NF- κ B inhibitors impair lung epithelial tight junctions in the absence of inflammation

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Keywords: alveolus, claudin, lung barrier, tight junction

Abbreviations: ARDS, Acute Respiratory Distress Syndrome; GM-CSF, Granulocyte Macrophage Colony Stimulating Factor; I κ B, Inhibitor of κ B; IL, interleukin; JAM-A, junctional adhesion molecule A; LPS, lipopolysaccharide; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; PBS, phosphate buffered saline; TER, transepithelial resistance; TNF, Tumor Necrosis Factor; ZO, zonula occludens

NF- κ B (p50/p65) is the best characterized transcription factor known to regulate cell responses to inflammation. However, NF- κ B is also constitutively expressed. We used inhibitors of the classical NF- κ B signaling pathway to determine whether this transcription factor has a role in regulating alveolar epithelial tight junctions. Primary rat type II alveolar epithelial cells were isolated and cultured on Transwell permeable supports coated with collagen for 5 d to generate a model type I cell monolayer. Treatment of alveolar epithelial monolayers overnight with one of 2 different I κ B kinase inhibitors (BAY 11-7082 or BMS-345541) resulted in a dose-dependent decrease in TER at concentrations that did not affect cell viability. In response to BMS-345541 treatment there was an increase in total claudin-4 and claudin-5 along with a decrease in claudin-18, as determined by immunoblot. However, there was little effect on the total amount of cell-associated claudin-7, occludin, junctional adhesion molecule A (JAM-A), zonula occludens (ZO)-1 or ZO-2. Moreover, treatment with BMS-345541 resulted in altered tight junction morphology as assessed by immunofluorescence microscopy. Cells treated with BMS-345541 had an increase in claudin-18 containing projections emanating from tight junctions ("spikes") that were less prominent in control cells. There also were several areas of cell-cell contact which lacked ZO-1 and ZO-2 localization as well as rearrangements to the actin cytoskeleton in response to BMS-345541. Consistent with an anti-inflammatory effect, BMS-345541 antagonized the deleterious effects of lipopolysaccharide (LPS) on alveolar epithelial barrier function. However, BMS-345541 also inhibited the ability of GM-CSF to increase alveolar epithelial TER. These data suggest a dual role for NF- κ B in regulating alveolar barrier function and that constitutive NF- κ B function is required for the integrity of alveolar epithelial tight junctions.

Introduction

Epithelial barrier function represents a first line of defense in innate immunity. It is well established that pro-inflammatory stimuli such as cytokines and bacterial endotoxin interact with epithelia to compromise barrier function by targeting tight junctions. In fact, the inflammatory response is a major factor which compromises the alveolar barrier in severe cases of Acute Respiratory Distress Syndrome (ARDS).¹ About 25% of patients with ARDS die as a result of failure of the alveolar epithelial air/liquid barrier, underscoring the need to control the inflammation which accompanies ARDS.^{2,3}

Moreover, chronic alcohol abuse further increases the severity of ARDS. In otherwise healthy alcoholics, the alveolar barrier is impaired^{4,5} although this is compensated by increased fluid clearance.^{6,7} Although this in and of itself does not cause ARDS, the alcoholic lung is more sensitive to a second "hit" like ventilator induced lung injury or sepsis, where barrier function is further impaired

which overwhelms fluid clearance, leading to increased airspace flooding and subsequent respiratory distress.⁸ Although it is well established that inflammation promotes alveolar leak following the second hit, roles for inflammation in alveolar leak in the otherwise healthy alcoholic at baseline are not well characterized.

The transcription factor NF- κ B is a heterodimer consisting of p50 and p65 subunits that is often considered a master regulator of inflammation.⁹ Inflammation is mediated by the classical NF- κ B pathway that is activated by several agents, including cytokines such as TNF- α and other stimuli (e.g. bacterial endotoxin). The pro-inflammatory pathway is downstream of I κ B Kinase, which can be targeted pharmacologically using several different compounds, suggesting the potential for anti-inflammatory treatment.^{10,11} In addition to the classical NF- κ B pathway there is also an alternative pathway that plays an important role in processes such as lymphoid cell development, although the alternative pathway does not require I κ B phosphorylation and activates

