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CFTR in alveolar type 1 cells contributes to lung liquid secretion and host defense

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28 **Conflict of interest statement:** The authors have declared that no conflict of interest exists.

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ABSTRACT

CFTR in the lung epithelium contributes to the secretion of a surface liquid layer that is essential to lung homeostasis and defense. The understanding of how liquid is secreted in the lung is derived largely from studies of the airway epithelium. Comparatively little is known about liquid secretion mechanisms in the alveolar epithelium, including its cellular source. To define which cell type drives alveolar liquid secretion, we generated transgenic mice that expressed a *Cftr* null allele in alveolar type 1 (AT1) cells, type 2 (AT2) cells, or both, then viewed liquid secretion in live alveoli using confocal microscopy of isolated, perfused lungs. Our findings show liquid secretion was blocked in alveoli of all three transgenic mice, indicating that both AT1 and AT2 cells contribute to alveolar liquid secretion. *Cftr* null expression in AT1 cells also blocked the secretion-mediated clearance of small particle and bacterial clusters from alveolar walls, indicating that AT1 cell CFTR contributes to alveolar defense. Together, these findings show alveolar liquid secretion depends on both AT1 and AT2 cell CFTR, and that CFTR in AT1 cells – a cell type not traditionally considered in liquid secretion mechanisms or CFTR-related lung diseases – contributes to lung liquid dynamics and host defense.

INTRODUCTION

The lung's air-facing epithelial surface is lined by an aqueous liquid layer that prevents epithelial cell desiccation and contributes to host defense. Loss of this liquid layer has a role in the pathogenesis of diverse lung diseases including cystic fibrosis (CF) (1, 2), chronic obstructive pulmonary disease (COPD) (3, 4), and lung infection (5-7). Much of the understanding of liquid secretion mechanisms in the lung is derived from studies of liquid transport in the airway epithelium. Although the alveolar epithelium also secretes liquid and comprises over 95% of the lung's air-facing surface area (5, 8, 9), comparatively little is known about liquid secretion in alveoli, including its cellular source.

Electron microscopic studies of rat lungs show the alveolar epithelium is lined by a continuous layer of surface liquid (10), and experiments in live alveoli of isolated, perfused lungs provide evidence that the liquid is secreted by the alveolar epithelium in a CFTR-dependent manner (5, 6, 11). CFTR is expressed in both alveolar type 1 (AT1) and type 2 (AT2) cells in adult human lungs (12, 13), raising the possibility that CFTR in both cell types contributes to liquid secretion in alveoli at homeostasis. In vitro studies support the possibility that both cell types contribute, since reports of cultured and freshly isolated alveolar epithelial cells show that both AT1 (14-18) and AT2 (19-25) cells transport Na^+ , Cl^- , and liquid. However, the extent to which CFTR in each cell type contributes to alveolar liquid secretion in vivo remains an open question. Whether CFTR in AT1 cells contributes is particularly unclear, since the extensive cell surface area that makes AT1 cells likely to contribute to alveolar liquid secretion has also made AT1 cells traditionally challenging to isolate and study.

In this work, we used recently-developed genetic tools to quantify the contribution of AT1 and AT2 cell CFTR to alveolar liquid secretion. We generated transgenic mice that express a *Cftr* null

allele in AT1 cells, AT2 cells, or both cell types, then used confocal microscopy to view liquid secretion in alveoli of live, perfused lungs. Our findings show alveolar liquid secretion was blocked in all three transgenic mice, indicating that CFTR in both AT1 and AT2 cells contributes to alveolar liquid secretion under baseline conditions. In addition, *Cftr* null expression in AT1 cells inhibited the secretion-mediated displacement of small clusters of particles and bacteria from alveolar walls, indicating that AT1 cell CFTR defends against particle and bacterial stabilization in alveoli. Together, these data provide the first evidence, of which we are aware, that CFTR in both AT1 and AT2 cells contributes to alveolar liquid secretion. We propose that AT1 cells are important for lung liquid secretion and host defense.

RESULTS

AT1 and AT2 cell-specific CFTR^{null} expression in transgenic mice

We bred widely-used transgenic mouse strains to generate mice in which a *Cftr* null allele and GFP expression were tamoxifen-induced in adulthood in an AT1 or AT2 cell-specific manner. Thus, AT2-CFTR^{null}-GFP mice carried *Sftpc-CreER^{T2}* (26), *Cftr^{f10/f10}* (27), and *ROSA^{mT/mG}*, a reporter allele that confers membrane-targeted TdTomato expression prior to Cre-mediated excision, and GFP expression after (28). Likewise, AT1-CFTR^{null}-GFP mice carried *Ager-CreER^{T2}* (29), *Cftr^{f10/f10}*, and *ROSA^{mT/mG}*; AT2-AT1-CFTR^{null}-GFP mice carried *Sftpc-CreER^{T2}*, *Ager-CreER^{T2}*, *Cftr^{f10/f10}*, and *ROSA^{mT/mG}*; and littermate control (LMC-TdTomato) mice carried *Cftr^{f10/f10}* and *ROSA^{mT/mG}*.

We used our established methods (6, 30) to view live alveoli of intact, perfused lungs of AT2-CFTR^{null}-GFP mice by real-time confocal microscopy (**Figure 1A**). GFP fluorescence was evident in plasma membranes of rounded cells that were located primarily at septal junctions (**Figure 1B**), consistent with AT2 cell morphology and location (31, 32). To test AT2 cell function, we used alveolar micropuncture to microinstill alveolar airspaces with LysoTracker dye, which is taken up by AT2 cells and localizes to AT2 cell lamellar bodies. Hyperinflation-induced LysoTracker fluorescence loss signals surfactant secretion (33). Our findings show lung hyperinflation caused time-dependent loss of LysoTracker fluorescence in GFP-positive cells (**Supplemental Figure 1, A-B**), affirming that GFP-positive cells in AT2-CFTR^{null}-GFP mice function as AT2 cells. To quantify *Cftr* deletion efficiency, we isolated AT2 cells (**Supplemental Figure 2, A-C**) and performed qPCR of *Cftr* RNA using our established methods (34, 35). AT2 cells of an AT2-CFTR^{null}-GFP mouse had 100-fold reduction of *Cftr* RNA compared with AT2 cells of an LMC-

TdTomato mouse (**Supplemental Tables 1-2**). Taking these findings together, we conclude that AT2 cells of AT2-CFTR^{null}-GFP mice are *Cftr*-deficient and marked by GFP fluorescence.

By contrast, GFP fluorescence in live lungs of AT1-CFTR^{null}-GFP mice was evident in thin cells that line alveolar walls (**Figure 1B**), consistent with AT1 cell morphology and location. AT2 cells of AT1-CFTR^{null}-GFP mice had abundant *Cftr* RNA (**Supplemental Figure 2D** and **Supplemental Table 1**). Taking these data together, we interpret that CFTR^{null} expression in AT1-CFTR^{null}-GFP mice is AT1 cell-specific.

In AT2-AT1-CFTR^{null}-GFP mice, GFP fluorescence was evident in plasma membranes of both AT2 and AT1 cells (**Figure 1B**), and AT2 cell qPCR showed more than 100-fold reduction of *Cftr* RNA (**Supplemental Figure 2E** and **Supplemental Table 1**). There was no GFP fluorescence in alveoli of LMC-TdTomato mice (**Figure 1B**). We interpret that AT2-AT1-CFTR^{null}-GFP mice are *Cftr*-deficient in both AT2 and AT1 cells, while LMC-TdTomato mice are *Cftr*-deficient in neither cell type.

CFTR^{null} expression is heterogeneous across alveoli of Ager-CreER^{T2}-expressing mice

Tamoxifen-treated *Ager-CreER^{T2}*-expressing mice show Cre recombination efficiency that varies across lung alveoli (29), leading to a heterogeneous distribution of Cre expression in which AT1 cells of some alveoli express Cre while AT1 cells of other alveoli do not. In line with the published data, our images show AT1 cell GFP fluorescence was heterogeneous across imaging fields of alveoli in lungs of AT1-CFTR^{null}-GFP mice (**Figure 2, A-B**), while TdTomato fluorescence was evident in all alveoli (**Figure 2A**). AT2-AT1-CFTR^{null}-GFP showed similar heterogeneity of GFP expression (data not shown). We categorized the proportion of GFP-expressing AT1 cells in imaging fields of AT1-CFTR^{null}-GFP mice as Low, Medium, or High if GFP expression was evident

in less than 40%, 40-80%, or more than 80%, respectively, of alveoli in the imaging field (**Figure 2, A-B**). We interpreted the GFP fluorescence categories to indicate sparse, moderate, and extensive CFTR^{null} expression, respectively, across imaging fields of alveoli.

CFTR in AT1 cells and AT2 cells contributes to alveolar liquid secretion

We next aimed to define the effect of AT1 and AT2 cell CFTR^{null} expression, and its heterogeneity, on alveolar function. To do this, we applied a technique that has been used previously to identify liquid secretion and absorption in alveoli of live, perfused lungs using upright confocal microscopy (5, 6). Briefly, the experiments are carried out by microinstilling alveolar airspaces with fluorophore-conjugated dextran in aqueous solution. The dextran-containing solution is visible in alveoli by confocal microscopy as a juxta-epithelial layer that pools at alveolar niches, where septa converge (5, 6). Since the fluorescence intensity of the dextran-containing solutions varies with dextran concentration (6), the solutions lose fluorescence in airspaces as they become diluted by non-fluorescent alveolar wall liquid (AWL) secreted by the alveolar epithelium (5, 6). Conversely, the solutions gain fluorescence when the epithelium absorbs liquid from airspaces (5, 6). Since secreted AWL flows toward airways and does not accumulate in alveoli (5), the widths of the dextran-containing solution pools are unchanged over time when AWL is secreted (5, 6). However, they diminish over time when airspace liquid is absorbed (5, 6).

Using this approach, we and others have reported alveoli of intact, perfused mouse lungs secrete AWL in a CFTR-dependent manner under baseline conditions (5, 6). A recent study from our group affirms CFTR dependence and adds that the direction of alveolar liquid transport depends on lung perfusion, since alveoli of lungs perfused at or near physiological levels secrete AWL, while alveoli of lungs in which perfusion is majorly reduced or halted absorb airspace liquid (36). Microvessel-to-airspace osmotic gradients do not seem to determine the direction of alveolar

liquid transport (36). Because fluid leak from microvessels into airspaces in perfused lungs could dilute the dextran-containing solutions, we ruled out alveolar leak by showing that airspace dextran fluorescence decreased, then increased in the same alveoli, under conditions of continuous perfusion, in response to changes of pulmonary artery pressure (36). Since airspace dextran fluorescence could not increase in alveoli with barrier leak, these data align with reports indicating alveolar barrier function is intact in isolated, perfused lungs (37, 38). Finally, we have used airspace instillation of fluorophore-tagged antibodies to show alveolar macrophages are present in airspaces of isolated, perfused lungs (36), indicating that expected non-epithelial cell types are present in our preparation. Neutrophils are absent in airspaces under baseline conditions but recruited after intranasal instillation of *S. aureus* (6).

We now applied these methods to test the hypothesis that AT1 and AT2 cell CFTR^{null} expression inhibits AWL secretion. We microinstilled alveolar airspaces of LMC-TdTomato, AT2-AT1-CFTR^{null}-GFP, AT2-CFTR^{null}-GFP, and AT1-CFTR^{null}-GFP mice with Alexa Fluor 647-conjugated dextran (AF-dextran; 10 kDa; 10 mg/mL) (**Figure 3A**). We used AF-dextran because its fluorescence intensity varies with concentration in aqueous solution (**Supplemental Figure 3, A-B**), and its excitation and emission spectra are distinct from that of GFP and TdTomato. The contents of the airspace instillate and lung perfusate solutions for our experiments are shown in **Supplemental Table 3**. In alveoli of AT1-CFTR^{null}-GFP and AT2-AT1-CFTR^{null}-GFP mice, we restricted our analysis to imaging fields that had High-GFP AT1 cell fluorescence, in order to avoid confounding the experiments with alveoli that lacked CFTR^{null} expression.

