Epithelial β1 integrin is required for lung branching morphogenesis and alveolarization

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INTRODUCTION
Embryonic lung development occurs as a result of epithelial-mesenchymal interactions that are initiated when the bronchial buds grow into the surrounding mesoderm. The buds undergo iterative branching morphogenesis to give rise to the respiratory tree, terminal sacculles and alveoli, whereas the mesoderm forms the lung fibroblasts and the pulmonary vasculature (Cardoso and Lu, 2006; Hogan, 1999). This complex pattern of development depends on a variety of factors, including expression of mesenchymal-derived signaling molecules and their receptors on epithelium, as well as cell-extracellular matrix (ECM) interactions (McGowan, 1992; Morrisey and Hogan, 2010).

The lung basement membrane (BM) is composed primarily of collagen IV, laminins, nidogens and proteoglycans (Thibault et al., 2002; Wasowicz et al., 1998), and several previous studies have shown that distinct laminin chains are required for normal lung branching morphogenesis, lobar septation and alveolarization (Nguyen et al., 2005, 2002; Nguyen and Senior, 2006; Willem et al., 2002). The major integrins that affect cell-ECM interactions during lung development are the laminin receptors α3β1, α6β1 and α6β4 (De Arcangelis et al., 1999; Georges-Labouesse et al., 1996; Has et al., 2012; Kim et al., 2009; Kreidberg et al., 1996; Nicolau et al., 2012). Of the laminin receptors, integrin α3β1 has been most extensively investigated. Lungs from constitutive integrin α3-null mice fail to branch into bronchioles (Kreidberg et al., 1996), and mice null for both integrin α3 and α6 have severe lung hypoplasia (De Arcangelis et al., 1999). Surprisingly, mice with a lung epithelial-specific deletion of α3 integrin have a normal lifespan with normal airway branching but subtle defects in epithelial differentiation (Kim et al., 2009). Recently, lung hypoplasia due to integrin α3 mutations has been identified as a cause of early childhood mortality (Has et al., 2012; Nicolau et al., 2012). The RGD-binding integrin α6β1 has also been shown to regulate lung development as constitutive integrin α6-null mice have abnormally fused medial and caudal lobes of the lung, as well as subtle abnormalities in airway division (Benjamin et al., 2009).

β1 integrin is the major isoform of the eight β-integrin subunits, and is the β-integrin subunit present in 12 of the 24 known α-β integrin heterodimers (Pozzi and Zent, 2011). When β1 integrin is selectively deleted in lung epithelium at the time of endodermal tracheal outgrowth (E9.5) using a Shh promoter-driven Cre, mice develop a severe branching morphogenesis defect that blocks development at the level of primary bronchi, making it impossible to determine the role of β1 integrin at later stages of development (Chen and Krasnow, 2012). To elucidate possible crucial functions of β1 integrin beyond the initial stages of lung development, we generated mice with an epithelial-targeted deletion of β1 integrin using a surfactant protein C (SP-C; also known as SFTPC) promoter-driven Cre, which is expressed in the lung epithelium at E10.5 (Okubo et al., 2005). Analysis of these mice showed that β1 integrin regulates a variety of crucial epithelial cell processes required for normal lung development and homeostasis.

**ABSTRACT**
Integrin-dependent interactions between cells and extracellular matrix regulate lung development; however, specific roles for β1-containing integrins in individual cell types, including epithelial cells, remain incompletely understood. In this study, the functional importance of β1 integrin in lung epithelium during mouse lung development was investigated by deleting the integrin from E10.5 onwards using surfactant protein C promoter-driven Cre. These mutant mice appeared normal at birth but failed to gain weight appropriately and died by 4 months of age with severe hypoxemia. Defects in airway branching morphogenesis in association with impaired epithelial cell adhesion and migration, as well as alveolarization defects and persistent macrophage-mediated inflammation were identified. Using an inducible system to delete β1 integrin after completion of airway branching, we showed that alveolarization defects, characterized by disrupted secondary septation, abnormal alveolar epithelial cell differentiation, excessive collagen I and elastin deposition, and hypercellularity of the mesenchyme occurred independently of airway branching defects. By depleting macrophages using liposomal clodronate, we found that alveolarization defects were secondary to persistent alveolar inflammation. β1 integrin-deficient alveolar epithelial cells produced excessive monocyte chemoattractant protein 1 and reactive oxygen species, suggesting a direct role for β1 integrin in regulating alveolar homeostasis. Taken together, these studies define distinct functions of epithelial β1 integrin during both early and late lung development that affect airway branching morphogenesis, epithelial cell differentiation, alveolar septation and regulation of alveolar homeostasis.

