

Regulation of Airway Tight Junctions by Proinflammatory Cytokines

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Epithelial tight junctions (TJs) provide an important route for passive electrolyte transport across airway epithelium and provide a barrier to the migration of toxic materials from the lumen to the interstitium. The possibility that TJ function may be perturbed by airway inflammation originated from studies reporting (1) increased levels of the proinflammatory cytokines interleukin-8 (IL-8), tumor necrosis factor α (TNF- α), interferon γ (IFN- γ), and IL-1 β in airway epithelia and secretions from cystic fibrosis (CF) patients and (2) abnormal TJ strands of CF airways as revealed by freeze-fracture electron microscopy. We measured the effects of cytokine exposure of CF and non-CF well-differentiated primary human airway epithelial cells on TJ properties, including transepithelial resistance, paracellular permeability to hydrophilic solutes, and the TJ proteins occludin, claudin-1, claudin-4, junctional adhesion molecule, and ZO-1. We found that whereas IL-1 β treatment led to alterations in TJ ion selectivity, combined treatment of TNF- α and IFN- γ induced profound effects on TJ barrier function, which could be blocked by inhibitors of protein kinase C. CF bronchi in vivo exhibited the same pattern of expression of TJ-associated proteins as cultures exposed in vitro to prolonged exposure to TNF- α and IFN- γ . These data indicate that the TJ of airway epithelia exposed to chronic inflammation may exhibit parallel changes in the barrier function to both solutes and ions.

INTRODUCTION

Tight junctions (TJs) are characteristically located at the apical-lateral borders of adjacent epithelial cells and are responsible for the selective regulation of the passage of ions and neutral molecules through the paracellular space. In addition to their key role in maintaining barrier function of the epithelium, TJs have been implicated in the pathogenesis of several diseases. One group of disorders, inflammatory bowel disease, displays a highly deregulated barrier function that appears to be a fundamental event in disease pathogenesis (Schmitz *et al.*, 1999a, 2000). Whether the alteration in TJ barrier function is a primary phenomenon associated with the disease or an acquired response caused by the high degree of inflammation associated with the disease is unclear. If, indeed, the TJ response is acquired, this would suggest that other diseases known to be associated with a

high level of inflammation may display a characteristic change in TJ barrier function.

Cystic fibrosis (CF) serves as an excellent model for the study of the effects of inflammation on TJ barrier function. The airway disease of CF is characterized by an inflammatory response with a marked influx of neutrophils and chronic bacterial infection with *Pseudomonas aeruginosa*. Proinflammatory cytokines detected in CF airways serve in part to perpetuate this inflammatory response. High levels of the proinflammatory cytokines tumor necrosis factor α (TNF- α), interleukin (IL)-8, and IL-1 β as well as the soluble intercellular adhesion molecule (ICAM)-1 have been measured in the airways of patients with CF (Salva *et al.*, 1996; Bonfield *et al.*, 1999; Osika *et al.*, 1999). The airway epithelium of patients with CF also expresses an increased level of signal transducer and activator of transcription-1 (STAT-1), a component of the interferon γ (IFN- γ) signaling cascade, indicating that IFN- γ may also be increased in CF airways (Kelley and Elmer, 2000). In addition, increased levels of IFN- γ mRNA have also been detected in the airway epithelium of CF patients (Wojnarowski *et al.*, 1999). Freeze-fracture electron microscopy (EM) has shown previously that the TJ strands of inflamed CF airways appear to be altered

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compared with those from non-CF patients (Carson *et al.*, 1990).

The cytokines IL-1, IL-4, IL-10, IL-13, TNF- α , and IFN- γ have all been shown to regulate the TJ of both epithelia and endothelia (Youakim and Ahdieh, 1999; Ahdieh *et al.*, 2001; Oshima *et al.*, 2001). In addition, IL-1 β has been shown to alter TJ permeability through an effect on the claudin family of transmembrane proteins thought to be important in maintaining junctional integrity in astrocytes (Duffy *et al.*, 2000). However, these studies have not focused on the effects of chronic cytokine exposure on airway epithelial TJ function.

The TJ is a complex structure composed of several protein components, some of whose function(s) remain largely unclear. The claudin family of transmembrane proteins are thought to be key components of the TJ, but the growing number of members of this family impedes a complete understanding of their function. Several other components, such as occludin, junctional adhesion molecule (JAM), and more recently the coxsackievirus and adenovirus 2/5 receptor, have been localized to the TJ (Cohen *et al.*, 2001). Occludin, the first transmembrane TJ protein identified, has been localized to the TJ strand with claudins. JAM has been identified as a component of the TJ necessary to allow the transmigration of monocytes through the paracellular space, which makes it a likely target for inflammation-induced alterations in TJ function. Which of these components is significant in maintaining the integrity of the airway TJ during inflammation is unknown.

The regulation of TJ function is incompletely understood and may vary among different cell types. Several protein kinase C (PKC) isoforms have been associated with changes in paracellular permeability. Previous studies have shown a correlation between the level of membrane-associated PKC α and the extent of paracellular permeability, and PKC β has been shown to play a role in the regulation of endothelial TJs (Clarke *et al.*, 2000). In addition, the atypical PKC isoforms PKC ζ have been localized to the TJ of MDCK cells (Dodane and Kachar, 1996). Atypical PKC isotype-specific interacting protein may also localize to the TJ via interactions with PKC ι/λ and PKC ζ , forming a complex that is tethered to the junction by a direct interaction with JAM (Ebnet *et al.*, 2001). Although PKC isoforms appear to play a functional role in the TJ, factors regulating their expression remain unclear, in particular, how these isoforms are affected by biological stimuli, including inflammation.

The difference in the morphology of CF airways as determined by freeze-fracture EM suggests that the environment of the CF airway might lead to disruption of the barrier function of the TJ. To determine whether the chronic inflammation of CF airways leads to modulation of airway TJs, we exposed CF and non-CF well-differentiated (WD) primary human airway epithelial (HAE) cells to cytokines that are upregulated in CF, including IL-1 β , TNF- α , and IFN- γ . We then assessed the effects of this treatment on TJ barrier function and on components of the TJ. To correlate the effects of cytokines on primary HAE cells *in vitro* with the effects seen *in vivo*, we performed immunofluorescence localization of TJ components in freshly excised human CF and non-CF large airways.

MATERIALS AND METHODS

Chemicals and Antibodies

TNF- α and IFN- γ were purchased from Sigma Chemical Inc. (St. Louis, MO). Rabbit polyclonal antibodies to ZO-1, claudin-1, and occludin and mouse monoclonal antibodies (mAbs) to occludin, ZO-1, and ICAM-1 were purchased from Zymed Laboratories (San Francisco, CA). Rabbit polyclonal and goat polyclonal antibodies to ICAM-1 were purchased from Santa Cruz Biochemical (Santa Cruz, CA). The 3D8 mouse mAb to JAM was kindly provided by Dr. Kenji Ishii (Kyoto, Japan). Rabbit polyclonal antibodies to both PKC ι/λ and PKC ζ were purchased from Santa Cruz. The mouse anti-claudin-4 mAb was generously provided by James M. Anderson (Yale University, New Haven, CT). The FITC-conjugated dextrans were purchased from Sigma. The PKC inhibitor H7 and the tyrosine kinase inhibitor genistein were purchased from Sigma. Chelerythrine chloride was purchased from Alexis Corp. (San Diego, CA).

Cell Culture

Primary airway cells from human subjects were obtained in accordance with guidelines approved by the Committee on the Protection of the Rights of Human Subjects. Bronchial cells of normal (non-CF) and CF type were isolated from surgical specimens, plated at a density of 2×10^5 cells/12-mm Transwell-Col (0.4- μ m pore size) insert, and maintained in a 50:50 mixture of LHC Basal Medium (Biofluids, Rockville, MD) and DMEM-H medium supplemented with growth factors, retinoic acid, and BSA as previously described (Yankaskas *et al.*, 1985). After cultures reached confluence, medium was aspirated from the apical surface, and cells were maintained at an air-liquid interface for 4 wk. Cultures with 10% cilia as determined by microscopy and a transepithelial resistance (R_T) of 600 Ω -cm² measured with an ohmmeter (EVOM; World Precision Instruments, Sarasota, FL) were selected for experiments. Cultures were exposed to cytokines on the basolateral surface for 24, 48, or 72 h.

Transepithelial Cell Permeability

Permeation of FITC-dextrans of 10 and 2000 kDa across primary HAE cells was measured after 24, 48, or 72 h exposure to cytokines. Compounds were added to the apical surface (200 μ L of a 5-mg/mL solution in HEPES-buffered Ringer's solution containing 1.3 mM CaCl₂), and samples (10 μ L) were removed from the apical and basolateral compartments at 10, 20, 30, 40, and 60 min. The rate of permeation was determined by measuring the sample fluorescence at 496 nm in a 96-well fluorescent plate reader. The paracellular permeability to hydrophilic solutes (P_{app}) coefficients were calculated as previously described (Stutts *et al.*, 1981).

Electrophysiological Measurements of Dilution Potential

Polarized CF primary HAE cultures were treated with IL-1 β for 72 h and mounted in modified Ussing chambers interfaced with an electrometer, in which transepithelial potential difference (V_T) and R_T were measured continuously under open-circuit conditions. The temperature of all solutions was maintained at 37°C, and pH was regulated by bubbling with 95% O₂-5% CO₂. Basal V_T , R_T , and current were recorded across cultures in a buffer containing high NaCl (in mM: 120 NaCl, 10 HEPES at pH 7.4, 10 NaHCO₃, 1.2 CaCl₂, 5 KCl, and 1 MgSO₄) and 10⁻⁴ M amiloride. The change in V_T (ΔV_T) in response to luminal substitution with a buffer containing low NaCl (in mM: 60 NaCl, 120 mannitol, 10 HEPES at pH 7.4, 10 NaHCO₃, 1.2 CaCl₂, 5 KCl, and 1 MgSO₄) and 10⁻⁴ M amiloride was subsequently recorded (Van Itallie *et al.*, 2001). Blank filters were used to determine background and subtracted from subsequent measurements. Dilution potentials (ΔV_T) and P_{Cl^-}/P_{Na^+} were calculated as previously described (Gorodeski *et al.*, 1996). All mea-

measurements were performed in a minimum of six total cultures isolated from two patients.

Immunofluorescence Labeling and Confocal Microscopy

Cells were permeabilized with methanol at -20°C for 30 min. Antibodies to ZO-1, occludin, claudin-1, claudin-4, PKC ι/λ , and JAM diluted to 1:1000 were added to the luminal surface for 1 h. Cells were washed with PBS, and Texas Red-labeled secondary antibodies (Amersham), diluted 1:600 in 10% goat serum/PBS, were added to the luminal surface. For occludin and claudin-1 double labeling, a mouse mAb to occludin conjugated to FITC was incubated with rabbit polyclonal antibody to claudin-1 and incubated for 1 h at room temperature. For JAM and ZO-1 double labeling, 3D8 mouse anti-JAM and rabbit anti-ZO-1 were added to the culture. After washing, anti-rabbit Texas Red and antimouse FITC were incubated in 10% goat serum/PBS for 1 h at room temperature. Cells were postfixated with 4% paraformaldehyde. Transwell-Col inserts were excised and mounted on slides with 100 μL Vectashield (Vector Laboratories, Burlingame, CA) containing 4',6-diamidino-2-phenylindole (DAPI). Images were captured with a confocal laser-scanning microscope (Leica, Exton, PA).

Freshly excised human bronchi from non-CF and CF ($\Delta\text{F}/\Delta\text{F}$) subjects were embedded in OCT, and frozen sections (10 μm) were cut. Sections were incubated in 95% ethanol followed by a brief wash in PBS. After incubation in acetone, sections were washed in 0.1% Triton X-100 in PBS, and then primary and secondary antibodies added as described above.

