

# Airway Epithelium Stimulates Smooth Muscle Proliferation

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Communication between the airway epithelium and stroma is evident during embryogenesis, and both epithelial shedding and increased smooth muscle proliferation are features of airway remodeling. Hence, we hypothesized that after injury the airway epithelium could modulate airway smooth muscle proliferation. Fully differentiated primary normal human bronchial epithelial (NHBE) cells at an air-liquid interface were co-cultured with serum-deprived normal primary human airway smooth muscle cells (HASM) using commercially available Transwells. In some co-cultures, the NHBE were repeatedly ( $\times 4$ ) scrape-injured. An *in vivo* model of tracheal injury consisted of gently denuding the tracheal epithelium ( $\times 3$ ) of a rabbit over 5 days and then examining the trachea by histology 3 days after the last injury. Our results show that HASM cell number increases 2.5-fold in the presence of NHBE, and 4.3-fold in the presence of injured NHBE compared with HASM alone after 8 days of *in vitro* co-culture. In addition, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1 and, more markedly, matrix metalloproteinase (MMP)-9 concentration increased in co-culture correlating with enhanced HASM growth. Inhibiting MMP-9 release significantly attenuated the NHBE-dependent HASM proliferation in co-culture. *In vivo*, the injured rabbit trachea demonstrated proliferation in the smooth muscle (trachealis) region and significant MMP-9 staining, which was absent in the uninjured control. The airway epithelium modulates smooth muscle cell proliferation via a mechanism that involves secretion of soluble mediators including potential smooth muscle mitogens such as IL-6, IL-8, and MCP-1, but also through a novel MMP-9-dependent mechanism.

**Keywords:** remodeling; NHBE; injury; MMP-9; HASM

Chronic tissue injury and inflammation triggered by exogenous stimuli can cause acute bronchoconstriction (1). Over time, structural changes such as goblet cell hyperplasia, subepithelial fibrosis, smooth muscle cell hypertrophy and hyperplasia, and increased vascularity and edema occur in the airway wall (airway remodeling) (2). An intensive effort is aimed at understanding the mechanisms that lead to airway remodeling, in

## CLINICAL RELEVANCE

An injured or compromised airway epithelium stimulates smooth muscle cell hyperplasia, in part, through IL-6, IL-8, monocyte chemoattractant protein-1, and matrix metalloproteinase-9-dependent mechanisms. This result provides new evidence supporting epithelium dysfunction as a potential therapeutic target.

hopes of not only slowing, but perhaps reversing, the structural alterations (3–5).

Airway epithelial cells play a critical role in the defense system of the lungs by providing an important barrier function to potentially toxic environmental agents that can promote epithelial damage, or induce bronchoconstriction in susceptible individuals (6, 7). Not surprisingly, epithelial shedding, marked by increased numbers of epithelial cell clumps (creola bodies) in sputum and bronchial epithelium desquamation, are features of airway injury. The sequelae is an airway epithelium in a chronic state of wound repair, which secretes soluble mediators (e.g., IGF) necessary for cell proliferation, migration, and extracellular matrix synthesis consistent with a healing wound environment (8–11).

Communication between the epithelium and the underlying fibroblast in the lamina propria is prevalent and normal during fetal lung development (9). In contrast, the airway smooth muscle lies adjacent to the lamina propria, and therefore a further distance ( $\sim 50$ – $200 \mu\text{m}$ ) from the epithelium (12). For the epithelium to influence smooth muscle cell behavior, soluble mediators would need to diffuse through the lamina propria. Hence, the vast majority of investigation has focused on the role of the epithelium in subepithelial fibrosis. Although progress has been made in identifying mitogenic stimuli of airway smooth muscle, the potential role of epithelium-derived mediators in smooth muscle proliferation remains unexplored.

We hypothesized that an injured airway epithelium secretes soluble mediators at biologically relevant concentrations to stimulate smooth muscle cell hyperplasia. To address this hypothesis, we used a co-culture model of primary normal bronchial epithelial cells (NHBE) and primary normal human airway smooth muscle cells (HASM), as well as an *in vivo* model of epithelial injury in the rabbit trachea. Our results demonstrate that an injured airway epithelium promotes HASM cell proliferation. Furthermore, proliferation in the smooth muscle (trachealis) region was observed in the rabbit trachea after repeated epithelial injury. To our knowledge, this is the first report demonstrating the role of the epithelium in airway smooth muscle cell proliferation. The results are consistent with an increasing interest in epithelial dysfunction as a target of therapeutic intervention in airway diseases.

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## MATERIALS AND METHODS

### Cell Culture

NHBE (lot no.: 4F1624, 4F1430, 5F1387; Lonza, Walkersville, MD) were seeded onto uncoated Costar Transwells inserts with 0.4  $\mu\text{m}$  pore size (Fisher Scientific, Pittsburgh, PA) and fully differentiated to a mucociliary phenotype at an air-liquid interface for 14 to 21 days, in media composed of 50% bronchial epithelium basal medium (BEBM; Lonza, Walkersville, MD) and 50% Dulbecco's modified Eagle's medium-F12 low glucose (Invitrogen, Carlsbad, CA) as previously described (13). This medium was supplemented with growth factors provided in the SingleQuot kits (Lonza) and retinoic acid at 50 nM, and will be referred to as "50:50 media."

