Monocyte/Macrophage Activation by Normal Bacteria and Bacterial Products

Implications for Altered Epithelial Function in Crohn's Disease

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Intestinal immune cells are less reactive than those in the peripheral blood; however, such cells from patients with Crohn's disease may be more responsive to bacterial products. Our study examined if non-pathogenic bacteria or lipopolysaccharide (LPS), can affect epithelial function in the presence of monocytes/macrophages. Lamina propria mononuclear cells (LPMCs) and peripheral blood monocytes (PBMs) were obtained from patients with Crohn's disease and control patients. Filter-grown T84 epithelial monolayers were co-cultured with nonactivated or LPS-activated LPMCs or PBMs for 48 hours. Epithelial secretory [baseline short-circuit current (Isc) and Δ Isc to forskolin] and barrier (transepithelial electrical resistance) parameters were measured in Ussing chambers. LPS-activated PBMs from both controls and patients with Crohn's disease significantly increased Isc (~300%) and reduced transepithelial electrical resistance (~40%). Epithelial function was not altered after co-culture with control LPMCs $\pm$ LPS. However, LPMCs from patients with Crohn's disease spontaneously secreted tumor necrosis factor- α , and induced epithelial changes similar to those produced by LPS-activated PBMs. Co-culture with control *Escherichia coli* and PBMs induced comparable changes in epithelial physiology, which were abrogated by anti-tumor necrosis factor- α antibody. We conclude that LPMCs of patients with Crohn's disease are spontaneously activated, possibly by gram-negative luminal bacteria, and can directly cause significant alterations in epithelial ion transport and barrier functions. (*Am J Pathol* 2001, 158:1101-1109)

Crohn's disease is a chronic inflammatory bowel disease (IBD) that can affect any part of the gastrointestinal tract. The precise nature of the initiating events involved in

Crohn's disease are unknown, although findings in animal models and human studies support the concept that either an inappropriate or exaggerated immune response is responsible for much of the pathogenesis of the disease.¹⁻⁶ This immune response may be driven by exposure to enteric bacteria (commensal or pathogenic) or bacterial products such as lipopolysaccharide (LPS).

Recently, attention has focused on cells of the monocyte/macrophage lineage and their inflammatory products. These phagocytic cells are scattered beneath the intestinal epithelium and represent a first line of defense against foreign antigens. Previous studies indicate that enteric inflammation can be associated with increased heterogeneity and activation of intestinal macrophages: there is a distinct population of recently recruited monocyte-like macrophages in actively inflamed IBD lesions.^{7,8} These cells are more reactive than resident macrophages, releasing bioactive products and cytokines such as tumor necrosis factor- α (TNF- α).⁹ Indeed, TNF- α has recently become the target of clinical investigations in studies inhibiting its effects using a human-murine chimeric monoclonal antibody, Remicade (previously known as cA2; Centocor Inc., Malvern, PA).¹⁰⁻¹²

The intestinal epithelium is a dynamic barrier that regulates absorption of nutrients and water and at the same time restricts uptake of microbes and other noxious material from the gut lumen. One of the distinct features of Crohn's disease is impaired intestinal epithelial function, characterized by increased permeability (altered barrier function) and ion secretion, often resulting in a lumenally directed driving force for water movement causing diarrhea. Previously we showed that co-culture of a model epithelium with LPS-activated normal monocytes resulted in epithelial abnormalities reminiscent of those observed in resected tissue from patients with IBD.¹³

Despite increasing evidence of an association between the commensal bacterial microflora and intestinal inflammation,¹⁴⁻¹⁶ little information is currently available

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Table 1. Description of Patient Population Used in this Study

Patient no.	Gender	Age	Diagnosis/disease severity	Medication prior to surgery	Tissue extracted
1	F	34	Severe Crohn's disease	Prednisone, 5-ASA, cyclosporine	Large bowel
2	F	19	Pan-colitis with terminal ileal involvement	Prednisone, metronidazole	Large bowel
3	F	34	Severe distal proctitis	Prednisone, 6-mercaptopurine	Large bowel
4	F	54	Severe disease, recurrent obstruction, strictures	Prednisone, cyclosporine	Large bowel
5	M	35	Crohn's colitis with terminal ileal involvement	Prednisone, 5-ASA, cyclosporine	Large bowel
6	M	14	Pan-colitis with terminal ileal involvement, total colectomy	5-ASA	Large bowel
7	F	25	Severe colitis/ileitis with stricturing	Prednisone	Small bowel
8	F	26	Severe extensive colitis/ileitis	Prednisone	Small bowel
9	M	28	Extensive ileal disease with stricturing	Prednisone, 5-ASA, cirpofloxacin	Small bowel