Western Blotting

Lysates of primary HAE cells were prepared with 0.1% Triton X-100 extraction buffer containing phenylmethanesulfonyl fluoride (PMSF) and dithiothreitol (DTT). Equal amounts of protein (30 μg) were loaded onto Tris-glycine gels (Novex, San Diego, CA). After electrophoresis for 1 h at 200 V, protein was transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membrane at 33 V and blocked in 5% fat-free milk. Membranes were probed at antibody dilutions of 1:1000 in Tris-buffered saline-Tween-20 (TBS-T). Protein was visualized with a peroxidase-conjugated secondary antibody (1:20,000) by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) or Supersignal west femto maximum sensitivity substrate for JAM blotting (Pierce, Rockford, IL). For immunoprecipitation of PKC ι/λ and ζ and JAM, lysates were incubated with rabbit polyclonal antibodies to PKC ι/λ or ζ or mouse mAb to JAM for 2 h (PKC) or overnight (JAM) at 4°C , and Sepharose G beads were added for an additional 1 h. After centrifugation, beads were washed in cell lysis buffer and then heated at 95°C for 15 min. Loading buffer was added and Western blotting performed as described above. Blots were stripped with Restore Western blot stripping buffer according to the manufacturer's protocol (Pierce).

Human bronchi from non-CF and CF ($\Delta\text{F}/\Delta\text{F}$) lungs were dissected immediately after removal from patients undergoing clinical lung transplantation (freshly excised) and placed in ice-cold PBS, and epithelium was removed with a scalpel. Protein was isolated in 7 M urea buffer containing SDS and β -mercaptoethanol and sonicated for 30 s. Protein concentration was determined with the RC/DC Protein Assay (Bio-Rad, Hercules, CA), 50 μg total protein run on Tris-glycine gels and transferred to PVDF membrane at 33 V for 2 h. Western blotting was performed as described above. For Western blotting of JAM, membranes were incubated with 3D8 antibody overnight at 4°C . Occludin Western blotting was performed with the rabbit polyclonal antibody as described above. Densitometry was performed with Scion Image for Windows (Scion, Frederick, MD) to determine average band intensity and percent difference between non-CF and CF. The intensity of lanes containing no bands was set to the background level.

RT-PCR and Quantitative RT-PCR

Total RNA was isolated with Qiagen RNeasy Protect according to the manufacturer's protocol (Qiagen, Valencia, CA). RNA was then treated with DNase (Ambion, Austin, TX). For complementary DNA synthesis, 1 μg total RNA was used in a 20- μL reaction containing 1 mM deoxynucleotide triphosphates (dNTPs), 2.5 mM random hexamers or oligo dT, 1000 U/ml RNase inhibitor, 0.1 volume 10X buffer (supplied by manufacturer), and 2500 U/ml murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). The reverse transcription (RT) reaction was carried out at 1 cycle in a thermal cycler at 42°C for 50 min, followed by 15 min incubation at 70°C . For competitive quantitative RT-PCR (cQRT-PCR), an internal standard was generated containing a 60-nucleotide deletion that would be recognized by the same primers as the target sequence (Ho *et al.*, 1989). RNA for this internal standard was generated by ligation of a T7 promoter followed by *in vitro* transcription. Before RT, the standard was added at a concentration range of 0.0001 to 1 ng, and cDNA was generated. For semiquantitative RT-PCR (QRT-PCR), primers to the gene of interest and those to GAPDH were added simultaneously to the PCR reaction. PCR was carried out with *Taq* DNA polymerase for 25 cycles. PCR products were separated on a 1.5% agarose gel containing ethidium bromide. The relative band intensities were then determined with Scion Image, and the amount of JAM mRNA expression was calculated as previously described (Kaufmann *et al.*, 1999). The amount of standard added to the reaction (in ng) versus the ratio of standard (ΔJAM) to transcript was plotted on a double logarithmic plot. The amount of transcript was then determined by measuring the point on the plot at which the y-intercept equals 1, indicating an equal ratio of standard to transcript, and then calculating the x-intercept.

Enzyme-linked Immunosorbent Assay

Primary HAE cells were treated with cytokines for 24, 48, or 72 h, and total protein was isolated as described above. For ICAM-1, a 96-well assay plate (Costar) was coated overnight with mouse monoclonal ICAM-1, then lysate was added at 1, 3, and 10 μg and incubated for 2 h. After washing, rabbit polyclonal anti-ICAM-1 was added for 2 h, followed by horseradish peroxidase (HRP)-labeled secondary antibody. For JAM, plates were coated overnight with 1, 3, 10, or 30 μg lysate and incubated with mouse monoclonal anti-JAM (3D8) for 2 h and visualized with antimouse HRP. Protein was detected by addition of a 1:1 ratio of H_2O_2 :tetramethylbenzidine. Optical density was determined at 450 nm. Data are expressed as percent change in JAM or ICAM-1 relative to vehicle controls.

Freeze-Fracture EM

Cultures treated with vehicle or cytokines were fixed in 2% glutaraldehyde/2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C overnight. The epithelium was gently removed from the Transwell-Col with a scalpel and rinsed in phosphate buffer containing 0.2 M sucrose at room temperature, followed by a 25% glycerol cryoprotectant solution. The epithelium was sandwiched between gold double-replica mounts and frozen in liquid nitrogen-cooled Freon. Specimens were fractured in a Balzers BAF 400T freeze-fracture plant at a stage temperature of -100°C , and replicas were made at a 30° angle with platinum/carbon shadowing. The replicas and adherent tissue were removed by placing in distilled water, followed by transferring to a solution of 5% sodium dichromate in 50% sulfuric acid for cleaning. Replicas were then moved again to distilled water, where they were retrieved and placed onto standard copper microscopy grids. The replicas were examined, and fields exhibiting TJs at a plate magnification of 20,000 \times were photographed with a Zeiss EM-10A at an accelerating voltage of 60 kV. Morphometric analysis of TJ strands was performed as previously described (Carson *et al.*, 1988). Photomicrographs were enlarged to a final magnification of 60,000 \times , and TJs were transected by lines perpendicular to the luminal border with adjacent transects no

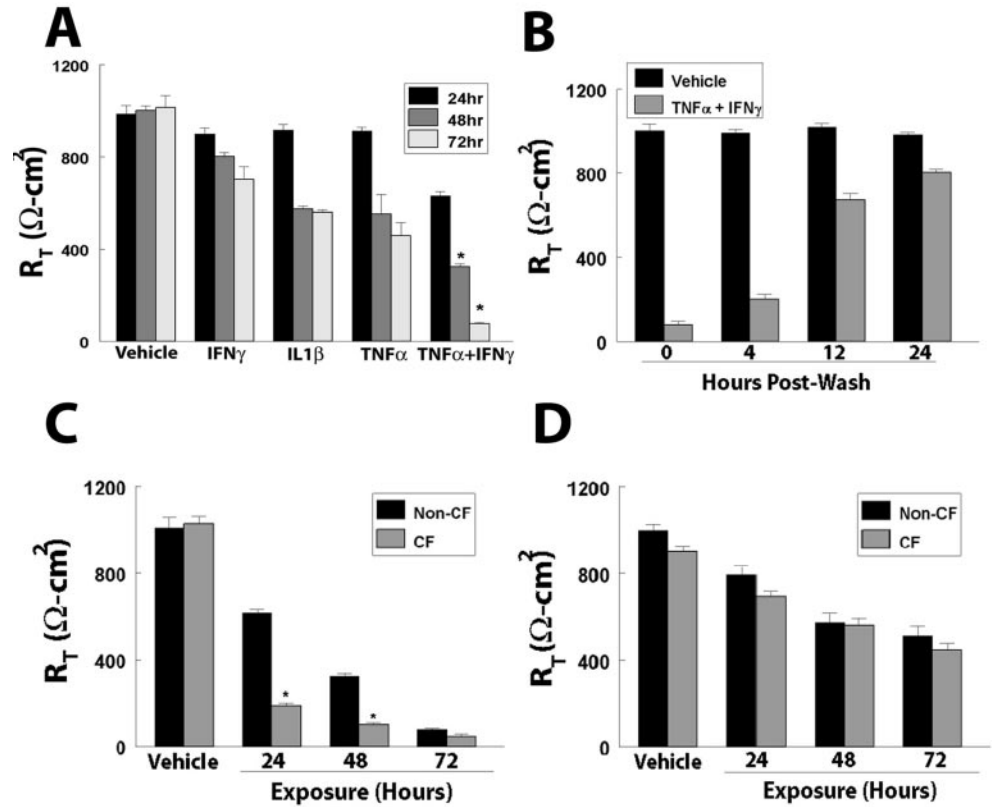


Figure 1. Effect of cytokines on R_T . (A) R_T of non-CF WD primary HAE cells exposed to $TNF-\alpha$, $IFN-\gamma$, or $IL-1\beta$ alone, or $TNF-\alpha$ and $IFN-\gamma$ in combination. (B) Recovery of R_T after removal of $TNF-\alpha$ and $IFN-\gamma$ at 4, 12, and 24 h after washing. (C) Effect of combined $TNF-\alpha$ and $IFN-\gamma$ treatment on R_T in non-CF and CF primary HAE cells. (D) Effect of $IL-1\beta$ on R_T in non-CF and CF primary HAE cells. *Significantly different from (A) vehicle and $TNF-\alpha$, $IFN-\gamma$, or $IL-1\beta$ alone or from (C) non-CF controls, at $p < 0.001$. Data presented are a minimum of $n = 12$ cultures from at least three patients.

closer than $1 \mu\text{m}$ apart. Strand depth was calculated at the transected lines by measuring the distance from the most luminal strand to the most distal strand.

Statistics

Data are presented as mean \pm SEM. A one-way analysis of variance (ANOVA) and Bonferroni's correction for multiple comparisons were used to determine statistical significance ($p < 0.05$).

RESULTS

Effect of Cytokine Treatment on R_T and P_{app}

WD primary HAE cells of non-CF and CF types were exposed to cytokines involved in CF inflammation, $IL-1\beta$, $IFN-\gamma$, and $TNF-\alpha$. To determine whether treatment with either cytokine could elicit effects on airway TJ permeability, we exposed the basolateral surface of HAE cells to $IL-1\beta$ (100 ng/ml), $TNF-\alpha$ (10 ng/ml), or $IFN-\gamma$ (100 ng/ml) alone or in combination and determined the effects on R_T and P_{app} at 24, 48, and 72 h. These concentrations were chosen because they are similar to levels detected in the airways of CF patients (Osika *et al.*, 1999). After 24 h exposure of non-CF WD HAE to $IL-1\beta$, $TNF-\alpha$, or $IFN-\gamma$ alone, there were no significant effects on R_T . However, in cultures exposed to combined treatment of $TNF-\alpha$ and $IFN-\gamma$, R_T at 24 h decreased to 65% of control (vehicle-treated) cultures (Figure 1A). After 48 h exposure, cultures exposed to $IFN-\gamma$ alone exhibited R_T values that were 80% of vehicle cultures, whereas R_T in those exposed to $TNF-\alpha$ or $IL-1\beta$ alone decreased to 60%. The R_T

of HAE cells treated with a combination of $TNF-\alpha$ and $IFN-\gamma$ decreased to 30% of control cultures (Figure 1A). The effect of cytokines on R_T was even more dramatic after 72 h exposure, with R_T of cultures treated with $TNF-\alpha$ and $IFN-\gamma$ in combination falling to 8% of control cultures (Figure 1A), whereas exposure to $IL-1\beta$, $TNF-\alpha$, or $IFN-\gamma$ alone decreased R_T to $\sim 60\%$ of vehicle controls. Exposure of cultures with $TNF-\alpha$, $IFN-\gamma$, and $IL-1\beta$ simultaneously did not alter the kinetics or magnitude of either R_T or P_{app} (data not shown). Upon removal of cytokines at 72 h, R_T returned to control levels by 24 h posttreatment (Figure 1B).

