

Factors Regulating the Effect of IL-4 on Intestinal Epithelial Barrier Function

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Key Words

Intestinal permeability · Macromolecular transport · Cytokines · Signal transduction

Abstract

Background: Inflammatory bowel disease is associated with an imbalance in cytokine production and defective intestinal barrier function. Previous studies indicate that IL-4, a cytokine increased in food allergy and in early Crohn's disease, enhances epithelial permeability. Here, we characterized the mechanism of action of IL-4 on cultured epithelial cells and examined if the anti-inflammatory cytokines, TGF- β or IL-10, can modulate the effects of IL-4. **Methods:** Confluent monolayers of human T84 epithelial cells were cultured with IL-4 alone or in combination with IL-10 or TGF- β or with inhibitors of protein synthesis and blockers of IL-4 receptor signalling pathways. Permeability was evaluated by measuring trans-epithelial resistance (TER), flux of ^3H -fMLP (a small bacterial tripeptide) and horseradish peroxidase (HRP) (a macromolecule). **Results:** T84 cells cultured with IL-4 showed a significant drop in TER as well as an increased flux of ^3H -fMLP and HRP. Co-treatment with IL-10 did not improve TER, whereas TGF- β attenuated the resistance drop. However, neither TGF- β nor IL-10 were able to correct the increased ^3H -fMLP flux. In contrast, the increased HRP flux caused by IL-4 was inhibited by both IL-10 and

TGF- β . TGF- β and IL-10 significantly reduced IL-4-enhanced values for endosomal area and paracellular spaces containing HRP. Inhibitor studies indicated the requirement for protein synthesis and the involvement of phosphatidylinositol 3-kinase. **Conclusions:** These results provide new insights into the regulation of intestinal barrier function and may suggest a novel approach in the treatment of intestinal inflammation.

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Introduction

The intestinal epithelium, besides its absorptive and secretory functions, plays an important role in modulating the immune response to intestinal contents, acting as a permeable but selective barrier to regulate the passage and uptake of luminal molecules. Luminal material can enter the mucosa by crossing the epithelium either between cells (the paracellular pathway) or through cells (the transcellular pathway). Passive movement of ions and small polar molecules occurs via the paracellular spaces between adjacent epithelial cells (enterocytes) which are controlled by the intercellular tight junctions. Under physiological conditions, these junctions form a structural barrier to prevent penetration of macromolecules [1]. Large molecules such as intact proteins (which have escaped enzymatic hydrolysis and are therefore potential-

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ly antigenic) are taken up by endocytosis at the apical membrane of the enterocytes. The typical transcellular pathway involves fusion of endosomes with lysosomes, where most proteins are degraded into non-antigenic amino acids or peptides. However, even under normal circumstances a small quantity of intact protein reaches the lamina propria and is thus capable of initiating an immune response [2].

Increased epithelial permeability is associated with intestinal inflammatory bowel diseases (IBD) [3] and often precedes clinical relapse [4]. Increased macromolecular permeability has been demonstrated even in non-inflamed regions of surgically resected intestine from Crohn's disease patients [5]. In animal models, increased intestinal permeability has been directly associated with onset of inflammation [6]. Intestinal epithelial cells express receptors for a variety of proinflammatory mediators, which influence epithelial function [7]. In particular, there are many reports that various cytokines act directly on enterocytes, altering epithelial functions including antigen transport [8–10].

Increased levels of IL-4 have been shown in allergy [11] and IBD [12], conditions associated with increased intestinal permeability [13]. In vitro, IL-4 affects epithelial integrity, altering transepithelial resistance (TER) and paracellular permeability of epithelial cell monolayers [14]. IL-4 also enhances macromolecular uptake. We have recently shown that in the T84 human epithelial cell line, IL-4 increases the transport of the model protein antigen, horseradish peroxidase (HRP), through both transcellular and paracellular pathways [15].

Concentrations of anti-inflammatory cytokines, IL-10 and TGF- β , which also have direct effects on epithelium [16, 17], are reduced in inflammatory conditions [18, 19]. In vitro, both of these cytokines improved intestinal epithelial pathophysiology in a co-culture model of inflammation [20, 21]. IL-10 also partially restored epithelial permeability during IFN- γ treatment [22]. Both IL-10 and TGF- β decreased endocytosis in macrophages [23, 24], suggesting that they may play a role in modulating antigen transport across the enterocytes. Therefore, the aims of our study were to further characterize the mechanism by which IL-4 adversely affects barrier function in a model human intestinal epithelium and to determine if IL-10 and TGF- β can inhibit this effect. Identification of mechanisms to interfere with epithelial barrier dysfunction may lead to novel therapeutic approaches for intestinal inflammatory conditions where increased permeability is thought to be involved in gut pathophysiology.

