Intestinal epithelial cells from inflammatory bowel disease patients preferentially stimulate CD4+ T cells to proliferate and secrete interferon-γ

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Submitted 5 July 2006; accepted in final form 6 March 2007

Inflammatotry bowel disease (IBD) is a chronic inflammatory intestinal disorder of unknown etiology comprised of two phenotypes: Crohn’s disease (CD) and ulcerative colitis (UC). Although the precise cause of inflammation is as yet undefined, it is believed that an antigen-driven response against nonpathogenic bowel flora is one of the initial events (15, 49). This has been clearly demonstrated in animal models of IBD (13, 52, 57) and has been suggested in human disease (4, 6, 7, 11, 54, 60). Unlike the normal state, in which an immunological response toward normal bowel flora and food antigens has been proposed to mediate controlled, “physiological,” 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) (3, 9, 10, 26, 45). In humans, however, these distinctions are not as clear and extrapolation from animal models is not straightforward (55). 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 (41, 47, 50, 51). For IFN-γ and IL-2, however, conflicting data exist, as both increased and decreased levels of these cytokines have been reported under various conditions (19, 21, 37). 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 (18, 19, 42). The role of regulatory Th3/Tr1 cells (8, 62), generally associated with tolerance, has not yet been defined in IBD, and both increased (28) and decreased (20) 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 (24, 33, 35, 58, 61). 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 major histocompatibility complex (MHC) class II molecules on IECs and their increased expression in IBD (39) make the direct interaction of IECs with CD4+ T cells a realistic possibility. Moreover, the demonstration of CD58 and CD1d, which associate and deliver costimulatory signals, thereby initiating active immunity. In the microenvironment of the IECs, however, costimulation may be less critical, because the vast majority of lamina propria lymphocytes (LPLs) are memory cells (56, 59). The demonstration, by Hershberg’s group (17, 23), of direct activation of IECs by CD4+ T cells a realistic possibility. Moreover, the demonstration of CD58 and CD86 on IECs in normal (17) and in IBD (UC) patients (44), respectively, and of B7H on normal as well as IBD IECs (22, 43) suggests that these cells are able to deliver costimulatory signals, thereby initiating active immunity. In the microenvironment of the IECs, however, costimulation may be less critical, because the vast majority of lamina propria lymphocytes (LPLs) are memory cells (56, 59). The demonstration, by Hershberg’s group (17, 23), of direct activation of CD4+ T cells by DR+ IEC lines and our own work demonstrating activation of CD4+ associated p56-lck by IBD IECs (63) 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 (2, 40, 63). This occurs through the IEC surface molecules gp180 (CD8 ligand) and CD1d, which associate and form a MHC class I-like complex (40) that stimulates CD8+ T cell activation, which is reflected in T cell proliferation (34, 48, 66). In IBD, gp180’s expression is decreased (63), so stimulation of CD8+ suppressor T cells does not occur (5), and the delicate balance between CD8+ T cell suppression and CD4+
T cell active immunity could be skewed toward 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 overactivity. To address this hypothesis, we compared the proliferation and cytokine secretion profiles of CD4+ T cells that were cocultured with fresh human IECs from normal and IBD tissues. In this study we demonstrate that IBD IECs preferentially stimulate CD4+ T cells 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 IECs**

Surgical specimens were obtained from colonic resections performed at the Mount Sinai Medical Center. Forty-five IEC-peripheral blood T cell (PB T cell) cocultures 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 (38). Briefly, specimens were washed with PBS, and the mucosa was stripped from the submucosa and placed in 1 mM dithiothreitol (Sigma, St. Louis, MO) for 10 min. The pieces were then washed with PBS and incubated twice in dispase (Boehringer Mannheim, Indianapolis, IN), 3 mg/ml streptomycin, 2 mM glutamine all from GIBCO BRL, Grand Island, NY) for coculture 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 antiepithelial cell-specific antigen (ESA, Biomeda) and flow cytometric analysis. IEC preparations were irradiated (3,000 rads) before coculture 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 by the RosetteSep procedure (StemCell Technologies, Vancouver, Canada) according to the manufacturer’s instructions. In brief, tetrameric antibody complexes directed against glycoporphin A on red blood cells 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 (50 μl/ml) and incubated at room temperature. After 20 min, samples were diluted with PBS-2% FBS, layered over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 20 min (the unwanted cells pellet along with the red blood cells). The interface was collected, washed twice with PBS-2% FBS and resuspended in serum-free medium (Aim-V, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine all from GIBCO BRL, Grand Island, NY) for coculture 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 antiepithelial cell-specific antigen (ESA, Biomeda) and flow cytometric analysis using Cell Quest software. IEC preparations were irradiated (3,000 rads) before coculture with T cells.

