Lung epithelial barrier function and wound healing are decreased by IL-4 and IL-13 and enhanced by IFN-γ

MINOO AHDIEH, TIM VANDENBOS, AND ADEL YOUAKIM

Departments of Biomolecular Screening and Protein Chemistry, Immunex Corporation, Seattle, Washington 98101

Received 12 March 2001; accepted in final form 31 July 2001

Asthma has traditionally been considered a disease of the immune system (35). T cells secreting Th2-type cytokines, such as interleukin (IL)-4 and IL-13, play a key role in disease initiation and propagation by recruiting and attracting a variety of cells, such as mast cells, eosinophils, and B cells, to the lung (for review see Ref. 35). The resulting cellular infiltration and inflammation are believed to contribute to other changes associated with lung function, such as airway remodeling and airway hyperresponsiveness (AHR).

An alternate view of asthma suggests that the epithelium, along with the immune system, is required for the disease to develop (11, 12). In the absence of this epithelial response, simple atopy, and not asthma, would ensue. According to this hypothesis, damage to epithelial cells that occurs during the initial stages of asthma stimulates the proliferation of fibroblasts and muscle cells, which, in turn, increases the production and secretion of extracellular matrix proteins. This increased cellular proliferation and matrix deposition give rise to the thickening of the submucosa and the fibrosis characteristic of asthma.

One of the major roles of the epithelium in the lung is its function as a barrier between the lumen and the underlying submucosa, which contains cells of the immune system. Histological analysis of lung biopsies from asthmatic individuals shows a disruption in epithelial cells and, in some instances, complete loss of these cells, indicating that perturbation of barrier function occurs in vivo (17, 21). Loss of barrier function results in the uncontrolled flux of allergens and other noxious substances from the lumen into the submucosa and subsequent activation of the immune system, culminating in inflammation in the lung. Until the barrier is restored, this situation persists and progresses, leading to more inflammation and epithelial damage. Restoration of the barrier involves healing of the wound formed by a loss of the cells and a reestablishment of the specialized cell adhesion complexes, which are crucial to epithelial barrier function. Thus the study of epithelial barrier regulation in the lung during inflammation may be crucial for understanding the pathogenesis of asthma and, ultimately, for the development of therapeutics to treat asthma. Unfortunately, little is known about pulmonary epithelial barrier regulation and how proinflammatory cytokines, such as IL-4 and IL-13, affect epithelial barrier function and restitution in asthma. IL-4 and IL-13 have been shown to disrupt epithelial barrier function in intestinal model systems (37), but similar work with lung cells has not been done.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
In this study, we have utilized Calu-3 lung epithelial cells as an in vitro model to determine the effects of IL-4 and IL-13 on barrier function and wound healing. We have also examined the effects of the Th1 cytokine interferon (IFN)-γ because of studies showing that asthma may be ameliorated by a Th1-type cytokine response (31). Our results show that treatment of Calu-3 cells with IL-4 and IL-13 decreases barrier function and reduces wound healing. In contrast, IFN-γ enhances barrier function and promotes wound healing.

MATERIALS AND METHODS

Cell culture. Calu-3 cells, derived from a human lung adenocarcinoma, were grown in minimal essential medium (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum, penicillin, streptomycin, and glutamine. Cells were passaged when they were ~80% confluent. For barrier function experiments, cells were plated at a density of 1 × 10⁶ cells per 300 cm² of medium (Nunc, Naperville, IL) coated with collagen I or fibronectin (10 µg/ml; both from Becton-Dickinson, Bedford, MA) and allowed to reach confluence. Wounds were made as previously described (5) by adding 1 µl of 1 N NaOH to the center of the well and then immediately rinsing with excess PBS. This treatment produced 3- to 6-mm-diameter circular wounds. Monolayers were allowed to recover for 24 h before the cytokine treatments. Cells were treated with various combinations of cytokines or cytokine receptor antagonists and photographed at 24-h intervals to record migration from the edge of the wound. Images were collected using a charge-coupled device camera (Sony) attached to an inverted phase contrast microscope (Nikon, Melville, NY). Images were analyzed using Metamorph Imaging software (Universal Imaging, West Chester, PA) to measure the area of the wounds at the indicated times. The results are expressed as the area of the repaired region, which is defined as the difference between wound area at time 0 and wound area at 24 or 48 h.