on the direct role of such bacteria in altering gut epithelial function. We recently described a co-culture model of epithelial cells and immune cells¹³ that was adapted for the current studies. The aim of the present study was twofold: 1) to compare the ability of peripheral blood monocytes (PBMs) and lamina propria mononuclear cells (LPMCs) from patients with Crohn's disease and control patients $\pm$ LPS activation to affect enteric epithelial ion transport and barrier functions; and 2) to assess the ability of a lumenally applied nonpathogenic ($\equiv$ commensal) strain of bacteria to modulate epithelial function directly and in the context of monocytes/macrophages in a subepithelial position. Our objective was to gain further knowledge regarding the role of commensal microorganisms and the responsiveness of gut-derived immune cells to bacterial products in affecting epithelial function.

Materials and Methods

Isolation of PBMs

Peripheral blood was collected from 12 ambulatory patients with mild to moderate Crohn's disease attending the IBD clinic at McMaster University Medical Center and 14 healthy volunteers. Volunteers were excluded if they showed any signs of illness (eg, common cold, flu, allergy, and so forth) or were currently on medication. Whole blood from each donor was diluted in phosphate-buffered saline (PBS) and subjected to one-step density centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). The interface containing mononuclear cells was collected, washed in PBS, and resuspended in fresh T84 culture medium [1:1 mixture of Dulbecco's modified Eagle's medium and F-12 medium supplemented with 1.5% HEPES, 2% penicillin-streptomycin, and 10% fetal calf serum (FCS)] at 10^6 cells/ml.¹⁶ The monocyte/macrophage population was obtained by plastic-plating of peripheral blood mononuclear cells (4 hours at 37°C) and subsequent removal of nonadherent T and B cells. Fresh medium was added to the adherent cells, which were then incubated for 18 hours at 37°C before use in co-culture studies.

Isolation of LPMCs

Surgical specimens of inflamed small or large intestine were obtained from nine patients undergoing resection for severe Crohn's disease (Table 1); four small bowel and six large bowel resections were used. Tissues supplied for this study were adjacent to the most severely inflamed or ulcerated region and were described by the pathologist as moderately inflamed, a decision made before full histological assessment. Noninflamed control small or large bowel was obtained from patients undergoing surgery for cancer ($n = 8$), and was taken at least 5 cm from the tumor margin. LPMCs were isolated by a modification of the technique described by Bull and Bookman.¹⁷ Briefly, surgical specimens were washed extensively in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 1 mmol/L dithiothreitol (Sigma Chemical Co., St. Louis, MO) and 5% FCS (Life Technologies, Inc.) and mucosa dissected free from the muscle layer. Tissue was then cut into small pieces, washed, and incubated for 20 minutes in RPMI containing dithiothreitol at 37°C. Epithelial cells were removed by two 30-minute incubations in Hanks' balanced salt solution (Life Technologies, Inc.) containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA; Sigma) and 10% FCS at 37°C. The mucosal samples were washed and incubated overnight with gentle stirring in RPMI 1640 containing 1 U/ml collagenase (Sigma), 6 U/ml DNase II (Sigma), and 10% FCS. The tissue digest was then washed and filtered through a 200- μ m steel mesh. The cells were washed twice in RPMI 1640 containing 10% FCS and subjected to Percoll density gradient centrifugation. The interface was carefully removed, washed, and resuspended in T84 culture medium. For each resection the size of tissue available was highly variable and thus total cell recovery was not a priority (most tissues yielded $\sim 2.5 \times 10^6$ cells per cm² of tissue). For comparative purposes with PBMs, LPMCs were resuspended and at 1×10^6 /ml and incubated for 18 hours at 37°C before use in co-culture studies.

The percentage of macrophages in LPMC preparations was determined by differential staining (HEMA-TEK 2000 slide stainer; Miles Inc., Diagnostic Division, Elkhart,

IN) to be 7 to 15% of the total cell population for both normal and Crohn's disease. The remainder of the cells in the LPMCs were predominantly T and B lymphocytes based on cell and nuclear morphology.

The use of patient blood samples and intestinal resections was approved by the Human Ethics Committee at McMaster University/Chedoke Hospital, Hamilton Health Sciences Corporation.