Materials and Methods

Epithelial Cells

The human intestinal epithelial cell line T84 was used. Cells were grown in a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F12 medium supplemented with 10% (vol:vol) fetal calf serum, 2% (vol:vol) penicillin-streptomycin and 1.5% (vol:vol) Hepes. All the reagents were from Gibco Laboratories, Grand Island, N.Y., USA. Cells were seeded at a density of 10^6 cells on 1.0 cm² polycarbonate tissue-culture-treated semipermeable filters (0.4 μ m pore size; Costar Inc., Cambridge, Mass., USA) and maintained under standard conditions for 7–10 days at 37°C, with daily replacement of media until the beginning of treatment. Monolayers with TER $\geq 1,500 \Omega/\text{cm}^2$ were used for the study.

Exposure of Epithelial Monolayers to Cytokines and Inhibitors

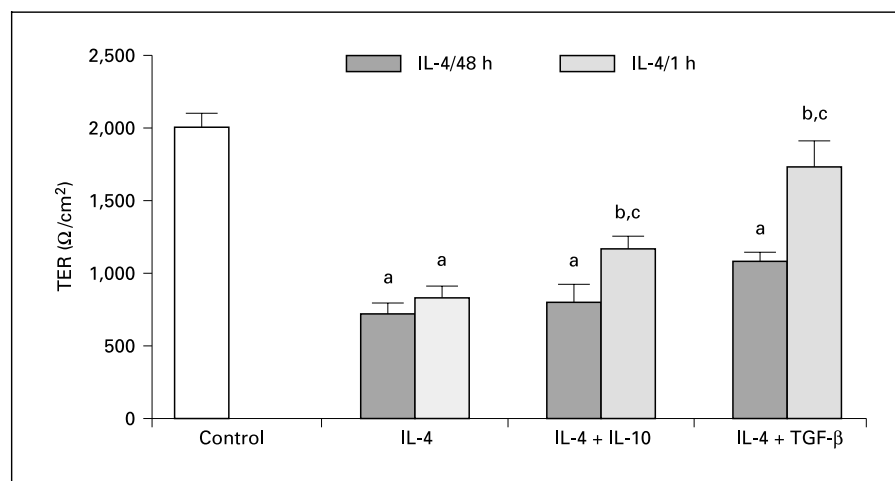
Confluent T84 monolayers were exposed to media containing 10 ng/ml recombinant human IL-4 in the basal compartment for 48 h. This dose was chosen since we previously showed that at this concentration IL-4 significantly reduces TER and selectively increases apical-to-basal macromolecular transport across epithelial cell monolayers [15].

To study the effect of IL-10 and TGF- β on IL-4-induced changes, monolayers were exposed to basal media containing IL-4 plus either IL-10 (10 ng/ml) or human TGF- β (10 ng/ml) for 48 h. In some of the wells, media containing IL-10 or TGF- β (but not IL-4) was applied after the cells had been treated for 1 h with IL-4. Finally, some monolayers were pre-treated for 48 h with TGF- β (10 ng/ml), before the addition of IL-4. Controls were untreated monolayers or monolayers treated with IL-10 or TGF- β alone. All cytokines were from R&D Systems, Minneapolis, Minn., USA.

In order to determine if the IL-4-induced changes in epithelial function were rapid physiological changes or involved structural alterations with new protein synthesis, in some experiments, media containing IL-4 was applied for 1 h, then replaced with fresh media and epithelial functions were evaluated after 48 h. In other experiments, the protein synthesis inhibitor, cycloheximide (ICN Biochemical Inc., Aurora, Ohio, USA), was added to the basal media at a concentration of 1 μ g/ml.

To determine the signal transduction pathway involved in the increase in macromolecular transport induced by IL-4, a pharmacological approach was used, treating the monolayers with inhibitors of two of the major pathways activated by IL-4. The activation of the STAT-6 pathway was blocked with transcription factor decoys (TFDs; MOBIX, McMaster University), double-stranded DNA with the same base sequence as the nuclear DNA to which STAT-6 binds. TFDs (0.2 μ M) were added to the basal compartment of the transwells in Opti-MEM I-reduced serum media (Sigma Chemical Co., St. Louis, Mich., USA) for 24 h. The monolayers were then washed with phosphate-buffered saline (3 $\times$), to remove non-incorporated TFDs, and received standard medium for 1 h prior to IL-4 treatment. Electrophoretic mobility shift assays [35] of nuclear protein extracts from T84 cells treated with IL-4 demonstrated induction of STAT-6 binding activity and reduction of the band by TGFs (data not shown). The specific phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (20 μ M; Sigma) was applied simultaneously with the cytokine and, therefore, was present in the media for 1 or 48 h.

Fig. 1. TER of T84 monolayer after treatment with 10 ng/ml IL-4, IL-4 plus IL-10 (10 ng/ml) or IL-4 plus TGF- β (10 ng/ml). Values indicate the mean $\pm$ SEM (n = 15–20 monolayers/group). ^a p < 0.001 compared to control; ^b p < 0.01 compared to IL-4 group; ^c p < 0.001 vs. IL-4/48 h group.



