**Introduction**

Integrins are heterodimeric transmembrane receptors consisting of α and β subunits that bind extracellular matrix (ECM) components; propagate bidirectional signaling (1–5); and regulate critical processes such as adhesion, migration and proliferation that are required for the development of multicellular organisms (6–10). Of the 24 known α-β integrin heterodimers, 12 integrins contain the β₁ subunit. β₁ Integrins are present in epithelial cells, where they mediate cell adhesion to basement membranes and facilitate epithelial tissue organogenesis (9, 11–24). Compared with organ development, the function of integrins in maintaining tissue homeostasis is poorly defined.

Alveoli are complex structures composed of epithelial cells attached to a basement membrane juxtaposed to capillaries and stromal fibroblasts. Epithelial cells are either cuboidal type 2 alveolar epithelial cells (AECs) expressing high levels of surfactant protein C (SP-C) or very thin type 1 AECs in close apposition to capillaries. We previously reported that β₁ integrin regulates branching morphogenesis and alveolarization during lung development (22). Moreover, we showed that genetically deleting β₁ integrin in the developing alveolus results in dilated airspaces, thickened alveolar septa, type 2 AEC hyperplasia, and increased numbers of alveolar macrophages. Macrophage depletion rescued the alveolarization defect in these mice (22). These findings suggest that epithelial β₁ integrin dysfunction has deleterious consequences in lung epithelium through regulation of innate immunity. The mechanisms whereby these epithelial-macrophage interactions occur are uncertain, and, perhaps more importantly, the function of β₁ integrin in the adult lung is not established.

In this study, we deleted β₁ integrin in type 2 AECs after completion of lung development, which occurs by P28. At 2 years of age, the mice developed emphysematous changes in the lung parenchyma, as
well as lymphoid aggregates and increased macrophage accumulation, which are characteristic of patients with advanced chronic obstructive pulmonary disease (COPD). This condition was preceded by proliferation of inflamed AECs that exhibited abnormal cell-cell junctions and excessive inflammation. Reduction of monocytes and monocyte-derived macrophages caused rapid onset of emphysema in young mice, suggesting that these cells limit inflammation and injury by clearance of deranged type 2 AECs. Thus, we conclude that under physiological conditions, β i integrin plays a critical homeostatic role in lung epithelial cells by suppressing inflammatory signaling.

**Results**

*Conditional β i integrin deletion in type 2 AECs results in emphysema and increased inflammation in aged mice.* To test the importance of β i integrin deletion after development, we crossed integrin β i;Cre (β iCre) mice with a doxycycline-inducible (dox-inducible) Cre recombinase under control of the surfactant protein-C (SP-C) promoter (designated as β i-tTA mice). β i Integrin deletion was induced in type 2 AECs by addition of dox to drinking water from day P28, at the completion of lung development, until 2 months of age. Mice were sacrificed at 3 and 24 months of age. Lungs of 24-month-old β i-tTA mice exhibited emphysema and increased numbers of macrophages (Figure 1, A and B). Lung morphometry quantification by mean linear intercept demonstrated a 60% airspace enlargement in β i-tTA lungs compared with both β iCre and β i+tTA mice that did not receive dox (Figure 1, C and D). Multiple lobes were sampled to minimize bias introduced by regional differences in alveolar size (25–27). There was evidence of bronchus-associated lymphoid tissue (BALT), which is characteristic of advanced COPD (arrows in Figure 1A, quantified in Figure 1E; and ref. 28). In addition to BALT lesions, histological examination revealed increased macrophages in β i-tTA lungs, identified by the pan-macrophage marker CD68 (Figure 1F), and an increase in bronchoalveolar lavage fluid (BALF) cell count (1.8 × 10^5 ± 0.2 × 10^5 cells/mL from β i-tTA lungs compared with 0.8 × 10^5 ± 0.1 × 10^5 cells/mL from β iCre lungs; Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.129259DS1).

Epithelial dysfunction precedes major morphological changes in β i-tTA mice. To determine the timing of the structural deficits in β i-tTA lungs relative to gene deletion, we performed histological examination of 3-month-old mice. We verified the efficiency of β i integrin deletion in the lungs of β i-tTA mice by immunohistochemistry and found it was removed in more than 90% of type 2 AECs (Figure 2, A and B). This finding was confirmed by immunoblotting of primary type 2 AEC lysates from β i-tTA and β iCre mice (Figure 2C). Microscopic examination showed no difference in airspace size in 3-month-old β i-tTA mice (Figure 3, A and B). By crossing β i-tTA mice to mice expressing the mTmG reporter (allowing visualization of GFP+ progeny derived from cells that had undergone Cre activation), we observed that β i-tTA; mTmG mice exhibited GFP+ type 1 AECs immediately adjacent to β i-deficient type 2 AECs, suggesting β i integrin is not required for type 2–to–type 1 AEC differentiation during homeostasis in the adult lung (Supplemental Figure 1B). β i-tTA did exhibit mild intrasepal edema (arrows in Figure 3C), increased BALF protein (Supplemental Figure 2A), and increased BALF macrophages (Supplemental Figure 2B). Transmission electron microscopy (TEM) revealed intact cell-matrix interactions (arrows in Figure 3D) and defects in tight junctions between type 1 and type 2 AECs. Rather than the normal dark stranded seal demarcating tight junctions at the apical cell-cell junction, β i-tTA lungs had a deep cleft (Figure 3, D and E, with tight junctions marked by asterisks in E). Consistent with these tight junction abnormalities, β i-tTA mice had decreased claudin-3 protein levels in primary type 2 AEC lysates (Figure 3F) and decreased mRNA expression of Claudin-4 but not Claudin-18 as measured by quantitative RT-PCR (qPCR) of type 2 AECs (Figure 3G).