Epithelial Cells

Human T84 colonic epithelial cells (passage 45 to 60) were seeded onto tissue culture-treated semipermeable filter supports (0.4- μ m pore size, 1.0-cm² surface area; Costar Corporation, Cambridge, MA) at a concentration of 10⁶ cells/ml and grown in T84 culture medium. Cells were grown at 37°C for 7 days to form polarized monolayers, displaying transepithelial electrical resistances >750 Ω .cm² as measured by a voltmeter and associated chop-stick electrodes (Millicell-RES; Millipore Corp., Bedford, MA). At the outset of the study, the stock epithelial cells were tested and found to be negative for mycoplasma contamination.

Bacterial Strain and Growth Conditions

Bacteria of the nonpathogenic, laboratory control strain of *Escherichia coli*, HB101^{18,19} were grown in nonaerated Trypticase soy broth for 6 hours at 37°C. Bacteria were pelleted by centrifugation at 2,400 $\times g$ for 15 minutes and then resuspended in sterile antibiotic-free T84 culture medium to a concentration of $\sim 1 \times 10^9$ colony forming units/ml. Viable bacteria were enumerated by serial 10-fold dilutions plated onto horse-blood agar plates.

Co-Culture Studies

Culture of Immune Cells with Epithelial Cells

Experiments with PBMs: PBMs were activated by addition of 10 μ g/ml LPS (either *E. coli* O111:B4, O127:B8, or *Salmonella minnesota*; Sigma) to the culture medium at the time of co-culture.²⁰ Our previous study¹³ showed that maximal changes in epithelial function occurred after 48 hours of co-culture with activated macrophages/monocytes. Thus, confluent T84 monolayers were co-cultured for 48 hours with normal or Crohn's disease PBMs ($\sim 1.5 \times 10^5$ cells/well) with or without LPS placed into the basal compartment of the culture wells. Controls were time-matched naive T84 monolayers and monolayers cultured with LPS or nonactivated PBMs only. The latter two treatments did not significantly impact on T84 ion transport or permeability and so naive monolayers only were predominantly used as the standard control condition.

Experiments with LPMCs: Use of LPMCs has the logistical problem of ensuring that a suitable T84 monolayer is available when surgical specimens are provided, the latter being at the discretion of the surgeon. We, and others, have shown that recombinant cytokines and con-

ditioned medium from activated immune cells evokes changes in epithelial ion transport and permeability that are very similar to those observed in co-culture studies.^{21–23} Thus, in some experiments LPMC-conditioned medium was made (culture 10⁶/ml LPMCs with 10 μ g/ml LPS for 24 hours, then collect cell-free conditioned medium) and stored at -70°C . Subsequently confluent monolayers were treated with 50% conditioned medium (diluted 1:1 in fresh culture medium), added into the basal compartment of the co-culture well, and epithelial function examined 48 hours later. Studies using LPMCs mirrored those with PBMs, with confluent monolayers being co-cultured with LPMCs (10⁶/ml) $\pm$ LPS for 48 hours. Of the 18 surgical specimens used, eight experiments with conditioned medium and 10 experiments with freshly isolated LPMCs were conducted.

Addition of Nonpathogenic *E. coli*

Confluent T84 monolayers were co-cultured for 48 hours with PBMs in the basal compartment, and 10⁵ *E. coli* strain HB101 added to the apical compartment (luminal side) of the transwells. Controls were naive T84 monolayers, those exposed to HB101 alone, or co-cultured with nonactivated PBMs.

Ussing Chamber Experiments

Epithelial Ion Transport

After culture with immune cells or conditioned medium, T84 monolayers on filter supports were mounted into Ussing chambers, as previously described.¹³ Briefly, monolayers were bathed in oxygenated Krebs buffer (pH 7.35, 37°C), containing 115 mmol/L NaCl, 8 mmol/L KCl, 1.2 mmol/L MgCl₂, 1.25 mmol/L CaCl₂, 2.0 mmol/L KH₂PO₄, 25 mmol/L NaHCO₃. The buffer bathing the serosal tissue surface contained 10 mmol/L of glucose as an energy source and this was osmotically balanced by inclusion of 10 mmol/L of mannitol in the buffer in the luminal side of the Ussing chamber. The epithelial spontaneous potential difference (in mV) was maintained at zero volts by the continuous injection of an external current by an automated voltage clamp (World Precision Instruments Inc., Sarasota, FL). This short-circuit current (Isc, in μ A/cm²) reflects net active ion transport across the preparation. Baseline Isc was recorded after a 15-minute equilibration period. Stimulated ion secretion was measured by adding the adenylate cyclase-activating agent, forskolin (10⁻⁵ mol/L; Sigma), to the serosal side of the T84 monolayers and recording the maximum increase in Isc.