In alveoli of LMC-TdTomato mice, microinstilled AF-dextran lined alveolar walls and pooled at alveolar niches within minutes of microinstillation (**Figure 3B**), as expected. AF-dextran fluorescence subsequently decreased in a time-dependent manner (**Figure 3, C-D**), suggesting the alveolar epithelium secreted AWL. The widths of AF-dextran-containing liquid pools in alveoli

were stable over time (**Figure 3, E-F**), also as expected, indicating that the volume of AF-dextran-containing liquid in airspaces did not increase during the imaging period. Stable airspace diameter during the same imaging period (**Figure 3, E-F**) indicates our measurements of AF-dextran pool width were not confounded by changes of alveolar morphology. To rule out the possibility that AF-dextran fluorescence loss was caused by leak of microvascular fluid into airspaces, we added fluorescein isothiocyanate (FITC)-tagged dextran (FITC-dextran, 20 kDa, 5 mg/mL) to the lung perfusate solution (**Figure 3A**), as we have done previously (6, 30). FITC-dextran fluorescence was visible in microvascular lumens but did not leak into alveolar airspaces (**Figure 3G**), indicating alveolar barrier function was intact during the imaging period. Together, these findings show alveoli secreted AWL in lungs of LMC-TdTomato mice.

By contrast, AF-dextran fluorescence loss in airspaces was blocked in alveoli of AT2-AT1-CFTR^{null}-GFP mice (**Figure 3, C-D**). Widths of AF-dextran-containing liquid pools and airspaces in alveoli did not change over time (**Figure 3, E-F**), and FITC-dextran in the lung perfusate solution did not leak into airspaces. These findings show CFTR^{null} expression in both AT1 and AT2 cells inhibits AWL secretion, and they confirm reports indicating that AWL secretion depends on CFTR in the alveolar epithelium (5, 6). Notably, AWL secretion was not completely lost in alveoli of AT2-AT1-CFTR^{null}-GFP mice (**Figure 3, C-D**). The residual secretion might reflect contributions to AWL secretion by non-CFTR Cl⁻ channels in the alveolar epithelium.

AF-dextran fluorescence loss was also blocked in alveoli of AT2-CFTR^{null}-GFP mice and AT1-CFTR^{null}-GFP mice (**Figure 3, C-D**). Widths of AF-dextran-containing liquid pools and airspaces in alveoli of both AT2-CFTR^{null}-GFP and AT1-CFTR^{null}-GFP mice were steady over time (**Figure 3, E-F**), and FITC-dextran that was added to the lung perfusate did not leak into airspaces. Together, these findings show CFTR^{null} expression in each of AT2 and AT1 cells inhibited liquid

secretion in alveoli. We conclude that CFTR in both AT2 and AT1 cells contributes to AWL secretion.

Heterogeneous recombination efficiency across alveoli of tamoxifen-treated *Ager-CreER^{T2}*-expressing mice (29) provides an opportunity to relate the magnitude of AT1 cell CFTR^{null} expression in imaging fields of alveoli to AWL secretion rates in lungs of AT1-CFTR^{null}-GFP mice. To determine whether AWL secretion varies with the extent of AT1 cell CFTR^{null} expression, we reanalyzed the AF-dextran images that we generated in AT1-CFTR^{null}-GFP mice by GFP expression category, as defined in **Figure 2**, rather than by mouse, as in **Figure 3**. **Figure 4, A-C shows** the relationship between AF-dextran fluorescence change and AT1 cell GFP expression. Our findings show that in Low-GFP imaging fields, airspace AF-dextran fluorescence decreased rapidly (**Figure 4, A-C**), indicating that sparse AT1 cell CFTR^{null} expression across alveoli had no impact on AWL secretion. However, AF-dextran fluorescence loss was slower in Medium-GFP imaging fields, and it was slower still in High-GFP imaging fields (**Figure 4, A-C**). These findings show the rate of AWL secretion depends on AT1 cell CFTR^{null} expression, such that imaged regions of alveoli that had the most CFTR^{null} expression had the least AWL secretion, and vice versa. We conclude that the extent of CFTR expression in AT1 cells determines AWL secretion. This direct, “dose-dependent” relationship between AT1 cell CFTR expression and AWL secretion strengthens our conclusion that AT1 cell CFTR is a driver of liquid secretion in alveoli.

We followed up by testing whether microvessel-to-airspace osmotic gradients promote AWL secretion and could have affected our interpretations. Our data show the AF-dextran-containing airspace solution that we used to generate **Figures 3-4** was hyperosmolar to the lung perfusate solution (**Supplemental Figure 4A** and **Supplemental Table 3**), raising the possibility that the microvessel-to-airspace osmotic gradient present in our preparation, which is favorable to AWL

secretion, drove the AF-dextran fluorescence loss we observed in our experiments (**Figures 3 and 4**). To test this possibility, we quantified AF-dextran fluorescence changes in alveoli of wild type mice that we microinstilled with modified AF-dextran solution that abolished the microvessel-to-airspace osmotic gradient by reducing the osmolality of the microinstillate solution (**Supplemental Figure 4, A-D and Supplemental Table 3**). Our data show the airspace fluorescence of the modified AF-dextran solution decreased, while the AF-dextran pool width and alveolar airspace width were steady (**Supplemental Figure 4, C-D**). These findings show AWL secretion occurred in the absence of a pro-secretion osmotic gradient between microvessels and airspaces and, thus, affirm findings included in a separate report from our group (36). We conclude that the direction of liquid transport in alveoli occurs independently of microvessel-to-airspace osmotic gradients. These data strengthen our conclusion that AT1 and AT2 cells contribute to AWL secretion at homeostasis.

AT1 cell CFTR promotes particle clearance in alveoli

AWL secretion defends against particle stabilization in alveoli by generating pleura-to-airway liquid flow along alveolar walls that convectively transports particles toward the airways (5). We considered that AWL secretion specifically by AT1 cells might contribute to particle clearance mechanisms. To test this possibility, we used an established method (5, 30) in which we microinstilled fluorescent polystyrene beads (1 μm diameter) into airspaces of Medium-GFP regions of AT1-CFTR^{null}-GFP mice (**Figure 5A**). We analyzed the initial organization and movement of 80 bead groups by generating confocal images of the beads from 30 minutes to 3 h after microinstillation, then measuring their displacement according to: (A) their initial location, defined as bead position against a flat alveolar septum or at an alveolar niche; and (B) their initial group size, defined as a small cluster (1-3 beads) or a microaggregate (4 or more beads).

Our findings show the beads formed small clusters and microaggregates at flat septa and niches within minutes of microinstillation (**Figure 5B** and **Supplemental Figure 5, A-B**). Across imaged regions of alveoli, there was no difference in the number of bead groups that initially localized to alveolar walls lined by TdTomato- or GFP-expressing AT1 cells (**Supplemental Figure 5A**). The distribution of bead subgroups – that is, small clusters at flat septa, microaggregates at flat septa, small clusters at niches, and microaggregates at niches – was also equivalent (**Supplemental Figure 5B**). Although mean bead group size at TdTomato-expressing AT1 cells was greater than the mean size of bead groups at GFP-expressing AT1 cells (**Supplemental Figure 5, C-D**), the difference seemed to be driven by a handful of large microaggregates (**Supplemental Figure 5E**). We interpret that AT1 cell CFTR^{null} expression did not have a major impact on how microinstilled beads organized against alveolar walls.

We next analyzed the movement of beads that were initially located at TdTomato-expressing AT1 cells which, we interpret, expressed WT CFTR. Taking all bead groups together, we found the mean distance of bead displacement was 4 µm along alveolar walls during the imaging period (**Figure 5, C-E**). However, the distance of bead displacement was not uniform across bead subgroups. The subgroup that moved the least was microaggregates at niches (**Supplemental Figure 5F**). Bead subgroups that were made up of small clusters or that were located at flat septa moved short distances (**Figure 5, F-G** and **Supplemental Figure 5G**), and the movement was either toward the airways or lateral. No bead groups moved toward the pleura. Together, these data largely support findings published by others that show the liquid flow generated by AWL secretion along alveolar walls transports beads in alveoli away from the pleura and toward the airways (5). In addition, our findings affirm our published data that show microaggregates at alveolar niches are stable in alveoli and resist alveolar clearance mechanisms (30).

We then considered the movement of beads that were located at GFP-expressing AT1 cells which, we interpret, expressed CFTR^{null}. As different from beads located at TdTomato-expressing AT1 cells, beads at GFP-expressing AT1 cells were largely immobile (**Figure 5, D-E**), indicating that AT1 cell CFTR^{null} expression blocked bead movement. Bead immobilization at CFTR^{null}-expressing AT1 cells was not caused by microenvironmental factors unrelated to CFTR^{null} expression, since in single alveoli lined on opposite walls by TdTomato- and GFP-expressing AT1 cells, only those beads located at TdTomato-expressing AT1 cells were displaced (**Figure 5F**). Like niche microaggregates at TdTomato-expressing AT1 cells, niche microaggregates at GFP-expressing AT1 cells moved little, but their relative immobilization was not different from other bead subgroups located at GFP-expressing AT1 cells (**Supplemental Figure 5F**). Notably, bead subgroups made up of small clusters or located at flat septa lined by GFP-expressing AT1 cells were almost totally immobile, and the distances of their displacements were markedly less than those of their counterparts at TdTomato-expressing AT1 cells (**Figure 5, F-G** and **Supplemental Figure 5G**). These findings show AT1 cell CFTR^{null} expression blocked the displacement of small bead clusters in alveoli, particularly at flat septal locations. We suggest that AT1 cell CFTR contributes to alveolar defense against inhaled particles.

AT1 cell CFTR promotes *S. aureus* clearance in alveoli

Since AT1 cell CFTR contributed to alveolar defense against particles, we considered it might also contribute to alveolar defense against inhaled bacteria. To test this possibility, we intranasally-instilled mice with GFP-expressing *S. aureus* (SA^{GFP}), then imaged the SA^{GFP} in alveoli of live, perfused lungs from 1-4 h after instillation (**Figure 6A**). We chose this timeframe because our published data show CD11b-expressing neutrophils and monocytes are absent from alveolar airspaces of SA-instilled lungs in this time period (6), reducing the possibility that innate immune responses would confound SA^{GFP} clearance measures. To avoid fluorescence overlap,

we instilled SA^{GFP} in non-fluorescent transgenic mice (AT1-CFTR^{null}) that carried *Ager-CreER*^{T2} and *Cftr*^{f10/f10} but not *ROSA*^{mT/mG}. Littermate control (LMC) mice carried *Cftr*^{f10/f10}.

Confocal images of live alveoli of AT1-CFTR^{null} and LMC mice at 1 h after SA^{GFP} instillation show inhaled SA^{GFP} organized against alveolar walls as small clusters of roughly 1-3 bacteria and microaggregates of 4 or more bacteria (**Figure 6B**). Microaggregates of SA^{GFP} were resistant to alveolar clearance in both AT1-CFTR^{null} and LMC mice (**Figure 6, B-C**) and, thus, unaffected by AT1 cell CFTR^{null} expression. However, AT1 cell CFTR^{null} expression blocked the clearance of small clusters of SA^{GFP} (**Figure 6, B-C**). These findings show AT1 cell CFTR contributes to the alveolar clearance of small bacterial clusters. We interpret that loss of AT1 cell CFTR caused retention of inhaled bacteria against alveolar walls, prolonging contact between small bacterial clusters and the alveolar epithelium.

DISCUSSION

Our findings reveal new roles for AT1 cells in lung homeostasis by providing evidence that AT1 cell CFTR contributes to alveolar liquid secretion and the alveolar clearance of particles and inhaled *S. aureus*. Specifically, we identified that CFTR^{null} expression in AT1 and AT2 cells each blocked homeostatic AWL secretion, and that AT1 cell CFTR^{null} expression disrupted the alveolar clearance of small clusters of particles and bacteria. Since AT1 cells comprise over 95% of the lung's air-facing surface (39), we interpret that AT1 cell CFTR is important to lung health and defense. These findings raise the possibility that alveoli, including AT1 cells, are a site of the CFTR dysfunction that underlies CFTR-related lung diseases.