Permeability Studies

Transepithelial Resistance. TER, a measure of the ability of T84 cells to restrict passive movement of ions, was monitored using a voltmeter (Millipore, Bedford, Mass., USA).

Uptake of Bacterial Products. The passage of small molecules through the paracellular space was evaluated by measuring the flux of tritiated f-Met-Leu-Phe (³H-fMLP; Nuclear Pharmacy, McMaster University), a bacterial pro-inflammatory tripeptide (MW 476 D). ³H-fMLP (10^{-5} M) was added to the media in the top well (luminal side). After a 30-min period of equilibration, a 'hot' sample (50 μ l) was obtained from the top well for determination of probe-specific activity and 'cold' samples (500 μ l) were taken from the bottom well at 30-min intervals over a 2-hour period and replaced with non-radioactive media containing fMLP. Radioactivity was determined in a scintillation counter and flux rates calculated as percent recovery of the total probe added to the apical side of the monolayers.

Macromolecular Transport. The uptake of antigen-sized proteins was assessed by the flux of HRP (MW 44,000 D, type VI-A; Sigma). Media containing HRP (10^{-5} M) was added to the top well and monolayers were incubated for 2 h at 37°C. Samples were taken from the apical and basal compartments and HRP concentration was measured by a kinetic enzymatic assay. Briefly, a 150- μ l aliquot of sample was added to 800 μ l of phosphate buffer containing 0.004% of H₂O₂ and 80 μ l/ml of o-dianisidine (both from Sigma). The increase in optical density was measured at 460 nm over a period of 2 min to determine enzyme activity. The amount of HRP that crossed the epithelium was expressed as percent recovery of HRP added to the apical side of the monolayers.

Electron Microscopy

To determine the pathway of transepithelial passage of HRP, T84 monolayers were examined by transmission electron microscopy. Two hours after the addition of HRP, filters were cut from their support and fixed with 2.5% glutaraldehyde buffer in 0.1 M sodium cacodylate buffer (pH 7.4). Cytochemistry was performed using 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H₂O₂ in Tris buffer. After dehydration through serial graded ethanol washes, filters were embedded in Epon and ultrathin sections were obtained and stained with uranyl acetate and lead salts. Exami-

nations were performed by a 'blinded' observer (P.-C.Y.), who was unaware of the experimental treatment of the cells. For each group, presence of HRP was quantified by determining the number of HRP-containing endosomes and total endosomal HRP area in windows (300 μ m², 20 groups). Paracellular regions were also examined for the presence of HRP [15].

Statistics

Data are expressed as means $\pm$ SEM. Statistical analysis was carried out using non-parametric ANOVA and Newman-Keuls post-hoc test. A p value of ≤ 0.05 was considered significant.

Results

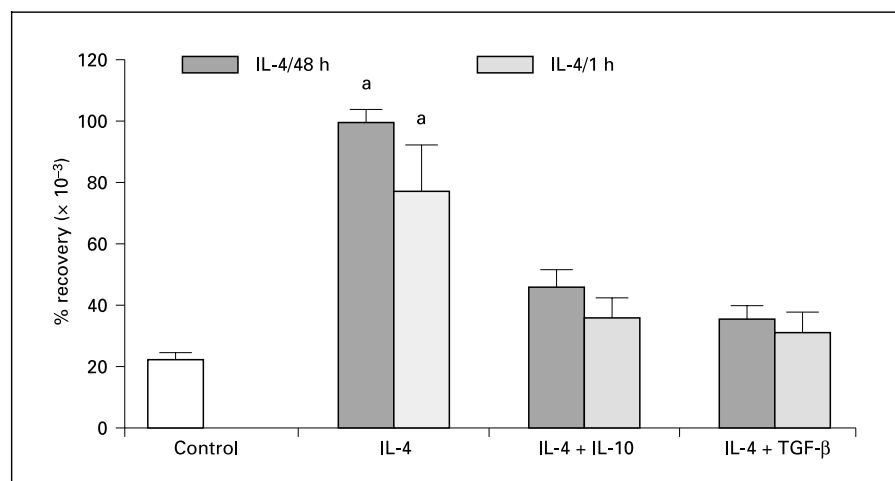
Effect of IL-4 on Monolayer Resistance and Permeability

The initial average TER was $\sim 2,000$ Ω /cm². During the experimental period, the resistance of control monolayers did not change significantly. IL-4-treated monolayers showed a significant drop in resistance after 48 h, irrespective of whether the exposure to cytokine was continuous ($38 \pm 1.5\%$ of controls) or for only 1 h ($44.3 \pm 2.3\%$ of controls) (fig. 1). IL-4 similarly increased monolayer permeability to fMLP from $0.6 \pm 0.1\%$ to 1.4 ± 0.3 (48 h) or $1.2 \pm 0.3\%$ (1 h); p < 0.001 vs. control. Transepithelial HRP flux was also dramatically enhanced by exposure of monolayers to IL-4, to $449 \pm 42\%$ (48 h) or $345 \pm 41\%$ (1 h) of control values (p < 0.001) (fig. 2).