Epithelial Barrier Function

Electrical resistance is a measure of the barrier function of the epithelium to passive ion movement. At intervals during each experiment, potential difference across the monolayer was clamped at 1.0 mV (differential pulse method, 1 pulse/30 seconds). The resulting change in

current was measured and the transepithelial electrical resistance (TER, in $\Omega \cdot \text{cm}^2$) was calculated according to Ohm's law. However, TER indicates the epithelial barrier to the flux of ions only and this need not be paralleled by increased transepithelial passage of larger molecules. Therefore, as an indication of epithelial permeability to larger molecules, in experiments conducted with normal PBMs ($\pm E. coli$) the mucosal-to-serosal movement of the inert probe, ^{51}Cr -EDTA (362.3 Daltons) was measured by adding 6.5 $\mu\text{Ci}/\text{ml}$ (Radiopharmacy, McMaster-Chedoke Hospital, Hamilton, Ontario, Canada) to the mucosal buffer. An equal concentration of nonradioactive Cr-EDTA was added to the serosal buffer to maintain the osmotic balance. After a 30-minute equilibration period 2×30 -minute fluxes were performed.²³

TNF- α

Basal or stimulated production of TNF- α by PBMs and LPMCs was measured at 24 hours by enzyme-linked immunosorbent assay with a sensitivity of 7.4 pg/ml (TNF- α -FLEXIA; BioSource International, Camarillo, CA).

The role of TNF- α in *E. coli*-PBM modulation of epithelial ion transport and barrier functions was assessed by inclusion of a neutralizing antibody to TNF- α , cA2 (1 $\mu\text{g}/\text{ml}$, >100-fold excess of the TNF- α measured in the conditioned medium; Centocor Inc., Malvern, PA). An irrelevant isotype matched antibody (anti-hepatoma IgG1, AF20; Centocor) was used as a control.

Data Presentation and Analyses

Results are presented as mean $\pm$ SEM. Because of variability in absolute values between different batches of T84 cells, data were normalized to control values in each experiment (expressed as percentage of control). The range of control responses is given in the figure legends; *n* values represent the number of experiments (different blood donors or tissue samples) in which two to four monolayers were examined per condition. Data were analyzed using one-way analysis of variance followed by Newman-Keuls comparison. Student's paired *t*-test was used to compare TNF- α production with or without LPS treatment. Statistically significant differences were accepted at $P < 0.05$.

Results

Effects of PBMs ($\pm$ LPS) on Epithelial Physiology

Epithelial Ion Transport

Control T84 epithelial monolayers displayed a baseline Isc of $2.2 \pm 0.4 \mu\text{A}/\text{cm}^2$ and a ΔIsc of $65 \pm 9 \mu\text{A}/\text{cm}^2$ in response to forskolin ($n = 14$). These indices of epithelial ion transport were not significantly altered after 48 hours of co-culture with nonactivated PBMs or LPS alone. However, co-culture with LPS-activated PBMs significantly ($P < 0.05$) elevated T84-baseline Isc to $251 \pm 16\%$ of control values ($5.4 \pm 0.5 \mu\text{A}/\text{cm}^2$; Figure 1A). Similarly,

Figure 1. Percent changes from control values (T84 cells alone) in epithelial baseline Isc (A), ΔIsc in response to forskolin (FSK) (B), and transepithelial resistance (C) of T84 monolayers after 48 hours of co-culture with normal or Crohn's disease (CD) PBMs in the presence or absence of LPS ($n = 12$ to 14 donors) [mean $\pm$ SEM, two to four monolayers per condition; *, $P < 0.05$ and **, $P < 0.005$ compared to control (100%)].

no change in T84-baseline Isc was observed after co-culture with nonactivated Crohn's disease PBMs, whereas LPS addition did evoke a significant ($P < 0.05$) increase in baseline Isc to $296 \pm 58\%$ of control values ($6.1 \pm 0.8 \mu\text{A}/\text{cm}^2$; $n = 12$). Also, T84 monolayers co-cultured with LPS-activated PBMs from patients with Crohn's disease displayed reduced ΔIsc to forskolin ($76 \pm 6\%$ of control values, $P < 0.05$), which was comparable to the changes induced by LPS-activated normal PBMs ($69 \pm 6\%$ of control values) (Figure 1B).