We next assessed whether there were abnormalities of type 2 AEC-ECM interactions by visualizing their adherence to the laminin-containing basement membrane. While the basal surface of type 2 AECs appeared to adhere normally to the basement membrane (Figure 4A), we noticed that there were more type 2 AECs in β i-tTA than β iCre mice (Figure 4, B and C). The excess of type 2 AECs, evidenced by pro–SP-C–positive staining, was due to increased cellular proliferation that was identified by Ki-67 immunostaining (Figure 4, D and E). In contrast, no differences in the number of apoptotic type 2 AECs between β i-tTA and β iCre lungs were observed, as demonstrated by dual TUNEL+pro–SP-C+ cells (Figure 4, F and G). Thus, deletion of β i integrin in AECs from 3-month-old adult mice caused subtle structural defects with abnormal tight junctions that likely allowed for paracellular fluid flux leak and type 2 AEC proliferation. Proliferation of type 2 AECs is a well-known feature of inflammatory lung diseases and a recognized consequence of lung injury (29–35); therefore, this finding suggests an ongoing injury-repair cycle.
β1 Integrin–deficient type 2 AECs induce increased efferocytosis. We next performed in-depth analysis of the inflammatory status of β1 integrin–deficient mice. When we examined aged β1rtTA mice, we noted that pro–SP-C staining often colocalized with CD68, suggesting that macrophages phagocytosed AECs in β1rtTA but not β1f/f lungs (Figure 5A). This observation is consistent with efferocytosis, a tightly regulated process by which phagocytic cells ingest diseased or dying cells, thereby minimizing inflammation in the microenvironment (36–39). To define the mechanisms whereby this occurred, we cultured primary type 2 AECs and measured secretion of CX3CL1, a “find me” chemokine that attracts phagocytes (40, 41). We also assessed the expression levels of Cd47, whose gene product is an inhibitory “don’t eat me” signal, in freshly isolated primary type 2 AECs (41, 42). We found increased CX3CL1 production and reduced Cd47 mRNA expression by β1rtTA type 2 AECs relative to type 2 AECs isolated from β1f/f mice (Figure 5, B and C). These findings support the conclusion that macrophage efferocytosis of β1 Integrin–deficient type 2 AECs is prominent in β1rtTA lungs.

Deleting CCL2-recruited monocytes/macrophages causes severe destruction of alveolar architecture in β1rtTA mice by decreasing AEC efferocytosis. Our histological examination of both aged and 3-month-old β1rtTA lungs suggested increased inflammation. Since β1rtTA lungs had increased macrophages, and β1-null type 2 AECs exhibited markers of efferocytosis, we tested whether impairment of macrophage recruitment would disrupt homeostasis in 3-month-old β1rtTA mice. To target these recruited immune cell populations, we crossed β1rtTA mice with Ccr2–null background. CCR2 is the receptor for CCL2, one of the primary monocyte chemokines in the lung. CCR2–/–;β1rtTA mice and their CCR2–/–;β1f/f littermate controls received dox from P28 until 2 months of age in the same manner as β1f/f and β1rtTA mice. In contrast to β1rtTA mice, 3-month-old CCR2–/–;β1rtTA mice exhibited dramatically enhanced lung pathology (Figure 6, A and B), with widespread emphysematous destruction; marked airspace enlargement, quantified by mean linear intercept (Figure 6C); increased inflammatory infiltrates (arrows in Figure 6B); and increased BALF cell counts (Figure 6D). CCR2–/–;β1rtTA mice exhibited a large number of CD68+ macrophages (Figure 6, E and F) despite loss of CCL2 recruitment due to excessive proliferation of existing resident macrophages as verified by increased Ki-67 staining (Supplemental Figure 3, A–C).

Figure 1. Deletion of β1 integrin in type 2 AECs results in emphysema and increased inflammation in aged mice. (A and B) H&E-stained paraffin lung sections show marked airspace enlargement and increased number of BALT lesions in 24-month-old β1rtTA mice compared with age-matched β1f/f mice and 24-month-old dox-naive β1rtTA mice. Arrows indicate BALT lesions. (C) High-power images: Airspace enlargement is visible in β1rtTA lungs; quantification in D shows increased mean linear intercept in β1rtTA lungs (10 sections/mouse; n = 11 β1f/f, n = 9 β1rtTA + dox mice, n = 5 β1rtTA – no dox mice). (E) Increased BALT lesions per lung section in β1rtTA mice. n = 19 β1f/f, n = 15 β1rtTA + dox mice; n = 5 β1rtTA – no dox mice; P = 0.0649 by 1-way ANOVA. (F) β1rtTA lungs contain increased numbers of CD68+ macrophages. Scale bars: 200 μm in A and B, 50 μm in C and F. *P < 0.05 by 1-way ANOVA with Tukey’s test for multiple comparison.
A large increase in pro–SP-C + type 2 AECs accompanied the expanded immune cell population (Figure 6E, quantified in Figure 6G). Immunostaining for Ki-67 demonstrated that depletion of CCL2-driven monocytes/macrophages did not change the proliferation rate of AECs compared with β1rtTA mice (Supplemental Figure 3, D and E). These findings indicate that the increase in epithelial cell numbers in β1rtTA mice was due to impaired AEC removal rather than increased AEC proliferation. Despite numerous macrophages and an overabundance of type 2 AECs, there was almost no colocalization of CD68 and pro–SP-C in CCR2–/–;β1rtTA mice (Figure 6H, quantified in Figure 6I), suggesting minimal efferocytosis in these mice. To directly test whether macrophages from CCR2–/–;β1rtTA mice were defective in efferocytosis, we collected macrophages from bronchoalveolar lavage and exposed these cells to fluorescently labeled primary type 2 AECs from β1rtTA mice (Figure 6J). While macrophages from β1rtTA and β1f/f lungs briskly engulfed β1-deficient AECs, macrophages from CCR2-deficient mice (both CCR2–/–;β1rtTA and CCR2–/–;β1f/f) ingested far fewer labeled AECs, demonstrating that CCR2-deficient macrophages were less efficient efferocytosis agents. These data strongly suggest that the more severe phenotype in the CCR2–/–;β1f/f mice is caused by their inability to remove deranged type 2 AECS and that the efferocytosis function of CCL2-recruited macrophages is to limit inflammation and mitigate lung damage in β1rtTA mice.

Figure 2. β1 Integrin is deleted in type 2 AECs in β1rtTA lungs. (A) Immunostaining for pro–SP-C (green) and β1 integrin (red) demonstrates type 2 AEC-specific deletion of β1 integrin in 3-month-old β1rtTA lungs. Arrows indicate the presence/absence of β1 integrin expression. Scale bar: 5 μm. (B) Type 2 AEC-specific deletion is represented as percentage of pro–SP-C+ cells that express β1 integrin. 100–120 type 2 AECs counted/mouse; n = 3 β1f/f, n = 4 β1rtTA mice. (C) Representative Western blot for β1 integrin on primary type 2 AEC lysate, normalized to GAPDH; representative of 3 separate experiments. *P < 0.05 by 2-tailed Student’s t test.

β1 Integrin regulates AEC inflammation. Our data thus far suggest that the β1 integrin–null cells provide an inflammatory stimulus resulting in monocyte-macrophage chemoattraction into the alveolus. These recruited cells function as effector cells but do not directly contribute to the inflammatory status of the lungs.