CF cultures displayed a different kinetic profile of cytokine-induced changes in R_T than did non-CF cultures. Whereas the R_T of non-CF cultures was only modestly affected by cytokine treatment at 24 h (61% of vehicle), the R_T of CF cultures exposed to combined $TNF-\alpha$ and $IFN-\gamma$ treatment was reduced to 18% of vehicle-treated controls (Figure 1C). This trend continued after 48 h exposure, with R_T in non-CF cultures reduced to 32%, compared with 10% of vehicle controls in CF cultures. However, by 72 h exposure, the R_T in both non-CF and CF cultures was reduced to 5–10% of vehicle controls (Figure 1C). In contrast, there was no significant difference between non-CF and CF cultures after exposure to $IL-1\beta$ at any time point (Figure 1D).

To correlate the changes in R_T induced after cytokine exposure to alterations in the barrier function of the TJ, the permeability coefficients of cultures to both a small solute, a 10-kDa FITC-labeled dextran, and a larger solute, 2000-kDa FITC-labeled dextran, were measured after cytokine expo-

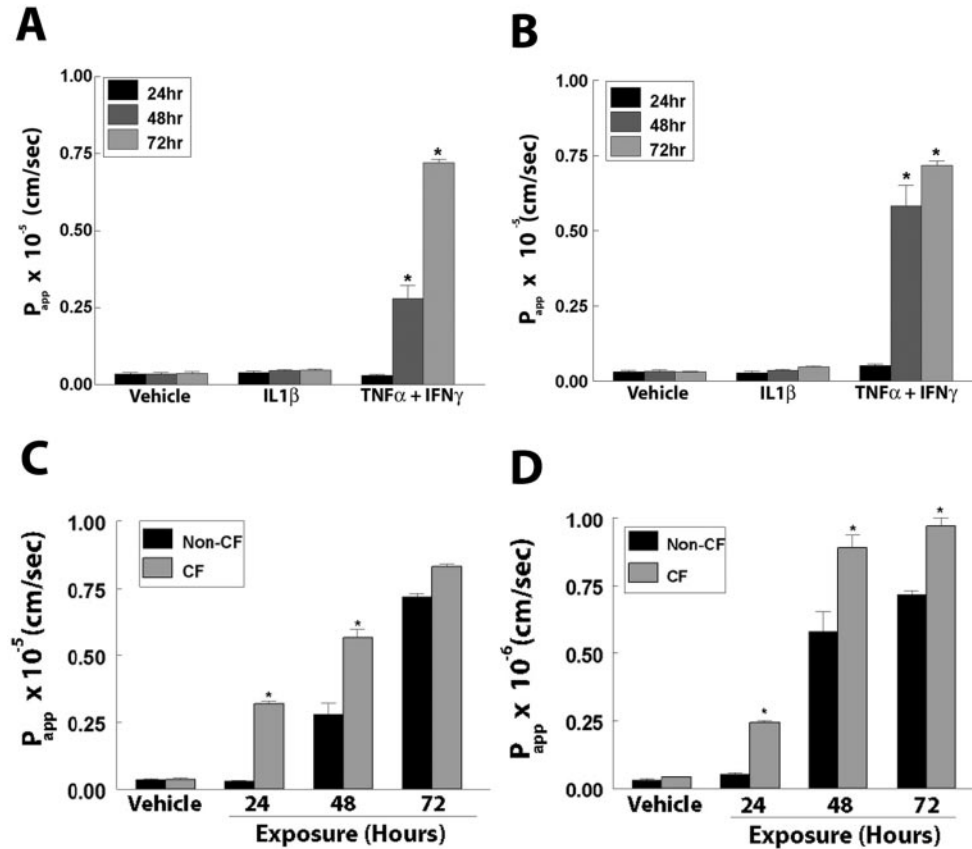


Figure 2. Effect of cytokines on P_{app} . P_{app} to (A) 10-kDa or (B) 2000-kDa FITC-labeled dextran in WD primary HAE exposed to IL-1 β alone or TNF- α and IFN- γ in combination for 24, 48, or 72 h. (C, D) Comparison of the effects of TNF- α and IFN- γ combined treatment on P_{app} in non-CF vs. CF WD primary HAE cells to (C) 10-kDa or (D) 2000-kDa FITC-labeled dextran. *Significantly different from vehicle (A, B) or from non-CF (C, D) controls at $p < 0.05$. Data presented are a minimum of $n = 12$ cultures from at least three patients.

sure. In non-CF cultures, there was no difference in the P_{app} to the 10- and 2000-kDa dextrans by 24 h (Figure 2, A and B) in cultures treated with TNF- α and IFN- γ in combination. However, pronounced increases in P_{app} after 48 and 72 h exposure were detected. The P_{app} to the 10-kDa dextran increased by 8-fold and that to the 2000-kDa 19-fold when treated with TNF- α and IFN- γ in combination for 48 h. By 72 h after treatment, P_{app} to the 10-kDa dextran was increased 17-fold and that to the 2000-kDa dextran 25-fold (Figure 2, A and B, respectively). There were no differences in the P_{app} between cultures treated with vehicle and those treated with IL-1 β alone at any time point (Figure 2, A and B). There was also no difference in cultures exposed to TNF- α or IFN- γ alone (data not shown). To provide evidence that cytokines were increasing P_{app} via passive mechanisms typical of the paracellular route, the permeability to solutes from the basolateral to apical compartments were measured and were equal to those from the apical to basolateral compartments (data not shown).

The kinetics of the changes in P_{app} of CF cultures treated with TNF- α and IFN- γ in combination were more significant than those of non-CF cultures. After 24 h exposure, P_{app} of CF cultures was 10-fold greater than both vehicle-control and non-CF cultures. At 48 h, the P_{app} s of CF cultures were ~twofold greater than those of non-CF cultures. However, like R_T , P_{app} after 72 h exposure was equal between non-CF and CF cultures (Figure 2C). In addition, P_{app} to the 2000-kDa dextran was also significantly increased in CF versus

non-CF cultures (Figure 2D). Although there was no increase in P_{app} in non-CF cultures after 24 h, there was a fivefold increase in CF cultures. In addition, at 48 and 72 h, there is an ~1.5-fold greater increase in P_{app} of CF cultures than non-CF (Figure 2D).

IL-1 β Effects on Na⁺ and Cl⁻ Permeability

Because treatment of primary HAE cells with IL-1 β did not induce alterations in P_{app} to dextrans but did lead to a modest decrease in R_T , we evaluated more subtle effects on TJ function by measuring the relative ion selectivity of the paracellular path in CF cultures at 72 h after IL-1 β exposure. The effect of NaCl ion substitution on the transepithelial dilution potentials were determined and used to calculate the ratio of the relative permeabilities of Cl⁻ to Na⁺ (P_{Cl^-}/P_{Na^+}). To exclude ion permeation via the transcellular pathway, experiments were performed in CF cultures that do not express the CF transmembrane conductance regulator (CFTR) channel on the apical membrane, thereby excluding Cl⁻ permeation via the transcellular route. To block Na⁺ transcellular permeation, all luminal solutions contained amiloride (10^{-4} M).

Cultures treated with IL-1 β relative to vehicle exhibited a decrease in ΔV_T in response to dilute NaCl solutions from 9.00 ± 0.28 mV (vehicle) to 3.50 ± 0.30 mV (IL-1 β), indicating an effect on ion permeation (Figure 3). This reduction in ΔV_T correlated with an increase in P_{Cl^-}/P_{Na^+} in cultures

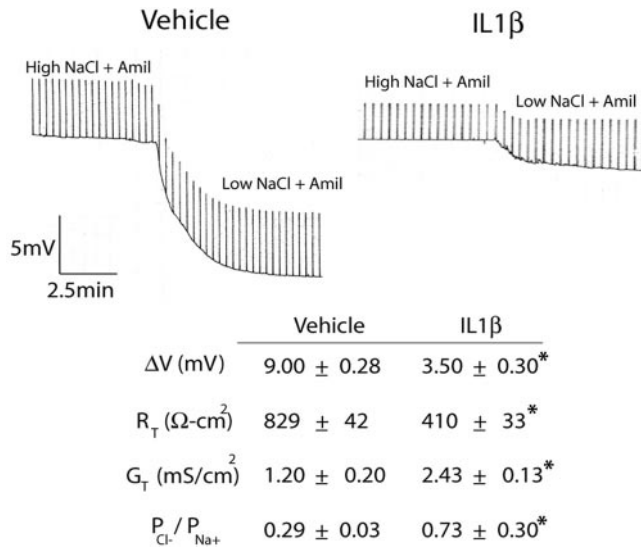


Figure 3. Effect of IL-1 β on relative ion selectivity. Polarized CF WD primary HAE cells were exposed to IL-1 β for 72 h. Electrophysiological measurements and dilution potential (ΔV_T) were measured and permeability ratio of Cl $^-$ /Na $^+$ ($P_{\text{Cl}^-}/P_{\text{Na}^+}$) calculated as described in MATERIALS AND METHODS. Shown are the transepithelial voltage recordings with intermittent current pulses in amiloride (Amil)-treated cultures before and after luminal substitution with a low-NaCl solution. Current pulses and voltage scales are the same. Summary data are presented in the table. Data presented are representative of recordings from 12 cultures from 2 patients. *Significantly different from vehicle-treated controls.

exposed to IL-1 β for 72 h, from 0.29 ± 0.03 to 0.73 ± 0.30 (Figure 3). The twofold increase in $P_{\text{Cl}^-}/P_{\text{Na}^+}$ in IL-1 β -treated cultures also correlated with a \sim twofold increase in conductance (G_T), an increase from 1.20 ± 0.20 mS/cm 2 (vehicle) to 2.43 ± 0.13 mS/cm 2 (IL-1 β). Because IL-1 β led to increased G_T and a hyperpolarization of V_T while exhibiting a net increase in $P_{\text{Cl}^-}/P_{\text{Na}^+}$, it can be concluded that IL-1 β leads to a selective increase in paracellular permeability to Cl $^-$.

Cytokines and TJ Morphology

To determine whether the effects of TNF- α and IFN- γ co-treatment on the distribution of TJ proteins also affected the morphology of TJ strands, we performed freeze-fracture EM and quantified the effect of cytokine treatment on junctional depth and strand number. Freeze fracture splits the cell membrane along a hydrophobic core, thereby allowing an en face view of the strands of the TJ. Electron micrographs of freeze-fracture replicas from non-CF cultures treated with TNF- α and IFN- γ for 72 h revealed that treatment decreased overall junctional depth and number of strands (Figure 4, A and C). In cultures exposed to cytokines, junctional depth was measured at 0.295 ± 0.020 μm , whereas that of vehicle controls was 0.469 ± 0.103 μm as determined by morphometric analysis (Figure 4C). Strand number was decreased from 6.90 ± 0.372 in vehicle controls to 4.85 ± 0.214 in cultures exposed to TNF- α and IFN- γ .