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Submitted: 07/15/2014; Revised: 10/24/2014; Accepted: 10/27/2014

<http://dx.doi.org/10.4161/21688370.2014.982424>

different NF- κ B related transcription factors than the p50/p65 heterodimer.¹²

In the adult lung, the alveolar epithelium is composed of a mixed monolayer of cells, type II and type I alveolar epithelial cells.^{13,14} Type II cells produce pulmonary surfactant and are the precursors to type I cells.¹⁵ However, type II cells cover only a small amount of the alveolar surface as opposed to type I cells, which are large flat cells that cover over 80% of the alveolar surface.¹⁶ Thus, tight junctions between type I cells form the main alveolar permeability barrier in the terminal airspaces of the lung.¹⁷ *In vitro* models using primary alveolar epithelial cells have successfully been used to study the alveolar barrier and tight junctions. Using this approach, roles for several tight junction proteins in regulating alveolar paracellular permeability have been identified. Specifically, claudin-4 and claudin-18 have been shown to promote barrier function while claudin-3 decreases barrier function, indicating specificity in regulation of tight junction permeability.¹⁸⁻²²

A role for NF- κ B in regulating the effects of cytokine stimulated decreases in alveolar barrier function was demonstrated by Lee, et al.,²⁰ using cells challenged with a mixture of pro-inflammatory cytokines (cytomix) consisting of TNF- α , IL-1 β and interferon- γ .²⁰ Specifically, cytomix caused a 4-fold increase in paracellular flux which correlated with a greater than 80 % decrease in total claudin-18. Treatment with the I κ B Kinase inhibitor BMS-345541 partially abrogated this effect, implicating the NF- κ B pathway in cytomix-induced alveolar epithelial leak.

However, NF- κ B does not act as a simple on/off switch regulating inflammation. For example, complete inhibition of classical NF- κ B signaling in NEMO knockout mice causes chronic intestinal inflammation, indicating a role for baseline NF- κ B activity in promoting a healthy gut epithelium.²³ Moreover, we found that healthy sheep intravenously administered the I κ B Kinase inhibitor BAY 11-7082 had catastrophic, severe pulmonary edema without inflammation.²⁴ This suggests that constitutive NF- κ B is required to preserve lung barrier function. Since lung barrier function is critically dependent on alveolar tight junctions, we hypothesized that NF- κ B inhibitors would increase alveolar epithelial permeability through dysregulation of tight junction proteins.

Using primary rat alveolar epithelial cells, we found that 2 different I κ B Kinase inhibitors caused a dose dependent decrease in alveolar epithelial barrier function. These alveolar epithelial cells showed a decrease in alveolar barrier function in response to NF- κ B inhibitors, regardless of whether the rats were fed alcohol, a liquid control diet or standard chow diet. Inhibiting NF- κ B reversed the deleterious effects of bacterial endotoxin (lipopolysaccharide; LPS) on alveolar epithelial cells, yet also inhibited the beneficial effect of granulocyte macrophage colony stimulating factor (GM-CSF), which is known to promote alveolar barrier function. This suggests a differential role for pro-inflammatory activation vs. constitutive NF- κ B in regulating alveolar tight junctions and suggests that part of the mechanism of action for the ability of GM-CSF to increase alveolar barrier function is mediated through the constitutive NF- κ B pathway. This also suggests that global inhibition of NF- κ B as a therapeutic target could have unexpected, deleterious consequences through inhibition of constitutive NF- κ B.

Methods

Recombinant rat GM-CSF was from PeproTech (Rocky Hill, NJ). Lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 was from Sigma (St. Louis, MO). The following antibodies were purchased from Life Technologies: Rabbit Anti-Claudin 4 36-4800, Rabbit anti-claudin-5 #341600, Rabbit anti-claudin-7 #34-9100, Rabbit Anti-claudin-18 #700178, Rabbit anti-ZO-1 #61-7300, Mouse Anti-ZO-1 #33-9100, Rabbit Anti-ZO-2 #30-3848, Mouse Anti-Occludin #331500 and Rabbit Anti-Jam-A #36-1700. Mouse-Anti-actin #A53116 was purchased from Sigma. Secondary horseradish peroxidase-conjugated goat anti-rabbit IgG #111-035-144 IgG or goat anti-mouse IgG 115-035-166 as well as secondary Cy2 or Cy3 conjugated antibodies Goat-Anti Mouse IgG #115-165-1660 or #115-225-166 or Goat-Anti Rabbit #111-225-1440 were obtained from Jackson Immuno Research laboratories.