Immunofluorescence and immunoblot analysis of integrin expression. Calu-3 cell monolayers from the wound assays were washed three times in ice-cold complete medium and incubated with anti-αvβ6 integrin antibody (10 µg/ml; Chemicon, Temecula, CA) in complete medium at 4°C for 60 min. Cells were washed in ice-cold PBS, fixed for 5 min in 4% paraformaldehyde in PBS, washed twice in PBS, and permeabilized with 0.1% Triton X-100 and 0.1% BSA (PBST) for 30 min. The sample was left on ice for 90 min with frequent trituration and centrifuged as described above. This supernatant contained the NP-40-insoluble proteins. Protein fractions were assayed by the micro-bicinchoninic acid procedure (Pierce Chemicals, Rockford, IL).

E-cadherin expression was analyzed using the NP-40-soluble protein fraction, and ZO-1 and occludin expression were analyzed using the NP-40-insoluble protein fraction. Proteins (25 µg) were separated on a 10% Tris-glycine polyacrylamide gel (Novex, San Diego, CA), transferred to nitrocellulose (0.45 µm; Bio-Rad, Richmond, CA), and blocked for 4–6 h in PBS-0.1% Tween 20–5% dried milk (PTM). The blots were then incubated overnight at 4°C with primary antibodies (ZO-1, occludin, and E-cadherin) at the indicated concentration. The blots were then washed in PBS, incubated at room temperature for 90 min with biotinylated secondary antibody (Molecular Probes, Eugene, OR) diluted 1:1,000 in PTM followed by horseradish peroxidase-conjugated streptavidin (Sigma, St. Louis, MO) diluted 1:10,000 in PTM, and then processed for chemiluminescence detection (ECL, Amersham, Arlington Heights, IL).

Wound assays. Calu-3 cells were plated on chamber slides (Nunc, Naperville, IL) coated with collagen I or fibronectin (10 µg/ml; both from Becton-Dickinson, Bedford, MA) and allowed to reach confluence. Wounds were made as previously described (5) by adding 1 µl of 1 N NaOH to the center of the well and then immediately rinsing with excess PBS. This treatment produced 3- to 6-mm-diameter circular wounds. Monolayers were allowed to recover for 24 h before the cytokine treatments. Cells were treated with various combinations of cytokines or cytokine receptor antagonists and photographed at 24-h intervals to record migration from the edge of the wound. Images were collected using a charge-coupled device camera (Sony) attached to an inverted phase contrast microscope (Nikon, Melville, NY). Images were analyzed using Metamorph Imaging software (Universal Imaging, West Chester, PA) to measure the area of the wounds at the indicated times. The results are expressed as the area of the repaired region, which is defined as the difference between wound area at time 0 and wound area at 24 or 48 h.

Immunofluorescence and immunoblot analysis of integrin expression. Calu-3 cell monolayers from the wound assays were washed three times in ice-cold complete medium and incubated with anti-αvβ6 integrin antibody (10 µg/ml; Chemicon, Temecula, CA) in complete medium at 4°C for 60 min. Cells were washed in ice-cold PBS, fixed for 5 min in 4% paraformaldehyde in PBS, washed twice in PBS, and incubated for 30 min in 50 mM NH₄Cl to quench unreacted aldehyde groups. The cells were washed twice more with PBS and then permeabilized and blocked in PBS containing 0.1% Triton X-100 and 0.5% BSA (PBST) for 30 min. Subsequently, the cells were incubated for 60 min with Alexa 488-conjugated goat anti-mouse IgG (10 µg/ml; Molecular Probes) and Alexa 568-conjugated phalloidin (10 µg/ml; Molecular Probes) diluted 1:1,000 in PTM followed by horseradish peroxidase-conjugated streptavidin (Sigma, St. Louis, MO) diluted 1:10,000 in PTM, and then processed for chemiluminescence detection (ECL, Amersham, Arlington Heights, IL).

E-cadherin expression was analyzed using the NP-40-soluble protein fraction, and ZO-1 and occludin expression were analyzed using the NP-40-insoluble protein fraction. Proteins (25 µg) were separated on a 10% Tris-glycine polyacrylamide gel (Novex, San Diego, CA), transferred to nitrocellulose (0.45 µm; Bio-Rad, Richmond, CA), and blocked for 4–6 h in PBS-0.1% Tween 20–5% dried milk (PTM). The blots were then incubated overnight at 4°C with primary antibodies (ZO-1, occludin, and E-cadherin) at the indicated concentration. The blots were then washed in PBS, incubated at room temperature for 90 min with biotinylated secondary antibody (Molecular Probes, Eugene, OR) diluted 1:1,000 in PTM followed by horseradish peroxidase-conjugated streptavidin (Sigma, St. Louis, MO) diluted 1:10,000 in PTM, and then processed for chemiluminescence detection (ECL, Amersham, Arlington Heights, IL).

