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Cytokines induce tight junction disassembly in airway cells via an EGFR-dependent MAPK/ERK1/2-pathway

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Epithelial barrier permeability is altered in inflammatory respiratory disorders by a variety of noxious agents through modifications of the epithelial cell structure that possibly involve tight junction (TJ) organization. To evaluate *in vitro* whether pro-inflammatory cytokines involved in the pathogenesis of respiratory disorders could alter TJ organization and epithelial barrier integrity, and to characterize the signal transduction pathway involved Calu-3 airway epithelial cells were exposed to TNF-α, IL-4 and IFN-γ to assess changes in: (a) TJ assembly, that is, occludin and zonula occludens (ZO)-1 expression and localization, evaluated by confocal microscopy; (b) apoptotic activity, quantified using terminal transferase deoxyuridine triphosphate nick-end labeling staining; (c) epithelial barrier integrity, detected as transmembrane electrical resistance and expressed as G_T values; (d) epidermal growth factor receptor (EGFR)-dependent mitogen-activated protein (MAP) kinase (MAPK)/extracellular signal-regulated kinases (ERK)1/2 phosphorylation, assessed by western blotting. Exposure to cytokines for 48 h induced a noticeable downregulation of the TJ transmembrane proteins. The degree ZO-1 and occludin colocalization was $62 \pm 2\%$ in control cultures and significantly decreased in the presence of TNF-α ($47 \pm 3\%$), IL-4 ($43 \pm 1\%$) and INF-γ ($35 \pm 3\%$). Although no apoptosis induction was detected following exposure to cytokines, changes in the epithelial barrier integrity were observed, with a significant enhancement in paracellular conductance. G_T values were, respectively, 1.030 ± 0.0, 1.300 ± 0.04, 1.260 ± 0.020 and 2.220 ± 0.015 (mS/cm^2) $\times$ 1000 in control cultures and in those exposed to TNF-α, IFN-γ and IL-4. The involvement of EGFR-dependent MAPK/ERK1/2 signaling pathway in cytokine-induced damage was demonstrated by a significant increase in threonine/tyrosine phosphorylation of ERK1/2, already detectable after 5 min incubation. All these cytokine-induced changes were markedly prevented when Calu-3 cells were cultured in the presence of an EGFR inhibitor (AG1478, 1 μM) or a MAP kinase inhibitor (U0126, 25 μM). In conclusion, cytokine-induced epithelial injury includes TJ disassembly and epithelial barrier permeability alteration and involves the EGFR-dependent MAPK/ERK1/2 signaling pathway.
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One of the major functions of the airway epithelium is to act as a selective barrier, maintaining the integrity of tissue compartments and impeding entry of inhaled microorganisms, irritants and allergens.[1] Epithelial barrier integrity is maintained by adhesive interactions occurring at the cell–cell and cell–matrix contacts by junctional proteins and focal adhesion complexes that are anchored to the cytoskeleton.[2] This intercellular junctional complex includes tight junctions (TJs), adherens junctions, desmosomes and gap junctions.[2,3] TJs are organized by the interactions between the transmembrane proteins (occludin, claudins and junction adhesion molecule) and the intracellular plaque zonula occludens (ZO)-1, ZO-2, ZO-3, cingulin, 7H6 and others and the actin cytoskeleton.[4–7] The plaque protein ZO-1 is shown to have a unique role in the formation of TJs during epithelial cellular polarization. At the initial stage of cell–cell contact of epithelial cells, primordial spot-like junctions are formed at the tips of thin cellular protrusions radiating from adjacent cells, where ZO-1 are precisely concentrated. In epithelial cells,[8] but also in mesenchymal cells,[9,10] occludin is accumulated at the ZO-1-positive spot-like junctions to form continuous TJ during the configuration of cell polarity.

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A significant body of evidence indicates that TJs are associated with numerous intracellular signaling molecules, and are regulated by the activity of signal transduction pathways.[4] Pro-inflammatory cytokines have been found to negatively affect epithelial barrier functions *in vitro* in experiments mainly conducted on the intestinal epithelial cell lines. Although epithelial damage and increased epithelial barrier permeability is also common in inflammatory conditions of the airways,[11–13] a limited information is available on the airway epithelial cells.[3]

Several mechanisms can be responsible for the cytokine-induced disruption of epithelial barriers, including expressional downregulation of junctional proteins. A variety of mediators possess the capability to alter TJ structure and barrier function on a variety of epithelial cell types,[11,14,15] but few studies have been performed on the bronchial epithelial cells,[16–23] exploring the signaling pathways involved.[19,21] Specifically, it has never been evaluated the possible engagement of the epidermal growth factor receptor (EGFR) signaling pathways, known to promote various cellular processes by phosphorylation of mitogen-activated protein (MAP) kinases.[24] Activation of the MAP kinases-extracellular signal-regulated kinases[25] (MAPK/ERK)1/2 has been associated not only with cell survival, proliferation, growth and differentiation[24] but also with TJ protein disruption.[12,26,27] A study was therefore designed to evaluate whether exposure to pro-inflammatory cytokines involved in the pathogenesis of infectious and allergic respiratory disorders could alter TJ and epithelial barrier integrity by dysregulation of ZO-1 and occludin and to define the possible involvement of EGFR/MAPK/ERK1/2 cascade.

MATERIALS AND METHODS

Epithelial Cell Culture

Calu-3[28] adenocarcinoma cells (a kind gift from Dr Diego Ferrone, University of Genova, Italy) were selected for these experiments because of their highly characterized intercellular adhesion molecules and ability to form monolayers that are highly impermeable to ionic and macromolecular flux.[29,30] Cells were grown in T-75 culture flasks in an atmosphere of 5% CO_2 at 37 °C, and used in passage numbers 20–30. For all experiments comparing different culture conditions, cells in similar passage numbers were used. The cells were maintained in a 1:1 mixture of DMEM/Ham's F12 medium (Euroclone, Europe) supplemented with fetal bovine serum, non-essential amino acids and penicillin/streptomycin (Euroclone, Europe). Cell viability was evaluated by trypan blue dye exclusion test (Euroclone).