Effect of IL-10 and TGF- β on IL-4-Induced Resistance Change

IL-10, when added alone, did not affect TER after 48 h of culture ($1,540 \pm 77$ vs. $1,893 \pm 40$ Ω /cm² of controls). When IL-4 was present in the media for 48 h, co-treat-

Fig. 2. Transport of HRP across T84 monolayers after treatment with IL-4, IL-4 plus IL-10 or IL-4 plus TGF- β . HRP (10^{-5} M) was added to the apical compartment of monolayers and samples of apical and basal media were taken after 2 h at 37°C. HRP concentration in basal compartment was expressed as percent recovery of apically added HRP. Values indicate the mean $\pm$ SEM (n = 15–20 monolayers/group). ^a p < 0.001 compared with the other groups.



ment with IL-10 did not reverse the TER change (IL-4 alone: $718 \pm 28 \Omega/\text{cm}^2$; IL-4 plus IL-10: $772 \pm 25 \Omega/\text{cm}^2$; $p < 0.001$ vs. controls) (fig. 1). In contrast, TGF- β alone significantly increased monolayer TER ($2,409 \pm 67$ vs. $1,893 \pm 39 \Omega/\text{cm}^2$ of controls; $p < 0.001$) and TGF- β added at the same time as IL-4 (or pre-treatment before IL-4), partially attenuated the drop in TER compared to values for monolayers treated with IL-4 alone ($1,084 \pm 37$ or $1,100 \pm 26 \Omega/\text{cm}^2$; $p < 0.001$ vs. IL-4 alone) (fig. 1). When IL-10 and TGF- β were added together, the effect on TER of IL-4-treated monolayers was similar to the values obtained when TGF- β was added alone ($1,250 \pm 23 \Omega/\text{cm}^2$). When IL-4 was removed after 1 h, subsequent exposure for 48 h to IL-10 was able to partially recover the TER values ($61.5 \pm 10\%$ of controls; $p < 0.01$ vs. IL-4), whereas TGF- β completely normalized TER ($92.3 \pm 18\%$ of controls) (fig. 1).

Effect of IL-10 and TGF- β on IL-4-Induced Enhanced Recovery of fMLP

When IL-10 or TGF- β was added at the same time as IL-4, permeability to fMLP was not improved at 48 h (IL-10 plus IL-4: $1.6 \pm 0.3\%$; TGF- β plus IL-4: $1.5 \pm 0.5\%$; IL-4 1.4 ± 0.3 vs. $0.6 \pm 0.1\%$ recovery for control monolayers). Comparable results were obtained for monolayers treated with IL-10 or TGF- β after 1 h of exposure to IL-4.

Effect of IL-10 and TGF- β on IL-4-Induced Enhanced Recovery of HRP

Recovery of intact HRP in the lower compartment of culture plates containing control, IL-10- or TGF- β -treated

monolayers was just above the detection limit. IL-4 treatment increased HRP transport 4.5 ± 0.4 -fold compared to control values when applied for 48 h and 3.5 ± 0.4 -fold in the short-term protocol. Both IL-10 and TGF- β significantly attenuated the permeability change caused by IL-4, to $211 \pm 14\%$ of control in IL-10 plus IL-4-treated monolayers and to $157 \pm 12\%$ of control for TGF- β plus IL-4 (fig. 2). Results were similar when IL-4 was present for only 1 h ($201 \pm 36\%$ of control for IL-10 plus IL-4 and $141 \pm 26\%$ of control for TGF- β plus IL-4) (fig. 2).

Pathways of IL-4-Induced Macromolecular Permeability

Quantitative analysis of paracellular and intracellular regions containing HRP (the latter by measuring the endosomal area positive for HRP) was performed by electron microscopy. Representative photomicrographs are shown in figure 3. Paracellular spaces in control monolayers did not contain HRP, whereas 40% of paracellular spaces in IL-4-treated monolayers were positive for HRP ($p < 0.05$ vs. control). A significant decrease was observed in IL-4 plus IL-10-treated monolayers (16%) as well as in IL-4 plus TGF- β (12%) (table 1). IL-4 increased the area of HRP in enterocytes from $1.07 \pm 0.2 \mu\text{m}^2/\text{window}$ in control monolayers to $6.03 \pm 1.3 \mu\text{m}^2/\text{window}$ ($p < 0.001$). Both IL-10 ($4.13 \pm 1.0 \mu\text{m}^2/\text{window}$) and TGF- β ($3.40 \pm 0.7 \mu\text{m}^2/\text{window}$) were able to reduce the HRP-positive endosomal area in IL-4-treated monolayers (fig. 4). However, even in the presence of IL-10 or TGF- β , IL-4-enhanced HRP transport through both pathways was significantly increased compared to untreated monolayers (fig. 4).