Our identification that AT1 cell CFTR has a role in AWL secretion builds on reports by others that suggest AT1 cells make important contributions to ion and water transport in the lung. For example, Folkesson et al. identified water channels in AT1 cells of sheep lungs and proposed that a major role of the AT1 cells is to transport water (14). Dobbs et al. showed freshly isolated AT1 cells are highly water-permeable and suggested AT1 cells account for the high water permeability of the lung (15). Johnson et al. showed freshly-isolated rat AT1 cells transport Na⁺ and Cl⁻ and express functional CFTR and ENaC proteins, and the authors proposed that, by virtue of their very large surface area, AT1 cells make major contributions to alveolar fluid balance (16, 18, 40). Our data support these proposals by providing evidence that AT1 cells are drivers of liquid transport in alveoli. Specifically, our finding that AT1 cell CFTR^{null} expression blocked AWL secretion in live alveoli of intact, perfused lungs demonstrates a role for AT1 cells and AT1 cell CFTR in liquid transport under conditions that approximate the structural and physiological complexity of alveoli in vivo. Hence, our findings join a cadre of reports by other groups that suggest AT1 cells are important to liquid and ion transport in the lungs. Since the published reports use data generated in fixed lung tissue and isolated AT1 cells, the advance represented

by our study stems from our application of genetic approaches and intact, perfused lungs to show an AT1 cell-specific role for CFTR in homeostatic liquid secretion in alveoli in situ.

Interestingly, the extent of the inhibition of AWL secretion we observed in AT1-CFTR^{null}-GFP mice matched the inhibition we observed in AT2-CFTR^{null}-GFP and AT2-AT1-CFTR^{null}-GFP mice. The equivalent inhibitory effect of CFTR^{null} expression in each of AT1 and AT2 cells shows that both AT1 and AT2 cells make major contributions to homeostatic AWL secretion. Why the inhibition in mice in which either AT1 or AT2 cells expressed CFTR^{null} was not less than the inhibition in mice in which both AT1 and AT2 cells expressed CFTR^{null} is not clear. One possibility is there was ectopic Cre expression in the *Ager-CreER^{T2}*- or *Sftpc-CreER^{T2}*-expressing mice, such that some AT2 cells expressed CFTR^{null} in AT1-CFTR^{null}-GFP mice, or some AT1 cells expressed CFTR^{null} in AT2-CFTR^{null}-GFP mice. This might exaggerate the inhibitory effect of CFTR^{null} expression in AT1-CFTR^{null}-GFP and AT2-CFTR^{null}-GFP mice. However, we do not consider ectopic Cre expression likely to be a major factor in our studies since two experiments – first, live lung imaging using the *ROSA^{mT/mG}* allele, and second, qPCR of *Cftr* RNA – provide evidence that CFTR^{null} expression in AT1-CFTR^{null}-GFP and AT2-CFTR^{null}-GFP mice was limited to the intended cell types. Another possibility is that CFTR^{null} expression simultaneously in AT1 and AT2 cells of AT2-AT1-CFTR^{null}-GFP mice stimulated a compensatory response in the alveolar epithelium, such as increased expression of non-CFTR Cl⁻ channels like SLC26A9 (41). This might attenuate the inhibitory effect of simultaneous CFTR^{null} expression in AT1 and AT2 cells of AT2-AT1-CFTR^{null}-GFP mice, thereby preventing complete inhibition of AWL secretion. Although these questions remain unanswered, we can conclude from our findings that CFTR in both AT1 and AT2 contributes to AWL secretion.

This study adds new understanding of alveolar liquid transport mechanisms in lungs at homeostasis. The literature is not clear as to whether alveoli secrete or absorb liquid under

homeostatic conditions. For example, data generated by live imaging of fluorophore-labeled dextran and Na⁺ and Cl⁻ indicator dyes in alveolar airspaces of intact, perfused lungs provide evidence that alveoli secrete Na⁺, Cl⁻, and liquid (5, 6, 11). By contrast, data generated by large airway instillation of radionuclides, fluorophore-tagged albumin, Evan's blue, and other tracers in whole lungs provide evidence that the distal lung epithelium is absorptive (20, 21, 24, 25, 38, 42-52). The reason for the discrepancy between the data generated by alveolar imaging and the data generated by whole lung approaches is not clear. One possibility is the reported liquid absorption in whole lung tissue includes liquid absorption by the airway epithelium, since the whole lung tracer data do not differentiate between airway and alveolar contributions to liquid transport. In line with this possibility, Martens and Ballard report that pig intrapulmonary bronchi secrete liquid when they are air-filled, but they absorb liquid when they are fluid-filled (53). Notably, the same report indicates that the rate of liquid secretion by air-filled intrapulmonary bronchi is $1.42 \pm 0.36 \mu\text{L} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ (53), which, if extrapolated across the 2,471 cm² of airway surface area in human lungs (8), would produce roughly 84 mL of liquid per day – a volume that is meaningfully smaller than that lost daily to respiration (54, 55). If the alveolar epithelium were absorptive under homeostatic conditions, the demand for airway liquid secretion to maintain the lung's epithelial lining layer would yet increase. Taken in context of the published literature, our findings support the idea that the alveolar epithelium secretes liquid under homeostatic conditions. In addition, our data add new evidence that alveolar liquid secretion in live alveoli of perfused lungs is dependent on CFTR in AT1 and AT2 cells.

Our data identify that AT1 cell CFTR displaces small particle clusters from alveolar walls, particularly at flat septa. The mean distance of bead displacement was a modest 4 μm over 2.5 h of live imaging, and the small distance calls into question the potential biological significance of the displacement. Since we started imaging bead movement 30 min after the beads were microinstilled, we might have missed an early period of particle displacement, if particle-epithelial

contact stimulates rapid liquid secretion in alveoli as bacteria-epithelial contact does in the airway (56). However, we note that the minimum distance of particle displacement needed to protect against particle-induced epithelial damage in alveoli is not known. Since particle-induced damage to AT2 cells in vitro associates with longer particle exposure times (57), AT1 cell functions like the modest particle displacement we identified could be protective by simply blocking particle stabilization against the alveolar epithelium or resisting particle-epithelial contact. Such resistance to particle contact might be important at flat septa, where the AWL is thin (10) and could bring inhaled particles and AT1 cells close together. Histological data that show CFTR expression on flat surfaces of human alveoli (12, 13) provide support for the idea that flat septa are physiologically significant sites of CFTR function. Keeping these issues in mind, we conclude from our findings that CFTR-dependent liquid secretion by AT1 cells defends against particle stabilization in alveoli by displacing small bead clusters from flat segments of alveolar walls.

Our data also show AT1 cell CFTR contributes to alveolar defense against inhaled bacteria, though the magnitude of the contribution may again be modest. We identified that CFTR^{null} expression in AT1 cells reduced the alveolar clearance of small clusters of inhaled SA^{GFP}, indicating that AT1 cell CFTR contributes to small cluster clearance in alveoli. As for particles, the potential biological significance of small bacterial cluster displacement is difficult to know. Since just minutes of contact between epithelial cells and *S. aureus* is sufficient to stimulate pro-inflammatory responses in vitro (58), displacement of small clusters of bacteria from alveolar walls may protect against bacteria-induced alveolar damage and lung inflammation. CFTR-dependent AWL secretion might offer additional epithelial protection by diluting secreted bacterial toxins such as alpha-hemolysin, which we have shown previously causes epithelial damage in alveoli (30). Hence, the modest bacterial clearance we attribute in this report to AT1 cell CFTR may serve a protective role in alveoli after bacterial inhalation.

The mechanism by which AT1 cell CFTR promoted bacterial clearance is not yet clear. One possibility is the liquid flow generated by AWL secretion directly cleared *S. aureus* by transporting the bacteria toward the airways, in the manner proposed by others for particle clearance (5). Another possibility is the secreted liquid indirectly contributed to bacterial clearance by promoting the antibacterial functions of alveolar macrophages, epithelium-derived antimicrobial peptides, and surfactant. Notably, AT1 cell CFTR^{null} expression had no impact on the alveolar clearance of SA^{GFP} that formed microaggregates at alveolar niches. These findings align with our previous reports indicating that microaggregation promotes bacterial stabilization in alveoli (30). The fate of the microaggregated bacteria is not clear, but they might be cleared by other innate immune mechanisms such as neutrophil- or macrophage-mediated bacterial killing. We conclude from our data that AT1 cell CFTR promoted the clearance of small bacterial clusters from alveoli, perhaps contributing to alveolar defense against bacteria-induced epithelial damage. Loss of this defense mechanism in lungs with AT1 cell CFTR dysfunction might prolong bacterial contact with AT1 cells and set the stage for lung infection and lung injury.

Our findings suggest therapies that are intended to promote or restore lung liquid secretion should consider targeting AT1 cells, in addition to other cell types. AT1 cell targeting might be achieved by delivering CFTR modulator drugs or expression vectors in preparations that bind AT1 cell surface proteins, like AGER (29) and EMP2 (59). Our data suggest that effective AT1-targeted inhaled therapies might be designed to not only deposit in alveoli (60, 61), but also to stabilize at flat septa, where we identified a role for AT1 cell CFTR in particle clearance. Although CFTR dysfunction in AT1 cells is not a known feature of CFTR-related lung diseases like CF and COPD, we note that seven groups have identified that lung alveoli in people with CF harbor tissue remodeling, acute inflammation, and bacteria (62-68), and data generated in lungs of humans with COPD and non-human primates with smoke exposure show evidence of alveolar damage (69-72).

If shown to be effective, AT1-targeted therapies for CFTR-related diseases have the potential to have an outsized effect by augmenting liquid secretion in a cell type with extensive surface area.

Our study has limitations. First, our findings were generated in mouse lungs, and mice differ from humans in the expression of some ion transport proteins (73). Future studies in human, non-human primate, pig, or ferret lungs are needed to define the extent to which our findings are broadly applicable. Second, we relied on male mice in our experiments because we achieved little GFP expression in lungs of female AT1-CFTR^{null}-GFP mice, making it challenging to define the effect of AT1 cell CFTR^{null} expression in females. Sex-related differences in responses to tamoxifen (74), which is required to induce CFTR^{null} expression in the transgenic mice used in this study, might underlie the low Cre recombination rates we observed in female mice. Follow up studies that compare the effects of cell-specific CFTR^{null} expression on alveolar liquid transport in male and female mice might illuminate whether differences in CFTR in AT1 and AT2 cells help explain sex disparities in CFTR-related diseases like CF (75). Data included in a separate report by our group provide evidence that alveoli of female mice secrete AWL at the same rate as males (36), indicating at least some aspects of alveolar liquid transport are preserved in female mice. Third, our studies do not identify the fate of secreted AWL. Publications by others raise the possibility that liquid secreted in alveoli moves onto small airway surfaces, where it might be, to some extent, absorbed (5, 76-78). Our observation that no beads moved toward the pleura in alveoli of LMC-TdTomato mice support the possibility that secreted AWL moves proximally, perhaps due to bulk flow as previously suggested (5). Finally, our experiments do not take into account physical forces of respiration that are applied to alveoli in vivo and that may influence alveolar fluid distribution and particle transport. Since hyperinflation-induced alveolar expansion affects flat segments of alveoli more than alveolar corners (79), respiration-related physical forces might impact the particle transport we identified, particularly at flat alveolar septa. Dedicated follow up studies might shed light on these important issues. Despite its limitations, this study

provides the first evidence, to our knowledge, that AT1 cell CFTR contributes to liquid transport in live alveoli of perfused lungs.

In conclusion, our findings suggest a shift in the understanding of the role of AT1 cells in lung health and disease. Our data reveal that AT1 cells, traditionally considered to be passive conduits for gas exchange, have important roles in homeostatic alveolar liquid secretion and alveolar defense, and they raise the possibility that loss of AT1 cell CFTR function contributes to CFTR-related lung diseases. Going forward, we suggest that new therapies that aim to augment lung liquid secretion consider AT1 cells as potential therapeutic targets.

METHODS

Experimental design. The experiments were designed and the manuscript was written according to ARRIVE guidelines. Sample sizes and outcome measures are indicated in figures and legends.

Fluorophores. We purchased LysoTracker Green (100 nM), AlexaFluor 647-conjugated dextran (AF-dextran; 10 kDa), fluorescein isothiocyanate (FITC)-conjugated dextran (FITC-dextran; 20 kDa; 5 mg/mL), and calcein red-orange AM (10 μ M) from ThermoFisher Scientific.

