 10). To assess the contribution of HLA-DR to IEC:CD4+ T cell interactions, IECs were incubated with the anti-HLA-DR blocking monoclonal antibody L243 or an isotype control mAb, washed and co-cultured with CD4+ T cells. As interferon-γ secretion was reproducibly demonstrated in IEC:CD4+ T cell co-cultures, we used interferon-γ secretion as a measure of CD4+ T cell stimulation by IECs. Pre-incubation of IECs with mAb L243, but not with an isotype control, significantly decreased interferon-γ secretion in the co-cultures by 80% (UC) to 99% (CD) (Fig. 11b and c). Interferon-γ mRNA expression, assessed by RT-PCR, supported the ELISA results (Fig. 12). Normal co-cultures, which, as shown, had significantly lower interferon-γ secretion, were less influenced by HLA-DR blockade (up to 50% decrease in interferon-γ secretion) (Fig. 11a, Fig. 12). Taken together, these data show that HLA-DR significantly contributes to CD4+ T cell stimulation by IBD IECs.

**Discussion**

In this study we determined the potential of intestinal epithelial cells (IECs) to stimulate *in vitro* proliferation of and cytokine secretion by CD4+ T cells. Preferential stimulation of CD4+ T cells by IECs originating from IBD vs. normal tissue was demonstrated. This was best reflected by the observation of increased interferon-γ secretion, which was significantly higher when CD4+ T cells were stimulated by IBD-IECs compared to normal IECs. We also demonstrated that MHC class II molecules, which are increased on IBD IECs, mediate the
interaction between IECs and CD4+ T cells. The data presented support the concept that IECs may be active participants in mucosal immune responses and suggest that IECs may bias T cell differentiation pathways either towards a controlled, regulated response in the normal state, or towards over-active immunity such as the uncontrolled inflammation seen in IBD.

We used co-cultures of freshly isolated human IECs (from IBD patients and normal controls) with allogeneic normal peripheral-blood T cells (PBTs) in order to investigate the effects of IECs on T cells in mucosal homeostasis and intestinal inflammation. This in vitro model system allows us to focus on the differences in proliferation and cytokine secretion by T cells induced by non-transformed IECs, while the target population (normal PBTs) is kept constant. The T cell stimulation thus generated may reflect the situation in the intestinal mucosa more than maximal stimulation protocols such as PHA/PMA, or antibodies against the TCR. Furthermore, the results produced by our co-culture experiments are consistent with previous tissue-mRNA- and LPL-stimulation studies, thus adding validity to this model.

Importantly, the proliferative responses induced by IECs were comparable in allogeneic as well as autologous normal T cells, as expected given the fact that these cells are CD1d restricted as previously reported [9]. This supports our hypothesis that IBD IECs have an increased stimulatory effect on normal T cells and that this effect is not merely the result of an allogeneic response. Moreover, as shown in the crossover studies presented, the enhanced T cell stimulation seen in IBD is at the level of the IECs, rather than the T cells, as the proliferation pattern of PB T cells from IBD or normal patients related to the stimulating IEC source.

Previous studies have reported increases in local interferon-γ production in tissues of patients with IBD (CD>UC). Increased interferon-γ mRNA expression was demonstrated in mucosal samples from IBD patients compared to normal controls [54], in chronic vs. early ileal lesions in CD patients [55], in active and inactive CD lesions, and in specimens of
pediatric CD patients compared to UC and normal controls [7, 56]. Increased interferon-γ mRNA has also been demonstrated in tissues from UC patients [56-58]. Results obtained directly from tissues and analyzed for cytokine mRNA content are assumed to reflect the cytokine content within the tissue at that specific point in time. While the strength of such studies is that they represent in vivo data, further analysis as to the source of the cytokine and the mechanisms involved in its production are not possible and mRNA expression does not necessarily reflect protein secretion. Other authors have, therefore, attempted to analyze cytokine profiles by immunohistochemical methods or by in vitro studies of LPLs. Similar results, i.e., increased interferon-γ production, with or without various stimuli, have been documented. LPLs from CD patients demonstrated significantly increased intracellular interferon-γ vs. LPLs from control patients [8], and increased interferon-γ secreting mononuclear cells were found in pediatric CD vs. UC and normal controls [7]. CD4+ LPLs from CD patients secreted more interferon-γ after stimulation via CD2/CD28 compared to UC or normal CD4+ LPLs [22] and spontaneous release of interferon-γ from cultured CD LPLs was also reported, in contrast to its absence in cultured normal LPLs [59]. Our finding of increased interferon-γ secretion in IBD-IEC stimulated CD4+ T cell co-cultures suggest one possible mechanism for the findings reported in these previously published studies, which is that IECs from IBD patients are capable of triggering interferon-γ secretion by CD4+ T cells.