E-cadherin expression was analyzed using the NP-40-soluble protein fraction, and ZO-1 and occludin expression were analyzed using the NP-40-insoluble protein fraction. Proteins (25 µg) were separated on a 10% Tris-glycine polyacrylamide gel (Novex, San Diego, CA), transferred to nitrocellulose (0.45 µm; Bio-Rad, Richmond, CA), and blocked for 4–6 h in PBS-0.1% Tween 20–5% dried milk (PTM). The blots were then incubated overnight at 4°C with primary antibodies (ZO-1, occludin, and E-cadherin) at the indicated concentration. The blots were then washed in PBS, incubated at room temperature for 90 min with biotinylated secondary antibody (Molecular Probes, Eugene, OR) diluted 1:1,000 in PTM followed by horseradish peroxidase-conjugated streptavidin (Sigma, St. Louis, MO) diluted 1:10,000 in PTM, and then processed for chemiluminescence detection (ECL, Amersham, Arlington Heights, IL).

Wound assays. Calu-3 cells were plated on chamber slides (Nunc, Naperville, IL) coated with collagen I or fibronectin (10 µg/ml; both from Becton-Dickinson, Bedford, MA) and allowed to reach confluence. Wounds were made as previously described (5) by adding 1 µl of 1 N NaOH to the center of the well and then immediately rinsing with excess PBS. This treatment produced 3- to 6-mm-diameter circular wounds. Monolayers were allowed to recover for 24 h before the cytokine treatments. Cells were treated with various combinations of cytokines or cytokine receptor antagonists and photographed at 24-h intervals to record migration from the edge of the wound. Images were collected using a charge-coupled device camera (Sony) attached to an inverted phase contrast microscope (Nikon, Melville, NY). Images were analyzed using Metamorph Imaging software (Universal Imaging, West Chester, PA) to measure the area of the wounds at the indicated times. The results are expressed as the area of the repaired region, which is defined as the difference between wound area at time 0 and wound area at 24 or 48 h.
RESULTS

IL-4 and IL-13 treatment of Calu-3 cells decreased TER. Calu-3 cells form polarized, relatively impermeable monolayers when grown on Transwell filters (34). These cells can be used to model epithelial barrier function in the lung. The effect of IL-4 and IL-13 on barrier function in Calu-3 cells was assessed by TER measurements. Calu-3 cells formed barriers with a TER of \( \sim 400 \, \Omega \cdot \text{cm}^2 \) and maintained that level throughout the course of the assay (Fig. 1). Treatment of the cells with 5 ng/ml of IL-4 or IL-13 resulted in a rapid, pronounced decrease (\( \sim 70-75\% \)) in TER by day 1 and remained at that level for the remainder of the assay. Treatment of the cells with 50 ng/ml of IL-4 or IL-13 showed similar results, whereas treatment with \( \leq 0.5 \, \text{ng/ml} \) of either cytokine had no effect on barrier function (data not shown). The cytokine concentrations used were the lowest doses that gave the maximal response in the barrier assay. In addition, these effects on barrier function were observed only when the cytokines were added to the basolateral side of the cells (data not shown). These results demonstrate that IL-4 and IL-13 caused a decrease in Calu-3 barrier function. Calu-3 cells grown on Transwell filters were treated with IL-4 (5 ng/ml) alone or IL-4 and soluble IL-4R (10 \( \mu \text{g/ml} \)) added basolaterally, and TER was measured. \( *P < 0.001 \) between control and IL-4 or IL-4 + control Ab (10 \( \mu \text{g/ml} \)). ** \( P > 0.1 \) between control and all other treatments.

![Fig. 1](image1.png)  
Fig. 1. Interleukin (IL)-4 and IL-13 treatment of Calu-3 cells decreases transepithelial electrical resistance (TER). Calu-3 cells grown on Transwell filters were treated with IL-4 (5 ng/ml) or IL-13 (5 ng/ml) added basolaterally, and TER was measured. Results represent an average of 3–4 filters per time point. \( *P < 0.001 \) between control and each treatment.

![Fig. 2](image2.png)  
Fig. 2. Soluble IL-4 receptor-\( \alpha \) (solIL-4R) and anti-IL-4 receptor-\( \alpha \) antibody (anti-IL-4R Ab) prevent IL-4-induced disruption of Calu-3 cell barrier function. Calu-3 cells grown on Transwell filters were treated with IL-4 (5 ng/ml) alone or IL-4 and soluble IL-4R (10 \( \mu \text{g/ml} \)) added basolaterally, and TER was measured. \( *P < 0.001 \) between control and IL-4 or IL-4 + control Ab (10 \( \mu \text{g/ml} \)). ** \( P > 0.1 \) between control and all other treatments.