Primary Alveolar Epithelial Cell Isolation

All animal experiments were performed with the approval of the Division of Animal Resources and Institutional Animal Care and Use Committee at Emory University, Atlanta GA. Adult male Sprague-Dawley rats (150–200 g, Charles River Laboratory, Wilmington, MA) were used as a source for alveolar epithelial cells. In most cases, rats were given a standard chow diet ad libitum. For some experiments, rats were fed for 6 weeks ad libitum an all liquid Lieber-DeCarli diet (Research Diets, New Brunswick, NJ) that contained either ethanol (36% of total calories; alcohol diet) or an equivalent isocaloric substitution of maltin-dextrin (control diet) as previously described.⁵ Type II alveolar epithelial cells were isolated from lungs lavaged and perfused with elastase using the method of Dobbs et al.²⁵ with modifications.²⁶ Preparations routinely contained greater than 90–95% type II alveolar epithelial cells. Freshly isolated cells were cultured in Dulbecco's Modified Eagle Media (Life Technologies, Rockville, MD) containing 10% FBS, 100 U/ml penicillin and 10 mg/ml streptomycin (Sigma), and 0.25 μ g/ml amphotericin B (Life Technologies) in Transwells coated with rat tail type I collagen (Roche Diagnostics, Mannheim, Germany) at 7.5×10^5 cells per well as previously described.²⁷

Inhibitor Treatment and Analysis

Cells were treated on Day 5 of culture as model type I alveolar epithelial cells. BMS-345541 and BAY-11-7082 were from Merck Millipore (Danvers, MA) and dissolved in DMSO as a concentrated stock prior to use. Inhibitors were added to tissue culture medium prior to application to cells and added so that the total DMSO concentration was less than 0.1% total. Controls consisted of cells treated with medium containing 0.1% DMSO. In either case, cells were incubated for 16 h in the presence of vehicle control or inhibitor.

Transepithelial resistance (TER) of cells in medium cultured on permeable supports was measured using an Ohmmeter

(World Precision Instruments, Sarasota, FL) as previously described.^{28,29} Statistical significance was calculated by t test. To measure the effects of agents on cell viability, treated cells were washed, incubated with 0.1 μ M calcein AM and 1 μ M ethidium (Live/Dead kit, Life Technologies/Molecular Probes) and scored by fluorescence microscopy as the percentage of surviving cells (calcein positive/ethidium negative).²⁸

Immunofluorescence Microscopy

Immunofluorescence staining of cells on Transwells was performed as previously described.^{28,29} Cells were washed with PBS 3 times, fixed in MeOH/Acetone 1:1 for 2 minutes at room temperature, washed 3 times with PBS, once with PBS + 0.5% TX-100, then once with PBS + 0.5% Triton X-100 + 2% normal goat serum. Cells were incubated with primary anti-rabbit and/or anti-mouse antibodies in PBS/GS for 1 h., washed, incubated with Cy2-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) in PBS/GS, washed, and then mounted in MOWIOL under a glass coverslip. Cells were imaged by phase-contrast and fluorescence microscopy using an Olympus IX70 with a U-MWIBA filter pack (BP460–490, DM505, BA515–550) or U-MNG filter pack (BP530–550, DM570, BA590–800+). Minimum and maximum intensity were adjusted for images in parallel so that the intensity scale remained linear in order to maximize dynamic range.

Immunoblot Analysis

After 6 d in culture, cells on permeable supports were harvested and lysed in 2 $\times$ sample buffer containing 50 mM dithiothreitol (DTT), resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to Immobilon membranes (Millipore, Billerica, MA), and blotted using primary antibodies and HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. Specific signals corresponding to a given protein were detected by immunoblot using enhanced chemiluminescence (ECL) reagent (GE Healthcare, Pittsburgh, PA) and quantified with the BioRad Image Lab system (Hercules CA). Normalization for protein content was done using parallel samples analyzed for actin. Statistical significance was calculated by t test.