**Coculture Assays**

A total of 1 x 10⁶ IECs from normal or IBD patients were cocultured with 1 x 10⁶ 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 cells, and 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 h after incubation and frozen at −70°C until ELISA assays were performed. In several experiments, IECs from normal and IBD patients were cocultured 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 crossover studies, in which IECs from a normal donor were cocultured with IBD peripheral blood T cells).

**Proliferation Assessment**

Membrane labeling of T cells with CFSE. Prior to the coculture assays, CD4+, CD8+ and whole T cells were resuspended in PBS at 5 x 10⁶/ml and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR). Cells were incubated for 10 min at 37°C with 1 mM (final concentration) CFSE, washed twice with RPMI, and used in the coculture experiments.

Flow cytometric analysis. Labeling of T cells with CFSE was performed before cocultures. Five days after incubation, T cells were resuspended in PBS and incubated for 30 min with antibodies. The following antibodies were used: anti-CD3, anti-CD4, and anti-CD8 (conjugated with phycoerythrin, peridinin chlorophyll protein, 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 (Becton Dickinson). Nonproliferating cells remain high in CFSE (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 CD8+ as well as those of CFSE-high and CFSE-low cells were determined. The data obtained by this approach was confirmed by standard [³H]thymidine incorporation assays.

[³H]Thymidine incorporation assays. Five days after incubation, cells were resuspended and placed in 100-μl triplicate microwell cultures for pulsing. Cells were pulsed with [³H]thymidine 1 μCi for 18 h, harvested (PHD Cambridge harvester, Cambridge Technologies, Cambridge, MA) and read by a β counter (Beckman 3801, Fullerton, CA).

ELISAs. IFN-γ, IL-2, and IL-10 production by cocultured 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 μg of RNA, extracted with Trizol reagent 24–36 h 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 μl. The reaction was stopped by heat inactivation and the cDNA was diluted to 100 μl. For each PCR reaction, 2.5 μl cDNA, 10 pM of forward and reverse primer, platinum Taq 0.2u with the supplied buffer, and 2 mM MgCl₂ and 200 μM dNTPs were used in a 25-μl final volume. The PCR cycle was 4 min at 95°C to activate the enzyme, then 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min for 30 cycles followed by 10-min extensions at 72°C.

The following primers were used (27): IFN-γ, 5’ primer 5’-ATGGATATCATGCCATACGCTGT-3’, 3’ primer 5’-GATCGTCTTTGCGACCTCCTGAAAACAGCAT-3’; β-actin, 5’ primer 5’-TGAAGGG-5’-TACCCCACTGTGCCCCATCTA-3’, 3’ primer 5’-CTAGAAAGCAT-TGGCGTGGACGATGGAGG-3’. Blocking assays. In some experiments, before the coculture, IECs were incubated with a purified blocking anti-DR monoclonal antibody (L243, ATCC, Rockville, MD) at 35 μg/ml for 1 h at 4°C, or an
isotype control (monoclonal antibody 204-Ig2b anti-B cell differentiation factor, which is not expressed by IECs). The cells were then washed twice with PBS and resuspended in AIM-V medium for coculture experiments. An aliquot of these IECs was stained by phycocerythrin-conjugated L243 (BD-Pharmingen) to assess human leukocyte antigen (HLA)-DR expression on fresh IECs. These cells were counterstained with FITC-conjugated ESA to confirm that HLA-DR expression was restricted to IECs. Nonviable IECs were excluded by propidium-iodide 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. Nonstimulated 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 cocultures, 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 cocultures 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 CD4+ T cell proliferation was compared between IBD and normal-IEC-stimulated cocultures, there was a higher proliferation of CD4+ T cells in IBD IECs T-cell cocultures (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 cocultured 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 IEC-stimulated cocultures. The percentage of CD4+ CFSE-low (proliferating) T cells in CD IEC:CD4+ T cell cocultures was 13.4 ± 1.9% (n = 7, P = 0.001 vs. normal) and 19.5 ± 7.2% in UC cocultures (n = 4, P = 0.02 vs. normal) vs. 3.3 ± 1.3% in normal IEC:CD4+ T cell cocultures (n = 6). Non-T cells induced even higher CD4+ T cell proliferation (21.7 ± 3.2%, n = 9, P = 0.03). To validate the CFSE data, as well as to 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 cocultures, mean CD4+ T cell proliferation was 5,864 ± 1,980 cpm (n = 22). This increased to 7,837 ± 1,966 cpm (n = 18) when CD IECs were the stimulator cells (P = 0.003 vs. normal) and to 17,450 ± 6,147 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:PBT cell cocultures are restricted by a complex of CEA and CD1d (7), one would predict that T cell responses to allogeneic vs. autologous