HASM were obtained from transplant donors with healthy lungs in accordance with procedures approved by University of Pennsylvania as described earlier and used between passages 1 and 5 (14). The HASM were cultured in HAM's F-12 (Invitrogen) medium supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen). Aliquots of HASM cell suspension were plated at a density of  $1.0 \times 10^4$  cells/cm<sup>2</sup> in medium supplemented with 10% FBS on 12-well culture plates (Fisher Scientific). After 24 hours, this medium was replaced with serum-free Ham's F-12 with 0.1% bovine serum albumin for 48 hours. After 48 hours the serum-deprived HASM were co-cultured with well-differentiated NHBE (E<sub>U</sub>S, subscript "u" for uninjured) in "50:50" epithelium medium (which has 0.1% serum), indicating start of co-culture (Day 0). In some co-cultures the NHBE were repeatedly injured (E<sub>I</sub>S, subscript "i" for "injured") using a 200-microliter pipette tip at Days 0, 2, 4, and 6. A final condition consisted of HASM cells alone (S) (i.e., without NHBE co-culture), uninjured NHBE alone (E<sub>U</sub>), or injured NHBE alone (E<sub>I</sub>). All conditions after Day 0 were maintained in 50:50 epithelium media replaced fresh every other day till Day 8 (this method is illustrated in Figure E1 in the online supplement). Three NHBE donors were used with 10 HASM donors in different combinations, and repeats were performed using at least two different NHBE-HASM combinations (additional details are available in the METHODS section of the online supplement).

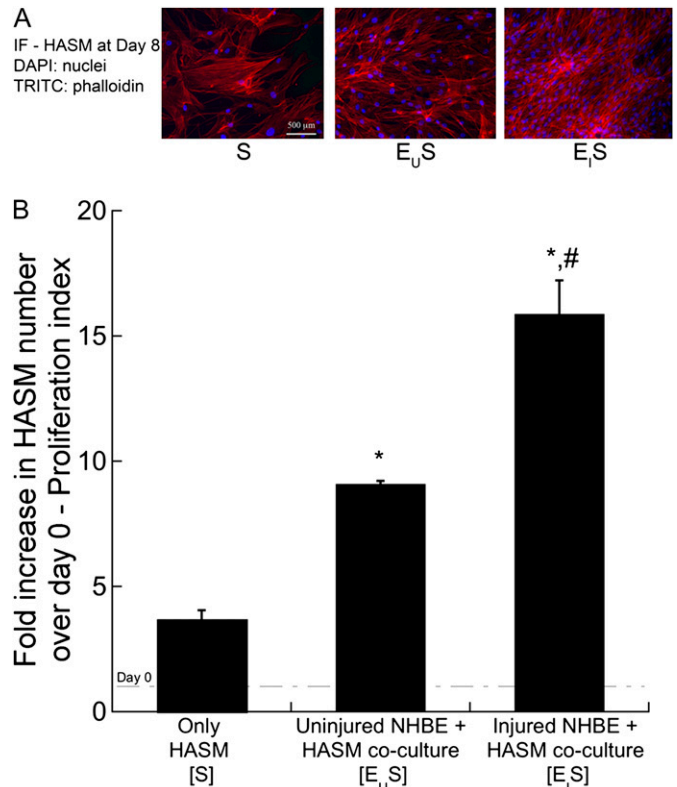
### Rabbit Tracheal Injury

Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine. To induce chronic tracheal epithelial damage similar to our *in vitro* model, we performed a triple scrape procedure on the tracheas of 3- to 5-kg male New Zealand White rabbits (Western Oregon Rabbit Co., Philomath, OR), with each scrape separated by 48 hours of recovery. Before each injury the animals were anesthetized, intubated, and mechanically ventilated as previously described (15). A 7.0-mm-diameter unsheathed cytology brush (Conmed, Utica, NY) was inserted just past the end of the endotracheal tube, and the exposed trachea between the tube and the carina was gently scraped using the following sequence: five vertical scrapes, five 360° brush revolutions along the entire length of exposed trachea, and five more vertical scrapes before brush removal. Three days after the last scrape, the rabbits with injured tracheas as well as healthy control animals (with no prior intubation or tracheal scrape) were killed as previously described (15). The tracheas were immediately removed, washed with ice-cold PBS, cut into sections, placed in standard cryomolds (Electron Microscopy Sciences, Hatfield, PA), immersed in embedding medium (Electron Microscopy Sciences), and snap-frozen in liquid nitrogen. For immunohistochemistry, the tissues were sectioned with a standard cryotome and mounted on Tissue Path Superfrost Plus Gold slides (Fisher Scientific).