Unlike non-CF cultures, which were unaffected by cytokines at 24 h, the effect of TNF- α and IFN- γ on R_T of CF

cultures was pronounced by 24 h after treatment (Figure 1C). For this reason, freeze-fracture analysis was also performed on CF cultures at 24 h. After exposure for 24 h, CF cultures displayed a significant change in both depth and strand number, with changes similar to those seen in non-CF cultures after 72 h cytokine exposure (Figure 4B). By 72 h, the number of strands had decreased to 1.97 ± 0.214 and junctional depth to 0.068 ± 0.007 μm , compared with a strand number of 6.7 ± 0.227 and a depth of 0.501 ± 0.170 μm in vehicle-controls. Although the R_T and P_{app} of CF cultures at 72 h after cytokine exposure do not differ significantly from non-CF, TJ morphology differs significantly, suggesting an increased sensitivity of CF cultures to TNF- α and IFN- γ .

Cytokine Effects on the Integrity of TJ-Associated Proteins

To determine whether exposure to TNF- α and IFN- γ disrupted the organization of TJ-associated proteins, we performed immunofluorescence localization of ZO-1, JAM, claudin-1, claudin-4, and occludin. When double-immunofluorescence localization was performed for the cytoplasmic protein ZO-1 and the integral membrane protein JAM, we saw redistribution of both proteins at 48 h and 72 h (Figure 5A), with less obvious alterations at 24 h (data not shown). The apparent disruption was detected only in cultures treated with the combination of TNF- α and IFN- γ and not in cultures treated with IL-1 β , TNF- α , or IFN- γ alone (data not shown). CF cultures at 24 h exposure to TNF- α and IFN- γ exhibited a similar pattern of reorganization of ZO-1 and JAM as non-CF cultures at 72 h (Figure 5A). CF cultures exposed to cytokines for 72 h displayed a similar relocalization of ZO-1 and JAM as non-CF at 72 h (data not shown). The apparent change in localization of ZO-1 and JAM shown by immunofluorescence was also associated with a decrease in ZO-1 and JAM expression by Western blot analysis of non-CF cultures exposed to TNF- α and IFN- γ for 72 h (Figure 5B).

However, immunofluorescence localization of claudin-1 showed no apparent change in either the intensity or distribution of fluorescent staining for either protein (Figure 6A). Occludin exists in both a high-molecular-weight (HMW) and low-molecular-weight (LMW) form. Although immunofluorescence did not reveal a change in the expression or localization of occludin, the antibody used recognized only the LMW form of the protein (Figure 6A). To reconcile whether there were TNF- α and IFN- γ -induced effects on either occludin expression or phosphorylation, we performed Western blots with two antibodies, one recognizing only the LMW and one recognizing both forms. Samples isolated from cultures exposed to TNF- α and IFN- γ for 72 h and probed with an antibody recognizing both the LMW and HMW forms appeared to express more of the 73-kDa occludin than vehicle-controls (Figure 6B, lanes 4–6). However, when the membrane was reprobed with an antibody that recognizes only the LMW form of the protein, there was no change in the 65-kDa occludin band after treatment (Figure 6C). This finding would indicate that the relative increase seen correlates with a shift from the LMW form to the HMW, indicating an increase in the phosphorylation state of the protein. Although there appeared to be effects on occludin levels, claudin-1 expression remained unchanged (Fig-

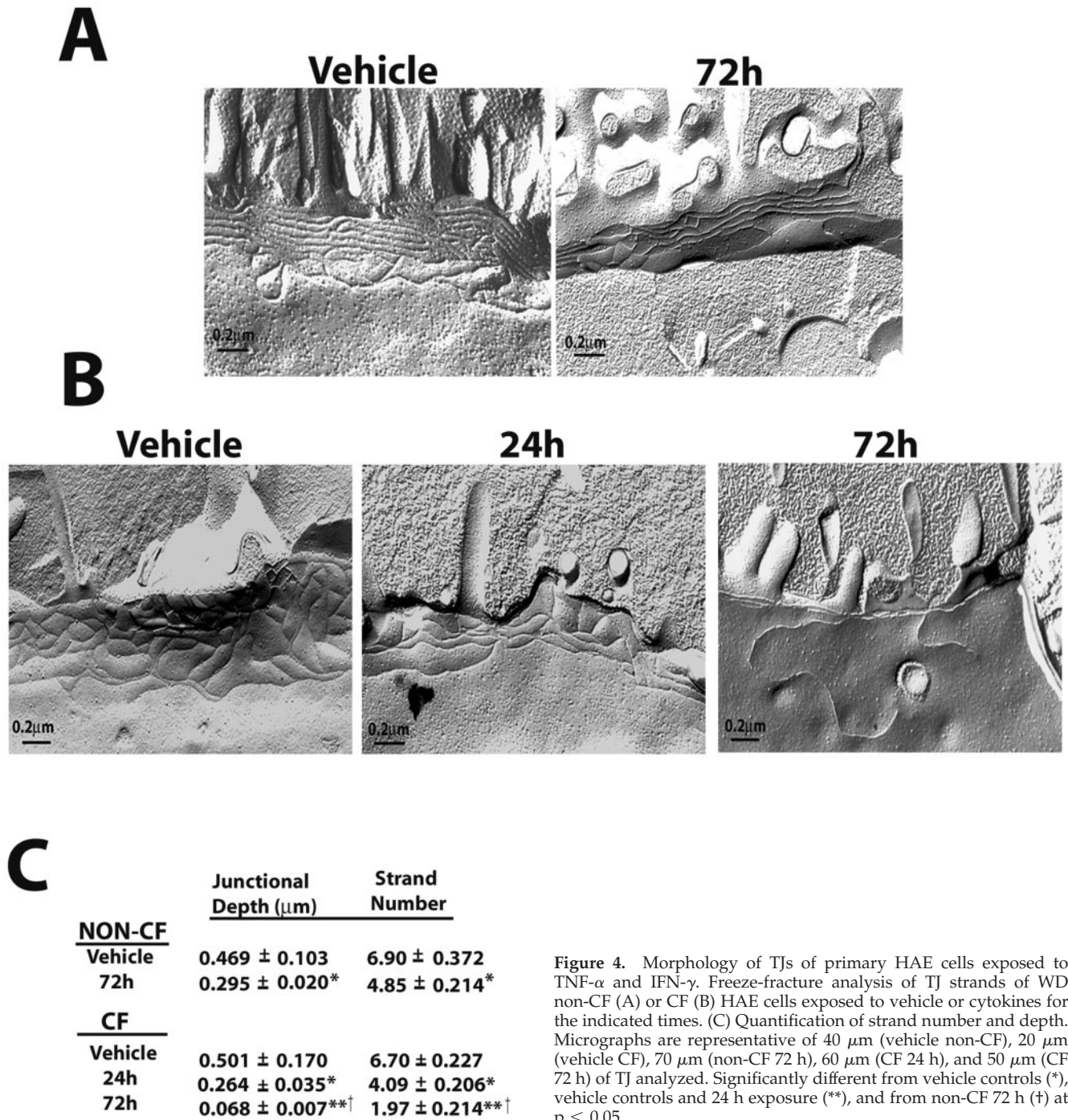


Figure 4. Morphology of TJs of primary HAE cells exposed to TNF- α and IFN- γ . Freeze-fracture analysis of TJ strands of WD non-CF (A) or CF (B) HAE cells exposed to vehicle or cytokines for the indicated times. (C) Quantification of strand number and depth. Micrographs are representative of 40 μm (vehicle non-CF), 20 μm (vehicle CF), 70 μm (non-CF 72 h), 60 μm (CF 24 h), and 50 μm (CF 72 h) of TJ analyzed. Significantly different from vehicle controls (*), vehicle controls and 24 h exposure (**), and from non-CF 72 h (†) at $p < 0.05$.

ure 6C). The distribution and expression of claudin-4, the other claudin species identified in our culture system, was also unaffected by TNF- α and IFN- γ treatment (Figure 6, B and C). The finding with regard to claudin-4 was confirmed by comparison of relative fluorescence intensity of immunofluorescence images and by densitometry of Western blots (data not shown).

Cytokine Exposure and JAM and ICAM-1 Expression

Redistribution of JAM by cytokines (Figure 5A) would be expected to decrease monocyte transmigration across the airway, whereas increased ICAM-1 expression would be required for neutrophil transmigration (Ozaki *et al.*, 1999; Kidney and Proud, 2000). Because neutrophils are the

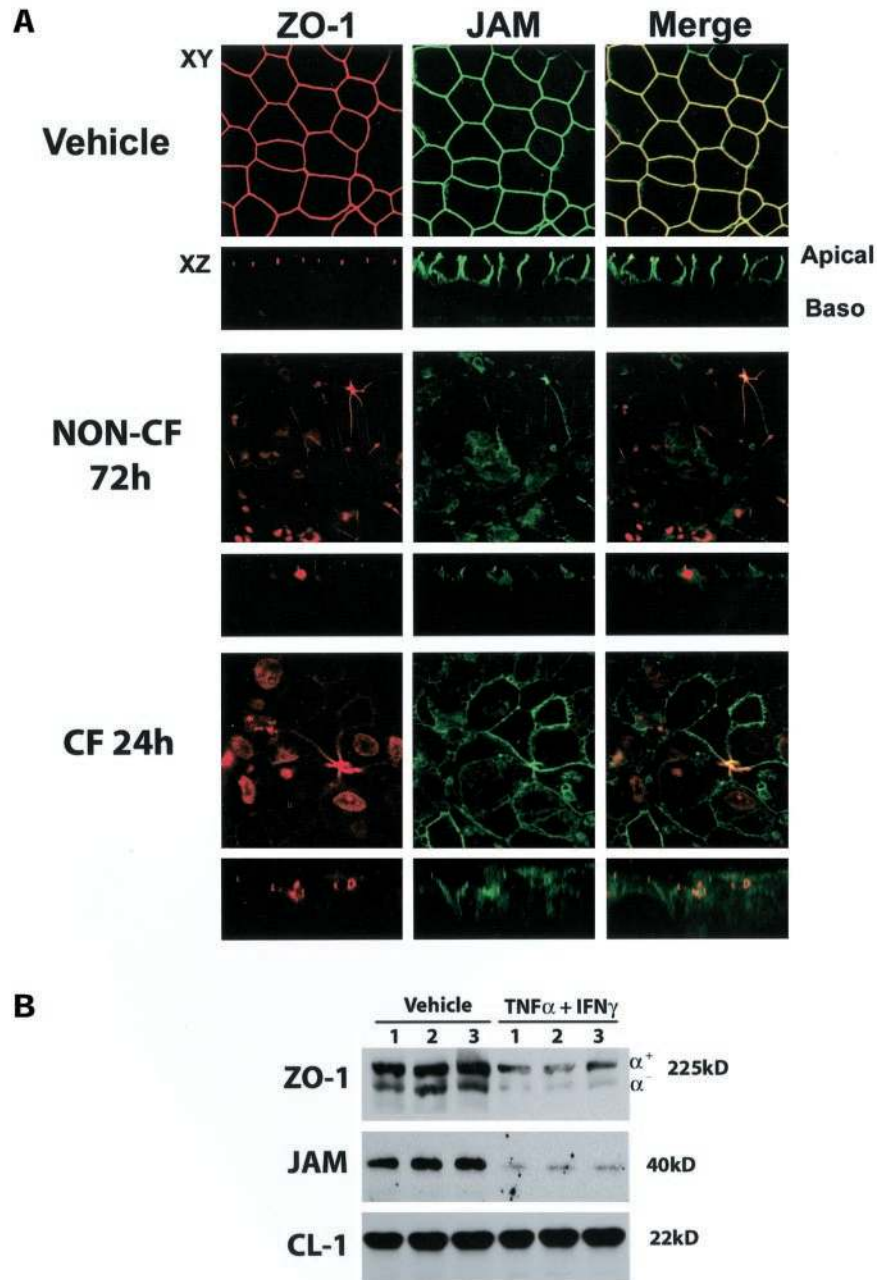


Figure 5. Effect of TNF- α and IFN- γ treatment on expression and localization of JAM and ZO-1. (A) Immunofluorescent staining for JAM and ZO-1 in non-CF and CF primary HAE cells exposed to TNF- α and IFN- γ for 72 or 24 h. Red staining (left) represents ZO-1, green staining (middle) is JAM, and (right) merged image of both ZO-1 and JAM. Areas of colocalization appear as yellow. Images are representative of a minimum of n = 6 cultures from two patients. (B) Western blotting of ZO-1 (top) and immunoprecipitation of JAM (middle) in non-CF cultures. Western blot for ZO-1 was stripped and reprobed with an antibody specific for claudin-1 to control for loading (bottom).

