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The Regulation and Functional Consequence of Proinflammatory Cytokine Binding on Human Intestinal Epithelial Cells

Asit Panja, Stan Goldberg,* Lars Eckmann,† Priya Krishen,* and Lloyd Mayer* 

Products of an activated immune system may affect cells within the immune system as well as nonlymphoid cells in the local environment. Given the immunologically activated state of the intestinal tract, it is conceivable that locally produced cytokines could regulate epithelial cell function. To assess whether epithelial cells are targets for particular cytokines, we initiated studies on the binding of a panel of proinflammatory cytokines in freshly isolated epithelial cells from normal and inflammatory bowel disease (IBD) patients as well as in cell lines. Isolated intestinal epithelial cells (IEC) were stained with phycoerythrin-conjugated or biotinylated cytokines to determine the expression and density of receptors for IL-1β, IL-6, granulocyte-macrophage-CSF (GM-CSF), and TNF-α. Receptors for IL-1β, IL-6, and GM-CSF were readily detectable in all epithelial cell preparations at levels equal to (GM-CSFR) or lower than those seen on monocytes. However TNFa-R were not detectable on freshly isolated IECs. Receptor density was greater in surface vs crypt epithelial cells, but no significant differences were seen between normal and IBD epithelial cells. Expression of IL-1R and IL-6R was enhanced by LPS and IFN-γ. Functionally, IL-1β enhanced proliferation of the IEC cell line, DLD1, whereas GM-CSF treatment of de-differentiated crypt-like DLD1 and HT29 cells resulted in enhanced expression of ICAM-1. Furthermore, TNF-α treatment enhanced the secretion of IL-8 and GRO-α in HT29 cells, but not in freshly isolated IEC cultures. The differential binding and function of proinflammatory cytokines on IEC support the hypothesis that these cytokines may be involved in normal physiological processes as well as in regulating mucosal immune responses. 


The pathophysiological mechanisms underlying the development of IBD are not fully understood. In ulcerative colitis (UC), the final target of the inflammatory process appears to be the epithelium, and epithelial cell dysfunction has been demonstrated. In Crohn’s disease (CD), inflammation extends transmurally, and the epithelium may be spared from destruction. However, there is evidence that epithelial cell dysfunction exists in this disease as well. Although separate cellular or molecular events may be involved in these inflammatory processes, it has been widely accepted that locally produced cytokines may play a critical role, comparable to their effects in a number of other chronic inflammatory disorders such as rheumatoid arthritis (1–7), psoriasis (8), and glomerulonephritis (9, 10). Proinflammatory cytokines such as IL-1, IL-6, granulocyte-macrophage CSF (GM-CSF), and TNF-α have been reported to be increased in serum (11), cultures of biopsy tissues (12–14), or isolated lamina propria lymphocytes (15–19) from inflammatory bowel disease (IBD) patients. There is increasing evidence that intestinal epithelial cells (IEC) may serve as targets for these locally produced cytokines. In this scenario, local cytokines may result in alterations of epithelial cell function or integrity. For example, the combination of TNF-α and IFN-γ is cytotoxic for the colonic epithelial cell line, HT29 (20), and IL-1β up-regulates IL-6 synthesis (a proinflammatory cytokine) by epithelial cells (21). Alternatively or additionally, cytokines may play a role in the normal regulatory mechanisms involved in epithelial cell growth and differentiation ensuring maintenance of homeostasis. An imbalance of the normal cytokine milieu seen in IBD might lead to the disruption of epithelial function. There is evidence for selective effects of various cytokines on IEC growth and differentiation, phenotype, and function through an autocrine/juxtacrine or paracrine pathway. One hypothetical model based on these scenarios might be that cytokine-mediated changes in the epithelial cell integrity might initiate pathologic events in the gut. The ability of cytokines to function in this manner depends on the presence of specific receptors expressed on IECs.

Therefore, we analyzed the binding of the proinflammatory cytokines IL-1β, IL-6, GM-CSF, and TNF-α on both freshly isolated intestinal epithelial cells and cell lines by flow cytometric analysis. Our results show that IECs from both IBD and normal control samples express receptors for GM-CSF, IL-1β, and IL-6 at comparable levels. TNFa-R were not detected on freshly isolated epithelial cells, although they were present on colonic adenocarcinoma cell lines. Functionally, IL-1 enhanced while GM-CSF inhibited IEC growth. Furthermore, GM-CSF could induce or up-regulate ICAM-1 expression preferentially on crypt-like epithelial cells. Taken together, these results support the potential for an autocrine or paracrine effect of proinflammatory and proapoptotic cytokines as potential modulators of epithelial cell integrity.
Materials and Methods
Isolation of IEC
Epithelial cells from resected bowel specimens were isolated by dispase or EDTA (TFNα-R) treatment as previously described (22, 23). Surgical specimens included those from “normal” controls, i.e., patients with adenocarcinoma (10 cm from tumor), colonic inertia, and familial polyposis, as well as those from patients with active and inactive UC and CD. In the latter cases, tissues were obtained from both areas of active inflammation and areas where no histologic inflammation was noted (where available). Briefly, after vigorous washing in PBS, the mucosal layer was dissected from the underlying submucosa. The dissected mucosal tissue was washed several times in HBSS supplemented with 1% penicillin-streptomycin, amphotericin, and gentamicin (50 μg/ml), minced into small pieces, and followed by treatment with the mucolytic agent DTT (1 mM) (Sigma, St. Louis, MO) in RPMI 1640 medium for 5 min at room temperature. Tissue pieces were washed in PBS and subjected to dispase (1 mg/ml; Boehringer Mannheim, Indianapolis, IN) or EDTA (0.75 mM; Sigma) treatment for 30 min at 37°C. During this 30-min incubation, tissue pieces were agitated every 5 min by vortex mixing, resulting in the liberation of epithelial cells and intraepithelial lymphocytes into the solution. This cell suspension was collected and immediately diluted 10-fold in PBS to minimize any effect of dispase on cytokine receptors (see below). Cells were spun down and washed twice in PBS. Epithelial cells were further separated from the contaminating intraepithelial lymphocytes and RBCs by percoll density gradient centrifugation (23). The cells on top of the 30% layer contained 95% pure epithelial cells (<1% CD45+ cells or CD14+ cells) with a viability of 95 to 100%. There was no difference in the viability of normal IECs vs those obtained from patients with IBD.