Occludin and ZO-1 Expression and Localization

To evaluate TJs (occludin and ZO-1) expression and localization, Calu-3 cells were grown to confluence on coverslips and incubated for 48 h with TNF-α (10 ng/ml),[31] IL-4 (5 ng/ml)[17] and IFN-γ (50 ng/ml)[17] (Invitrogen, Milano, Italy). At these possibly clinically relevant doses,[18] these cytokines were reported to induce barrier breakdown in a rapid, dose-dependent fashion in Calu-3.[16–19,31] Preliminary experiments in our lab confirmed the results reported by others. Cells were then permeabilized and stained as previously described.[4,12]

After blocking, the preparations were incubated with mouse anti-human occludin and anti-rabbit human ZO-1 (Zymed Laboratories San Francisco, CA, USA), washed and incubated with Alexa-fluor-488 (green) goat antimouse and Alexa-fluor 647 (red) goat antirabbit (Invitrogen SRL, Italy).

For the negative control, normal mouse (for occludin) or rabbit (for ZO-1) non-specific immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at the same protein concentration as the primary antibody. Both green and red fluorescence were analyzed by confocal laser scanning microscopy (TCS SL microscope; Leica; Mannheim, Germany). Images were collected at the height of the layer where TJs are known to be expected.[17]

The quantitative estimation of colocalized proteins in immunocytochemical studies has been performed calculating the colocalization coefficients[32] from the red- and green-channel scatterplot.

Colocalization coefficients express the fraction of colocalizing molecular species in each component of a dual-colour image and are based on the Pearson's correlation coefficient, a standard procedure for matching one image with another in pattern recognition.[33] If two molecular species are colocalized, the overlay of their spatial distributions has a correlation value higher than what would be expected by chance alone. Costes *et al*,[34] developed an automated procedure to evaluate correlation between the green and red channels with a significance level $>95\%$. The same procedure automatically determines an intensity threshold for each colour channel based on a linear least-square fit of the green and red intensities in the image's two-dimensional correlation cytofluorogram. Costes'[34] approach has been accomplished by macro routines integrated as plugins (WCIF Colocalization Plugins, Wright Cell Imaging Facility, Toronto Western Research Institute, Canada) in the ImageJ 1.45*g* software (Wayne Rasband, NIH, USA).

Determination of DNA Damage

Briefly, DNA damage was evaluated using terminal transferase deoxyuridine triphosphate nick-end labeling (TUNEL) staining following manufacturer's instructions (Roche Diagnostics GmbH, Germany), Briefly, Calu-3 cells (5×10^{-4} per well) were plated in 24-well till subconfluency, and then exposed for 48 h to TNF-α (10 ng/ml), IL-4 (5 ng/ml) and IFN-γ (50 ng/ml).

As apoptotic cells are extruded from epithelia 'anoikis' fairly rapidly[35] 48 h following cytokine exposure, the apical surfaces of epithelia were washed to collect extruded cells while Calu-3 cells were trypsinized. The PBS-washed and -trypsinized cells were then loaded into a cytospin apparatus, cytocentrifuged onto polysine glass slides, fixed in

4% paraformaldehyde in PBS for 1 h at room temperature and washed with PBS. Cells were then permeabilized and labeled as previously described.[12] Rinsed slides were then coverslipped with a mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) for nuclear counterstaining. TUNEL$^+$ apoptotic cells, which fluoresce bright green, were viewed with a Nikon Eclipse E1000 fluorescent microscope (Nikon, Tokyo) equipped with a FITC filter. To avoid erroneously false–positive results, Calu-3 cells treated with DNase I were used as a positive control.

Measurement of Transepithelial Electrical Resistance (TER)

To assess epithelial barrier integrity, Calu-3 were plated (0.5×10^6cells per well) and cultured for 5 days and then exposed to TNF-α (10 ng/ml), IL-4 (5 ng/ml) and IFN-γ (50 ng/ml) for 48 h. As a positive control, the Ca^{2+} chelator EDTA, 1 mM, was used to increase cell permeability and added to the apical site of the Calu-3 cell cultures for 2 h.[36] TER ($\Omega.cm^2$) or total conductance (G_T) were measured with an epithelial voltmeter (Millipore-ERS, Millipore) using chopstick-like electrodes as a measurement of tight junctional barrier formation. For experiments, cells were seeded at high density on Snapwell inserts (Costar, Corning) and maintained at 37 °C in a 5% CO2, 95% air atmosphere, as previously described.[37]

TER values shown as G_T ($1/TER \times 1000$, mS/cm^2)[37] were obtained by subtracting blank filter resistance ($30\,\Omega.cm^2$) from all readings.

Immunoblotting for Native and Activated ERK1/2

Calu-3 cells (0.5×10^6 cells per well) were cultured in Petri culture dishes to 90% confluence and then exposed to TNF-α (10 ng/ml), IL-4 (5 ng/ml) and IFN-γ (50 ng/ml) for 5 min. The same amount of protein from each line (50 μg) was separated by 10% SDS-PAGE, transferred to PVDF membranes. Membrane was blotted for rabbit anti-human total ERK1/2 (Tot-ERK1/2) and phosphorylated ERK1/2 (P-ERK1/2; Cell Signalling Technology, Beverly, MA, USA).[17] After incubation with HPR-conjugated anti-rabbit antibody (Cell Signaling Technology), the bands were detected using enhanced chemoluminescence (ECL, Pierce, Celbio, Italy) and relevant band intensities were quantified using a Versadoc Imaging System model 3000 (Biorad Laboratories, Hercules, CA, USA).