predominant inflammatory cell in the CF airway, we investigated whether treatment of primary non-CF HAE cells with TNF- α and IFN- γ altered the levels of expression of JAM and ICAM-1. After exposure of cultures for 24, 48, or 72 h with TNF- α and IFN- γ , the level of JAM and ICAM-1 mRNA was determined by cQRT-PCR or semi-quantitative RT-PCR (QRT-PCR), respectively, and compared with the expression in untreated cultures. Two methods of mRNA quantification were chosen because more subtle changes were expected in JAM expression level. The level of JAM mRNA decreased to $58 \pm 12\%$ of vehicle controls after 72 h cytokine exposure as deter-

mined by cQRT-PCR (Figure 7A). Similar results were found after QRT-PCR (data not shown). In contrast, ICAM-1 mRNA expression increased with increasing duration of exposure to cytokines (Figure 7B). By 48 and 72 h exposure, the level of ICAM-1 mRNA was significantly greater in cytokine-treated than in control cultures. No ICAM-1 was detectable in vehicle-treated HAE cultures, consistent with previously published data (Striz *et al.*, 1999). After 24 h exposure to cytokines, there was a significant increase in ICAM-1 mRNA expression, which increased further to $214 \pm 14.5\%$ of the 24-h level by 48 h and $242 \pm 8.5\%$ at 72 h.

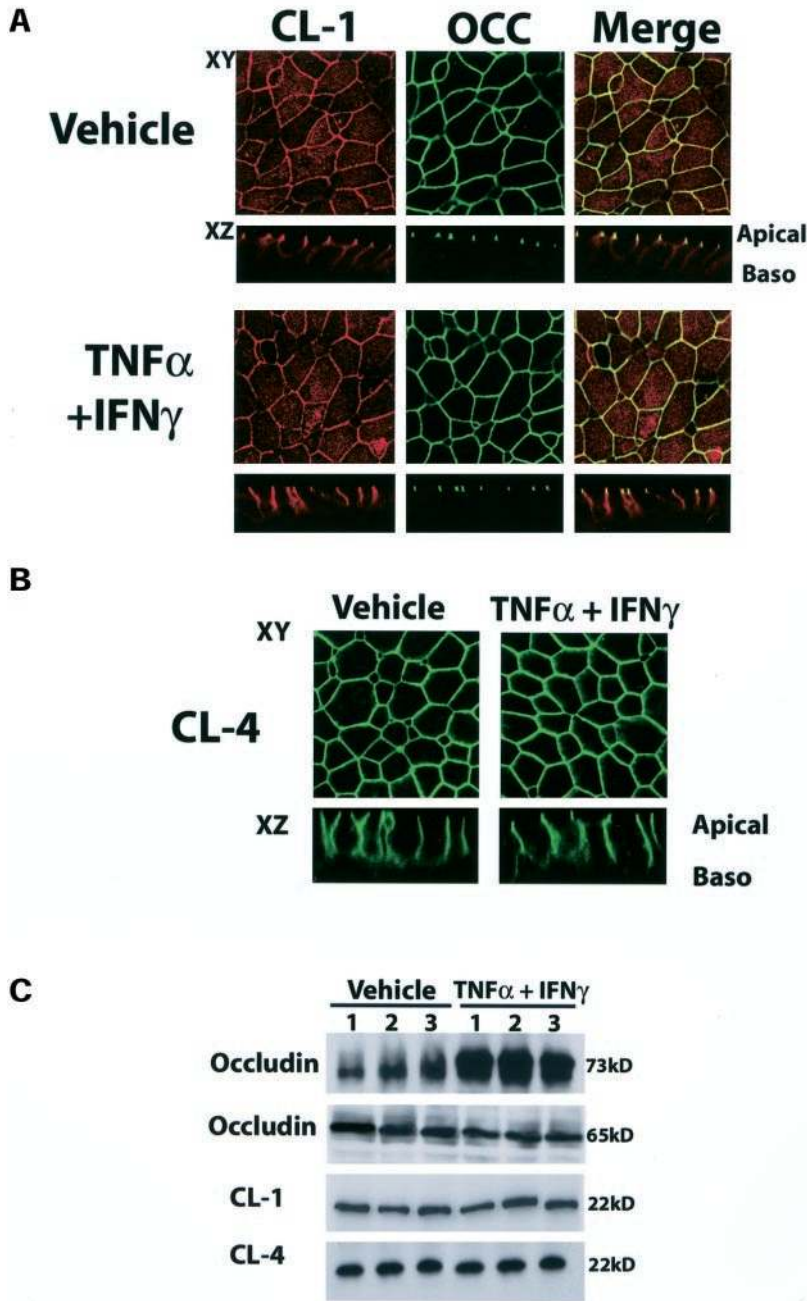


Figure 6. TNF- α and IFN- γ effect on occludin, claudin-1, and claudin-4 distribution and expression. (A) Immunofluorescent staining for occludin and claudin-1 in primary HAE cells exposed to TNF- α and IFN- γ for 72 h. Occludin staining in green (middle), claudin-1 in red (left), and merged image (right). Colocalization appears as yellow. (B) Immunofluorescence staining of claudin-4 in vehicle-treated cultures and in cultures exposed to TNF- α and IFN- γ for 72 h. (C) Western blot analysis of occludin, claudin-1, and claudin-4. Top, Probed with an antibody recognizing both the HMW and LMW forms of occludin. This blot was then stripped and reprobed with an antibody recognizing only the LMW form of the protein. Claudin-1 and claudin-4 expression are below. Lanes 1-3, vehicle; 4-6, exposed to TNF- α and IFN- γ for 72 h. Images are representative of a minimum of n = 6 cultures from two patients.

To correlate the changes in mRNA expression after cytokine exposure with protein levels, we performed an enzyme-linked immunosorbent assay (ELISA) on vehicle and cytokine-treated cultures for 24, 48, or 72 h (Figure 7). The level of JAM expression decreased to $80 \pm 10\%$, $70 \pm 10\%$, and $40 \pm 4\%$ of control cultures at 24, 48, and 72 h, respectively. In contrast, ICAM-1 expression increased to $141 \pm 9\%$, $227 \pm 7\%$, and $356 \pm 22\%$ of controls at 24, 48, and 72 h, respectively. Thus, TNF- α and IFN- γ downregulate JAM expression while upregulating ICAM-1 expression.

Mechanism of Cytokine-induced Alterations in Permeability

Cytokine-induced changes in TJ permeability have previously been linked to alterations in tyrosine kinases and PKC (Ratcliffe *et al.*, 1999; Schmitz *et al.*, 1999b). We examined the ability of a tyrosine kinase inhibitor, genistein, and the non-specific PKC inhibitor H7 to block the effect of TNF- α and IFN- γ on R_T and P_{app} to small and large solutes, respectively. Cultures were pretreated bilaterally with the inhibitor

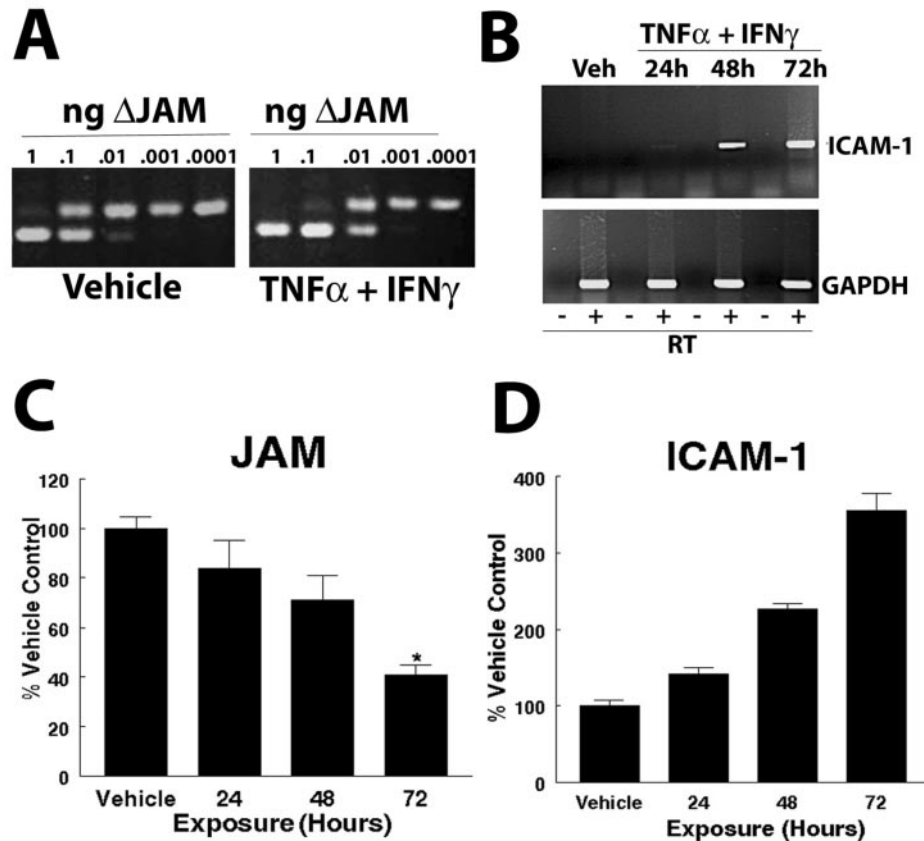


Figure 7. Coordinate regulation of JAM and ICAM-1 in response to TNF- α and IFN- γ exposure. (A) JAM mRNA expression as determined by cQRT-PCR with an internal standard (Δ JAM) at the indicated concentration (1 to 0.0001 ng). Left, Vehicle, and right, cultures exposed to TNF- α and IFN- γ for 72 h. (B) Semiquantitative RT-PCR (QRT-PCR) analysis of ICAM-1 mRNA expression normalized to GAPDH. Top, ICAM-1 expression in vehicle or at 24, 48, or 72 h after exposure to TNF- α and IFN- γ . Bottom, GAPDH expression. (C, D) JAM (C) and ICAM-1 (D) protein expression determined by ELISA at 24, 48, and 72 h after exposure to TNF- α and IFN- γ . Data are from a minimum of $n = 6$ cultures from at least three patients.

at concentrations ranging from 1 to 300 μ M for 1 h. After this initial incubation, TNF- α and IFN- γ in the presence of the inhibitor were incubated for 72 h, the time point at which the greatest effect of cytokine was observed (Figure 1). Genistein did not inhibit the effect of cytokines on R_T or P_{app} at any concentration (data not shown), whereas H7 (10 and 30 μ M) significantly inhibited the effect on both R_T and P_{app} . At 72 h, H7 (10 μ M) displayed partial inhibition of the TNF- α and IFN- γ -induced changes in R_T (Figure 8A).

We next determined whether H7 would also induce a partial inhibition of the cytokine-mediated increase in P_{app} by measuring P_{app} of the 10-kDa FITC-dextran at 72 h after cytokine exposure. P_{app} in cultures treated in the absence of H7 was increased 17-fold over vehicle controls. In contrast, cultures treated with TNF- α and IFN- γ in the presence of H7 (10 μ M) displayed a negligible increase in P_{app} , with P_{app} to the FITC-dextran only twofold greater than that of vehicle controls (Figure 8B).