Results

NF- κ B is a key transcription factor that regulates pro-inflammatory responses of several classes of cells including

epithelial cells.⁹ Since inhibiting NF- κ B caused rapid and severe pulmonary edema in the absence of inflammation in sheep,²⁴ we examined the effect of NF- κ B inhibitors on barrier function of primary rat alveolar epithelial cells cultured on Transwell permeable supports. We found that 2 different I κ B Kinase inhibitors, BAY-11-7082 or BMS-345541,^{10,11} inhibited alveolar epithelial barrier function in a dose dependent manner (Fig. 1A,C). Alveolar epithelial cells showed a $38.4 \pm 9.9\%$ ($n = 6$) decrease in TER after overnight treatment with 0.2 μ M BAY-11-7082 (Fig. 1A). Overnight treatment with 4 μ M BMS-345541 resulted in a comparable decrease in TER ($29.4 \pm 6.3\%$ ($n = 8$); Fig. 1C). For both inhibitors, these concentrations did not affect cell viability (Fig. 1B, D). However, we did note that at higher concentrations, primary alveolar epithelial cells were more sensitive to BAY-11-7082 (Fig. 1B) than BMS-345541 (Fig. 1D). Given the increased toxicity of BAY-11-7082, we focused our additional studies on examining the effects of BMS-345541 on alveolar epithelial barrier function.

Tight junctions provide the physical molecular basis for paracellular epithelial barrier function. Thus, we examined the effects of BMS-345541 on a subset of the alveolar epithelial tight junction proteome. As shown in Figure 2, 10 μ M BMS-345541 significantly altered claudin expression, where claudin-4 and claudin-5 increased and claudin-18 decreased. Claudin-7 was

Figure 1. NF- κ B inhibitors decrease alveolar epithelial cell barrier function. Primary rat alveolar epithelial cells were isolated and cultured on Transwell permeable supports for 5 d to produce model type I cell monolayers. The cells were then further incubated overnight with varying amounts of either BAY 11-7082 (A) or BMS-345541 (C) or vehicle control (0) and transepithelial resistance (TER) was measured using an Evohm voltmeter. For both agents, there was a statistically significant decrease in TER with increasing levels of drug treatment (* $P < 0.05$, $n = 3-8$). To rule out cell death as a possible cause of compromised barrier function, we assessed parallel cultures using the Invitrogen Live/Dead assay (B, D). Although there was considerable cell death at 0.4 and 1.0 μ M BAY 11-7082 and some loss of viability at 10 μ M BMS-345541 (B), TER also significantly decreased at lower concentrations of BAY 11-7082 and BMS-345541 where cell viability was maintained (* $P < 0.05$, $n = 5$).

Figure 2. Effect of BMS-345541 on alveolar tight junction protein expression. Primary rat alveolar epithelial cells were isolated and cultured on Transwell permeable supports for 5 d to produce model type I cell monolayers. The cells were then incubated overnight with varying amounts of either BMS-345541 or vehicle control (0), harvested and analyzed by immunoblot for tight junction proteins. We found that with increasing BMS-345541 concentration there was a significant increase in claudin-4 and claudin-5 and a significant decrease in claudin-18 (* $p < 0.05$, $n = 3$). The other proteins examined showed no significant changes.

unchanged. A comparable trend in alveolar claudin composition was observed at 4 μ M BMS-345541 although these changes in alveolar epithelial claudin content did not reach statistical significance. These changes in claudin expression induced by BMS-345541 were not due to effects on cell viability, since cells treated with concentrations of BAY-11-7082 which had considerable impact on cell viability (0.4 or 1.0 μ M), had little if any effect on claudin expression (Fig. 3). Moreover, there was little change in total occludin, JAM-A, ZO-1 and ZO-2, indicating that changes in alveolar barrier function induced by BMS-345541 were not due to extensive alterations in tight junction composition, particularly at 4 μ M concentration.