KEY WORDS: Extracellular matrix, Branching morphogenesis, Alveolar epithelial cell, Differentiation, Inflammation, Reactive oxygen species

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RESULTS

Epithelial-specific β1 integrin deficiency results in abnormal lung structure and reduced lifespan

We deleted β1 integrin in the lung epithelium from E10.5 onwards by crossing SP-C-Cre mice with integrin β1f/f (β1f/f) mice (hereafter called β1SP-C-Cre mice). By E13, β1 integrin was selectively deleted in the proximal and distal lung epithelium as shown by immunostaining of embryonic airways (Fig. 1A,B). β1SP-C-Cre mice were indistinguishable from littermate n of embryonic airways (Fig. 1A,B). The proximal and distal lung epithelium as shown by immunostaining β1 integrin immunostaining showing lung epithelial-specific deletion in E13 β1SP-C-Cre mice were in the normal Mendelian ratio; however, few survived longer than 4 months (Fig. 1C). β1SP-C-Cre mice grew poorly and were significantly smaller than age-matched littermate control β1f/f (Fig. 1D) and heterozygote SP-C-Cre; β1f/f WT mice (data not shown). Interestingly, β1SP-C-Cre had normal oxygen saturation at birth, but by 8 weeks they were severely hypoxic with a mean oxygen saturation of 79% (Fig. 1E). Thus, deleting β1 integrin in the lung epithelium results in a lethal phenotype with severe hypoxia.

Despite having lower body weight, adult β1SP-C-Cre mouse lungs were larger and weighed more (35.8±3 mg versus 24.2±2 mg) than littermate β1f/f mice (Fig. 2A,B). Histological examination of lung parenchyma from P28 and older β1SP-C-Cre mice revealed dilated airspaces surrounded by thickened, hypercellular alveolar septa with type II epithelial cell hyperplasia and increased numbers of macrophages (Fig. 2C-K). β1SP-C-Cre lungs showed sustained airspace enlargement at 3 months of age (Fig. 2H-K). Alveolar size, as measured by calculating the mean linear intercept, in P28 β1SP-C-Cre mice was approximately double the value in controls (Fig. 2G). Heterozygotes were indistinguishable from littermate control β1SP-C-Cre mice in histological examinations at both time points (data not shown). Thus deleting β1 integrin from the lung epithelium results in large and dilated airspaces, thickened inter-alveolar septa, abnormal epithelial cell differentiation and influx of alveolar macrophages, probably explaining the hypoxemia and premature mortality observed in these mice.

Deleting β1 integrin in lung epithelium results in a branching morphogenesis defect

Analysis of the airways in adult β1SP-C-Cre mice revealed a paucity of terminal bronchioles, indicative of a branching morphogenesis defect. We therefore examined lung histology near the initiation, mid-point and completion of branching morphogenesis. Although no branching defect was identified at E13 during early branching morphogenesis (Fig. 3A,B), abnormal branching was seen at E15. At E15, β1SP-C-Cre airways were similar in size to controls but fewer in number and separated by a thickened interstitium (Fig. 3C,D). This branching defect was even more evident by E18, where there was a marked reduction of airways in β1SP-C-Cre mice (Fig. 3E,F) as verified by quantification of histological sections (147±14 airways per mm² in β1SP-C-Cre lungs compared with 259±13 airways per mm² in β1f/f mice; mean±s.e.m.) (Fig. 3I). In contrast to the proximal to distal airway narrowing seen in normal mice, there were a number of large airways found in the peripheral lung of the β1SP-C-Cre mice (Fig. 3F, asterisks). Consistent with observations indicating fewer airways in β1SP-C-Cre mice, the airspace volume density of E18 β1SP-C-Cre lungs was significantly less (29±1%) than β1f/f lungs (46±1%) (Fig. 3J). Airspaces in lungs of β1SP-C-Cre mice at P0 were fewer in number and larger in histological examinations (Fig. 3G,H).

In addition to abnormal airway branching at E18 in β1SP-C-Cre mice, hypercellularity of the interstitium was evident. By Ki67 (also known as MKI67) staining, we identified increased cellular proliferation in the interstitium of E18 β1SP-C-Cre lungs compared with β1f/f lungs (29±2% versus 7±1% Ki67-positive cells) (Fig. 3K-M). The proliferating cell population was primarily non-epithelial in β1SP-C-Cre lungs as verified by co-immunostaining for the mitotic marker phosphohistone H3 (PHH3) and the epithelial marker E-cadherin (Fig. 3N-P). A total of 58±7±0.5% and 39±2±0.5% of proliferating (PHH3+) cells were non-epithelial in β1SP-C-Cre and β1f/f lungs respectively (P<0.05). By contrast, there was no significant difference in apoptosis between β1SP-C-Cre and β1f/f lungs at this time point (supplementary material Fig. S1E). Thus, deleting β1 integrin in the lung epithelium results in a branching morphogenesis defect and increased interstitial cell proliferation.

To better define the mechanism of branching defects in β1SP-C-Cre mice, we examined E15 fetal lung explants. Compared with controls, significantly less new branching occurred in β1SP-C-Cre lung explants cultured for 12 and 48 h (Fig. 4A-E). When the velocity of the leading epithelial edge of peripheral airways was tracked in these cultures using time-lapse microscopy, we measured a decreased outward velocity of the epithelial edge in β1SP-C-Cre explants (3.2±0.05 μm/h versus 2.9±0.06 μm/h in β1f/f explants) (Fig. 4F). Although no difference in explant size was identified between β1SP-C-Cre and β1f/f lung explants at 48 h (data not shown), the distance from the epithelial basement membrane to the explant edge was greater in β1SP-C-Cre lung explants compared with controls (2.08±0.5 μm versus 0.13±0.6 μm), indicative of decreased outward epithelial growth and/or increased mesenchymal proliferation (Fig. 4G-I). These data suggest that loss of epithelial β1 integrin disrupts epithelial migration, a key cellular process during branching morphogenesis. To further investigate the migratory phenotype of β1 integrin-null lung epithelial cells, we isolated type II epithelial cells from P28 β1SP-C-Cre and β1f/f mice and
confirmed β1 integrin deletion by immunoblotting (Fig. 4J). These cells had a marked adhesion defect (74±11% β1FT versus 18±18% β1SP-C.Cre cells adhered) on purified laminin 511, a major laminin isoform found in the fetal lung basement membrane that is required for normal lung development (Nguyen et al., 2005) (Fig. 4K). In addition, these cells had a major haptotactic migration defect on laminin 511 (Fig. 4L). Taken together, these data indicate a role for β1 integrin in alveolar epithelial adhesion and migration, and suggest a potential mechanism for the branching defect seen in β1SP-C.Cre mice.