Involvement of EGFR/MAPK/ERK1/2 Pathway in Cytokine-Induced Changes

To demonstrate the involvement of the EGFR-dependent MAPK/ERK1/2 signaling pathway, in some experiments, cells were pre-incubated for 1 h with the EGFR tyrosine kinase inhibitor AG1478 (1 μM; Calbiochem Inalco SpA; Milan, Italy) or the MAP kinase (MEK) inhibitor, U0126 (25 μM; Sigma-Aldrich, St Louis, MO, USA)[12] before being exposed to TNF-α, IL-4 and IFN-γ.

Statistical Analysis

Statistical evaluation was performed using the statistical software package GraphPad Prism 3.02 (GraphPad Software, San Diego, CA, USA). The results from relevant band intensities and colocalization analysis were expressed as mean ± s.e.m. and Paired *t* test or Unpaired *t* test were used. Probability values ($P<0.05$) were considered as statistically significant.

RESULTS

TJ Expression in Calu-3 Cells

Calu-3 cells cultures showed a strong positivity for occludin (green fluorescence) (Figure 1a) and ZO-1 (red fluorescence) (Figure 1b) appearing as contiguous rings around the peripheral margins of the cells, colocalized at sites of cell–cell contact (Figure 1c). Figures 1d–f show the negative control. Exposure to TNF-α, IL-4 or INF-γ for 48 h induced a noticeable downregulation of TJs, with a stronger suppression of ZO-1 than of occludin (Figures 1a–q) confirmed by quantitative analysis (Figure 2). The degree ZO-1 and occludin colocalization detected in control cultures (62 ± 2%) was decreased in the presence of TNF-α (47 ± 3%; $P<0.05$), IL-4 (43 ± 1%; $P<0.01$) and INF-γ (35 ± 3%; $P<0.001$), with a significant difference between the damage induced by INF-γ, as compared with TNF-α ($P<0.01$) and IL-4 ($P<0.05$).

Determination of DNA Damage by TUNEL Assay

To evaluate whether the disassembly of TJs could be related to pro-apoptotic effects, modifications of cell viability were tested as DNA damage by TUNEL assay. As shown in Figure 3, only a very low number of cells exhibiting a faint fluorescent signal was detected in control cultures (Figure 3a). We treated Calu 3 cells with Dnase I as a positive control (Figure 3b). Neither significant morphological changes nor DNA damage was observed in Calu-3 after exposure to TNF-α, IL-4 or IFN-γ (Figures 3c–e).

Transepithelial Electrical Resistance

As expected, exposure to Ca^{2+} chelator EDTA for 2 h caused a significant increase in paracellular conductance, G_T levels being 0.705 ± 0.06 (mS/cm^2) × 1000 in control cultures and 1.513 ± 0.05 (mS/cm^2) × 1000 in EDTA exposed cultures ($P<0.001$) (Figure 4a). Similarly, also exposure to TNF-α, IL-4 and IFN-γ for 48 h resulted in a detectable reduction in barrier function, associated with an enhancement in paracellular conductance (Figure 4b). G_T values, 1.030 ± 0.03 (mS/cm^2) × 1000 in control cultures, were increased at similar levels after exposure to TNF-α and IFN-γ (1.300 ± 0.04 (mS/cm^2) × 1000 and 1.260 ± 0.020 (mS/cm^2) × 1000, respectively; $P<0.0001$, each comparison), and even more in cells exposed to IL-4 (2.220 ± 0.015 (mS/cm^2) × 1000; $P<0.001$)).