Because the nonspecific inhibitor H7 may also inhibit protein kinase A (PKA), we also evaluated the effect of the specific PKC inhibitor chelerythrine, which inhibits the catalytic domain of all PKC isoforms on R_T and P_{app} . Like H7, chelerythrine (1 μ M) inhibited the cytokine-induced decrease in R_T and the associated increase in P_{app} to a similar degree as H7 (Figure 8, A and B), suggesting that the effects on R_T and P_{app} were mediated through PKC signaling pathways.

Effect of H7 on Cytokine-induced Alterations JAM and ICAM-1 Expression

In addition to eliciting a profound effect on R_T and P_{app} , exposure of WD primary HAE cells to combined treatment of TNF- α and IFN- γ caused changes in the expression of JAM and ICAM-1 (Figure 7). To determine whether cotreatment of H7 with cytokines could inhibit the effect on JAM and ICAM-1 protein expression, we performed ELISA analysis after 72 h exposure to TNF- α and IFN- γ in the absence or presence of H7. JAM and ICAM-1 expression were $44 \pm 6\%$ and $378 \pm 7\%$ of vehicle controls after treatment in the absence of H7 (Figure 8, C and D). However, JAM expression in cultures treated in the presence of H7, 10 and 30 μ M, was $65 \pm 20\%$ and $90 \pm 16\%$ of control, respectively (Figure 8C). ICAM-1 expression in the presence of H7 also remained at or near control levels, $189 \pm 15\%$ and $102 \pm 15\%$ in cultures treated with 10 and 30 μ M H7, respectively (Figure 8D).

Effect of PKC Inhibitors on Distribution of TJ-associated Components

The nonspecific (H7) and specific (chelerythrine) PKC inhibitors prevented the cytokine-induced changes in junctional barrier function (as assessed by R_T and P_{app} measurements) and the changes in the reciprocal regulation of JAM and ICAM-1 expression (Figure 8). To determine whether these inhibitors could also inhibit the changes in the distribution

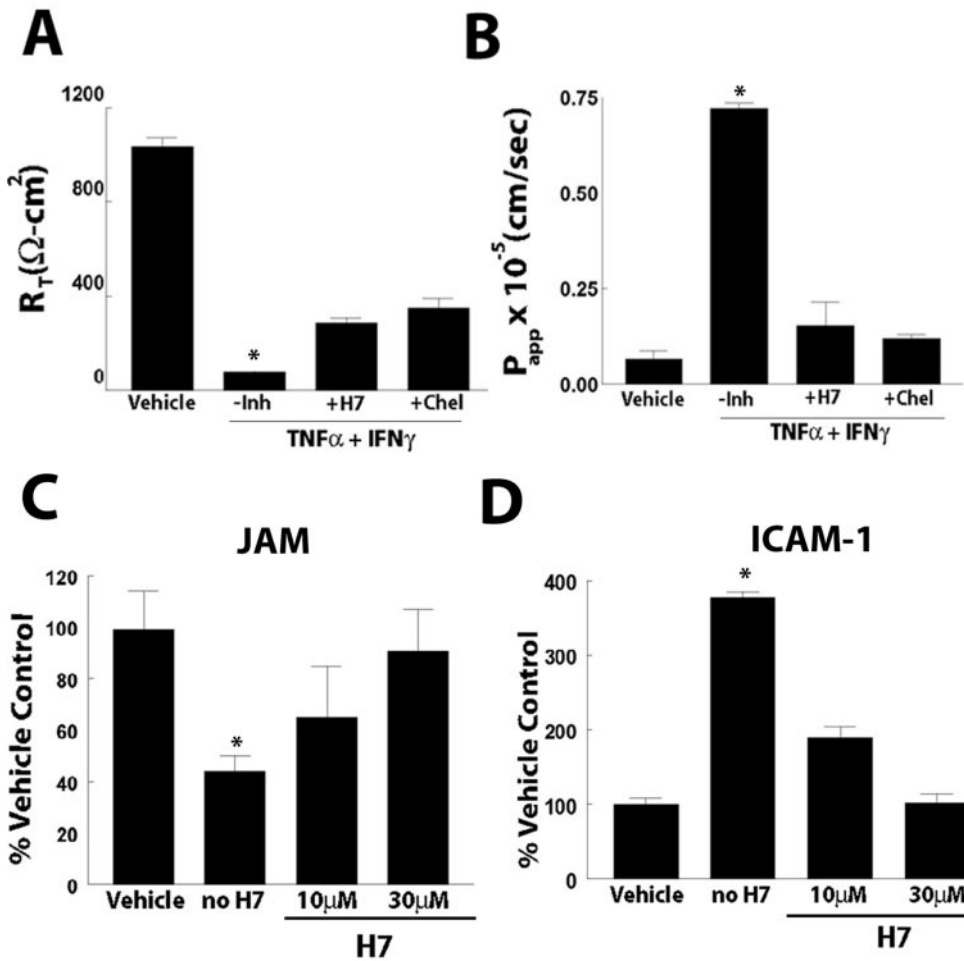


Figure 8. Effect of selective and nonselective inhibitors of PKC on cytokine-induced changes in R_T , P_{app} , and ICAM-1 expression. Cultures were exposed to TNF- α and IFN- γ in the presence or absence of H7 (nonselective) or chelerythrine chloride (selective) inhibitors of PKC. (A) R_T and (B) P_{app} to 10-kDa FITC-dextran. JAM (C) and ICAM-1 (D) protein expression by ELISA in cultures treated with TNF- α and IFN- γ for 72 h in the absence or presence of 10 μ M or 30 μ M H7. *Significantly different from vehicle at $p < 0.05$. Data presented are a minimum of $n = 12$ cultures from at least three patients.

of ZO-1 and JAM, which occurred after 72 h TNF- α and IFN- γ treatment, we performed immunofluorescence localization after treatment of cultures in the presence of H7 and chelerythrine. Although a subtle redistribution of JAM and ZO-1 was detected after cytokine exposure in the presence of H7 or chelerythrine, the extent of this relocalization was less pronounced than with cytokine exposure alone (Figure 9). Claudin-1 and occludin distribution was not effected either in the presence or absence of the inhibitors (data not shown).

Characterization of PKC Isomers Mediating Cytokine-induced Changes in TJ Permeability

Because H7 and chelerythrine inhibited the effects of TNF- α and IFN- γ on R_T and P_{app} , we determined which PKC isoform was involved in the cytokine-mediated alteration of the TJ. We focused on the atypical PKC isoforms ι/λ and ζ because of their link to the TJ (Ebnet *et al.*, 2001). In particular, because JAM expression is highly regulated by cytokine exposure, we examined whether expression of PKC ι/λ , an isoform thought to bind directly to JAM, would be altered in the same manner. After 72 h exposure, PKC ι/λ protein expression was greatly increased in cultures exposed to TNF- α and IFN- γ (Figure 10). Immunofluorescence re-

vealed an increase in PKC ι/λ expression, correlating with enhanced staining of the nuclei, plasma membrane, and the apical portion of the cytoplasm (Figure 10A). In addition, immunoprecipitation of PKC ι/λ and PKC ζ followed by Western blot analysis demonstrated an increase in the PKC ι/λ isoform after cytokine exposure, but not PKC ζ (Figure 10B). Treatment of cells with cytokines in the presence of H7, which prevented the changes in barrier function and JAM and ICAM-1 expression (Figures 7 and 9), also partially inhibited the increase in PKC ι/λ at 72 h (Figure 10B). In contrast, the specific PKC inhibitor chelerythrine, which prevented the cytokine-induced alteration in R_T and P_{app} (Figure 7), did not inhibit the increase in PKC ι/λ expression (Figure 10B), suggesting that the reduction in PKC ι/λ expression by H7 was mediated through PKA. PKA is known to regulate expression of aPKCs (see below) in cells that express adenosine receptors (Huang *et al.*, 2001b).

Expression of Junctional Components and ICAM-1 In Vivo

We have demonstrated that prolonged cytokine treatment induces alteration in the barrier function of the TJ, presumably via PKC-induced changes in protein components of the

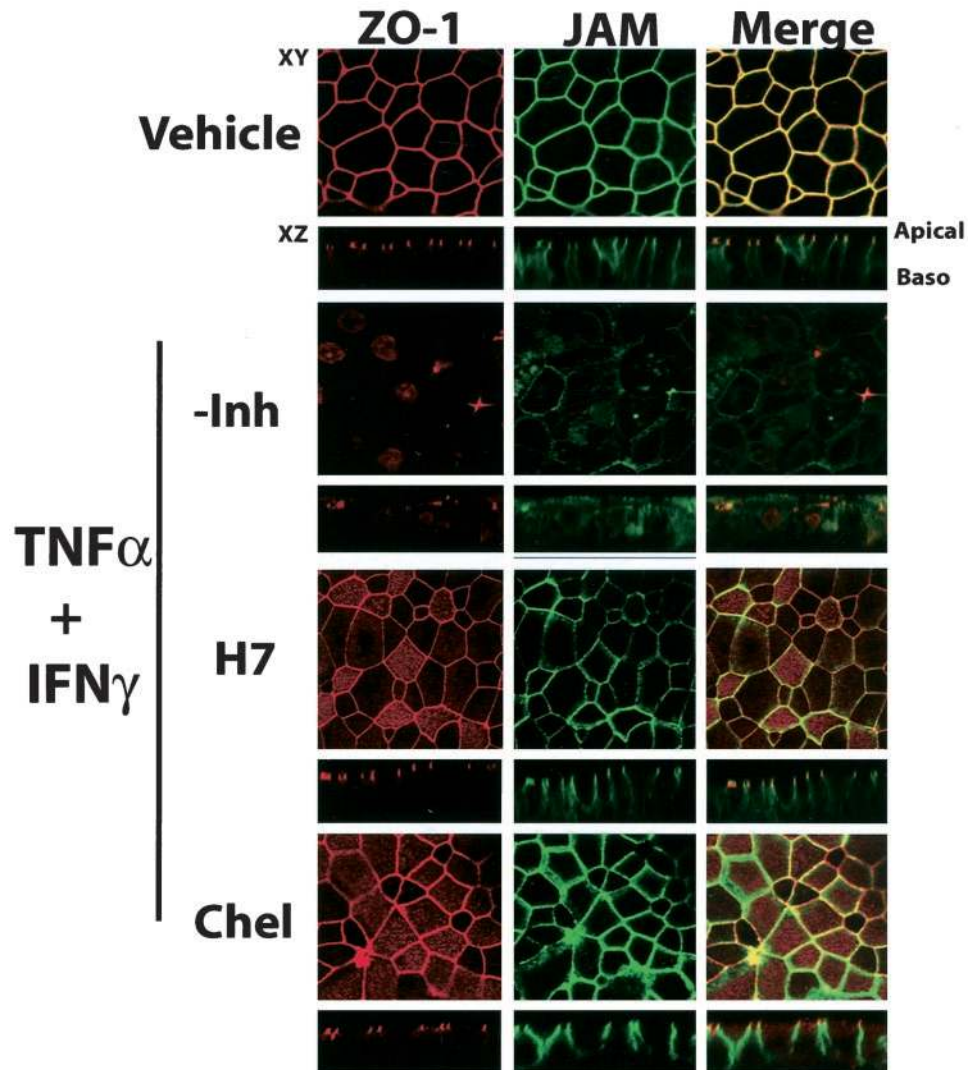


Figure 9. Effect of H7 and chelerythrine on the reorganization of ZO-1 and JAM. Immunofluorescent staining for JAM and ZO-1 in primary HAE cells exposed to $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ for 72 h in the presence or absence of H7 (10 μM) or chelerythrine (10 μM). Red staining (left) represents ZO-1, green staining (middle) is JAM, and right is a merged image of both ZO-1 and JAM. Areas of colocalization appear yellow.

