ORIGINAL RESEARCH

SMAD Signaling in the Airways of Healthy Rhesus Macaques versus Rhesus Macaques with Asthma Highlights a Relationship Between Inflammation and Bone Morphogenetic Proteins

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Abstract

Bone morphogenetic protein (BMP) signaling is important for correct lung morphogenesis, and there is evidence of BMP signaling reactivation in lung diseases. However, little is known about BMP signaling patterns in healthy airway homeostasis and inflammatory airway disease and during epithelial repair. In this study, a rhesus macaque (*Macaca mulatta*) model of allergic airway disease was used to investigate BMP signaling throughout the airways in health, disease, and regeneration. Stereologic quantification of immunofluorescent images was used to determine the expression of BMP receptor (BMPR) Ia and phosphorylated SMAD (pSMAD) 1/5/8 in the airway epithelium. A pSMAD 1/5/8 expression gradient was found along the airways of healthy juvenile rhesus macaques ($n = 3, P < 0.005$). Membrane-localized BMPRIa expression was also present in the epithelium of the healthy animals. After exposure to house dust mite allergen and ozone, significant down-regulation of nuclear pSMAD 1/5/8 occurs in the epithelium. When the animals were provided with a recovery period in filtered air, proliferating cell nuclear antigen, pSMAD 1/5/8, and membrane-localized BMPRIa expression were significantly increased in the epithelium of conducting airways ($P < 0.005$). Furthermore, in the asthmatic airways, altered BMPRIa localization was evident. Because of the elevated eosinophil presence in these airways, we investigated the effect of eosinophil-derived proteins on BMPRIa trafficking in epithelial cells. Eosinophil-derived proteins (eosinophil-derived neurotoxin, eosinophil peroxidase, and major basic protein) induced transient nuclear translocation of membrane-bound BMPRIa. This work mapping SMAD signaling in the airways of nonhuman primates highlights a potential mechanistic relationship between inflammatory mediators and BMP signaling and provides evidence that basal expression of the BMP signaling pathway may be important for maintaining healthy airways.

Keywords: BMP; asthma; airways; homeostasis; repair

Clinical Relevance

Bone morphogenetic protein (BMP) signaling is essential for correct lung morphogenesis, and the pathway becomes reactivated in inflammatory and fibrotic lung diseases. Whether and how BMP signaling is involved in airway homeostasis is poorly understood. Here, we investigate BMP signaling in a nonhuman primate model of allergic airway disease. Our results indicate that BMP signaling is involved in epithelial homeostasis and is altered throughout the airways in asthma and during recovery. Accompanying *in vitro* studies highlight a mechanistic relationship involving eosinophil-induced trafficking of BMP receptor Ia in airway epithelial cells. This study highlights an important role of BMP signaling in both healthy and asthmatic airways during inflammation and repair.

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Both the architecture of the lungs and the epithelial cell types that line the lungs change throughout the descending respiratory tract to reflect specialized functions therein. The trachea and bronchi are lined with ciliated and secretory cells in the form of a pseudostratified epithelium to facilitate the mucociliary escalator and the clearance of inhaled pathogens. At the terminal end of the bronchioles, a single layer of alveolar epithelial cells type I and type II line the alveolar sacs. These cells facilitate the bidirectional movement of gases between the airways and the blood (1). Basal cells are differentially distributed along the proximal-distal axis with a higher frequency found in the upper airways (2). In addition, distinct niches populated with epithelial cell progenitors, such as the bronchioalveolar stem cell and alveolar surfactant protein-C-negative progenitors of the distal airways, are found in the respiratory tract (3, 4).

It is known that the morphology of the developing respiratory tract and the distribution of cell phenotypes throughout the developing airways are tightly controlled by signaling molecules, growth factors, and morphogens. Fibroblast growth factors, Wnt-β-catenin, Notch, and bone morphogenetic proteins (BMPs) produced by the developing endoderm and underlying mesoderm form a complex signaling network coordinating these developmental processes (5). These signaling factors communicate across the epithelial-mesenchymal-trophic unit, which is made up of opposing layers of epithelial and mesenchymal cells encompassing a network of basement membrane, fibroblasts, nerves, and endothelial cells (6). In adult lungs, the epithelial-mesenchymal-trophic unit is challenged constantly by irritants, pathogens, and disease-causing agents. It is believed that the signaling factors governing lung morphogenesis are reactivated after an injury. This causes the lung epithelium to initiate proliferation and the differentiation pathways to correctly restore epithelial integrity. However, in situations of chronic injury or prolonged inflammation, the epithelium will likely undergo incorrect repair, such as abnormal epithelial cell differentiation and remodeling events. Although both normal repair and abnormal disease processes have been studied extensively, the underlying signaling processes governing lung phenotypic changes, remodeling, inflammation, and restitution of epithelial homeostasis remain unclear (7, 8).

Previous studies have shown that BMP signaling is essential for correct lung formation during embryogenesis, governing the pattern of lung bud formation and epithelial cell distribution along the proximal distal axis (9–11). Studies inhibiting the BMP pathway using antagonists, reporter genes, and dominant-negative overexpression systems in mice have highlighted the important role this specific family of patterning molecules plays in both lung bud morphogenesis and epithelial cell distribution along the proximal distal axis. BMP signaling is also perturbed in airway diseases such as cancer and pulmonary hypertension (12, 13). Increased understanding of the cross talk between BMP and other developmental signaling pathways has led to the successful induction of epithelial cell differentiation from progenitor cells and dedifferentiation of committed epithelial cells to stem cells, and BMP signaling plays a key role in this plastic process. Furthermore, inhibition of BMP, Wnt, and transforming growth factor-β pathways in definitive endoderm cells followed by the supply of a ventralizing cocktail containing BMP-4 and other growth factors is essential for correct lung specification *in vitro* (14). These studies demonstrate that targeted manipulation of the signaling pathways governing lung morphogenesis holds significant potential for regenerative medicine approaches and highlight BMP as a key morphogen involved in lung bud formation and epithelial cell patterning (15, 16).

We are interested in the concept of signaling gradients throughout the airways and the ways in which these may relate to homeostasis, repair, regeneration, and inflammatory disease. Seminal studies in the past have revealed cross talk between the mesoderm and the developing lung endoderm and have demonstrated that the mesenchymal signals contributing to correct epithelial cell differentiation and airway branching are spatially regulated. These studies showed that when tracheal and distal mesenchyme were transplanted onto opposing denuded airway regions, branching morphogenesis and epithelial cell expansion were altered (17–19). It is now widely accepted that expression gradients of morphogens and signaling components exist along the proximal-distal axis in the developing airways, as evidenced by the Sox-2/Sox-9 expression patterns that exist during development (20). Furthermore, BMP-4 and fibroblast growth factor 10 in the mesoderm of the distal bud tip regulate branching morphogenesis, whereas reduced concentrations of these factors are present in more proximal airway regions (9, 11, 21). A recent study investigating the development of the vertebrate mucociliated epithelium provides further evidence of BMP gradients in the airways (22). Although gradients are clearly important during airway development, little is known about localized expression profiles and the effects of morphogen gradients in healthy, fully developed airways and during fibrotic or inflammatory diseases.

We hypothesized that a BMP signaling gradient exists in healthy adult airways and that this gradient may be altered during inflammatory airway disease and regeneration. To investigate this, we examined the expression pattern of certain BMP signaling pathway components in healthy rhesus macaques airways compared with an allergic airway disease model in these nonhuman primates. Previous studies of this rhesus macaque allergic airway disease model showed immunological and structural responses similar to those in human asthmatic lungs (23, 24). The allergic monkey airways displayed elevated eosinophil accumulation in both proximal and distal airways, in addition to an increased eosinophil presence in the bronchoalveolar fluid. Furthermore, extensive airway remodeling was observed. These changes indicate that the expression of growth factors and morphogens instrumental in both airway development and homeostasis may have been disturbed. Here, we report the existence of a BMP signaling gradient along the airways. We further report that this signaling pattern is altered during allergic airway disease and provide evidence that eosinophils can modulate BMP signaling in airway epithelial cells.

