Prenatal retinoid deficiency leads to airway hyperresponsiveness in adult mice

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Introduction

Vitamin A deficiency (VAD) is the most common dietary deficiency in the developing world and is associated with increased infant mortality and morbidity (1–3). It has been clear for decades that retinoic acid (RA), the active metabolite of vitamin A (retinol, ROH), plays a vital role in organogenesis and homeostasis, particularly in the lung (4, 5). Altered vitamin A or RA status has been linked to defects in embryonic and postnatal pulmonary development as diverse as lung agenesis, tracheoesophageal fistula, and bronchopulmonary dysplasia (6–10). Numerous clinical studies have demonstrated a positive relationship between vitamin A status and lung function. There is increasing evidence of an association between VAD and airway hyperresponsiveness, defined as an exaggerated contraction of the bronchial smooth muscle (SM) in response to environmental or pharmacological stimuli (11–14). These studies have also shown that maternal VAD has a negative impact on postnatal lung function in children, which can be alleviated by proper supplementation during gestation (15, 16).

SM is a major component of vascular and visceral structures, including airways, and the mechanisms that control growth and differentiation of SM in the embryonic lung remain poorly understood. Airway SM originates from various sources, including mesenchymal cells of the developing lung during formation of the bronchial tree (17–20). During development, the airway SM is responsible for phasic contractility of the liquid-filled epithelial tubules and production of growth factors, both critical for normal lung growth (21, 22). Changes in structural and mechanical properties of airway SM are found in conditions such as asthma that lead to airway hyperresponsiveness, remodeling, and airflow obstruction (23, 24). Interestingly, low serum levels of vitamin A have been reported in children with persistent asthma, correlating with the severity of disease (11, 25). Although inflammation plays a key role in triggering or exacerbating these responses, there is strong evidence that airway hyperresponsiveness can be uncoupled from inflammation and, at least in part, can result from fundamental changes in the SM phenotype itself (26, 27). How vitamin A/RA signaling regulates the program of SM differentiation in the lung, however, is unclear. The role of RA-dependent prenatal events in this process has been difficult to explore, since in most animal models, disruption of RA signaling leads to embryonic or neonatal lethality (28, 29). Thus, there is a paucity of information about the impact of early exposure of the fetal lung to insufficient levels of vitamin A/RA in postnatal life.

In the process of identifying genome-wide RA targets in early lung development, we found an abnormal increase in the expression of SM markers associated with RA-deficient status. This intriguing observation led us to further investigate the impact of RA signaling in SM development and lung function during pre- and postnatal life. We used cell and organ cultures and animal models in vivo and combined pharmacologic, genetic, and dietary approaches to address this issue. Here, we provide evidence that during lung development, RA has a key role in restricting the SM differentiation program of the forming airways. Our data show that prenatal vitamin A/RA deficiency fosters the development of an aberrant SM phenotype in the murine airways, leading to adverse effects on lung structure and function lasting well into adulthood.

Results

RA signaling influences SM marker expression during early lung development. Using pharmacologic and genetic models of RA deficiency and gene expression profiling, we previously identified an RA-dependent gene network in the embryonic foregut critical for lung formation (30–33). A large number of these genes were shown to be present in the mesoderm and to be associated with a repressive function in multiple pathways (33). Surprisingly, we found that genes typically associated with SM differentiation were overrepresented and significantly upregulated in RA-deficient foreguts.

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compared with those of the RA-sufficient controls. These included the following genes: actin, α2, smooth muscle, aorta (Acta2); actin, γ2, smooth muscle, enteric (Actg2); cysteine- and glycine-rich proteins 1 and 2 (Csrp1 and Csrp2); transgelin (Tagln); myosin heavy chain 11, smooth muscle (Myh11); and calponin 2 (Cnn2) (ref. 34 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI70291DS1). These data raised the intriguing possibility that the RA status at a relatively early stage of lung organogenesis could influence and alter the program of mesenchymal differentiation.

