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Intestinal barrier function: regulation of epithelial permeability and mucin expression

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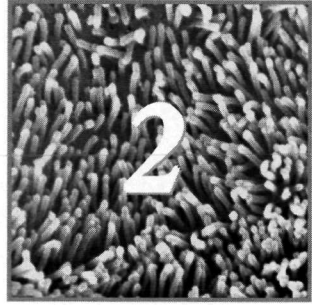
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***A coculture model mimicking
the intestinal mucosa reveals
a regulatory role for myofibroblasts
in immune mediated barrier disruption***

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Abstract

The pathogenesis of Crohn's disease involves a mucosal inflammatory response affecting the barrier function of the gut. Myofibroblasts directly underlining the intestinal epithelium may have a regulatory role in immune-mediated barrier disruption. A coculture system of T84 epithelial and CCD-18Co myofibroblasts was established in order to mimic the *in situ* spatial interactions between these cell types and to evaluate their role in barrier integrity. Lamina propria mononuclear cells (LPMC) were introduced in co- and monocultures. Effects of immune cells on barrier integrity was determined by measuring resistance and permeability for macromolecules. Introduction of LPMC in both culture systems caused a time- dependent decrease in barrier integrity. This was found to be less pronounced in cocultures indicating a regulatory role for mesenchymal cells. The effects were also found to depend on the route of LPMC stimulation. Additional analyses suggested that the regulatory role of myofibroblasts in barrier integrity involves production of growth factors.

Introduction

A prominent hypothesis for the pathogenesis of Crohn's disease describes a dysregulated immune response against luminal microbial factors in a genetically susceptible host¹⁻³. Increased exposure of epithelial and mucosal immune cells to dietary and microbial antigens may initiate and/or perpetuate an inflammatory response affecting barrier integrity¹. This chronic inflammation may cause tissue destruction and loss of intestinal absorptive functions. In this respect increased intestinal permeability may be associated with development of Crohn's disease^{4,7}. Interestingly, increased permeability was found to be predictive for relapse in Crohn's disease patients in remission⁵. Various investigators have shown that key inflammatory cytokines such as IFN- γ and TNF- α , which are up-regulated in Crohn's disease, increase permeability *in vitro* by affecting tight junctions⁸⁻¹⁰. These *in vitro* models, designed to study intestinal permeability in response to inflammatory mediators, make use of monolayers of intestinal epithelial cell lines. However, there is increasing evidence that in the intestinal mucosa there is a close spatial and functional relationship between the epithelium and other cell types. In particular, subepithelial myofibroblasts are known to control epithelial cell function¹¹⁻¹⁴. This cross-talk between intestinal epithelial cells and subepithelial myofibroblasts not only is important for secretory activity but also affects epithelial regeneration, motility, and barrier function¹⁵⁻¹⁷. In a coculture model in which we integrated intestinal epithelial, mesenchymal, and immune cells, the contribution of mesenchymal cells in regulation of the barrier integrity was evaluated. Here we describe functional properties of this CCD-18Co/T84 coculture in comparison with the conventional T84 monolayer system in the presence of differentially stimulated lamina propria mononuclear cells (LPMC). Hence we found a regulatory role of mesenchymal cells in intestinal barrier integrity.

Materials and methods

Cell culture

Monolayers (MC) of T84 (ATCC, Maryland, USA; passages 57-64) intestinal epithelial cells and cocultures (CC) of CCD-18Co (ATCC; passages 10-14) intestinal myofibroblasts with T84 cells were cultured on 12-mm transwell inserts (0.4 μ m, Corning, Costar B.V., The Netherlands). Both cell lines were cultured in DMEM/F12 glutamax I (Life Technologies, The Netherlands) with penicillin (100 IU/ml), streptomycin (100 μ g/ml) (Life Technologies) and 5% heat-inactivated fetal bovine serum (Greiner, The Netherlands). CCD-18Co cells were seeded on transwell inserts. After 7 days the CCD-18Co cells had grown confluent and T84 cells were seeded on top of this layer (CC) or as a monolayer (MC) in a separate plate. Medium was refreshed every two days. T84 cells grew confluent in 7 days and differentiated

in culture, developing functional tight junctions as measured by resistance. In general both CC and MC were used two weeks after confluence of the epithelial layer.