CD11b+CD11c+ monocytes/macrophages efferocytose type 2 AECs in β1rtTA mice. We next examined the immune cell population in the whole lung by flow cytometry. β1rtTA lungs contained increased CD45+CD11b+CD11c+ immune cells, markers consistent with recently recruited monocyte–early macrophages (Figure 7A, gating strategy in Supplemental Figure 4; and refs. 43, 44). We identified this as a mixed population, as cells expressed the monocyte marker Ly6C, the macrophage marker CD64, or both (Figure 7B). Since the CD11b+CD11c+ immune cells were differentially enriched in β1rtTA mice, we collected this population by FACS, cytopspun the cells, and immunostained for pro–SP-C and CD68 to determine whether these cells contributed to the increased efferocytosis seen in β1rtTA mice. We found that 68% ± 4% of monocytes/macrophages collected from β1rtTA lungs contained pro–SP-C+ material compared with 14% ± 2% of these cells from β1f/f lungs (Figure 7, C and D). To functionally phenotype these cells in β1rtTA mice, we collected media from cultured monocytes/macrophages and assayed for cytokine production by cytokine multiplex. The β1rtTA monocyte-macrophage population secreted only scant amounts of inflammatory cytokines/chemokines, equivalent to expression levels by cells from β1f/f mice (Supplemental Table 1). Taken together, these data demonstrate that CD11b+CD11c+ monocytes/macrophages are critical effector cells for efferocytosis but do not directly contribute to the inflammatory state of β1rtTA mice.
Next, we tested whether β1-deficient AECs drive the inflammatory phenotype in lungs of β1rtTA mice. Ten of 32 cytokines (31%), including mediators of macrophage chemotaxis and maturation, were significantly increased in the culture media of β1rtTA AECs compared with that of β1f/f AECs (Figure 8A and Supplemental Table 2). To define the consequence of increased AEC inflammatory signaling in the whole lung, we performed multiplex analysis on tissue lysates (Figure 8B and Supplemental Table 3). Multiple inflammatory mediators were increased in lungs of β1rtTA mice compared with β1f/f controls. Even further increases were seen in CCR2−/−;β1rtTA, where inflamed β1-deficient type 2 AECs remained unchecked by efferocytosis. Since many of the cytokines increased in β1rtTA and CCR2−/−;β1rtTA lungs were recognizable gene products of NF-κB signaling (including KC, IL-6, MIP-2, and G-CSF), we

Figure 3. In the absence of aging, deletion of β1 integrin in type 2 AECs minimally alters gross alveolar structure but results in epithelial dysfunction. (A and B) H&E-stained paraffin lung sections from 3-month-old β1f/f and β1rtTA mice demonstrate equal airspace size. (C) H&E-stained paraffin lung sections show increased intraseptal edema (arrows) in β1rtTA lungs. (D and insets in E) Transmission electron microscopic images of β1f/f and β1rtTA lungs show intact cell-matrix interactions (arrows in D), but clefts at the cell-cell junctions in β1rtTA lungs (junctions marked by asterisks in E). (F) Representative Western blot for claudin-3 on primary type 2 AEC lysate, with densitometry. n = 6 mice/group, normalized to GAPDH. (G) Gene expression for Claudin-4 and Claudin-18 by qPCR. n = 6 mice/group, normalized to GAPDH. RQ, relative quantitation. Scale bars: 200 μm in A, 25 μm in B, 50 μm in C, 500 nm in D, 250 nm in E. *P < 0.05 by 2-tailed Student’s t test. Images in A–C are representative of 6 mice/group.
performed immunohistochemistry for activated NF-κB in β1 integrin–deficient AECs (Figure 8, C and D). Immunofluorescence staining for phospho-p65 (S276), a well-recognized marker of NF-κB activation (45), revealed numerous phospho-p65+pro–SP-C+ type 2 AECs in lungs from β1rtTA mice and CCR2–/–;β1rtTA mice. Other cell types, in addition to type 2 AECs, exhibited NF-κB activation in β1rtTA and CCR2–/–;β1rtTA lungs. These findings indicate that β1 integrin deficiency results in a pervasive inflammatory environment in the distal lung with contributions from retained β1-deficient type 2 AECs.

β1-Deficient AEC inflammatory mediators are produced as a consequence of ROS generation. Since the generation of ROS has been linked to NF-κB–dependent cytokine expression in epithelial cells and β1-containing integrins have been shown to modulate ROS signaling (46–50), we measured ROS production in cultured type 2 AECs. We found that β1rtTA type 2 AECs produced more superoxide (O2–) and hydrogen peroxide (H2O2) than β1f/f cells (Figure 9, A and B); however, no differences in mitochondria-derived ROS were detected (Figure 9C). Given the increase in O2– generation, we investigated whether the NADPH oxidase (NOX) system was upregulated in cells from β1rtTA mice and CCR2–/–;β1rtTA mice. Other cell types, in addition to type 2 AECs, exhibited NF-κB activation in β1rtTA and CCR2–/–;β1rtTA lungs. These findings indicate that β1 integrin deficiency results in a pervasive inflammatory environment in the distal lung with contributions from retained β1-deficient type 2 AECs.

Figure 4. Loss of type 2 AEC β1 integrin results in epithelial proliferation. (A) ABCA3+ type 2 AECs (green) remain adherent to the basement membrane (laminin in red) in β1rtTA lungs. (B) Lung sections immunostained for type 2 AEC marker pro–SP-C (red) and laminin γ1 (green) show increased numbers of type 2 AECs in β1rtTA lungs, as quantified in C. 20 sections/mouse; n = 6 mice/group. (D) Lung sections immunostained for pro–SP-C (red) and the proliferation marker Ki-67 (green) show increased type 2 AEC proliferation in β1rtTA lungs, as quantified in E. 20 sections/mouse; n = 6 mice/group. (F) No difference in the number of apoptotic type 2 AECs in β1rtTA and β1f/f lungs, as identified by TUNEL and pro–SP-C costained type 2 AECs, as quantified in G. n = 6 β1f/f, n = 5 β1rtTA mice. Scale bars: 5 μm in A, 50 μm in B, D (10 μm for insets), and F. *P < 0.05 by 2-tailed Student’s t test.
treatment decreased CCL2 secretion by β1rtTA type 2 AECs (Figure 9, E and F). Although a specific Duox1 inhibitor is not available, we narrowed down the NOX subunits potentially regulated by β1 integrin using the NOX1/4 inhibitor GKT137831 (Figure 9G). In contrast to the pan-NOX inhibitor DPI, treatment with GKT137831 did not reduce CCL2 secretion from β1 rtTA type 2 AECs, implicating NOX2, Duox1, and/or Duox2 as the source of increased ROS in β1rtTA mice. As Duox1 was the only NOX isoform with increased expression, these data suggest that β1 integrin regulates ROS production through this isoform in AECs.