## RESULTS

### NHBE (Uninjured and Injured) Induce Airway Smooth Muscle Proliferation

Co-culture of NHBE with HASM for 8 days stimulated HASM proliferation significantly ( $P < 0.05$ ) as evidenced qualitatively by an increase in DAPI staining by immunofluorescence (Figure 1A), and quantitatively by cell counts and MTT assay (Figure 1B) compared with HASM cell alone (2.5-fold).

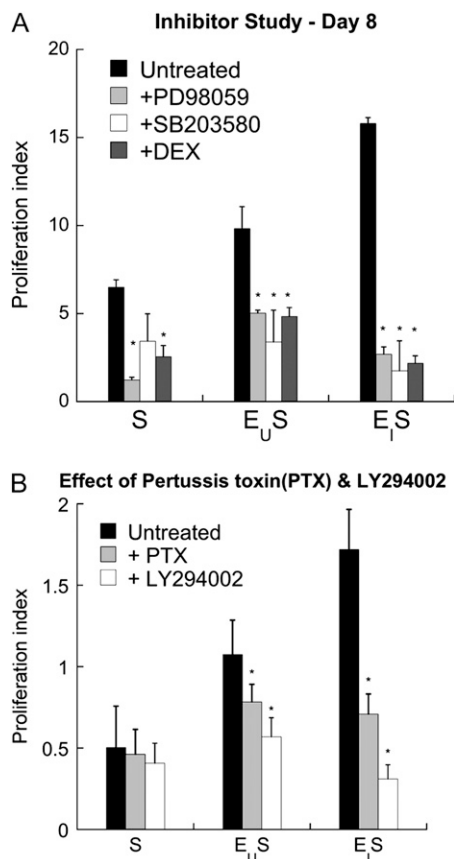


**Figure 1.** Co-culture of uninjured and injured normal human bronchial epithelial cells (NHBE) with human airway smooth muscle cells (HASM) at Day 8. (A) Immunofluorescence (IF) staining of HASM cells after 8 days of co-culture. (B) An increase in the proliferation index (PI) of HASM when co-cultured with uninjured NHBE (E<sub>U</sub>S) as compared with HASM cells alone (S); and an increase in PI in the injured NHBE-HASM co-culture (E<sub>I</sub>S) compared with E<sub>U</sub>S and S is observed. \* $P < 0.05$  as compared with S and # $P < 0.05$  as compared with E<sub>U</sub>S.

Furthermore, at Day 8 the proliferation index (i.e., fold increase in HASM number at Day 8 over Day 0) of HASM in co-culture with injured NHBE was significantly ( $P < 0.05$ ) augmented compared with uninjured NHBE-HASM co-culture (1.7-fold) and HASM cells alone (i.e., HASM single culture) (4.3-fold). The increase from S to E<sub>U</sub>S was observed in 9 of the 10 donor combinations, and the increase from E<sub>U</sub>S to E<sub>I</sub>S was observed in 8 of the 10 donor combinations used (Table E1).

### Mitogen-Activated Protein Kinase (Extracellular Signal-Regulate Kinase 1/2 and p38), Phosphoinositide-3-Kinase, and Gi Pathways Are Activated in HASM

Mitogens (e.g., growth factors and cytokines) activate several transduction pathways in the HASM (16–18). Thus, we examined the possible cellular mechanism (in HASM) for NHBE-induced HASM proliferation. We observed a significant abrogation in proliferation on incubation with either an inhibitor of extracellular signal-regulated kinase (ERK) 1/2 (PD98059, 30  $\mu\text{M}$ ) or p38 (SB203580, 10  $\mu\text{M}$ ) MAPK pathways in some or all of the culture conditions (Figure 2A). In addition, incubation with pertussis toxin (Gi inhibitor, PTX-100 ng/ml) or an inhibitor of PI3K (LY294002, 10  $\mu\text{M}$ ) also decreased proliferation of both co-culture conditions (E<sub>U</sub>S and E<sub>I</sub>S), as shown in Figure 2B. All doses were selected to maximally inhibit the target molecules in HASM based on previous studies (16, 19). Furthermore, incubating with dexamethasone (1  $\mu\text{M}$ ) every other day significantly reduced HASM proliferation at Day 8 in



**Figure 2.** The mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase 1/2 and p38, Gi and PI3K pathways are stimulated in the proliferation of HASM at Day 8. (A) A decrease in the proliferation index (PI) in one or all the inhibitor-incubated (PD98059, SB203580, and dexamethasone) conditions was noted. (B) Pertussis toxin (PTX) and LY294002 reduced proliferation of HASM in E<sub>U</sub>S and E<sub>I</sub>S. \* $P < 0.05$  compared with the untreated condition in the same group.

co-culture with uninjured and injured NHBE. DMSO-only control did not affect proliferation (data not shown). Figure E2 shows inhibition of phosphorylated protein levels upon incubation with respective inhibitors.