Histology

CCD-18Co/T84 cocultures were grown on filters in transwell inserts until two weeks after confluence and fixed in 2.5% glutaraldehyde/PBS solution, pH 7. Filters were cut out, washed in PBS, and embedded in Tissue-Tek (Sakura, Adamas, The Netherlands). Cryostat sections (7 μm) were stained with hematoxylin-eosin (Sigma-Aldrich Chemie, The Netherlands), embedded in Clearmount (Zymed, Sanbio BV, The Netherlands) and studied by light microscopy.

Isolation of lamina propria mononuclear cells

LPMC were isolated according to a method previously described¹⁸. In brief, surgical resection specimens from nonpathological areas in the ileum (n=7) or colon (n=2) from patients with intestinal carcinomas (n=5) or inflammatory disease (n=4) were collected. Mucosal tissue was dissected from underlying muscular tissue and submucosa was incubated with HBBS/1 mM DTT (ICN Biochemicals BV, The Netherlands) for 20 min at room temperature in a Wheaton stirring bottle. The tissue was rinsed and put in HBBS/0.75 mM EDTA (Life Technologies) for 4-5 sequential 45 minute incubations at 37°C. Subsequently the tissue was cut into smaller fragments and incubated overnight with DNase (100 U/ml) and collagenase (20 U/ml) (Interchema, The Netherlands) in RPMI 1640, 10% fetal calf serum, 1 mM pyruvate, 2 mM glutamine and 2.5 $\mu\text{g}/\text{ml}$ fungizone/penicillin (100 IU/ml)/streptomycin (100 $\mu\text{g}/\text{ml}$) (Life Technologies) while stirring at 37°C. Further purification of mononuclear cells was achieved using Ficoll (1.079 g/ml, Amersham Pharmacia, The Netherlands) gradient centrifugation. Isolated and purified LPMC were stored in liquid nitrogen until use in the CC and MC model.

Incubation with LPMC, CCD18-Co/LPMC supernatants or recombinant cytokines

LPMC were thawed and 1×10^6 /ml vital cells were added to the serosal compartment of the CC and MC. Every 24 h total luminal and 0.5 ml serosal supernatant was refreshed. The serosal supernatants were stored at -20°C. LPMC were stimulated with PMA (50 ng/ml) or $\alpha\text{CD}2/\alpha\text{CD}28$ (1:1000) (CLB, The Netherlands). CC and MC were exposed to LPMC for 72 h. In additional experiments CCD18-Co cells without epithelial cells were incubated with LPMC (CCD18-Co/LPMC) and 0.5 ml serosal supernatant was collected every 24 h. These supernatants were incubated with T84 monolayers to assess whether myofibroblast- derived soluble factors contribute to the observed effects on barrier integrity. In separate experiments recombinant cytokines IFN- γ (100 U/ml, Hycult Biotechnology BV, The Netherlands) and/or TNF- α (10 ng/ml, PreproTech, The Netherlands) were added to the serosal compartment.

Measurement of IFN- γ , TNF- α , IL-10, KGF and TGF- β_1

LPMC supernatants collected from the serosal compartment of CC and MC were analyzed for cytokine and growth factor production using specific ELISA's for IFN- γ , TNF- α , IL-10, KGF and TGF- β_1 (CLB or R&D, ITK Diagnostics, The Netherlands). Substantial TGF- β_1 background levels present in culture media were subtracted.

Measurement of resistance

Transepithelial resistance (TER; ohm.cm²) across CC and MC was measured using an epithelial voltohmmeter (EVOM; World Precision Instruments).