To determine whether SM precursors in the embryonic lung are sensitive to changes in endogenous RA activation when airways are forming, we modulated RA signaling in lung explant cultures and examined the phenotype. E11.5 lungs from WT mice carrying one copy of the RA reporter RARElacZ were cultured for 48 hours under control conditions or in the presence of the RA receptor antagonist BMS493 (hereafter referred to as BMS) (35, 36). Disruption of RA signaling was confirmed by significant downregulation of Rarb and RARElacZ (A) and increased expression of Tagln and Myh11 (F) and their products SM22α and SMMHC2, respectively) (G). (B–E) Whole-mount ISH of Acta2 (B and C) and Myh11 (D and E) increased signals in the mesenchyme of proximal (pr) and distal (di) airways in BMS-treated lungs compared with those of controls (arrowheads). Strong ectopic Acta2 expression was seen in stalks of distal buds in BMS-treated lungs. (H) Increased ratio of pMYL2/tMYL2 in BMS-treated lungs. (I) PCR detection of RA pathway components in MLg cells: RA-synthesizing enzymes (ALDH1A1, -2, and -3) and RA receptors (RARα, RARβ, and RARγ). (J–L) BMS treatment (24 hours) disrupted RA signaling (downregulation of Rarb) and increased expression of SM marker genes (Tagln and Myh11) and products (SM22α) and of the pMYL2/tMYL2 ratio. (A, F, I, and K) PCR and (G, H, and L) Western blot analysis. n = 3–4 per condition. *P < 0.05. tr, trachea. Scale bar: 200 μm.

Figure 1
Disruption of RA signaling leads to aberrant expression of SM markers in mouse embryonic lung explant (A–H) and MLg cell (I–L) cultures. BMS-treated RARElacZ lungs (48 hours): significant downregulation of Rarb and RARElacZ (A) and increased expression of Tagln and Myh11 (F) and their products SM22α and SMMHC2, respectively) (G). (B–E) Whole-mount ISH of Acta2 (B and C) and Myh11 (D and E) increased signals in the mesenchyme of proximal (pr) and distal (di) airways in BMS-treated lungs compared with those of controls (arrowheads). Strong ectopic Acta2 expression was seen in stalks of distal buds in BMS-treated lungs. (H) Increased ratio of pMYL2/tMYL2 in BMS-treated lungs. (I) PCR detection of RA pathway components in MLg cells: RA-synthesizing enzymes (ALDH1A1, -2, and -3) and RA receptors (RARα, RARβ, and RARγ). (J–L) BMS treatment (24 hours) disrupted RA signaling (downregulation of Rarb) and increased expression of SM marker genes (Tagln and Myh11) and products (SM22α) and of the pMYL2/tMYL2 ratio. (A, F, I, and K) PCR and (G, H, and L) Western blot analysis. n = 3–4 per condition. *P < 0.05. tr, trachea. Scale bar: 200 μm.
The aberrant SM marker expression in these lungs was confirmed by a significant upregulation of Tagln, Myh11, and their protein products (SM22α and SMMHC2, respectively), as revealed by quantitative real-time PCR (qPCR) and Western blot analysis (Figure 1, F and G). Since phosphorylation of myosin regulatory light chain 2 (MYL2) is required for SM contraction and its level correlates strongly with airway SM shortening velocity (37), the increase in phospho-MYL2 (pMYL2) over total MYL2 (tMYL2) in BMS-treated lungs suggests that the SM in these lungs is hypercontractile (Figure 1H).

To investigate whether the effect of RA signaling in the SM program was independent of signals originating from other cell types, we modulated signaling directly in the well-characterized mouse lung mesenchymal cell line MLg, which has previously been shown to have a response to RA similar to that of embryonic lung mesenchyme (33, 38). PCR analysis of RA pathway components revealed that under control conditions, MLg cells express the RA-synthesizing enzymes aldehyde dehydrogenase 1 family ALDH1A1, ALDH1A2, and ALDH1A3 and RA receptors RARα, RARβ, and RARγ (Figure 1I). This suggests that they are able to produce active ligand from a retinoid precursor (in the media) and turn on RA signaling endogenously. Indeed, BMS treatment of MLg cells for 24 hours showed significantly lower levels of the early RA response gene Rarb compared with levels in cells.
cultured under control conditions (Figure 1J). Consistent with the phenomenon seen in organ cultures, we observed that disruption of RA signaling led to an increased expression of SM markers both at the message and protein levels, as well as an increased pMYL2/tMYL2 ratio in these cells (Figure 1, K and L). Western blotting of Ki67, a marker of cellular proliferation, showed no difference between groups, suggesting a similar proliferative capacity (Supplemental Figure 2D). Thus, MLg cells seem to retain their capacity to activate endogenous RA signaling in the presence of a retinoid precursor and to control expression of SM markers on a per-cell basis.

Overall, our data suggest that the airway SM phenotype is strongly influenced by RA during lung development.