Materials and Methods

Rhesus Macaque Allergic Airway Exposure Model

Rhesus macaques (*Macaca mulatta*) were chosen from the breeding colony at the California National Primate Research

Centre, University of California, Davis, CA. All animal housing and care were in compliance with the Institute of Laboratory Animal Resources and the Association for Assessment and Accreditation of Laboratory Animal Care. House dust mite allergen (HDMA) and ozone (O_3) exposure experiments were performed as described previously (25–27). The Institute of Animal Use and Care Committee reviewed and approved experimental protocols prior to commencement.

Immunofluorescence

After deparaffinization, rehydration, and antigen retrieval on tissue sections, both tissue and methanol-fixed cells were stained as described previously (28). Primary antibodies were phosphorylated using SMAD (pSMAD)-1/5/8 (sc-12353-R; SantaCruz Biotechnology, Santa Cruz, CA), BMP receptor (BMPR) Ia (sc-20736; SantaCruz), and proliferating cell nuclear antigen (PCNA) (sc-7907; SantaCruz Biotechnology). An isotype control was prepared for each section. Images were captured using an Olympus BX61 (Olympus, Center Valley, PA). BMPRIa nuclear localization was quantified using an Olympus FluorViewFV1000 confocal microscope.

Stereology

Using point and intercept counting on a cycloid grid, the approximate abundance of fluorescent cells per unit basal lamina of the epithelium was quantified for each section, as described previously (29). By counting the number of positive cells per unit volume of interstitial epithelium on the fluorescent image (Vv) and the surface area of basal lamina per unit volume of interstitial epithelium (Sv) on the corresponding differential interference contrast (DIC) image, the number of positive cells per unit area of basal lamina was calculated (Vs). (P, number of points counted; bl, basal lamina; epi, epithelium; I, intersection; l/p, length/point, 86 μm; txred, Alexa568 positive cells).

$$\mathrm{Vv}_{\mathrm{txred/epi}} = P_{\mathrm{txred}} / \left(P_{\mathrm{epi}} + P_{\mathrm{txred}}\right)$$
$$\mathrm{Sv}_{\mathrm{bl/epi}} = \left[(\Pi \times \mathrm{I}_{\mathrm{bl}})/2\right] / \left[(l/p) \times P_{\mathrm{epi}}\right]$$
$$\mathrm{Vs}_{\mathrm{txred/bl}} = \mathrm{Vv}_{\mathrm{txred/epi}} / \mathrm{Sv}_{\mathrm{bl/epi}}$$

Quantification was carried out using the Stereology Toolbox (Morphometrix, University of California, Davis, CA). Statistical analysis was calculated using one-way analysis of variance tests and paired two-tailed t test in GraphPad Prism version 5.00 (GraphPad, San Diego, CA). $P < 0.05$ was considered significant.

Air–Liquid Interface Differentiation and Culture of Primary Tracheobronchial Epithelium

Tracheal epithelial cells were harvested from rhesus monkeys after necropsy as described previously (30). Once the cells were confluent and differentiated at the air–liquid interface (ALI), BMP-4 (R&D Systems, Oxan, UK) at 100 ng/ml was administered apically. After 72 hours, whole-cell protein was harvested as outlined previously (28). Blots were probed using pSMAD 1/5/8 antibody.

Isolation of Eosinophil-Derived Proteins

Purified eosinophil proteins were a gift from Dr. G. Gleich and were prepared as described previously (31).

Isolation of Mouse Airway Epithelial Cells and Coculture with Eosinophil-Derived Proteins

Mouse airway epithelial cells (MAECs) were isolated from female C3H-Hen mice as described previously (32). For the eosinophil-derived protein treatment, MAECs were seeded onto fibronectin-coated chamber slides overnight. Serum-free media containing eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), or major basic protein (MBP) at 0.5 μg/ml was added for 2, 17, or 48 hours and was maintained at 37°C, 5% carbon dioxide. Cultures were washed and fixed with methanol for immunostaining.

Results

pSMAD 1/5/8 Signaling Gradient in Healthy Juvenile Rhesus Macaque Airways

We hypothesized that a BMP signaling gradient exists in healthy airways and that this gradient may be altered during inflammatory airway disease and regeneration. To investigate this hypothesis, we used immunofluorescence to examine the expression of BMP signaling pathway components in the airway epithelium of healthy juvenile rhesus macaques aged 6 and 12 months that had been housed in chambers supplied with filtered air. Tissue sections from previous studies were used for this study (23, 26). As outlined in Figure 1, rhesus macaque models of asthma and recovery were developed previously. All monkeys in this study were male. Group 1 monkeys were exposed to filtered air for 6 months and served as healthy control animals. Group 2 monkeys were sensitized to HDMA allergen and were housed in specialized air chambers supplied with cyclic doses of aerosolized HDMA and O_3 for 5 months. These monkeys developed asthma and were sent to necropsy at 6 months old. A recovery model, group 4, was also established whereby monkeys were exposed to cyclic doses of HDMA and O_3 for 5 months followed by 6 months of filtered air, including one dose of aerosolized HDMA per month. These monkeys were killed at 12 months old. Control animals, group 3, were housed in filtered air for 12 months. Tissue from the trachea of 6-month-old monkeys was no longer available and so could not be included in this study. We chose to examine expression of BMPRIa as an indicator of potential cell responsiveness to BMP ligands. We used an antibody that recognizes pSMADs 1, 5, and 8 to detect nuclear localized SMADs as an indicator of active BMP signaling. We also examined the expression of PCNA as an indicator of regeneration in the airway epithelium.

Immunofluorescence was performed to examine PCNA, BMPRIa, and pSMAD 1/5/8 expression in lung tissue sections from airway epithelium of healthy 6-month-old (group 1) and 12-monthold (group 3) rhesus macaques (Figure 1A). The volume of positively labeled epithelial cells per unit basal lamina was counted after randomized sampling of airway sections. Expression of PCNA was observed in the nuclei of epithelial cells throughout the airways of these animals (Figures 2A and 2B; *see* Figure E1 in the online supplement). No significant difference in the level of expression of PCNA in the airways was detected in either age group. Expression of BMPRIa was observed in all regions examined, and no significant difference in the expression of membrane-bound BMPRIa in the conducting airways and distal bronchioles in 6- and 12-month-old monkeys was detected (Figure 2). Expression of nuclear-localized pSMAD 1/5/8 was also evident throughout the airways of rhesus macaques at both 6 and 12 months old (Figure 2). In the 12-month-old monkey group, the incidence of nuclear