TJ. Because the airways of CF patients are chronically exposed to an inflammatory milieu, we examined the expression of these same TJ components in large airways excised from non-CF and CF patients after lung transplantation. We found that the changes in ICAM-1, JAM, and ZO-1 induced by cytokine treatment in our in vitro system were also present in vivo. Bronchi from CF patients expressed increased ICAM-1, particularly at the basolateral surface, and decreased ZO-1 and JAM staining as assessed by immunofluorescence (Figure 11). However, no apparent change in the fluorescence intensity of occludin, claudin-1, or claudin-4 was detected.

To further quantify these changes, we performed Western blot analysis of protein isolated from bronchi of non-CF donors and CF patients after transplant. We found that, as occurs with cytokine exposure in our in vitro system, bronchial epithelia from CF patients in vivo exhibit a decrease in ZO-1 and JAM and a marked increase in ICAM-1 and occludin (Figure 12). CF bronchial epithelia in vivo expressed $46 \pm 14\%$ less ZO-1 and $54 \pm 19\%$ less JAM than non-CF

donors. In contrast, CF bronchial epithelia in vivo demonstrated a $196 \pm 18\%$ increase in ICAM-1 expression over non-CF. These data are similar to those of previously published reports measuring ICAM-1 expression in CF and non-CF epithelium (Hubeau *et al.*, 2001). Similarly, occludin Western blotting showed an increase of $234 \pm 36\%$ over non-CF donor epithelium. When the polyclonal antibody to occludin was used in Western blotting, only a single band was detected in the range of 65–73 kDa. There was no significant change in the expression of either claudin-1 or -4 in CF epithelium ($97 \pm 14\%$ and $96 \pm 6\%$ of non-CF, respectively), consistent with our in vitro data.

DISCUSSION

We hypothesized that the chronic inflammation in the CF lung may lead to changes in the integrity of the TJ in CF patients, leading to decreased barrier function and alterations in ion selectivity. To address this hypothesis, we

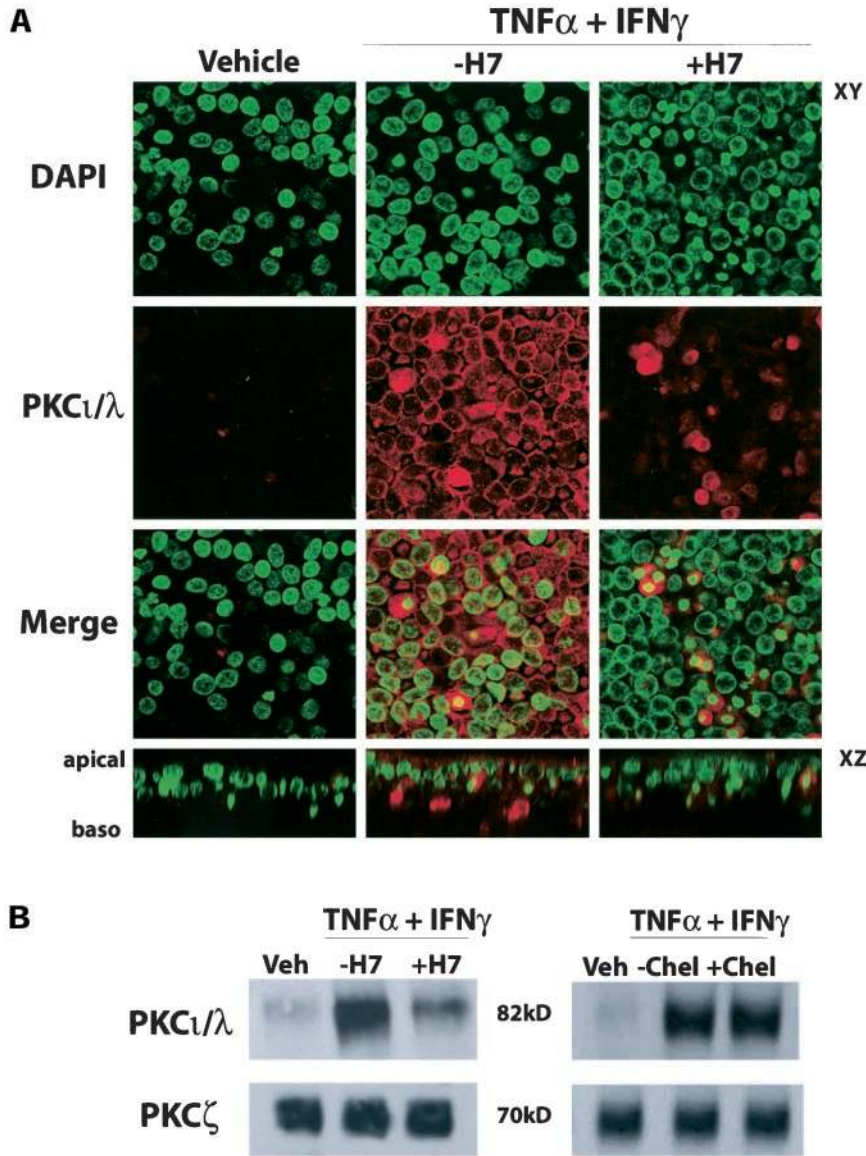


Figure 10. Cytokine effects on PKC isomer expression. WD primary HAEs were exposed to TNF- α and IFN- γ in the absence or presence of H7, and expression of PKC ι/λ and PKC ζ was determined. (A) Immunofluorescence localization of PKC ι/λ after 72 h exposure to TNF- α and IFN- γ , with or without H7. Increased staining in cultures exposed to TNF- α and IFN- γ is evident. (B) Immunoprecipitation followed by Western blot analysis of PKC ι/λ (top) and PKC ζ (bottom) after 72 h exposure to TNF- α and IFN- γ , with or without H7 (left) or chelerythrine (right). Images and gel are representative of a minimum of $n = 6$ cultures from at least 3 patients.

exposed non-CF and CF WD primary HAE cultures to cytokines known to be upregulated in CF. We found that exposure of WD primary HAE to TNF- α and IFN- γ combined, but not separately, induced significant changes in the barrier function and rearrangement of structural components of the TJ. In addition, it appears that these cytokine-induced changes may be regulated via a signaling cascade involving upregulation of PKC ι/λ , an atypical PKC isoform.

Prolonged exposure of the airway to a combination of TNF- α and IFN- γ led to significant changes in TJ barrier properties, with significant alterations in both R_T and P_{app} (Figures 1 and 2). However, there was a less pronounced effect when cultures were treated with either of these cytokines alone or with IL-1 β . In addition, the kinetics of the effects of TNF- α and IFN- γ on R_T and P_{app} were different in CF WD primary HAE cells from those in non-CF controls. Although cultures of the non-CF type were resistant to the

effects of cytokine treatment until 48 h of exposure, CF cultures showed a significant decrease in R_T and an increase in P_{app} by 24 h (Figure 2, C and D), indicating some properties of the CF epithelium make it respond more rapidly to the effects of cytokines on the TJ. Perhaps primary cells isolated from CF airways are already sensitized to the effects of cytokines because of retention of changes in gene expression induced by the inflammatory environment present in vivo.

Our data measuring the effects of cytokines on R_T and P_{app} for noncharged solutes and dilution potentials allowed us to identify differences in TJ responses to individual cytokines. For example, IL-1 β treatment modestly decreased R_T , while exhibiting no effect on P_{app} to small or large noncharged solutes, suggesting that the barrier function of the TJ to noncharged solutes was unchanged, whereas the ion permeability of the junction was altered. Measurements of

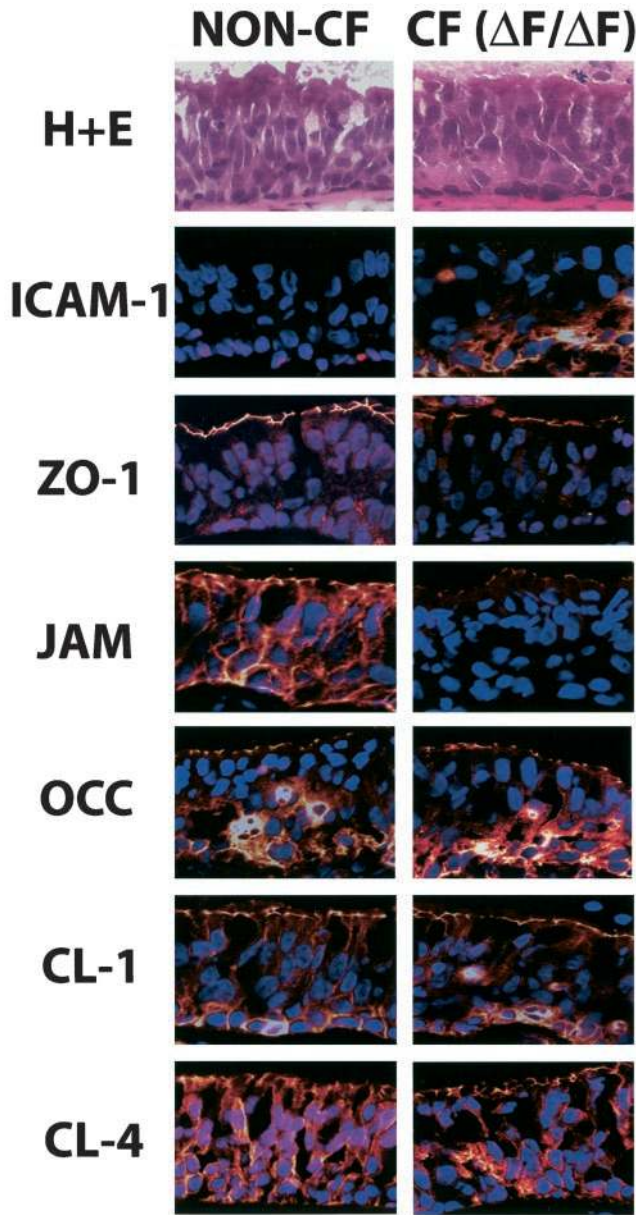


Figure 11. In vivo localization of ICAM-1 and junctional components in freshly excised non-CF and CF ($\Delta F/\Delta F$) bronchus. Bronchi from non-CF and CF patients were isolated and prepared for staining as described in MATERIALS AND METHODS. Frozen sections were stained for ICAM-1, ZO-1, JAM, claudin-1, occludin, and claudin-4. Blue, DAPI-stained nuclei and red, positive protein staining. Images are representative of sections from a total of four patients of non-CF and CF ($\Delta F/\Delta F$) type each. Top, Hematoxylin and eosin (H+E) staining of a representative section of non-CF or CF type to illustrate section morphology.

the effects of 72 h IL-1 β treatment on G_T and transepithelial dilution potentials suggested that the increase in G_T (or reduction in R_T) could be accounted for solely by the increase in relative permeability for Cl $^-$ across the paracellular pathway (Figure 3). Previously published data have estab-