To determine whether the effects of IL-4 on the Calu-3 cells were the result of binding to the IL-4 receptor complex found on other cells (23), the cells were treated with IL-4 and a monoclonal antibody to IL-4 receptor-\( \alpha \). This antibody blocks IL-4 and IL-13 signaling (25). When added to IL-4-treated cells, the antibody prevented IL-4-induced barrier disruption, inasmuch as the TER values were similar to control cells (Fig. 2); an isotype-matched control antibody (directed to human CD3) did not alter the effects of IL-4. Similar results were obtained using soluble IL-4 receptor-\( \alpha \) as an IL-4 receptor antagonist (Fig. 2). This protein binds IL-4 and prevents it from interacting with the cell surface IL-4 receptor complex (22). Treatment of the cells with anti-IL-4 receptor antibody or soluble IL-4 receptor alone had no effect on TER (Fig. 2). These results show that IL-4 is exerting its effects on barrier function in Calu-3 cells through the classic IL-4 receptor complex.

The anti-IL-4 receptor-\( \alpha \) monoclonal antibody was also used to determine whether the effect of IL-13 on barrier function was mediated by the IL-4 receptor complex. As shown in Fig. 3, the addition of this antibody to IL-13-treated cells prevented the rapid decrease in TER induced by IL-13 and maintained barrier levels comparable to controls cells. A control antibody had no effect on IL-13 treatment of the cells. In contrast, soluble IL-4 receptor-\( \alpha \) did not affect the IL-13-induced decrease in barrier function. These observations are consistent with the hypothesis that IL-13 does not bind directly to IL-4 receptor-\( \alpha \) but,
rather, binds to an IL-13 receptor that requires IL-4 receptor-α for signaling (38). Thus IL-13 signals through an IL-4 receptor-α-dependent pathway in Calu-3 cells.

**IFN-γ protects barrier function in IL-4- and IL-13-treated cells.** IFN-γ was shown previously to disrupt the barrier function in an intestinal epithelial barrier model (19, 36). To determine whether IFN-γ possessed the same activity in lung epithelial cells, the Calu-3 cells were treated with 50 ng/ml of IFN-γ, a concentration that was shown to be very effective at disrupting barrier function in T84 colonic epithelial cells (36). Quite unexpectedly, IFN-γ treatment of the Calu-3 cells enhanced barrier function about threefold (Fig. 4). At early time points, IFN-γ-treated cells had barrier levels comparable to control cells, but by day 2, a slight enhancement became apparent. By day 3 of treatment, barrier function increased by greater than threefold compared with control cells.

To determine whether IFN-γ could protect the Calu-3 cells from the barrier-disrupting activity of IL-4 and IL-13, cells were treated with combinations of IFN-γ and IL-4 or IL-13 (Fig. 4). On day 1, cells treated with both IL-4 and IFN-γ showed a decrease in TER similar to that observed in cells treated with IL-4 alone. However, by day 2, the difference became more apparent, and the cells treated with both IFN-γ and IL-4 had TER values significantly higher than cells treated with IL-4 (58% vs. 27% of day 0, P = 0.001). After 3 days, barriers in the cells treated with both IFN-γ and IL-4 were similar to those in control cells, suggesting that IFN-γ had completely reversed the damage to the epithelial barrier induced by IL-4. IFN-γ was also able to restore IL-13-disrupted barriers to control levels with similar kinetics and to the same extent as in IL-4-treated cells (Fig. 4). These results demonstrate that IFN-γ is a potent stimulator of lung epithelial barrier function and that it can reverse IL-4- and IL-13-induced barrier disruption.

**Mannitol flux measurements confirm the TER results.** To confirm that the changes in TER induced by the various cytokine treatments were a reflection of altered paracellular flow and not changes in ion flux, [14C]mannitol flux was measured in cells after cytokine treatment. Mannitol is not transported by cells, so when added to the apical chamber in a barrier assay, its appearance on the basolateral side is a measure of paracellular flow, i.e., barrier leakiness (19). Treatment of the cells with IL-4 and IL-13 resulted in a ~2.5-fold increase in mannitol flux into the basolateral compartment compared with control cells (Fig. 5). The addition of anti-IL-4 receptor antibody or soluble IL-4 receptor to IL-4-treated cells reduced mannitol flux to the same level as controls. In the case of IL-13, only the anti-IL-4 receptor antibody was able to achieve this effect; the soluble receptor had no effect on mannitol flux. IFN-γ treatment of the cells reduced mannitol flux to about half that of control cells. These results confirm that barrier function is indeed decreased by IL-4 and IL-13 and increased by IFN-γ, in agreement with the electrophysiological measurements.