We then examined the effects of BMS-345541 on alveolar tight junction morphology, using claudin-18 as a marker since this protein is the predominant claudin expressed by alveolar epithelial cells²². As shown in Figure 3A, C, untreated alveolar epithelial cells showed claudin-18 coherently localized to junctions at cell-cell interfaces, as previously described.^{21,28,29} By contrast, cells treated with 4 μ M BMS-345541 showed altered claudin-18 morphology, where there were projections that appeared to be perpendicular to the orientation of the intercellular junction (Fig. 4 B, D). We refer to these projections as “tight junction spikes.” By scoring the number of cells which contained 5 or more tight junction spikes, we found that BMS-345541

treated cells had significantly more cells forming spikes than untreated control cells. Moreover, BMS-345541 also altered the localization of ZO-1 and ZO-2 in alveolar epithelial cells (Fig. 5), where there were discontinuities in localization of these scaffold proteins to junctions induced by the NF- κ B inhibitor. This was also accompanied by a profound change in the organization of the actin cytoskeleton, where there was an overall decrease in F-actin, especially cortical actin along the plasma membrane (Fig. 6). This was consistent with a previous report demonstrating that BAY 11-7082 has a comparable effect on the actin cytoskeleton.³⁰ We also examined β -catenin, which is particularly enriched in areas where cells overlap (Fig. 7). BMS-345541 decreased the overall extent of β -catenin labeling (Fig. 7C, E), although the distribution was comparable to control cells. Interestingly,

most β -catenin appeared opposite to areas enriched with tight junction spikes. However, there also was some spike localized β -catenin as well, correlating with changes to the actin cytoskeleton in spike-enriched areas. Taken together the changes in localization of claudin-18, ZO-1, ZO-2 and F-actin which accompany decreased TER suggest that BMS-345541 inhibits alveolar barrier function by destabilizing tight junctions.

Several conditions that impact alveolar barrier function, including chronic alcohol ingestion and sepsis, have been found to persist in alveolar epithelial cells isolated from affected animal models.^{5,31} The mechanism for this is not known at present, however alveolar epithelial cells isolated from alcohol fed rodents provide a model system where the impact of chronic alcohol ingestion on alveolar tight junctions can be studied *in vitro*. To determine whether an alcohol related decrease in alveolar TER is affected by inhibiting NF- κ B signaling, we examined the effects of BMS-345541 on alveolar epithelial cells isolated from alcohol fed rats. We found that in response to BMS-345541 treatment alveolar epithelial cells from alcohol fed rats showed a further decrease in TER in response to BMS-345541 as did cells from rats fed a control liquid diet (Fig. 8A, B). This was despite the fact that the overall TER of cells from alcohol fed rats was lower than that of cells from control fed rats. Note, however, that cells from mice fed either the control or alcohol Lieber-DeCarli diet

Figure 3. Effect of BAY 11-7082 on alveolar tight junction protein expression. Primary rat alveolar epithelial cells were isolated and cultured on Transwell permeable supports for 5 d to produce model type I cell monolayers. The cells were then incubated overnight with varying amounts of either BAY 11-7082 or vehicle control (0), harvested and analyzed by immunoblot for tight junction proteins. We found that BMS-345541 did not cause significant changes to claudin-4, claudin-5 or claudin-18.

were less sensitive to BMS-345541 than chow fed mice (e.g., Fig. 1C). At 4 μ M BMS-345541 inhibited TER of alveolar epithelial cells from chow fed mice by $29.4 \pm 6.3\%$ ($n = 8$), whereas cells from rats fed control or alcohol Lieber-DeCarli liquid diet showed $11.0 \pm 0.9\%$ ($n = 3$) or $12.7 \pm 1.8\%$ ($n = 3$) decreases in TER respectively in response to 4 μ M BMS-345541. These data underscore the potential effects of diet on alveolar epithelial function, particularly since the Lieber DeCarli diet is enriched for simple sugars vs chow which has more complex carbohydrates.³²

To further investigate the effects of BMS-345541 on alveolar epithelial cells, we used a well characterized pro-inflammatory stimulus, lipopolysaccharide (LPS), to decrease barrier function.³³ As shown in Figure 8C, cells treated with 1 μ g/ml LPS showed a $24.8 \pm 3.5\%$ ($n = 3$) decrease in TER, consistent with LPS being a pro-inflammatory stimulus. However, co-incubation with 4 μ g/ml BMS-345541 reversed the effects of LPS and preserved alveolar barrier function, implicating NF- κ B mediated signaling in the effects of LPS on barrier function.