Epithelial Barrier Function

Control T84 monolayers displayed a TER of $1,784 \pm 179 \Omega \cdot \text{cm}^2$ ($n = 14$). Co-culture with nonactivated PBMs produced a small drop in TER (to $78 \pm 6\%$ and $75 \pm 10\%$ of control values; normal PBMs and Crohn's disease PBMs, respectively; Figure 1C). This change in epithelial barrier function was enhanced after LPS activation of

Figure 2. Basal (–LPS) or stimulated (+LPS) TNF- α production by PBMs (A) and LPMCs (B) from normals or patients with Crohn's disease (CD) after 24 hours of co-culture ($n = 6$ to 8 donors). Each symbol represents an individual cell donor.

PBMs. After 48 hours of co-culture with activated PBMs, T84 TER was significantly ($P < 0.05$) reduced to $37 \pm 5\%$ and $49 \pm 7\%$ of control values (normal PBMs and Crohn's disease PBMs, respectively; $n = 12$).

TNF- α Production by PBMs

Nonactivated normal PBMs produced no detectable TNF- α . LPS activation of PBMs caused a significant increase in TNF- α secretion after 24 hours (range, 346 to 4,595 pg/ml; mean, 2,332 pg/ml; $n = 10$). Similar to normal PBMs, PBMs isolated from Crohn's disease patients' blood did not secrete TNF- α under basal conditions, but did demonstrate TNF- α production when activated by LPS (range, 590–3,304 pg/ml; mean, 1,921 pg/ml; $n = 6$) (Figure 2A). This increase in TNF- α production was not statistically significantly different from that observed in LPS-stimulated PBMs from normal volunteers.

Figure 3. Percent changes from control values (T84 cells alone) in epithelial baseline Isc (A), Δ Isc in response to forskolin (FSK) (B), and transepithelial resistance (C) of T84 monolayers after 48 hours of co-culture with normal or Crohn's disease LPMCs in the presence or absence of LPS ($n = 8$ to 10 specimens per condition). Data for small bowel (SB) and large bowel (LB) are shown separately and combined as Crohn's total; statistical analyses performed on grouped Crohn's total data only [mean $\pm$ SEM, two to four monolayers per condition; *, $P < 0.05$ and **, $P < 0.005$ compared to control (100%); control values: baseline Isc range = 0 to 6 (mean, 2.4) μ A/cm 2 ; Δ Isc to FSK range = 30 to 154 (mean, 70) μ A/cm 2 ; TER range = 770 to 3,333 (mean, 1,784) Ω /cm 2].

Effects of LPMCs ($\pm$ LPS) on Epithelial Physiology

Results obtained with LPMCs $\pm$ LPS and LPMC-conditioned mediums were not statistically significantly different (eg, T84 TER was reduced to $27 \pm 11\%$ and $29 \pm 5\%$ of control values, respectively) and so the data are considered together.

Epithelial Ion Transport

The baseline Isc of T84 monolayers was unchanged after co-culture with LPMCs isolated from normal mucosa (Figure 3A) and remained comparable to control levels

after adding LPS to the co-culture ($86 \pm 6\%$ and $102 \pm 16\%$ of control values, normal LPMCs and normal LPMCs plus LPS, respectively). In contrast, 48 hours of co-culture with LPMCs isolated from Crohn's disease mucosa resulted in an elevated T84-baseline Isc ($198 \pm 40\%$ of control values, $P < 0.05$), and LPS addition produced no further increase in the baseline Isc ($188 \pm 21\%$ of control values; $n = 8$ to 10).

As shown in Figure 3B, the Δ Isc evoked by forskolin was unaltered by co-culture with normal LPMCs ($94 \pm 8\%$ of control) even in the presence of LPS ($98 \pm 10\%$ of control). However, there was a significantly diminished response to forskolin after co-culture with LPMCs from Crohn's disease mucosa, and inclusion of LPS resulted in no additional effects on T84 responses ($70 \pm 8\%$ and $72 \pm 8\%$ of control values; Crohn's disease LPMCs and Crohn's disease LPMCs plus LPS, respectively; $n = 8$ to 10).

Epithelial Barrier Function

T84 monolayers co-cultured with normal LPMCs, in the absence or presence of LPS, displayed TERs that were not different from control monolayers (Figure 3C). In contrast, co-culture with LPMCs from Crohn's disease mucosa, led to a decrease in TER ($34 \pm 9\%$ of control values, $P < 0.005$) with no further reduction when LPS was added ($28 \pm 5\%$ of control values; $n = 8$ to 10).

TNF- α Production by LPMCs

The spontaneous secretion of TNF- α by LPMCs isolated from normal mucosa was below the detection limit (<7.4 pg/ml) for all samples ($n = 6$) and stimulation by LPS had no effect on TNF- α secretion. In contrast, the spontaneous secretion of TNF- α by LPMCs obtained from Crohn's disease tissue was markedly elevated (range, 29 to 236 pg/ml; mean, 168 pg/ml; $n = 8$) compared to normal LPMCs and was further stimulated by LPS by approximately twofold (range, 76 to 590 pg/ml; mean, 354 pg/ml; Figure 2B).