**Analysis of tight junction proteins.** To examine the effects of the cytokines on proteins associated with tight junctions, ZO-1 and occludin were examined by immunoblotting. ZO-1 is a cytoplasmic protein that plays a central role in tight junction organization by linking the transmembrane components of tight junc-

---

**Fig. 3.** Anti-IL-4 Ab, but not solIL-4R, prevents IL-13-induced disruption of Calu-3 cell barrier function. Calu-3 cells grown on Transwell filters were treated with IL-13 (5 ng/ml) alone or IL-13 and solIL-4R (10 μg/ml) or anti-IL-4 Ab (10 μg/ml) added basolaterally, and TER was measured. *P < 0.001 between control and IL-13, IL-13 + solIL-4R, or IL-13 + control Ab (10 μg/ml). **P > 0.1 between control and IL-13 + anti-IL-4 Ab.

**Fig. 4.** Interferon-γ (IFN-γ) enhances barrier function and can also prevent IL-4- and IL-13-induced disruption of Calu-3 barrier function. Calu-3 cells grown on Transwell filters were treated with IFN (50 ng/ml) alone or IFN and IL-4 (5 ng/ml) or IL-13 (5 ng/ml) added basolaterally, and TER was measured. *P > 0.2 between control and IFN-γ at days 1 and 2. **P < 0.0001 between control and IFN-γ at day 3 or between control and IL-4 or IL-13 at days 1–3 and between control and IL-4 + IFN-γ or IL-13 + IFN-γ at day 1. *P < 0.001 between IL-4 + IFN-γ or IL-13 + IFN-γ and IL-4 or IL-13 at days 2 and 3; **P > 0.1 between control and IL-4 + IFN-γ or IL-13 + IFN-γ at day 3.
effects of various cytokine treatments on cell migration, an in vitro wound-healing assay of Calu-3 cells was performed. The wounds were generated by NaOH-induced damage to the monolayers (5), resulting in ~3- to 6-mm-diameter circular wounds. Cytokines were administered 24 h after the wounds were generated, and the rate of cell migration was measured subsequently at 24-h intervals for 2 days. The amount of wound repair was expressed as the difference between the area of the wound at time 0 and that at 24 or 48 h. Migration was measured on fibronectin- or collagen-I-coated slides, because the expression of these two matrix components is elevated in the asthmatic lung (14). When these assays were performed on fibronectin-treated slides, no difference was seen between any of the treatments (data not shown). However, when the wound assays were performed on collagen-I-coated slides, differences in migration rate between the various cytokine treatments were observed (Fig. 7). Untreated cells showed a time-dependent increase in the area of the repaired region from 3,211 at 24 h to 5,691 at 48 h. IFN-γ-treated cells showed an even greater amount of repair (6,400 at 24 h to 14,117 at 48 h), indicating that IFN-γ stimulated cell migration and wound repair compared with control cells. In contrast, in IL-4- and IL-13-treated cells, wound repair was reduced by 55–65% compared with control cells at 24 and 48 h. Coincubation of the cells with IL-4 or IL-13 and IFN-γ stimulated the cells to migrate at rates comparable to untreated cells, but less than with IFN-γ alone. Similarly, addition of anti-IL-4 receptor antibody or soluble IL-4 receptor-α to the IL-4-treated cells produced wound repair similar to control cells. As was the case in the barrier assay, only anti-IL-4 receptor antibody, and not soluble IL-4 receptor-α, had an effect on IL-13-treated cells, restoring the rate of wound repair to control levels. Treatment of the cells with anti-IL-4 receptor antibody or soluble IL-4 receptor alone had no effect on cell migration, inasmuch as the rate of wound repair was comparable to control cells (Fig. 7). These results demonstrate that IFN-γ stimulates cell migration and wound healing, whereas IL-4...
or IL-13 inhibits these processes. Furthermore, treatment of the cells with IFN-γ and IL-4 or IL-13 overcomes the inhibitory effects of IL-4 and IL-13 on cell migration.

Expression of integrins on cells in the wound assay.