Thus, we had conditions where BMS-345541 at the same concentration could either promote or decrease alveolar barrier function. To help address this apparent paradox, we identified stimuli known to promote alveolar epithelial barrier function and determined which might have an NF- κ B dependent signaling component. Chief among these is GM-CSF which we previously found directly stimulates alveolar epithelial cells to have

Figure 4. BMS-345541 destabilized tight junction associated claudin-18. Primary rat alveolar epithelial cells were isolated and cultured on Transwell permeable supports for 5 d to produce model type I cell monolayers. The cells were then incubated overnight with vehicle control (A, C) or 4 μ M BMS-345541 (B, D), fixed and processed to image claudin-18 by immunofluorescence microscopy. Magnified insets are shown in C, D. In contrast to control cells, where claudin-18 was strongly localized to tight junctions at the plasma membrane, cells treated with BMS-345541 showed linear trails emanating from the plasma membrane (tight junction spikes; arrowheads). Bar – 10 micron.

increased barrier function.^{34,35} Critically, GM-CSF has been shown to activate several epithelial signal transduction pathways, including an NF- κ B dependent pathway.^{36,37} So we hypothesized that BMS-345541 would antagonize the effects of GM-CSF on alveolar epithelial cells. As shown in Figure 8C, this was indeed the case. Alveolar epithelial cells treated with 20 μ g/ml GM-CSF showed a $23.0 \pm 13.6\%$ ($n = 9$) increase in TER, co-treatment with 4 μ g/ml BMS-345541 abrogated this effect.

Figure 5. BMS-345541 disrupts association of ZO-1 and ZO-2 with tight junctions. Model type I cell monolayers were incubated overnight with vehicle control (A, B) or 4 μ M BMS-345541 (C, D), fixed and processed to image ZO-1 (A, C) or ZO-2 (B, D) by immunofluorescence microscopy. In contrast to control cells, BMS-345541 treated cells had large areas of cell-cell contact which lacked strong ZO-1 or ZO-2 labeling (arrowheads). Bar – 10 micron.

Taken together these data demonstrate that BMS-345541 can both decrease and increase TER and suggest a dual role for NF- κ B in regulating alveolar epithelial barrier function.

Discussion

The central finding of this study was that, at baseline, I κ B kinase inhibitors had a deleterious effect on alveolar epithelial barrier function (Fig. 1, Fig. 8 A, B). This is in apparent contrast to the preponderance of evidence demonstrating that NF- κ B is a key mediator of pro-inflammatory responses in several cell types⁹ and that inflammation is classically associated with a failure of epithelial barrier function.³⁸ Nonetheless, baseline constitutive NF- κ B activity has been shown to be critical for preventing inflammatory bowel disease²³ and in preventing pulmonary edema.²⁴ Thus, our data finding that I κ B Kinase activity is required for alveolar barrier function suggests that constitutive NF- κ B signaling pathway functions at baseline to promote alveolar tight junction formation and that inhibiting this pathway can lead ultimately to pulmonary edema, even in an otherwise unperturbed lung. In addition, since BMS-345541 did not reverse the deleterious effects of chronic alcohol on alveolar epithelial barrier function, our data suggest that activation of NF- κ B does not induce the barrier dysfunction that occurs in the uninjured alcoholic lung.

GM-CSF is highly expressed in the lung where it has several effects on lung function that can be both beneficial and deleterious.³⁹ Roles for GM-CSF in promoting alveolar epithelial homeostasis were revealed by GM-CSF deficient mice which

Figure 6. BMS-345541 induces actin cytoskeletal rearrangements. Model type I cell monolayers were incubated overnight with vehicle control (A-C, G-I) or 4 μ M BMS-345541 (D-F, J-L), fixed and processed to image claudin-18 (A, D, G, J) and actin (B, E, H, K) by immunofluorescence microscopy using rhodamine-phalloidin to label F-actin. Merged images are in C, F, I, L. Images in G-L represent magnifications of the regions in C, F denoted by the box. Cells treated with BMS-345541 showed decreases in cortical actin as compared with control cells (arrowheads). Actin frequently co-localized with claudin-18 localized to tight junction spikes (arrows), particularly in cells treated with BMS-345541. Bar – 10 micron (A-F), 2 micron (G-L).