For the purpose of obtaining crypt epithelial cells, minced tissue pieces were incubated with dispase for up to five treatments (5 min for the first treatment, 30 min for the second and third treatments, and then 40 min each for the next two treatments). At each interval, sample tissue (for histologic examination to detect the disappearance of surface epithelium and subsequent crypt cell liberation) and cell suspensions were collected. Epithelial cells were separated by percoll density gradient, and purity of IECs was >95% (as evaluated by staining with anti-CD3, CD14, CD20, CD45 mAbs) and L12 (anti-epithelial cell) Abs. Hematoxylin and eosin staining of histologic sections of the treated tissue samples demonstrated that the surface epithelium was completely removed by the first three dispase treatments with maintenance of the crypt epithelium. After the fifth treatment histologic sections revealed the absence of crypt epithelium. The IEC from the fourth and fifth dispase treatments were used as a source of crypt cells whereas the IEC from the first and second dispase treatments were used as the source of surface epithelium. Purity of the surface or crypt cell populations in the cell suspension from each treatment was determined by morphology (by phase contrast microscopy and histochemical staining), measurement of alkaline phosphatase (as a measure of surface epithelium) (24, 25), and [3H]Thymidine uptake incorporation (as a measure of the crypt cell population) for 4, 16, and 24 h. Consistent with the findings in the histologic sections, alkaline phosphatase was detectable only in the lysates from the cells isolated in the first three dispase treatments, suggesting that these fractions contained an enriched population of surface epithelial cells. [3H]Thymidine uptake incorporation was minimal in these cells, whereas the cells liberated in the fourth and fifth dispase treatments were able to proliferate with a 4- to 5-fold increase in thymidine uptake seen at 24 h. These data suggest that the latter fractions are enriched for crypt epithelial cells. No alkaline phosphatase activity was noted in lysates of cells from the fourth and fifth dispase treatments. In some experiments intact crypts were liberated during the treatment, and these could be visualized by conventional light microscopy. Such crypts were teased apart by incubation with 0.15 mol/liter NaCl, nmol/liter tetraphenyl borate (26, 27) and subjected to analysis.

Isolation of peripheral blood monocytes
Peripheral venous blood was obtained from the same individual from whom the epithelial cells were derived and monocytes were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation and plastic adherence, as described (28). The nonadherent cells were removed, and the adherent cells were washed with PBS to remove all loosely bound contaminating cells. Adherent monocytes were harvested by a rubber policeman. Purity and viability were assessed by staining with the anti-CD14-14 mAb Leu M3 (Becton Dickinson, San Jose, CA) and propidium iodide (29), respectively. In all cases purity was >95% and viability was 100%.

Cytokine Binding by Intestinal Epithelial Cells
Cell lines
Cell lines (EL-4, L929, DLD1, HT-29, and Caco2) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The IL-6 receptor (IL-6R) from B9, was kindly provided by Dr. Lester May (New York Medical College, Valhalla, NY). Cell lines were either used as positive controls for receptor assays or as sources of intestinal epithelium. Cells were thawed and grown in T75 tissue culture flasks (Nunc, Naperville, IL). DLD1, HT29, and L-929 cell lines were grown in RPMI 1640 medium supplemented with 10% FCS (Life Technologies, Grand Island, NY), Caco2 in DMEM with 10% FCS, EL-4 in DMEM with 10% horse serum, and B9 cells in RPMI 1640 with 10% FCS and 20 U/ml rIL-6 (kind gift of Dr. Edward Siden, Mount Sinai Medical Center, NY). All culture media contained 1% penicillin-streptomycin (ICN-Flow, McLean, VA) and L-glutamine (ICN-Flow). Cells from suspension cultures (EL-4, B9) or confluent monolayers (DLD1, HT29, Caco2), dissociated from the plastic surface by treating with a nonenzymatic cell dissociation solution (Sigma), were washed and used for staining. In some experiments, control cell lines or IEC lines were treated with dispase or EDTA to assess the effects of the isolating procedure on cytokine binding. For receptor regulation studies secondary cultures were generated in the presence or absence of modulatory agents for varying time periods. Receptor density was compared between stimulated and unstimulated cells by analysis of mean fluorescence intensity or peak channel (see below).