Fig. 1. T cell proliferation induced by normal (NL), Crohn’s disease (CD), or ulcerative colitis (UC) intestinal epithelial cells (IECs). IECs from inflammatory bowel disease (IBD) or normal mucosa were cocultured with carboxyfluorescein diacetate succinimidyl ester (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 (nonproliferating) vs. CFSE-low (proliferating) CD3+CD4+ vs. CD3+CD4− T cells. T cells incubated alone (negative control) are represented in A. Cocultures with conventional antigen presenting cells (non-T cells, positive control) are represented in E. The proliferation of PBT induced by IECs that were isolated from normal, CD, and UC mucosa is represented in B, C, and D, respectively. A representative example of 7 independent experiments is depicted.
IBD IECs Stimulate 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 IFN-γ secretion, above background, starting at 24–48 h after incubation and reaching a plateau at days 5–6. Thus, IFN-γ concentrations were determined at two points in time (36 and 120 h). IECs and non-stimulated CD4+ or CD8+ T cells incubated alone secreted <40 pg/ml IFN-γ throughout the incubation period. There was no significant difference between IFN-γ secretion seen in normal and IBD IECs (control for contaminating intraepithelial lymphocytes) or by CD4+ or CD8+ T cells alone used in the different cocultures. At 36 h, IFN-γ secretion by CD4+ T cells cocultured with normal IECs was similar to background levels. However, at 120 h an increase to a mean concentration of 205.6 ± 54.9 pg/ml was observed (P = 0.008 vs. 36 h). In contrast, IBD IECs stimulated significantly higher IFN-γ secretion by CD4+ T cells. At 36 h, the IFN-γ concentration in CD, UC, and normal IEC:CD4+ T cell cocultures 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. normal and 0.002 for UC vs. normal). This increased secretion persisted in both CD and UC vs. normal IEC-stimulated cocultures over a 5-day period (Fig. 8A).

Fig. 2. CD3+CD4+ vs. CD3+CD4− T cell proliferation in IEC:T cell cocultures. The averages of CD3+CD4+ vs. CD3+CD4− proliferation in IEC:T cell cocultures (such as the representative experiment in Fig. 1) are summarized. Coculture conditions (7 experiments for each condition) are depicted on the left column. Error bars represent SEM. *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 cocultured with allogeneic CD4+ T cells that were purified by negative selection from whole blood of healthy volunteers. Purified CD4+ T cell preparations were >98% CD3+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 (nonproliferating) vs. CFSE-low (proliferating) CD3+CD4+ T cells. A representative example of 9 independent experiments is depicted.
CD4+ T cell proliferation induced by normal IECs was stronger than the one induced by normal IECs. As seen in Fig. 8, normal or IBD IECs also contribute to the overall production of IFN-γ, in contrast to IEC-stimulated CD4+ T cells. As demonstrated in Fig. 9, CD4+ T cells from the same healthy donor were stimulated by the same IEC preparation, IFN-γ secretion by the stimulated CD4+ T cells after 120 h was 2.4 higher than the stimulated CD8+ T cells in the normal cocultures, five times higher in UC cocultures (P = 0.002 vs. normal), and 15 times higher in CD cocultures (P = 0.008 vs. normal), suggesting a greater contribution to IFN-γ secretion by CD8+ T cells in the normal IEC-stimulated cocultures (Fig. 8C).

Interleukin-2 Secretion

To determine whether the increase in IFN-γ production by IBD IECs stimulated CD4+ T cells paralleled a more classic

Fig. 4. CD4+ T cell proliferation in IEC-purified CD4+ T cell cocultures (CFSE). The averages of CD3+CD4+ T cell proliferation in IEC-purified CD4+ T cell cocultures (such as the representative experiment in Fig. 3) are summarized. Coculture conditions (7 representative experiments for each condition) are depicted at left. Solid 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 normal IECs.