To understand why cells treated with the various cytokines showed differences in migration rate, the expression of the integrins that mediate attachment to fibronectin and collagen I was examined by immunofluorescence and immunoblotting. Lung epithelial cells utilize α2β1-integrin to bind fibronectin (3) and α2β1-integrin to bind collagen I (16). In addition to these adhesion receptors, the organization of actin was examined by immunofluorescence because of the critical role of the cytoskeleton in cell migration. Calu-3 cells plated on fibronectin-coated slides and stained with anti-αβ6 antibodies showed a similar staining pattern regardless of cytokine treatment (data not shown). The staining was of uniform intensity on all cells in the monolayer, including those at the leading edge of the wound (data not shown). In contrast, expression of α2β1-integrin on the cells plated on collagen showed differences in staining patterns depending on the cytokine treatment (Fig. 8, A–D). In IL-4- and IL-13-treated cells, staining for α2β1-integrin was uniformly bright throughout the monolayer (Fig. 8, B and C). In particular, the leading edge of some cells at the wound interface showed moderate to intense staining for α2β1-integrin (arrowheads, Fig. 8, B and C). Control cells also showed staining of all the cells in the monolayer (Fig. 8A); however, staining intensity on the leading edge of cells at the wound front was reduced compared with IL-4- and IL-13-treated cells (cf. Fig. 8A with Fig. 8, B and C). Cells in this region showed faint to moderate staining, and in some instances, no α2β1 staining was detectable. IFN-γ-treated cells showed staining similar to the control and IL-4- and IL-13-treated cells in the monolayer distal to the wound edge but differed considerably in cells at the leading edge of the wound (Fig. 8D). The cells at the wound front showed very low to nondetectable staining for α2β1-integrin at their leading edge. The fact that reduced α2β1-integrin expression was confined to the leading edge of IFN-γ-treated cells suggests that downregulation or altered targeting of this receptor may contribute to the increased migration rate and wound healing observed. To begin to address this issue, the levels of α2β1-integrin were examined semiquantitatively by immunoblotting extracts of cells from the wound assays using antibodies specific for each subunit of α2β1. The results showed that the levels of α2- and β1-subunits were similar to or only modestly different from the control (Fig. 9, A and B). Thus the reduction in α2β1-integrin seen in the IFN-γ-treated cells was limited to the leading edge and does not represent a global effect on the expression of this integrin.

The organization of the actin cytoskeleton was examined by fluoresceinated phalloidin staining (Fig. 8, E–H). In control cells, actin localized to the cell periphery and was detectable throughout the monolayer and, in contrast to α2β1-integrin, was intense and continuous along the leading edge of the wound (Fig. 8E). IL-4- and IL-13-treated cells showed staining similar to control cells, in which actin was expressed uniformly throughout the monolayer and along the leading edge (Fig. 8, F and G). In IFN-treated cells, the actin staining in the monolayer distal to the wound edge resembled that of the other treatments, but the staining at the leading edge of the wound appeared less intense than in the other treatments (Fig. 8H).

These results suggest a correlation between decreased α2β1-integrin expression on the leading edge of cells and increased migratory ability. How this alteration in integrin expression contributes to cell migration remains to be determined.

DISCUSSION

Epithelial cells in the lung form a restrictive barrier between the lumen and the underlying submucosa. In asthma, this barrier is disrupted, possibly contributing to the initiation and/or exacerbation of the inflammatory process that occurs in this disease (12). Although the effects of proinflammatory cytokines in modulating immune cell function and activation are well characterized, the role they play in regulation of the lung epithelial barrier is poorly understood. In this report, using the Calu-3 cells as an in vitro model of barrier function, we show that Th1 and Th2 cytokines have distinct effects on lung epithelial cell function. The Th1 cytokine IFN-γ has beneficial effects on barrier function and wound healing. In contrast, IL-4 and IL-13, Th2 cytokines with elevated expression in asthma,
have detrimental effects on these same biological processes.

Epithelial barrier function is mediated by a complex of proteins, including ZO-1 and occludin, which constitute the tight junctions (for reviews see Refs. 1 and 20). ZO-1 plays a pivotal role in tight junction formation and organization by linking the transmembrane protein occludin to other cytoplasmic components of the tight junctions and to the actin cytoskeleton (7). Occludin binds to cells on apposing cells and is linked to ZO-1 and the cytoskeleton (9). The regulation of epithelial barrier function by cytokines has been studied most extensively in the gut using the T84 intestinal epithelial cell line. In these cells, IL-4, IL-13, and IFN-γ induce barrier breakdown in a rapid, dose-dependent fashion (19, 36, 37). In IFN-γ-treated T84 cells, disruption of barrier function correlates with a loss of ZO-1 and reorganization of the apical actin cytoskeleton (36). The effects of IFN-γ, IL-4, and IL-13 on Calu-3 barrier function are the first such analysis using lung epithelial cells. IL-4 and IL-13 treatment of Calu-3 cells shows reduced barrier function similar to that observed in T84 cells treated with these cytokines and with IFN-γ (19, 36, 37). However, IFN-γ treatment of Calu-3 cells enhances barrier function, while it decreases it in T84 cells. The effects of IFN-γ on Calu-3 and T84 cells are a striking example of how epithelial cells from different tissues respond differently to an identical stimulus.