#### Levels of Known Mitogens Do Not Account for Proliferation Patterns

We next sought to identify possible soluble mediators, which might be responsible for activating the cell proliferation pathways. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF-1), platelet-derived growth factor (PDGF)-BB, endothelin (ET)-1, and vascular endothelial growth factor (VEGF) (Figure E3) are known to induce HASM proliferation (20–23). Figure E3 depicts the concentrations of these growth factors for S, E<sub>U</sub>S, and E<sub>I</sub>S conditions at Days 2 and 8 of co-culture. While the concentration of several growth factors changed between conditions, there was no single growth factor whose pattern mirrored that of smooth muscle proliferation, suggesting that additional factors may also be involved.

#### IL-6, IL-8, Monocyte Chemotactic Protein-1, and Matrix Metalloproteinase-9 Concentrations Are Increased in NHBE-HASM Co-Culture

We next examined an array of cytokines, chemokines, and proteases known to play roles in airway remodeling and

inflammation, and we found that IL-6, IL-8, monocyte chemotactic protein (MCP)-1, and more dramatically matrix metalloproteinase (MMP)-9 were all significantly ( $P < 0.05$ ) increased in a pattern consistent with HASM proliferation (Figure 3A). Furthermore, we evaluated IL-6, IL-8, MCP-1, and MMP-9 levels released by the NHBE alone either uninjured and injured without any HASM co-culture (Figure 3B), and confirmed MMP-9 levels in all conditions with gelatin zymography (Figure 3C). Our results indicate elevated levels of MMP-9 were secreted by the uninjured and injured epithelium alone and in co-culture with HASM beginning by Day 2 and persisting through Day 8. While IL-6 and IL-8 are primarily secreted by the epithelium, MCP-1 is produced by the HASM, and cross-talk during NHBE-HASM co-culture plays a vital role, as observed by the difference in levels in Figures 3A and 3B. Quantification of equal volumes of cell culture medium demonstrated equal protein levels (data not shown).

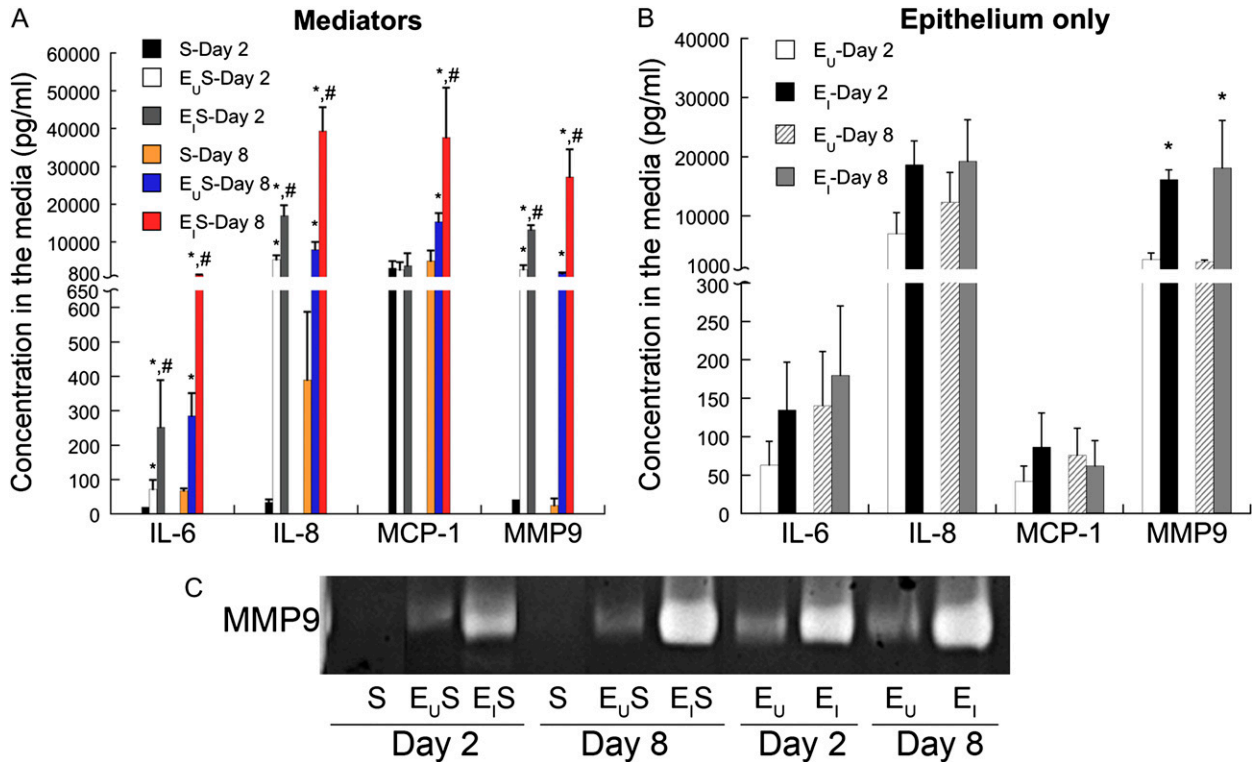
#### Inhibiting MMP-9 Abrogates Proliferation of HASM