RA is active in the developing SM layer of newly formed airways during branching. The observations above prompted us to further investigate the relationship between activation of RA signaling and establishment of the SM program in forming airways. Previous analysis of the RARElacZ reporter showed that during branching morphogenesis, RA signaling becomes largely undetectable in the epithelium of forming buds. Signals are still maintained in the mesenchymal subpleural region and in mesenchymal derivatives, such as lymphatics, cartilage precursors, and some proximal epithelial cells (36, 39).

We performed X-gal staining of the embryonic lungs from a RARElacZ reporter mouse and confirmed the mesenchymal expression, which was particularly prominent in the distal lung (Figure 2, A–C). Diffuse mesenchymal signals were also present proximally in regions encompassing connective tissue and structures of the pulmonary hilum, such as large vessels and extrapulmonary airways, as previously reported (ref. 39 and data not shown). To better visualize how the mesenchymal pattern of RA activation is established in branching airways, we investigated the spatial distribution of RARElacZ in E11.5 lung explants growing under RA-sufficient (control) conditions. In these cultures, airway branching and differentiation can be easily followed, as the explant flattens and blood vessels, including vascular SM, do not develop. Our analysis of these explants up to 72 hours in culture revealed β-gal activity in a highly focal pattern in the subepithelial mesenchyme associated with the stalk region of the nascent buds (Figure 2, D–G). We found that the expression appeared to be dynamic, as it was most prominent in newly formed distal airways and was declining in preceding airway generations. This distal pattern of RARElacZ was highly dependent on endogenous RA, since it was completely abolished in BMS-treated cultures (Figure 2H).

Interestingly, the local sites of RA activation in the distal mesenchyme coincide with the sites of SM differentiation in newly formed airways (40–42). This was supported by the strong local expression of myocardin (Myocd), a serum response factor (SRF)
cofactor critical for regulation of SM-specific gene transcription as well as expression of myocardin-related factors, as previously reported (Figure 2I, data not shown, and refs. 43–45).

To further investigate the presence of RA activity in SM cells of the developing lung, we crossed RARElacZ mice with an Acta2-GFP reporter mouse line, in which GFP is expressed under the control of an Acta2 promoter (46). GFP-positive cells isolated and sorted from E13.5 Acta2-GFP reporter mouse line, in which GFP is expressed under the control of the Acta2 promoter (46). GFP-positive cells isolated and sorted from E13.5 Acta2-GFP lungs (not shown) and lung explant cultures (Figure 2J) expressed RA pathway components, including key RA-synthesizing enzymes ALDH1A1 and ALDH1A2 and RA receptors RARα, RARβ, and RARγ. Furthermore, GFP-positive cells from Acta2-GFP/RARElacZ double-transgenic lungs expressed lacZ by PCR analysis (Figure 2, K and L), confirming the presence of RA signaling in SM cells of the embryonic lung. The observations above and the results from our functional assays in organ and cell culture were consistent with the hypothesis that endogenous RA restricts SM differentiation in the distal lung.

VAD in utero results in aberrant airway SM differentiation during development in vivo. We asked whether the SM phenotype observed in lung cell and organ cultures could also occur in vivo. Thus, we investigated the impact of maternal VAD in the development of airway SM in the embryonic lung. We disrupted RA signaling by restricting maternal vitamin A dietary intake and by using a mouse mutant strain lacking both Lrat (lecithin retinol acyltransferase) and Rbp4 (retinol-binding protein 4, plasma) genes. Lrat is the main enzyme required for the formation of retinyl esters (RE), the storage form of vitamin A, in both adult and developing tissues; Rbp4 is the sole specific transport protein for ROH in the bloodstream. Being unable to store vitamin A or to mobilize it from the liver as RBP4-bound retinol, these mice rely on dietary vitamin A to support their retinoid-dependent functions, such as embryogenesis (47). Rbp4/Lrat double-null (hereafter referred to as DKO) mutants on a normal vitamin A–sufficient (VAS) diet have negligible RE levels and substantially lower ROH levels than WT mice, but do not show the developmental defects or lethality of severely RA-deficient mice unless fed a VAD diet (<0.2 IU vitamin A/g) from the earliest developmental stages. Thus, DKO mice have been used as a tunable model of vitamin A/RA deficiency. Since these animals have chronically low retinoid levels despite a VAS diet, they also serve as a model of mild chronic VAD when compared with WT mice (47).