Reagents. All reagents were freshly constituted for experiments. We purchased HEPES and Ca^{2+} - and Mg^{2+} -containing PBS from ThermoFisher and NaCl, KCl, glucose, CaCl_2 , MgCl_2 , and sucrose from Sigma. Tamoxifen was purchased from Sigma, reconstituted at 20 mg/mL in sunflower oil (Sigma), and administered within 30 min of reconstitution. Fluorescent microspheres (1.00 μ m, Flash Red, cat. no. FSFR004) were purchased from Bangs Laboratories, Inc.

Solutions. Calculated and measured values for solution contents are detailed in **Supplemental Table 3** and compared with reported normal values for mouse blood pH (80), plasma osmolality (81), and plasma Na^+ , K^+ , Cl^- , ionized Ca^{2+} , and non-fasting glucose (82-84). We prepared HEPES-buffered solution to serve as the vehicle for the lung perfusate and alveolar instillations. A pH meter (Mettler Toledo Seven Excellence) and NaOH were used to titrate the solution pH. Osmolality was measured by freezing point depression using a micro-osmometer (Precision Systems 6002 Touch Micro Osmette). To generate the lung perfusate solution, we modified the HEPES-buffered vehicle solution by adding dextran (70 kDa; Molecular Probes; 4% w/v) and FBS (Gemini Bio-Products; 1% w/v). FITC-dextran (5 mg/mL) was added to the lung perfusate solution to generate the perfusate solution used for alveolar barrier determinations.

Bacterial strain and preparation. *S. aureus* was GFP-expressing USA300 LAC (SA^{GFP}). SA^{GFP} were stored at -80°C in 25% glycerol in autoclaved Luria-Bertani (LB) broth media (MP Biomedicals) and propagated on LB-agar plates containing chloramphenicol (10 ug/mL). Plates were refreshed from frozen stock every 2 weeks. Single colonies of SA^{GFP} were propagated in autoclaved LB media containing chloramphenicol (10 µg/mL) in a shaking incubator at 37°C and 200 rpm (New Brunswick Scientific) for 18 h (stationary growth phase), then the bacterial solution was prepared for intranasal instillation by diluting 1.3 mL in 200 µL of PBS containing Ca²⁺ and Mg²⁺.

Animals. Wild type mice were C57BL/6J (Charles River), and transgenic mice were on a C57BL/6J background. Transgenic strains include: tamoxifen-inducible, *Sftpc-CreER^{T2}* (26) (Jackson Laboratory strain #028054); tamoxifen-inducible, *Ager-CreER^{T2}* (29) (Jackson Laboratory strain #032771); *Cftr^{fl10}* (27) (Case Western Reserve University strain *Cftr^{tm1Cwr}*); and *ROSA^{mT/mG}* (28) (Jackson Laboratory strain #007676). *Cre* was transmitted through female mice, as recommended by the Case Western Reserve University Cystic Fibrosis Mouse Resource Center. Tail biopsy was performed within 2 weeks of age and sent to Transnetyx for genotyping. All transgenic mice were genotyped. To induce *Cre* expression, we gave intraperitoneal injections of 100 µL of tamoxifen solution in sunflower oil at 8 weeks of age. Mice used for experiments were 16-18 weeks old for AWL studies, 18-24 weeks for PCR studies, and 28-31 weeks old for beads and bacterial studies.

Sex as a biological variable. Our study examined male mice because male animals exhibited more extensive GFP fluorescence in the alveolar epithelium by live lung imaging. It is unknown whether the findings are relevant for female mice.

Intranasal instillation. SA^{GFP} were prepared for intranasal instillation and instilled within 10 min of bacterial removal from the incubator. Mice were anesthetized with inhaled isoflurane (4%) and intraperitoneal injections of ketamine (up to 1 mg) and xylazine (up to 0.1 mg). Each mouse was instilled with 30 μ L of SA^{GFP}-containing solution to deliver 1×10^8 CFU per mouse. Instillation quality was recorded at the time of instillation by the performing investigator and considered acceptable for experiments if no loss of instillate was observed. Mice woke from anesthesia within 3 min of instillation. Within 10 min of intranasal instillation, mice were re-anesthetized with inhaled isoflurane (4%) and intraperitoneal injections of ketamine (up to 100 mg/kg) and xylazine (up to 5 mg/kg) for euthanasia and surgical preparation of isolated, perfused lungs.

Preparation of isolated, perfused lungs for imaging. We anesthetized mice with inhaled isoflurane (4%) and intraperitoneal injections of ketamine (up to 100 mg/kg) and xylazine (up to 5 mg/kg), then gave intracardiac injections of heparin (50 units; Mylan) and exsanguinated the mice by cardiac puncture. Using our reported methods (6, 30), we cannulated the trachea, pulmonary artery, and left atrium of the heart, then excised the heart, lungs, and cannulas en bloc. The lungs were positioned to enable micropuncture and imaging of the diaphragmatic surface of the right middle lobe, right caudal lobe, or left lung. Then, the lungs were inflated with room air through the tracheal cannula and perfused through the pulmonary arterial and left atrial cannulas at 0.5-1.0 mL/min with autologous blood diluted in the lung perfusate solution (see **Supplemental Table 3**) and warmed to 37°C. We used in-line pressure transducers (ADInstruments) to maintain constant airway pressure 6 cm H₂O via a continuous positive airway pressure machine (Philips Respironics) and pulmonary artery and left atrial pressures 10 and 3 cm H₂O, respectively, via a roller pump (Ismatec). Portions of the lung surface that were not used for micropuncture and imaging were covered with plastic wrap to prevent desiccation.

Alveolar microinstillation. We hand-beveled glass micropipettes (Sutter Instruments) to micropuncture single alveoli under bright-field microscopy, as we have done previously (6, 30). Micropunctured alveoli were instilled with fluorophores and reagents in solution, resulting in their spread from the micropunctured alveolus to neighboring alveoli. Microinstillations were performed in 1-3 alveoli bordering each imaging field.

Live lung imaging and analysis. By our established methods (6, 30), we viewed alveoli by confocal microscopy (LSM800; Zeiss) with a 20x water immersion objective (NA 1.0; Zeiss) and coverslip. We used bright-field microscopy to randomly select regions of 30-50 alveoli for microinstillation and imaging. All images were acquired as single images using Zen (v.2.6; Zeiss) and recorded as Z-sections. Analyzed images were 4-8 μm below the pleura. Optical thickness was 1 μm for beads imaging and 2 μm for all other experiments, and frame size was 512 x 512 pixels. We established laser, filter, pinhole, and detector settings at the beginning of each imaging experiment to optimize alveolar fluorescence and avoid fluorescence saturation, then maintained the settings for the duration of the experiment. We confirmed absence of bleed-through between fluorescence emission channels. Images were analyzed using ImageJ (NIH; v.2.0.0-rc-69/1.52n). Linear adjustments of brightness and contrast were applied to individual color channels of entire images and equally to all experiment groups. We did not apply downstream processing or averaging.

Determinations of alveolar liquid transport. We used alveolar micropuncture to microinstill alveolar airspaces with AF-dextran in HEPES-buffered vehicle solution as described in *Solutions* and **Supplemental Table 1**. Provided alveolar barrier function is intact, time-dependent loss of fluorophore-tagged dextran fluorescence intensity signals dextran dilution by secreted AWL, while time-dependent increase of dextran fluorescence intensity signals dextran concentration by airspace liquid absorption (5, 6). To measure dextran fluorescence changes, we used ImageJ

software (NIH; v.2.0.0-rc-69/1.52n) to quantify AF-dextran gray levels in alveolar airspaces at 2 and 30 min after dextran microinstillation. To account for minor heterogeneity of AF-dextran fluorescence changes across imaged alveolar regions, AF-dextran gray levels were quantified as a mean of dextran fluorescence in representative alveoli located at the image top left, top right, bottom left, bottom right, and center. Measurement locations were the same across time points. All experiments were followed by determinations of alveolar barrier permeability (see *Determinations of alveolar barrier permeability*). We excluded imaged regions of alveoli from the AF-dextran analysis if: initial AF-dextran gray levels were less than 20; TdTomato fluorescence intensity increased by more than 10% during the imaging period; and in the rare event that permeability determinations showed leak of microvascular fluid into alveolar airspaces.

Determinations of alveolar barrier permeability. To determine alveolar barrier properties, we added FITC-dextran (20 kDa; 5 mg/mL) to the intact lung perfusate solution using our established methods (6, 30). FITC-dextran was circulated in the perfusate for 10 min prior to image acquisition. Details of the FITC-dextran-containing perfusate solution are included in *Solutions* and **Supplemental Table 3**.

Beads quantifications. We microcentrifuged 1 mL of fluorescent microspheres ("beads"; cat. no. FSFR004; Bangs Laboratories, Inc.) and resuspended them in 20 μ L of HEPES-buffered vehicle solution. Beads in HEPES-buffered solution were microinstilled into alveolar airspaces by alveolar micropuncture under bright field microscopy, then we used confocal microscopy to generate image Z-stacks from 30 min to 3 h post-instillation. *ROSA^{mT/mG}* fluorescence delineated alveolar walls. To determine bead group size, we used ImageJ (NIH; v.2.0.0-rc-69/1.52n) software to measure the two-dimensional area of bead groups in single Z-stack images, selecting the Z-stack image in which the bead group was the largest. To define bead group displacement, we used ImageJ to measure the distance from the location of the center of the beads group on

the 30 min image to the location of the center of the beads group on the 3 h image. Lateral displacement was defined as the distance (μm) moved between Z-section images that were generated at the same sub-pleural depth; vertical displacement was defined as the distance (μm) moved between Z-section images generated at different depths, with each Z-section image set at 1 μm apart; and total displacement was the sum of the lateral and vertical displacements.

Bacterial quantification. We added calcein red-orange (10 μM) to the lung perfusate solution for 30 min, then replaced the perfusate with fluorophore-free perfusate solution. Within 1 h of intranasal SA^{GFP} instillation, we identified live alveoli with SA^{GFP} and generated confocal images as Z-sections. Additional Z-sections were generated in the same locations at 4 h post-instillation, then we compared SA^{GFP} fluorescence in the 1 and 4 h post-instillation images at 6 μM below the pleura. We restricted the analysis of SA^{GFP} to alveoli in which airspace diameters were stable from 1-4 h post-instillation. SA^{GFP} were considered to be retained in alveoli if, on the 4 h post-instillation image, they were visible on the same Z-section and located within 5 μM of their original location on the 1 h post-instillation image. SA^{GFP} that merged with calcein fluorescence were considered to be intracellular and excluded from the analysis.

Mouse lung epithelial cell preparation and fluorescence-activated cell sorting (FACS) of AT2 cells from mouse lungs. We applied our standard methods (34, 35) to isolate AT2 cells from lung homogenate. Single cell suspension of mouse lungs was created as previously described (85). Briefly, lungs of euthanized mice were instilled with 1 mL of 15 U/mL Dispase II (ThermoFisher) and incubated with additional 2 mL/mouse of Dispase for 45 minutes at room temperature with agitation. Single cell suspensions were prepared from digested lungs by incubating with DNase I (10 min at 37°C) and red blood lysis buffer (2 min at room temperature) and filtration through 100 μm , 70 μm , and 40 μm filters. For FACS analysis, single cell preparations were incubated with the following antibodies for 45 min at 4°C in DMEM without phenol red + 2% FBS: Rat anti-

mouse CD45 (1:200 dilution, BD Biosciences clone 30-F11), rat anti-mouse CD31 (1:200 dilution; BioLegend clone 390, cat. no. 102434), rat anti-mouse EpCAM (1:500 dilution, BioLegend clone G8.8), and rat anti-mouse integrin β 4 (1:75 dilution, BD Biosciences clone 346-11A). Cells were then washed twice with 1X PBS and flash-frozen for RNA isolation. Sorting and subsequent analyses were performed using BD FACS Aria cytometers and FlowJo software.

qPCR of Cfr RNA. RNA was extracted from cells using PicoPure RNA Isolation Kit (Applied Biosystems cat. no. KIT0204). cDNA was synthesized from total RNA using SuperScript Strand Synthesis System (ThermoFisher cat. no. 18080044). qPCR was performed using SYBR Green (ThermoFisher cat. no. F415L). Relative gene expression levels were defined using the $\Delta\Delta C_t$ method. qPCR primers were acquired from Integrated DNA Technologies and are listed in Table S2.