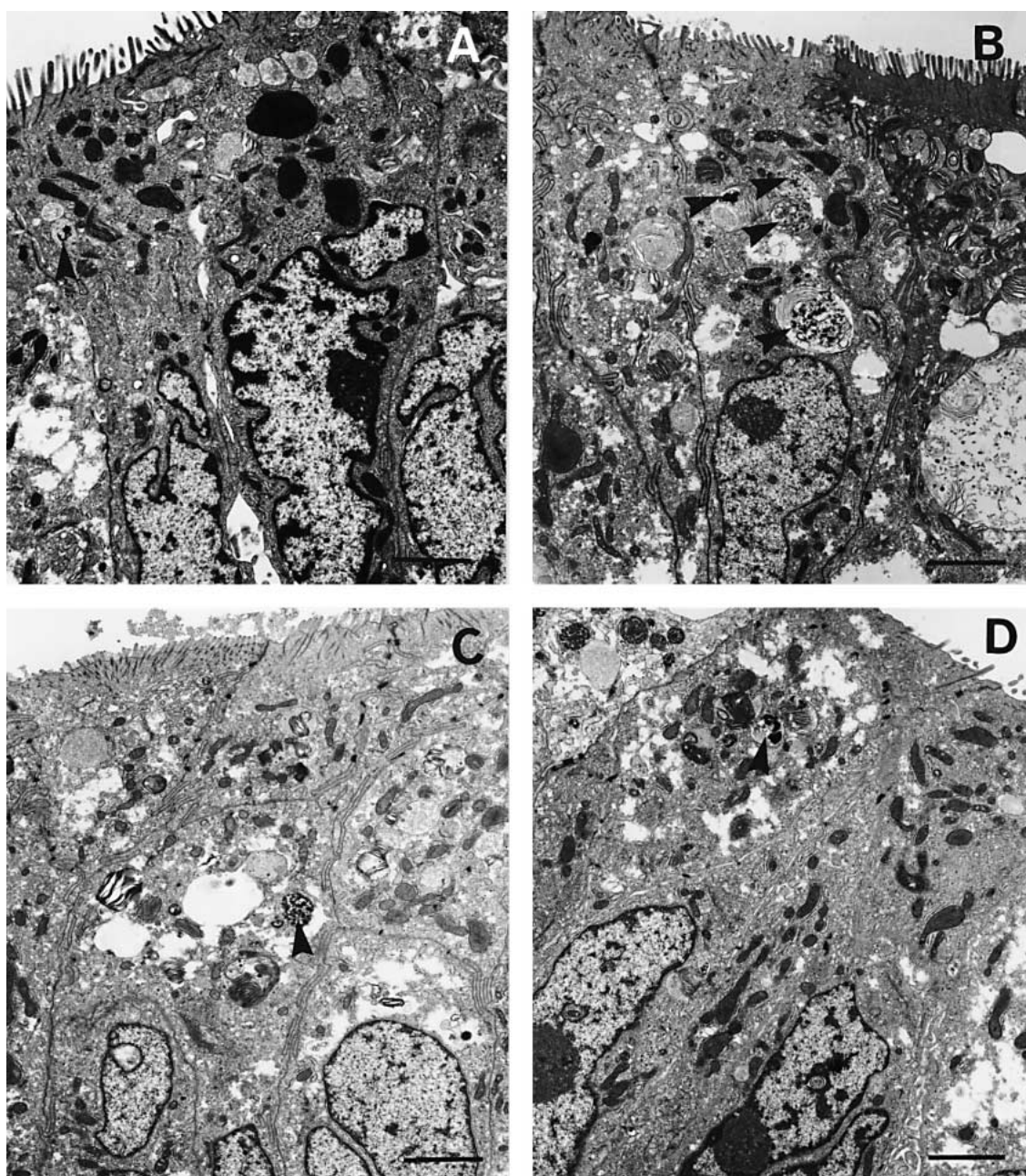


Fig. 3. Representative photomicrographs of T84 cells treated with media (A), IL-4 (B), IL-4 plus IL-10 (C) or IL-4 plus TGF- β (D) for 48 h. T84 monolayers were exposed to 10^{-5} M HRP for 2 h at 37°C before fixation for electron microscopy. Arrowheads indicate HRP-containing endosomes. Magnification $\times 4,000$; bar = 2 μ m.

Role of Protein Synthesis in IL-4-Induced TER and Permeability Changes

The protein synthesis inhibitor cycloheximide (1 μ g/ml) alone did not decrease the TER of monolayers ($2,366 \pm 90$ vs. $1,893 \pm 30$ Ω/cm^2 of controls). In the presence

of the inhibitor, IL-4 caused no significant reduction in TER ($2,200 \pm 70$ Ω/cm^2). Cycloheximide also prevented the increased permeability to fMLP caused by IL-4 ($0.4 \pm 0.01\%$ for IL-4 plus cycloheximide vs. $1.4 \pm 0.3\%$ for IL-4 alone; $p < 0.001$). Cycloheximide was equally effec-