Comparison of LPS-Activated PBMs and Nonactivated LPMCs from Crohn's Disease

Striking similarities were found between the T84 functional changes induced by Crohn's disease LPMCs in the absence of LPS and those induced by LPS-activated PBMs (Table 2). These data suggest that macrophages in the mucosa of patients with Crohn's disease are spontaneously activated, possibly by bacteria or their products, and directly influence intestinal epithelial physiology in a manner similar to LPS-activated circulating PBMs. Therefore, we used normal PBMs to examine if a non-pathogenic control strain of *E. coli* (HB101), added to the luminal aspect (physiological side) of T84 cell monolayers, could induce changes in epithelial function.

Table 2. Comparison of Epithelial Pathophysiology Induced by LPS-Activated PBMs and Spontaneously Activated LPMCs from Crohn's Disease

	Properties of T84 Monolayers (% of control)		
	Baseline Isc	Δ Isc to forskolin (10^{-5} mol/L)	TER
Normal PBM + LPS	$251 \pm 16^*$	$69 \pm 6^*$	$37 \pm 5^+$
Crohn's disease LPMC	$198 \pm 40^*$	$70 \pm 8^*$	$34 \pm 9^+$

Mean $\pm$ SEM of 10 to 14 experiments. Confluent T84 monolayers were co-cultured for 48 hours with peripheral blood monocytes (PBMs) + LPS ($10 \mu\text{g/ml}$), or lamina propria mononuclear cells (LPMCs) without LPS from Crohn's disease mucosa.

* $P < 0.05$ and $^+P < 0.005$ compared to control (T84 monolayers alone) at 100%.

Effects of PBMs and Luminal Bacteria on Epithelial Physiology

Epithelial Ion Transport

The baseline Isc of T84 monolayers was not significantly altered by co-culture with luminally applied *E. coli* only (ie, $123 \pm 8\%$ of control values; Figure 4A). However, co-culture with luminal bacteria in the presence of basal PBMs resulted in a significantly ($P < 0.05$) elevated T84-baseline Isc ($217 \pm 39\%$ of control values). Also, the Δ Isc evoked by forskolin remained unchanged after co-culture with *E. coli* alone ($93 \pm 11\%$ of control values) but was significantly ($P < 0.05$) diminished in the presence of basal PBMs ($61 \pm 7\%$ of control values) (Figure 4B).

Epithelial Barrier Function

Co-culture of the T84 monolayers with *E. coli* alone had no effect on the TER. However, TER was reduced ($54 \pm 7\%$ of control values; $P < 0.05$) after 48 hours of co-culture in the presence of PBMs (Figure 5A). Similarly, after 48 hours co-culture with *E. coli* plus PBMs (but not bacteria alone), the serosal-to-mucosal flux of ^{51}Cr -EDTA across T84 monolayers was significantly ($P < 0.05$) elevated compared with control monolayers ($n = 6$ to 8; Figure 5B). This increase in epithelial permeability is in agreement with our previous study, in which normal PBMs activated by bacterial products induced an increase in the flux of ^{51}Cr -EDTA.¹³

TNF- α Production and Effect of Anti-TNF- α Treatment

PBMs stimulated indirectly by co-culture with the non-pathogenic *E. coli* secreted substantial amounts of TNF- α after 24 hours (range, 984 to 2,907 pg/ml; mean, 2,240 pg/ml; $n = 5$). Addition of anti-TNF- α antibody at the start of the co-culture completely prevented the increase in T84-baseline Isc ($113 \pm 19\%$ of control values) and restored the diminished Δ Isc response to forskolin (to $98 \pm 12\%$ of control values) (Figure 4). In addition, anti-TNF- α ameliorated both the changes in the TER and the in-

Figure 4. Percent changes from control values (T84 cells alone) in epithelial secretory responses; baseline Isc (A) and Δ Isc in response to forskolin (FSK) (B) of T84 monolayers after 48 hours of co-culture with control *E. coli* alone or *E. coli* and PBMs in the presence or absence of anti-TNF- α antibody (aTNF- α) ($n = 6$ to 8 donors) [mean $\pm$ SEM, two to four monolayers per condition; *, $P < 0.05$ compared to control (100%); control values: baseline Isc range = 1 to 3 (mean, 1.4) μ A/cm 2 ; Δ Isc to FSK range = 66 to 118 (mean, 92) μ A/cm 2].

creased flux of 51 Cr-EDTA caused by *E. coli* infection in the presence of PBMs (Figure 5). Use of an irrelevant control antibody did not prevent the changes in epithelial responsiveness to forskolin or TER, that were reduced to 66% and 52% of control values, respectively ($n = 4$ to 6 monolayers from two experiments).

