Fatal COVID-19 ARDS associated with incomplete AEC1 differentiation from the transitional state without senescence or fibrosis

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Abstract

COVID-19 ARDS is associated with prolonged respiratory failure and high mortality, but the underlying mechanisms are unknown. ARDS results from injury to the alveolar epithelial cell (AEC) barrier; clinical recovery requires epithelial regeneration. During physiologic regeneration, AEC2s proliferate, exit the cell cycle, and transiently assume a transitional state before differentiating into AEC1s; transitional cells persist with ineffectual AEC1 differentiation in pulmonary fibrosis. It is unknown why transitional cells differentiate into AEC1s during physiologic regeneration but persist with ensuing scar in fibrosis and whether incomplete AEC1 differentiation from transitional cells without fibrosis may underlie prolonged respiratory failure in COVID-19 ARDS. Immunostaining of postmortem COVID-19 ARDS lungs revealed abundant transitional cells. They were typically cuboidal or partially spread, occasionally flat, but rarely expressed AEC1 markers. They formed organized monolayers on alveolar septa without fibrosis. Immunostaining and/or meta-analysis of scRNAseq datasets revealed that transitional cells in two mouse models of physiologic regeneration, COVID-19 ARDS, and fibrosis express markers of cell cycle exit but only in fibrosis express a specific senescence marker. These data suggest that in COVID-19 ARDS, physiologic AEC1 differentiation from transitional cells is incomplete, thus underlying prolonged barrier permeability and respiratory failure, but as in physiologic regeneration, is ongoing without fibrosis.

Introduction

COVID-19 remains a global scourge, claiming thousands of lives daily and straining healthcare systems worldwide. In its most severe form, COVID-19 manifests as the acute respiratory distress syndrome (ARDS). COVID-19 ARDS causes acute respiratory failure associated with prolonged ventilator dependence and high mortality rates (1-8). However, the underlying mechanism by which COVID-19 ARDS results in prolonged ventilator dependence and high mortality is unknown.

Critical to the pathogenesis of ARDS is injury to the alveolar epithelium, which forms a tight barrier that maintains dry airspaces and permits gas exchange (9). The alveolar epithelium is comprised of AEC1s and AEC2s. AEC1s cover >95% of the alveolar surface, thus playing a critical role in barrier integrity, and are exquisitely thin, permitting efficient gas exchange (10, 11). AEC2s, are cuboidal, produce surfactant, and serve as progenitors (12). During ARDS, AEC death results in barrier permeability, leading to flooding of the airspaces with proteinaceous edema fluid, which in turn causes severe hypoxemia necessitating mechanical ventilation and death (9). Conversely, regeneration of the alveolar epithelium is critical for the restoration of barrier integrity, resolution of edema, liberation from the ventilator, and survival (13, 14). Therefore, we speculated that incomplete alveolar regeneration may underlie the prolonged ventilator dependence and high mortality rates observed in COVID-19 ARDS.

The principal progenitor responsible for alveolar regeneration is the AEC2 (12, 15-17), though other progenitors may contribute (18-20). AEC2s proliferate and then differentiate into AEC1s to restore normal alveolar architecture and barrier integrity. Using a mouse model of physiologic regeneration, we made the seminal discovery that AEC2-to-AEC1 differentiation occurs in a nongradual manner (21). After proliferation, AEC2s exit the cell cycle and assume a transitional state before differentiating into AEC1s. The transitional state is characterized by markers of cell cycle exit, downregulation of AEC2 markers, a cuboidal or partially spread morphology, and expression of unique signature genes including *Krt8*. This transitional state was subsequently identified in diverse mouse models of lung injury, suggesting that it is a conserved stage of regeneration regardless of the cause of injury (22-25). During physiologic regeneration in mice, the transitional state is transient; lineage tracing studies confirmed that transitional cells differentiate into AEC1s, restoring normal alveolar architecture and function (10, 21, 22, 26) (Graphic Abstract). Which stage of alveolar regeneration, AEC2 proliferation, transitional, or AEC1 differentiation, may be impaired in COVID-19 is unknown. The histology of COVID-19 ARDS is classic diffuse alveolar damage (DAD) and includes AEC2 hyperplasia (27-30), suggesting that proliferation may be preserved. Therefore, we speculated that the prolonged barrier dysfunction and poor clinical outcomes in COVID-19

ARDS may be specifically attributable to incomplete conversion of AEC2s into the transitional state or differentiation of transitional cells into AEC1s.