To test whether ROS-dependent CCL2 production by β1 rtTA type 2 AECs was in part responsible for increased macrophage infiltration in β1rtTA mice, we performed chemotaxis assays using WT macrophages collected by bronchoalveolar lavage and conditioned media from cultured type 2 AECs from β1f/f and β1rtTA mice. Macrophage migration toward media from β1rtTA type 2 AECs was greatly enhanced compared with media from control cells, and this increase was completely abrogated by treatment with DPI or neutralizing antibodies to CCL2 (Figure 9H). These findings support the conclusion that β1rtTA type 2 AECs have persistent ROS production that contributes to CCL2 secretion that induces macrophage migration into the airspaces of β1rtTA mice.

Discussion

While numerous studies have defined the critical role of integrins in organ morphogenesis, few have examined their role in tissue homeostasis in adults. In the setting of development, phenotype severity is highly correlated with timing of integrin deletion after conception and is primarily ascribed to defects in cell adhesion and migration. In this study, we defined the role of β1 integrin in the structurally stable, fully formed alveolus of the lung, where epithelial cells undergo slow turnover and are tightly bound to the basement membrane. We show that deleting β1 integrin in AECs under these circumstances results in emphysema, a condition characterized by destruction/loss of gas exchange units and chronic inflammation. Surprisingly, there were no adhesion defects in the AECs in our model; however, these cells were highly inflamed, with excessive ROS production that caused increased NF-κB–dependent cytokine production. Thus, β1 integrin in alveolar epithelial cells has an antiinflammatory role and is required for alveolar homeostasis in the lung.

Our studies provide direct evidence that mice with a targeted deletion of β1 integrin in type 2 AECs develop aging-related, spontaneous emphysema as quantified by mean linear intercept. This method easily captures one component of the emphysematous phenotype, enlargement of airspaces. Although we did not perform stereological analysis to address alveolar number specifically, we took precautions
in our studies to minimize bias in our 2D morphological measurements from sampling (25–27). Loss of β1 integrin in AECs stimulates ROS production and NF-κB signaling, and subsequently released inflammatory mediators recruit and activate a mixed population of monocytes/macrophages that efferocytose the β1-deficient AECs. One possible mechanism for the development of emphysema is that macrophages mediate lung destruction via altered protease/antiprotease balance (54–56). These observations are consistent with studies demonstrating a role for excessive ROS and NF-κB activation as initiators of macrophage accumulation and subsequent alveolar injury, resulting in emphysema (57–59). In addition, epithelial apoptosis in combination with ineffective efferocytosis could contribute to the development of emphysema. Both epithelial and endothelial apoptosis can contribute to emphysema independent of inflammation (60–62). Consistent with these potential explanations for development of emphysema in our model, blocking efferocytosis has been shown to potentiate alveolar destruction in murine models of elastase-induced emphysema associated with increased MMP2 and -12 expression (63). It is unclear whether loss of efferocytosis with its antiinflammatory effects or the retention of inflamed β1-deficient AECs causes emphysema. However, our data indicating that there is no phenotypical difference in the efferocytosing monocytes/macrophages suggest that retained β1-deficient AECs are the primary driver of emphysema in β1rtTA mice. Our data also confirmed and extended studies that indirectly implicated β1-containing integrins in the pathogenesis of emphysema. Mice with impaired fucosylation exhibit an emphysematous lung phenotype, and fucosylation is required for normal α3β1 integrin–dependent migration and signaling, suggesting that the phenotype is due to impaired α3β1 integrin function (64, 65).

Similarly, fibulin 5−/− mice have enlarged airspaces at birth that progressively dilate into adulthood (66). Fibulin 5 is a ligand for αvβ3, αvβ5, and α9β1 integrins, participates in outside-in integrin signaling, and is crucial for proper assembly of elastic fibers (66, 67).

Figure 6. Recruited monocytes/macrophages maintain structural homeostasis in β1rtTA mice through efferocytosis. (A and B) CCR2−/−;β1rtTA lungs show severe remodeling and increased inflammatory infiltrate (arrows) in low-power (A) and high-power (B) images of H&E-stained sections. (C) Increased mean linear intercept in 3-month-old CCR2−/−;β1rtTA lungs. 6–10 sections/mouse; n = 6 β1f/f, n = 6 CCR2−/−;β1f/f, n = 6 β1rtTA, n = 7 CCR2−/−;β1rtTA mice/group. (D) Increased BALF cell counts in CCR2−/−;β1rtTA mice. n = 17 β1f/f, n = 6 CCR2−/−;β1f/f, n = 21 β1rtTA, n = 6 CCR2−/−;β1rtTA mice/group. (E) Immunostaining for CD68 (green) and pro–SP-C (red) demonstrates increased macrophages and type 2 AECs in CCR2−/−;β1rtTA lungs. (F and G) Immunostaining demonstrates minimal colocalization of CD68− and pro–SP-C− cells in CCR2−/−;β1f/f and CCR2−/−;β1rtTA lungs, whereas abundant colocalization was present in β1rtTA lungs. 20 sections/mouse; n = 4–6 mice/group. (H and I) Immunostaining demonstrates minimal colocalization of CD68− and pro–SP-C− cells in CCR2−/−;β1f/f and CCR2−/−;β1rtTA lungs, whereas abundant colocalization was present in β1rtTA lungs. 20 sections/mouse; n = 4–6 mice/group. (J) Quantification of alveolar macrophage (Alv mac) efferocytosis of fluorescently labeled primary type 2 AECs. n = 8–12 mice/group. Scale bar: 200 μm in A, 50 μm in B and E, 5 μm in H. *P < 0.05 by ordinary 1-way ANOVA with secondary analysis by Tukey’s test for multiple comparisons as indicated.
We show that the inflammatory phenotype of β1 integrin–deficient AECs, as manifested by increased NF-κB signaling and cytokine production, is mediated at least in part by excessive ROS production. While this phenomenon is well documented in multiple other cell types (68–70), the mechanisms whereby integrins regulate ROS production are poorly understood. Our studies implicate β1 integrin as a critical negative regulator of the NOX isoform Duox1 in AECs. Previous studies reported that β1 integrin negatively regulates ROS production through NOX2 in chondrocytes and kidney mesangial cells (46, 47, 71, 72). Thus, integrins play a critical role in regulating ROS production in multiple cell types; however, the mechanisms appear to be cell type specific.