Do IBD-IECs stimulate classic Th1 cells? The data presented here do not support this possibility. In general, while most studies have reported increased levels of interferon-γ and Th1-promoting cytokines in IBD tissues [60], results relating to IL-2 production are inconsistent, showing both increased (in CD) [7, 19, 54], and decreased [61] levels in IBD vs. normal. In support of the latter observations, IL-2 levels <200 pg/ml were demonstrated in both IBD and normal co-cultures. Moreover, when IBD IECs were the stimulator cells,
interferon-γ secretion by CD4+ T cells was ~70% of that seen in conventional MLRs. In contrast, CD4+ T cell secretion of IL-2 was only ~20% of that seen in conventional MLRs (in the same co-cultures) when the stimulator cells were IBD IECs. The discordant secretion of IL-2 and interferon-γ raises further questions regarding classical Th1 cytokine profiles in the human intestinal mucosal immune system.

In contrast to the mouse, in whom IL-10 deficiency is strongly associated with ileocolonic inflammation reminiscent of human CD [62], in human IBD tissues IL-10 mRNA is reported to be increased rather than decreased [56, 57]. When attempts were made to identify the source of IL-10, IL-10 expression by LPLs was low. This was further supported by IL-10 mRNA and in vitro IL-10 secretion studies of LPLs from IBD patients [30]. In our study, IL-10 levels secreted in the co-cultures were low, ~10% of those secreted in conventional MLRs. Nevertheless, higher mean values were demonstrated in normal-IEC stimulated CD4+ T cell co-cultures (p<0.0001 vs. UC IECs-stimulated IL-10 secretion by CD4+ T cells) hinting at a possible loss of tolerance in the inflamed epithelium vs. normal. Current data suggest that other suppressor cytokines such as TGF-β may also contribute to tolerance in the normal intestinal mucosal [27, 63]. However we were unable to detect the presence of TGF-β mRNA in normal IEC:CD4+ T cell co-cultures (data not shown).

By what mechanism do IECs stimulate CD4+ T cells? In a manner similar to their professional APC counterparts, the non-professional APCs, IECs, express several surface molecules that enable antigen presentation and lymphocyte stimulation. As these molecules are differentially expressed in normal and inflamed intestinal mucosa, this may explain the different CD4+ T cell subpopulation activation patterns seen. MHC class II molecules are constitutively expressed on normal IECs and their expression is increased on IBD epithelium [36]. The co-stimulatory molecule CD58 is found on normal epithelium while CD86 has been demonstrated only on UC epithelium [38, 40]. An attractive hypothesis would therefore be
that in the normal state CD4+ T cells are activated via TCR/CD3 by MHC class II molecules expressed on IECs. In IBD, stronger activation of TCR/CD3 by the increased levels of class II and CD2/CD28 by CD58/CD86 (in UC) molecules may shift CD4+ T cell subpopulations from relatively hypoproliferative cells that produce low levels of cytokines to proliferating, high cytokine secreting ones. In the present study we show that when HLA-DR expression on IBD IECs is blocked prior to co-culture with CD4+ T cells a reduction of interferon-γ production, as demonstrated by protein secretion and mRNA expression, is seen. Thus, stimulation by class II+ IECs may be part of the mechanism of CD4+ T cell activation in IBD.

An interesting observation is that normal IECs are capable of stimulating CD4+ T cells to secrete a low but detectable amount of interferon-γ. The contribution of MHC class II molecules to normal IEC:CD4+ T cell interaction was less apparent than in IBD. This may suggest that other co-stimulatory molecules on normal IECs are responsible for interferon-γ secretion by these cells and also that a low level of interferon-γ may be important in normal mucosal homeostasis. The intriguing possibility that interferon-γ is not always a pro-inflammatory cytokine is supported by studies reporting the regulatory effects that interferon-γ has in models of inflammation and in oral tolerance induction in vitro and in vivo [64-66]. Thus, it is tempting to speculate that interferon-γ may also play a role in normal mucosal immunoregulation. There may be a fine line between the level of interferon-γ required to maintain tolerance and the level required to promote active inflammation.