Idiopathic pulmonary fibrosis (IPF) is a fatal disease caused by irreversible scarring of the lungs (22-26, 31, 32). Pulmonary fibrosis is widely believed to arise from impaired alveolar regeneration after repetitive injury, but the specific regenerative defect underlying the pathogenesis of pulmonary fibrosis has remained elusive. We and others recently discovered that IPF is characterized by an abundance of transitional cells with a paucity of mature AEC1s, suggesting that ineffectual differentiation of transitional cells into AEC1s may be the specific regenerative defect underlying the pathogenesis of fibrosis. Since the transitional state has a transcriptomic signature of cell cycle exit (21, 22, 31, 32), the persistence of transitional cells in IPF is reminiscent of prior literature suggesting that epithelial cells in IPF are senescent (33-37). Recent case reports identified transitional AECs in the fibrosis that develops after months of nonresolving COVID-19 ARDS, so-called “fibroproliferative ARDS” (38, 39). In these cases, the transitional cells exist in the milieu of extensive matrix deposition and architectural distortion that was deemed irreversible, necessitating lung transplantation. In fact, the presence of transitional cells on biopsy was proposed as a potential biomarker of irreversible fibrosis (38). However, it is unknown whether transitional cells are inevitably associated with fibrosis in humans. Moreover, why transitional cells differentiate into AEC1s to restore normal alveolar architecture and function in certain circumstances, while they persist and may beget fibrosis in others, is a fundamental unanswered question in the field.

We hypothesized that in COVID-19 ARDS, after severe epithelial injury, AEC2s proliferate and assume the transitional state, but that AEC1 differentiation from the transitional state is incomplete, resulting in prolonged barrier permeability, pulmonary edema, ventilator dependence, and mortality. We further speculated that in early human ARDS, as in mouse models of physiologic regeneration, proliferating AEC2s exit the cell cycle and *transiently* adopt the transitional state but retain the capacity to differentiate into AEC1s, restoring normal alveolar architecture without fibrosis, whereas in IPF and fibroproliferative ARDS, AECs undergo *permanent* cell cycle arrest, or senescence, losing capacity for an AEC1 fate, and fibrosis ensues (22, 31-36). To explore these hypotheses, we obtained postmortem lung tissue of COVID-19 ARDS patients who died of acute respiratory failure within 14 days of presentation. Tissue was examined for evidence of AEC2 proliferation, transitional cells, AEC1 differentiation, senescence, and fibrosis. Using scRNAseq datasets, we compared the gene expression profiles of transitional cells in two mouse models of physiologic regeneration without fibrosis, lipopolysaccharide (LPS) and pneumonectomy, human COVID-19 ARDS, and human IPF, focusing on markers of cell cycle exit and senescence. The findings advance our basic understanding of physiologic and pathologic alveolar regeneration and have implications for clinical prognosis and management. Ultimately, investigation of the cellular and

molecular mechanisms underlying ineffectual alveolar regeneration in ARDS and fibrosis may lead to novel therapies to promote physiologic regeneration, thus accelerating restoration of barrier integrity, resolution of edema, liberation from the ventilator, and survival in ARDS and preventing fibrosis in fibroproliferative ARDS and IPF.

Results

Clinical Presentation

All patients presented with progressive hypoxemia and radiographic evidence of pulmonary edema, leading to acute respiratory failure refractory to maximal ventilatory support and death (Supplemental Figure 1, Supplemental Table, Supplemental Text).

Severe Epithelial Damage in COVID-19 ARDS

To characterize epithelial injury in COVID-19 ARDS, we first examined histologic lung sections stained with hematoxylin and eosin (H&E). Consistent with prior reports (27-30), the histology of COVID-19 ARDS was acute DAD. There was diffuse airspace filling with edema, fibrin, and hyaline membranes (Figure 1A-D, Supplemental Figures 2,3), indicative epithelial permeability. Interstitial and alveolar inflammation was present. Notably, normal septal architecture was preserved (Supplemental Figure 2B). Desquamated epithelial cells with elongated morphology were observed (Figure 1D, Supplemental Figure 3B2). Such cells are commonly designated as AEC1s (40) but are often, and in this case were, thicker and cover less surface area than AEC1s (41), raising doubt as to their identity. Regardless, the radiographic and histologic findings observed in COVID-19 ARDS revealed the influx of proteinaceous edema into the alveolar space, indicating permeability of the alveolar epithelium.