Macromolecular permeability

To relate barrier integrity to functional permeability for macromolecules, we determined horseradish peroxidase (HRP) fluxes. Permeability for HRP was determined in both CC and MC, which were incubated with nonstimulated and stimulated LPMC or MC incubated with CCD18-Co/ LPMC culture supernatants. To the luminal compartment, 5 μ l 1 mM HRP (type VI-A, 40 kDa, Sigma) was added. Samples were taken from the serosal compartment at 30 min. HRP concentrations were determined in an enzymatic assay using tetramethylbenzidin (TMB; Merck, The Netherlands) substrate. Detection was done by spectrophotometer (Biorad, The Netherlands) at 450 nm absorption and HRP fluxes were calculated.

Viability measurements

To exclude that impaired cell function or cell death contributes to the change of barrier integrity, we also measured viability of CC and MC using a WST-1 assay. This assay measures mitochondrial enzyme activity reflecting cell viability (Boehringer Mannheim, The Netherlands). After 72 h of incubation, 30 μ l of WST-1 solution was added to 300 μ l refreshed medium on the mucosal part of the cell layer. Cell layers incubated with DMSO were used as positive control for loss of cell viability. After 45 min, a 100 μ l sample of the supernatant was measured at A450- A655.

Data analysis

All data are presented as mean \pm SEM. Data were analyzed with univariate ANOVA using SPSS version 10 software.

Results

Histological features of coculture CCD-18Co/T84

In the coculture (CC) model T84 cells were grown on a transwell insert filter on top of a confluent layer of CCD-18Co cells (figure 1). Two days after seeding, large clusters of

outgrowing epithelial cells were found on top of the confluent CCD-18Co layer. Histological examination of a coculture at two weeks after confluency clearly showed a polarized monolayer of columnar epithelial cells supported by a thin confluent layer of elongated mesenchymal cells, mimicking the spatial distribution of these cell types as found in the intestinal mucosa.

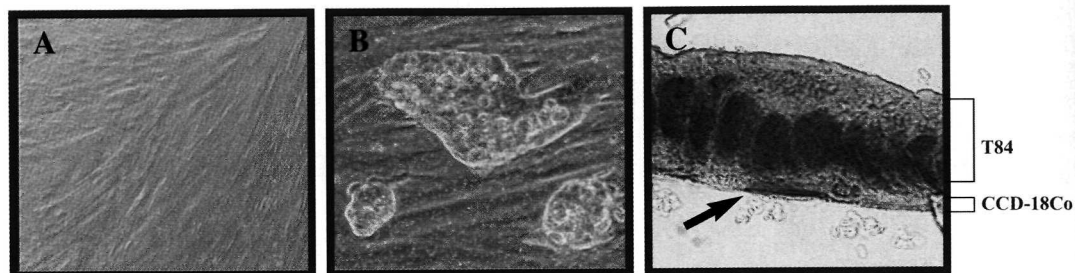


Figure 1 CCD-18Co cells were grown to confluency on a porous filter of a transwell insert (A). Clusters of outgrowing T84 cells can be seen two days after seeding on top of a confluent CCD-18Co layer (B). Cryostat cross sections (1000X, H&E) of CCD-18Co/T84 cocultures two weeks after confluence show a tightly organized monolayer of polarized columnar epithelium on top of a thin layer of elongated myofibroblasts (arrow; C).

Barrier building properties of CC and MC; TER and flux

Resistance (TER) was measured to evaluate the formation of an adequate barrier in both CC and MC (monoculture T84 cells). Solid barrier resistance of both CC and MC could be measured up to 3 weeks after confluence (figure 2). HRP fluxes in control CC and MC were consistently low, indicating solid barrier function (<2 pmol/cm²/h). In additional experiments we confirmed that the CCD18-Co layer alone does not contribute to the barrier integrity as revealed by low TER (127.5 ± 2.2 ; filters alone $114 \Omega \cdot \text{cm}^2$) and high HRP fluxes (>100 pmol/cm²/h).