Based on the data presented in the current study, as well as previous work from our laboratory and others, we propose the following model for the interaction of normal IECs and lymphocytes (Fig.13): Normal IECs predominantly activate CD8+ regulatory T cells via the MHC class I-like complex gp180:CD1d, which binds to CD8 and the TCR, respectively [9]. CD8+ T cell proliferation and low level interferon-γ production ensues. These suppressor
CD8+ T cells, together with CD4+ T regulatory cells already described in humans [15], are responsible for the dominant suppressive tone in the healthy intestine, which may be mediated, in part, by interferon-γ. In IBD, gp180 expression on IECs is decreased, as reported previously [44]. This leads to the defective formation of the gp180:CD1d complex leading to decreased suppressor CD8+ T cell activation [51]. In parallel, the direct interaction of IECs with CD4+ T cells leads to increased CD4+ T cell activation resulting in increased proliferation and interferon-γ secretion. This interaction, which is mediated by MHC class II molecules, as has been shown in the present study, may further contribute to skewing of the delicate balance between suppression and over-active immunity towards the latter.
**Text footnotes**

1) **Grant Support:** This work was supported by NIH grants AI 23504, AI 24671, AI 44236.

Iris Dotan was supported by a Crohn’s and Colitis Foundation of America (CCFA) fellowship award. Matthieu Allez was supported by a CCFA fellowship award, Societe Nationale Francaise de Gastro-Enterologie (SNFGE), Institut de Recherche des Maladies de l’Appareil Digestif (IRMAD) and Laboratoire Glaxo Wellcome. Jens Brimnes was supported by the Danish Medical Research Agency.

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3) **Abbreviations used in this paper:** IECs: intestinal epithelial cells, IBD: inflammatory bowel disease, CD: Crohn’s disease, UC: ulcerative colitis, CFSE: carboxyfluorescein diacetate succinimidyl ester, PBT: peripheral blood T cells, MLR: mixed lymphocyte reaction, ESA: epithelial specific antigen, NL: normal.
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Figure legends:

Fig. 1. T cell proliferation induced by normal, CD or UC IECs. IECs from IBD or normal mucosa were co-cultured with CFSE labeled allogeneic normal peripheral-blood T cells (PBT). T cell proliferation was assessed 5-6 days after incubation by flow cytometric analysis. Viable lymphocytes were gated by their forward and side scatter properties and then separated to CFSE-high (non-proliferating) vs. CFSE-low (proliferating) CD3+CD4+ vs. CD3+CD4- T cells. T cells incubated alone (Fig. 1a, negative control) are represented in Fig. 1a. Co-cultures with conventional antigen presenting cells (non-T cells, positive control) are represented in Fig. 1e. The proliferation of PBT induced by IECs that were isolated from normal, CD and UC mucosa is represented in Fig. 1b, c, and d, respectively. A representative example of 7 independent experiments is depicted.

Fig. 2. CD3+CD4+ vs. CD3+CD4- T cell proliferation in IEC:T cell co-cultures. The averages of CD3+CD4+ vs. CD3+CD4- proliferation in IEC:T cell co-cultures (such as the representative experiment in Fig. 1) are summarized. Co-culture conditions (7 experiments for each condition) are depicted on the left column. Black bars: CD3+CD4+ T cells, grey bars: CD3+CD4- (CD8+) T cells. Values are mean±SE. * p=0.04, † p=0.009, ‡ p=0.04, and § p=0.01 vs. CD3+CD4- T cell proliferation;

Fig. 3. Purified CD4+ T cell proliferation induced by normal, CD or UC IECs. IECs from IBD or normal mucosa were co-cultured with allogeneic CD4+ T cells that were purified by negative selection from whole blood of healthy volunteers. Purified CD4+ T cell preparations were >98% CD4+CD4+. Proliferation was assessed 5-6 days after incubation by flow cytometry. Viable lymphocytes were gated by their forward and side scatter properties and then separated into CFSE-high (non-
proliferating) vs. CFSE-low (proliferating) CD3+CD4+ T cells. A representative example of 9 independent experiments is depicted.