To assess the biological activity of the epithelial-derived mediators, we incubated the cell cultures with neutralizing antibodies to IL-6, IL-8, and MCP-1, as well as GM6001 (a global MMP inhibitor-10  $\mu$ M), and a mixture of all four in separate wells. The neutralizing antibodies to IL-6 (10  $\mu$ g/ml), IL-8 (20  $\mu$ g/ml), and MCP-1 (50  $\mu$ g/ml) did not impact proliferation of E<sub>U</sub>S, but did reduce proliferation when the epithelial cells were injured (E<sub>I</sub>S). In contrast, the global MMP inhibitor significantly reduced proliferation of both co-culture conditions, and to a larger degree (Figure 4A). Purified goat IgG at equivalent concentrations and DMSO were used as controls and did not impact proliferation. All doses were selected such that they maximally inhibit the target molecules based on manufacturers' recommendations. The mixture of all the inhibitors decreased proliferation in a pattern consistent with an additive effect observed from each individual inhibitor.

Because of the more dramatic effect of GM6001 and the significant changes in MMP-9 (Figures 3A–3C), we inhibited MMP-9 specifically by employing chemical inhibition and siRNA-mediated knockdown. siRNA for MMP-9 reduced (irrelevant siRNA did not affect the MMP-9 levels; Figure E5A), but did not eliminate, the activity of MMP-9 (Figures 4B and E5B). This resulted in a decrease in the proliferation response of the smooth muscle cells (Figure 4C). Furthermore, specific chemical inhibition of MMP-9 (1  $\mu$ M, AZ11557517) did not impact proliferation of smooth muscle cells alone, but significantly reduced the proliferation response in both co-culture conditions (Figure 4D).

#### Epithelial Scrape Injury to Rabbit Trachea Stimulates Proliferation in the Airway Smooth Muscle (Trachealis) Region

*In vivo*, the airway smooth muscle is separated from the epithelium by the lamina propria; hence, soluble mediators from the epithelium must travel through this diffusion barrier to stimulate smooth muscle proliferation. We next developed an *in vivo* model of airway epithelial injury to determine if our *in vitro* observations were active *in vivo*. Repeated airway epithelial injury to the rabbit trachea increased Ki67 staining (a marker of cell proliferation; Figures 5 and E6) in the airway smooth muscle region compared with the uninjured control. Furthermore, there is evidence of an increase in diffuse MMP-9 staining in the submucosa of the injured (Figure 5E) trachea section of the rabbit compared with uninjured (Figure 5D), and is consistent with our *in vitro* observations.



**Figure 3.** Concentrations of IL-6, IL-8, monocyte chemotactic protein (MCP)-1, and matrix metalloproteinase (MMP)-9 are elevated in co-culture. (A) Significant increase in the concentration of IL-6, IL-8, MCP-1, and MMP-9 in the E<sub>U</sub>S and E<sub>I</sub>S conditions was noted at Day 8 of co-culture. (B) IL-6, IL-8, MCP-1, and augmented MMP9 levels were measured in the uninjured epithelium (E<sub>U</sub>) and injured epithelium (E<sub>I</sub>) alone. (C) MMP-9 levels were confirmed by gelatin zymography. \*P < 0.05 as compared with S or E<sub>U</sub> (B); #P < 0.05 as compared with E<sub>U</sub>S.