At 120 h, the IFN-γ concentration in CD, UC, and normal IEC:CD4+ T cell cocultures was 1,014 ± 238, 1,139 ± 263.4, and 205.6 ± 54.9 pg/ml, respectively (P = 0.001 for CD vs. normal and 0.0005 for UC vs. normal). Interestingly, this secretion was ~80% of the maximal IFN-γ secretion stimulated by professional APCs (non-T cells). IFN-γ 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 IFN-γ mRNA expression, assessed by RT-PCR (see Fig. 12). These results suggest that the difference between normal and IBD cocultures is not merely kinetic, but that the signal for IFN-γ 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 IFN-γ. As seen in Fig. 8B, in contrast to IEC-stimulated CD4+ T cells (Fig. 8A), CD8+ T cells secreted low concentrations of IFN-γ (<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, IFN-γ secretion by the stimulated CD4+ T cells after 120 h was 2.4 higher than the stimulated CD8+ T cells in the normal cocultures, five times higher in UC cocultures (P = 0.002 vs. normal), and 15 times higher in CD cocultures (P = 0.008 vs. normal), suggesting a greater contribution to IFN-γ secretion by CD8+ T cells in the normal IEC-stimulated cocultures (Fig. 8C).

The different PBT cell preparations that are used in the cocultures may have different responses in allogeneic cocultures. 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 IFN-γ when IBD IECs (compared with normal IECs) were the stimulator cells.

Interleukin-2 Secretion

To determine whether the increase in IFN-γ production by IBD IECs stimulated CD4+ T cells paralleled a more classic

Fig. 5. CD4+ T cell proliferation in IEC-purified CD4+ T cell cocultures. (°[3H]thymidine). To validate and quantify the proliferative responses seen in the CFSE studies, °[3H]thymidine was used. IECs from normal or IBD patients were cocultured with purified allogeneic CD4+ T cells in 100-μl triplicate microwell cultures. After 5 days cells were pulsed with 1 μCi °[3H]thymidine, harvested, and counted. Three experiments using normal IECs are depicted. Coculture conditions are depicted at left. Comparable results were generated in CD IECs auto/allo T cell cocultures using CFSE (see Fig. 7). P = not significant.

Fig. 6. T cell proliferation in IEC:autologous vs. allogeneic T cell cocultures. IECs from normal or IBD patients were cocultured with allogeneic or autologous T cells in 100-μl triplicate microwell cultures. After 5 days cells were pulsed with 1 μCi °[3H]thymidine, harvested, and counted. Three experiments using normal IECs are depicted. Coculture conditions are depicted at left. Comparable results were generated in CD IECs auto/allo T cell cocultures using CFSE (see Fig. 7). P = not significant.

Fig. 7. T cell proliferation in IEC:autologous vs. allogeneic T cell cocultures. IECs from normal or IBD patients were cocultured with allogeneic or autologous T cells in 100-μl triplicate microwell cultures. After 5 days cells were pulsed with 1 μCi °[3H]thymidine, harvested, and counted. Three experiments using normal IECs are depicted. Coculture conditions are depicted at left. Comparable results were generated in CD IECs auto/allo T cell cocultures using CFSE (see Fig. 7). P = not significant.

Fig. 8. T cell proliferation in IEC:autologous vs. allogeneic T cell cocultures. IECs from normal or IBD patients were cocultured with allogeneic or autologous T cells in 100-μl triplicate microwell cultures. After 5 days cells were pulsed with 1 μCi °[3H]thymidine, harvested, and counted. Three experiments using normal IECs are depicted. Coculture conditions are depicted at left. Comparable results were generated in CD IECs auto/allo T cell cocultures using CFSE (see Fig. 7). P = not significant.
Th1 cytokine profile, IL-2 secretion was measured in the same coculture supernatants. IL-2 levels at 120 h in normal, CD, and UC cocultures were $34 \pm 10.2$, $66.4 \pm 39$, and $185.3 \pm 77$ pg/ml, respectively (Table 1) ($P =$ not significant). When CD4$^+$ T cells were stimulated by conventional APCs (non-T cells), maximal stimulation resulted in IL-2 levels of $664 \pm 199$ pg/ml at 120 h ($P =$ 0.02 vs. normal IEC-induced IL-2 secretion by CD4$^+$ T cells).

IFN-$\gamma$ concentrations that were secreted in the same normal, CD, and UC cocultures were $205.6 \pm 54.9$, $1,014 \pm 238$, and $1,139 \pm 263.4$ pg/ml, respectively, and $1,556 \pm 90$ in non-T-stimulated CD4$^+$ T cell cocultures. Thus IEC-stimulated IFN-$\gamma$ secretion by CD4$^+$ T cells is relatively higher than the corresponding value for IL-2.