**Fig. 4. CD4+ T cell proliferation in IEC: purified CD4+ T cell co-cultures (CFSE).** The averages of CD3+CD4+ T cell proliferation in IEC: purified CD4+ T cell co-cultures (such as the representative experiment in Fig. 3a) are summarized. Co-culture conditions (7 representative experiments for each condition) are depicted in the left column. Black bars: CD3+CD4+ T cells. Values are mean±SE. *p=0.002, †p= 0.03 and ‡p=0.03 vs. CD4+ T cell proliferation induced by NL IECs;

**Fig. 5. CD4+ T cell proliferation in IEC: purified CD4+ T cell co-cultures (3H-thymidine).** In order to validate and quantitate the proliferative responses seen in the CFSE studies, 3H-thymidine was used. IECs from normal or IBD patients were co-cultured with purified allogeneic CD4+ T cells in 100ul triplicate microwell cultures. After 5 days cells were pulsed with 1 uCi 3H-thymidine, harvested and counted. Co-culture conditions (n=22) are depicted in the left column. Values are mean±SE. *p=0.003 vs. normal, †p<0.00008 vs. normal.

**Fig. 6. T cell proliferation in IEC: autologous vs. allogeneic T cell co-cultures.** IECs from normal or IBD patients were co-cultured with allogeneic or autologous T cells in 100ul triplicate microwell cultures. After 5 days cells were pulsed with 1 uCi 3H-thymidine, harvested and counted. Three experiments using normal IECs are depicted. Co-culture conditions are depicted in the left column. Comparable results were generated in CD IECs auto/allo T cell co-cultures using CFSE (see Fig. 7). p=ns.

**Fig. 7. T cell proliferation in cross over IEC: T cell co-cultures.** Co cultures of normal IECs with CFSE labeled T cells from CD patients were established, and compared to normal autologous T cell proliferation, as well as to normal T cell proliferation after
co-culture with CD IECs. Proliferation after 5 days was assessed using flow
cytometry. The results of one set of crossover experiments, representing 3 similar
experiments are depicted. Co-culture conditions are depicted in the left column.
Numbers in brackets (1, 2, and 3) represent a different patient each.

**Fig. 8. Interferon-γ secretion by CD4+ and CD8+ T cells.** IECs were isolated from normal, CD or UC tissues and co-cultured with CD4+ (Fig. 8a) or CD8+ (Fig. 8b) T cells. The concentration of interferon-γ in co-culture supernatants was determined at two time points (36 and 120 hours) by ELISA. The source of IECs and incubation time with CD4+ T cells are depicted in the left column. The mean±SE of 12 independent experiments is depicted. a p=0.01, b p=0.002, and c p<0.0001 in IBD IEC or non T:CD4+ T cell co-cultures vs. interferon-γ secretion by NL IEC:CD4+ T cell co-cultures at 36 hours. d p=0.001 e p=0.0005 and f p<0.0001 in IBD IEC or non T:CD4+ T cell co-cultures vs. interferon-γ secretion by NL CD4+ T cells at 120 hours. Grey bars: 36 hours, black bars: 120 hours. Fig. 8c: The ratio of interferon-γ secretion by CD4+ vs. CD8+ T cells from the same donor stimulated by IECs from NL, CD or UC donor was assessed at two time points (36 and 120 hours) in four independent experiments. The mean ratio±SE is depicted. *p=0.008, †p= 0.002 vs. the ratio of CD4/CD8 interferon-γ secretion at 120 hours. Grey bars: ratio at 36 hours, black bars: ratio at 120 hours.

**Fig. 9. CD4+ T cells from a single donor exhibit differential proliferation and interferon-γ expression and secretion patterns in response to normal vs. CD IECs.** Purified CD4+ T cells from a single donor were co-cultured with IEC preparations from either normal or IBD patients that were isolated on the same day. CD4+ T cells were stained with CFSE prior to the co-culture and proliferation was assessed 5 days after stimulation by flow cytometry. Interferon-γ secretion was
determined by ELISA of co-culture supernatants harvested 120 hours after stimulation, and interferon-γ mRNA expression was determined by RT-PCR using mRNA that was extracted 24 hours after stimulation. A representative example of two independent experiments is depicted.

**Fig. 10. HLA-DR expression on freshly isolated normal, CD and UC IECs.** HLA-DR expression was determined using staining freshly isolated IECs from normal; IBD (CD and UC) and non-IBD inflammatory control tissues with PE-conjugated anti-HLA-DR mAb (L243) and flow cytometric analysis. Gated cells were viable epithelial cells (PI-negative, ESA positive). A representative example of 5 independent experiments is depicted. The average MFI and percentages of cells expressing HLA-DR for each patient group are reported in table 2.