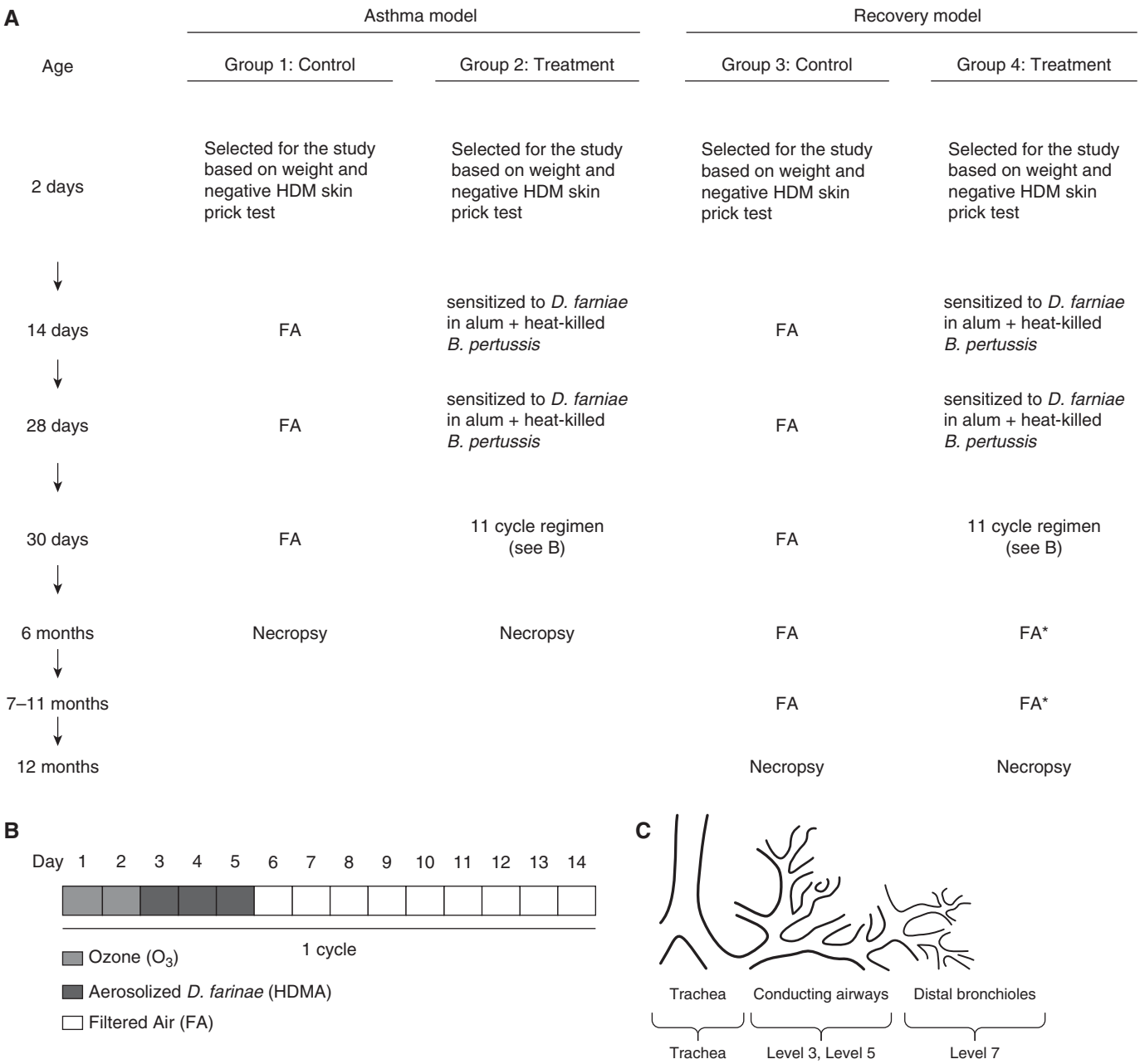

Figure 1. Experimental outline. (*A*) Animals were chosen for the study on the basis of a negative intradermal skin sensitivity test to *Dermatophaoides farinae,* a common house dust mite allergen (HDMA). Male rhesus macaques were included in the study. At 14 days old, *D. farinae* in alum was injected subcutaneously, in addition to an intramuscular injection of heat-killed *Bortetella pertussis*. At 28 days old, a repeated dose of *D. farinae* was given in alum. When the rhesus macaques were 30 days old, they were moved to specially regulated air chambers. The asthma model cohort received 11 repeated doses of ozone and aerosolized HDMA and were sent to necropsy at 6 months old, as outlined in *B*. In the recovery model, the monkeys received the same cyclic exposure. After the 11th cycle, FA was supplied to the chambers for an additional 6 months. These monkeys underwent necropsy when they were 12 months old. *HDMA + ozone (O_3) was given for 2 hours on a monthly basis to maintain sensitivity. (*B*) Aerosolized ozone was supplied for 5 days (*solid gray*), with aerosolized HMDA (*solid dark gray*) on Days 3 to 5. FA was supplied subsequently for 9 days. This regimen was repeated 11 times. Treatment lasted for 5 months. (*C*) The left cranial lobe was harvested, and the mainstem bronchus was dissected into consecutive sections for paraffin wax embedding. For the purpose of this study, airway levels 3 and 5 were classified as the conducting airways with a ciliated pseudostratified columnar epithelium. Airway level 7 sections were classified as distal bronchioles. The epithelial wall is made of simple ciliated columnar epithelium. FA, filtered air.

pSMAD 1/5/8 was significantly greater in the trachea compared with either the conducting airways or the distal bronchioles (Figure 2B; $P = 0.0023$ and 0.0064, respectively). The unavailability of tracheal tissue from the 6-month-old cohort meant that pSMAD 1/5/8 expression could not be examined in these tissues and there was no significant difference in pSMAD 1/5/8 in conducting airways compared with distal bronchioles in this group.