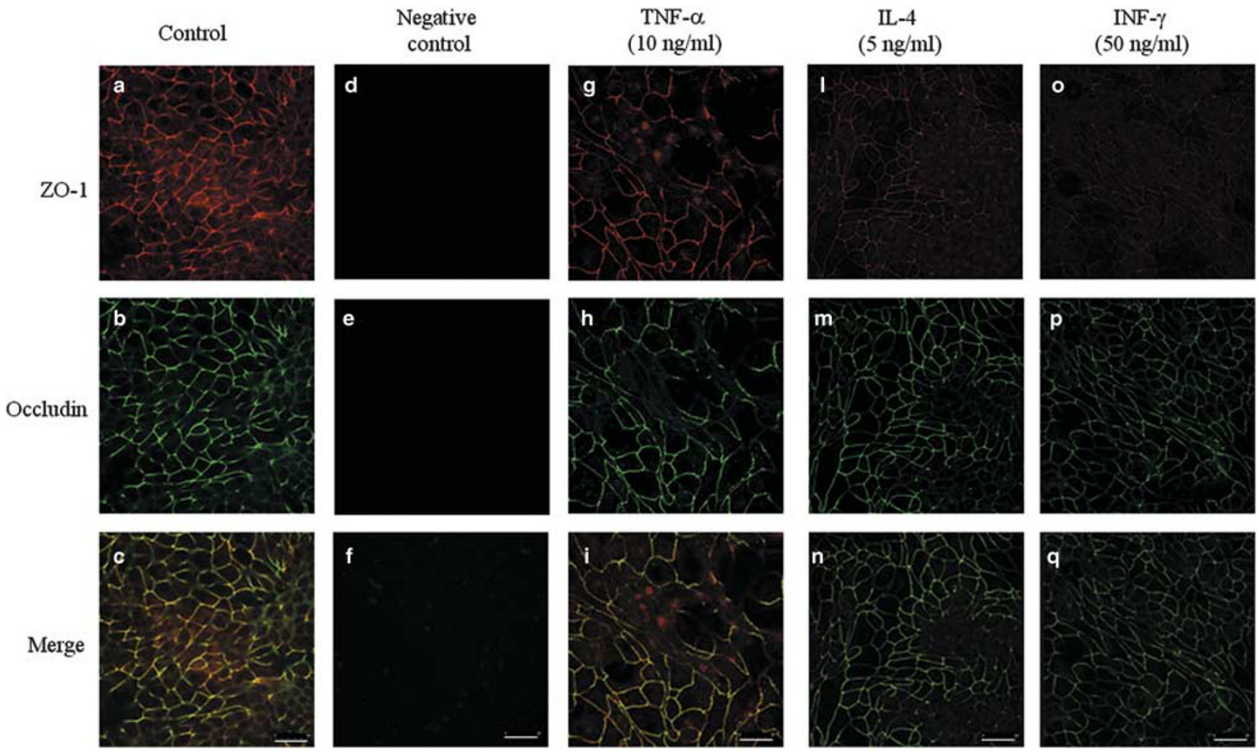

Figure 1 Representative photomicrographs obtained by fluorescent microscopy after immunofluorescence staining of zonula occludens (ZO)-1 and occludin expression in Calu-3 cells under basal culture conditions (**a**–**f**) or after stimulation for 48 h with TNF-α, (**g**–**i**), IL-4 (**l**–**n**) or INF-γ (**o**–**q**). Red staining (top panels) represents ZO-1, green staining (middle panels) occludin and (bottom panels) merged images of both occludin and ZO-1, where areas of colocalization appear as yellow. Unstimulated cells (**a**–**c**) stained with normal mouse (for occludin) or rabbit (for ZO-1) non-specific immunoglobulins were used as negative control. Original magnification $\times 63$. Each picture is representing one confocal layer. The results shown are representative of the results obtained in six independent experiments. Scale bar: 50 μm.

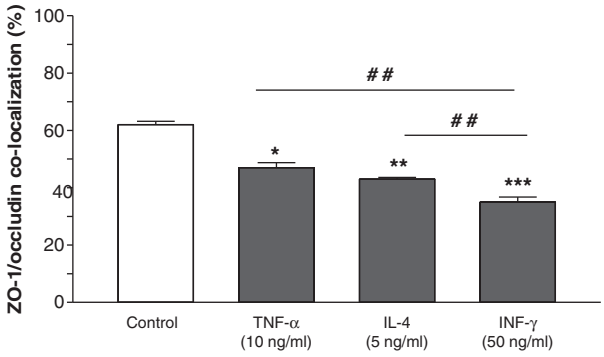

Figure 2 Quantitative evaluation of the percentage of zonula occludens (ZO)-1/occludin colocalization in Calu-3 cells under basal culture conditions (Control) or after exposure to TNF-α, IL-4 or INF-γ. Data are reported as mean ± s.e.m.. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, *versus* control; $^{\#\#}P<0.01$ *versus* INF-γ-treated cells.

ERK1/2 Pathway Activation

To evaluate the possible involvement ERK1/2 pathway, Calu-3 cells were exposed to TNF-α, IL-4 and IFN-γ for 5 min. An increased threonine/tyrosine phosphorylation of ERK1/2 was already detectable by western blotting at 5 min (Figure 5a), with a detectable increase of the phosphorylated/Tot-ERK1/2 ratio, as compared with unstimulated cultures. Quantitative analysis showed a similar increase of the phosphorylated/Tot-ERK1/2 ratio in cell cultures exposed to TNF-α, IL-4 and IFN-γ ($P<0.01$, each comparison) (Figure 5b).

Involvement of EGFR in Cytokine-Induced Activation of the ERK1/2 Pathway

To determine whether EGFR is required for cytokine-induced ERK1/2 activation, Calu-3 cells were pre-incubated with AG1478 or with U0126. Pre-incubation of the cells for 1 h with AG1478 almost completely prevented the cytokine-induced ERK1/2 phosphorylation (Figure 6a) and the associated changes in the phosphorylated/Tot-ERK1/2 ratio. The quantitative analysis showed that the degree of inhibition was similar in cell cultures stimulated with TNF-α, IL-4 and IFN-γ ($P<0.001$, each comparison) (Figure 6b). Similar results were observed when the cells were pre-incubated with U0126 (Figure 6).

Involvement of ERK1/2 Pathway Activation via EGFR-MEK in the Cytokine-Induced Damage

Confocal microscopy showed that pre-exposure of Calu-3 cells to AG1478 or U0126 almost completely prevented ZO-1 and occludin junctional organization induced by TNF-α