One of the most interesting observations in our study was that genetic depletion of CCR2, which blocks CCL2-mediated recruitment of monocyte-derived macrophages, exacerbates alveolar remodeling in adult β1−/− mice. This contrasts with our previous observation that chemical depletion of macrophages using intranasal instillation of clodronate during lung development rescues alveolarization defects (22). These findings expose differential functions of macrophage subtypes and their potentially paradoxical roles in the adult versus developing lung. In development, fetal lung macrophages are essential for normal lung morphogenesis. They actively clear mesenchymal cells through phagocytosis during sacculation, and their response to inflammatory stimuli regulates airway branching through modulation of developmental signals (36–39, 73–75). During homeostasis, macrophages are required for regulation of inflammatory signaling, host defense, and wound healing (76, 77). The majority of effecrocytosis activity following injury is accomplished by macrophages, but more recent data suggest that monocytes significantly contribute to effecrocytosis and antigen presentation in the presence of apoptotic cells (78). Although monocytes and macrophages effecrocytose dying cells during acute injury, their role in chronic inflammation is less well defined (78, 79). In our model, deranged type 2 AECs are effecrocytosed by the CD11b+CD11c– monocyte/macrophage population. This is likely a mix of newly recruited monocytes and monocytes transitioning into macrophages. In β1−/− mice, homeostatic compensation fails with loss of CCL2-driven monocyte-macrophage recruitment, resulting in an escalation of inflammation associated with diminished effecrocytosis. Although determining why CCL2-recruited monocytes/macrophages are necessary for efficient effecrocytosis of type 2 AECs will require further study, this finding could have direct implications for human lung diseases, including COPD, in which ineffective effecrocytosis has been suggested to be a contributor to pathogenesis (80–86).

Aside from regulating inflammation, β1 integrin is required for other critical cellular processes in type 2 AECs, including maintenance of tight junctions and control of proliferation. Microscopic examination of

Figure 7. CD11b+CD11c– monocytes/macrophages effecrocytose type 2 AECs in β1RTA mice. (A) β1RTA lungs contain increased CD45+CD11b+CD11c– monocytes/macrophages by flow cytometry. n = 7 β1RTA, n = 4 CCR2−/−β1RTA, n = 6 β1TNA, and n = 7 CCR2−/−β1TNA mice. (B) CD45+CD11b+CD11c– population consists of cells that are both Ly6C− and CD64+ in β1RTA lungs. n = 6 β1RTA mice. (C) Immunostained cytospins from CD45+CD11b+CD11c– monocytes/macrophages show increased numbers of CD68+pro–SP-C+ cells in β1RTA lungs, as quantified in D. n = 6 mice/group. Scale bar: 40 μm in C, 10 μm for inset. *P < 0.05 by 2-tailed Student’s t test.
**β1rtTA** mice revealed abnormal tight junctions associated with decreased Claudin-3 and Claudin-4 expression. Consistent with this observation, proximal kidney tubule cells with β1 integrin deletion exhibit altered Claudin-2 expression (87). Our epithelial β1-null mice also share many phenotypic similarities with claudin-deficient mice. Unchallenged claudin-4-deficient mice have normal lung histology but exhibit significantly...
increased BALF cell counts with hyperoxia exposure and increased CCL2 signaling with mechanical ventilation (88). In addition, claudin-18–null mice develop AEC hyperplasia and macrophage accumulation over time (89–91). Although a causal relationship between β1 integrin and claudins has not been described, previous in vitro studies demonstrated that ROS can disrupt tight junctions (92–94), suggesting that β1 integrin–mediated ROS could regulate tight junctions via effects on claudin expression. Another phenotype identified in β1rtTA mice is AEC proliferation. Developmental deletion of β1 integrin decreases epithelial

**Figure 9.** β1-Deficient AECs generate excess inflammatory mediators via ROS production. (A and B) Primary β1rtTA type 2 AECs produce increased superoxide and H2O2 by LumiMax assay (A; n = 7 mice/group) and Amplex Red assay (B; n = 5 β1f/f, n = 7 β1rtTA mice), respectively. (C) No difference in mitochondrial ROS production in β1rtTA and β1f/f type 2 AECs by MitoSOX assay. n = 8–9 mice/group. ex, excitation; em, emission. (D) Increased gene expression of Duox1 in primary type 2 AECs isolated from β1rtTA mice. n = 6 mice/group. (E) TEMPO treatment decreases CCL2 secretion by primary type 2 AECs isolated from β1rtTA mice. 2 mM; n = 4 mice/group. (F) DPI treatment decreases CCL2 secretion by primary type 2 AECs isolated from β1rtTA mice. 10 μM; n = 7 β1rtTA mice. (G) CCL2 secretion is not different between β1f/f and β1rtTA type 2 AECs after treatment with the NOX1/4 inhibitor GKT137831. 10 μM; n = 5 β1f/f, n = 5 β1rtTA mice. (H) Macrophage migration in response to β1rtTA type 2 AEC media is decreased by treatment with CCL2-neutralizing antibody (NAb) and DPI. n = 4–8 mice/group. *P < 0.05 by 2-tailed Student’s t test. CCR2, recombinant CCR2.
proliferation in the kidney, mammary, and submandibular glands (11, 15, 19, 21, 22), whereas increased epithelial proliferation has been reported when β1 integrin was deleted in the intestine or skin (14, 17). The mechanisms whereby β1 integrin regulates cell number/density in fully formed organs is unknown; however, this could also be ROS mediated, since multiple investigations have shown that ROS can stimulate cell proliferation.

In conclusion, this study shows that loss of β1 integrin in type 2 AECs promotes persistent lung inflammation and emphysematous remodeling, which is mitigated by efferocytosis of inflamed AECs by CD11b+CD11c+ monocytes/macrophages. Thus, regulation of inflammation is the major function of β1 integrin in alveolar homeostasis in the lung.