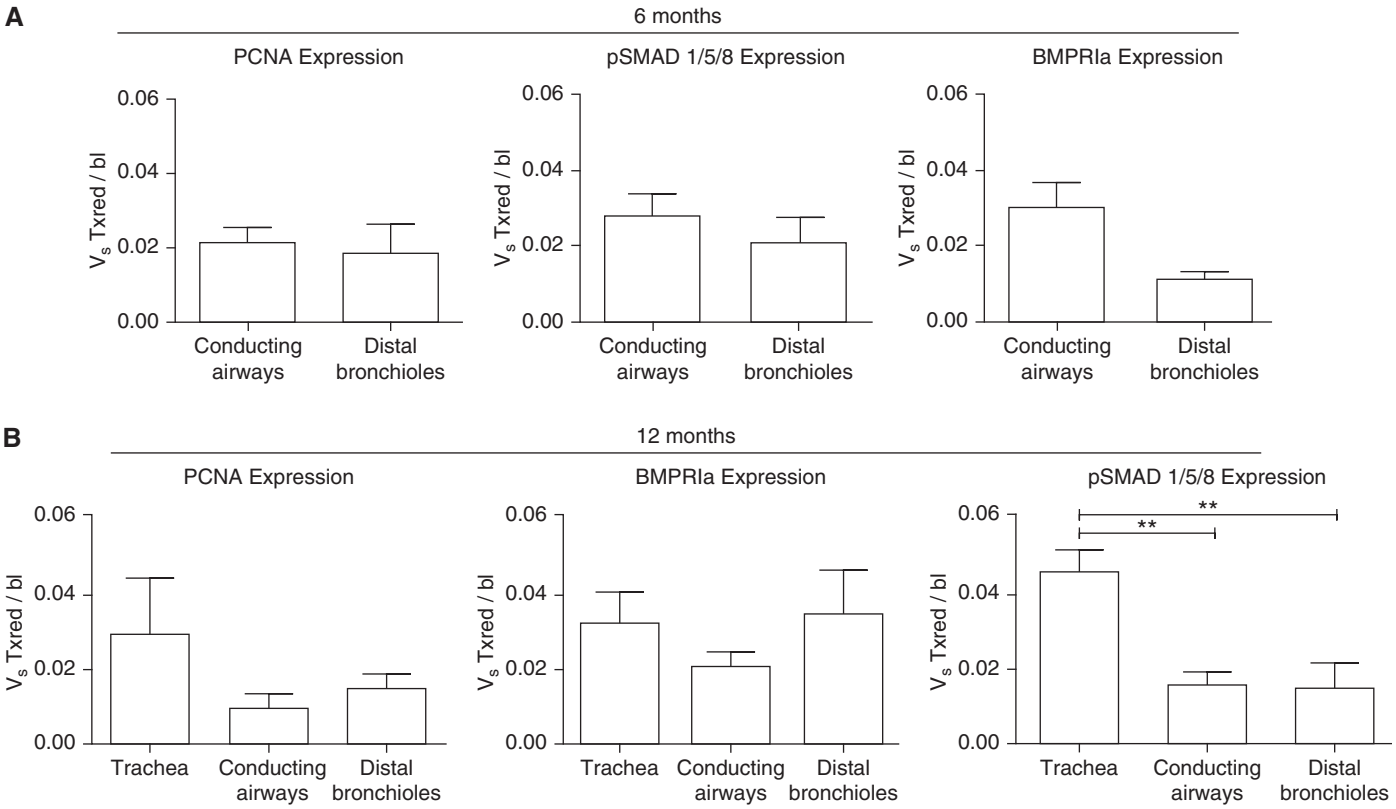

Figure 2. Bone morphogenetic protein (BMP) expression gradients in healthy airways at 6 and 12 months. (*A*) Membrane-localized expression of BMP receptor (BMPR) Ia and nuclear expression of proliferating cell nuclear antigen (PCNA) and phosphorylated SMAD (pSMAD) 1/5/8 were quantified using stereologic techniques throughout the airways of healthy rhesus macaques. There was no significant difference in PCNA, BMPRIa, or pSMAD 1/5/8 expression in the epithelium of conducting airways and distal bronchioles in 6-month-old monkeys. (*B*) PCNA and BMPRIa expression was unchanged along the airways of 12-month-old rhesus macaques. pSMAD 1/5/8 expression was significantly different in the tracheal epithelium of 12-month-old monkeys compared with the conducting airways and distal bronchioles. $n = 3$ for all targets; $^{**}P < 0.005$. V_STxred/bl, the number of positive cells per unit volume of interstitial epithelium.

Reduced Levels of Nuclear pSMAD 1/5/8 Expression in Conducting Airways of Monkeys with Asthma

Repeat exposure of sensitized rhesus macaques to HDMA/O_3 was performed over a 6-month period to induce allergic airway disease as reported previously (23). Immunofluorescence analysis was performed on tissues from these 6-month-old animals. No significant difference in the expression levels of PCNA was detected in 6-month-old animals with asthma (Figure 2A; group 2) compared with healthy control animals (group 1) at either airway level examined (Figures 3A and 3B). Similarly, no difference was detected in expression levels of BMPRIa. However, translocation of BMPRIa from the membrane and cytoplasm to the nucleus of some epithelial cells in the conducting airways of animals with asthma was observed (Figures 3D and E3). Levels of nuclear pSMAD 1/5/8 were significantly reduced in the conducting airways of animals with asthma compared with healthy animals (Figure 3C).

Increased Expression of PCNA, BMPRIa, and pSMAD 1/5/8 in Conducting Airways of Recovering Lungs

After cyclic exposure to HDMA/O_3 over a 6-month period, a group of animals was subsequently housed in chambers supplied with filtered air for an additional 6 months to investigate the extent to which recovery, if any, could occur after a chronic lung injury, as reported previously (26). Immunohistochemical analysis was performed on tissues from these 12-month-old animals. The expression of PCNA was significantly elevated ($P = 0.0013$) in the conducting airways of these animals (Figure 1A; group 4) compared with healthy animals (group 3) (Figure 4A). The expression of BMPRIa and nuclear SMAD 1/5/8 was also increased significantly ($P = 0.0037$ and 0.045, respectively) in the conducting airways of recovering animals (Figures 4B–4D).

Primary Tracheobronchial Cells Are Responsive to BMP-4 Ligand

Given the observation that pSMAD 1/5/8 is elevated in the epithelium of conducting airways of recovering lungs, we sought to investigate which specific BMP ligands could be driving this increase in pathway activation. Our laboratory has shown previously that primary MAECs are responsive to exogenous BMP-2, -4, and -7 *in vitro*, and given that BMP-4 has been reported in both healthy and diseased airway tissue, we hypothesized that this ligand may activate SMAD signaling in rhesus macaque airway epithelial cells (33, 34).