Effects of recombinant cytokines on barrier integrity

In order to examine the effects of recombinant cytokines IFN- γ and TNF- α in barrier integrity, we incubated CC and MC with 100U (5 ng)/ml IFN- γ and/or 10 ng/ml TNF- α in the serosal compartment for 72 h (figure 3). An IFN- γ mediated decrease in TER was found in both CC and MC ($p < 0.005$). After 72 h of IFN- γ incubation the TER levels in CC and MC were similar, although the reduction in barrier resistance was more pronounced in MC as compared to CC. Incubation with TNF- α resulted in only minor reduction in MC ($p < 0.05$). Moreover no synergistic effects were found using IFN- γ and TNF- α .

Effects of stimulated LPMC on barrier integrity

LPMC were introduced to the serosal side of transwell culture systems. Both CC and MC were incubated with 1×10^6 LPMC/ml with or without PMA or α CD2/ α CD28 stimulation. Control experiments revealed that stimuli alone, in the absence of LPMC, did not affect TER. Incubation with nonstimulated LPMC resulted in both CC and MC in a reduction of TER levels as compared to controls at all time points (figure 4; $p < 0.01$). Again the reduction in resistance was found to be more pronounced in MC as compared to CC. Incubation with stimulated LPMC resulted in a further decrease in TER over time as compared to the nonstimulated LPMC, whereas differences were found between the routes of stimulation ($p < 0.05$).

In MC incubations with PMA- or α CD2/ α CD28-stimulated LPMC, the resistance was significantly decreased starting as early as 24 h as compared to nonstimulated LPMC ($p < 0.05$). In CC, however, this was only the case for incubation with PMA-stimulated LPMC where the resistance of CC incubated with α CD2/ α CD28-stimulated LPMC was not changed until 72 h. Incubation with α CD2/ α CD28-stimulated LPMC resulted in a relatively smaller reduction in TER and significantly higher TER values for CC compared with similarly treated MC ($t = 72$ h, 470 ± 32 vs $320 \pm 23 \Omega \cdot \text{cm}^2$; $p < 0.001$). Furthermore there was no difference in effects on barrier disruption between LPMC isolated from nonpathological areas from patients with intestinal carcinomas or inflammatory disease (data not shown).

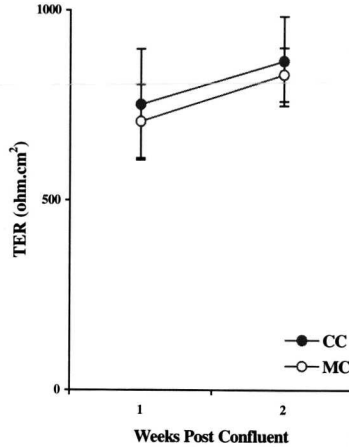


Figure 2 CC and MC follow the same pattern in barrier formation as measured by means of resistance (n=12).

In MC incubations with PMA- or α CD2/ α CD28-stimulated LPMC, the resistance was significantly decreased starting as early as 24 h as compared to nonstimulated LPMC ($p < 0.05$). In CC, however, this was only the case for incubation with PMA-stimulated LPMC where the resistance of CC incubated with α CD2/ α CD28-stimulated LPMC was not changed until 72 h. Incubation with α CD2/ α CD28-stimulated LPMC resulted in a relatively smaller reduction in TER and significantly higher TER values for CC compared with similarly treated MC ($t = 72$ h, 470 ± 32 vs $320 \pm 23 \Omega \cdot \text{cm}^2$; $p < 0.001$). Furthermore there was no difference in effects on barrier disruption between LPMC isolated from nonpathological areas from patients with intestinal carcinomas or inflammatory disease (data not shown).