**Lung epithelial-specific deletion of β1 integrin disrupts alveolarization**

In addition to an airway branching defect, adult β1SP-C.Cre mice had dilated alveoli with thickened alveolar septa, suggestive of an additional alveolarization defect. We therefore examined β1SP-C.Cre lungs at P7 and P14 to identify defects in secondary septation. Airspace enlargement was seen by P7 (Fig. 5A,B) in β1SP-C.Cre lungs and this was even more obvious at P14 (Fig. 5C,D). In addition, P14 β1SP-C.Cre alveolar septa were hypercellular and thickened suggesting increased ECM deposition. We quantified the differences in alveolar size by measuring the average alveolar diameter in P14 β1SP-C.Cre and β1FT mice (50±2 μm in β1SP-C.Cre lungs versus 30±3.5 μm in β1FT lungs; mean±s.e.m.) (Fig. 5G). To determine whether loss of β1 integrin disrupted secondary septation, we quantified the number of secondary crests and found that β1SP-C.Cre lungs had significantly fewer secondary crests per high-power field than β1FT control littermates (26±2 crests in β1SP-C.Cre lungs versus 92±5 crests in β1FT lungs) (Fig. 5H). Similar to the above studies in embryonic lungs, we found that the hypercellularity noted in the interalveolar spaces during alveolarization was due to increased cell proliferation and not decreased apoptosis, as Ki67 staining was increased in β1SP-C.Cre mice compared with β1FT controls (25±2% versus 10±1%), whereas immunostaining for cleaved caspase 3 was not different between the two genotypes (Fig. 5I,J). When we defined the composition of interstitial ECM at P14, we noted a marked increase in elastin and collagen I, but not collagen IV, in β1SP-C.Cre alveolar septa compared with that amount in alveolar septa from β1FT lungs (Fig. 5K-P). Despite disruptions to the epithelium and ECM, the vasculature developed in close proximity to the alveolar epithelium in β1SP-C.Cre mice because staining for the endothelial marker CD31 demonstrated that there were intact capillary networks adjacent to the alveolar surface in both β1FT and β1SP-C.Cre mice (supplementary material Fig. S1C,D). Taken together, these data suggest that deleting β1 integrin disrupts alveolar septal structure by reducing secondary septation and increasing mesenchymal cell proliferation and ECM deposition.

**Loss of epithelial β1 integrin results in abnormal alveolar epithelial cell differentiation**

The histological features of β1SP-C.Cre lungs suggested that there were β1 integrin-dependent defects in epithelial cell differentiation. Abundant cuboidal epithelial cells lined the peripheral airways of E18 β1SP-C.Cre lungs (Fig. 6A,B), compared with a more differentiated flattened epithelium in control mice (Fig. 6A, arrowheads). After birth, periodic acid schiff staining revealed that there were epithelial cells with a less differentiated phenotype (i.e. between that of a type I and type II cell), we diminished cytoplasm. As these cells appeared to have a hybrid appearance of P28 β1SP-C.Cre and β1FT mice (labeled 600×). H&E-stained paraffin sections of P28 β1SP-C.Cre mice (labeled 600×). (H,I) Dilated airspaces and thickened alveolar septa persist throughout the lifespan of β1SP-C.Cre mice (labeled 200×). (J,K) Type II cell (arrows) hyperplasia demonstrated in 3-month-old (3 mo) β1SP-C.Cre mice (labeled 600×). *P<0.05 between β1SP-C.Cre and β1FT mice. Scale bars: in D, 100 μm for C,D; in I, 100 μm for H,I; in F, 25 μm for E,F; in K, 25 μm for J,K.
β1 integrin plays a distinct role in lung alveolarization

To address whether the β1 integrin-dependent airway branching and alveolarization defects were separable, we generated triple transgenic mice (SP-C rtTA; tetO-Cre; β1f/f) with a doxycycline-inducible lung epithelial-specific β1 integrin deletion at P0, which is after the completion of branching but prior to the start of alveologenesis. These mice retained the alveolarization defect seen in β1SP-C.Cre mice at P28, characterized by dilated airspaces, thickened alveolar septa, type II cell hyperplasia and increased alveolar macrophages (Fig. 7A-D). As seen in Fig. 7B, the subpleural airspaces in the peripheral lung were most severely affected, with more modest dilation of the proximal airspaces, consistent with proximal-distal maturation of the lung. These findings indicate that regulation of alveolarization by epithelial β1 integrin is independent of its role in branching morphogenesis.