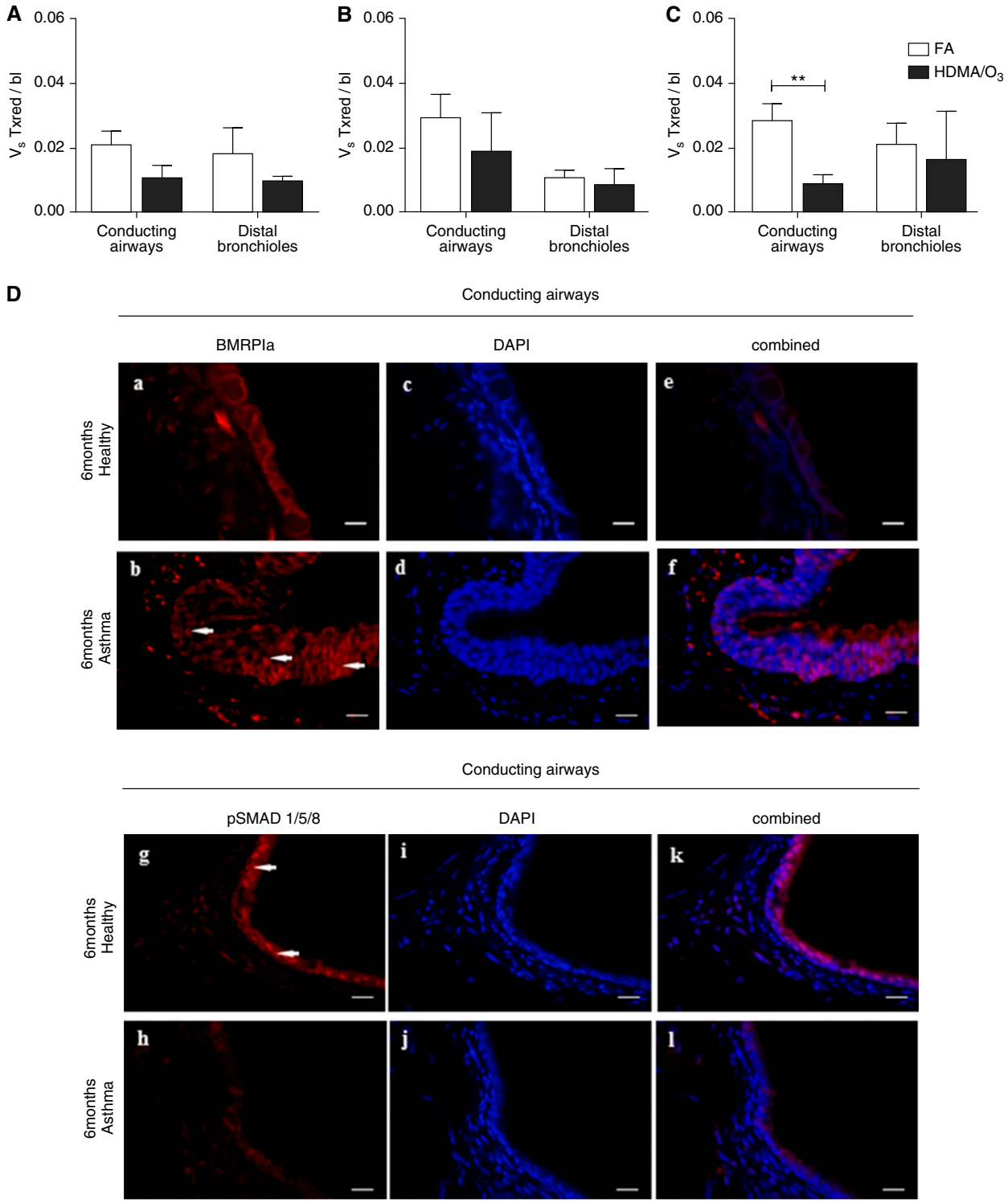

Figure 3. Suppression of PCNA, BMPRIa, and pSMAD 1/5/8 in conducting airways of monkeys with active asthma. (*A*) No significant difference in PCNA expression was detected in the airways of healthy rhesus macaques versus rhesus macaques with asthma. (*B*) There was no significant difference in membrane-bound BMPRIa expression in the airways of healthy rhesus macaques versus rhesus macaques with asthma. (*C*) A significant decrease in nuclear pSMAD 1/5/8 expression was evident in healthy monkeys versus monkeys with asthma. Immunofluorescent images were quantified using stereologic techniques. $n = 3$ for all targets; $^{**}P < 0.005$; FA = 6 months of FA; HDMA/ozone (O_3) = 6 months of exposure to HDMA/O_3. (*D*) BMPRIa immunofluorescent images displayed nuclear localization in the epithelium of asthmatic conducting airways compared with healthy airways, as indicated by the *white arrows* (*a–f*). Significant down-regulation of nuclear pSMAD 1/5/8 was seen in asthmatic conducting airways compared with healthy epithelium (*g–l*). Combined images of Alexa568-stained targets and 4',6-diamidino-2-phenylindole (DAPI) are shown (*e*, *f*, *k*, *l*). *Scale bars* represent 20 μm.

Primary tracheobronchial epithelial cells were harvested from healthy rhesus macaques and grown in an ALI culture system. After 72 hours of treatment with BMP-4, levels of pSMAD 1/5/8 were increased 1.5-fold ($n = 3$, $P < 0.05$) in these cells (Figures 5A and 5B).

Nuclear Translocation of BMPRIa in Primary MAECs Cocultured with Eosinophils and Eosinophil-Derived Proteins

We were interested in the observation of nuclear translocation of BMPRIa in some epithelial cells in the conducting airways of 6-month-old animals with asthma. Previous studies of this rhesus macaque allergic airway disease model showed immunological and structural responses in the airways similar to those in human asthmatic lungs with elevated eosinophil accumulation in both proximal and distal airways, in addition to an increased eosinophil presence in the bronchoalveolar fluid (23). Extensive airway remodeling was also observed in these animals.

During inflammation, eosinophils elicit their effector response in part by the release of cytotoxic proteins such as EDN, EPO, and MBP. Given that eosinophils-derived proteins contribute to airway hyperresponsiveness and remodeling in inflammatory airways diseases and that BMP ligands have been shown to colocalize with MBP in asthmatic airways, we examined whether these eosinophil-derived proteins could modulate BMP signaling in epithelial cells (34, 35). MAECs were used in culture on the basis of the similarities between human and murine eosinophilic responses (36). MAECs were exposed to eosinophil-derived proteins for 2, 17, or 48 hours and BMPRIa expression was examined by immunofluorescence (Figure 6). At 2 hours, BMPRIa appeared localized to the cytoplasm. However at 17 hours, nuclear localization of BMPRIa was observed in 30, 56, and 36% of MAECs exposed to EDN, EPO, MBP, respectively. The translocation was transient, however, because at 48 hours, BMPRIa was localized primarily in the cytoplasm. Nuclear localization was quantified at the 17-hour time point (Figure 6B).

Discussion

Lung development relies on the complex interplay of signaling pathways such as those regulated by the BMP family. It is believed that gradients of morphogens, such as BMP-4, are critical in forming the lung architecture as well as in directing the spatial distribution of epithelial cells types throughout the airways (9, 37). These pathways are believed to be reactivated in injured and diseased adult lungs to orchestrate epithelial repair and to restore healthy airway homeostasis. Our group and others have shown that BMP signaling is activated during repair in rodent models of airway injury using ovalbumin, nitronaphthalene, and bleomycin exposure (28, 33, 38). Furthermore, BMP signaling is activated in lung stem cell niches after injury, highlighting the importance of the BMP pathway in epithelial cell differentiation and proliferation (39). Studies of human bronchial biopsies have also shown that BMPR expression is attenuated in atopic patients with mild asthma compared with control subjects and that after allergen exposure, BMP pathway activity is elevated, shown by increased pSMAD 1/5/8 expression and BMP-7 ligand expression in the airway epithelium (34). However, to date, the concept of signaling gradients along the respiratory axis has not been investigated in healthy adult lungs.

In our study, the presence of nuclear pSMAD 1/5/8 in the conducting airways and distal bronchioles of healthy 6- and 12-month-old monkeys demonstrates constitutive activation of BMP signaling in these regions. Similar results have been reported by us and others in rat and human airway epithelium, although pSMAD 1/5/8 was reported absent from the bronchial epithelium of phosphate-buffered saline–treated mouse lungs (38, 39). In the current study, we also detected a significant gradient in nuclear pSMAD 1/5/8 localization in the distal airways of healthy 12-month-old nonhuman primates. This suggests that BMP signaling may play a role in the maintenance of homeostasis throughout the airways and suggests a more active role in the trachea compared with the lower airways. Interestingly, BMPRIa expression did not vary significantly throughout the airways. This indicates that positive regulators of BMP signaling, such as BMP ligands themselves, may be more active in the trachea, or that negative regulators, such as noggin and gremlin, may be more active in the lower airways. We confirmed here that BMP-4 is capable of signaling via SMADs in primary ALI cultures of monkey tracheal epithelial cells. The levels of nuclear PCNA in the airways did not vary significantly, consistent with health and homeostasis. BMP signaling is involved in muscle, skeletal, and adipose tissue homeostasis, and our results indicate a role for BMP signaling in airway homeostasis (40). Given that BMP signaling governs cell cycle progression *in vitro* and orchestrates correct epithelial cell differentiation during lung morphogenesis, we speculate that BMP signaling is involved in epithelial cell turnover in healthy airways and in mediating epithelial homeostasis (9, 28, 33).

Repeat exposure of HDMA/O_3 was used to induce allergic airway disease in the 6-month treatment group of rhesus macaques. Previous studies of these monkeys demonstrated both immune and structural responses in the airways (23, 24). The animals displayed elevated eosinophil accumulation in both proximal and distal airways and increased eosinophil presence in bronchoalveolar fluid. Furthermore, the lungs displayed extensive remodeling, which directly affected the functionality of the airways. Conducting airways and terminal bronchioles exhibited elevated mucous cell mass, and a negative correlation between baseline airway resistance and the ratio of conducting airways to lung parenchyma was evident (23). This suggests that the cyclic exposure model causing allergic airway disease alters the rudimental growth factor and morphogen patterns that orchestrate correct respiratory tract repair and epithelial cell distribution. By examining the expression profile of BMP components along the airways, we have shown that the normal BMP pathway signals are indeed perturbed as a result of HDMA/O_3 exposure. A significant decrease in nuclear pSMAD 1/5/8 in the lung epithelium of conducting airways was evident. Furthermore, although the results proved not to be statistically significant, a decreasing trend of BMPRIa expression could be seen in epithelium of both the conducting airways and the distal bronchioles.