We characterized SM development in E14.5 lungs from WT and DKO embryos whose mothers were fed a VAS diet throughout life or a VAD diet from gestation days E9.5 to E14.5 (Figure 3A). This window of VAD was selected because it encompasses the initial stages of lung morphogenesis and airway SM development (41, 48) and prevents early embryonic lethality (47). Reverse HPLC analysis was used to determine RE and ROH levels in embryonic lungs (ref. 49 and Figure 3B). WT embryos responded to the maternal VAD diet by exhibiting lower ROH and RE contents in the lung (P < 0.05). We found that lungs from DKO mice showed a major reduction in ROH content compared with that in seen WT mice, with DKO-VAD mice being the most severely affected. RE was nearly undetectable in both DKO-VAS and DKO-VAD lungs, consistent with the critical role of Lrat in RE formation.

We then generated reporter lines to assess overall RA activity in these lungs by breeding WT or DKO mice with RARElacZ mice. Decreased lacZ expression, as demonstrated by qPCR in lung homogenates of E14.5 WT;RARElacZ VAD, DKO;RARElacZ VAD, and DKO;RARElacZ VAD mice relative to that in WT;RARElacZ VAD mice, confirmed the downregulation of RA signaling (Figure 3C). Remarkably, ISH of E14.5 lungs from DKO embryos showed a thicker layer of subepithelial mesenchymal cells in the proximal airways expressing Tagln and Myh11 compared with that seen in WT lungs. The aberrant expression of SM markers in proximal airways was most severe in the DKO-VAD embryos (Figure 3, D–G).

Furthermore, we found that a maternal VAD diet in both WT and DKO embryos resulted in a strong ectopic expression of Tagln and Myh11 that extended to the most distal airways (Figure 3, D–J). We observed that both proximal and distal features of the aberrant phe-
Figure 5
Consequences of prenatal VAD in the SM phenotype of the adult lung. (A) Diagram of experimental design. (B) HPLC measurements of retinoids in the lung homogenates of adult mice: no difference between VAS and VAD groups, but significantly lower levels in DKO compared with WT mice. (C–E) Significantly lower levels of RARElacZ (C) correlating with Tagln and Myh11 upregulation (D) by qPCR in DKO lungs compared with WT. (E) Significant increase in SM22α, SMMHC2, and pMYL2/MYL2 ratio levels in lung homogenates of WT-VAD, DKO-VAS, and DKO-VAD mice compared with those in WT-VAS lung homogenates by Western blot analysis. IHC of SM markers in adult proximal airways (F–K: Acta2; M–N: Sm22α) showing increased expression in VAD groups (double arrows). Quantitative analysis of Acta2 (L) and Sm22α (O) IHC: signal intensity, relative volume of staining per SA, and relative number of labeled cells per SA in proximal airways (large and medium size, see Methods) suggest a significant increase in SM mass in VAD lungs relative to VAS lungs. Data represent the mean ± SEM, n = 3 per condition. *P < 0.05 compared with WT-VAS; **P < 0.05 compared with DKO-VAS. Scale bars: 30 μm (I) and 20 μm (M). AW, airway.
notypes were also evident by Acta2 immunohistochemistry (IHC) of DKO-VAD lungs compared with DKO-VAS lungs (Figure 4, A–F, and Supplemental Figure 2, A–C). qPCR and Western blot analyses of SM markers confirmed the increase in \( \text{Tagln} \), \( \text{Myh11} \), \( \text{SM22} \alpha \), and SMMHC2 as well as in the pMYL2/tMYL2 ratio, correlating inversely with retinoid content and RA activity in these lungs (Figure 4, G and H). We found that the changes in the SM phenotype were not accompanied by differences in cell proliferation, as assessed by Ki67 or PCNA staining (Supplemental Figure 2, E–J). Moreover, the pattern of SM gene expression in lung blood vessels was essentially the same in all groups (Supplemental Figure 4, A and B). We detected no gross morphological abnormalities in these mice other than a slight decrease in the size of the DKO embryos and lungs compared with those of WT animals. IHC staining of SRY-BOX 2 (SOX2) and SRY-BOX 9 (SOX9), markers of proximal and distal lung epithelium, respectively (50–52), showed no difference in proximal-distal (P-D) patterning among groups (Supplemental Figure 3).

Overall, our data suggest that prenatal VAD profoundly alters the airway SM differentiation program in vivo.