In vitro models of crypt and surface epithelial cells
In vitro models of fully differentiated or undifferentiated human colonic epithelial cell lines were developed by using glucose free or high glucose containing conditioned medium, respectively, as previously described by Wicke et al. (30). Briefly, HT29 (obtained from Dr. Burton M. Wise, Washington University School of Medicine, St, Louis, MO) and DLD1 cells (from ATCC) were cultured in glucose-free DMEM with 10% dialyzed FCS (Summit Biotechnology, Fort Collins, CO) supplemented with 2.5 mM inosine (Sigma) (for differentiated surface-like cells designated HT29ino or DLD1ino in the text) or 25 mM glucose (for de-differentiated crypt-like cells designated HT29glu or DLD1glu in the text). Cells were grown for at least four passages and maintained in a postconfluent state for at least 10 days with daily replacement of fresh medium before their use in the experiments. Although the features of full differentiation (in inosine cells) or de-differentiation (in glucose cells) of these cells have been characterized previously by Wicke et al. (30, 31) under these conditions, we reconfirmed the differentiation states of these cells by demonstrating lower levels of alkaline phosphate and sucrase isomaltase (markers of a mature enterocyte phenotype) in the “glucose-conditioned” cells compared with the higher levels of these enzymes in the “inosine-conditioned” cells (data not shown).

Cytokines and chemicals
Human recombinant IL-1β was obtained from Regeneron (Boston, MA), IL-6 was a generous gift from Dr. Edward Siden, human GM-CSF was purchased from R&D Systems (Minneapolis, MN), TNF-α from Cetus (Emeryville, CA), IFN-y from Genentech (South San Francisco, CA), and Escherichia coli LPS and phospho-12-myristate-13-acetate (PMA) from Sigma.

Identification of cytokine binding
Cytokine receptors were identified using fluorochrome-labeled cytokines (Fluorokine; R&D Systems) and analyzed by flow cytometry. We utilized phycocerythrin (PE) or biotin (followed by avidin-FITC) conjugated cytokines as the signal-to-noise ratio was greater than that seen with fluorescein. Briefly, purified epithelial cells were washed twice in RDF-1 buffer (wash buffer for fluorokine staining from R&D Systems) and resuspended at 106 cells/ml. A total of 25 μl of cell suspension (105 cells) was incubated with 10 μl (chosen from serial dilution studies in normal control cells) of biotin-conjugated cytokines or soybean trypsin inhibitor (control) and incubated for 45 min at 4°C. The cells were then incubated with 10 μl of biotin-conjugated avidin-FITC for another hour. The cells were incubated with 10 μl of biotin-conjugated streptavidin for 1 h on ice. In the cases where staining was performed with biotinylated cytokines, 5 μl (chosen from serial dilution studies in normal control cells) of biotin-conjugated cytokines or soybean trypsin inhibitor (control) was used. The cells were then incubated with 5 μl avidin-FITC for another hour. After this incubation, cells were washed twice with 2 ml of RFD-1 buffer. Cells were resuspended in 200 μl of PBS and analyzed by an Epics Profile-I flow cytometer (Couler, Hialeah, FL). At least 3000 cells were counted per analysis. In preliminary studies, specificity of these conjugated cytokines was determined by the inhibition of specific staining on receptor bearing control cells by unlabeled cytokines. Cells were first incubated with unlabeled cytokine at 100-fold excess molar concentration (or the fluorescent cytokines
were neutralized by incubation with specific mAbs against the cytokines for 10 min before their incubation with cells) and then incubated with labeled cytokine for another hour on ice following the protocol described above. To assess purity of the cell preparations, in each set of experiments parallel immunofluorescence staining was performed as described previously (21) with Abs against HLA-class I (W6/32), CD14 (Leu-M3), CD20, CD45 (HL-6-1), and an epithelial cell specific mAb L12 (32) followed by FITC-conjugated F(ab')2 goat anti-mouse IgG. An irrelevant mouse IgG1 mAb served as a negative control. This allowed us to gate on the L12 positive epithelial cells, thus avoiding any false positive cytokine binding from contaminating non epithelial cells. In another series of experiments, dual labeling analyses were performed. Cells were first stained with L-12/ goat anti-mouse FITC as described above and then incubated with PE- conjugated cytokines to discriminate between epithelial cells and other contaminating cells in our IBD epithelial cell preparations. In each assay, positive control cells for IL-6R (B9), IL-1R (EL-4), GM-CSFR (monocytes), and TNFR-a (L929) binding were included to compare receptor density and reliability of the technique.

**Measurement of IL-8 and GRO-a**

Freshly isolated IEC and HT29 colonic adenocarcinoma cell lines were cultured in the presence or absence of TNF-a (33) for 48 h, and culture supernatants were assayed for IL-8 and GRO-a production by ELISA as described previously (34). Briefly, a polyclonal goat anti-human (GAH) IL-8- or a monoclonal GAH GRO-a (R&D Systems) Ab were used as capturing Abs with a polyclonal rabbit anti-human IL-8 and monoclonal mouse anti-human GRO-a as the detecting Ab. The IL-8 and GRO-a ELISAs were sensitive to 20 and 50 pg/ml, respectively.

**Determination of the cytokine effect on epithelial cell growth**

DLD1 cell lines were seeded (5 x 10^5 cells per well) in 6-well plates. Cytokines were added at the time of adherence to the culture well. Cells were removed at different time points (24 and 48 h) by treatment with cell dissociation solution and counted in a Neubauer chamber (Haussner Scientific, Horsham, PA). Three wells were used for each data point and counting was performed by three investigators independently. The results from direct cell counts were validated in a parallel series of [3H]thymidine uptake assays. IEC cell lines were grown in the presence or absence of varying concentrations of cytokines in 96-well (5000 cells/well) flat-bottom tissue culture plates in a humidified 5% CO2 incubator. After 48 h of culture, 1 µCi [3H]thymidine was added, and after 6 h of further incubation the culture medium was aspirated and thymidine incorporation was measured in a 1450 Microbeta Plus counter (Wallac, Gaithersburg, MD).