Interestingly, in addition to reduced pSMAD 1/5/8, nuclear localization of BMPRIa was evident in the conducting airway epithelium of the 6-month asthma group. Infiltrating eosinophils were shown previously to be significantly elevated in the

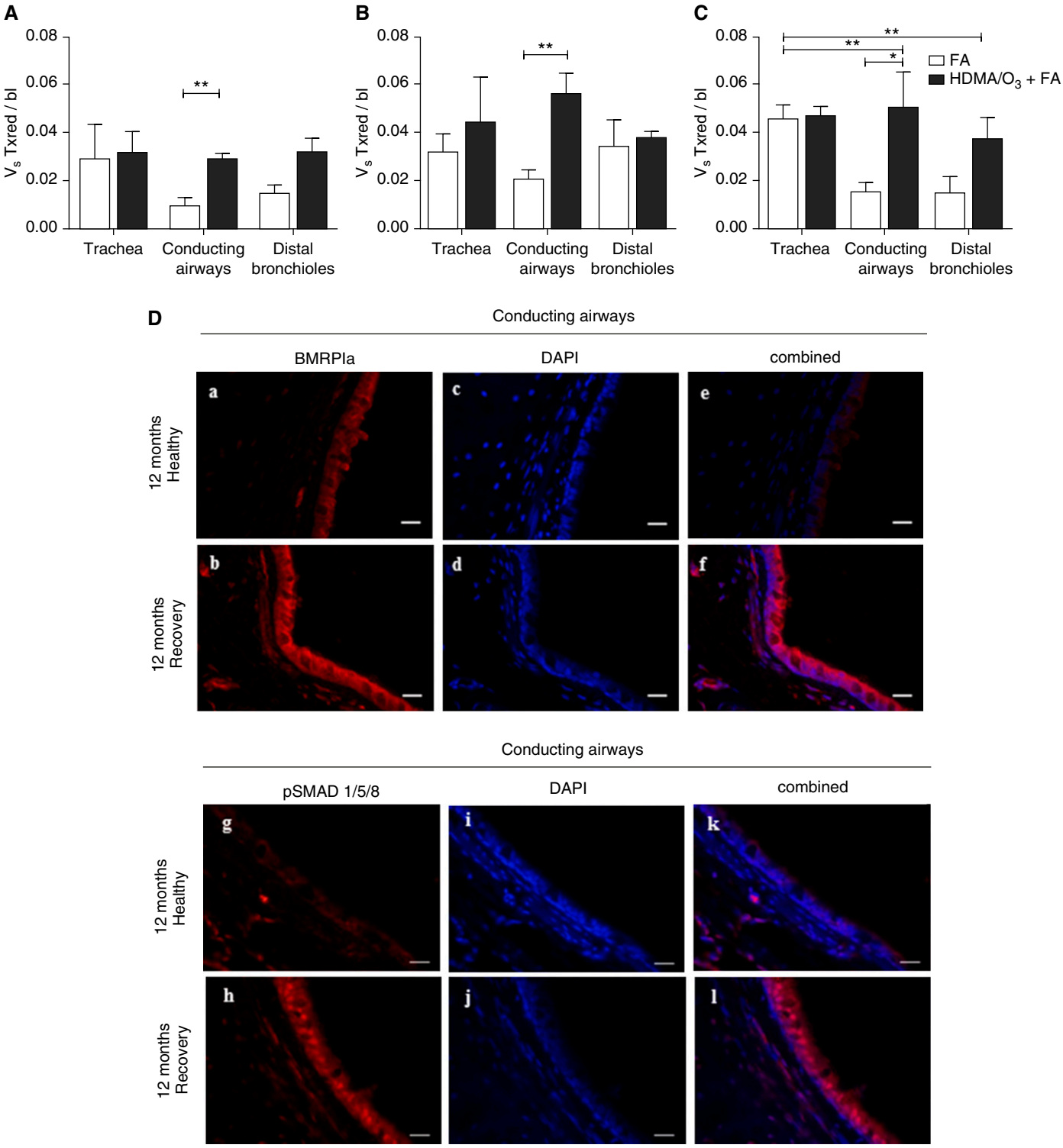

Figure 4. Elevated expression of PCNA, BMPRIa, and pSMAD 1/5/8 in conducting airways of recovering monkeys with asthma. (*A*) PCNA expression was elevated in the conducting airways when a period of 6 months' recovery in FA was provided after 6 months of exposure to HDMA and ozone (O_3). (*B*) Membrane-localized BMPRIa expression was elevated in the epithelium of the conducting airways of the recovery group of animals. (*C*) Nuclear pSMAD 1/5/8 expression was elevated in the epithelium of conducting airways in the recovery group of animals. Quantified immunofluorescence using stereologic techniques is shown. $n = 3$ for all targets; $^{*}P < 0.05$; $^{**}P < 0.005$. FA = 12 months of FA; HDMA/O_3 + FA = 6 months of exposure to HDMA/O_3 + 6 months of FA. (*D*) Representative immunofluorescent images display elevated membrane-localized BMPRIA expression in recovering airways (*b*) compared with healthy airways (*a*). Images show an increase in nuclear pSMAD 1/5/8 in recovering airways (*g*) compared with those in healthy animals (*h*). Combined images of Alexa568-stained targets and DAPI are shown (*e*, *f*, *k*, *l*). *Scale bars* represent 20 μm.

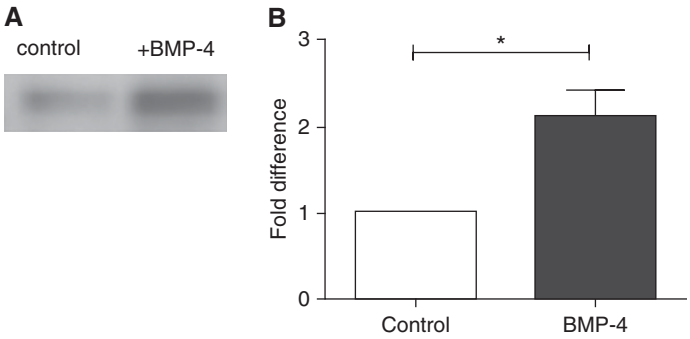

Figure 5. Primary monkey tracheobronchial epithelial cells are responsive to BMP-4 ligand stimulation. (*A*) Western blot of primary tracheobronchial epithelial cells differentiated at air–liquid interface and subsequently stimulated with 100 ng/ml BMP-4 for 74 hours showed elevated pSMAD 1/5/8 expression. (*B*) Densitometry analysis of the blots showed that pSMAD 1/5/8 expression was increased ~2.1-fold after treatment with BMP-4, compared with control cells. Cultures were performed with epithelial cells isolated from three individual rhesus macaque monkeys. $^*P < 0.05$.

conducting airways and distal bronchioles of these monkeys, and we hypothesized a link between these inflammatory cells and the BMP pathway. Many studies have shown that eosinophils, and specifically their secondary derived proteins, play a central role in airway remodeling, perpetuating airway hyperresponsiveness in asthma and inflammatory airway diseases, but the signaling pathways governing these events remain poorly understood (41–43). Our results using primary MAECs indicate a possible mechanism whereby the eosinophil-derived proteins may influence BMP signaling by altering the localization of BMPRIa. Moreover, if reduced pSMAD 1/5/8 signaling is a consequence of this modulation, this could explain the significant reduction in levels of nuclear pSMAD 1/5/8 in the conducting airways in the 6-month asthma group. This possibility is further supported by Kariyawasam and colleagues, who demonstrated that eosinophils are the predominate producer of BMP-7 in the lung epithelium of asthmatic airways (34). Colocalization of BMP-7 with MBP-positive eosinophils was observed in these asthmatic airways. Because BMP-7 is known to act as an antiinflammatory ligand in other organs, the authors speculated that the increase in ligand expression could be an attempt to regulate inflammation as part of the reparative processes in the airways (34, 44). In addition, an *in vitro* study using the neuroblast IMR32 cell line reported a similar down-regulation of BMPRIa gene expression after a 4-hour exposure to eosinophil-derived MBP. Furthermore, coincubation of the cells with BMP-7 and MBP decreased the induction of inhibitor of differentiation (ID)-1, an established downstream target of the BMP pathway (42). Taken together with our results, we hypothesize that the attenuated expression levels of BMP signaling present in this nonhuman primate model of asthma are caused by eosinophil-induced nuclear translocation of membrane-bound BMPRIa. Subsequent inhibition of downstream BMP signaling pathways interferes with the appropriate epithelial repair processes mediated by BMP and its signaling partners such as fibroblast growth factor 7 (45). This could contribute to a chronic disease state promoting incorrect epithelial turnover, an inflammatory phenotype, and epithelial remodeling, all of which are well-established hallmarks of asthma. Although BMPR processing and trafficking between the nucleus and the membrane are essential for correct signal transduction, incorrect receptor trafficking can contribute to disease, as evidenced by BMPRII mutations in pulmonary hypertension (46, 47). Our results highlight a potential novel mechanistic relationship between the granule proteins secreted by eosinophils and BMPRIa trafficking in asthma.