Statistics. Statistics are indicated in figures and legends. In general, paired comparisons were analyzed using two-tailed *t* tests, and multiple comparisons were made using ANOVA with post hoc testing. We considered statistical significance at $p < 0.05$. Data were analyzed and figures were prepared using Microsoft Excel, StatPlus:mac Pro (AnalystSoft, Inc., Build 7.5.0.0/Core v7.6.11), and SigmaPlot (Systat, version 14.5).

Study approval. The Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai approved the animal procedures.

Data availability. All data are available in the manuscript, supplemental materials, or supporting data values file.

AUTHOR CONTRIBUTIONS

J.L.H. designed the study and was responsible for the overall project. J.L.H., S.S., S.H., D.C., S.T., S.M., and J.K. contributed to data collection and data analysis. All authors contributed to the experimental design and interpretation of results. J.H. wrote the first draft, and all authors edited the manuscript. The order of the co-first authors was determined based on the greater contribution by S.S. to manuscript formatting and editing.

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REFERENCES

1. Matsui H, et al. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell*. 1998;95(7):1005-15.
2. Boucher RC. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu Rev Med*. 2007;58:157-70.
3. Clunes LA, et al. Cigarette smoke exposure induces CFTR internalization and insolubility, leading to airway surface liquid dehydration. *FASEB J*. 2012;26(2):533-45.
4. Lin VY, et al. Excess mucus viscosity and airway dehydration impact COPD airway clearance. *Eur Respir J*. 2020;55(1).
5. Lindert J, et al. Chloride-dependent secretion of alveolar wall liquid determined by optical-sectioning microscopy. *Am J Respir Cell Mol Biol*. 2007;36(6):688-96.
6. Tang S, et al. Rescue of alveolar wall liquid secretion blocks fatal lung injury by influenza-staphylococcal coinfection. *J Clin Invest*. 2023;133(19):e163402.
7. Hook JL, and Kuebler WM. CFTR as a therapeutic target for severe lung infection. *Am J Physiol Lung Cell Mol Physiol*. 2025;328(2):L229-L38.
8. Mercer RR, et al. Cell number and distribution in human and rat airways. *Am J Respir Cell Mol Biol*. 1994;10(6):613-24.
9. Dobbs LG, et al. The great big alveolar TI cell: evolving concepts and paradigms. *Cell Physiol Biochem*. 2010;25(1):55-62.
10. Bastacky J, et al. Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung. *J Appl Physiol (1985)*. 1995;79(5):1615-28.
11. Jiang J, et al. Pleural surface fluorescence measurement of Na⁺ and Cl⁻ transport across the air space-capillary barrier. *J Appl Physiol (1985)*. 2003;94(1):343-52.
12. Engelhardt JF, et al. Expression of the cystic fibrosis gene in adult human lung. *J Clin Invest*. 1994;93(2):737-49.

- 730 13. Regnier A, et al. Expression of cystic fibrosis transmembrane conductance regulator in
731 the human distal lung. *Hum Pathol.* 2008;39(3):368-76.
- 732 14. Folkesson HG, et al. Transcellular water transport in lung alveolar epithelium through
733 mercury-sensitive water channels. *Proc Natl Acad Sci U S A.* 1994;91(11):4970-4.
- 734 15. Dobbs LG, et al. Highly water-permeable type I alveolar epithelial cells confer high water
735 permeability between the airspace and vasculature in rat lung. *Proc Natl Acad Sci U S A.*
736 1998;95(6):2991-6.
- 737 16. Johnson MD, et al. Alveolar epithelial type I cells contain transport proteins and transport
738 sodium, supporting an active role for type I cells in regulation of lung liquid homeostasis.
739 *Proc Natl Acad Sci U S A.* 2002;99(4):1966-71.
- 740 17. Ridge KM, et al. Alveolar type 1 cells express the alpha2 Na,K-ATPase, which contributes
741 to lung liquid clearance. *Circ Res.* 2003;92(4):453-60.
- 742 18. Johnson M, et al. Characteristics of Cl⁻ uptake in rat alveolar type I cells. *Am J Physiol*
743 *Lung Cell Mol Physiol.* 2009;297(5):L816-27.
- 744 19. Jiang X, et al. Adrenergic stimulation of Na⁺ transport across alveolar epithelial cells
745 involves activation of apical Cl⁻ channels. *Am J Physiol.* 1998;275(6):C1610-20.
- 746 20. Frank J, et al. Transforming growth factor-beta1 decreases expression of the epithelial
747 sodium channel alphaENaC and alveolar epithelial vectorial sodium and fluid transport via
748 an ERK1/2-dependent mechanism. *J Biol Chem.* 2003;278(45):43939-50.
- 749 21. McAuley DF, et al. Clinically relevant concentrations of beta2-adrenergic agonists
750 stimulate maximal cyclic adenosine monophosphate-dependent airspace fluid clearance
751 and decrease pulmonary edema in experimental acid-induced lung injury. *Crit Care Med.*
752 2004;32(7):1470-6.
- 753 22. Fang X, et al. Contribution of CFTR to apical-basolateral fluid transport in cultured human
754 alveolar epithelial type II cells. *Am J Physiol Lung Cell Mol Physiol.* 2006;290(2):L242-9.

23. Bove PF, et al. Human alveolar type II cells secrete and absorb liquid in response to local nucleotide signaling. *J Biol Chem*. 2010;285(45):34939-49.
24. Li X, et al. CFTR is required for maximal transepithelial liquid transport in pig alveolar epithelia. *Am J Physiol Lung Cell Mol Physiol*. 2012;303(2):L152-60.
25. Li X, et al. Cystic Fibrosis Transmembrane Conductance Regulator potentiation as a therapeutic strategy for pulmonary edema: a proof-of-concept study in pigs. *Crit Care Med*. 2017;45(12):e1240-e6.
26. Rock JR, et al. Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. *Proc Natl Acad Sci U S A*. 2011;108(52):E1475-83.
27. Hodges CA, et al. Generation of a conditional null allele for *Cftr* in mice. *Genesis*. 2008;46(10):546-52.
28. Muzumdar MD, et al. A global double-fluorescent Cre reporter mouse. *Genesis*. 2007;45(9):593-605.
29. Chung MI, and Hogan BLM. Ager-CreER(T2): a new genetic tool for studying lung alveolar development, homeostasis, and repair. *Am J Respir Cell Mol Biol*. 2018;59(6):706-12.
30. Hook JL, et al. Disruption of staphylococcal aggregation protects against lethal lung injury. *J Clin Invest*. 2018;128(3):1074-86.
31. Mensah EA, et al. Distribution of alveolar type II cells in neonatal and adult rat lung revealed by RT-PCR in situ. *Am J Physiol*. 1996;271(1 Pt 1):L178-85.
32. Weibel ER. On the tricks alveolar epithelial cells play to make a good lung. *Am J Respir Crit Care Med*. 2015;191(5):504-13.
33. Islam MN, et al. F-actin scaffold stabilizes lamellar bodies during surfactant secretion. *Am J Physiol Lung Cell Mol Physiol*. 2014;306(1):L50-7.
34. Kathiriya JJ, et al. Distinct airway epithelial stem cells hide among club cells but mobilize to promote alveolar regeneration. *Cell Stem Cell*. 2020;26(3):346-58 e4.

- 781 35. Kathiriya JJ, et al. Human alveolar type 2 epithelium transdifferentiates into metaplastic
782 KRT5(+) basal cells. *Nat Cell Biol.* 2022;24(1):10-23.
- 783 36. Zhang J, et al. Lung hypoperfusion stimulates liquid absorption in alveoli. *bioRxiv.*
784 2026:2026.06.29.735362.
- 785 37. Czartolomna J, et al. Permeability characteristics of isolated perfused rat lungs. *J Appl*
786 *Physiol (1985).* 1991;70(4):1854-60.
- 787 38. Saldias FJ, et al. Time course of active and passive liquid and solute movement in the
788 isolated perfused rat lung model. *J Appl Physiol (1985).* 1998;85(4):1572-7.
- 789 39. Haies DM, et al. Morphometric study of rat lung cells. I. Numerical and dimensional
790 characteristics of parenchymal cell population. *Am Rev Respir Dis.* 1981;123(5):533-41.
- 791 40. Johnson MD, et al. Functional ion channels in pulmonary alveolar type I cells support a
792 role for type I cells in lung ion transport. *Proc Natl Acad Sci U S A.* 2006;103(13):4964-9.
- 793 41. Lohi H, et al. Functional characterization of three novel tissue-specific anion exchangers
794 SLC26A7, -A8, and -A9. *J Biol Chem.* 2002;277(16):14246-54.
- 795 42. Crandall ED, et al. Effects of terbutaline on sodium transport in isolated perfused rat lung.
796 *J Appl Physiol (1985).* 1986;60(1):289-94.
- 797 43. Effros RM, et al. Fluid reabsorption and glucose consumption in edematous rat lungs. *Circ*
798 *Res.* 1987;60(5):708-19.
- 799 44. Basset G, et al. Significance of active ion transport in transalveolar water absorption: a
800 study on isolated rat lung. *J Physiol.* 1987;384:311-24.
- 801 45. Goodman BE, et al. Evidence for active sodium transport across alveolar epithelium of
802 isolated rat lung. *J Appl Physiol (1985).* 1987;62(6):2460-6.
- 803 46. Sakuma T, et al. Alveolar fluid clearance in the resected human lung. *Am J Respir Crit*
804 *Care Med.* 1994;150(2):305-10.
- 805 47. Lasnier JM, et al. Terbutaline stimulates alveolar fluid resorption in hyperoxic lung injury.
806 *J Appl Physiol (1985).* 1996;81(4):1723-9.

- 807 48. Sakuma T, et al. Beta-adrenergic agonist stimulated alveolar fluid clearance in ex vivo
808 human and rat lungs. *Am J Respir Crit Care Med*. 1997;155(2):506-12.
- 809 49. Fukuda N, et al. Relationship of interstitial fluid volume to alveolar fluid clearance in mice:
810 ventilated vs. in situ studies. *J Appl Physiol (1985)*. 2000;89(2):672-9.
- 811 50. Fang X, et al. Novel role for CFTR in fluid absorption from the distal airspaces of the lung.
812 *J Gen Physiol*. 2002;119(2):199-207.
- 813 51. Kaestle SM, et al. Nitric oxide-dependent inhibition of alveolar fluid clearance in
814 hydrostatic lung edema. *Am J Physiol Lung Cell Mol Physiol*. 2007;293(4):L859-69.
- 815 52. Peteranderl C, et al. Macrophage-epithelial paracrine crosstalk inhibits lung edema
816 clearance during influenza infection. *J Clin Invest*. 2016;126(4):1566-80.
- 817 53. Martens CJ, and Ballard ST. Effects of secretagogues on net and unidirectional liquid
818 fluxes across porcine bronchial airways. *Am J Physiol Lung Cell Mol Physiol*.
819 2010;298(2):L270-6.
- 820 54. Burch GE. Rate of water and heat loss from the respiratory tract of normal subjects in a
821 subtropical climate. *Arch Intern Med (Chic)*. 1945;76:315-27.
- 822 55. Zielinski J, and Przybylski J. [How much water is lost during breathing?]. *Pneumonol*
823 *Alergol Pol*. 2012;80(4):339-42.
- 824 56. Luan X, et al. Cystic fibrosis swine fail to secrete airway surface liquid in response to
825 inhalation of pathogens. *Nat Commun*. 2017;8(1):786.
- 826 57. Kim YH, et al. Alveolar epithelial cell injury due to zinc oxide nanoparticle exposure. *Am J*
827 *Respir Crit Care Med*. 2010;182(11):1398-409.
- 828 58. Kumar A, et al. Innate immune response of corneal epithelial cells to *Staphylococcus*
829 *aureus* infection: role of peptidoglycan in stimulating proinflammatory cytokine secretion.
830 *Invest Ophthalmol Vis Sci*. 2004;45(10):3513-22.
- 831 59. Lin WC, et al. Epithelial membrane protein 2 governs transepithelial migration of
832 neutrophils into the airspace. *J Clin Invest*. 2020;130(1):157-70.