**Detection of cytokine-induced ICAM-1 on in vitro models of surface and crypt epithelial cells**

Conditionally differentiated (inosine medium) or de-differentiated (glucose medium) cell lines (DLD1 and HT29) were grown in T25 tissue culture flasks (Falcon, Franklin Lakes, NJ) as described above. Each type of cell line or freshly isolated crypt and surface IEC were cultured either with recombinant human GM-CSF (20 ng/ml) or medium alone for 24 h at 37°C. The cell lines were dissociated by treatment with nonenzymatic cell dissociation solution (freshly isolated IEC do not adhere to the plastic surface and did not require this cell dissociation procedure after stimulation), washed three times in PBS/1% BSA/0.02% sodium azide, and processed for immunofluorescence staining using an anti-ICAM-1 mAb (Becton Dickinson) or an irrelevant mouse IgG1 control mAb.

**Flow cytometric analysis**

Stained cell suspensions were analyzed on a Epics Profile flow cytometer (Coulter) as previously described (35), gating on viable cells. Mean channel fluorescence (MCF), which correlates with fluorescence intensity, was determined from the peak of positively stained cells and is recorded on a log scale.

**Stimulation of epithelial cells**

Freshly isolated epithelial cells or cell lines were cultured in the presence or absence of IL-1β (100 ng/ml and 1 µg/ml), INF-γ (250 U/ml), LPS (1 µg/ml), GM-CSF (20 ng/ml), or PMA (10 ng/ml) for 24 h in culture medium in a 5% CO2 humidified 37°C incubator. These concentrations were optimal for induction of specific receptors in conventional APCs. Cells were washed and checked for viability, and receptor expression was analyzed by the method described above.

**Results**

**IL-6, GM-CSF, and IL-1β receptors are constitutively expressed on IECs**

Freshly isolated epithelial cells and appropriate positive control cells were stained with either PE-conjugated IL-1β, IL-6, GM-CSF, TNF-α, or streptavidin to detect the constitutive expression of receptors for these cytokines. The flow cytometric analysis for one representative experiment is shown in Figure 1. A peak shift in MCF demonstrates the presence and density of receptors compared with the streptavidin-PE negative control. In each case binding of PE-labeled cytokines was specific since the binding could be inhibited by the corresponding unlabeled human recombinant cytokine.

IL-1βR was constitutively expressed on freshly isolated IECs from normal tissues (Fig. 1A, upper). The specificity of IL-1β PE binding to IL-1βR was demonstrated by inhibition of binding of the labeled cytokine to EL-4 cells in a blocking assay using 100 fold excess unlabeled IL-1β (Fig. 1A, middle) but not with IL-6 or GM-CSF (data not shown). Blocking experiments were also performed in a series of experiments with freshly isolated IECs (data not shown) with similar results. Disperse treatment of EL-4 cells had no effect on IL-1βR expression (Fig. 1A, lower).

As seen in Figure 1B, IL-6R was also expressed on IECs (upper) but at a lower density compared with IL-1βR. Because we previously demonstrated that IL-6 is secreted by IEC (21), the lower density of IL-6R may relate to autocrine blockade of IL-6R by IEC-secreted IL-6. Similar to IL-1βR, IL-6-PE binding was specific (Fig. 1B, middle) and disperse treatment had no effect on IL-6R expression (Fig. 1B, lower) on the IL-6-dependent B9 cell line.

We next examined expression of receptors for GM-CSF, which enhances phagocytosis in macrophages (36). Figure 1C, upper, demonstrates that GM-CSFRs are expressed on epithelial cells at high density. Since, in human, GM-CSF and IL-3 have been reported to share a common β subunit, we performed blocking experiments with cold IL-3 as well. Binding of GM-CSF PE was partially inhibited by both cold GM-CSF (Fig. 1C, middle) and IL3 (data not shown). However, the ability of GM-CSF but not IL-3 to regulate surface Ags on IECs (37, 38) confirms that the staining relates to GM-CSFR and not IL-3R. Dispare had no effect on GM-CSF expression (Fig. 1C, lower).

Interestingly, receptors for TNF-α were not detected in any epithelial cell preparation (Fig. 1D, upper), although TNFα-R were clearly demonstrated in the colonic adenocarcinoma cell lines (see Table I below). In this case, TNFα-R expression in control cell lines was reduced by disperse treatment; therefore, EDTA was utilized to isolate IECs in this series of experiments. EDTA had minimal effects on TNFα-R expression. The variability in the staining of the L929 cells related to a reduced background in this experiment.

**Comparison of cytokine receptor expression on IECs and conventional APCs**

Since IECs possess some of the functional properties ascribed to APCs, it was of interest to compare cytokine receptor expression on IECs with that of monocytes. Therefore we performed studies using paired samples of epithelial cells (n = 5) and isolated monocytes (n = 5) from the same individuals. As can be seen in Figure 2, with the exception of GM-CSF, the density of receptor expression in IECs for all cytokines, was lower than that of monocytes. As described earlier (Fig. 1), no TNFα-R was detected in any of the epithelial cell preparations, whereas TNFα-R were present in the monocytes. GM-CSFR expression was the highest of any of the
Cytokine receptors tested and was comparable to monocytes. Cytokine receptor expression and density on IEC was similar regardless of the site from which the IEC were isolated (e.g. ileum vs colon, right colon vs left colon). The variability seen in receptor expression between the different individuals likely relates to individual variation rather than differences in isolation technique.