Treatment of the Calu-3 cells with IL-4 and IL-13 only modestly affects occludin expression, whereas IFN-γ dramatically reduces it. Given the important role of occludin in tight junction formation, the effects of IL-4 and IL-13 on occludin expression are consistent
permeability properties of an epithelium are the products of the tight junctions and that the barrier activity is mediated by the claudins. In fact, it is possible that these proteins still possess enough of the receptor for adhesion despite the profound reduction in expression of occludin. One explanation may be the redundancy of function provided by claudins, a new family of transmembrane proteins that also localize to tight junctions (32). There are 20 known claudins, several of which interact with many of the same proteins as occludin (32). The existence of proteins with functional redundancy to occludin has been inferred from studies with occludin-deficient embryonic stem cells that retain the ability to form polarized epithelia with normal barrier function (27). This occludin-like activity is mediated by the claudins. In fact, it is becoming apparent that claudins are the major transmembrane components of tight junctions and that the permeability properties of an epithelium are the product of claudin composition within those cells (20).

IFN-γ treatment of the Calu-3 cells also dramatically reduces ZO-1 expression, yet barrier function is enhanced. This result, too, was unexpected because of the importance ascribed to ZO-1 in the regulation of tight junction function. In IFN-γ-treated T84 cells, ZO-1 levels are significantly reduced, with a concomitant loss of barrier function, supporting the concept that this protein plays a crucial role in tight junctions. The reduction in ZO-1 levels and the associated decrease in epithelial barrier observed with IL-4 and IL-13 treatment of Calu-3 cells are also consistent with a critical role of ZO-1 in barrier function. Although no adequate explanation can be provided to account for the paradoxical effect of IFN-γ on barrier function and ZO-1 expression, one intriguing possibility that we are exploring is that Calu-3 cells express a protein with functional redundancy to ZO-1 that may differ in regulation by IFN-γ and IL-4 and IL-13.

Although the tight junction is the major cellular component of barrier permeability, an intact epithelial monolayer is required for barrier function at the tissue level. Damage and loss of epithelial cells that occur in asthma result in denuded areas of epithelium and exposure of the submucosa to the external environment (17, 21). The process of epithelial repair may be abnormal in the asthmatic lung, resulting in prolonged exposure of the submucosa to antigens and irritants found in the lung lumen (12). In this study, we have shown that IL-4 and IL-13 decrease lung epithelial cell migration, which, if it occurs in vivo, would reduce the rate of repair and restitution of the epithelium. As is the case with barrier function, IFN-γ has the opposite effect of IL-4 and IL-13 and enhances migration of the Calu-3 cells. The effects of the cytokines on migration are specific for collagen I and do not affect migration on fibronectin. These observations may be significant in an in vivo setting, since increased collagen I deposition, leading to fibrosis, is a hallmark of the asthmatic lung (6). The migration effects correlate with altered expression of the collagen I-specific integrin, αβ1, one of the major collagen-binding integrins expressed in the lung (16). Previous studies have suggested that αβ6, the fibronectin-binding integrin in lung epithelial cells, modulates inflammation in a murine asthma model (13). However, in the Calu-3 cells, there is no difference in migration rate of the cells on fibronectin, nor is there any change in αβ6-integrin expression with any of the three cytokines examined. Migration of the Calu-3 cells on collagen I is associated with changes in the levels of αβ1-integrin expression on the leading edge of the migrating cells; cells distal to the wound edge show no differences in αβ1-integrin expression regardless of cytokine treatment. The rate of migration is inversely related to αβ1-integrin expression; IFN-γ-treated cells migrate the fastest and have no detectable αβ1-integrin on their leading edge, whereas IL-4 and IL-13-treated cells migrate the slowest and have the highest levels of αβ1-integrin on their leading edge. This result is somewhat unexpected, since migration has been associated with integrin expression on the leading edge of cells (18). It is unclear how IFN-γ-treated Calu-3 cells migrate on collagen I if they have reduced levels of αβ1-integrin on their leading edge, although it is possible that they still possess enough of the receptor for migration. Alternatively, high levels of integrin expression at the leading edge of IL-4- and IL-13-treated cells may decrease motility, because the cells become too adhesive to detach from the matrix. In addition to changes in αβ1-integrin expression, actin at the leading edge of IL-4- and IL-13-treated Calu-3 cells was brightly stained by rhodamine-phalloidin, possibly indicating increased stability of the cytoskeleton in this region of the cells. Because lamellipodial extension involved in cell migration requires actin reorganization (26), the increased actin staining at the leading edge of IL-4- and IL-13-treated cells may also be a reflection of the increased adhesiveness induced by these cytokines. In contrast to IL-4 and IL-13, IFN-γ-treated cells showed no detectable actin at the leading edge, sug-