Loss of epithelial β1 integrin results in lung inflammation which modulates lung alveolarization

An increase in alveolar macrophage numbers was confirmed in the bronchoalveolar lavage fluid (BALF) of 8-week-old β1SP-C.Cre mice (Fig. 8A-C). Increased macrophage accumulation, as measured by CD68 immunostaining, was seen as early as E18 in β1SP-C.Cre lungs (Fig. 8D-G); however, there were no difference in macrophage numbers in E15 β1f/f and β1SP-C.Cre lungs (supplementary material Fig. S1A,B). No differences in lung neutrophils or lymphocytes were identified between genotypes on immunostained lung sections (Fig. 8H-K).

The massive recruitment of macrophages, but not other types of immune or inflammatory cells, seen in adult β1SP-C.Cre lungs prompted us to investigate the contribution of increased alveolar macrophages to the alveolarization defect seen in β1SP-C.Cre mice. We therefore depleted macrophages during alveolarization by treating β1f/f and β1SP-C.Cre mice with intranasal clodronate from P5 to harvest at P14 or P28. Although clodronate-treated P28 β1SP-C.Cre mice still had airway branching defects (asterisks in Fig. 9B), this treatment rescued the alveolarization defect seen in β1SP-C.Cre mice. In contrast, P28 β1SP-C.Cre mice treated with vehicle (liposomes containing PBS) retained the phenotype of dilated airspaces, thickened alveolar septa, type II cell hyperplasia and increased alveolar macrophages (Fig. 9A-F). Normalization of the β1SP-C.Cre phenotype was quantified by measuring the average alveolar diameter (Fig. 9G) and quantification of secondary crests at P14 (Fig. 9H). Taken together, these data indicate that deficiency of β1 integrin disrupts alveolarization through recruitment and/or activation of macrophages.
Given that inflammation that is predominantly mediated by macrophages impairs alveolarization in β1SP-C.Cre mice, we next sought to identify the mechanism of alveolar macrophage recruitment to the lungs of mice with epithelial β1 integrin deficiency. We first measured the BALF levels of the chemoattractant monocyte chemoattractant protein 1 (MCP-1; also known as CCL2), the major chemokine responsible for monocyte and macrophage recruitment. We found that MCP-1 levels were markedly increased in BALF from β1f/f mice (n=7 β1SP-C.Cre mice, n=6 β1f/f mice, five explants per mouse) (Fig. 10A). In contrast, there was no difference in the BALF levels of KC (also known as CXCL1), a neutrophil-specific chemoattractant protein 1 (MCP-1; also known as CCL2), the major chemokine responsible for monocyte and macrophage recruitment. We found that MCP-1 levels were markedly increased in BALF from β1f/f mice (n=3 β1SP-C.Cre mice, n=6 β1f/f mice, five explants per mouse). (F) Outward epithelial velocity was decreased in β1SP-C.Cre explants compared with β1f/f explants over a 6 h time period (n=9 β1SP-C.Cre mice, n=5 β1f/f mice, one explant per mouse, ten consecutive airways per explant for F and I). (G-I) The distance from the epithelial edge to explant edge (yellow lines) was greater in β1SP-C.Cre compared with β1f/f explants. The difference is quantified in I. (J) Immunoblot for β1 integrin and SP-C on primary epithelial cell lysates showing epithelial-specific deletion in β1SP-C.Cre mice (blot representative of three separate experiments). (K) Decreased cell adhesion of isolated β1SP-C.Cre epithelial cells plated on laminin-511 compared with β1f/f epithelial cells (epithelial cells isolated from n=7 β1SP-C.Cre mice, n=6 β1f/f mice, completed in four separate assays each performed in triplicate). (L) Isolated β1SP-C.Cre epithelial cells have decreased migration across a laminin-511-coated filter compared with β1f/f epithelial cells (n=6 from each group, completed in three separate assays, five sections analyzed per mouse). *P<0.05 between β1SP-C.Cre and β1f/f mice.

**Fig. 4. Deleting β1 integrin in lung epithelium causes adhesion and migration defects.**

(A-D) E15 fetal lung explants have decreased branching after 48 h. (E) Quantification of new branches in β1SP-C.Cre explants compared to β1f/f explants (n=3 β1SP-C.Cre mice, n=6 β1f/f mice, five explants per mouse). (F) Outward epithelial velocity was decreased in β1SP-C.Cre explants compared with β1f/f explants over a 6 h time period (n=9 β1SP-C.Cre mice, n=5 β1f/f mice, one explant per mouse, ten consecutive airways per explant for F and I). (G-I) The distance from the epithelial edge to explant edge (yellow lines) was greater in β1SP-C.Cre compared with β1f/f explants. The difference is quantified in I. (J) Immunoblot for β1 integrin and SP-C on primary epithelial cell lysates showing epithelial-specific deletion in β1SP-C.Cre mice (blot representative of three separate experiments). (K) Decreased cell adhesion of isolated β1SP-C.Cre epithelial cells plated on laminin-511 compared with β1f/f epithelial cells (epithelial cells isolated from n=7 β1SP-C.Cre mice, n=6 β1f/f mice, completed in four separate assays each performed in triplicate). (L) Isolated β1SP-C.Cre epithelial cells have decreased migration across a laminin-511-coated filter compared with β1f/f epithelial cells (n=6 from each group, completed in three separate assays, five sections analyzed per mouse). *P<0.05 between β1SP-C.Cre and β1f/f mice.