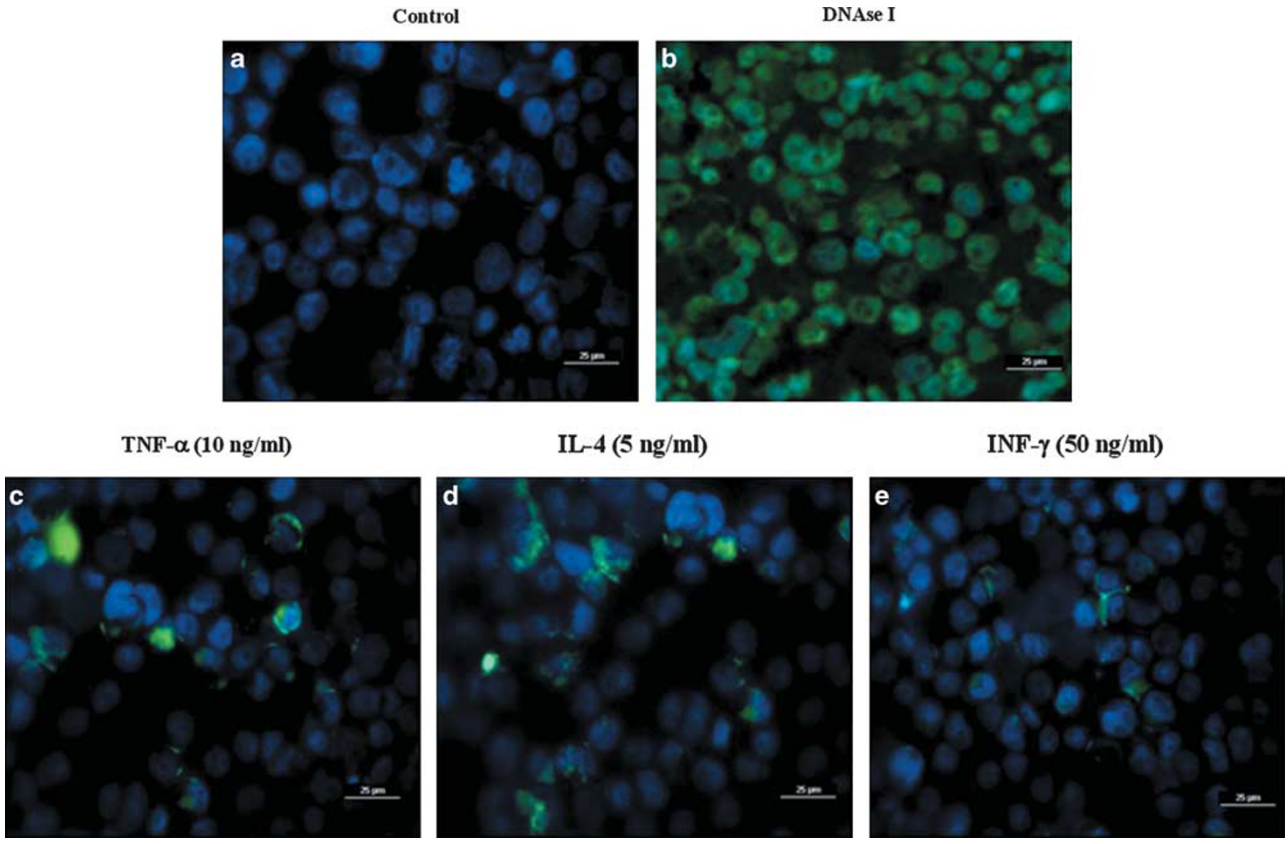

Figure 3 Representative photomicrographs obtained by fluorescent microscopy after immunofluorescence staining of the analysis of DNA cleavage by terminal transferase deoxyuridine triphosphate nick-end labeling (TUNEL) assay in Calu-3 cultured in medium alone (Control), (**a**) with DNAse I (positive control) (**b**) or in the presence of TNF-α, (**c**) IL-4 (**d**) and INF-γ (**e**) for 48 h. Apoptotic nuclei are displayed by green fluorescence whereas viable DAPI counterstained nuclei appeared blue. Original magnification × 40. The results shown are representative of five independent experiments. Scale bar: 25 μm.

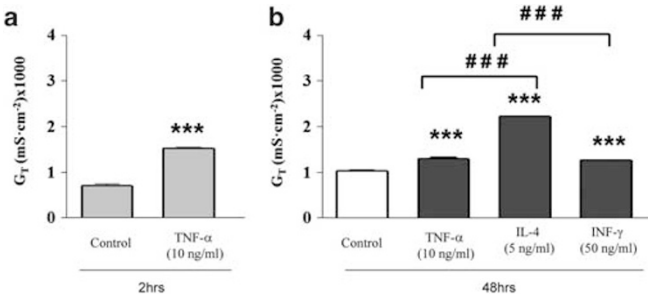

Figure 4 Quantitative evaluation of the effects of EDTA, TNF-α, IL-4 and INF-γ on transepithelial electrical resistance (TER) in Calu-3 cells under basal culture conditions (Control) or after stimulation for 2 h with EDTA (1 mM) (**a**) and for 48 h TNF-α, IL-4 and INF-γ (**b**). Data are reported as mean ± s.e., ($n = 3$). The results shown are representative of five independent experiments. $^{***}P < 0.001$ *versus* control; $^{\#\#\#}P < 0.01$ *versus* IL-4-treated cells.

(Figures 7a–c), IL-4 (Figures 7d–f) and INF-γ (Figures 7g–i). These modifications were confirmed by quantitative analysis, showing that both AG1478 and U0126 significantly and similarly inhibited the degree of TJ damage induced by TNF-α ($P < 0.05$), IL-4 ($P < 0.01$) and INF-γ ($P < 0.05$) (Figure 8). The addition of AG1478 or U0126 to confluent Calu-3 before cytokine exposure also completely restored the barrier dysfunction induced by TNF-α ($P < 0.05$), IL-4 ($P < 0.01$) and INF-γ ($P < 0.05$) (Figure 9).

DISCUSSION

The present study shows that exposure to TNF-α, IL-4 or IFN-γ induced a remarkable reduction in the expression of transmembrane TJ components, occludin and ZO-1, associated with their spatial dislocation, through an EGFR-dependent MAPK/ERK1/2 signaling pathway. The disassembly of TJs observed was associated with modification of the epithelial barrier integrity leading to a decrease in TER. Cell apoptosis was not detected by TUNEL assay, suggesting that TJ disassembly may be an 'early event,' a sensitive indicator of initial bronchial epithelial cells injury, before DNA damage can be perceived. Apoptotic cells are extruded from epithelia 'anoikis' fairly rapidly. The quantification of DNA damage, or evaluation of apoptosis can be missed at any given time if the assays are done in the epithelia.[35] Epithelial barrier integrity and functions are determined by lateral adhesive plasma membrane structures known as intercellular junctions, including ZO-1 and occludin, which constitute the TJs[38] ZO-1 has a pivotal role in TJ formation and organization by linking