Epithelial permeability can be due to cell death and/or paracellular permeability. To assess the extent of structural damage to AECs, we stained sections for AEC1 and AEC2 markers. AEC1 markers appeared diffuse and speckled throughout the lungs, consistent with extensive AEC1 injury (Figure 1E, Supplemental Figure 4). Notably, the septa were thickened, likely due to the presence edema and inflammatory cells in the interstitial space (asterisks in Figure 1E, Supplemental Figure 4). Small defects in staining were frequent (Figure 1E2). Complete absence of staining from one or both sides of the alveolar septa were infrequent (Figure 1F, Supplemental Figure 5) and were interpreted as extensive

AEC1 loss resulting in denuded septa. Staining for the AEC2 marker surfactant protein (SP) C revealed vast areas devoid of mature AEC2s with only rare AEC2s remaining (Figure 1G, Supplemental Figure 6). The absence of SPC+ cells may indicate AEC2 death or SPC downregulation. Taken together, the clinical, radiographic, and histologic data indicated severe, extensive epithelial damage, resulting in alveolar edema, ultimately leading to respiratory failure.

AEC2s Proliferate and Assume the KRT8^{hi} Transitional State in COVID-19 ARDS

To assess whether surviving AEC2s are mobilized to regenerate the injured epithelium, we first examined histologic sections stained with H&E. We observed hyperplastic cuboidal epithelial cells (Figure 2A). Hyperplasia of cuboidal epithelial cells is a common histologic feature of DAD and has historically been termed “AEC2 hyperplasia” (40). Indeed, based on SPC staining, we did observe some hyperplastic AEC2s (Figures 2B, Supplemental Figure 7). The hyperplastic AEC2s were hypertrophic, consistent with previous observations in mouse models of lung injury (42). However, AEC2 hyperplasia was rare amidst vast areas devoid of mature AEC2s (Figures 1G, Supplemental Figures 6,7), suggesting that the majority of the hyperplastic epithelial cells observed by H&E staining do not retain the AEC2 phenotype. Regardless, the presence of hyperplastic cuboidal epithelial cells suggested that AEC2s had successfully proliferated to replace damaged AECs (Graphic Abstract).

To determine whether regenerating AEC2s were able to assume the transitional state, we stained sections for the transitional state marker KRT8. KRT8^{hi} transitional cells were abundant throughout the COVID-19 lungs but absent in normal lungs (Figure 2C, Supplemental Figures 8,9). [AEC1s and AEC2s in normal lungs express KRT8 at much lower levels than transitional cells in injured lungs (Supplemental Figure 10) (21-26, 31, 32).] Some KRT8^{hi} cells were cuboidal; some existed as single cells while others were doublets or hyperplastic suggesting recent cell division (Figure 2C, Supplemental Figure 8). However, the morphology of transitional cells was often partially spread and occasionally flat approaching AEC1 morphology (Figure 2C4-5). The cuboidal hyperplastic, partially spread, and flat KRT8^{hi} cells typically existed as monolayers along structurally normal septa (Figure 2C, Supplemental Figure 8), consistent with organized proliferation and ongoing differentiation into AEC1s. Occasional transitional cells were hypertrophic, bizarrely-shaped, and disorganized (Figure 2C6, Supplemental Figure 9). KRT8^{hi} transitional cells did not express SPC (Figure 2D), consistent with known downregulation of AEC2 markers as cells assume the transitional state (21-25, 31, 32). Moreover, transitional cells were abundant in areas devoid of SPC+ cells (Figure 2D). In rare areas of hyperplastic mature AEC2s, KRT8^{hi} SPC- transitional cells coexisted (Figure 2D), suggesting that while some AEC2s proliferate, others assume the transitional

state. [Although transitional cells may arise from club-like progenitors (18, 23, 31, 32), bronchiolization and honeycomb cysts were not observed (Supplemental Figure 2), and transitional cells did not express the club cell marker CCSP (Supplemental Figure 11).]. Taken together, these data demonstrate that transitional cells arise in early human ARDS. The abundance of hyperplastic transitional cells (Figure 1G, Supplemental Figure 6) suggests that the paucity of SPC+ cells represents not massive AEC2 death but successful acquisition of the transitional state. Thus, the progressive edema, prolonged ventilator dependence and high mortality observed in COVID-19 ARDS are not due to an inability of AEC2s to proliferate or assume the transitional state (Graphic Abstract).

Incomplete Differentiation of Transitional Cells into Mature AEC1s in COVID-19 ARDS