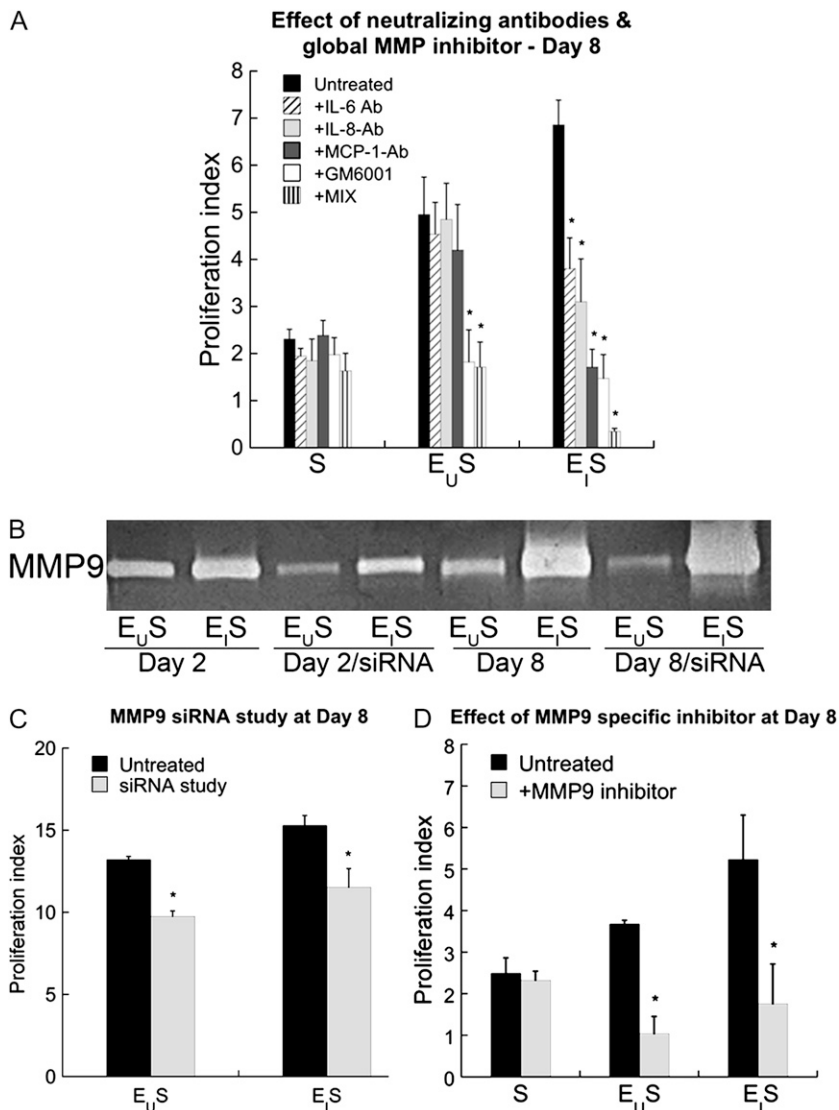
**DISCUSSION**

Tissue injury along with inflammation is associated with remodeling as observed in several airway diseases including asthma, chronic obstructive pulmonary disease, and fibrosing alveolitis (24). One feature of airway remodeling is smooth muscle cell hyperplasia, which impacts airway caliber and decreases lung function (25, 26). Current anti-inflammatory strategies do not reverse smooth muscle hyperplasia, creating a need for alternative therapies (27). Using both a novel *in vitro* co-culture model as well as an *in vivo* model of epithelial injury in the trachea, our study demonstrates that epithelial-derived mediators stimulate airway smooth muscle proliferation at baseline and after injury. We identified IL-6, IL-8, and MCP-1 as mediators, which contribute significantly to proliferation after injury, and MMP-9 as a novel mediator involved in both baseline and injury-induced proliferation. IL-6, IL-8, and MMP-9 levels are affected by the co-culture of NHBE and HASM, while MCP-1 is mainly produced by HASM. While not all diseases with epithelial injury manifest airway smooth muscle proliferation, our results provide potentially new targets to limit smooth muscle hyperplasia in the airways, and are consistent with a growing interest in the health of the airway epithelium.

IL-6, IL-8, and MCP-1 expression and release from airway epithelial cells has been demonstrated by numerous groups with interest in the response to infectious diseases (1, 28, 29). MMP-9 expression in NHBE cells has also been reported in airways after injury and in infectious diseases (30, 31). Airway epithelium-derived bFGF (FGF-2), IGF-1, PDGF-BB, VEGF, HB-EGF, MMP-2, and ET-1 have demonstrated a role in cell proliferation, myofibroblast induction, angiogenesis migration, matrix degradation, and the immune response (8, 9, 13, 32–34);

however, a role in airway smooth muscle proliferation has not been demonstrated. During epithelial injury, the absence of neighboring cells at the wound edge and hence loss of tight junctions can activate mechanosensors present on the cell surface. The mechanical signals are converted into a chemical response via site-specific integration with signal-transducing molecules (e.g., MAPK) that initiate a cascade of signaling events within the cells to release cytokines and chemokines (e.g., MCP-1, IL-6, IL-8) (35–38).

Mitogenic stimuli for human airway smooth muscle include polypeptide growth factors (e.g., EGF), plasma-derived or inflammatory cell-derived mediators (e.g., spingosine-1-phosphate), reactive oxygen species, and components of the extracellular matrix (e.g., collagen), stretch, as well as contractile agents (e.g., endothelin-1) (17, 18, 39–42). Growth factors activate intrinsic receptor tyrosine kinase (RTK) activity, whereas contractile agonists are linked to heterotrimeric guanosine triphosphate-binding proteins (GPCR proteins), and proinflammatory cytokines signal through glycoprotein complexes to stimulate airway smooth muscle proliferation. PI3K and MAPK are the major transduction pathways for the RTK, GPCR, or cytokine-stimulated proliferation of HASM (17, 18, 39–42). Since specific inhibitors to MAPK, PI3K, and Gi pathways significantly reduced proliferation of HASM in E<sub>U</sub>S and E<sub>I</sub>S, we infer that the epithelium stimulates smooth muscle cell proliferation through multiple pathways used by growth factors, contractile agonists, and/or inflammatory cytokines. Furthermore, the proliferation of HASM on co-culture with the NHBE is sensitive to dexamethasone. Previous studies have demonstrated that selective growth factor-induced proliferation of HASM is not dexamethasone sensitive (43). This is consistent with our observations that MMP-9, IL-6, IL-8, and MCP-1 from the