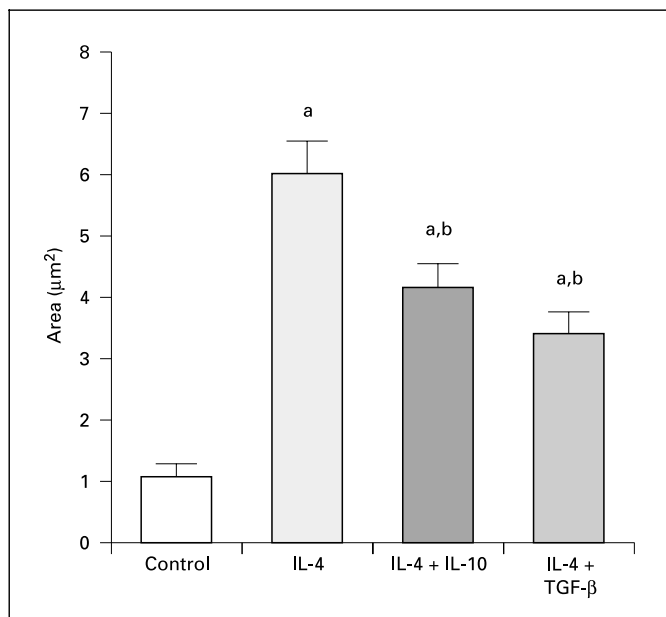


Fig. 4. Quantification of intracellular HRP in T84 cells after treatment with IL-4, IL-4 plus IL-10 or IL-4 plus TGF- β for 48 h. HRP (10^{-5} M) was added to luminal side for 2 h at 37°C before fixation for electron microscopy. The area of HRP containing endosomes was quantified in 300- μm^2 windows using 20 photomicrographs/treatment group. Values indicate the mean $\pm$ SEM (2 monolayers/group). ^a $p < 0.001$ compared to controls; ^b $p < 0.001$ compared to IL-4 group.

Table 1. Quantification of HRP within paracellular regions of T84 monolayers

	Total regions examined	HRP-positive regions	% HRP-positive
Control	400	0	0
IL-4	400	150	40 ^a
IL-4 + IL-10	400	64	16 ^{a,b}
IL-4 + TGF- β	400	48	12 ^{a,b}

T84 were cultured for 48 h in presence of IL-4 (10 ng/ml), IL-4 plus IL-10 (10 ng/ml) or IL-4 or TGF- β (10 ng/ml) or with media alone (control). Monolayers were fixed for electron microscopy 2 h after addition of HRP to luminal side. ^a $p < 0.05$ vs. controls; ^b $p < 0.05$ vs. IL-4.

tive with respect to macromolecular barrier function, since the HRP flux was not increased by IL-4 when protein synthesis was inhibited (table 2). Comparable results were obtained in the short-term treatment protocol (table 3).

Role of Other Intracellular Signals in IL-4-Induced Effects

Pre-treatment with TFDs did not affect IL-4-induced events. Similar to IL-4 alone, the TER of monolayers treated with IL-4 plus TFDs was reduced significantly to $40 \pm 5\%$ of controls ($p < 0.01$) and HRP transport was increased to 3.7 ± 2.9 -fold ($p < 0.01$) (table 2). However, when IL-4 was added in the presence of the PI3K inhibitor LY294002, both TER and HRP transport were completely normalized (tables 2, 3). LY294002 alone enhanced TER ($2,200 \pm 79$ vs. $1,893 \pm 39$ Ω/cm^2 of controls; $p < 0.05$) with no significant effect on monolayer permeability (tables 2, 3). In addition, both cycloheximide and LY294002 added to IL-4-treated monolayers completely abolished the deleterious effect of IL-4 on fMLP flux (tables 2, 3).

Discussion

Intestinal epithelial permeability is a central factor in the development and maintenance of immune responses in the gut. During the early stages of life, the intestinal epithelium is more permeable, allowing the induction of tolerance, whereas, later on, only a limited amount of immunogenic material from the intestinal lumen is able to penetrate the epithelial barrier [2]. Under physiological conditions, transepithelial passage of antigenic molecules, mainly proteins, occurs via the transcellular pathway within endosomes, since the intercellular spaces are 'sealed' by tight junctions restricting passage of molecules to those with a MW < 5 kD. The majority of internalized proteins are degraded to smaller fragments by lysosomal enzymes, thus blunting the antigenic load reaching the lamina propria. Therefore, the passage of intact macromolecules accounts for less than 3% of those ingested [25]. However, in certain conditions, an increased rate of macromolecular uptake can occur, resulting in abnormally enhanced antigenic stimulation of gut immune cells. Immune products, such as cytokines, play a significant role in regulating the intestinal barrier function [26]; delineating their mechanisms of action and studying their interaction can provide important information with therapeutic potential.