Since progenitors successfully proliferated and assumed the transitional state, we hypothesized that incomplete differentiation of transitional cells into mature AEC1s may underlie the persistent barrier permeability and poor clinical outcomes in COVID-19. Supporting this hypothesis, although the transitional cells appeared to be in the process of differentiating into AEC1s, they were typically partially spread (Figure 2C3,4), only occasionally assuming the flat morphology of AEC1s (Figure 2C5). To further elucidate the extent to which the transitional cells differentiated into AEC1s, we costained lung sections for KRT8 and AEC1 markers. In most cases, transitional cells expanded along alveolar septa filling gaps denuded of AEC1s (Figure 3A,B, Supplemental Figures 12-15). Again, most cells were partially spread (open white arrowheads in Figure 3A), but even those with a flat morphology rarely expressed AEC1 markers (closed white arrowheads in Figure 3A). In areas of severe injury, transitional cells occasionally spread on top of damaged AEC1s (open green arrowheads in Figure 3B). Flat cells expressing AEC1 markers but not KRT8 were interpreted as native AEC1s that withstood injury (closed green arrowheads in Figure 3B), although we cannot exclude nascent AEC1s that downregulated KRT8 after differentiation. Rarely, transitional cells assumed bizarre morphologies and/or sloughed off the septa into the airspaces (Figure 3C, Supplemental Figure 16). The predominant organization of transitional cells in a monolayer on alveolar septa filling gaps denuded of AEC1s and displaying increasingly spread morphologies without AEC1 marker expression suggested that differentiation along a linear trajectory into mature AEC1s is ongoing but incomplete (Figure 3A; Supplemental Figure 12,13). Thus, the progressive edema, prolonged ventilator dependence and high mortality observed in COVID-19 ARDS may be due to incomplete differentiation of transitional cells into AEC1s (Graphic Abstract).

Absence of Fibrosis and AEC Senescence in COVID-19 ARDS

In humans, persistence of the transitional state has previously been observed only in the setting of fibrosis (22, 23, 25, 26, 31, 32, 38, 39). However, in mouse models of physiologic regeneration such as LPS and pneumonectomy, transitional cells appear transiently and then differentiate into AEC1s to restore normal alveolar architecture without fibrosis (21, 24). It is unknown whether transitional cells are inevitably associated with and/or cause fibrosis in humans and thus whether fibrosis may be the cause of acute respiratory failure in COVID-19 ARDS (38, 39). We hypothesized that in early COVID-19 ARDS, transitional cells, which appear to be in the process of physiologic AEC1 differentiation AEC1s (Figure 3A), exist without fibrosis. Indeed, in contrast to IPF, we observed no excessive collagen deposition, myofibroblast accumulation, or architectural distortion in COVID-19 ARDS (Figure 4A,B, Supplemental Figures 2B, 17-20). Moreover, whereas in IPF the transitional cells overlie fibroblastic foci (Figure 4C) and line honeycomb cysts (25), the transitional cells in COVID-19 ARDS overlie structurally normal alveolar septa (Figure 3). Finally, in COVID-19 ARDS, nascent AEC1s appear to be emerging from transitional cells (Figure 2C5, 3A), whereas IPF is characterized by a paucity of AEC1s (Figure 4B).

It is unknown why in mouse models of physiologic alveolar regeneration and human ARDS, transitional cells appear capable of differentiating into AEC1s with restoration normal alveolar architecture, whereas in IPF, transitional cells persist with a paucity of AEC1s and fibrosis ensues. To confirm that transitional cells were highly conserved across diverse mouse models of physiologic alveolar regeneration, human ARDS, and human IPF, we assessed expression of transitional state markers by immunostaining and meta-analysis of existing single cell RNA sequencing (scRNAseq) datasets. Transitional cells in the LPS and PNX mouse models of physiologic alveolar regeneration, human ARDS, and human IPF shared the unique gene expression signature of the transitional state (Figure 5A,B). That the transcriptome of the transitional state was highly conserved across two mouse models of physiologic alveolar regeneration and IPF underscores the enigma of the vastly divergent pathologic outcomes. During physiologic regeneration, transitional cells emerge as proliferating AEC2s exit the cell cycle and express markers of cell cycle arrest, but ultimately differentiate into AEC1s (21, 24), whereas AECs in IPF have previously been identified as senescent (31-36, 38). Therefore, we speculated that the critical difference underlying the divergent outcomes between physiologic regeneration and fibrosis may be that while transitional cells exist in a transient state of cell cycle arrest in physiologic regeneration, they assume a permanent state of cell cycle arrest, or senescence, in fibrosis. To confirm that transitional cells in fibrosis but not mouse or human ARDS are senescent, we assessed expression of general markers of CCA, p21 (CDKN1A), p15 (CDKN2B), p53 (TP53), and cyclin D1 (CCND1) and a more specific marker of senescence, p16/CDKN2A (43), in mouse models,

human ARDS, and human IPF. We found that general markers of CCA are expressed in all three conditions (Figure 5B,C), but p16 is expressed only in IPF (Figure 5D,E). Taken together, these data suggest that in mouse and human ARDS, proliferating AEC2s undergo cell cycle exit and adopt the transitional state *transiently* before differentiating into AEC1s to restore normal alveolar architecture, whereas, in fibrosis, transitional AECs become *permanently* arrested, or senescent, losing capacity for an AEC1 fate and begetting fibrosis (Graphic Abstract).