- 833 60. Brand P, et al. Intrapulmonary distribution of deposited particles. *J Aerosol Med.*
834 1999;12(4):275-84.
- 835 61. Heyder J. Deposition of inhaled particles in the human respiratory tract and consequences
836 for regional targeting in respiratory drug delivery. *Proc Am Thorac Soc.* 2004;1(4):315-20.
- 837 62. Bedrossian CW, et al. The lung in cystic fibrosis. A quantitative study including prevalence
838 of pathologic findings among different age groups. *Hum Pathol.* 1976;7(2):195-204.
- 839 63. Lam J, et al. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within
840 infected lungs in cystic fibrosis. *Infect Immun.* 1980;28(2):546-56.
- 841 64. Baltimore RS, et al. Immunohistopathologic localization of *Pseudomonas aeruginosa* in
842 lungs from patients with cystic fibrosis. Implications for the pathogenesis of progressive
843 lung deterioration. *Am Rev Respir Dis.* 1989;140(6):1650-61.
- 844 65. Moller LV, et al. *Haemophilus influenzae* in lung explants of patients with end-stage
845 pulmonary disease. *Am J Respir Crit Care Med.* 1998;157(3 Pt 1):950-6.
- 846 66. Bjarnsholt T, et al. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic
847 fibrosis patients. *Pediatr Pulmonol.* 2009;44(6):547-58.
- 848 67. Ulrich M, et al. Alveolar inflammation in cystic fibrosis. *J Cyst Fibros.* 2010;9(3):217-27.
- 849 68. Malet K, et al. Intracellular *Pseudomonas aeruginosa* within the airway epithelium of cystic
850 fibrosis lung tissues. *Am J Respir Crit Care Med.* 2024;209(12):1453-62.
- 851 69. Black PN, et al. Changes in elastic fibres in the small airways and alveoli in COPD. *Eur*
852 *Respir J.* 2008;31(5):998-1004.
- 853 70. Abraham T, and Hogg J. Extracellular matrix remodeling of lung alveolar walls in three
854 dimensional space identified using second harmonic generation and multiphoton
855 excitation fluorescence. *J Struct Biol.* 2010;171(2):189-96.
- 856 71. Polverino F, et al. A novel nonhuman primate model of cigarette smoke-induced airway
857 disease. *Am J Pathol.* 2015;185(3):741-55.

858 72. Sauler M, et al. Characterization of the COPD alveolar niche using single-cell RNA
859 sequencing. *Nat Commun.* 2022;13(1):494.

860 73. Shah VS, et al. Airway acidification initiates host defense abnormalities in cystic fibrosis
861 mice. *Science.* 2016;351(6272):503-7.

862 74. Blum KM, et al. Sex and Tamoxifen confound murine experimental studies in
863 cardiovascular tissue engineering. *Sci Rep.* 2021;11(1):8037.

864 75. Rosenfeld M, et al. Gender gap in cystic fibrosis mortality. *Am J Epidemiol.*
865 1997;145(9):794-803.

866 76. Macklin CC. Pulmonary sumps, dust accumulations, alveolar fluid and lymph vessels. *Acta*
867 *Anat (Basel).* 1955;23(1):1-33.

868 77. Kilburn KH. A hypothesis for pulmonary clearance and its implications. *Am Rev Respir*
869 *Dis.* 1968;98(3):449-63.

870 78. Hill DB, et al. Physiology and pathophysiology of human airway mucus. *Physiol Rev.*
871 2022;102(4):1757-836.

872 79. Perlman CE, and Bhattacharya J. Alveolar expansion imaged by optical sectioning
873 microscopy. *J Appl Physiol (1985).* 2007;103(3):1037-44.

874 80. Iversen NK, et al. The normal acid-base status of mice. *Respir Physiol Neurobiol.*
875 2012;180(2-3):252-7.

876 81. Bekkevold CM, et al. Dehydration parameters and standards for laboratory mice. *J Am*
877 *Assoc Lab Anim Sci.* 2013;52(3):233-9.

878 82. Surwit RS, et al. Diet-induced type II diabetes in C57BL/6J mice. *Diabetes.*
879 1988;37(9):1163-7.

880 83. Tordoff MG, et al. Forty mouse strain survey of voluntary calcium intake, blood calcium,
881 and bone mineral content. *Physiol Behav.* 2007;91(5):632-43.

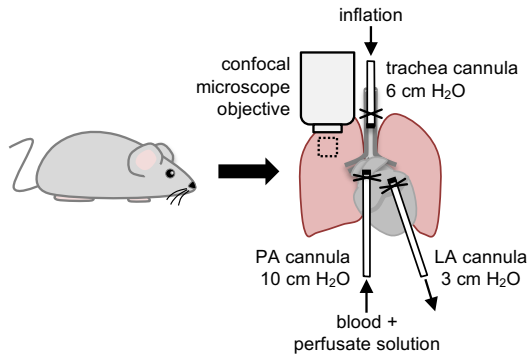
882 84. Otto GP, et al. Clinical chemistry reference intervals for C57BL/6J, C57BL/6N, and
883 C3HeB/FeJ mice (*Mus musculus*). *J Am Assoc Lab Anim Sci.* 2016;55(4):375-86.

884 85. Chapman HA, et al. Integrin alpha6beta4 identifies an adult distal lung epithelial population
885 with regenerative potential in mice. *J Clin Invest*. 2011;121(7):2855-62.
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FIGURES

Figure 1

A



B

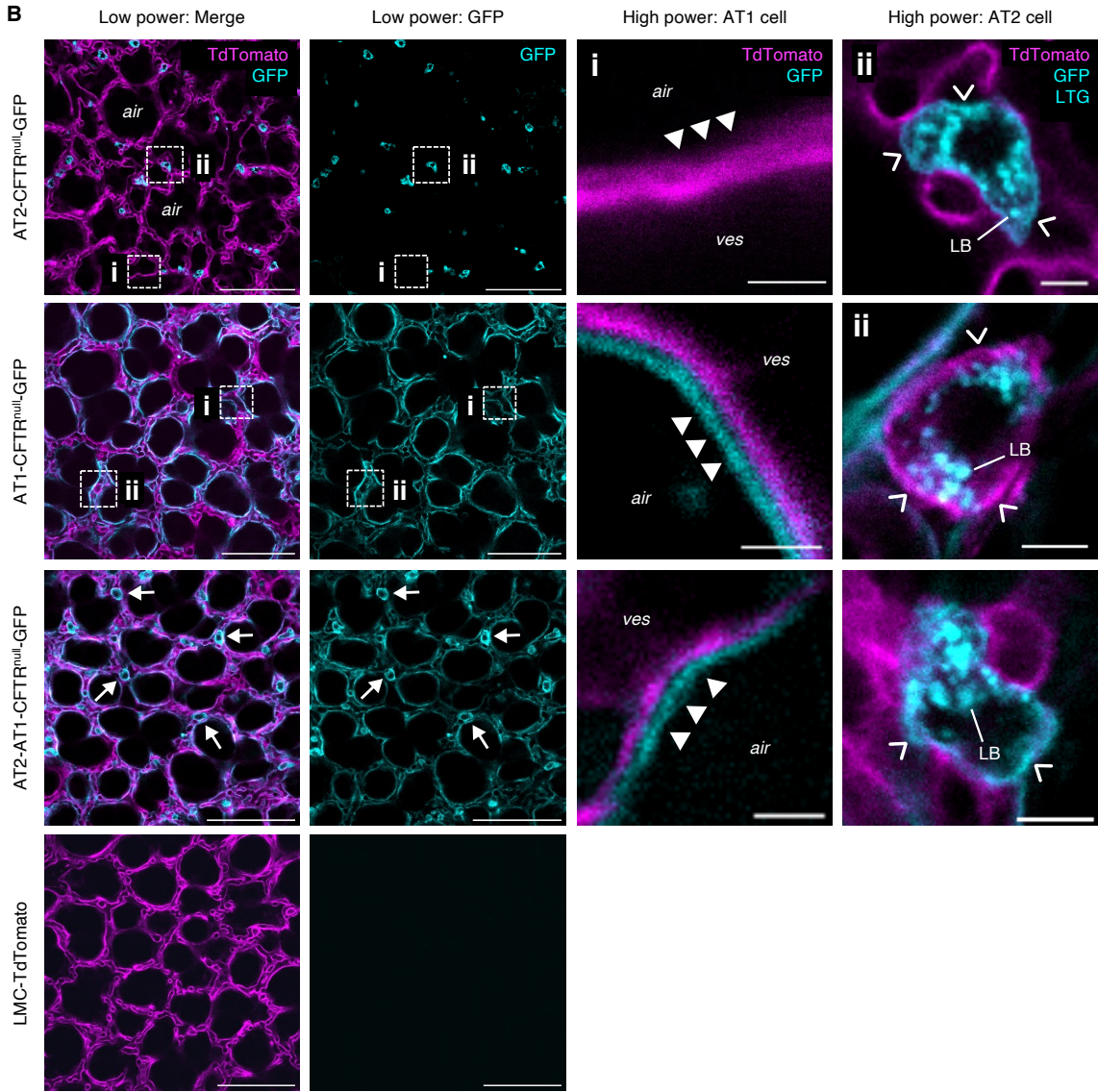


Figure 1. Alveolar epithelial cell type-specificity of GFP expression. (A) The cartoon shows the experimental design of confocal imaging studies of live, intact, perfused lungs of transgenic mice. (B) Confocal images show plasma membrane *GFP* (cyan) and *TdTomato* (magenta) fluorescence in live lungs of mice of the indicated genotype. *Dashed squares* show locations of the high power views shown in the third and fourth columns. *Closed arrowheads* point out AT1 cell fluorescence, which is GFP+ (cyan) and distinct from neighboring TdTomato+ (magenta) endothelial cells only in AT1-CFTR^{null}-GFP and AT2-AT1-CFTR^{null}-GFP mice. *Open arrowheads* show plasma membrane fluorescence of AT2 cells, which is GFP+ (cyan) only in AT2-CFTR^{null}-GFP and AT2-AT1-CFTR^{null}-GFP mice. In the fourth column, cytosolic fluorescence of lysotracker green (*LTG*) delineates lamellar bodies (*LB*) and confirms AT2 cell identity. In the third row, *arrows* show example AT2 cells in an AT2-AT1-CFTR^{null}-GFP mouse. *Air*, airspace; *ves*, microvessel lumen. Scale bars: 100 (*first and second columns*), 3 (*third columns*), and 5 (*fourth columns*) μ m. Images were replicated in lungs of 3 mice per genotype. Note, in the fourth column, both *LTG* and *GFP* are pseudocolored cyan.