developed a form of pulmonary alveolar proteinosis.⁴⁰ ARDS survival correlates with higher levels of GM-CSF in lung lavage fluid consistent with a protective role for this cytokine in promoting lung function.⁴¹ In the alcoholic lung, GM-CSF signaling is blunted which renders the lung more susceptible to injury; recombinant GM-CSF in vivo ameliorates the effects of alcohol by increasing lung barrier function to alcohol-fed rats and improving lung liquid clearance during endotoxemia.³⁵ In other words, GM-CSF can antagonize the deleterious effects of LPS. However, GM-CSF is not strictly protective since GM-CSF deficient mice are resistant to endotoxin induced lung injury.⁴² This is partially due to a decrease in the ability of LPS to induce a complete NF- κ B response in alveolar macrophages, implicating GM-CSF signaling in endotoxemia.⁴³ This led Trapnell and co-workers to propose a rheostat model where the relative concentration of GM-CSF could either have pro-inflammatory or protective effects on the lung, when synergistically acting with other stimuli.⁴³

Figure 7. BMS-345541 alters the extent of β -catenin expression.

Model type I cell monolayers were incubated overnight with vehicle control (A–C) or 4 μ M BMS-345541 (D–F), fixed and processed to image claudin-18 (A, D) and β -catenin (B, E) by immunofluorescence microscopy. Merged images are in C, F. Cells treated with BMS-345541 showed an overall decrease in β -catenin as compared with controls although the overall appearance was comparable. Note that the majority of β -catenin was opposed to areas which have tight junction spikes (arrowheads), although spikes did have some β -catenin as well. Bar – 10 micron.

GM-CSF is mostly associated with the JAK/STAT signal transduction pathway which, in turn activates the transcription factor PU.1.⁴⁴ In fact, PU.1 signaling is significantly reduced in the alcoholic lung.⁴⁵ However, GM-CSF receptors also activate several other signaling pathways, including the MAP kinase ERK1/2.³⁷ Recently, it was discovered that GM-CSF receptors directly interact with IKK β to activate I κ B Kinase and thus NF- κ B signaling.³⁶ Our data suggest that in the absence of other pro-inflammatory insults, the GM-CSF NF- κ B signaling pathway promotes alveolar epithelial barrier function. Moreover, this pathway is active at baseline since barrier function of naïve cells was also sensitive to NF- κ B inhibition. This opens up the possibility that alveolar epithelial cells may be activating this pathway via constitutive GM-CSF production and paracrine signaling. Whether this is the case or another pathway is responsible for constitutive NF- κ B signaling remains to be determined.

In response to BMS-345541, alveolar epithelial cells showed altered expression of several tight junction proteins including increased claudin-4 and claudin-5 and decreased claudin-18 (Fig. 2). Although these changes in alveolar epithelial claudin content were only statistically significant at 10 μ M BMS-345541, which begins to have an impact on cell viability, these claudins trended toward a comparable change in cells treated with 4 μ M BMS-345541 where cell viability was unaffected (Fig. 1D). This also underscores the importance of tight junction destabilization vs. overt changes to claudin expression as a mechanism for the effects of inhibiting NF- κ B on alveolar barrier function.

Decreased alveolar epithelial claudin-18 has been shown to correlate with impaired barrier function in several systems, including isolated primary human alveolar epithelial cells²⁰ in claudin-18-deficient mice.^{18,19} The finding that claudin-4 was

increased by inhibiting NF- κ B was somewhat surprising, in light of studies by Frank and coworkers, demonstrating that claudin-4 is specifically upregulated in response to ventilator-induced lung injury which would be expected to be a pro-inflammatory environment.⁴⁶ Given that alveolar barrier function remains impaired despite increased claudin-4 content, our data suggest that in response to BMS-345541 there is not a sufficient level of claudin-4 produced to adequately compensate for decreased claudin-18, even when cell viability is unaffected.

We also found that BMS-345541 increased claudin-5 expression. This is consistent with our previous findings on alveolar epithelial cells where increased claudin-5 correlated with increased paracellular leak in alveolar epithelial cells exposed to methanandamide.²⁸ Moreover claudin-5 is also increased as a result of chronic alcohol exposure as well.⁵ The finding that BMS-345541 further impaired the barrier function of alveolar epithelial cells isolated from alcohol fed rats suggests that claudin-5 may be further increased in these cells by inhibiting NF- κ B. Whether this is the case or whether BMS-345541 is affecting tight junction assembly via another mechanism remains to be determined.