Methods

Mice. For timed deletion of β1 integrin, we crossed transgenic mice with inducible Cre recombinase expression by the dox-inducible reverse tetracycline transactivator under control of the SP-C promoter (SP-C rtTA;Tet-O-Cre) with integrin β1f/f mice (95, 96). Postdevelopmental type 2 AEC deletion was induced on P28 in these triple transgenic SP-C rtTA;Tet-O-Cre; β1f/f mice (called β1rtTA mice) using dox in drinking water (2 g/L × 4 weeks). Control littermate β1f/f mice received identical dox treatment. To test the role of β1 integrin in epithelial differentiation during alveolar homeostasis, we crossed β1rtTA mice to the mTmG Cre recombinase reporter. To test the role of CCL2-recruited monocytes/macrophages in β1 integrin-regulated alveolar homeostasis, we crossed β1rtTA and β1f/f mice onto a homozygous null background for Ccr2, the CCL2 receptor. The resulting transgenic CCR2−/−;SP-C rtTA;Tet-O-Cre; β1f/f mice (termed CCR2−/−;β1rtTA mice) and control littermate CCR2−/−;β1f/f mice, received identical dox treatment on P28 to induce β1 integrin deletion. Integrin β1f/f mice were a gift from Elaine Fuchs (Howard Hughes Medical Institute, The Rockefeller University, New York, New York, USA). SP-C rtTA, Tet-O-Cre, Ccr2 homozygous null, and mTmG Cre recombinase reporter mice were purchased from the Jackson Laboratory. All mice were on a C57BL/6 background.

Histology and morphological analysis. Lungs from β1rtTA, β1f/f, CCR2−/−;β1rtTA, and CCR2−/−;β1f/f mice were harvested for histological examination. Mice were sacrificed, right ventricle flushed with PBS, and lung inflation fixed at 25 cm with 10% formalin for more than 24 hours prior to paraffin embedding and sectioning. Multiple lobes were sectioned to reduce bias in morphological analysis generated from regional differences in alveolar size (25–27). Mean linear intercept was calculated from images obtained using a Keyence BZ-X710 inverted fluorescence phase contrast microscope with ×40 objective for 6–10 nonoverlapping sections per mouse. For immunohistochemistry stains, paraffin sections were deparaffinized, antigen retrieved, blocked, and incubated with the indicated primary antibody, followed by colorimetric detection by Vector Red (Vector Laboratories). Stained paraffin sections were imaged using a Keyence BZ-X710 inverted fluorescence phase contrast microscope with ×20 objective (low-power images) or an Olympus BX41 with ×60 objective lenses (high-power images). Immunofluorescence staining was performed on frozen lung sections that were inflation fixed with 2:1 PBS/O.C.T. mixture (Tissue-Tek), embedded, and sectioned at 8-μm thickness. Slides were subsequently fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X, blocked with 5% donkey serum, incubated in primary antibody overnight at 4°C, incubated in secondary antibody for 2 hours at room temperature, incubated with DAPI nuclear stain (Vector Laboratories), and anti-rabbit Alexa Fluor 594 (Life Technologies A21207), anti-rat Alexa Fluor 488 (Life Technologies A21206), anti-rabbit Alexa Fluor 594 (Life Technologies A21207), anti-mouse Alexa Fluor 488 (Life Technologies A11001), and anti-rat Alexa Fluor 488 (Life Technologies A21208). TUNEL staining was performed per the manufacturer’s instructions (Roche 11684795910). Quantification of immunostained sections was performed on 20 nonoverlapping images obtained with a ×20 objective.

TEM. Lungs were harvested from 3-month-old β1rtTA and β1f/f mice, processed, postfixed with potassium ferrous cyanide, dehydrated with graded acetone, thick sectioned at 1 μm, then sectioned at 80 nm in the region of interest, and imaged using a Philips FEI T-12 transmission electron microscope in the Vanderbilt Cell Imaging Shared Resource core.
Bronchoalveolar lavage. Sterile saline lavages were performed with 1 mL PBS after sacrifice. Lavage fluid was centrifuged at 270 g at 4°C, and cells were resuspended and counted. The Pierce BCA Protein Assay kit (Thermo Fisher Scientific, 23225) was used to test for BALF protein per the manufacturer’s instructions. For immunofluorescence analysis of immune cells collected by bronchoalveolar lavage, 40,000 cells were spun onto Shandon cytoslides (Thermo Fisher Scientific) at 240 g for 7 minutes, dried, and immunostained per the above protocol.

AEC isolation and collection of conditioned medium. Type 2 AECs were isolated from 3-month-old β1rtTA and β1f/f mice as previously described, yielding more than 90% type 2 AECs (22, 97, 98). Briefly, a single-cell suspension was generated with a 40-minute dispase digestion and 100-μm, 40-μm, and-20 μm serial filtration. The suspension was then incubated at 37°C for 2 hours in anti-CD45 (BD 553076) and anti-CD32 (BD 553142) antibody–coated plates for negative selection. The medium containing epithelial cells was collected and spun down, and AECs were plated in 5% bronchial epithelial cell growth medium (BEGM) on Matrigel-coated wells with or without the indicated treatment. Treatment reagents included TEMPO (Sigma-Aldrich 176141) and DPI (Sigma-Aldrich D2926). Medium was collected at 24 hours for analysis.

Western blotting. Protein (60 μg) collected from type 2 AEC isolations was electrophoresed in a 10% gel and transferred onto nitrocellulose membranes. Membranes were blocked, incubated with primary antibody (anti–β1 integrin [Millipore MAB1997], anti–claudin-3 [Invitrogen 341700], anti-GAPDH [Invitrogen MA5-15738]), and incubated with Odyssey IRDye 800CW and 680RD secondary antibodies. Signal was detected using a LI-COR Odyssey CLx Near-Infrared Western Blot Detection system.

qPCR. RNA was isolated from freshly isolated primary type 2 AECs using the RNEasy Plus Mini Kit (QIAGEN) and cDNA synthesized using the SuperScript VILO Master Mix kit (Thermo Fisher Scientific). qPCR reactions were performed in triplicate using TaqMan PCR Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher Scientific) on a StepOne Plus PCR System (Applied Biosystems) using the following TaqMan probes (Applied Biosystems, Thermo Fisher Scientific): Claudin-4 Mm00515514_s1, Claudin-18 Mm00517321_m1, CD47 Mm00495011_m1, Duox1 Mm01326865_m1, Duox2 Mm01326247_m1, Nox1 Mm00549170_m1, Nox2 Mm01287743_m1, Nox4 Mm00479246_m1, and GAPDH Mm99999915_g1. Data were normalized to the housekeeping gene GAPDH. Relative quantities were analyzed using β1f/f values as control.