Permeability (HRP flux) in cultures with LPMC

Functional permeability was assessed by measuring HRP fluxes after 72 h of LPMC incubation (figure 5). HRP fluxes of control or PMA- or α CD2/ α CD28-treated cultures without LPMC were consistently low ($< 2 \text{ pmol/cm}^2/\text{h}$). Fluxes of CC and MC incubated with nonstimulated LPMC were not increased. Incubation with PMA-stimulated LPMC resulted in increased fluxes in both CC and MC ($p < 0.05$) as compared to nonstimulated LPMC. On the other hand incubation, with α CD2/ α CD28-stimulated LPMC resulted in dramatically increased fluxes only in the MC ($p < 0.005$); CC fluxes were not increased but rather significantly lower than similarly treated MC (3.5 ± 1.4 vs $24.5 \pm 2.3 \text{ pmol/cm}^2/\text{h}$; $p < 0.005$). This correlates with the resistance that was significantly higher in CC as compared to MC.

Moreover, fluxes in the CC with α CD2/ α CD28-stimulated LPMC were significantly lower than in the CC where PMA-stimulated LPMC were introduced ($p < 0.05$).

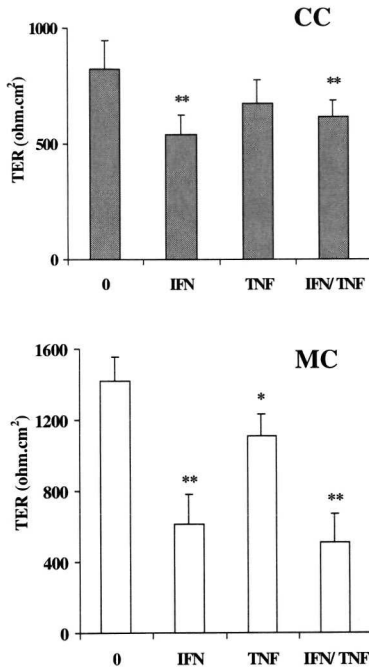


Figure 3 Effects of recombinant IFN- γ (100 U/ml) and TNF- α (10 ng/ml) on CC and MC after 72 h of incubation ($n=6$). IFN- γ reduced barrier resistance in both CC and MC (** $p < 0.005$). TNF- α was only slightly effective in MC (* $p < 0.05$). No synergistic effects of IFN- γ and TNF- α were observed.

stimulated LPMC (23.0 ± 8.9 vs 6.1 ± 3.1 pg/ml; $p < 0.05$). This finding correlated with the low permeability in these experiments.

Effects of soluble factors released by CCD18-Co on MC barrier integrity

In order to test whether protection in the CC was mediated by soluble factors, we incubated MC with serosal supernatants collected from CCD18-Co cells incubated with LPMC. These

Cytokine production under different culture conditions

Inflammatory cytokines IFN- γ , TNF- α , and IL-10, which might be involved in LPMC-mediated barrier disruption were measured in the 72 h culture supernatants (figure 6). Despite obvious differences between PMA or α CD2/ α CD28 stimulation pathways, there were no significant differences in cytokine levels between CC and MC. Activation of LPMC with PMA or α CD2/ α CD28 resulted in significantly higher levels of IFN- γ and TNF- α compared to nonstimulated LPMC ($p < 0.05$). IL-10 levels were higher in both CC and MC incubated with PMA-stimulated LPMC as compared to the nonstimulated LPMC ($p < 0.01$).

Growth factor production in CC or MC

As levels of inflammatory cytokines could not explain the observed differences between CC and MC incubated with α CD2/ α CD28-stimulated LPMC, we also measured growth factors (figure 7). TGF- β_1 levels were significantly increased in CC as compared to MC ($p < 0.05$, § $p = 0.068$; $n=6$). KGF could only be detected in CC since epithelial cells do not produce KGF. In CC incubated with α CD2/ α CD28-stimulated LPMC ($n=4$) KGF was found to be significantly increased compared to CC incubated with PMA-