Cytokine receptor expression on IECs does not correlate with severe histologic inflammation in IBD

Given the above data, it is clear that epithelial cells might be targets for proinflammatory cytokines released by the epithelial cells themselves as well as by other mucosal cells during the process of inflammation. Receptor-cytokine interactions could alter the phenotype of the epithelial cells resulting in abnormal cytokine receptor expression, alteration in function, growth, or induction of specific costimulatory signals for T cell activation. To determine whether such a phenomenon may be responsible for the epithelial cell dysfunction seen in IBD [39], we studied the presence and density of receptor expression for the four cytokines in epithelial cells isolated from inflamed (UC, \( n = 11 \); CD, \( n = 10 \)) and uninfamed (UC, \( n = 2 \); CD, \( n = 8 \)) tissues from patients with IBD and compared them with normal control epithelial cells (\( n = 16 \)).

As shown in Table I, receptor expression on epithelial cells derived from inflamed tissues was not significantly different from normal control IECs, although there was a trend toward an increase in GM-CSFR in uninflamed UC and CD. However, when we directly compared the cytokine receptor expression and density on epithelial cells derived from inflamed and uninflamed areas in the bowel of the same IBD patient, we found that cytokine receptor expression did not correlate with inflammation in either UC or CD (data not shown).

### Table I. Comparison of cytokine binding on IEC: normal vs IBD

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Cytokine Receptor (log MCF ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1β</td>
</tr>
<tr>
<td>Normal (( n = 16 ))</td>
<td>1.444 ± 0.173</td>
</tr>
<tr>
<td>UC inv (( n = 11 ))</td>
<td>0.946 ± 0.210</td>
</tr>
<tr>
<td>UC uninfl (( n = 2 ))</td>
<td>1.655 ± 1.225</td>
</tr>
<tr>
<td>CD inv (( n = 10 ))</td>
<td>1.035 ± 0.226</td>
</tr>
<tr>
<td>CD uninfl (( n = 8 ))</td>
<td>1.144 ± 0.281</td>
</tr>
</tbody>
</table>

FIGURE 1. Flow cytometric analysis of cytokine receptor expression on freshly isolated intestinal epithelial cells. PE-conjugated cytokines (10 \( \mu l \)) or streptavidin (negative control) were added to \( 1 \times 10^5 \) freshly isolated IEC (upper) or positive control cells (middle). A, IL-1β PE, EL4 cells are the positive control. B, IL-6-PE, B9 cells are the positive control. C, GM-CSF PE, monocytes are the positive control. D, TNF-α PE, L929 cells are the positive control. The lower panels represent the lack of effect of dispase (for IL-1, IL-6, GM-CSF) or EDTA (for TNF) treatment of the control cells in terms of cytokine binding.

FIGURE 2. Comparison of cytokine receptor density in monocytes (professional APC) vs IEC (nonprofessional APC). Comparison of mean channel fluorescence of cytokine receptors expressed on monocytes/macrophages (M) vs epithelium (E) in paired samples. With the exception of GM-CSF, all cytokine receptors are expressed at lower (or absent—TNF) density on IEC.
Table II. Cytokine receptor expression on intestinal epithelial cell lines

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>HT29</th>
<th>DLD1</th>
<th>CaCo-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin PE</td>
<td>1.850 ± 0.091</td>
<td>1.483 ± 0.552</td>
<td>1.486 ± 0.672</td>
</tr>
<tr>
<td>IL-6 PE</td>
<td>2.376 ± 0.233</td>
<td>6.703 ± 2.15</td>
<td>4.381 ± 3.001</td>
</tr>
<tr>
<td>IL-1β PE</td>
<td>3.442 ± 1.293</td>
<td>6.161 ± 2.786</td>
<td>5.071 ± 3.850</td>
</tr>
<tr>
<td>GM-CSF PE</td>
<td>5.863 ± 2.370</td>
<td>7.742 ± 5.321</td>
<td>3.754 ± 1.00</td>
</tr>
<tr>
<td>TNF-α PE</td>
<td>6.063 ± 2.139</td>
<td>1.429 ± 0.653</td>
<td>2.250 ± 1.163</td>
</tr>
</tbody>
</table>

Cell lines differ from freshly isolated epithelial cells in cytokine receptor expression

To develop a model for assessing the regulation of cytokine receptor expression in IECs, we examined receptor expression in the epithelial cell lines DLD1, CaCO2, and HT29. Receptors for IL-1β, IL-6, and GM-CSF were expressed on these cell lines at levels greater than those seen in freshly isolated IECs (Table II). Furthermore, unlike freshly isolated IECs, TNFα-R were detected on all three cell lines.

The presence of TNFα-R in the cell lines but not in freshly isolated IECs raised concerns about the possibility that TNFα-R on the epithelial surface might be blocked by the binding of endogenous TNF-α, resulting in negative staining by the PE-conjugated cytokine. Such a possibility was rendered less likely by the finding of an absence of TNF-α protein or mRNA in isolated IEC (21). To ensure that our isolation procedure was not responsible for stripping off TNFα-R, we subjected macrophage and control cell lines to the same isolation procedure. As discussed earlier, dispase treatment did not change receptor density on either macrophages or the cell lines for any cytokine except for TNFα-R. However, we were still unable to detect TNFα-R on IEC when the IEC were isolated using EDTA (which had no inhibitory effect on TNFR expression and in fact had a modest stimulatory effect). These data suggest that the absence of TNFα-R in freshly isolated epithelial cells is not due to the isolation procedure but that tumor bearing epithelial cell lines differ from fresh IECs in their cytokine receptor expression.