Figure 2

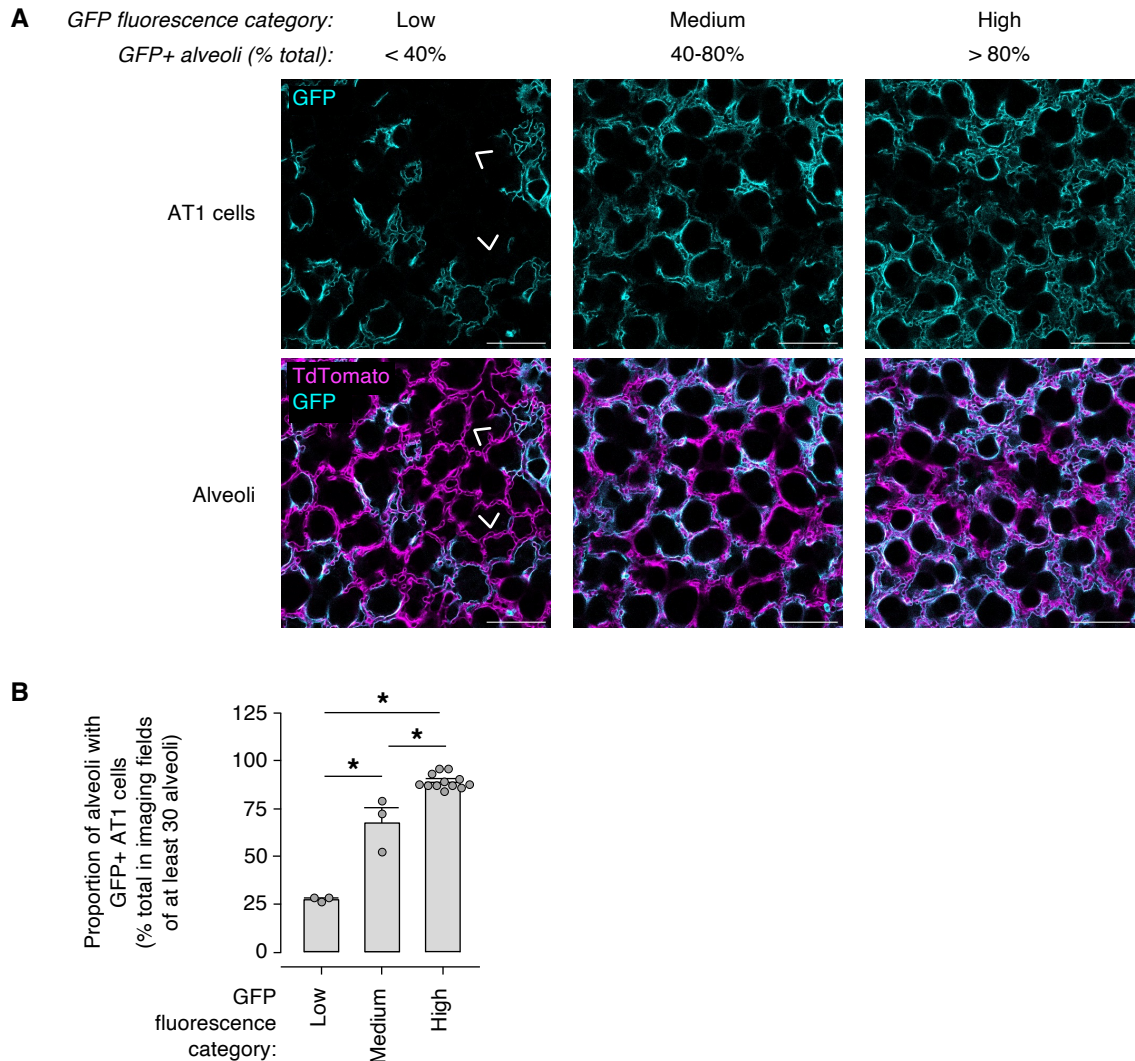


Figure 2. Cre recombination is spatially heterogeneous across alveoli of AT1-CFTR^{null}-GFP mice. (A-B) Confocal images (A) and group data (B) show *GFP* fluorescence (cyan) across imaging fields of at least 30 alveoli in live, perfused lungs of AT1-CFTR^{null}-GFP mice. In the images in A, cyan fluorescence marks Cre-expressing AT1 cells. Note, Cre expression is heterogeneous across alveoli, since some AT1 cells expressed TdTomato, but not GFP, as evident by TdTomato (magenta) fluorescence in GFP-free alveoli (examples: arrowheads). The group data in B quantify the heterogeneity by indicating the proportion of alveoli with GFP+ cells as a percentage of the total number of alveoli per imaging field. Circles in B indicate *n* and each represent one imaging field of at least 30 alveoli. Imaging fields were generated in lungs of 3 mice but presented according to their GFP fluorescence category. Bars: mean $\pm$ SEM; **p* < 0.05 by ANOVA with post hoc Tukey testing. Scale bars: 50 μ m.

Figure 3

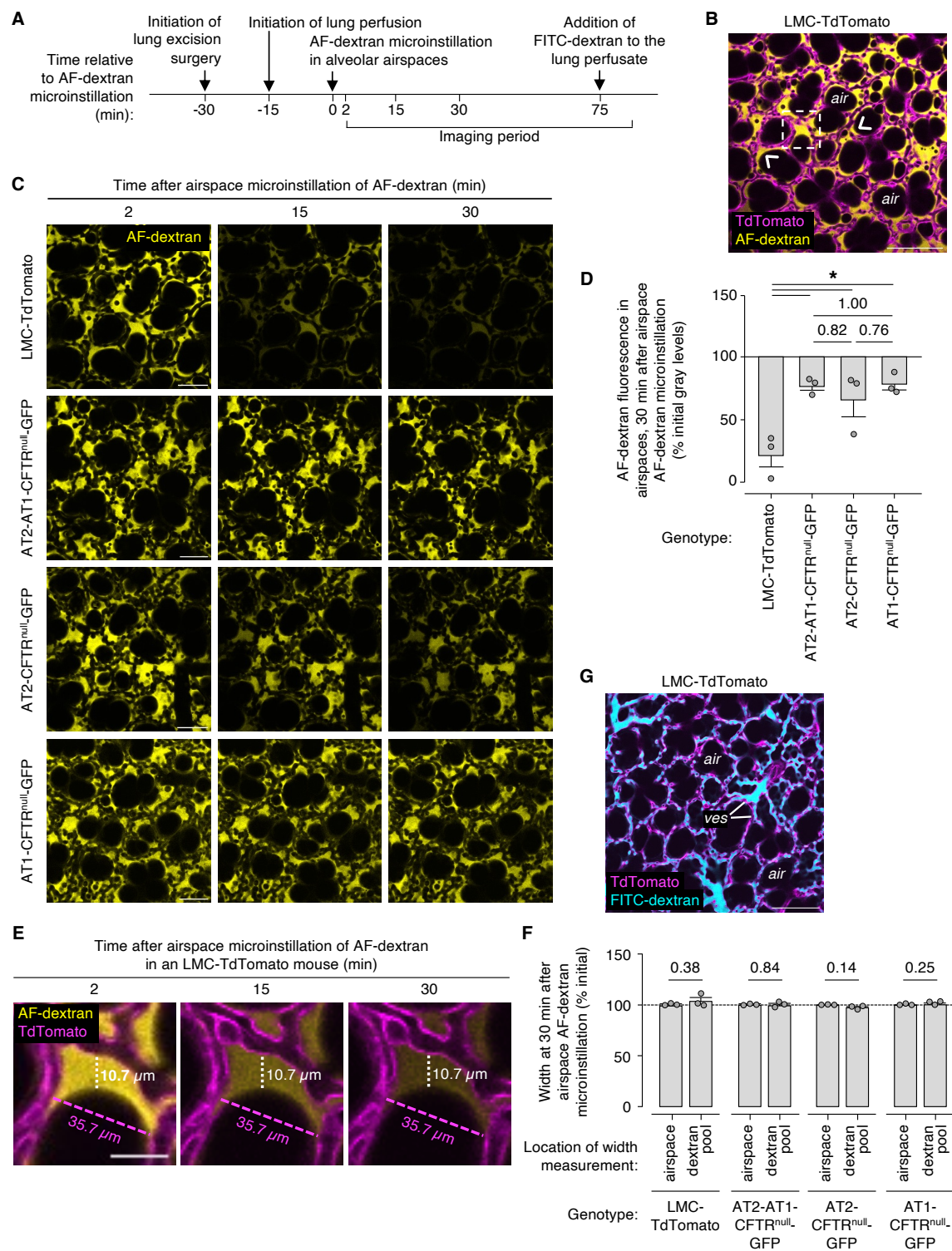


Figure 3. Real-time confocal imaging of liquid secretion in live alveoli. (A-G) Cartoon in **A** shows the design of confocal imaging studies of liquid transport in live alveoli of intact, perfused lungs of transgenic mice of the genotypes indicated in **B-G**. As indicated in **A**, we microinstilled

alveolar airspaces with Alexa Fluor 647-conjugated dextran (*AF-dextran*, yellow, 10 kDa, 10 mg/mL) to carry out the determinations of liquid transport shown in B-F. At the conclusion of each experiment, we added fluorescein isothiocyanate-conjugated dextran (*FITC-dextran*, cyan, 20 kDa, 5 mg/mL) to the lung perfusate solution to define alveolar barrier properties. The confocal image in B shows AF-dextran fluorescence in airspaces of alveoli (*magenta*) of a TdTomato-expressing LMC-TdTomato mouse at 2 min after AF-dextran microinstillation. *Air*, example airspace. *Arrowheads* point out example AF-dextran pools at alveolar niches. Images and group data in C-D show change of AF-dextran fluorescence over time. Fluorescence of alveolar walls is not shown. In D, *circles* indicate *n* and each represent one mouse in which mean fluorescence change was quantified in imaging fields of at least 30 alveoli; *bars*: mean $\pm$ SEM; *p* as indicated or < 0.05 (*) by ANOVA with post hoc Tukey testing. High power views in E show widths of the example AF-dextran pool (*white dashed lines and text*) and airspace (*magenta dashed lines and text*) indicated by the *dashed square* in B. Group data in E show pool and airspace widths over time. In F, *circles* and *bars* are as in D; *p* is as indicated by two-tailed *t* test. The confocal image in G shows fluorescence of FITC-dextran added to the lung perfusate solution. Images in B and G represent images generated in 3 mice each of LMC-TdTomato, AT2-AT1-CFTR^{null}-GFP, AT2-CFTR^{null}-GFP, and AT1-CFTR^{null}-GFP mouse groups. Scale bars: 100 (B), 50 (C,G), 20 (E) μ m.

Figure 4

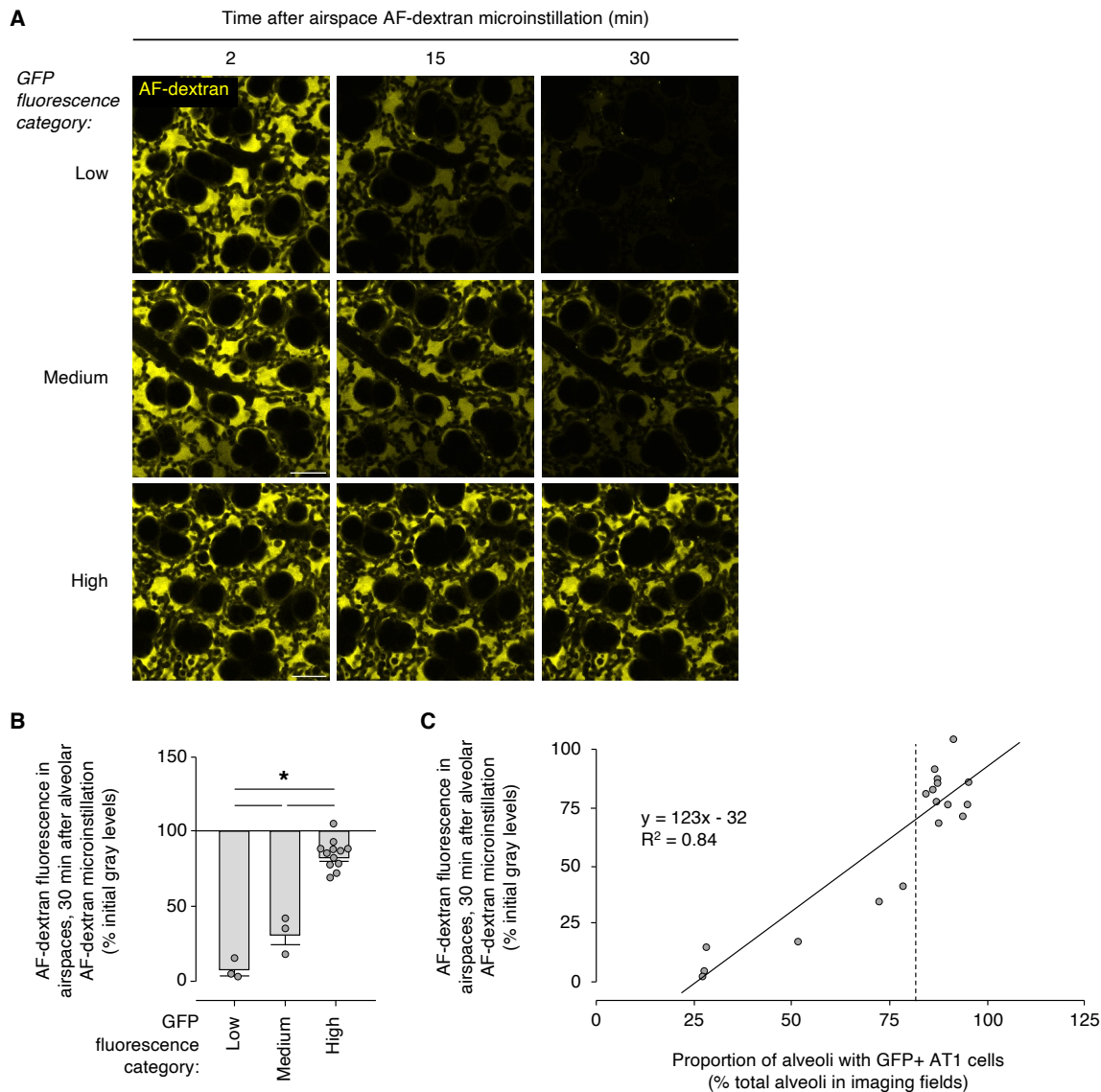


Figure 4. “Dose-dependent” relationship between AT1 cell *Cfr* deficiency and inhibition of AWL secretion in lungs of AT1-CFTR^{null}-GFP mice. (A-C) Confocal images (A) and group data (B-C) show AF-dextran fluorescence (yellow; 10 kDa, 10 mg/mL) after microinstillation in alveolar airspaces of lungs of AT1-CFTR^{null}-GFP mice. Experiments were carried out as indicated in Figure 3A. GFP fluorescence is not shown but was categorized in imaging fields of at least 30 alveoli as Low, Medium, and High as indicated in Figure 2. Images shown in the *bottom row* in A are reproduced from Figure 3C. In B-C, *circles* indicate *n* and each represent one trial of AF-dextran microinstillation in which mean fluorescence change was quantified in an imaging field of at least 30 alveoli. Trials were carried out in lungs of 3 mice but are presented according to the GFP fluorescence category (B) or the proportion of alveoli with GFP+ cells as a percentage of the total number of alveoli (C) in the imaging field. In C, the *dashed line* sets apart the 12 imaging fields in the High GFP fluorescence category, and the *solid line* was calculated by linear regression. Bars: mean ± SEM; **p* < 0.05 by ANOVA with post hoc Tukey testing. Scale bars: 50 μm.