supernatants caused a decrease in TER and increased fluxes in MC (data not shown). Incubation with supernatants of CCD18-Co/LPMC stimulated with α CD2/ α CD28 did not result in higher TER levels as compared to CCD18-Co/LPMC stimulated with PMA (356 ± 20 vs $329 \pm 13 \Omega \cdot \text{cm}^2$; $n=4$). CCD18-Co/LPMC stimulated with PMA significantly increased HRP fluxes as compared to nonstimulated LPMC supernatants (6.4 ± 2.2 vs 2.8 ± 0.8 pmol/cm²/h; $p<0.05$) while supernatants of CCD18-Co/LPMC stimulated with α CD2/ α CD28 did not (5.8 ± 2.2 pmol/cm²/h) resembling effects found in CC stimulated with life LPMC. This suggests barrier protection as found in CC at least in part to be mediated by soluble factors.

Cell viability; WST and pH measurement

In order to exclude that changes in viability of the cells in the different culture systems are contributing to the effects on barrier integrity, we measured mitochondrial enzyme activity (WST). In none of the culture systems were WST levels different from control incubations (data not shown). Furthermore the pH of the cultures remained within the physiological range (7.2-7.5).

Discussion

Immune mediators released during inflammation may impair the barrier integrity of the intestinal mucosa. Levels of proinflammatory cytokines IFN- γ and TNF- α are known to be elevated in the mucosa of Crohn's patients possibly, resulting in leaky tight junctions and increased permeability of the gut barrier. This may result in increased antigen exposure to the underlying immune cells

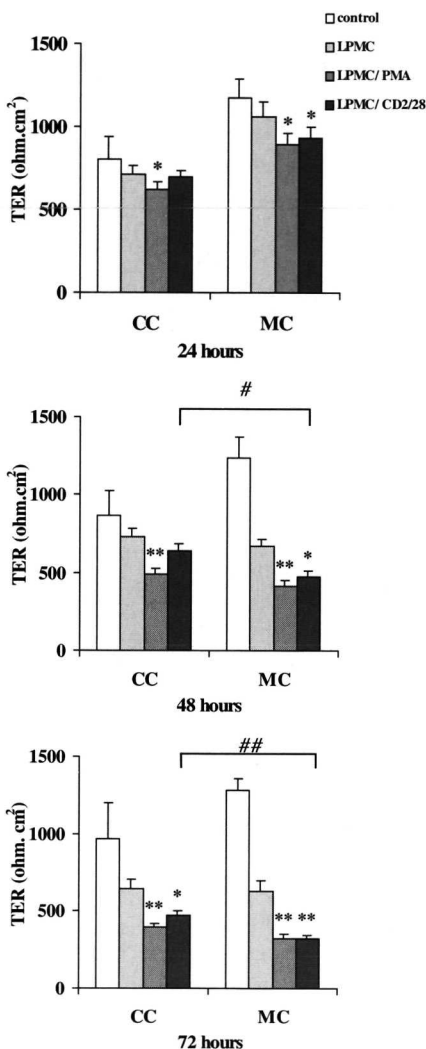


Figure 4 Decrease in TER was found in both CC and MC after serosal incubation with stimulated or nonstimulated LPMC at all time points ($p<0.01$; $n=5$). In both CC and MC the incubation with stimulated LPMC resulted in significantly lower TER levels as compared to incubation with nonstimulated LPMC at all time points, except for CC incubated with α CD2/ α CD28-stimulated LPMC (* $p<0.05$; ** $p<0.005$; $n=9$). Resistance of CC incubated with α CD2/ α CD28-stimulated LPMC remained significantly higher than MC at 48 h and 72 h of incubation (# $p<0.05$; ## $p<0.005$).

in the mucosa. *In vitro* models of intestinal permeability focus on monolayers of epithelial cell lines. However, other cell types including subepithelial myofibroblasts may have a key regulatory role in intestinal epithelial cell function *in vivo*. Subepithelial myofibroblasts may play an important role in the processes of tissue regeneration and mucosal immunoregulation. Furthermore myofibroblasts may affect epithelial function by inducing differentiation and regulating chloride secretion^{12,19,20}. We studied barrier integrity in an *in vitro* model mimicking spatial and functional integration of human intestinal subepithelial myofibroblasts and epithelial cells.