Discussion

In summary, the lungs of COVID-19 ARDS patients who died of acute respiratory failure were characterized by extensive epithelial damage and a regenerative response in which AEC2s proliferated and assumed the transitional state. Residual mature AEC2s were rare. Some transitional cells were hyperplastic and cuboidal. However, most displayed a partially spread morphology, filling gaps on alveolar septa denuded of AEC1s, suggesting they were in the process of AEC1 differentiation. However, the transitional cells rarely assumed a flat AEC1 morphology and almost never expressed AEC1 markers, suggesting that AEC1 differentiation is incomplete. Rarely, the transitional cells appeared to have sloughed off into the airspaces. However, similar to mouse models of physiologic regeneration and in contrast to IPF, transitional cells existed on structurally normal alveolar septa without fibrosis and expressed markers of cell cycle exit but not senescence. Taken together, these data suggest that AEC1 differentiation from the transitional state with restoration of normal alveolar architecture and barrier integrity is ongoing but incomplete in early fatal COVID-19 ARDS, whereas impaired AEC1 differentiation and ensuing fibrosis are irreversible in IPF.

The cause of prolonged ventilator dependence and high mortality in COVID-19 ARDS has remained elusive. In ARDS, injury to the alveolar epithelial barrier results in pulmonary edema, which causes severe hypoxemia necessitating mechanical ventilation and death (9). Conversely, epithelial repair is associated with clinical recovery and survival (13, 14). However, whether poor clinical outcomes in COVID-19 ARDS may be linked to ineffectual alveolar regeneration and if so, the specific stage of alveolar regeneration that is ineffectual, are unknown. Our data reveal that the ability of progenitors to proliferate and assume the transitional state is preserved but AEC1 differentiation from the transitional state is incomplete. We propose that incomplete AEC1 differentiation from the transitional state may result in persistent barrier permeability, ongoing pulmonary edema, prolonged ventilator dependence, and high mortality in COVID-19 ARDS. In fact, we suspect that the pathology observed here is common to severe ARDS regardless of etiology. Since the histology of COVID-19 ARDS is classic DAD [Figs. 1A-D, Supplemental Figs. 2,3 and (27, 29, 30)], immunostaining of ARDS lungs would likely yield similar results regardless of underlying etiology. In addition, although initial studies suggested astronomical mortality rates for COVID-19 ARDS (8), and COVID-19 is certainly more likely to progress to ARDS than other viral infections, new evidence indicates that clinical outcomes of COVID-19 ARDS may be equivalent to those of ARDS from other etiologies (44). The sheer number of patients with COVID-19 ARDS and the availability of postmortem lung tissue has provided an unprecedented opportunity to study ARDS in general. Since ARDS will remain a prevalent and devastating cause of morbidity and mortality long beyond the COVID-19 pandemic (9), we believe the findings presented here will have enduring impact. Alveolar regeneration is the subject of active investigation in the context of

fibrosis but rarely in the context of barrier restitution in ARDS. We propose that incomplete AEC1 differentiation from the transitional state may underlie the persistent barrier permeability, pulmonary edema, refractory hypoxemia, prolonged ventilator dependence, and mortality in COVID-19 ARDS and likely in severe ARDS of other etiologies.

Although AEC1 differentiation from the transitional state was insufficient to restore barrier integrity, the appearance of cuboidal, partially spread, and flat transitional cells organized in a monolayer on structurally normal alveoli without fibrosis suggests that physiologic AEC1 differentiation was ongoing, although this has not been proven. Bizarrely shaped, disorganized, and/or desquamated transitional cells were present but rare. That transitional cells may retain the capacity for AEC1 differentiation with restoration of normal alveolar architecture and barrier integrity, as occurs in mouse models of physiologic regeneration (21, 24), provides a pathophysiologic rationale for our clinical intuition that recovery is possible and justifies ongoing aggressive supportive care, including extracorporeal membrane oxygenation (ECMO), to allow time for complete regeneration. Moreover, this finding underscores the critical need to elucidate the mechanisms by which transitional cells differentiate into AEC1s. Signaling pathways differentially activated in the transitional and AEC1 states, such as TGFβ, Sox4 and TNF (21-24), are plausible candidates that merit further investigation. In addition, future research should explore whether specific factors actively delay AEC1 regeneration in ARDS, including but not limited to factors inherent to SARS-CoV-2. More fundamentally, it will be important understand why even physiologic AEC2-to-AEC1 differentiation is nongradual, pausing in a transitional state that upregulates genes expressed in neither AEC2s or AEC1s, and whether AEC2s can differentiate into AEC1s without passing through the transitional state. In sum, elucidation of the cellular and molecular mechanisms underlying AEC1 differentiation and how it may be impaired in ARDS will address fundamental questions of physiologic and pathologic alveolar regeneration and may ultimately lead to novel therapies to accelerate AEC1 differentiation, barrier restitution, liberation from the ventilator, and survival.