The regenerating airway model used here provided further evidence for the involvement of BMP signaling in inflammatory airway disease and repair processes. The increased incidence of nuclear PCNA is consistent with repair processes in the conducting airways after a period of chronic injury and inflammation. Increased expression of membrane-localized BMPRIa in these regions indicates a role for BMP signaling in the repair/regeneration processes. In other mouse and human asthma models, similar increases in BMP signaling evidenced by nuclear pSMAD1/5/8 were observed in bronchial epithelium challenged with ovalbumin or an aeroallergen, respectively (34, 38). Previous studies of this rhesus macaque model showed that these monkeys displayed modest signs of repair and airway regeneration. Perlecan was reintroduced at the basement membrane and there was evidence for the reestablishment of correct mucous cell expression in the proximal airways (48). We speculate that this recovering phenotype of the airways is further promoted by increased BMPRIa expression at the membrane, promoting elevated ligand-induced nuclear translocation of pSMAD 1/5/8 protein. This causes the epithelial cells to enter a state of active repair and differentiation in an attempt to restore an appropriate homeostatic environment in the airways. This occurs perhaps through the expression of ID genes and elevated production of BMP-7 ligand in an attempt to counteract the antimitogenic properties of transforming growth factor-β to regulate cell proliferation, remodeling, and epithelial differentiation (49, 50).

Conclusions

We speculate that the higher BMPRIa membrane staining in the recovery monkeys is a result of reduced eosinophil infiltration in the airways. This is supported by previous human asthmatic airway studies that showed attenuated cellular inflammation after the removal of airway insult and a reduced eosinophil presence in the BAL fluid of the recovery monkeys compared with the monkeys with asthma (Figure E4) (51). Lowered levels of eosinophilic granule proteins could decrease the nuclear translocation of BMPRIa and thus account for the elevated membrane-localized BMPRIa expression and nuclear pSMAD 1/5/8 signaling seen in the cells. As a result, an important feedback mechanism in the lung epithelium may exist whereby the removal of airway insult reduces eosinophil infiltration and eosinophil-induced down-regulation of BMP signaling. This hypothesis is supported by recent studies showing for the first time a definitive antiinflammatory

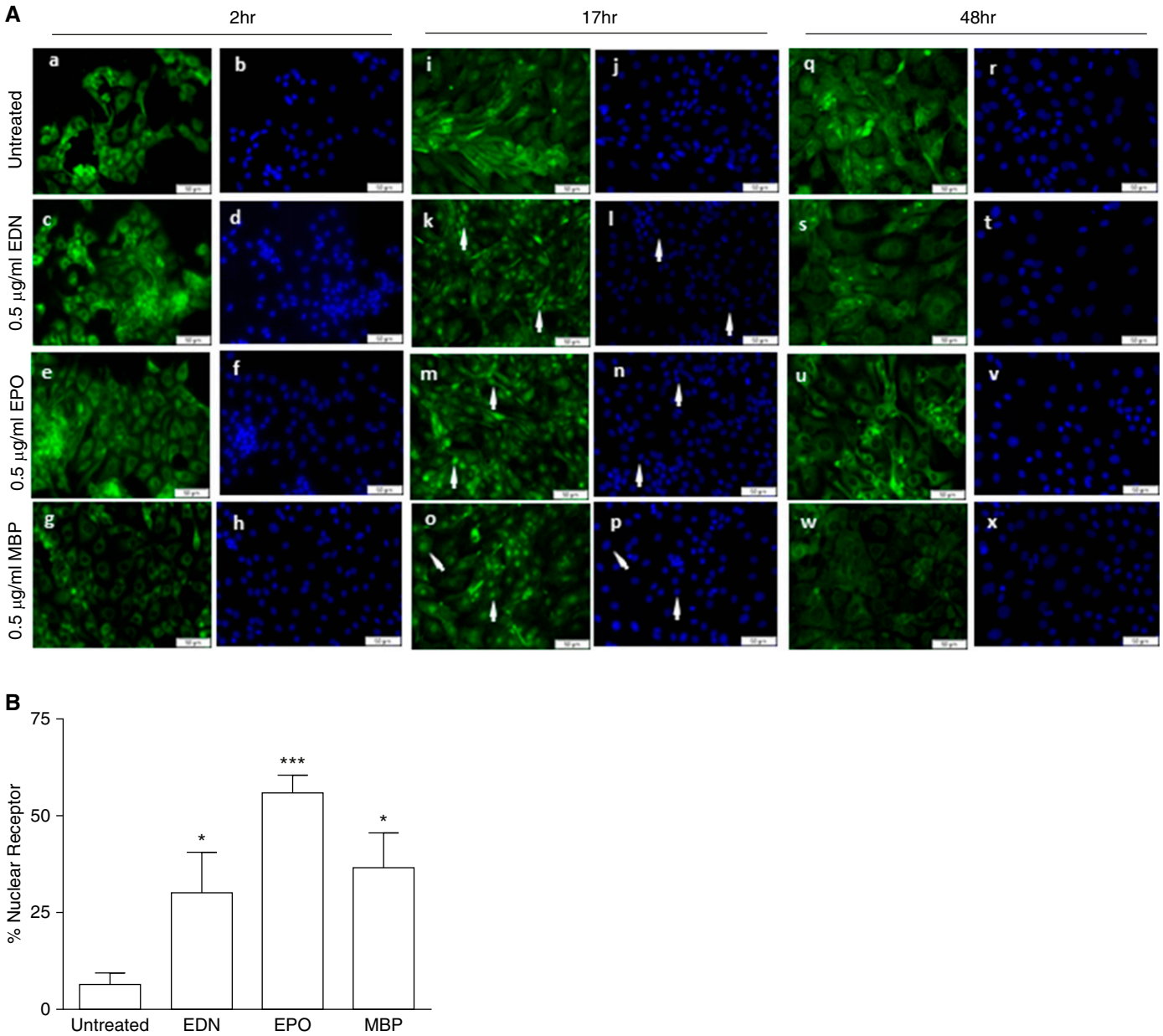

Figure 6. BMPRIa nuclear translocation in mouse airway epithelial cells (MAECs) treated with eosinophil derived granule proteins. (*A*) MAECs were exposed to human eosinophil-derived proteins over a time course of 2, 17, and 48 hours. BMPRIa appeared localized to the cytoplasm of untreated cells at 2, 17, and 48 hours. At 2 hours, BMPRIa was localized to the cytoplasm of MAECs exposed to eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), or major basic protein (MBP) at a concentration of 0.5 μg/ml (*a–h*). At 17 hours, BMPRIa was localized to the nuclei of cells exposed to 0.5 μg/ml EDN, EPO, and MBP (*i–p*). BMPRIa was relocalized to the cytoplasm with no nuclear localization apparent after 48 hours of exposure to EDN, EPO, and MBP. *Arrows* denote BMPRIa nuclear localization (*q–x*). *Scale bars* represent 50 μm. (*B*) BMPRIa nuclear localization was quantified by counting positively immunostained nuclei. Graph illustrates mean expression of BMPRIa in 30, 56, or 36% of MAECs with exposure to EDN, EPO, or MBP, respectively. Results are representative of counts obtained from three separate experiments. Approximately 800 cells were counted in at least three fields of view. Cells were counterstained with DAPI. $^{*}P < 0.05$; $^{***}P < 0.0005$ after Student *t* test.