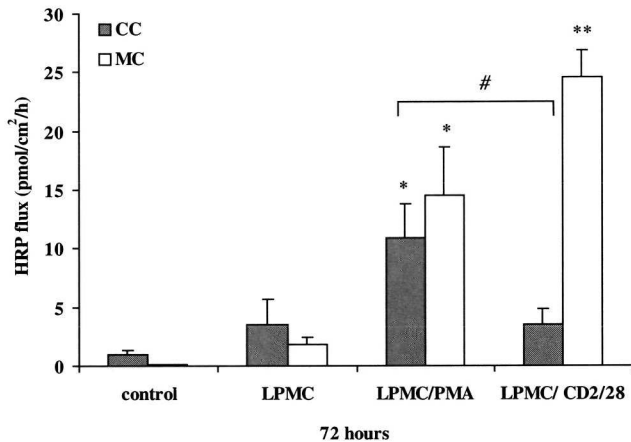


Figure 5 Fluxes of CC (n=6) and MC (n=4) incubated with nonstimulated LPMC did not differ from control fluxes. PMA activation of LPMC resulted in increased flux in both CC and MC as compared to control cultures incubated with nonstimulated LPMC (* $p < 0.05$). Incubation with α CD2/ α CD28-stimulated LPMC resulted in dramatically higher HRP fluxes in MC (** $p < 0.005$), whereas in CC the fluxes were not found to differ from controls and were even lower than fluxes of CC incubated with PMA-stimulated LPMC (# $p < 0.05$).

Coculture models were described earlier to study physiological mechanisms rather than immune-mediated barrier disruption. Furthermore in most cases the cells were not grown in direct physical contact¹⁹⁻²¹. In our study both CC and MC developed good barrier integrity. Addition of IFN- γ in both CC and MC was found to decrease resistance. However, in CC the decrease in resistance was less pronounced than MC, indicating a regulatory role for mesenchymal cells.

Using this model, we further explored the role of myofibroblasts in processes of immune-mediated barrier disruption by introducing differentially stimulated immune cells. LPMC isolated from resection specimens were introduced to the serosal compartment and stimulated through different routes of activation. Of these stimuli α CD2/ α CD28 specifically

activates mucosal T-cell receptor, mimicking the *in vivo* antigenic stimulation route while PMA is a mitogen nonselectively activating LPMC. It has been previously shown that cytokines like IFN- γ and TNF- α or nonstimulated immune cells can decrease barrier resistance in T84 monolayers^{8-10,22-26}. In our hands stimulated LPMC more dramatically diminished barrier integrity than nonstimulated LPMC. Again the effect was less pronounced in CC. The effect observed with nonstimulated LPMC can be explained by the fact that LPMC from gut mucosa are activated under physiological conditions²⁷⁻³¹. During the introduction of α CD2/ α CD28 stimulated LPMC in the models, barrier integrity was better preserved in CC as compared to MC revealing pronounced differences between the culture systems, and pointing to a regulatory role for mesenchymal cells. Activation of LPMC through α CD2/ α CD28 stimulation might be more representative for antigen-driven inflammation *in vivo* because it selectively targets the T-cell function in stead of generally activating immune cells.