**Fig. 11. The effect of HLA-DR blockade on interferon-γ secretion in IEC: T co-cultures.** IECs from normal (**Fig. 11a**), CD (**Fig. 11b**) and UC (**Fig. 11c**) tissues were incubated with the anti-HLA-DR blocking monoclonal antibody L243 or with an isotype control, washed and co-cultured with CD4+ T cells. Interferon-γ secretion after 120 hours was used as a measurement of CD4+ T cell stimulation by IECs. CD4+ T cells incubated alone or with non T cells served as negative and positive controls, respectively. A representative example of four independent experiments is depicted.

**Fig. 12. The effect of HLA-DR blockade on interferon-γ mRNA expression in IEC stimulated T cells.** IECs isolated from normal and IBD tissues were co-cultured with purified CD4+ T cells with or without prior blocking by anti-HLA-DR (mAb L243). RNA was extracted after 24-36 hours and interferon-γ expression was assessed by RT-PCR. An example of interferon-γ expression in co-cultures of
normal, CD or UC IECs and CD4+ T cells without (left lane) or with (right lane) prior HLA-DR blockade from 4 independent experiments is depicted.

**Fig. 13. IEC:CD4+ T cell interactions – a proposed *in vitro* model.** A proposed model for the interaction of IECs and CD4+ T cells is presented, based upon the results of the current study along with previous data from our laboratory and others.
Table 1. IL-2 and IL-10 production by CD4+ T cells in normal and IBD co-cultures.

<table>
<thead>
<tr>
<th></th>
<th>NL IEC:CD4</th>
<th>CD IEC:CD4</th>
<th>UC IEC:CD4</th>
<th>Non-T:CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-2 (pg/ml)</strong></td>
<td>34±10.2</td>
<td>66.4±39</td>
<td>185.3±77</td>
<td>864±199*</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td>(n=4)</td>
<td>(n=5)</td>
<td>(n=3)</td>
</tr>
<tr>
<td><strong>IL-10 (pg/ml)</strong></td>
<td>21.1±4.1</td>
<td>8.8±5.3</td>
<td>4.5±0.2‡</td>
<td>340±170†</td>
</tr>
<tr>
<td>(n=10)</td>
<td>(n=8)</td>
<td>(n=6)</td>
<td>(n=4)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SE

* p=0.02 vs. NL IEC:CD4

†p=0.03 vs. NL IEC:CD4

‡p<0.0001 vs. NL IEC:CD4
Table 2: HLA-DR expression by fresh normal, CD and UC IECs.

<table>
<thead>
<tr>
<th>Origin of IECs</th>
<th>MFI</th>
<th>DR+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL (n=4)</td>
<td>32±11</td>
<td>3.8±0.7</td>
</tr>
<tr>
<td>CD (n=4)</td>
<td>1052±406‡</td>
<td>66±12.2 †</td>
</tr>
<tr>
<td>UC (n=5)</td>
<td>385±114‡</td>
<td>46.3±5.6*</td>
</tr>
</tbody>
</table>

Values are mean±SE

* p=0.003 vs. NL
† p =0.002 vs. NL
‡ p=0.03 vs. NL
Cells in culture

<table>
<thead>
<tr>
<th>Cells in culture</th>
<th>CD4+ T cell proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL T cells alone</td>
<td>□</td>
</tr>
<tr>
<td>NL IECs:</td>
<td>□</td>
</tr>
<tr>
<td>NL CD4+ T cells</td>
<td>□</td>
</tr>
<tr>
<td>CD IECs:</td>
<td>*</td>
</tr>
<tr>
<td>NL CD4+ T cells</td>
<td></td>
</tr>
<tr>
<td>UC IECs:</td>
<td>†</td>
</tr>
<tr>
<td>NL CD4+ T cells</td>
<td></td>
</tr>
<tr>
<td>Non-T:</td>
<td>‡</td>
</tr>
<tr>
<td>NL CD4+ T cells</td>
<td></td>
</tr>
</tbody>
</table>

% Proliferation

0  5  10  15  20  25  30  35  40  45  50
Cells in culture

NL T cells alone
NL IECs:
NL CD4+ T cells
CD IECs:
NL CD4+ T cells
UC IECs:
NL CD4+ T cells
Non-T:
NL CD4+ T cells

CD4+ T cell proliferation

Proliferation (CPM)
Cells in culture

T cell proliferation

- NL T cells alone
- NL IECs: autologous NL T cells
- NL IECs: allogeneic NL T cells
- Non-T: NL T cells

Proliferation (CPM)
Cells in culture

NL(2) IECs: CD(1) T Cells
NL(3) IECs: CD(1) T Cells
NL(3) IECs: NL(3) T Cells (autologous)
CD(1) IECs: CD(1) T Cells (autologous)
CD(1) IECs: NL(3) T Cells