![Fig. 9. Cytokine treatment does not alter levels of α2- or β1-subunits in Calu-3 cells. Calu-3 cells from wound assays were treated with cytokines (IL-4, IL-13, and IFN-γ) for 48 h and then extracted. Proteins were fractionated by SDS-PAGE and immunoblotted with rabbit serum to the α2-subunit (A), β1-subunit (B), or control rabbit serum (C). RI, intensity of each band relative to control (control = 1).](image)
suggesting that it may be in a more dynamic state required to promote lamellipodial extension and motility.

The differential effects of Th1 and Th2 cytokines on Calu-3 cells may have important implications in how we consider treatments for asthma. Because asthma is associated with an increased Th2-type immune profile, one therapeutic strategy being considered is the stimulation of a Th1 cytokine response to inhibit the Th2 response (31). This approach has been validated in a number of experimental models of asthma using IL-12 (15, 29) or CpG oligonucleotides (30) to enhance the Th1 character of the immune system. The general interpretation of these studies is that skewing the cytokine profile from Th2 to Th1 affects the activation and recruitment of mast cells, eosinophils, and B cells and that these changes in immune cells subsequently alter disease progression. However, as the results of this study with the Calu-3 cells showed, lung epithelial cells can also alter their biological responses to Th1 and Th2 cytokines. More important is the fact that a Th1 cytokine (IFN-γ) can inhibit the effects of Th2 cytokines (IL-4 and IL-13) in the Calu-3 cells, resulting in the maintenance of barrier function and a normal rate of wound healing. By extrapolation to the in vivo setting, a Th2 cytokine-rich environment in the lung would result in stimulation and activation of immune cells and epithelial cells, the latter contributing to disease exacerbation through disruption of the epithelial barrier and reduction in wound healing. Conversely, the net result of Th1 cytokine induction in an asthmatic lung would be a reduction in Th2 immune cell response and additional dampening of inflammation by enhanced epithelial barrier function and wound healing.

Therapeutic agents that are able to affect epithelial cell function may prove to be of great benefit for both lung inflammation and AHR associated with asthma. In the case of inflammation, restitution of the epithelial barrier would eliminate a source of stimulatory antigens from further activating the immune system, thus helping to decrease tissue inflammation (24). AHR, which is associated with permanent alterations in lung architecture, results in enhanced sensitivity to noxious agents that enter the lung (6). As with inflammation, a restoration of the epithelial barrier could act to eliminate contact between these irritants and resident cells in the submucosa. In addition, the restitution of the epithelial cells may restore the levels of relaxing factors that can inhibit bronchoconstriction associated with AHR (8). In this study, IFN-γ showed very potent epithelial restitution activity and, more importantly, was able to inhibit the deleterious effects of IL-4 and IL-13, suggesting that it may prove beneficial in a clinical setting. Recently, however, the results of a clinical trial of IL-12, a stimulator of IFN-γ production, on AHR in asthmatic patients showed no clinical efficacy, although it is unclear whether IFN-γ levels in patients were increased as a result of the treatment (4). Soluble IL-4 receptor-α was also able to inhibit the effects of IL-4 in the barrier and wound-healing assays. Although soluble IL-4 receptor-α is ineffective against IL-13, it is possible that inhibition of IL-4 in vivo may indirectly reduce IL-13 levels by decreasing the number of IL-13-producing cells (33). Soluble IL-4 receptor is currently in clinical trials for asthma, and the results of phase II/III trials indicate that patients with moderate asthma showed improvement in respiratory parameters (2).

In summary, the results of this study demonstrate that Th1 and Th2 cytokines have distinct activities in lung epithelial cells that are consistent with the role of these cytokines in asthma. IL-4 and IL-13 disrupt epithelial barrier function and wound healing, both of which would be expected to exacerbate inflammation. In contrast, IFN-γ enhances epithelial barrier function and wound healing, which would contribute to a reduction in inflammation.

We thank Joel Tocker and Kathy Anderson for many helpful discussions, Lori Whittaker, Helen Hathaway, Stewart Chipman, and Douglas Williams for critical reading of the manuscript, Ann Aumell for editorial assistance, and Gary Carlton for assistance with graphics.

REFERENCES