In the present study, we examined if IL-10 and/or TGF- β could counteract the ability of IL-4 to enhance epithelial permeability. In addition, we characterized the effect of IL-4 on a model intestinal epithelium with a focus on macromolecular transport. We confirmed that IL-4 was able to disrupt the intestinal epithelial barrier

Table 2. Effect of cyclohexamide (CMX), transcription factor decoys (TFDs) and LY294002 on epithelial physiology during 48 h treatment with IL-4

	Control	IL-4	CMX	TFDs	LY294002	IL-4 + CMX	IL-4 + TFDs	IL-4 + LY294002
Resistance Ω/cm^2	1,893 $\pm$ 39 (20)	720 $\pm$ 58 ^a (25)	2,366 $\pm$ 90 ^b (6)	1,800 $\pm$ 78 (6)	2,200 $\pm$ 79 ^b (6)	2,200 $\pm$ 70 ^b (10)	800 $\pm$ 40 ^a (7)	1,904 $\pm$ 122 (12)
³ H-fLMP % accumulation	0.6 $\pm$ 0.1 (10)	1.4 $\pm$ 0.3 ^a (13)	0.3 $\pm$ 0.01 (6)	n.t.	0.6 $\pm$ 0.2 (6)	0.4 $\pm$ 0.01 (8)	n.t.	0.7 $\pm$ 0.3 (13)
HRP flux % of recovery	0.0016 $\pm$ 0.0003 (20)	0.020 $\pm$ 0.001 ^a (20)	0.0010 $\pm$ 0.0001 (6)	0.0012 $\pm$ 0.0001 (6)	0.0012 $\pm$ 0.0001 (6)	0.0010 $\pm$ 0.0004 (10)	0.020 $\pm$ 0.003 ^a (8)	0.002 $\pm$ 0.0002 (12)

T84 monolayers were cultured for 48 h in presence of IL-4 plus CMX (1 mg/ml) or IL-4 plus LY294002 (20 μM) in the basal well. TFDs were added to the basal well in Opti-MEM I-reduced serum media for 24 h before 48 h treatment with IL-4. Values are expressed as means $\pm$ SEM. ^a $p < 0.001$ vs. all the other groups; ^b $p < 0.05$ vs. control.

Table 3. Effect of cyclohexamide (CMX) and LY294002 on epithelial physiology during 1 h treatment with IL-4

	Control	IL-4	CMX	LY294002	IL-4 + CMX	IL-4 + LY294002
Resistance, Ω/cm^2	1,893 $\pm$ 39 (20)	835 $\pm$ 44 ^a (18)	2,249 $\pm$ 70 ^b (6)	2,400 $\pm$ 110 ^b (6)	2,345 $\pm$ 70 ^b (6)	2,004 $\pm$ 92 (8)
³ H-fLMP, % accumulation	0.61 $\pm$ 0.1 (15)	1.2 $\pm$ 0.3 ^a (12)	0.4 $\pm$ 0.01 (6)	0.5 $\pm$ 0.01 (6)	0.39 $\pm$ 0.01 (8)	0.4 $\pm$ 0.01 (8)
HRP flux, % of recovery	0.0016 $\pm$ 0.0003 (20)	0.010 $\pm$ 0.001 ^a (20)	0.002 $\pm$ 0.0001 (6)	0.002 $\pm$ 0.0001 (6)	0.0010 $\pm$ 0.0004 (6)	0.002 $\pm$ 0.0002 (8)

T84 monolayers were cultured for 1 h in presence of IL-4 plus CMX (1 mg/ml) or IL-4 plus LY294002 (20 μM) in the basal well. Values are expressed as means $\pm$ SEM. ^a $p < 0.001$ vs. all the other groups; ^b $p < 0.05$ vs. control.

function, acting both on the paracellular and transcellular pathways, as demonstrated by decreased TER and increased flux of ³H-fMLP and HRP. IL-10 and TGF- β were able to partially inhibit the deleterious effect of IL-4 on the epithelium, particularly with respect to macromolecular transport.

IL-4 is a Th2-type cytokine produced by T cells, mast cells and eosinophils. Among its functions, IL-4 is a growth factor for B cells, enhances the expression of MHC class II molecules and CD23, is required for IgE production and for the generation of a Th2 cytokine profile [27]. As for the intestine, several studies have identified that high tissue levels of IL-4 are associated with onset or relapse of inflammation. In human studies, increased levels of IL-4 have been shown in the early phase of relapse in the intestine of Crohn's disease patients [28] as well as in the active phase of ulcerative colitis [12]. In addition to immunological roles, IL-4 has been shown to act directly on the intestinal epithelium, decreasing epithelial resistance [14] and increasing macromolecular transport [15]. We confirmed these findings and also demonstrated that a short-term exposure (i.e. 1 h) to IL-4 is sufficient to

induce changes in the epithelium detectable after 48 h. In general, a cytokine binds a cell surface receptor to initiate signals that are transmitted to the nucleus and lead to either a rapid primary response or a delayed secondary response requiring new protein synthesis [29]. In our system, a consistent response to IL-4 was evident only after 48 h. This finding suggested the involvement of newly synthesized molecules. Indeed, cycloheximide, an inhibitor of protein synthesis, completely abolished the effect of IL-4 on T84 monolayers.