Discussion

Inflammatory conditions of the gut are associated with increased heterogeneity and activation of intestinal macrophages. Unlike macrophages in normal mucosa, many of those isolated from the mucosa of patients with Crohn's disease strongly express the LPS receptor, CD14, and the myelomonocytic marker L1, suggesting recent recruitment from peripheral blood.^{7,8,24} Functional studies have shown that IBD macrophages seem to have an increased ability to undergo respiratory burst and produce cytokines in response to LPS.^{7,25} However, to our knowledge, data have not been published on the direct effects of macrophages obtained from affected mucosa of patients with Crohn's disease on epithelial cell physi-

Figure 5. Changes from control values (T84 cells alone) in epithelial barrier function measured by transepithelial resistance (A) and mucosal-to-serosal flux of 51 Cr-EDTA across T84 monolayers (B) after 48 hours of co-culture with control *E. coli* alone or *E. coli* and PBMs in the presence or absence of anti-TNF- α antibody (aTNF- α) ($n = 6$ to 8 donors) [mean $\pm$ SEM, two to four monolayers per condition; *, $P < 0.05$ compared to control (100%); control TER range = 860 to 1,620 (mean, 1,093) Ω /cm 2].

ology. Moreover, comparisons have not been made between LPMCs and PBMs isolated from normal patients and patients with Crohn's disease in regards to their ability to modulate enteric epithelial function.

Here, we show that PBMs from normal patients and those from patients with Crohn's disease were not spontaneously active with respect to TNF- α production and, apart from a slight decrease in TER, did not alter epithelial physiology. LPS-induced activation, however, resulted in comparable changes in T84 cell function after co-culture with PBMs from both groups—increased baseline Isc, reduced responses to forskolin and reduced TER were observed. Similarly, and in accordance with Bouma and colleagues,²⁶ there was no significant difference in the intrinsic capacity of normal PBMs and Crohn's disease PBMs to produce TNF- α after stimulation with LPS. We have reported that the increase in T84-baseline Isc elicited by co-culture with activated monocytes/macrophages is because of active Cl $^-$ secretion¹³ and this is mostly likely the cause of the increased Isc

observed here. Also, the magnitude of the reduction in the ΔI_{sc} response to forskolin suggests that it is not solely a consequence of the concomitant increase in baseline I_{sc} , and likely reflects a specific perturbation in ion transport signaling. Oprins and colleagues²⁷ recently reported that TNF- α potentiates the I_{sc} response elicited in the HT-29 epithelial cell line in response to cholinergic stimulation. These data complement our findings, demonstrating that TNF- α can affect enteric epithelial ion transport, however our data indicate that TNF- α (in the context of other mediators) reduces the response to forskolin, indicating distinct effects of this cytokine on Ca^{2+} - and cAMP-driven Cl^{-} transport.

We also document, for the first time, that LPMCs from Crohn's disease differ from those of normal patients in their ability to directly influence enteric epithelial physiology. LPMCs isolated from normal intestinal mucosa induced neither secretory (baseline I_{sc} , ΔI_{sc} to forskolin) nor permeability (TER) changes in the T84 monolayers after a 48-hour co-culture period. These cells did not respond directly to LPS (using TNF- α production as an indication of activity) nor did they induce epithelial physiological changes on LPS exposure. In contrast, LPMCs isolated from affected Crohn's disease mucosa displayed spontaneous TNF- α production and evoked significant changes in epithelial function, as evidenced by a marked increase in T84-baseline I_{sc} , diminution of the response to forskolin and impairment of barrier function (ie, reduced TER) after co-culture.

The fact that normal LPMCs were incapable of activation by LPS could be explained by the lack of CD14⁺ macrophages.⁸ This limitation of the macrophage response is important in avoiding an inflammatory reaction to every day exposure of the epithelium to LPS from commensal bacteria in the normal intestine. In the inflamed intestine, however, the continuing influx of blood monocytes to the mucosa results in the presence of a population of macrophages that is strongly CD14⁺ and, consequently, sensitive to LPS. This sensitivity to LPS exposure becomes particularly important when associated with the damaged epithelial cells in active IBD or in the presence of a primary abnormality in epithelial tight junctions,²⁸ when large quantities of LPS may leak across the intestinal epithelium and gain access to the underlying mucosa. Exposure to LPS could activate newly recruited macrophages in Crohn's disease mucosa, thus causing changes in epithelial function (ie, ion secretion and permeability) analogous to those observed in this *in vitro* model system.