function of BMP signaling in the stomach and airway epithelium (52, 53). The enhanced BMP pathway activity could induce the putative antiinflammatory function of BMPs, preventing further inflammation and damage to the epithelium and facilitating the restoration of correct epithelial cell turnover and repair. Our work using this nonhuman primate model of allergic airway disease supports this hypothesis and furthermore, our results suggest that not only are developmental pathways reactivated during inflammatory airway disease involving epithelial injury, but that basal expression of the BMP signaling pathway may be important for maintaining healthy airways. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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SMAD signaling in the airways of healthy versus asthmatic rhesus macaques highlights a relationship between inflammation and BMPs

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ONLINE DATA SUPPLEMENT

Supplemental figures

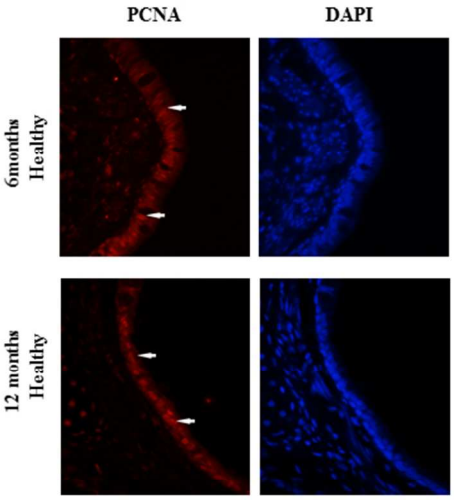
Figure 1 PCNA expression in healthy six and twelve month old airways: Immunofluorescence images showed nuclear PCNA in the distal airways of healthy six and twelve month old monkeys. Arrows denote nuclear localization.

Figure 2 Quantified expression of PCNA, BMPRIa and pSMAD 1/5/8 from all treatment groups: Compiled data comparing all treatment groups at six and twelve months for PCNA (a), BMPRIa (b) and pSMAD 1/5/8 (c).

Figure 3 BMPRIa expression in six month old healthy and asthmatic monkeys: Analysis was carried out on immunofluorescent images using Olympus cellSens Dimension 1.9 software. Analysis shows TxRed and DAPI expression patterns in epithelial cells in 6 month old healthy monkeys compared to HDMA/O_3 exposed monkeys. There was evidence of nuclear BMPRIa staining in the asthmatic monkeys.

Figure 4 Percentage of eosinophils in bronchoalveolar lavage Group1 and group2 data from cell differentials contained in table 2 [25]. Group3 and group4 data from personal communication with Dr. Lisa Miller (n=3). Data representing mean ± standard error, * $p<0.05$.

Supplemental images



Supplemental Figure 1. PCNA expression in healthy six and twelve month old airways: Immunofluorescence images show nuclear PCNA in the distal airways of healthy six and twelve month old monkeys.
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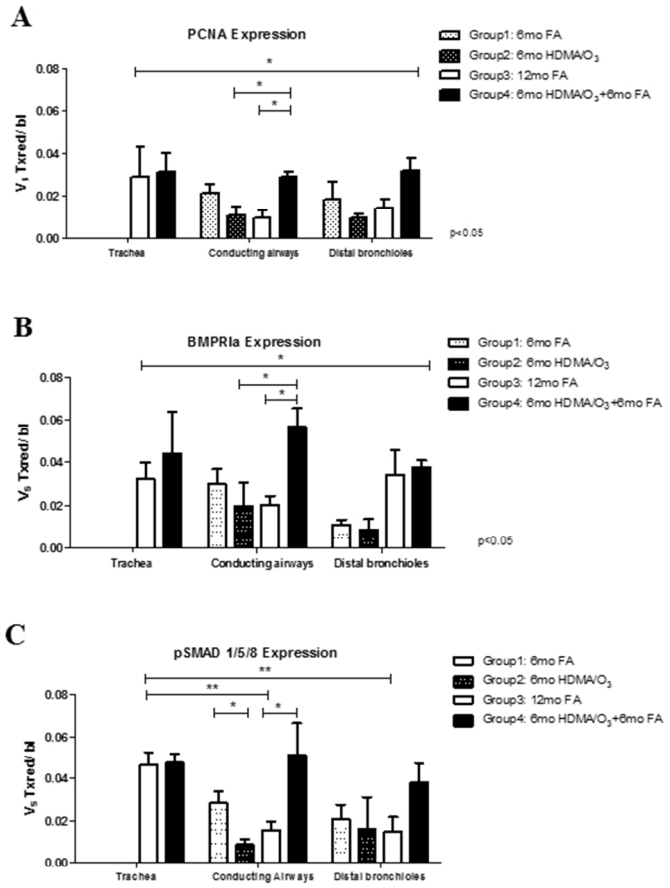

Supplemental Figure 2. Quantified expression of PCNA, BMPRIa and pSMAD 1/5/8 from all treatment groups: Compiled data comparing all treatment groups at six and twelve months for PCNA (a), BMPRIa (b) and pSMAD 1/5/8 (c).
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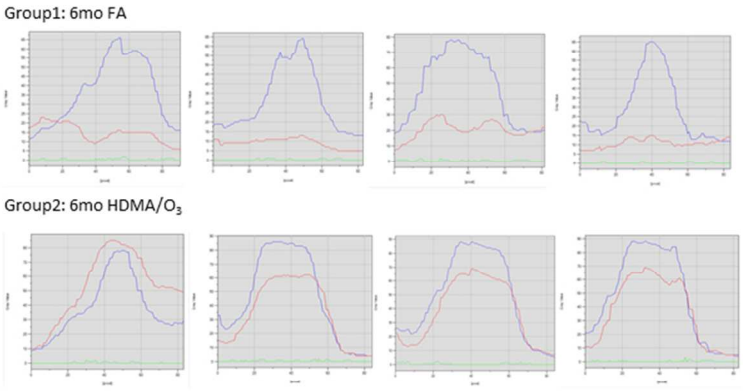

Supplemental Figure 3. BMPRIa expression in six month old healthy and asthmatic monkeys: Image analysis of immunofluorescent images was carried out using Olympus cellSens Dimension 1.9 software. Analysis shows TxRed and DAPI expression patterns on epithelial cells in 6 month old healthy monkeys compared to HDMA/O3 exposed monkeys. There is nuclear BMPRIa staining present in the epithelial cells of asthmatic monkeys.
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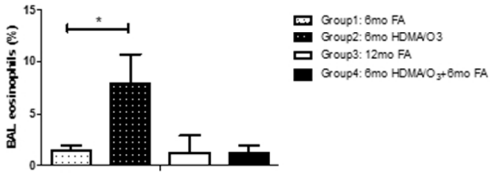

Supplemental Figure 4. Percentage of eosinophils in bronchoalveolar lavage Group1 and group2 data from cell differentials contained in table 2 [25]. Group3 and group4 data from personal communication with Dr. Lisa Miller (n=3). Data representing mean ± standard error, * $p<0.05$.
190x254mm (96 x 96 DPI)