Since IL-10, TNF- α , and IFN- γ concentrations did not differ in CC and MC, the observed differences in resistance and flux could hereby not be explained. The barrier protective effects as found in the coculture pointed to a role for other factors, such as soluble factors, matrix deposition, or direct cell-cell contact^{16,32-34}. It is known that growth factors such as TGF- β_1 and KGF can promote barrier integrity by inducing epithelial restitution, proliferation, differentiation, or stabilization of the epithelial cytoskeleton^{26,34-37}. Interestingly, intraperitoneal administration of KGF promotes healing in a rat TNBS model of colitis^{36,38}. We found TGF- β_1 production to be increased in cocultures as compared to monolayers reflecting the contribution of myofibroblasts. Elevated KGF

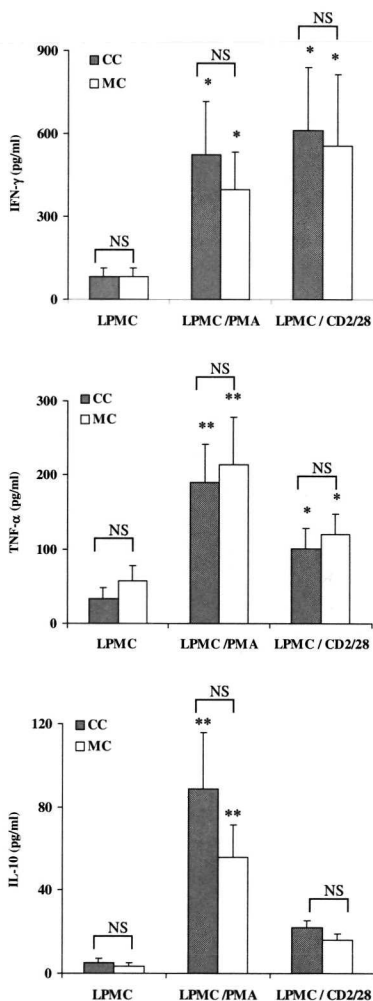


Figure 6 No differences in IFN- γ , TNF- α , and IL-10 levels were observed between CC and MC in the different culture systems. IFN- γ and TNF- α levels were significantly higher in cultures incubated with stimulated LPMC for 72 h as compared to nonstimulated LPMC. IL-10 was significantly increased in both CC and MC with PMA-stimulated LPMC (* $p < 0.05$, ** $p < 0.005$).

levels were only found in CC incubated with α CD2/ α CD28-stimulated LPMC, corresponding with low HRP fluxes in this system. KGF can be an important mediator by which myofibroblasts either directly or indirectly regulate potential barrier disrupting effects due to inflammatory reactions. However CCD18-Co supernatants only partly reproduce the barrier protection as found in CC, suggesting that growth factor production is not solely responsible for barrier protection. Hence, direct cell-cell contact or matrix deposition can not be excluded. Further studies using this model will be performed to address these aspects. Our present study supports the role for human intestinal mesenchymal cells in the regulation of the mucosal barrier integrity, which is of particular relevance considering the strategic localization of these cells underneath the epithelium.

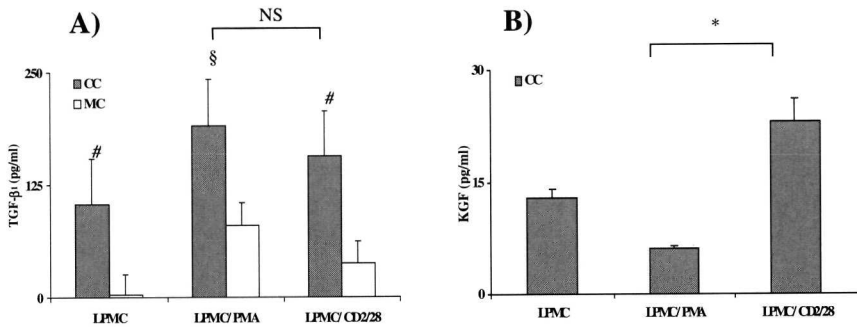


Figure 7 TGF- β_1 levels were higher in CC as compared to MC, reflecting the contribution of mesenchymal cells (A; # p <0.05, § p =0.068; n =6). Significantly higher KGF levels were detected in CC incubated with α CD2/ α CD28-stimulated LPMC as compared to PMA-stimulated LPMC (B; * p <0.05; n =4).

Acknowledgements

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