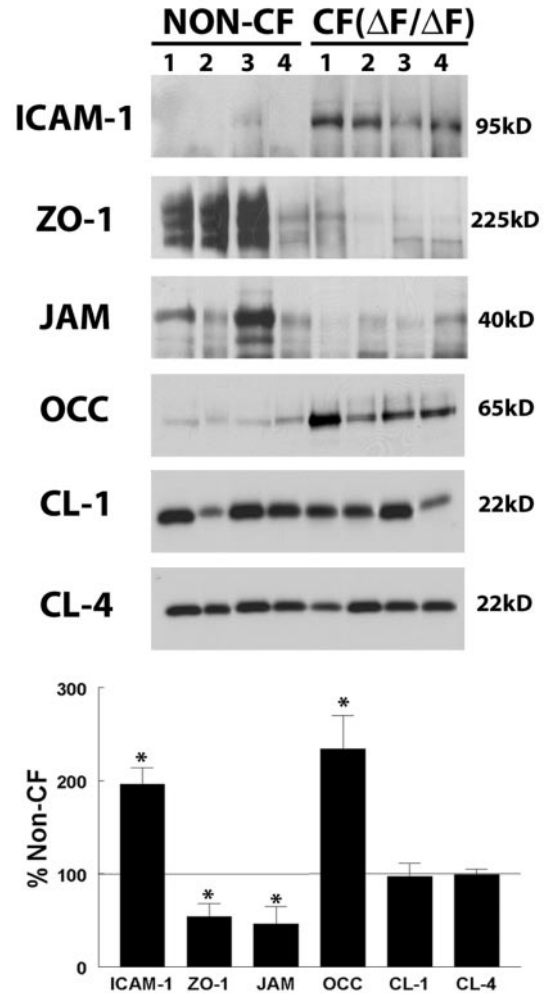


Figure 12. In vivo expression of ICAM-1 and junctional components in non-CF and CF ($\Delta F/\Delta F$) freshly excised bronchus. Total protein isolated from the bronchi of non-CF and CF patients after transplant was subjected to Western blot analysis of ICAM-1 and TJ components as described in MATERIALS AND METHODS. Band intensity was analyzed and expression in CF patients presented as a percentage of non-CF (bottom). *Significantly different from non-CF ($p < 0.05$).

lished a pattern of ion transport defects in CF epithelium both in vitro and in vivo (for review, see Boucher, 1994a, 1994b). Although CF epithelia are impermeable to Cl $^-$ ions via the transcellular pathway, because of the CFTR defect, they exhibit an increased rate of Cl $^-$ absorption in vivo, which would reflect increased permeability of the paracellular pathway to Cl $^-$ ions in CF (Boucher *et al.*, 1986; Wil-lumsen and Boucher, 1989). Because IL-1 β levels have been shown to be markedly increased in CF sputum, our data showing increased P_{Cl^-}/P_{Na^+} ratio induced by IL-1 β may in part explain some of the enhanced absorption of Cl $^-$ ions in CF epithelia. Isotonic transcellular hyperabsorption of sodium (Na $^+$), which occurs because of absent or defective apical membrane CFTR Cl $^-$ channels that are responsible for inhibiting epithelial sodium channels, is accompanied by

hyperabsorption of Cl^- via the paracellular path in CF epithelia and has been proposed as a mechanism by which defective cellular Cl^- transport leads to decreased airway surface liquid height and volume, decreased mucociliary clearance, bacterial infection, and bronchiectasis in CF (Matsui *et al.*, 1998; Wine, 1999; Tarran *et al.*, 2001). This finding might also suggest the induction of a claudin with selective Cl^- permeability, which is in contrast to the recently reported decreased Na^+ permeability induced by expression of claudin-4 (Van Itallie *et al.*, 2001). In contrast, the large changes in R_T and in large solute permeability (P_{app}) induced by $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ suggest that these cytokines are acting via a distinct pathway that either degrades the TJ barrier function greatly or alters the properties of a claudin member with very large equivalent pore radii.

The claudin family of transmembrane proteins play a critical role in maintaining TJ integrity and may be responsible for the selective passage of ions and molecules through the paracellular space (for review, see Tsukita and Furuse, 1999; Tsukita *et al.*, 2001). However, claudin-1 and claudin-4 do not appear to be affected by the exposure of primary HAE cells to $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ (Figure 6), suggesting that claudin-1 and -4 are not involved in the regulation of the TJ mediated by cytokines in the airway epithelium.

Freeze-fracture EM revealed that as a correlate to altered barrier properties of the TJ to ions and solutes, there was also an effect on the morphology of the TJ strands. Non-CF cultures exposed to cytokines had a decrease in the extent of their junctional depth and a modest decrease in strand number (Figure 4A). These data are consistent with previously published data suggesting that cytokine exposure altered TJ ultrastructure in intestinal cell lines (Schmitz *et al.*, 1999b). In contrast to non-CF cultures, those of the CF type appear to exhibit a unique kinetic profile, as evidenced by a significant decrease in R_T and P_{app} after 24 h $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ treatment (Figure 1, C and D). The more rapid effect of cytokines on CF cultures was also seen in freeze-fracture EM at 24 and 72 h after treatment. At 24 h after treatment, the depth and strand number of CF strands resembled those of the non-CF type exposed to cytokines for 72 h. Similarly, JAM and ZO-1 redistribution in CF cultures at 24 h was similar to that of non-CF cultures at 72 h (Figure 5).

To determine whether $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ exposure of primary HAE cells altered the organization of TJ components, we performed QRT-PCR, Western blotting, ELISA, and immunofluorescence studies. We found that several components of the TJ are affected by cytokine exposure. In particular, ZO-1 and JAM were downregulated and redistributed after cytokine exposure (Figure 5). Previous studies of the effects of cytokines on proteins involved in the maintenance of TJ integrity have focused primarily on cell lines of intestinal origin. Previous work in HT-29/B6 cells, a human intestinal cell line, suggested that the effects of $\text{TNF-}\alpha$ on the TJ are mediated via downregulation of occludin (Mankertz *et al.*, 2000). Occludin exists in both an LMW and an HMW form, with the HMW form corresponding to an increased state of phosphorylation. In our study, the expression of the LMW form of occludin remained unchanged, whereas the expression of the HMW form increased, indicating a role for occludin phosphorylation in the cytokine-mediated effects on the TJ, perhaps as an attempt to reseal leaky junctions (Wong, 1997).

JAM is a member of the immunoglobulin superfamily localized to the TJ and may play a key role in monocyte transmigration across the paracellular pathway (Ozaki *et al.*, 1999). Therefore, redistribution from the TJ and decreased expression of JAM after $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ exposure may suggest a decreased ability of monocytes to penetrate to the lumen and perpetuate the inflammatory response. Because CF airways are filled with neutrophils, we postulated that reciprocal regulation of JAM and ICAM-1 might occur with cytokine treatment. Chronic $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ treatment increased ICAM-1 expression at both the mRNA and protein levels, with a relative decrease in JAM (Figure 7). These data indicated a shift in the epithelium chronically exposed to $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$, from that which allows the migration of monocytes to one that is much more prone to the passage of neutrophils via the paracellular pathway. This observation was supported by published data showing increased levels of neutrophils and soluble ICAM-1 in CF sputum, consistent with a predominant influx of neutrophils through the paracellular pathway into the lumen (Salva *et al.*, 1996). The *in vivo* results presented here in CF freshly excised airways are consistent with this hypothesis (Figures 11 and 12).

The effect of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ on the airway TJ appears to reflect widespread effects on several protein components. To investigate which signaling pathways activated by the combined treatment of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ may be relevant to TJ function, we tested agents reported to inhibit two pathways known to regulate TJ permeability, tyrosine kinases and PKC. The tyrosine kinase inhibitor genistein was ineffective in blocking the cytokine-mediated effect on TJ barrier function. In contrast, the PKC inhibitors H7 and chelerythrine significantly inhibited the increase in P_{app} to both intermediate- and large-sized molecules induced by cytokines (Figure 8). However, the inhibition of the decrease in R_T was less complete, indicating that whereas PKC is involved in the pathway regulating the cytokine-induced effects on permeability to solutes, it may have limited effects on ionic permeability. These data support a model of cytokine regulation of TJ barrier function in which there are distinct properties for the regulation of permeability to solutes and ions, one involving PKC.

The family of aPKC kinases consists of two distinct members, $\text{PKC}\iota/\lambda$ and $\text{PKC}\zeta$, that are insensitive to Ca^{2+} , diacylglycerol, and phorbol esters, which are known to be potent activators of the calcium-dependent (cPKCs) and novel (nPKCs) subfamilies but are instead regulated by lipid cofactors (Dempsey *et al.*, 2000). We focused on the effects of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ on the expression of both $\text{PKC}\iota/\lambda$ and $\text{PKC}\zeta$, because of its reported interaction with JAM and our data indicating that cytokine exposure affected JAM localization and expression. After cytokine treatment, there was an increase in the expression of $\text{PKC}\iota/\lambda$, as revealed by Western blot analysis and immunofluorescence, while $\text{PKC}\zeta$ remained constant (Figure 10). Of note, the diffuse cytoplasmic staining for $\text{PKC}\iota/\lambda$ in the apical region of the cells is consistent with studies of $\text{PKC}\iota/\lambda$ in MDCK cells (Dodane and Kachar, 1996). In addition, this increase was blocked when cells were cotreated with H7, presumably because of the chronic exposure of the epithelium to H7 for 72 h. Because H7 also inhibits PKA, we also examined the effect of chelerythrine on the increase in $\text{PKC}\iota/\lambda$ expression. Whereas H7 partially inhibited the increase in expression of

PKC ι/λ , chelerythrine did not, presumably indicating the inhibition of PKA by H7 upstream of PKC ι/λ . This conclusion is supported by published data implicating a role for PKA-mediated regulation of aPKCs by adenosine receptors (Huang *et al.*, 2001a), which are known to be expressed in HAE cells.

The aPKC isoform PKC ι/λ has also been identified as a potential regulator of apoptosis in several cell types, and inhibition of this isoform may be necessary for the induction of apoptosis (Murray and Fields, 1997; Xie *et al.*, 2000). We did detect a modest increase in lactate dehydrogenase (LDH) levels after treatment with TNF- α and IFN- γ in combination (data not shown), consistent with the possible apoptotic events. However, the reversible effect of TNF- α and IFN- γ treatment, with R_T levels returning to normal levels within 24 h (Figure 1B) and specific effects on TJ proteins, would suggest a role for PKC ι/λ independent of apoptosis in the cytokine-induced alteration at the TJ, perhaps via its interactions with JAM. In addition, LDH levels were similar in cultures treated with TNF- α and IFN- γ alone to those treated with TNF- α and IFN- γ in combination, although only combined treatment elicits effects on junctional components (data not shown).

In summary, we examined the effects on TJ structure and function of exposure of primary HAE cultures to cytokines known to be present in CF. Chronic exposure of TNF- α and IFN- γ caused significant effects on both R_T and P_{app}. The mechanism by which TNF- α and IFN- γ altered TJ permeability may involve signaling via PKC ι/λ , because inhibitors of PKC, H7 and chelerythrine, inhibited the effects of cytokines on R_T and P_{app} and prevented the increase in expression of ICAM-1 and decrease in JAM. Although the majority of our studies were conducted in a model system of WD polarized airway epithelium, changes in expression of JAM, ZO-1, and ICAM-1 induced by cytokines correlated with the expression of these proteins in chronically inflamed excised CF airways. In addition, the distinct effects induced by TNF- α and IFN- γ synergistically compared with IL-1 β suggest that epithelium exposed to a mixture of cytokines, as is the CF airway, may exhibit extreme alterations in the barrier of the TJ to solutes and ion species.

In our study, the cytokine-induced alteration in the TJ may help explain several associated findings in CF, namely, increased Cl⁻ absorption through the paracellular pathway in response to accelerated Na⁺ absorption and the ability to increase neutrophil transmigration caused by increased ICAM-1 levels. With regard to other inflammatory diseases, the pattern of cytokine-induced alteration of TJs may similarly contribute to the severity and type of inflammation observed. Given the ability of H7 and chelerythrine to inhibit TJ effects on permeability and ICAM-1 expression, a clearer understanding of cytokine effects on TJs may therefore permit the ability to pharmacologically modulate the severity of chronic inflammation.

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