ELISA and multiplex assay. ELISA for CCL2 and CX3CL1 on AEC conditioned media was performed in triplicate according to the manufacturer’s instructions (R&D Systems, MJE00 and MCX310, respectively). Cytokine/chemokine Magnetic Bead 32-Multiplex Panel (Millipore MCYT MAG-70K-PSX2) assay was performed on monocyte/macrophage conditioned media, AEC conditioned media, and whole lung tissue lysates in triplicate per the manufacturer’s instructions. Tissue lysates were generated by sonication the right upper lobe, centrifuging the tissue mixture, collecting the supernatant, and normalizing to protein. The Multiplex assay was read on the Luminex MAGPIX platform in the Vanderbilt Hormone and Analytical Services Core.

Flow cytometry. We used collagenase XI (Sigma-Aldrich C7657, 0.7 mg/mL) and type IV DSNase (Sigma-Aldrich D5025, 30 μg/mL) digestion and 40-μm filtration to obtain a single-cell whole lung suspension for flow cytometry analysis. Briefly, cells were blocked with anti-CD32 antibody (BD 553142), incubated with conjugated primary antibody, and analyzed using a 5-laser BD LSR II analytical flow cytometer (BD Biosciences) and FlowJo analysis software (Becton, Dickinson, and Co.). Both single antibody and fluorescence-minus-one controls were used for compensation. The following primary conjugated antibodies were used in flow cytometry experiments: CD45-BV650 (BioLegend 103151), CD64-APC (BioLegend 139306), CD11b–PE-Cy7 (BD 561098), CD11c–PE-Cy5 (eBioscience 15-0114-82), and Ly6C-APC-Cy7 (BD 560596).

Efferocytosis assay. Macrophages collected by bronchoalveolar lavage (5 × 10⁴ cells/well) were plated for 4 hours in serum-free media and exposed to fluorescently labeled primary type 2 AECs (Millipore 382065) from β1rtTA mice (1 × 10⁵ cell/well) for 1 hour. After incubation, nonadherent cells were removed by careful washing, and fluorescence was detected on a Molecular Devices SpectraMax M5 Plate Reader.

Macrophage migration assay. Conditioned medium from primary β1rtTA and β1f/f AECs was placed in the bottom chamber of a 5-μm Transwell insert (Corning 3422). WT macrophages (40,000 per insert) were obtained from pooled BALF and placed in the top chamber, incubated at 37°C for 4 hours. Unmigrated macrophages were removed from the top chamber, while migrated macrophages were fixed to the underside of the Transwell membrane and stained using the spHema 3 Manual Staining System.
Five nonoverlapping images of stained migrated macrophages were taken, and the number of migrated cells/field was quantified.

**ROS assays.** LumiMax Superoxide Anion Detection Kit (Agilent Technologies 204525) was used to detect superoxide from primary type 2 AECs per the manufacturer’s instructions. Amplex Red assay (Invitrogen, Thermo Fisher Scientific A22188) was used to detect H$_2$O$_2$ released from primary type 2 AECs per the manufacturer’s instructions. MitoSOX assay (Thermo Fisher Scientific M36008) was performed on primary type 2 AECs per the manufacturer’s instructions. For inhibitor studies, we used TEMPOL (Sigma-Aldrich 176141, 2 mM) and DPI (Sigma-Aldrich D2926, 10 μM).

**Statistics.** A 2-tailed Student’s t test was used for comparisons between 2 groups, with results representing mean SEM. For comparisons between 3 or 4 groups, an ordinary 1-way ANOVA was used with secondary analysis by Tukey’s test for multiple comparisons as indicated. For both statistical analyses, $P < 0.05$ was considered statistically significant.

**Study approval.** All animal experiments were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

**Author contributions**

EJP, JTB, JMS, PMG, LAG, SK, SMH, VVP, and WH designed and performed experiments and analyzed data. EJP, TSB, RZ, and LRY conceived the study and designed experiments. EJP, TSB, LRY, RZ, AP, and SHG wrote and edited the manuscript.