In vivo, we assayed in lungs of β1SP-C.Cre and control mice for ROS production by bioluminescence imaging following injection of a modified luminol derivative (Han et al., 2013). We found increased ROS production over the chest of β1SP-C.Cre mice compared with β1f/f control mice (Fig. 10E-G). To define whether the β1 integrin-deficient epithelium directly contributed to ROS production, we examined epithelial lipid peroxidation, as an indicator of oxidative stress. Primary type II alveolar epithelial cells isolated from β1SP-C.Cre mice had a fivefold increase in lipid peroxidation over β1f/f epithelial cells (Fig. 10H). These findings suggest that deleting β1 integrin in epithelial cells alters ROS production, which in turn regulates macrophage influx.

**DISCUSSION**

We found that deleting β1 integrin in lung epithelium beginning at E10.5 results in abnormal lung development with mortality by 4 months of age. A variety of specific defects were identified, including altered branching morphogenesis, impaired alveolarization with epithelial cell differentiation defects and persistent macrophage-mediated inflammation, indicating that β1 integrin expression in the lung epithelium plays multiple roles in distinct phases of lung development.

Our findings are complementary to a prior study that found that epithelial depletion of β1 integrin beginning at E9.5 causes a serious branching defect and neonatal death (Chen and Krasnow, 2012).
Although the disparity in these phenotypes could be due to differences in potency between the Cre-expressing mice (Shh and the SP-C), it is more likely that β1 integrin has a more profound role in early rather than late lung development. This is consistent with the crucial role for β1 integrin in the early development of other branched organs. In the kidney, β1 integrin deletion at initiation (E10.5) of ureteric bud development results in a lethal phenotype but has no effect when it is removed at E18.5 (Zhang et al., 2009). In addition, fetal β1 deletion in the basal epithelial cells in the mammary gland causes a branching defect, whereas postpubertal deletions do not (Faraldo et al., 1997; Naylor et al., 2005; Taddei et al., 1999). Consistent with findings in constitutive integrin α3-null mice, functional mutations of integrin α3 in humans cause pulmonary hypoplasia, severe fibrosis with thickened alveolar septa lined with reactive type II pneumocytes, and increased numbers of alveolar macrophages (Has et al., 2012; Nicolaou et al., 2012). Although integrins expressed by the epithelial and mesenchymal compartments of the lung probably play distinct roles in different aspects of lung development, available information suggests that several of the α-β1 integrin heterodimers expressed in lung epithelium regulate lung branching morphogenesis and alveolarization.

We show that integrin β1-null type II alveolar epithelial cells are increased in number and morphologically abnormal with enlarged nuclei and fewer mitochondria and lamellar bodies, suggesting abnormal type II alveolar epithelial cell differentiation. Consistent with this abnormal morphology, the epithelial cells lining dilated airspaces in β1F/C-Cre lungs are positive for both elastin and collagen IV on P14 β1F/C-Cre (L,N,P) and β1F (K,M,O) paraffin sections. Scale bars: in A, 250 μm for A,B; in C, 100 μm for C-F; in K, 50 μm for K-P. *P<0.05 between β1F/C-Cre and β1F mice.
through integrin-ECM interactions regulate lung epithelial cell differentiation in vitro through unknown mechanisms (Huang et al., 2012; Sanchez-Esteban et al., 2004; Wang et al., 2013, 2006, 2009). Thus, it is likely that abnormally differentiated type II cells in β1SP-C.Cre mice result from impaired integrin-dependent attachment and altered interactions with the ECM. This explanation is consistent with abnormalities in epithelial cell differentiation induced by deletion of integrin β1 in other organs, such as the kidney proximal tubule (Elias et al., 2014), enterocytes (Jones et al., 2006), keratinocytes (Brakebusch et al., 2000), mammary epithelium (Naylor et al., 2005) and the submandibular gland (Menko et al., 2001).

Many studies have focused on the role of mesenchymal growth factors and epithelial receptor signaling in lung development, and there are many similarities between mice with growth factor and/or receptor deletions and mice lacking β1 integrin in the lung epithelium. Lungs from FGF10- and FGFR2-null mice fail to branch beyond the trachea (Min et al., 1998).

![Fig. 6. β1SP-C.Cre mice have defects in proximal and distal epithelial differentiation.](image)

(A,B) Cuboidal epithelial cells (arrows) seen in E18 β1SP-C.Cre H&E-stained lungs. (C,D) Increase in periodic acid schiff (PAS)-positive epithelial cells in β1SP-C.Cre lungs at P14. (E,F) Immunostaining for the type II cell marker Nkx2.1 (red) and E-cadherin (green). (G) The number of Nkx2.1+ cells in β1SP-C.Cre lungs was greater than in β1f/f lungs (n=5 β1SP-C.Cre mice, n=5 β1f/f mice, eight sections per mouse). (H,I) Immunostaining for type II cell marker SP-C (red) and E-cadherin (green). (J) The number of SP-C+ cells in β1SP-C.Cre lungs was greater than in β1f/f lungs (n=4 β1SP-C.Cre mice, n=4 β1f/f mice, eight sections per mouse). (K-M) Paucity of lamellar bodies (arrowheads) in β1SP-C.Cre type II cells as assessed in transmission electron microscopy images (taken at 11,000×). Type II cell (arrows) hyperplasia along thickened alveolar septa in β1SP-C.Cre mice (L). (N,O) Dual positive epithelial cells are seen in β1SP-C.Cre lungs, demonstrated by 25× confocal images of co-immunostaining for the type I marker T1α (red) and type II marker pro-SP-C (green). Insets are 100× images. *P≤0.05 between β1SP-C.Cre and β1f/f mice. Scale bars: in I, 50 µm for E,F,H,I; in M, 2 µm for K-M; in O, 25 µm for N,O.