### IL-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 cocultures could help to explain the observed differences in cytokine secretion profiles. Low (<35 pg/ml) IL-10 levels were demonstrated in all cocultures (Table 1), compared with concentrations of $340 \pm 170$ pg/ml at 120 h, 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 IFN-$\gamma$ secretion. In previous studies, increased HLA-DR expression on IBD IECs and a low constitutive HLA-DR expression on normal IECs was demonstrated (39). Therefore, we hypothesized that HLA-DR molecules, which are a prerequisite for the interaction of APCs 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). From 2.5 to 5.5% [mean fluorescence intensity (MFI) = 32 ± 11] of normal IECs expressed HLA-DR. In contrast, 45–97% of CD (MFI = 1,052 ± 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 cocultured with CD4$^+$ T cells. Because IFN-$\gamma$ secretion was reproducibly demonstrated in IEC:CD4$^+$ T cell cocultures, we used IFN-$\gamma$ secretion as a measure of CD4$^+$ T cell stimulation by IECs. Preincubation of IECs with MAb L243, but not with an isotype control, significantly decreased IFN-$\gamma$ secretion in the cocultures by 80% (UC) to 99% (CD) (Fig. 11, B and C). IFN-$\gamma$ mRNA expression, assessed by RT-PCR, supported the ELISA results (Fig. 12). Normal cocultures, which, as shown, had significantly lower IFN-$\gamma$ secretion, were less influenced by HLA-DR blockade (up to 50% decrease in IFN-$\gamma$ secretion).
best reflected by the observation of increased IFN-γ expression and secretion patterns in response to normal vs. CD IECs. Purified CD4+ T cells from a single donor were cocultured with IEC preparations from either normal or IBD patients that were isolated on the same day. CD4+ T cells were stained with CFSE before the coculture and proliferation was assessed 5 days after stimulation by flow cytometry. IFN-γ secretion was determined by ELISA of coculture supernatants harvested 120 h after stimulation, and IFN-γ mRNA expression was determined by RT-PCR using mRNA that was extracted 24 h after stimulation. A representative example of 2 independent experiments is depicted.

(Figs. 11A and 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 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 IFN-γ secretion, which was significantly higher when CD4+ T cells were stimulated by IBD IECs compared with 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 toward a controlled, regulated response in the normal state, or toward overactive immunity such as the uncontrolled inflammation seen in IBD.

We used cocultures of freshly isolated human IECs (from IBD patients and normal controls) with allogeneic normal PBTs 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 nontransformed IECs, whereas 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 phytohemagglutinin/PMA, or antibodies against the T cell receptor (TCR). Furthermore, the results produced by our coculture 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 (7). 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 PBT cells from IBD or normal patients related to the stimulating IEC source.

Previous studies have reported increases in local IFN-γ production in tissues of patients with IBD (CD > UC). Increased IFN-γ mRNA expression was demonstrated in mucosal samples from IBD patients compared with normal controls (46), in chronic vs. early ileal lesions in CD patients (12), in active and inactive CD lesions, and in specimens of pediatric CD patients compared with UC and normal controls (1, 4). Increased IFN-γ mRNA has also been demonstrated in tissues from UC patients (1, 25, 64). Results obtained directly from

Table 1. IL-2 and IL-10 production by CD4+ T cells in normal and IBD cocultures

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

Values are means ± SE. IBD, inflammatory bowel disease; NL, normal; CD, Crohn’s disease; UC, ulcerative colitis; IEC, Intestinal epithelial cells. *P = 0.02 vs. NL IEC:CD4; †P = 0.03 vs. NL IEC:CD4; ‡P < 0.0001 vs. NL IEC:CD4.
tissues and analyzed for cytokine mRNA content are assumed to reflect the cytokine content within the tissue at that specific point in time. Although 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 is 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 IFN-γ production, with or without various stimuli, have been documented. LPLs from CD patients demonstrated significantly increased intracellular IFN-γ vs. LPLs from control patients (6), and increased IFN-γ secreting mononuclear cells were found in pediatric CD vs. UC and normal controls (4). CD4+ LPLs from CD patients secreted more IFN-γ after stimulation via CD2/CD28 compared with UC or normal CD4+ LPLs (19), and spontaneous release of IFN-γ from cultured CD LPLs was also reported, in contrast to its absence in cultured normal LPLs (14). Our finding of increased IFN-γ secretion in IBD IEC stimulated CD4+ T cell cocultures suggest one possible mechanism for the findings reported in these previously published studies, which is that IECs from IBD patients are capable of triggering IFN-γ secretion by CD4+ T cells.