Figure 5

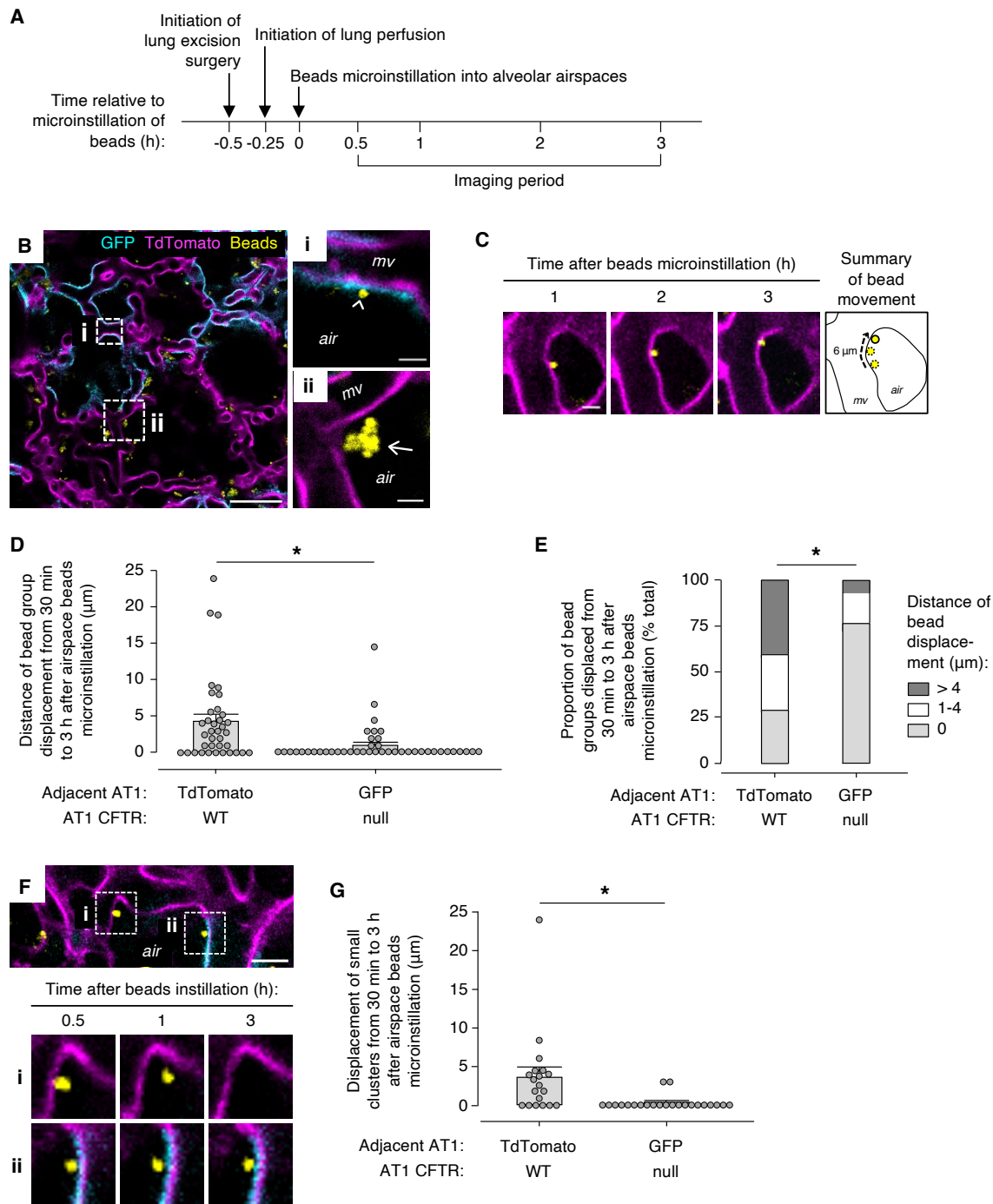


Figure 5. CFTR in AT1 cells promotes particle clearance in alveoli. (A-G) Cartoon in **A** shows the design of the confocal imaging studies shown in B-G. As indicated in A, we microinstilled fluorescent beads (yellow) in airspaces of 3 imaging fields of at least 30 alveoli in live, perfused lungs of an AT1-CFTR^{null}-GFP mouse, then generated images over 2.5 h to define bead location, group formation, and movement. GFP fluorescence of the imaging fields was in the Medium category. Images in **B** show the initial locations and size distributions of example bead groups

that formed within 0.5 h of beads microinstillation. *Dashed squares* show locations of the high-power views shown in *i* and *ii*. The *arrowhead* shows a small cluster of beads located adjacent to a GFP-expressing (*cyan*) flat alveolar septum, while the *arrow* points out a microaggregate located at a TdTomato-expressing (*magenta*) alveolar niche. *mv*, microvessel; *air*, airspace. Images and group data in C-G show spontaneous movement of all bead groups (C-E) or small clusters (F-G) in the 3 imaging fields over the first 3 h after beads microinstillation. Bead groups were categorized according to whether the *adjacent AT1* cell expressed *WT* CFTR (signaled by TdTomato fluorescence) or *CFTR^{null}* (signaled by GFP fluorescence), as indicated. Note in the images in C and F, beads located adjacent to WT CFTR-expressing AT1 cells moved (C and *i* in F), but beads adjacent to *CFTR^{null}*-expressing AT1 cells did not (*ii* in F). In D and G, *circles* indicate *n* and each represent one bead group; *bars*: mean $\pm$ SEM; **p* < 0.05 by two-tailed *t* test. In E, *stacked bars* represent bead groups as proportions of the total number; **p* < 0.05 by Chi-squared test. Scale bars: 30 (B), 3 (*i* and *ii* in B, C), and 15 (F) μ m.

Figure 6

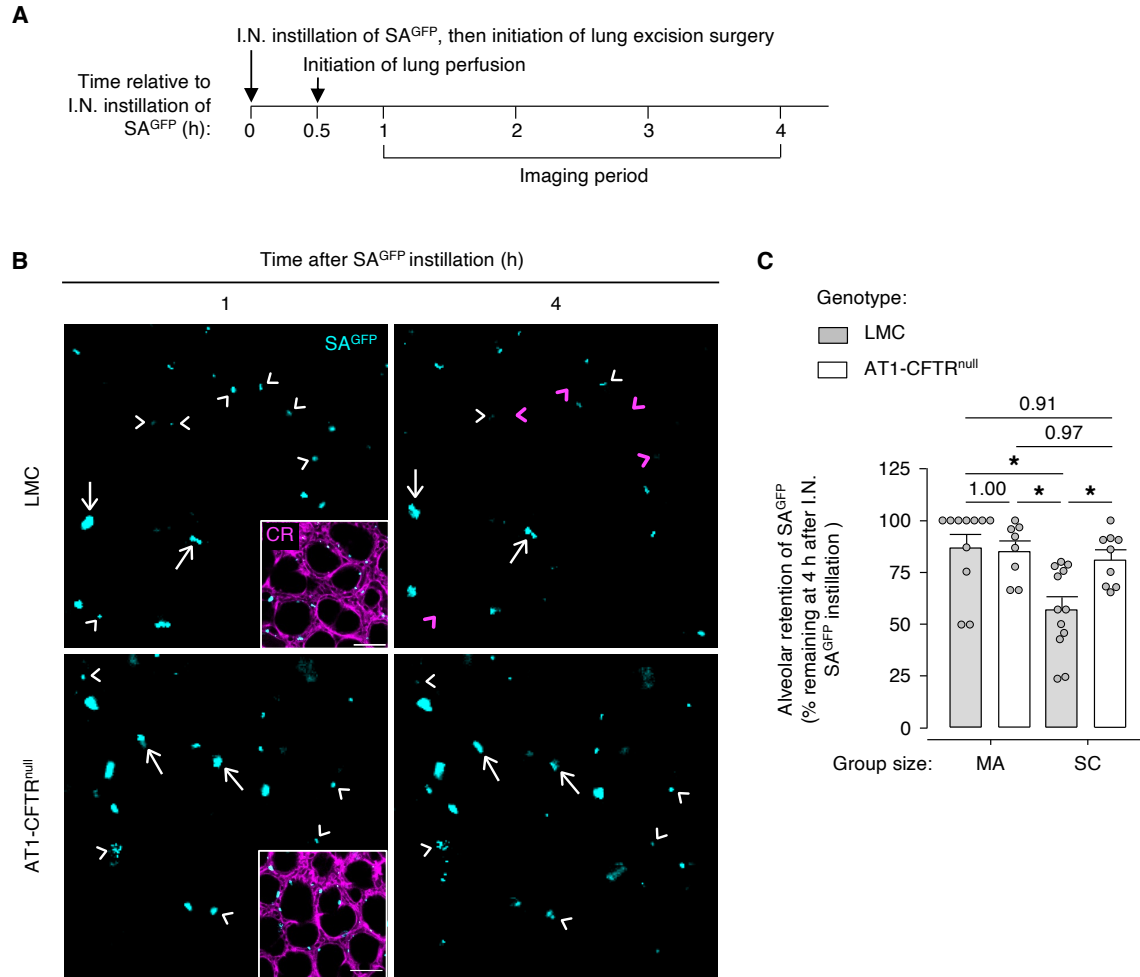


Figure 6. CFTR in AT1 cells promotes bacterial clearance in alveoli. (A-C) The cartoon in A shows the experimental design of the confocal imaging studies of bacterial clearance shown in B-C. As indicated in A, mice were given intranasal instillations (I.N.) of SA^{GFP}, then the lungs were excised for live imaging 10 min later. Confocal images (B) and group data (C) show microaggregates (arrows; MA) and small clusters (arrowheads; SC) of GFP-expressing *S. aureus* (SA^{GFP}) in live alveoli of intact, perfused lungs of LMC or AT1-CFTR^{null} mice as indicated. Calcein red-orange (CR) dye was added to the perfusate solution to delineate alveolar walls (inset). Images included at least 30 alveoli and were generated in the same location at 1 and 4 h post-instillation. In the 4 h images, magenta arrows and arrowheads point out the initial locations of microaggregates and small clusters that were displaced during the imaging period or were not found in the 4 h images. White arrows and arrowheads in the 4 h images indicate microaggregates and small clusters that did not change. In C, circles indicate *n* and each represent the change of all small clusters or microaggregates present in one imaging field from 1 to 4 h after I.N. SA^{GFP} instillation. Imaging fields were generated in the lungs of 3 LMC and 3 AT1-CFTR^{null} mice. Since imaging fields had to be excluded from the analysis if no small clusters or microaggregates were present in the 1 h image, the data are presented on a per-imaging-field basis rather than a per-mouse basis. Bars: mean ± SEM; *p* as indicated or < 0.05 (*) by ANOVA with post hoc Tukey testing. Scale bar: 50 μm.