This unprecedented immunostaining of DAD lungs for AEC2, transitional cell, and AEC1 markers, yielded some unanticipated discoveries that are clinically relevant and may shift paradigms in our basic understanding of the pathology of DAD. We found that both sloughed epithelial cells long believed to be AEC1s and hyperplastic epithelial cells long believed to be AEC2s are actually transitional cells. These discoveries not only rectify a longstanding misidentification of epithelial cells in DAD, but provide additional insight into clinical outcomes in ARDS. The extensive acquisition of the SPC negative transitional state, with few mature, surfactant-producing AEC2s remaining, likely contributes to surfactant deficiency in ARDS. Surfactant deficiency in ARDS causes atelectasis, which exacerbates hypoxemia and poor lung compliance and leads to compensatory alveolar overdistension and ventilator-associated lung injury (VILI) (9). That robust regeneration with extensive acquisition of the transitional state may paradoxically exacerbate atelectasis and VILI is a

novel and provocative concept. Moreover, that the desquamated and hyperplastic epithelial cells long believed to be AEC1s and AEC2s, respectively, are actually transitional cells suggests that the histologic entity known as “acute DAD” represents a significantly more advanced state of regeneration than previously recognized. The acute injury and AEC2 proliferation phases of DAD probably occur within the first few hours to days of clinically manifest ARDS and are unlikely to be captured on autopsy specimens. Assuming the findings presented here are representative of ARDS in general, they radically alter our understanding of DAD.

By comparing and contrasting the transitional state in mouse models of physiologic regeneration, human ARDS, and human IPF, our study provides insight into physiologic regeneration and the mechanism by which it is impaired in the pathogenesis of fibrosis. During physiologic regeneration in mice, proliferating AEC2s exit the cell cycle and transiently assume the transitional state before differentiating into AEC1s (21, 24). Persistence of the transitional state with impaired AEC1 differentiation has been proposed to be pathogenic in fibrosis (22-25). Here, we demonstrate for the first time that human ARDS is characterized by abundant transitional cells *without fibrosis*. We confirmed that the transcriptomes of the transitional state in two mouse models of physiologic regeneration and human IPF are highly conserved. Taken together, these findings raise the pivotal question of why transitional cells maintain the capacity to differentiate into AEC1s and restore normal alveolar structure in mouse models of physiologic regeneration and early human ARDS but persist in the transitional state, leading to fibrosis, in IPF and fibroproliferative ARDS. While transitional cells in physiologic regeneration in mice, early human ARDS, and IPF all express markers of cell cycle exit, but only in IPF do they express a specific marker of senescence. Therefore, we propose the novel paradigm that in mouse models of physiologic regeneration and early human ARDS, proliferating AEC2s exit the cell cycle and *transiently* adopt the transitional state but retain the capacity to differentiate into AEC1s, restoring normal alveolar architecture without fibrosis, whereas in IPF and fibroproliferative ARDS, transitional AECs evolve into a *permanent* state of cell cycle arrest, or senescence, losing capacity for an AEC1 fate and promoting fibrosis [(22, 31-36), Graphical Abstract]. Additional investigation will be necessary to determine whether senescent transitional cells actually cause fibrosis and the underlying mechanisms. Since transitional cells lie in close spatial proximity to fibroblasts in the fibroblastic foci [(Figure 4C), (25, 31)]) and express profibrotic genes including TGFβ, CTGF, and PDGFA [Figure 5A, (21, 31, 32)], we further speculate that transitional cells may promote fibrosis by directly stimulating fibroblasts to become myofibroblasts and deposit matrix. If evolution from a transient state of cell cycle exit into a permanent state of senescence proves to be the critical switch that irreversibly diverts physiologic regeneration towards fibrosis, elucidation of the mechanisms by which transitional cells become senescent and by which senescent transitional cells activate fibroblasts may be the holy grail of fibrosis research and lead

to the development of novel therapies to promote physiologic regeneration and prevent fibrosis in IPF and fibroproliferative ARDS.