Fig. 7. Lung epithelial β1 integrin deletion at P0 retains alveolarization defect. (A-D) H&E-stained paraffin sections of P28 SP-C rtTA; tetO-Cre; β1f/f (B,D) and littermate β1f/f (A,C) lungs from mice given doxycycline (dox)-containing drinking water from P0 to P28. Mice with a lung epithelial β1 integrin deletion induced at P0 have an alveolarization defect similar to β1SP-C.Cre mice. Representative low magnification (taken at ×100) images are shown in A,B, and high-magnification (taken at ×600) images in C,D. Scale bars: in B, 250 µm for A,B; in D, 25 µm for C,D.
Sekine et al., 1999), FGFR3/4-null mice have an alveolarization defect (Weinstein et al., 1998), and mice null for both FGFR3 and FGFR4 have a mild branching defect and thickened alveolar septa, similar to the β1SP-C.Cre mice (Miettinen et al., 1995). We have previously shown in the kidney that β1 integrin is required for FGF2 and FGF10 signaling in ureteric bud development (Zhang et al., 2009), and that β1 integrin regulates FGF- and EGF-dependent signaling in renal collecting duct cells (Mathew et al., 2012). Thus many of the phenotypical characteristics observed in the β1SP-C.Cre mice might be caused by both alterations in integrin-dependent growth factor signaling as well as adhesion and migration defects.

Our studies point to an important role for β1 integrin in maintaining alveolar homeostasis, which is required for normal alveolarization during the early post-natal period. In the mammary gland, epithelial β1 integrin deletion results in epithelial detachment from the basement membrane without inflammation (Li et al., 2005; Naylor et al., 2005). In contrast, increased numbers of macrophages were observed in the lungs of mice with laminin α3 chain mutations (Urich et al., 2011) and in the lungs of humans with integrin α3 mutations (Nicolaou et al., 2012), suggesting that β1 integrin-mediated regulation of inflammation is specific to the lung epithelium. Whereas β1 integrin deficiency results in increased ROS production and MCP-1 secretion from alveolar macrophages, clodronate treatment rescues the alveolarization defect in β1SP-C.Cre mice.

**Fig. 8.** β1SP-C.Cre mice have increased alveolar macrophages. (A,B) Microscopy of cytospin from β1SP-C.Cre and β1f/f mice. (C) The increased number of macrophages in β1SP-C.Cre BALF was quantified (n=4 mice in each group). (D,E) P14 frozen sections immunostained for CD68 (green) and E-cadherin (green). (H,J) E18 frozen sections immunostained for CD3 (red). (J,K) E18 frozen sections immunostained for Gr1 (green). *P<0.05 between β1SP-C.Cre and β1f/f mice. Scale bar: in K, 50 μm for D-K.

**Fig. 9.** Macrophage depletion rescues alveolarization defect in β1SP-C.Cre mice. (A-F) H&E-stained paraffin sections of clodronate-treated β1f/f lungs (A,D) and β1SP-C.Cre lungs treated with either clodronate (B,E) or lipid vehicle (C,F), harvested at P28 (A-C) and P14 (D-F). Lungs from clodronate-treated β1SP-C.Cre mice (B,E) have normal septation but demonstrate a branching defect with large airways that fail to taper at the lung periphery (asterisks in B), whereas vehicle-treated β1SP-C.Cre lungs retain the original β1SP-C.Cre phenotype (C,F). (G,H) Lung morphometry on P14 H&E-stained sections. Alveolar diameter (G) and number of secondary crests per high-power field (H) in P14 β1SP-C.Cre lungs are normalized to the levels in β1f/f with depletion of macrophages from P5 to P14 (n=4 β1f/f mice, n=4 β1SP-C.Cre mice, six sections per mouse). *P<0.05 between vehicle-treated β1SP-C.Cre mice and each of the other groups. Scale bars: in C, 250 μm for A-C; in F, 100 μm for D-F.
epithelial cells, the molecular mechanisms accounting for these findings will require further study. Increased ROS production has been described in integrin αι-null glomerular mesangial cells (Chen et al., 2007); however, this has not previously been shown to occur in epithelial cells, and β1 integrins have not been linked to MCP-1 expression or ROS production in other systems. Although the role of macrophages during alveolarization is not well understood, we and others have shown that macrophage and macrophage-derived products disrupt branching morphogenesis (Blackwell et al., 2011; Nold et al., 2013). Specifically, we have found that products of activated macrophages can impair expression of molecules by epithelial and mesenchymal cells that are important for control of airway branching, including BMP4, Wnt7b and FGF10 (Benjamin et al., 2010; Blackwell et al., 2011; Carver et al., 2013). We speculate that mediators secreted by activated macrophages might also disrupt crucial epithelial-mesenchymal interactions required for normal septation and remodeling of the interstitium.