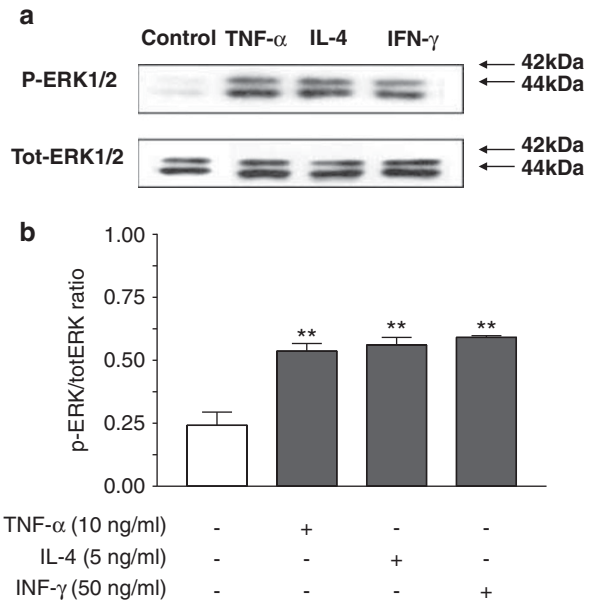

Figure 5 Cytokine-induced phosphorylation of extracellular signal-regulated kinases (ERK)1/2. (**a**) Representative western blots for total (Tot-ERK1/2) and phosphorylated ERK1/2 (P-ERK1/2) levels in Calu-3 cultured with medium alone (Control) or exposed to TNF-α, IL-4 and INF-γ for 5 min. Arrows indicate the molecular size of the ERK1 (42 kDa), ERK2 (44 kDa). (**b**) After densitometric analysis, P-ERK 1/2 data were normalized to Tot-ERK1/2 and plotted as means ± s.e.m.. The results shown are representative of five independent experiments. $^{**}P < 0.01$ *versus* control.

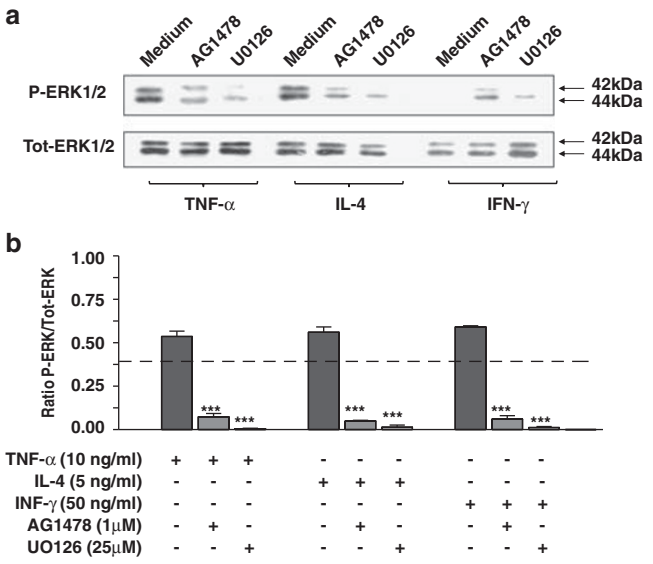

Figure 6 Detection by western blotting of the effect of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (AG1478) and mitogen-activated protein kinase kinase (MEK) inhibitor (U0126) on cytokine-induced extracellular signal-regulated kinases (ERK)1/2 phosphorylation. (**a**) Representative western blots for total (Tot-ERK1/2) and phosphorylated ERK1/2 (P-ERK1/2) levels in Calu-3 cultured with medium alone (Medium) or exposed to TNF-α, IL-4 and INF-γ for 5 min, in the presence of AG1478, 1 μM or U0126, 25 μM. (**b**) Densitometric analysis of P-ERK1/2 data, normalized to Tot-ERK1/2 and plotted as means ± s.e.m.. Dotted line represents P-ERK/Tot-ERK ratio under basal conditions (Control). The results shown are representative of five independent experiments. $^{***}P < 0.001$ *versus* cells exposed to cytokines in cultures without AG1478 or U0126.

the transmembrane protein occludin to other cytoplasmic components of the TJ complex and to the actin cytoskeleton.[2] Occludin is an integral membrane protein localizing at TJ with four transmembrane domains and a long COOH-terminal cytoplasmic domain, necessary for links to ZO-1 and the cytoskeleton.[2]

However, it has been shown that occludin is not required for the formation of TJ strands in the tissues such as the intestine, liver and kidney of knockout mice, but it is suggested that claudins, another transmembrane TJ protein, take over the role of occludin.[39]

TJs possess significant adhesive strength to maintain the integrity of epithelial layers and to form a tight barrier to paracellular flux of macromolecules, but these junctional complexes are also characterized by an intrinsic plasticity manifested as the ability to rapidly disassemble and reestablish their structures.[40] This junctional plasticity is essential in normal condition, but if occurs at a higher rate, as in disease states, may result in TJ disassembly with breakdown of epithelial barrier integrity.[41,42] Disruption of TJ structure and increased epithelial permeability, induced by pollutants, microbial products, oxidative agents, proteases and cytokines, is a common event in acute and chronic inflammatory conditions.[4,11,12,38,43] Elevated levels of pro-inflammatory cytokines involved in the pathogenesis of a variety of acute and chronic inflammatory diseases have been implicated in the endothelial and epithelial barrier dysfunction through cytoskeletal rearrangement in a variety of tissues.[3,4,11–17,44–46] However, variable effects of these cytokines on the TJ cytoplasmic plaque protein ZO-1 and TJ structure and on epithelial permeability have been described in the few studies performed on airway epithelial cells. Evaluating 16HBE14o-cells by transmission electron microscopy, no effect of IL-4, IL-8, IL-13 or IFN-γ on ZO-1 expression was reported,[16] whereas the combination of IL-4 and IFN-γ appeared to be able to induce gaps in TJs.[16] Exposure of Calu-3 cells to IL-4, IL-13 and IFN-γ gave contrasting results, with dissociation between TJ structure and function.[17] Indeed, analysis of the TJ-associated proteins by immunoblotting showed that IL-4 and IL-13 significantly reduced ZO-1 expression and modestly decreased occludin, whereas IFN-γ downregulated ZO-1 and occludin expression to almost undetectable levels.[17] Surprisingly, whereas IL-4 and IL-13 treatment resulted in a 70–75% decrease in barrier function at 24, 48 and 72 h incubation, in the presence of IFN-γ no changes were observed at 24 and 48 h incubation followed by a threefold enhancement at 72 h.[17] No adequate explanation was provided by the authors to account for the paradoxical effect of IFN-γ on barrier function and ZO-1 expression.[17]