Our study has several limitations. Although a failure of barrier restitution due to incomplete AEC1 differentiation likely contributes to poor clinical outcomes, causality has not been established. The patients studied here died of acute respiratory failure due to pulmonary edema, and the histologic appearance of transitional cells (Figs. 2,3, Supplemental Figs. 12-15) certainly does not seem compatible with barrier integrity. Still, other factors likely contribute to poor clinical outcomes, including unchecked inflammation (45). However, the mechanism by which unchecked inflammation perpetuates respiratory failure due to pulmonary edema likely involves its effects on the alveolo-capillary barrier. One intriguing possibility is that unchecked inflammation may via specific mediators delay AEC1 differentiation from the transitional state, a notion that merits future investigation. As mentioned, we have not excluded the possibility that some transitional cells arise from progenitors other than AEC2s (18-20). Distinguishing senescence from transient cell cycle arrest is not straightforward, but p16 typically indicates senescence (43). Finally, though we speculate that transitional cells retain the ability to differentiate into AEC1s in human ARDS and lose the capacity for AEC1 differentiation in IPF, the potential fates of these cells remain speculative. Without the ability to acquire serial tissue samples, intervene, and perform lineage tracing, it is difficult to definitively establish causality and cell fate in observational human studies.

In conclusion, the findings presented here suggest that in severe ARDS, physiologic AEC1 differentiation from transitional cells is incomplete, thus underlying prolonged barrier permeability and respiratory failure, but as in physiologic regeneration, is ongoing without fibrosis. These findings establish a foundation for future mechanistic studies to dissect the molecular mechanisms by which transitional cells can differentiate into AEC1s during physiologic regeneration and ARDS and may lose capacity for an AEC1 fate and/or promote fibrogenesis in pulmonary fibrosis. Such mechanistic studies may ultimately lead to novel therapies to promote AEC1 differentiation, thus accelerating restoration of barrier integrity, clearance of edema fluid, liberation from the ventilator, and survival in severe ARDS and prevent fibrosis in fibroproliferative ARDS and fibrosis.

Methods

This study was approved by the University of Michigan Institutional Review Board. Methods are available in the Supplement.

Figure Legends

Figure 1. Epithelial Injury and Permeability in COVID-19 ARDS. H&E staining of lungs from COVID-19 patients reveals (A-D) acute DAD with (A) edema (asterisks), (B) fibrin (star), (C) hyaline membranes (arrowheads), and (D) desquamated epithelial cells (arrows) (200x). Immunostaining for the AEC1 markers HTI-56 and tomato lectin revealed a speckled pattern diffusely (E) with frequent small defects in staining (arrowhead) and septal thickening consistent with interstitial edema and/or inflammation (asterisk). Less frequent were areas in which staining was completely absent from one or both sides of the alveolar septa (F), consistent with denudation (indicated by red dashed tracing). Immunostaining for the AEC2 marker proSPC revealed vast areas devoid of mature AEC2s with rare AEC2s remaining (G). Scale bars = 50 μm. n=3.

Figure 2. Epithelial Proliferation Yields Abundant Transitional Cells in COVID-19 ARDS. Sections were stained with (A) H&E or (B-D) the AEC2 marker proSPC and/or the transitional cell marker KRT8. Staining revealed (A) hyperplastic cuboidal epithelial cells (arrow). Hyperplastic AECs were rarely mature AEC2s (B); the vast majority were KRT8hi transitional cells (C,D). C) Some KRT8hi transitional cells were cuboidal and isolated (open yellow arrowheads) but many occurred in pairs (closed yellow arrowheads) or were hyperplastic (2), suggestive of recent cell division. Transitional cells were most often partially spread (open white arrowheads in 3,4) and occasionally flat (closed white arrowheads in 5). D) Transitional cells did not express proSPC. Transitional cells were abundant in areas devoid of SPC+ cells (left panel); in rare areas of hyperplastic mature AEC2s, KRT8hi SPC- transitional cells coexisted (right panel). Scale bar = 50 μm. n=3.

Figure 3. Persistence of KRT8hi Transitional Cells with Ineffectual Differentiation into AEC1s in COVID-19 ARDS. Sections were stained for KRT8 and the AEC1 marker HTI-56. A) KRT8hi transitional cells filled gaps denuded of AEC1s on alveolar septa. Some were cuboidal (open yellow arrowheads); most were partially spread (open white arrowheads). Occasional transitional cells approached a flat AEC1 morphology but did not express AEC1 markers (closed white arrowheads) with rare exception (closed yellow arrowheads). Flat cells that expressed AEC1 markers but not KRT8 (closed green arrowheads) were interpreted as native AEC1s that were not damaged during lung injury. Findings suggest ongoing organized, albeit incomplete, AEC1 regeneration. B) In areas of severe injury, some KRT8hi transitional cells

spread on top of damaged AEC1s (open green arrowheads). C) Rare cells displayed bizarre morphologies and/or sloughed into the airspaces (orange arrowheads), consistent with haphazard regeneration. Scale bars = 50 µm. n=3.