**Figure 4.** Effect of neutralizing antibodies to IL-6, IL-8, MCP-1, and global (GM6001) or specific MMP-9 inhibition (chemical or siRNA) on HASM proliferation. (A) A decrease in PI in GM6001 or mix incubated condition in E<sub>0</sub>S and a decrease in PI in all inhibited conditions in the E<sub>1</sub>S group was observed. (B) Representative gelatin zymogram showing decrease in MMP-9 levels in the silenced case (siRNA) compared with the unsilenced condition. (C) The PI of “pre-transfected NHBE with MMP-9 siRNA co-cultured with HASM” is markedly reduced in the E<sub>0</sub>S and E<sub>1</sub>S conditions. (D) MMP-9-specific inhibitor in the E<sub>0</sub>S and E<sub>1</sub>S conditions significantly decreased the PI. \*P < 0.05 as compared with untreated condition in the same group.

epithelium contribute to the proliferative response of the smooth muscle cells.

Levels of IL-6 and IL-8 are increased in asthmatic bronchoalveolar lavage (BAL) fluid, and an increase in MCP-1 expression in the bronchial epithelium of individuals with asthma has also been noted (37). IL-6, IL-8, and MCP-1 have been shown to play a role in vascular smooth muscle proliferation (36, 44); however, there are limited and variable data on their effects on HASM growth. Our study suggests a role of the above inflammatory cytokines in HASM proliferation after epithelial injury.

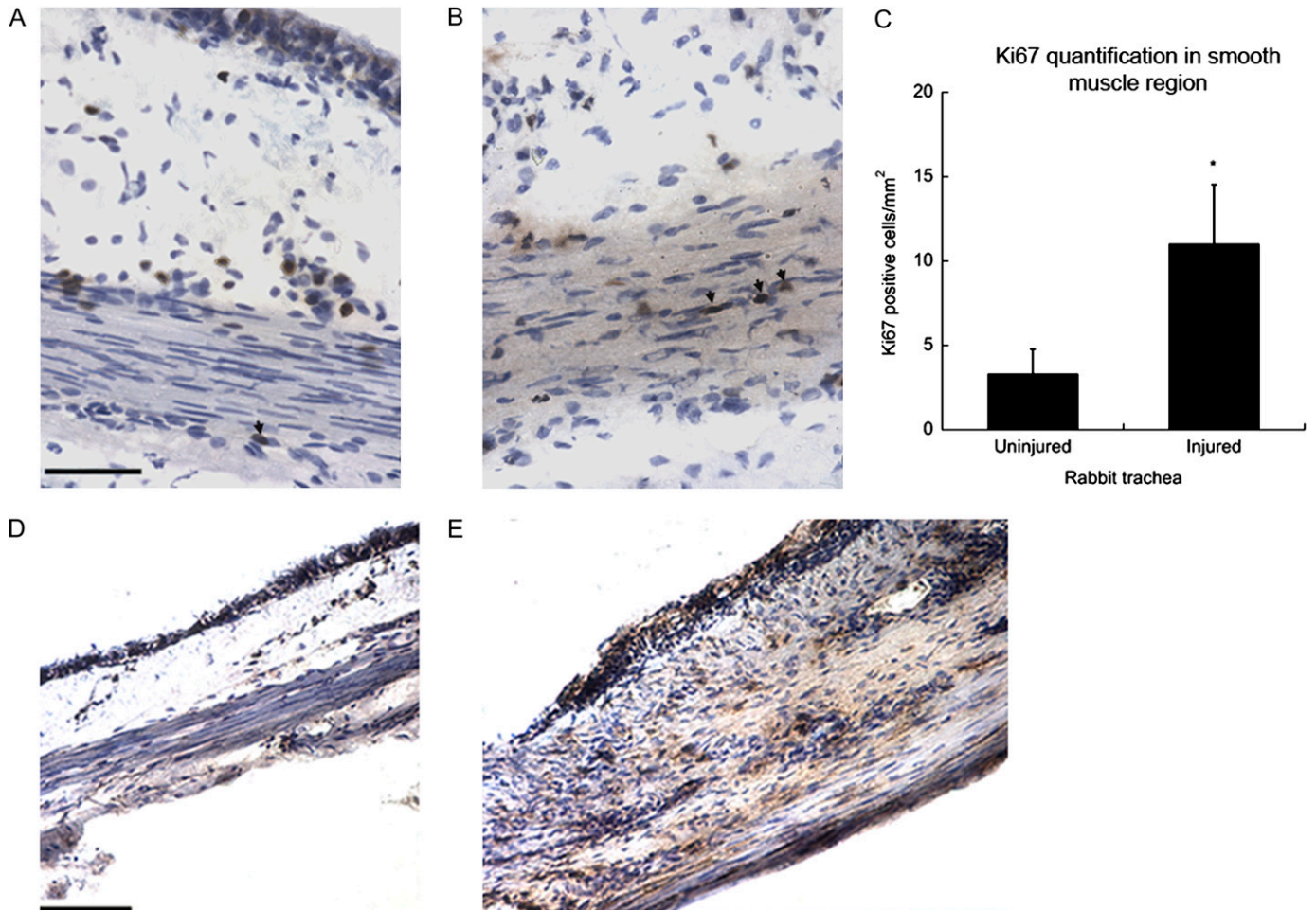
MMPs are proteolytic enzymes believed to be essential for development, turnover, and degradation of ECM proteins and denatured collagens (also known as gelatins) (45, 46). A role for MMP-9 has been implicated in the re-epithelization (i.e., regrowth of denuded epithelium at the wound site) of wounds. This requires the epithelial cells at the wound edge to lose their cell-cell contacts and migrates across the wound (47–50); thus, increased MMP-9 levels secreted by the epithelium after injury could impact new tissue growth and cell proliferation.