In conclusion, this study shows that β1 integrin expression in lung epithelium is required during different stages of lung development for airway branching morphogenesis, alveolarization and maintenance of homeostasis. In addition to its well-known functional role in epithelial cell adhesion and migration, β1 integrin modulates alveolar cell differentiation and alveolar septal ECM deposition. Finally, we have also shown that β1 integrin regulates epithelial chemokine and ROS production, which appears to be a new mechanism whereby cell-ECM interactions regulate lung inflammation and alveolarization.

MATERIALS AND METHODS

Generation of β1<sup>SP-C.Cre</sup> mice

All experiments were approved by the Vanderbilt University Institutional Animal Use and Care Committee. Transgenic mice expressing Cre recombinase under control of the surfactant protein C promoter (SP-C-Cre) were generated by Brigid Hogan (Duke University, Durham, NC, USA) (Okubo et al., 2005) and integrin β1<sup>Dox/fox</sup> mice were a generous gift from Elaine Fuchs (Howard Hughes Medical Institute, The Rockefeller University, New York, NY) (Raghavan et al., 2000). β1<sup>SP-C.Cre</sup> mice (C57BL/6J background) were generated by crossing a male heterozygote (SP-C-Cre; β1<sup>F<sup>T</sup>T</sup>) with a female control (β1<sup>F<sup>T</sup></sup>). This mating strategy deletes β1 integrin specifically from the lung epithelium from E10.5 in SP-C-Cre; β1<sup>F<sup>T</sup></sup> mice (called β1<sup>SP-C.Cre</sup> mice). Littermate β1<sup>F<sup>T</sup></sup> mice were used as controls.

Generation of inducible β1 integrin deletion

Triple transgenic mice (SP-C-Cre; tetO-Cre; β1<sup>F<sup>T</sup></sup>) were obtained by breeding (Perl et al., 2002; Tanjore et al., 2013) and lung epithelial-specific deletion of β1 integrin was induced by the ingestion of doxycycline in the drinking water (2 g/dl). β1<sup>F<sup>T</sup></sup> littermates given identical dosage of doxycycline were used as controls.

Morphological analysis

Lungs were dried overnight at 75°C. Non-anesthetized oxygen saturations were obtained using MouseOx pulse oxymetry (Starr Life Sciences, Holliston, MA). Lungs were embedded, sectioned and stained as indicated. Ki67 staining was used for analysis of proliferation (Abcam ab16667, Cambridge, MA). Lung morphometry was performed on images viewed using a 40× objective for six sections per mouse, with a minimum of four mice in each group (Kauffman, 1977) using Image Pro Plus software. The airspace volume density was measured by dividing the sum of the airspace area by the total area. P14 alveolar secondary crests were quantified as previously described (Nicola et al., 2009). For electron microscopy, adult lungs were processed, post-fixed with potassium ferrous cyanide, dehydrated with graded acetone, sectioned at 1 μm and imaged using a Philips FEI T-12 transmission electron microscope.

Lung explants

Lung explants from E15 β1<sup>SP-C.Cre</sup> and β1<sup>F<sup>T</sup></sup> mice were cultured on permeable supports and on an air-liquid interface at 37°C in 95% air with 5% CO<sub>2</sub> overnight to allow adherence to the filter, as previously described (Blackwell et al., 2011; Prince et al., 2005; Prince et al., 2004). Peripheral branch counts (all branches touching the edge of the explant) were obtained from images taken at 12 and 48 h in culture using a Leica DFC450 microscope. Time-lapse images were obtained every 10 min for 8 h using a Nikon Ti Eclipse microscope. The epithelial edge was tracked on the first ten consecutive peripheral airways from the 12:00 position using ImageJ analysis software.

Depletion of macrophages with clodronate

Clodronate (dichloromethylene diphosphonic acid; Sigma-Aldrich) and sterile PBS-containing liposomes (vehicle) were prepared as previously described (Everhart et al., 2005; Zaynagutdinov et al., 2011). β1<sup>F<sup>T</sup></sup> and β1<sup>SP-C.Cre</sup> mice were treated with intranasal clodronate or PBS-containing liposome vehicle every 5 days from P5 until harvest at P14 or P28. Intranasal clodronate was given at doses of 12 μl, 15 μl, 18 μl, 21 μl and 24 μl at P5, P10, P15, P20 and P25, respectively. Duplicate experiments were performed using PBS-containing liposome vehicle control.