Using Calu-3 cells, we also found a more remarkable reduction of ZO-1 than of occludin expression in response to TNF-α, IL-4 and IFN-γ, and a more remarkable occludin and ZO-1 spatial dislocation in response to IFN-γ than to TNF-α.

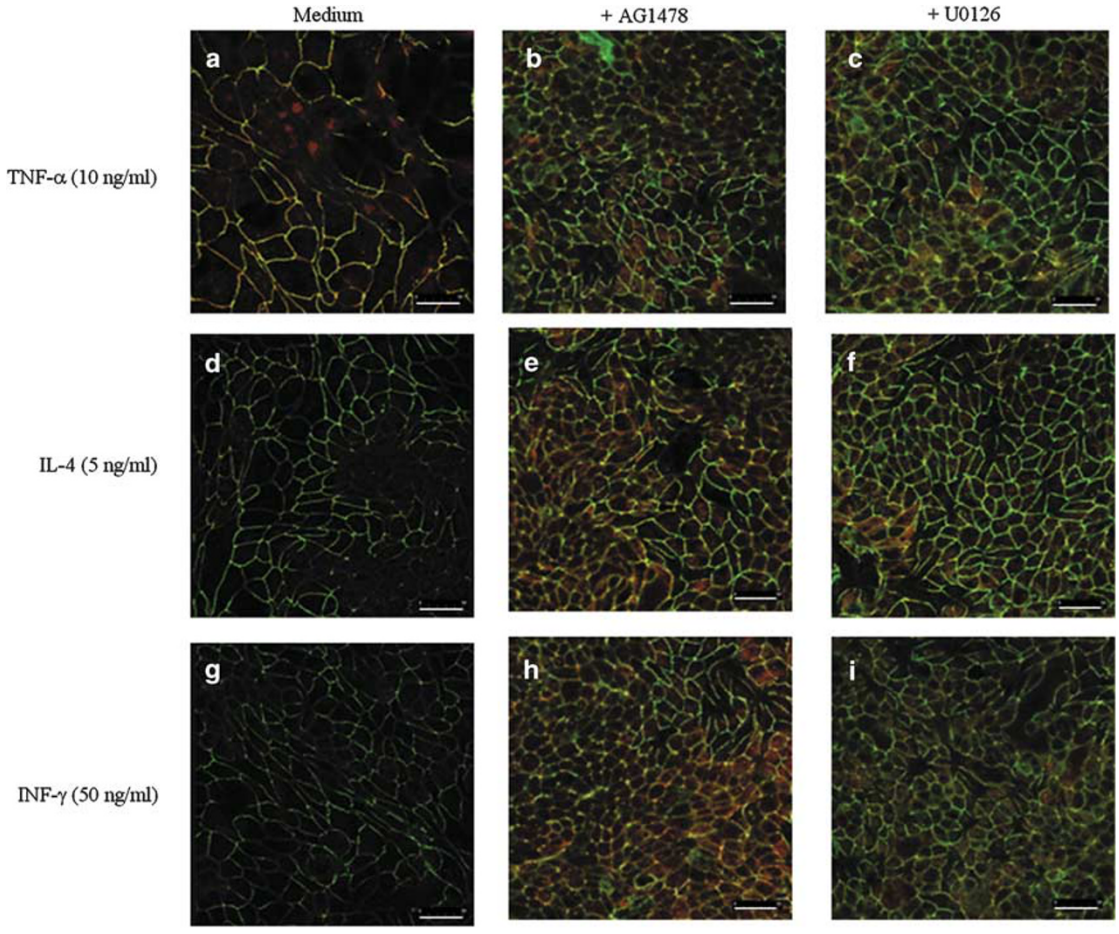

Figure 7 Effects of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinases (ERK)1/2 inhibitors on tight junction (TJ) disassembly induced by 48-h exposure to cytokines. Representative merged photomicrographs obtained by confocal microscopy after immunofluorescence staining, showing the inhibitory effect of pre-exposure of Calu-3 cells to AG1478 or UO126 on zonula occludens (ZO)-1 and occludin junctional disruption induced by TNF-α (**a**–**c**), IL-4 (**d**–**f**) and INF-γ (**g**–**i**). Original magnification $\times 63$. The results shown are representative of the results obtained in four independent experiments. Scale bar: 50 μm.