Demonstration of a lack of functional response to TNF-α by freshly isolated IEC

Although our results of TNF-α binding (receptor expression) by malignant epithelial cell lines have been consistent with previously described functional effects of TNF-α on these cell lines, the lack of TNF-α binding on freshly isolated IEC raises the concern as to whether the nonbinding of TNF-α on isolated IEC is an artifact of either endogenous TNF-α production or the isolation procedure. To address this issue we compared the functional response of the HT29 cell line and freshly isolated IEC to TNF-α stimulation by examining chemokine (IL-8 and GRO-α) production by these cells. As can be seen in Table III, consistent with previously reported results, TNF-α stimulation of HT29 cells resulted increased production of both IL-8 and GRO-α in a dose-dependent fashion, whereas the freshly isolated IECs from two different individuals showed no response to TNF-α stimulation even at the higher concentration of 10 ng/ml. Furthermore, it appeared less likely that endogenous TNF-α was present since baseline IL-8 and GRO-α was comparable to the HT29 cell line. These data support the lack of TNFα-R expression on freshly isolated IEC.

Table III. TNF-α stimulation does not affect chemokine secretion by freshly isolated IECs

<table>
<thead>
<tr>
<th>Cells</th>
<th>Added TNF-α (ng/ml)</th>
<th>IL-8 (ng/ml)</th>
<th>GRO-α (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>0</td>
<td>0.79</td>
<td>2.31</td>
</tr>
<tr>
<td>IEC no. 1</td>
<td>0</td>
<td>26.89</td>
<td>13.28</td>
</tr>
<tr>
<td>IEC no. 2</td>
<td>1</td>
<td>6.09</td>
<td>7.49</td>
</tr>
<tr>
<td>IEC no. 3</td>
<td>10</td>
<td>5.55</td>
<td>0.85</td>
</tr>
<tr>
<td>IEC no. 4</td>
<td>10</td>
<td>5.75</td>
<td>0.99</td>
</tr>
<tr>
<td>IEC no. 5</td>
<td>1</td>
<td>5.32</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Comparison of cytokine binding (receptor expression) between surface and crypt epithelium

The differences in cytokine receptor expression between the different epithelial cell lines raised the possibility that cytokine receptor expression may vary depending upon the differentiation state of the cells. Previous studies have shown that the response to various hormones and physiological modulators differs in crypt and surface epithelial cells. Therefore, to determine whether the two cell types (crypt and surface IEC) display any difference in the cytokine receptor expression, we isolated surface and crypt epithelial cells from 4 individual specimens. As can be seen in Figure 3, the surface epithelial cells expressed cytokine receptors (IL-1β and IL-6) at a higher density than crypt epithelium. These findings were reconfirmed in an in vitro cell line model system of crypt and surface epithelial cells (see Materials and Methods). HT29 and DLD1 cell lines were conditionally differentiated (HT29glu and DLD1glu, comparable to crypt-like) by using glucose free or high glucose containing medium (see Materials and Methods). After growth under such conditions, experiments were performed to detect cytokine receptor expression on these cells. Consistent with our results with freshly isolated crypt and surface epithelial cells, IL-1β and IL-6R expression was greater in the differentiated (HT29glu and DLD1glu) cells than the de-differentiated (HT29gdu and DLD1gdu) crypt-like cells (data not shown). As seen with freshly isolated IEC, the GM-CSFR density was similar in both differentiated and dedifferentiated cell lines. The finding of cytokine receptor heterogeneity in the crypt vs surface epithelial cells may relate to specific cytokine requirements.
for the modulation of growth, differentiation, function, or apoptosis of epithelial cells as they migrate from the crypt to the surface.

Regulation of cytokine binding (receptor expression)

Several laboratories have recently shown that inflammatory cytokine production is elevated in IBD mucosa and that these cytokines can modulate expression of various cytokine receptors. Therefore we assessed the regulation of cytokine receptor expression in IECs. Based on the receptor profile of the epithelial cell lines, we chose DLD1 cells for these studies since it most closely mimicked normal IECs. A total of 10^6 DLD1 cells/well were cultured in the presence or absence of LPS (1 μg/ml), IFN-γ (250 U/ml), or PMA (10 ng/ml) for 18 h and stained with PE conjugated IL-1β (A), IL-6 (B), and GM-CSF (C) as described in Figure 1. The results are expressed as fold increase over baseline expression (stimulated MCF/baseline MCF). This figure is representative of three separate experiments. Similar results were obtained by using freshly isolated IECs.

Figure 4 (average of five experiments), IL-1βR expression was enhanced by all of the stimuli used, although this did not achieve statistical significance (Fig. 4A). Unlike monocytes (40), IL-6Rs on IEC (Fig. 4B) were up-regulated by LPS and unchanged with PMA, suggesting a different regulatory pathway for IL-6R expression in both conventional and mucosal APCs. This lack of induction of IL-6R cannot relate to the production of (unlabeled) IL-6 in the cell line as we have previously seen that DLD1 cells do not secrete this cytokine (A. Panja and L. Mayer, unpublished data).

GM-CSFR expression was unaffected by PMA, LPS, or IFN-γ (Fig. 4C). Finally, TNFα-R was neither altered in DLD1 cells nor induced in freshly isolated epithelial cells (data not shown) by any of these stimuli.