The abnormal SM phenotype due to prenatal VAD is carried throughout life and is prominent in the adult lung. We used DKO mice, which are viable and fertile when fed a VAS diet, to investigate the postnatal effects of the embryonic VAD diet. WT and DKO embryos were subjected to VAS or VAD regimens in utero, as described above. However, instead of being terminated on E14.5, embryos were allowed to develop to adulthood with a VAS diet (Figure 5A and Methods).

Reverse HPLC analysis of WT adult lungs showed RE as the predominant form of retinoid. Lungs from all DKO adult mice had a markedly lower level of ROH compared with that in WT mice and essentially undetectable RE (Figure 5B). As expected, a VAD diet between E9.5 and E14.5 did not influence retinoid levels in either WT or DKO models. However, our analysis of the mutant mice carrying the \( \text{RARElacZ} \) transgene showed that the very low retinoid content in DKO adult lungs only reduced RA activity by approximately 50% (Figure 5C). We investigated the impact of this reduction in RA activity on the SM phenotype of adult DKO lungs. qPCR showed a 1.6- to 1.8-fold increase in \( \text{Tagln} \) and \( \text{Myh11} \) relative to that in WT lungs, suggesting that the chronic RA deficiency in DKO mice, although not severe, still influenced SM gene transcription (Figure 5D). The levels of SM22\( \alpha \) and SMMHC2, as revealed by Western blot analyses, were significantly increased in the adult DKO lungs compared with levels in WT lungs. Notably, while lungs from VAS and VAD mice had similar levels of \( \text{Tagln} \) and
Myh11 in each group, the protein products were increased in those exposed to a gestational VAD diet. The abnormal SM phenotype associated with fetal VAD exposure was further supported by the increased pMYL2/tMYL2 ratio in these lungs (Figure 5E).

Immunostaining and morphometric analyses of Acta2 (Figure 5, F–L) showed a marked increase in signal intensity, relative volume, and number of Acta2-labeled cells per surface area (SA) in large- and medium-sized airways of VAD versus VAS lungs. Terminal bronchioles of VAD lungs appeared unaffected, likely due to the limited exposure time to the VAD diet influencing only a few initial generations of branches. Analysis of Sm22α IHC showed similar findings (Figure 5, M–O). Vascular SM appeared to be unaffected by gestational VAD status, consistent with our previous observations in E14.5 lungs (Supplemental Figure 4, A and B). Importantly, the SM abnormalities from the embryonic VAD exposure of WT mice (Figure 3, H and I) indicate that the aberrant SM phenotype is present despite normal adult RA status. Thus, prenatal disruption of RA signaling leads to long-lasting structural changes in the airways.

**Prenatal disruption of RA signaling results in increased postnatal airway responsiveness.** Increased airway SM mass and pMYL2/tMYL2 ratio correlate with increased SM contractility and responsiveness to bronchoconstrictive stimuli (24). We reasoned that the structural changes in the adult airway resulting from prenatal RA deficiency were accompanied by abnormal bronchial responsiveness. Thus, we assessed the degree of airflow obstruction in adult WT or DKO mice under VAS or gestational VAD conditions using a flexiVent ventilator. We measured airway resistance in a nonchallenged state to prevent precocious and excessive SM differentiation while the VAD and VAS groups were still evident (Figure 6E). Overall, the data support the hypothesis that insufficient levels of vitamin A prenatally predispose to airway hyperresponsiveness.

**Discussion**

Studies in cell culture and injury models show that SM cells are highly diverse and plastic, with features ranging from a more mature phenotype characterized by an abundance of SM markers and contractile apparatus, to a less differentiated phenotype, marked by a tendency to proliferate and synthesize bioactive proteins and ECM. The balance of these phenotypes during development or injury/repair is largely dependent on environmental cues acting in a network of intracellular signals and, ultimately, phenotype-specific genes and their products (54). We have previously identified RA as a major regulatory signal integrating multiple pathways in the foregut to control formation of the lung primordium (30–33). Here, we provide evidence that during lung development, endogenous RA has an additional key role in restricting the SM differentiation program when airways are forming and branching. It is remarkable that disruption of RA signaling in all of the models examined here consistently resulted in upregulation of SM contractility markers. The developmental defect caused by prenatal RA deficiency appeared to result in an overall increase in airway SM mass that persisted throughout life and manifested in the adult as abnormal airway SM accumulation and hyperresponsivenes.