Immunostaining

For immunofluorescence, 8 μm frozen sections were fixed with 10% formalin for 20 min at room temperature, permeabilized with 0.1% Triton X-100, blocked in 5% donkey serum in PBS, and stained with the
following primary antibodies: anti-β1 integrin (1:250, Millipore MAB1997), anti-Ε-cadherin (1:500, Invitrogen 131900), anti-cleaved caspase 3 (1:500, Cell Signaling 9661), anti-Nxα2.1 (1:250, Santa Cruz Biotechnology sc-13040), anti-pro-SP-C (1:500, Abcam ab90716), anti-Tiα (1:250, Developmental Studies Hybridoma Bank 8.1.1), anti-CD68 (1:500, Abcam ab53444), anti-CD31 (1:500, BD Pharmingen 550274), anti-CD3 (1:500, Abcam ab5690) and FITC-conjugated anti-Gr1 (also known as Gsr; 1:500, eBioscience 11-5931) antibodies. Detection of bound antibodies was accomplished with Alexa Fluor 488-conjugated donkey anti-rat IgG or Alexa Fluor 555-conjugated donkey anti-rabbit IgG (both 1:500, Life Technologies A31572). Slides were analyzed using a Leica SPE confocal microscope.

Isolation of type II epithelial cells and collection of conditioned medium
Type II epithelial cells were isolated from P28 β1SP-C.Cre and β1f/f mice as previously described, yielding >90% type II cells (Rice et al., 2002; Young et al., 2012). Tissue was digested using dispase and the epithelial cell population was separated from leukocyte and monocyte populations using antibody-coated plates (CD45 and CD32, BD Pharmingen) for negative selection. After incubation for 2 h at 37°C in 5% CO2, type II cells were harvested, centrifuged and were resuspended in lysis buffer or cultured. Conditioned medium was collected at 24 h from 106 isolated epithelial cells per well cultured in serum-free medium on laminin-511.

Cell adhesion and migration assays
Cell adhesion and migration assays were performed with isolated epithelial cells from β1SP-C.Cre and β1f/f mice in serum-free bronchial epithelial growth medium (BEGM) (Chen et al., 2004a). Plates and filters were coated with purified laminin 511 (1 μg/ml, made by Eugenia Yazlovitskaya), a prominent lung basement membrane component, which we purified from HEK cells stably transfected with the o5β1γ1 using methods previously described (McKee et al., 2009).

For the adhesion assay, 105 cells were added per well, and cells that were adherent at 6 h were fixed, stained with Crystal Violet and solubilized. The optical density was read at 540 nm. Each mouse was analyzed in triplicate and five independent experiments were averaged. For the migration assay, 2×105 cells were added to the upper chamber of a filter. After 6 h, cells that migrated through the filter were stained and counted. Five random fields were analyzed and three independent experiments were performed.

Bronchoalveolar lavage
Sterile saline lavages were performed with 35 μl/g of PBS after killing, using a 20 g blunt-tipped needle inserted into the trachea. Samples were centrifuged at 400 g for 10 min, and the supernatant was collected. Cell counts were performed using a Bio-Rad TC10 Automated Cell Counter.

Immunoblotting
Total protein (40 μg) collected from isolated type II cell lysate was electrophoresed in a 10% gel and transferred onto nitrocellulose membranes. Membranes were blocked and incubated with different primary antibodies [anti-SP-C (1:5000, Abcam 90716), anti-β1 integrin from Millipore (1:10,000, 1952) and anti-β-actin (1:5000, Sigma A5316) antibodies], followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Immune-reactive bands were identified using enhanced chemiluminescence according to the manufacturer’s instructions.

Enzyme-linked immunosorbent assay
Enzyme-linked immunosorbent assay (ELISA) for MCP-1 and KC on BALF and medium was performed according to the manufacturer’s instructions (R&D Systems, MJE00 and MKC00B, respectively). Each sample was run in triplicate.

Thiobarbituric acid reactive substances assay
Lipid peroxidation in freshly isolated epithelial cells from β1SP-C.Cre and β1f/f mice was detected by measuring the reaction of thiobarbituric acid with malondialdehyde colorimetrically at 540 nm (Cayman Chemical Company).

In vivo ROS assay
ROS production was detected in β1SP-C.Cre and β1f/f mice in vivo as previously described (Han et al., 2013). L-012 (Wako Chemicals), a luminol analog, was injected retro-orbitally. Upon reaction with superoxide, luminescence was produced and detected by an IVIS system device (Xenogen). Luminescence data was collected using Living Image software v.4.1 (Xenogen).

Statistics
The Student’s t-test was used for comparisons between two groups with results representing mean±s.e.m. P<0.05 was considered statistically significant.

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Competing interests
The authors declare no competing financial interests.

Author contributions
E.J.P., L.R.Y., L.S.P., T.S.B. and R.Z. developed the concepts, performed experiments and data analysis, and prepared or edited the manuscript prior to submission. P.M.G., V.V.P., R.Z., J.T.B., A.M.I., R.v.d.M., L.A.G., N.B. and W.H. performed experiments for the manuscript.

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Supplementary material
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References


Supplemental Figure 1. Epithelial β1 integrin deletion results in increased macrophage accumulation after E15 and retains an intact capillary network at P14.  (A, B) E15 frozen sections immunostained for CD68 (green) show similar numbers of macrophages present in control (A) and β1<sup>SP-C.Cre</sup> (B) lungs.  (C, D) P14 frozen sections immunostained for the vascular marker CD31 (green) and Nkx2.1 (red) demonstrate an intact capillary network in close proximity to the alveolar surface in both control (C) and β1<sup>SP-C.Cre</sup> (D) lungs.  (E) The number of cleaved caspase-3 (CC3) + cells/mm<sup>2</sup> is not significantly different between E18 β1<sup>SP-C Cre</sup> and β1<sup>ff</sup> lungs.