We also observed that alveolar tight junctions exhibited morphological changes in response to BMS-345541, where this agent promoted formation of tight junction spikes that were perpendicular to the axis of the cell-cell junction (Fig. 4). This morphological change suggests tight junction destabilization which is expected to decrease paracellular barrier function. Destabilized claudin-18 could be responsible for the net decrease in claudin-18 observed in cells treated with BMS-345541 (Fig. 2). Spike formation correlated with disrupted localization of ZO-1 and ZO-2 (Fig. 5) and changes in cytoskeletal organization (Fig. 6). In fact, the effects of BMS-345541 on actin were consistent with the BAY 11-7082 treatment, which also induced cytoskeletal disassembly.³⁰ This concordant destabilization of tight junction is in good agreement with data supporting roles for ZO-1 and ZO-2 scaffold proteins in promoting claudin assembly into tight junctions as well as their stability by regulating interactions with F actin.⁴⁷⁻⁴⁹ What is not known is whether these morphological changes are caused by alterations in scaffold protein activity or whether they are instead driven by changes in tight junction protein composition. Future studies defining roles for claudins in regulating ZO-1 and vice versa would enable these 2 possibilities to be distinguished, whether they are in response to NF- κ B inhibition or other stimuli which destabilize alveolar epithelial tight junctions.

This study focused on the effects of two NF- κ B inhibitors, which had comparable effects on tight junction organization and function. BMS-345541 and BAY 11-7082 both impaired alveolar epithelial barrier function, however they did differ in relative toxicity and effect on claudin expression. Although they both act as IKK inhibitors, BMS-345541 and BAY 11-7082 have distinct mechanisms of action,^{10,11} which may explain this difference in the way they affect tight junctions. Moreover, these agents may have off target effects beyond NF- κ B signaling that may affect other pathways required for

Figure 8. Differential effects of BMS-345541 on alveolar epithelial barrier function. Primary rat alveolar epithelial cells were isolated from rats fed either a control Lieber-DeCarli diet (A) or a Lieber-DeCarli diet containing dietary alcohol (B). The cells were cultured on Transwell permeable supports for 5 d to produce model type I cell monolayers and then incubated overnight with varying amounts of either BMS-345541 or vehicle control (0) and TER was measured. There was a statistically significant decrease in TER with increasing levels of drug treatment (* $P < 0.05$, $n = 3$). At all but the highest concentration of BMS-345541 cells from alcohol fed rats had significantly lower TER than cells isolated from control rats (# $P < 0.05$, $n = 3$). C. Cells from alcohol fed rats were further incubated with either 1 μ g lipopolysaccharide (LPS) or 20 μ g/ml GM-CSF overnight in either the presence or absence of 4 μ g/ml BMS-345541. LPS significantly decreased TER while GM-CSF significantly increased TER. BMS-345541 reversed the effects of LPS and GM-CSF. *— $P < 0.05$, $n = 3$ vs. untreated and BMS-345541 co-treated cells.

scenario is that the effects of these agents on tight junctions is via an NF- κ B dependent pathway.

Assuming that the effects we see of these inhibitors are indeed due to an NF- κ B dependent pathway, assigning a specific link between NF- κ B and regulation of tight junctions is a challenge, given the multiplicity of cell functions that are associated with this transcription factor.⁹ Despite limitations to our approach, the findings that IKK inhibitors impair alveolar epithelial barrier function in the absence of inflammation suggest a previously unappreciated role for NF- κ B in normal alveolar epithelial barrier function. Moreover, one consequence of our study is that NF- κ B inhibitors to control inflammation may have limited utility, particularly in the treatment of ARDS, since they may actually have a deleterious effect on lung barrier function and thus exacerbate the severity of pulmonary edema and lung injury.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Samuel Molina and William Koval for critical reading of the manuscript.

Funding

Supported by NIH R01-HL116958, R01-HL070891, P50-AA013757, and T32-AA013528.

tight junction assembly. However, considering the protective effects of BMS-345541 on alveolar epithelial cells subjected to pro-inflammatory stimuli (Fig. 8C) and,²⁰ the most likely

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