TJ assembly disruption was associated with modification of the epithelial barrier integrity. In fact, the specific different concentrations of TNF-α, IL-4 or INF-γ used to exanimate the effect on TJ protein reduction demonstrated a significant different decrease in TER in response to all three cytokines. In agreement with the results of the present paper, treatment of mouse tracheal epithelial cell monolayers with TNF-α, IFN-γ and IL-1β resulted in a markedly decreased TER after 24 h.[19] However, we also found a dissociation between the degree of cytokine-induced TJ disruption (highest for IFN-γ) and TER decrease (highest for IL-4), unexpected because of the importance ascribed to ZO-1 and occludin in the regulation of TJ function. In a different experimental model, exposure of primary human airway epithelial cells (16HBE14o-) to the nitric oxide donor (DETA-NO) resulted in an altered distribution and expression of TJ proteins (claudin-1 and occludin), not associated with significant barrier disruption.[19]

One explanation may be the redundancy of function provided by claudins, a new family of transmembrane proteins that also localize to TJs.[47] There are $\geq$20 known claudins, several of which interact with many of the same proteins as occludin.[47] The existence of proteins with functional redundancy to occludin has been inferred from studies with occludin-deficient knockout mice that retain the ability to form morphologically TJs possessing normal barrier function.[39] This occludin-like activity is mediated by the claudins. In fact, it is becoming apparent that claudins are the major transmembrane components of TJs and that the permeability properties of an epithelium are the product of claudin composition in these mutant animals.[48] However, further detailed analyses of the functions of claudin at the cellular level are required for a better understanding of the molecular mechanism behind the epithelial barrier integrity.

Cell culture conditions may also be critical. In an air–liquid interface co-culture system of human lung epithelial cells and of primary human pulmonary microvascular endothelial cells, it was shown that responses to several cytokines, activation of transcription factors and TNF reduction

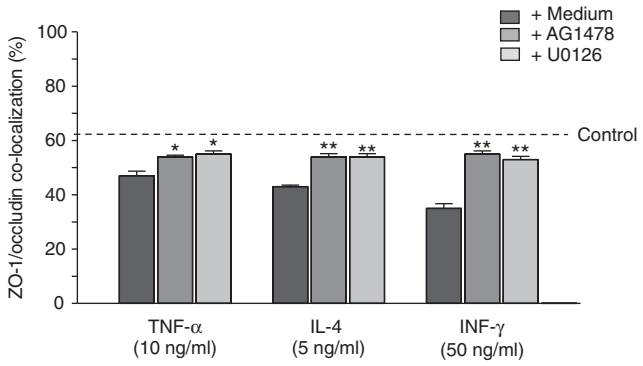

Figure 8 Quantitative evaluation of the activity epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinases (ERK)1/2 inhibitors on tight junction (TJ) disassembly induced by exposure to cytokines. The percentage of zonula occludens (ZO)-1/occludin colocalization in Calu-3 cells is expressed on the ordinate and the various culture conditions on the abscissa. Dotted line represents ZO-1/occludin colocalization under basal culture condition (Control). Data reported are representative of the results obtained in four independent experiments and are expressed as mean ± s.e.m.. $^{*}P<0.05$ and $^{**}P<0.01$ *versus* cells exposed to cytokines in cultures without AG1478 or UO126.

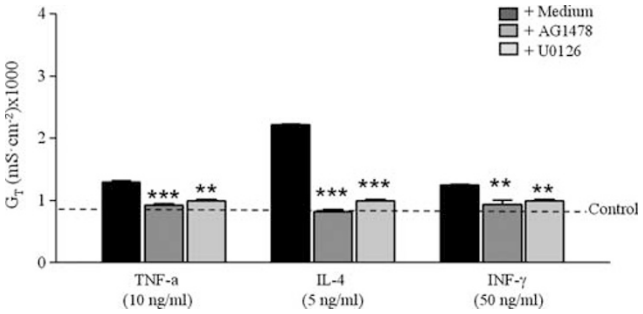

Figure 9 The effects of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinases (ERK)1/2 inhibitors on cytokine-induced changes in total conductance (G_T). Dotted line represents total conductance (G_T) under basal culture condition (Control). Data reported are representative of the results obtained in four independent experiments and are expressed as mean ± s.e.m.. $^{**}P<0.01$ and $^{***}P<0.001$ *versus* cells exposed to cytokines in cultures without AG1478 (1 μM) or UO126 (25 μM).

of TER values require basolateral mediator application, a condition favoring *in vivo* exposure to mediators released by immunoeffector cells in the airway walls.[21,22] Epithelial permeability regulation is regulated by complex mechanisms, with a wide array of growth factors, cytokines, drugs and hormones exerting opposite effects on TJ organization and barrier function.[1,2,21,22] Glucocorticoids, prolactin and unsaturated fatty acids, all enhance TJ barrier partially by increasing the expression of TJs whereas cytokines, oxidants and growth factors, as also reported here, increase permeability and mediate ZO-1 and/or occludin disruption in endothelial and epithelial cells.[2,13,14] TJ permeability may be regulated directly through the modification of TJ proteins, but also indirectly through effects on the cytoskeleton, with the involvement of a variety of intracellular pathways, depending on the stimulus and on the cell type.[2,20] TJ integrity in different epithelia is regulated by G-proteins, protein kinase C, c-Src, phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ and protein phosphatase 2A.[2,49–51] Evidence suggests that these signaling activities may affect TJs by inducing phosphorylation and regulation of protein–protein interactions.[50] In bronchial epithelial cells, various cellular processes are regulated by epidermal growth factor-like factors and the EGFR signaling pathways.[12,23,24,27] EGFR activation turns on the MAPK/ERK1/2 pathway and mediates the promotion of cell proliferation, growth and differentiation.[12,51,52] Various other external stimuli, including pro-inflammatory cytokines, that have the potential to disrupt epithelial junctions are known to activate the MAPK/ERK1/2 pathway, and previous studies have implicated ERK1/2 in disassembly of epithelial junctions at corneal level by phorbol 12-myristate 13-acetate[53] at renal level by cyclosporine A[54] and at airway level by cigarette smoke condensate.[12] This has been confirmed in the present study where we showed on a bronchial epithelial cell line that MAPK/ERK pathway has a crucial role on protection of cytokines-induced TJ disruption and function.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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