CD4+ T cell proliferation

% Proliferation
### Incubation time with CD4+ T cells (hours)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL IECs</td>
<td>36</td>
</tr>
<tr>
<td>NL IECs</td>
<td>120</td>
</tr>
<tr>
<td>CD IECs</td>
<td>36</td>
</tr>
<tr>
<td>CD IECs</td>
<td>120</td>
</tr>
<tr>
<td>UC IECs</td>
<td>36</td>
</tr>
<tr>
<td>UC IECs</td>
<td>120</td>
</tr>
<tr>
<td>Non-T cells</td>
<td>36</td>
</tr>
<tr>
<td>Non-T cells</td>
<td>120</td>
</tr>
</tbody>
</table>

**Graph:**
- **Interferon-γ pg/ml**
- **Legend:**
  - a
  - b
  - c
  - d
  - e
  - f

**Note:** The graph illustrates the levels of Interferon-γ pg/ml for different cell types and incubation times.
<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Incubation time with CD8+ T cells (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL IECs</td>
<td>36</td>
</tr>
<tr>
<td>NL IECs</td>
<td>120</td>
</tr>
<tr>
<td>CD IECs</td>
<td>36</td>
</tr>
<tr>
<td>CD IECs</td>
<td>120</td>
</tr>
<tr>
<td>UC IECs</td>
<td>36</td>
</tr>
<tr>
<td>UC IECs</td>
<td>120</td>
</tr>
<tr>
<td>Non-T cells</td>
<td>36</td>
</tr>
<tr>
<td>Non-T cells</td>
<td>120</td>
</tr>
</tbody>
</table>

**Interferon-γ pg/ml**
Stimulation Incubation time with CD4+ or CD8+ T cells

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL IECs</td>
<td>36</td>
</tr>
<tr>
<td>NL IECs</td>
<td>120</td>
</tr>
<tr>
<td>CD IECs</td>
<td>36</td>
</tr>
<tr>
<td>CD IECs</td>
<td>120</td>
</tr>
<tr>
<td>UC IECs</td>
<td>36</td>
</tr>
<tr>
<td>UC IECs</td>
<td>120</td>
</tr>
</tbody>
</table>

![Graph showing the ratio for different tissue types and incubation times.](image)
11a: Co-culture conditions: Normal IECs, Normal CD4+ T cells

IECs alone
CD4+ T cells alone
Non-T cells alone
IEC:CD4+ T cells
IEC αHLA class II:CD4+ T cells
IEC Isotype:CD4+ T cells
Non-T:CD4+ T cells
Non-T αHLA class II:CD4+ T cells
Non-T Isotype:CD4+ T cells

Interferon-γ pg/ml
11b: Co-culture conditions: CD IECs, Normal CD4+ T cells

IECs alone
CD4+ T cells alone
Non-T cells alone
IEC:CD4+ T cells
IEC αHLA class II:CD4+ T cells
IEC Isotype:CD4+ T cells
Non-T:CD4+ T cells
Non-T αHLA class II:CD4+ T cells
Non-T Isotype:CD4+ T cells

Interferon-γ pg/ml
11c: Co-culture conditions: UC IECs, Normal CD4+ T cells

IECs alone
CD4+ T cells alone
Non-T cells alone
IEC:CD4+ T cells
IEC αHLA class II:CD4+ T cells
IEC Isotype:CD4+ T cells
Non-T:CD4+ T cells
Non-T αHLA class II:CD4+ T cells
Non-T Isotype:CD4+ T cells

Interferon-γ pg/ml
<table>
<thead>
<tr>
<th></th>
<th>UC</th>
<th>CD</th>
<th>NL</th>
</tr>
</thead>
<tbody>
<tr>
<td>αHLA class II</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ</td>
<td><img src="image" alt="IFN-γ UC" /></td>
<td><img src="image" alt="IFN-γ CD" /></td>
<td><img src="image" alt="IFN-γ NL" /></td>
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<tr>
<td>Actin</td>
<td><img src="image" alt="Actin UC" /></td>
<td><img src="image" alt="Actin CD" /></td>
<td><img src="image" alt="Actin NL" /></td>
</tr>
</tbody>
</table>
Normal IECs

- gp-180, CD1d
- Treg?
- MHC II

IBD IECs

- No gp-180, CD1d
- MHC II CD86

CD8+ T cell

- Low proliferation & Interferon-γ secretion

CD4+ T cell

- High proliferation interferon-γ secretion

Treg?