We next assessed the functional role of the cytokine receptors by assessing the effect of recombinant human cytokines on IEC growth and surface molecule (ICAM-1) expression.

**IL-1β, IL-6, and GM-CSF differentially regulate epithelial cell growth**

The effect of cytokines on epithelial cell growth was studied by culturing DLD1 cells in the continuous presence of IL-1β (10–100 ng/ml), IL-6 (60 U/ml), GM-CSF (20 ng/ml), or medium only. Cell growth at various time points was quantitated by direct cell counts. As can be seen in Figure 5, IL-1β enhanced the cell growth by 3- to 4-fold, whereas GM-CSF and to a lesser extent IL-6 inhibited growth. In a second series of experiments cultures were grown in 96-well flat-bottom plates (5000 cells/well) for 24 h in 1% FCS with 0.5% BSA. Cells were then treated with various concentrations of cytokines in the presence of 2% FCS for 72 h, and cell proliferation was assessed by a thymidine incorporation assay. Again, a stimulatory effect of IL-1β and an inhibitory effect of GM-CSF was seen in a dose-dependent fashion (data not shown). There was minimal or no effect of IL-6 in this system.

**GM-CSF treatment of crypt IEC induces ICAM-1 expression**

To further evaluate the physiological role of the cytokine receptors on IEC, we examined whether treatment of IECs with GM-CSF
(since GM-CSF production by IECs is increased in active inflammation (71) and GM-CSFR are present in both crypt and surface IEC) could regulate ICAM-1 expression. This integrin has been shown to be of importance in Ag-specific T cell responses and in lymphocyte homing to endothelium with subsequent migration of lymphocytes into inflammatory sites (41). As shown in Figure 6, de-differentiated crypt-like cell lines (DLD1glu and HT29glu) lack constitutive ICAM-1 expression. Treatment with GM-CSF induced ICAM-1 expression in both of these cell lines (significant level of expression in DLD1glu and a very low level of expression in HT29glu). However, the differentiated surface-like (DLD1ino and HT29ino) cell lines constitutively expressed ICAM-1 (low in HT29ino and high in DLD1ino) and this was not further enhanced by GM-CSF treatment in either HT29ino or DLD1ino cells. Similar results were seen in experiments ($n = 2$) with freshly isolated crypt epithelial cells although the response in these cells was weaker than in the cell lines and there was no constitutive expression in either cell type (Fig. 6C).

**Discussion**

The results of this study have implications for both normal and inflammatory states in the bowel. One the one hand, the expression of proinflammatory cytokine receptors by normal IEC may point toward a role for these cytokines in normal growth and differentiation processes, e.g., crypt to villus maturation. Alterations in either cytokine receptor expression or local cytokine concentrations would induce an imbalance in normal homeostatic mechanisms, potentially leading to epithelial barrier dysfunction. On the other hand, proinflammatory cytokine receptor expression renders the epithelium susceptible as a target for the negative effects of these cytokines as well, potentially promoting IEC destruction, ineffective repair, or altered APC function. In this case, the increase in cytokine production within the mucosa would adversely affect the IEC contributing to the clinical expression of disease. Thus the cytokine milieu within the intestinal epithelium has the potential to affect normal physiologic as well as immunoregulatory processes.

The possibility that IECs could serve as a target for these proinflammatory cytokines is supported by similar studies with endothelial cells in vascular processes. Several groups have demonstrated that IL-1 induces PGE$_2$ secretion by fibroblasts, smooth muscle, and endothelial cells (42), which may have a role in promoting local cytoprotection or in some cases tissue destruction. In addition, IL-1, as well as other cytokines, has been shown to induce the expression of adhesion molecules (ICAM, ELAM-1) on endothelial cells (43), which promotes cell-cell interactions and contributes to the inflammatory response. Proinflammatory cytokine receptor expression by various epithelial cells has also been reported. IL-1R expression has been documented in the rat intestinal epithelial cell line, IEC-18 (44, 45), whereas Krueger and coworkers (46) have reported mitogenic effect of GM-CSF on basal keratinocytes, suggesting the presence of GM-CSFRs on these cells. This latter observation is of interest since GM-CSF is a potent stimulator of phagocytosis and class II Ag expression in APCs (macrophages) (34). Specific examples of IECs serving as targets for such cytokines include studies by Shanahan and coworkers (20), who demonstrated that the combination of IFN-γ and TNF-α is cytotoxic for the colonic epithelial cell line HT-29; Madara et al. (47), who demonstrated that IFN-γ alters the permeability of tight junctions in T84 cells allowing for neutrophil transmigration; Chang et al. (48), who reported that IL-1 induces chloride secretion and short circuit current by chicken enterocytes when added to the serosal side of chicken intestine.