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50. Wang H, et al. p47(phox) contributes to albuminuria and kidney fibrosis in mice. *Kidney Int*. 2015;87(5):948–962.
51. Boekhoudt GH, Guo Z, Beresford GW, Boss JM. Communication between NF-kappa B and Sp1 controls histone acetylation within the proximal promoter of the monocyte chemoattractant protein 1 gene. *J Immunol*. 2003;170(8):4139–4147.
52. Martin T, Cardarelli PM, Parry GC, Felts KA, Cobb RR. Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF-kappa B and AP-1. *Eur J Immunol*. 1997;27(5):1091–1097.
53. Ping D, Boekhoudt GH, Rogers EM, Boss JM. Nuclear factor-kappa B p65 mediates the assembly and activation of the TNF-receptor element of the murine monocyte chemoattractant-1 gene. *J Immunol*. 1999;162(2):727–734.
54. Abboud RT, Vimalathanan S. Pathogenesis of COPD. Part I. The role of protease-antiprotease imbalance in emphysema. *Int J Tuberc Lung Dis*. 2008;12(4):361–367.
55. Hendrix AJ, Kheradmand F. The role of matrix metalloproteinases in development, repair, and destruction of the lungs. *Prog Mol Biol Transl Sci*. 2017;148:1–29.
56. Janoff A, et al. Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *Am Rev Respir Dis*. 1977;115(3):461–478.
57. Cheng DS, et al. Airway epithelium controls lung inflammation and injury through the NF-kappa B pathway. *J Immunol*. 2007;178(10):6504–6513.
58. Zaynagetdinov R, et al. Chronic NF-kB activation links COPD and lung cancer through generation of an immunosuppressive macroenvironment in the lungs. *Oncotarget*. 2016;7(5):5470–5482.
59. Kim C, et al. Attenuation of cigarette smoke-induced emphysema in mice by apolipoprotein A-1 overexpression. *Am J Respir Cell Mol Biol*. 2016;54(1):91–102.
60. Tuder RM, Petracek I, Elias JA, Voelkel NF, Henson PM. Apoptosis and emphysema: the missing link. *Am J Respir Cell Mol Biol*. 2003;28(5):551–554.
61. Aoshita K, Yokohori N, Nagai A. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol*. 2003;28(5):555–562.
62. Kasahara Y, et al. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest*. 2000;106(11):1311–1319.
63. Yoshida S, et al. Annexin V decreases PS-mediated macrophage efferocytosis and deteriorates elastase-induced pulmonary emphysema in mice. *Am J Physiol Lung Cell Mol Physiol*. 2012;303(10):L852–L860.
64. Wang X, et al. Dysregulation of TGF-beta1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. *Proc Natl Acad Sci U S A*. 2005;102(44):15791–15796.
65. Zhao Y, et al. Deletion of core fucosylation on alpha3beta1 integrin down-regulates its functions. *J Biol Chem*. 2006;281(50):38343–38350.
66. Nakamura T, et al. Fibulin-3/DANCE is essential for lung matrix assembly and disease. *Matrix Biol*. 2011;30(5):3048–3058.
67. Kresch MJ, Christian C, Wu F, Hussain N. Ontogeny of apoptosis during lung development. *Pediatr Res*. 2018;73:21–33.
68. Janssen-Heininger YM, et al. Nuclear factor-kappa B, airway epithelium, and asthma: avenues for redox control. *Proc Am Thorac Soc*. 2009;6(3):249–255.
69. Pantano C, Reynaert NL, van der Vliet A, Janssen-Heininger YM. Redox-sensitive kinases of the nuclear factor-kappaB signaling pathway. *Antioxid Redox Signal*. 2006;8(9-10):1791–1806.
70. Moodie FM, et al. Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. *FASEB J*. 2004;18(15):1897–1899.
71. Sheng ZG, Huang W, Liu YX, Yuan Y, Zhu BZ. Olfaxacin induces apoptosis via beta1 integrin-EGFR-Rac1-Notch2 pathway in microencapsulated chondrocytes. *Toxicol Appl Pharmacol*. 2013;267(1):74–87.
72. Chen X, et al. Integrin alphaB eta1 regulates epithelial growth factor receptor activation by controlling peroxisome proliferator-activated receptor gamma-dependent cavelin-1 expression. *J Biol Chem*. 2010;383(12):3048–3058.
73. Blackwell TS, et al. NF-kB signaling in fetal lung macrophages disrupts airway morphogenesis. *J Immunol*. 2011;187(5):2740–2747.
74. Henson PM, Hume DA. Apoptotic cell removal in development and tissue homeostasis. *Trends Immunol*. 2006;27(5):244–250.
75. Kresch MJ, Christian C, Wu F, Hussain N. Ontogeny of apoptosis during lung development. *Pediatr Res*. 1998;43(3):426–431.
76. Winter C, et al. Important role for CC chemokine ligand 2-dependent lung mononuclear phagocyte recruitment to inhibit sepsis in mice infected with Streptococcus pneumoniae. *J Immunol*. 2009;182(8):4931–4937.
77. Boniakowski AE, et al. Murine macrophage chemokine receptor CCR2 plays a crucial role in macrophage recruitment and regulated inflammation in wound healing. *Eur J Immunol*. 2018;48(9):1445–1455.
78. Larson SR, et al. Ly6C(+)-monocyte efferocytosis and cross-presentation of cell-associated antigens. *Cell Death Differ*. 2013;20(6):997–1003.
79. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. *Nat Immunol*. 2005;6(12):1191–1197.
80. Morimoto K, Janssen WJ, Terada M. Defective efferocytosis by alveolar macrophages in IFP patients. *Respir Med*. 2012;106(12):1800–1803.
81. Vandivier RW, et al. Impaired clearance of apoptotic cells from cystic fibrosis airways. *Chest*. 2002;121(3 suppl):895.
82. Vandivier RW, Henson PM, Douglas IS. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest*. 2006;129(6):1673–1682.
83. Vandivier RW, et al. Dysfunctional cystic fibrosis transmembrane conductance regulator inhibits phagocytosis of apoptotic cells with proinflammatory consequences. *Am J Physiol Lung Cell Mol Physiol*. 2009;297(6):L677–L686.
84. Huyhn ML, et al. Defective apoptotic cell phagocytosis attenuates prostaglandin E2 and 15-hydroxyecosatetraenoic acid in severe asthma alveolar macrophages. *Am J Respir Crit Care Med*. 2005;172(8):972–979.
85. Hodge S, Hodge G, Scicchitano R, Reynolds PN, Holmes M. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunol Cell Biol*. 2003;81(4):289–296.
86. Kirkham PA, Spooner G, Rahman I, Ross AG. Macrophage phagocytosis of apoptotic neutrophils is compromised by matrix proteins modified by cigarette smoke and lipid peroxidation products. *Biochem Biophys Res Commun*. 2004;318(1):32–37.
87. Elias BC, et al. The integrin beta1 subunit regulates paracellular permeability of kidney proximal tubule cells. *J Biol Chem*. 2010;285(19):14137–14143.
88. Kage H, et al. Claudin 4 knockout mice: normal physiological phenotype with increased susceptibility to lung injury. *Am J Physiol Lung Cell Mol Physiol*. 2014;307(7):L524–L536.

89. Li G, et al. Knockout mice reveal key roles for claudin 18 in alveolar barrier properties and fluid homeostasis. *Am J Respir Cell Mol Biol*. 2014;51(2):210–222.

90. Zhou B, et al. Claudin-18-mediated YAP activity regulates lung stem and progenitor cell homeostasis and tumorigenesis. *J Clin Invest*. 2018;128(3):970–984.

91. LaFemina MJ, et al. Claudin-18 deficiency results in alveolar barrier dysfunction and impaired alveologenesis in mice. *Am J Respir Cell Mol Biol*. 2014;51(4):550–558.

92. Kim KA, Jung JH, Kang IG, Choi YS, Kim ST. ROS is involved in disruption of tight junctions of human nasal epithelial cells induced by HRV16. *Laryngoscope*. 2018;128(12):E393–E401.

93. Yu L, Gan X, Liu X, An R. Calcium oxalate crystals induces tight junction disruption in distal renal tubular epithelial cells by activating ROS/Akt/p38 MAPK signaling pathway. *Ren Fail*. 2017;39(1):440–451.

94. Gangwar R, et al. Calcium-mediated oxidative stress: a common mechanism in tight junction disruption by different types of cellular stress. *Biochem J*. 2017;474(5):731–749.

95. Perl AK, Tichelaar JW, Whitsett JA. Conditional gene expression in the respiratory epithelium of the mouse. *Transgenic Res*. 2002;11(1):21–29.

96. Raghavan S, Bauer C, Mundschau G, Li Q, Fuchs E. Conditional ablation of beta1 integrin in skin. Severe defects in epidermal proliferation, basement membrane formation, and hair follicle invagination. *J Cell Biol*. 2000;150(5):1149–1160.

97. Rice WR, Conkright JJ, Na CL, Ikegami M, Shannon JM, Weaver TE. Maintenance of the mouse type II cell phenotype in vitro. *Am J Physiol Lung Cell Mol Physiol*. 2002;283(2):L256–L264.

98. Young LR, et al. The alveolar epithelium determines susceptibility to lung fibrosis in Hermansky-Pudlak syndrome. *Am J Respir Crit Care Med*. 2012;186(10):1014–1024.