We had no evidence that the excess in airway SM in retinoid-deficient lungs resulted from excessive proliferation of SM cells. Instead, we speculate that this phenotype may arise from increased recruitment of putative mesenchymal precursors into the SM program or from uninhibited differentiation of cells already committed to the SM phenotype in newly formed airways. Our findings of RA pathway components and RARElacZ expression in Acta2-GFP-labeled cells favor the latter scenario. Future lineage studies can help clarify this issue. We propose that in the developing lung, endogenous RA maintains airway SM cells in a less differentiated state to prevent precocious and excessive SM differentiation while distal airways are forming (Figure 7). In this regard, the mechanism we describe is similar to that reported in the developing cor-

**Figure 7** Proposed mechanism of SM regulation by endogenous RA. During branching of the developing airways, a program of differentiation of SM takes place in the distal mesenchyme at the newly formed stalks of the lung buds (white circled area). RA signaling is activated in these areas, as evidenced by locally enriched RARElacZ expression (inset) at these sites. Endogenous RA regulates this SM program, restricting SM gene expression and preventing excessive formation of SM while airways are branching. As new generations of airways arise, the process is reiterated, and the RA restriction of the SM program becomes less crucial proximally (left panel, dashed yellow lines). RA fine-tunes the SM gene expression program, likely by inhibiting the expression of a key activator of SM transcription (in green) or by inducing the expression of the transcriptional repressor (in blue), ultimately resulting in the downregulation of SM gene expression.
VAD is limited to a defined prenatal developmental window or stage (15). How do the observations described here relate to those that link maternal smoking and abnormal lung function in neonates (64) and an increased risk of asthma later in life (63) and the link between prenatal exposure to maternal smoking and maternal smoking and abnormal lung function in neonates (64) and an increased risk of asthma later in life (63? We believe that the present study establishes for the first time a link between prenatal exposure to maternal smoking and maternal smoking and abnormal lung function in neonates (64) and an increased risk of asthma later in life (63) and the link between prenatal exposure to maternal smoking and maternal smoking and abnormal lung function in neonates (64) and an increased risk of asthma later in life (63).
turers. For IHC, sections were incubated with HRP- or AP-conjugated antibodies (α-SMA, 1:1,000, Santa Cruz Biotechnology Inc.; SM22α, 1:1,000, Abcam; SMMHC2, 1:1,000, Abcam; and Ki67, 1:200, Cell Signaling Technology) and developed using kits and protocols provided by Vector Laboratories. ISH was performed in 96-well plates (whole-mount specimens) or in 5-μm paraffin sections, as reported previously (32).

Isolation and sorting of Acta2-GFP–positive cells. Cell suspensions were obtained by digesting lungs and lung explants with 0.1% collagenase A (Roche Diagnostics), 2.4 units/ml dispase (Roche Diagnostics), and 6 units/ml DNase I (QIAGEN) at 37°C for 1 hour. GFP-positive cells were collected using a MoFlo cell sorter (Beckman Coulter). The GFP-positive cells were then processed for qPCR and Western blotting.

 Morphometry. α-SMA IHC images of lungs from WT and DKO mice (×20 and ×40 magnification) were ImageJ software following established protocols (67). α-SMA–labeled airway SM cells were counted in 15 large- and 15 medium-sized airways and normalized to the surface of their basement membranes. The intensity of the α-SMA signals was estimated by changing images to 8-bit grayscale and determining the mean gray value of the IHC signal in 15 random regions around the airways sampled by the ImageJ oval tool, excluding the vasculature. Background subtraction was done by sampling 15 random, α-SMA–negative regions from the same lung. The volume occupied by the eSMA–positive cells in the airways was outlined (freehand tool), and the value was expressed as SA of the associated basement membrane.

Methacholine challenge and airway resistance measurements. Adult mice were anesthetized and paralyzed (i.p. pentobarbital, 100 μg/g BW), intubated (tracheostomy), placed on a mechanical ventilator (flexiVent; SCIREQ), and ventilated at 300 breaths/minute (tidal volume: 6–7 ml/kg; 3 cm H2O positive end expiratory pressure [PEEP]). Baseline airway resistance and elastance were measured after airway delivery of nebulized vehicle (0 mg/ml of methacholine) and, thereafter, with delivery of increasing concentrations of nebulized methacholine (5 and 10 mg/ml) (53). Lungs were then harvested and processed for various analyses. flexiVent data were presented as absolute airway resistance (Rn) and relative resistance from baseline.

Statistics. Statistical analyses were performed using 2-tailed Student’s t tests (minimum of three samples per condition). Differences were considered significant if the P value was less than 0.05.

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