Our study also examined the effects of two anti-inflammatory cytokines, IL-10 and TGF- β , on IL-4-induced changes in barrier function. Numerous reports indicate that these cytokines play an important role in antigen processing. In particular, it has been shown that the induction of oral tolerance requires the presence of IL-10 and TGF- β together with IL-4 [30, 31]. This observation suggests that the local concentration of these soluble mediators, besides affecting immune cells, may also have an effect on intestinal barrier function and, specifically, on macromolecular transport. It has previously been shown that IL-10 and TGF- β can reduce the effect of IFN- γ and other pro-

inflammatory cytokines on epithelial pathophysiology [20, 22]. In our experiments, both IL-10 and TGF- β were able to inhibit the action of IL-4, although the degree of inhibition was different for TER and macromolecular transport. The effect of cytokine co-treatment on TER seems to be related to the time of exposure to IL-4. When monolayers were exposed to IL-10 in conjunction with IL-4, there was no improvement in TER; whereas IL-10 partially maintained resistance values if IL-4 was removed after 1 h. TGF- β , on the other hand, was able to significantly improve the fall of TER induced by IL-4 regardless of the duration of treatment. The effects of the cytokines on the flux of fMLP, a bacterial tripeptide (MW 476 D) whose passage is mainly paracellular, were mostly similar to the results for TER. Both cytokines were able to normalize HRP transport in both treatment protocols.

In previous studies, IL-10 and TGF- β were shown to partially reverse the effect of IFN- γ or TNF- α on epithelial permeability in a co-culture model of inflammation [21, 22], although there was no improvement of cytokine-induced ion secretion. In our system, IL-10 was less efficient in reversing changes in permeability compared to TGF- β . IL-10 alone did not have a direct effect on barrier function, whereas TGF- β -treated monolayers had higher resistance compared to untreated controls. Thus, it is possible that in the epithelium IL-10 does not interfere directly with IL-4-induced events, despite that in T84 cells IL-10 ameliorates other cytokine-induced dysfunction [22]. Another possibility is that TGF- β may counteract the IL-4-induced effect because of its own stabilizing properties on tight junctions and cell membranes.

Here, we showed that the effect of IL-4 on macromolecular transport and paracellular permeability is PI3K-dependent and requires *de novo* synthesis of protein mediators. Signalling through the IL-4 receptor activates both the STAT-6 and the PI3K pathways [32]. STAT-6 activation regulates genes for CD23 and MHC class II expression and for IgE production [33], while the PI3K pathway is involved in vesicle trafficking [34] and, specifically in intestinal epithelial cells, in the regulation of TER [35]. Previously, we demonstrated that the IL-4-mediated events on intestinal epithelium could be abolished by the tyrosine kinase inhibitor genistein [15], which, by blocking the phosphorylation of IL-4 receptor, may prevent the activation of the STAT-6 signalling cascade [36]. In the present study, we used a pharmacological approach to further identify the pathways involved in the regulation of macromolecular transport. IL-4-induced changes in transcellular transport as well as in paracellular permeability were abolished by LY294002, a specific inhibitor of

PI3K, but not by the inhibition of STAT-6 by TFDs, supporting the central role of the PI3K in the regulation of intestinal epithelial barrier function. The function of the PI3K pathway in the modulation of epithelial tight junctions has been shown by Ceponis et al. [35], who demonstrated the efficacy of TFDs to reduce STAT-6 activation and also showed that the event was not affected by the inhibition of the PI3K pathway. Inhibition of both pathways did not have a major effect on monolayer physiology, suggesting that neither are required for the basal control of epithelial permeability. Finally, although we demonstrated that IL-10 and TGF- β were able to ameliorate the epithelial barrier dysfunction induced by IL-4, it seems probable that they do not interfere with the PI3K pathway, as the inhibition of this pathway completely abolished the effect of IL-4.

In our system, IL-4 increased the flux of both HRP, an antigen-sized protein, and fMLP, a small tripeptide, which crosses the epithelium mainly through the paracellular route. Examination of monolayers treated with IL-4 by electron microscopy showed the presence of HRP in the spaces between the enterocytes and in endosomes within the cells. The finding that both IL-10 and TGF- β were able to inhibit the transport of HRP even in presence of decreased TER indicates that these two pathways are regulated independently. Increased macromolecular transport as an initiating event in the pathogenesis of intestinal diseases has been recently indicated. Abnormal transcellular passage of macromolecules has been shown in non-inflamed intestine of Crohn's disease patients at the time of surgery [5]. More than 70% of such patients develop recurrence of disease, indicated by endoscopy, 6 months after surgery [37], so it is likely that an increased antigen load may lead to the onset of intestinal inflammation. In animal models of hypersensitivity, increased macromolecular transport has been shown to precede inflammatory events [6] and increased paracellular permeability [38]. Based on these data, it is possible that correcting enhanced macromolecular transport may represent a valid target for therapeutic intervention in preventing or treating intestinal inflammation.

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