Elevated levels of MMP-2 and MMP-9 have been observed in BAL fluid and induced sputum of subjects with asthma (46). A role for MMPs in smooth muscle proliferation remains unclear (31, 45, 46). Evidence suggests that HASM require

MMP-2 for the proliferative response to mitogens (8, 51, 52); however, although MMP-2 was present in our system and thus may contribute to smooth muscle proliferation in our model, there were no significant changes in levels of MMP-2 after co-culture or epithelial injury (Figure E4). In other words, MMP-2 may be necessary but not sufficient to stimulate HASM in our *in vitro* system. For example, MMP-9 can be activated by MMP-2 (53).

We also demonstrated that chemical inhibition and silencing of MMP-9 expression significantly abrogated the epithelium-dependent increase in smooth muscle proliferation both at baseline and after injury. The silencing as observed in Figure 4 is transient and the MMP-9 levels start creeping back to normal after 8 days, as assessed by zymography. However, there is significant decrease in HASM proliferation at Day 8 in the silenced versus unsilenced co-culture. Interestingly, we did not detect any active MMP-9 by gelatin zymography. This observation may be due to rapid consumption; however, enzyme activity is also possible before the propeptide is cleaved using chaotropes (54).

The exact mechanism by which MMP-9 stimulates smooth muscle proliferation remains unclear (55, 56). One possible pathway could include the activation of mitogenic signaling pathways by activating latent or matrix-bound growth factors



**Figure 5.** Injury to the rabbit airway epithelium stimulates an increase in Ki67 levels in HASM. (A, B) An increase in the Ki67 levels (brown peroxidase staining, black arrows) in (B) the injured rabbit as compared with (A) the uninjured control is observed. Scale bar is 50  $\mu$ m. (C) Quantification of Ki67-positive cells in the smooth muscle region. (D, E) Furthermore, MMP-9 levels in the stroma of tissue sections from (E) the injured trachea are increased compared with (D) uninjured control. Scale bar is 200  $\mu$ m. \*Significantly different from uninjured.

(46). Alternatively, MMPs may cleave and thus directly activate GPCRs such as the protease activated receptors (PARs) (57). GPCR agonists (e.g., thromboxane A<sub>2</sub>, prostaglandin D<sub>2</sub>) have been shown to influence HASM proliferation (58); however, their levels did not change significantly in our co-culture model (data not shown).

Epithelial-derived mediators must diffuse through the lamina propria to reach the airway smooth muscle. The extracellular matrix of the lamina propria contains numerous binding sites for epithelial-derived proteins including proteoglycans, fibrin, fibronectin, and collagen (2). These features of the diffusion pathway are not part of our *in vitro* model. In addition, our *in vitro* model does not incorporate inflammatory cells, which can migrate to the source of injury, or other stromal cells (e.g., fibroblasts), both of which are potential sources of mediators such as MMP-9 (59). Thus, we developed an *in vivo* model of epithelial injury in the rabbit trachea. We mimicked the injury pattern by repeatedly denuding the epithelium over an 8-day period. We observed significant proliferation in the smooth muscle region, as indicated by increased Ki67 staining, and also increased MMP-9 levels within the lamina propria as determined by immunohistochemical staining. Our *in vivo* model does not allow us to identify the specific cellular source of MMP-9 (e.g., epithelium, inflammatory cells such as neutrophils, or stromal cells), or directly link epithelial release of MMP-9 to

airway smooth muscle cell proliferation. In fact, the airway smooth muscle has recently been shown to be a significant source of MMP-9 in fatal asthma (59). Thus, our results do not allow us to quantitatively predict the contribution of epithelial-derived MMP-9 toward airway smooth muscle proliferation *in vivo*. Nonetheless, our *in vivo* observations are consistent with our *in vitro* results that repetitive epithelial injury can increase the release of biologically relevant levels of MMP-9, and stimulate airway smooth muscle cell proliferation. Future studies might consider developing an *in vitro* model of the extracellular matrix to investigate its role in modulating the transport of epithelial-derived mediators.

Our results implicate the airway epithelium as a source of soluble mediators capable of stimulating airway smooth muscle cell proliferation. The soluble mediators include cytokines/chemokines IL-6, IL-8, and MCP-1 as well as the matrix metalloproteinase MMP-9. Our results suggest new avenues to develop therapeutic agents targeting airway smooth muscle cell proliferation via altering airway epithelial function.

**Conflict of Interest Statement:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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