TNF- α was not detected in the supernatants of normal LPMCs under basal or LPS-stimulated conditions. In contrast, LPMCs obtained from affected Crohn's disease mucosa spontaneously produced TNF- α and were further stimulated by LPS to produce greater amounts of this cytokine. Infiltrating monocyte-like macrophages are an important source of TNF- α in Crohn's disease.⁹ Currently, there is much interest in the role of TNF- α in Crohn's disease. Several studies have shown increased TNF- α protein and mRNA levels in biopsies obtained from patients with Crohn's disease.^{25,29,30} The increased level of TNF- α may lead to enhanced activation of multiple mu-

cosal cells, including macrophages, lymphocytes, and epithelial cells, and thereby contribute to mucosal damage in the intestine. In our experiments, a key role for TNF- α in the pathogenesis of the epithelial abnormalities was found—both ion transport and barrier defects were prevented by inclusion of an anti-TNF- α antibody in the co-culture.¹³ The importance of TNF- α in the pathophysiology of Crohn's disease is further supported by recent clinical trials demonstrating the efficacy of anti-TNF- α antibody in treatment of Crohn's disease.^{10–12}

There is considerable evidence from both animal models and clinical investigations supporting a pivotal role for bacteria in the initiation or exacerbation of the intestinal inflammation. For instance, bacteria, or their products, have been detected in inflamed mucosa of patients with Crohn's disease,¹⁵ antibiotic treatment³¹ or diversion of the fecal stream¹⁴ can reduce disease severity in some patients with Crohn's disease, and in the majority of the spontaneous models of murine colitis the inflammation does not occur when the animals are housed under germ-free conditions.^{16,32–34} However, little data are available on the role of commensal bacteria in altering epithelial function. Because LPMCs from patients with Crohn's disease evoke epithelial abnormalities virtually identical to those elicited by LPS-activated PBMs (Table 2), we used PBMs (a convenient and readily available source of monocytes) to explore the affect of a nonpathogenic strain of *E. coli* (HB101) on epithelial physiology.

Our investigations revealed that a nonpathogenic strain of bacteria could indeed, via activation of monocytes/macrophages, induce irregularities in enteric epithelial function that are characteristic of those observed in tissue resections from patients with IBD. We showed a marked increase in T84-baseline I_{sc} , reduced responsiveness to forskolin and a simultaneous disruption of barrier function (ie, reduced TER and increased ⁵¹Cr-EDTA flux) after co-culture with luminal bacteria, but only in the presence of PBMs. These results indicate that nonpathogenic commensal bacteria, in the presence of CD14⁺ macrophages, have the potential to alter intestinal epithelial secretory and barrier functions via immune activation. *In vivo*, similar events could result in a cascade of immune events, the consequences of which may be tissue damage leading to chronic inflammation. In this context, Duchmann and colleagues^{1,5} have shown that LPMCs isolated from patients with IBD, or from mice with a chemically induced colitis, proliferate when exposed to autologous intestinal bacteria. Collectively these data support the concept that the tolerance toward autologous intestinal flora in healthy individuals is lost during inflammation associated with IBD. It is also noteworthy that the altered epithelial functions observed in our *in vitro* tripartite model were negated by use of an anti-TNF- α antibody, adding further support for the current hypothesis that an inappropriate immune response to bacteria contributes to the pathophysiology of IBD.

In summary, this study extends our previous findings,^{13,20} illustrating the ability of PBMs from patients with Crohn's disease exposed directly to LPS, or indirectly (ie, separated by an epithelial layer) to nonpathogenic bacteria to affect the transport and barrier properties of a

model gut epithelium. Moreover, we have identified that co-culture with LPMCs from patients with Crohn's disease, unlike those from controls, resulted in increased epithelial permeability and altered ion transport in the absence of LPS stimulation and that inclusion of LPS did not accentuate the changes in epithelial function. We conclude that LPMCs from patients with Crohn's disease are spontaneously altered compared to controls, and that their response to bacteria/bacterial products may lead to epithelial dysfunction through a TNF- α -dependent mechanism. Finally, while one must always bear in mind the inherent cellular complexity of the gut, we present an easily manipulated tripartite model that can be used to dissect signaling pathways between enterocytes, immune cells, and microflora in the regulation of gut physiological and pathophysiological reactions.

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