**FIGURE 6.** Induction of ICAM-1 in IEC lines by GM-CSF. Conditionally de-differentiated (glu) or fully differentiated (ino) HT29 (A), DLD1 (B) colonic epithelial cell lines and freshly isolated surface and crypt IECs (C) were cultured for 24 h with either medium alone (upper) or with medium containing 20 ng/ml GM-CSF (lower). ICAM-1 expression was detected by immunofluorescence staining using an anti-ICAM-1 mAb (dotted line) or an isotype control (solid line). Flow cytometric analysis demonstrates induction of ICAM-1 in HT29glu and freshly isolated crypt (although very weak) cells or enhanced expression of ICAM-1 in DLD1glu by GM-CSF treatment. A and B are representative of five separate experiments. C is representative of two separate experiments.
mounted in an Ussing chamber (although these effects may have been mediated through stromal cells); and Molmenti et al. (49), who documented acute phase response regulation by IL-1 and IL-6 binding on Caco2 cells. Such data support the potential for autocrine and paracrine effects of cytokines produced by epithelial cells and/or lamina propria mononuclear cells. Given the potential implications of such possibilities, we studied the expression and density of IL-1β, IL-6, TNF-α, and GM-CSF receptors on epithelial cells from normal and IBD tissues. These cytokines were chosen because they have significant effects on the APC function of monocytes and have been reported to be increased in the mucosa of patients with IBD. The findings reported here demonstrate that receptors for IL-1β, IL-6, and GM-CSF were comparably expressed in both normal and IBD epithelial cells. However, considerable differences did exist in receptor density within differentiated and undifferentiated epithelial cells. TNFα-R were not detected in any of the freshly isolated epithelial cell preparations. In contrast, conventional APCs from the same individuals as well as malignant epithelial cell lines expressed receptors for all four cytokines including receptors for TNF-α. Thus freshly isolated epithelial cells differ from professional APCs in their inability to express receptors for TNF-α. This latter finding is of importance as TNF has been shown to be an important factor in regulating cytokine production by macrophages and as a potentially cytotoxic agent for IEC. The absence of TNFα-R on IEC suggests that these cells may not be targets for killing by TNF-α in diseases such as IBD where TNF-α levels are high.

Proinflammatory and phagocytic cytokines have been known to regulate APC functions as well. However, different APCs display a distinct range of responses to these cytokines. For example IFN-γ up-regulates class II MHC expression on murine macrophages but fails to do so on B cells, although non-class II Ag presentation is enhanced (50). Similar differences have been observed with other cytokines (e.g. IL-4) (51, 52). Thus disparate effects of cytokines on different APCs are well described. Intestinal epithelial cells like conventional APCs express class II MHC molecules on their cell surface (53, 54) and are capable of taking up, processing, and presenting Ag to immunocompetent T cells (55–61). However, several differences exist as well. These include a lower density of class II molecules on the IEC surface (62), a limited cytokine profile (lack of IL-1 production) (15, 21), preferential stimulation of suppressor T cells in mixed cell culture systems (52), and a slower rate of Ag processing by IECs (57, 61). These differences may relate to cytokines in the local environment and their effect on IEC function. Such correlations have not been addressed in vivo systems.

It is well described that cytokines or bacterial products can modulate cytokine receptor expression (62, 63). This may in turn influence immune and inflammatory events by promoting or suppressing activation, surface molecule expression, or costimulatory signals for T cell activation. The regulatory mechanisms governing cytokine receptor expression in IECs are not known. Our data suggest that the regulation of IL-6R is different from that of monocytes, i.e., LPS up-regulates IL-6R expression in DLD1, a colonic epithelial cell line, whereas it down-regulates expression in macrophages. This differential regulation of cytokine receptors at a site where LPS is abundant may reflect a compensatory mechanism. Still these cytokine receptors may play an important role in intestinal homeostasis. Reinecker and Podolsky (64) have recently identified the presence of receptors for T cell-derived cytokines on IEC describing the expression of the common γ-chain of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors in a rat IEC cell line. Taken together, one could develop a model where locally produced cytokines by resident T cells, macrophages, myofibroblasts, as well as epithelial cells might regulate the normal growth and differentiation of IEC. It is of interest to note that many of the cytokine knockout mice that develop intestinal inflammation display histologic pictures characterized by marked crypt cell proliferation. Thus alterations in cytokine production could alter normal physiologic processes (i.e., growth and differentiation) and result in epithelial cell dysfunction. Such a scenario may be true in IBD where both T cell-derived as well as proinflammatory cytokines are increased in inflamed tissues. The effects of these cytokines on IEC phenotype and function may account for some of the clinically expressed components of these diseases. Since IBD IEC express similar profiles of cytokine receptors to those seen on normal IEC, the epithelial cell dysfunction seen would relate to the higher concentrations of local cytokine.

Cytokines might be important for the growth, differentiation, and migration of epithelial cells during maturation from crypt cells as well. Alteration in any of these events may cause serious pathologic consequences. Specific roles of cytokines in each of these events require further exploration, but the identification of candidate cytokines by virtue of receptor expression is the first step. That cytokines alter epithelial cell function is well described. IL-4 enhances IEC-6 cell line proliferation (65) whereas the proinflammatory cytokines IL-1 and GM-CSF either enhance or inhibit DLD1 cell line growth. IFN-γ, IL-1, and TNF-α have been shown up-regulate ICAM-1 expression on the surface of many cells (66, 67) including intestinal epithelial cells (68). Although the role of ICAM-1 expression in intestinal inflammation has been controversial (69, 70), our in vitro data suggest that an increased GM-CSF concentration in the epithelium can induce ICAM-1 expression by crypt epithelial cells enhancing the chances of adhesion between these cells and CD4+ lamina propria lymphocytes. This finding may represent an important mechanism for the immune responses seen in IBD.

In conclusion, our data support the concept that IEC can be affected by cytokines in their local environment. Alterations in cytokine concentrations as a consequence of inflammation may therefore have profound effects on epithelial cell growth, phenotype, and function. Selective regulatory roles of proinflammatory cytokines on IEC growth and surface molecule expression may provide evidence for an unrecognized mechanism responsible for inflammation, ulceration, or malignant transformation seen in IBD. Further elucidation of the functional interactions between distinct cytokines would be of importance in the understanding of cytokine mediated regulation of mucosal immune responses.

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