Do IBD IECs stimulate classic Th1 cells? The data presented here do not support this possibility. In general, whereas most studies have reported increased levels of IFN-γ and Th1-promoting cytokines in IBD tissues (36), results relating to IL-2 production are inconsistent, showing both increased (in CD) (4, 41, 46), and decreased (30) levels in IBD vs. normal. In support of the latter observations, IL-2 levels were demonstrated in both IBD and normal cocultures. Moreover, when IBD IECs were the stimulator cells, IFN-γ secretion by CD4+ T cells was ~70% of that seen in conventional mixed lymphocyte reactions (MLRs). In contrast, CD4+ T cell secretion of IL-2 was only ~20% of that seen in conventional MLRs (in the same cocultures) when the stimulator cells were IBD IECs. The discordant secretion of IL-2 and IFN-γ raises further questions regarding classical Th1 cytokine profiles in the human intestinal mucosal immune system.

**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 ± 12.5‡</td>
</tr>
<tr>
<td>UC (n = 5)</td>
<td>385 ± 114‡</td>
<td>46.3 ± 5.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE. MFI, mean fluorescence intensity. HLA, human leukocyte antigen. *P = 0.003 vs. NL; †P = 0.002 vs. NL; ‡P = 0.03 vs. NL.
In contrast to the mouse, in whom IL-10 deficiency is strongly associated with ileocolonic inflammation reminiscent of human CD (29), in human IBD tissues IL-10 mRNA is reported to be increased rather than decreased (1, 25). 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 (20). In our study, IL-10 levels secreted in the cocultures were low, ~10% of those secreted in conventional MLRs. Nevertheless, higher mean values were demonstrated in normal IEC-stimulated CD4+ T cell cocultures (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. Present data suggest that other suppressor cytokines such as TGF-β may also contribute to tolerance in the normal intestinal mucosa (8, 65). However, we were unable to detect the presence of TGF-β mRNA in normal IEC:CD4+ T cell cocultures (data not shown).

By what mechanism do IECs stimulate CD4+ T cells? In a manner similar to their professional APC counterparts, the nonprofessional 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 (39). The costimulatory molecule CD58 is found on normal epithelium whereas CD86 has been demonstrated only on UC epithelium (44, 43). 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 before coculture with CD4+ T cells a reduction of IFN-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 IFN-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 costimulatory molecules on normal IECs are responsible for IFN-γ secretion by these cells and also that a low level of IFN-γ may be important in normal mucosal homeostasis. The intriguing possibility that IFN-γ is not always a proinflammatory cytokine is supported by studies reporting the regulatory effects that IFN-γ has in models of inflammation and in oral tolerance induction in vitro and in vivo (16, 31, 32). Thus it is tempting to speculate that IFN-γ may also play a role in normal mucosal immunoregulation. There may be a fine line between the level of IFN-γ required to maintain tolerance and the level required to promote active inflammation.

On the basis of the data presented in the present 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 (7). CD8+ T cell proliferation and low-level IFN-γ production ensue. These suppressor CD8+ T cells, together with CD4+ T regulatory cells already described in humans (26), are responsible for the dominant suppressive tone in the healthy intestine, which may be mediated, in part, by IFN-γ. In IBD, gp180 expression on IECs is decreased, as reported previously (63). This leads to the defective formation of the gp180:CD1d complex leading to decreased suppressor CD8+ T cell activation (38). In parallel, the direct interaction of IECs with CD4+ T cells leads to increased CD4+ T cell activation resulting in increased proliferation and IFN-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 overactive immunity toward the latter.

**GRANTS**

This work was supported by National Institute of Allergy and Infectious Diseases Grants AI-23504, AI-24671, and AI-44236. I. Dotan was supported by a Crohn’s and Colitis Foundation of America (CCFA) fellowship award. M. Allez was supported by a CCFA fellowship award, Societe Nationale Francaise de Gastro-Enterologie, Institut de Recherche des Maladies de l’Appareil Intestinal, and Societe Francaise de Pathologie Digestive.

**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 on the results of the present study along with previous data from our laboratory and others. MHC, major histocompatibility complex; Treg, regulating T cells.
Digestif, and Laboratoire Glaxo Wellcome. J. Brimnes was supported by the Danish Medical Research Agency.

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