Figure 4. Absence of Fibrosis in Acute COVID-19 ARDS. A) Trichrome highlights (in blue) basement membranes and vascular adventitia in normal and COVID-19 lungs and collagen deposition with marked fibrosis in IPF. B) Immunostaining demonstrates abundant myofibroblasts in IPF but not COVID-19. Open arrowheads indicate smooth muscle cells; closed arrowheads indicate myofibroblasts. C) Transitional cells (arrowheads) overlying fibroblastic focus (asterisks). Scale bars = 50 µm. n=3.

Figure 5. Transitional Cells in Mouse Models of Physiologic Regeneration, COVID-19 ARDS, and IPF Exist in a State of Cell Cycle Exit but only in IPF are Senescent. A,B,D) Single cell RNA sequencing datasets from two mouse model of physiologic regeneration, LPS (21) and pneumonectomy (PNX) (24) and human IPF (31, 32) were interrogated. C,E) Lung sections were immunostained. A) As AEC2s assume the transitional state, they downregulate AEC2 markers and upregulate transitional markers, which are conserved in mouse models of physiologic regeneration and human IPF. AEC1s express low levels of transitional markers and high levels of AEC1 markers. In mouse models of physiologic regeneration, COVID-19 ARDS, and IPF, transitional cells express general markers of cell cycle exit (B,C), but only in IPF do they express the highly specific marker of senescence CDKN2A/p16 (D,E). Scale bars = 50 µm. n=3.

Author Contributions

Conception and design: RLZ. Data acquisition and analysis: CT, MA, NV, SH, KAR, FW, CF. Interpretation of Data: CT, CF, RLZ. Drafting or revising the manuscript: CT, RLZ. Final approval: All authors.

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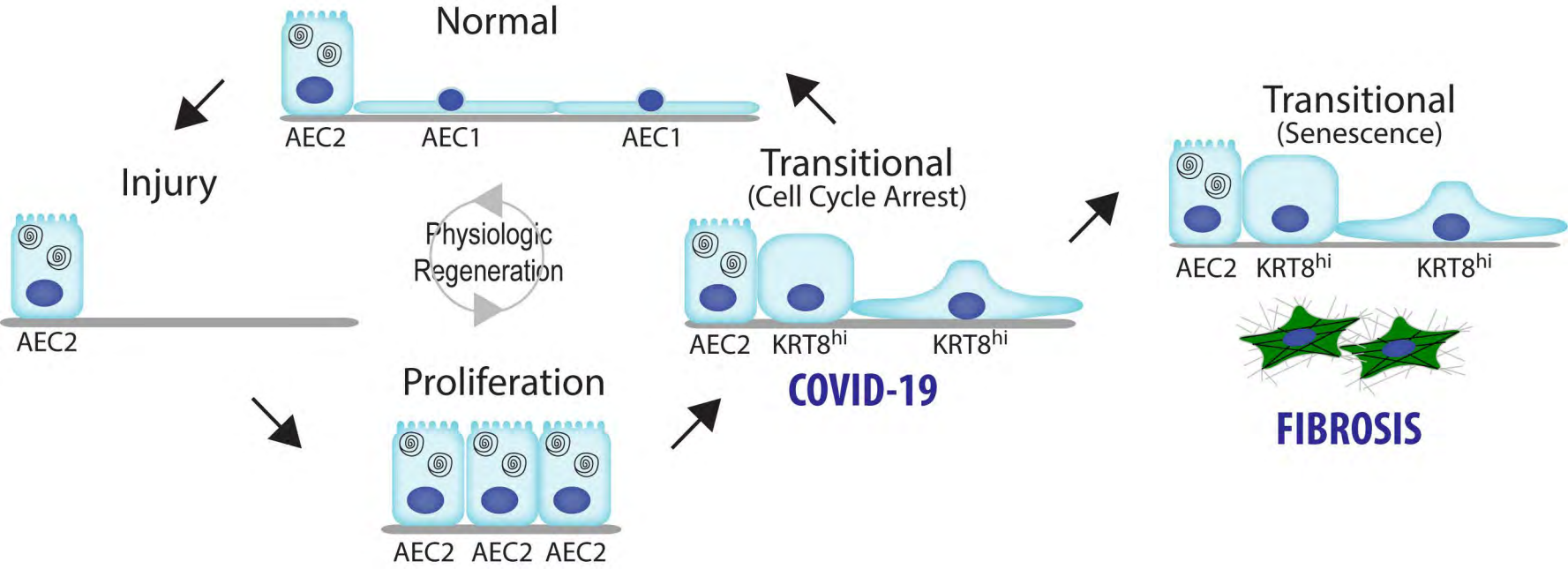
Normal
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COVID-19
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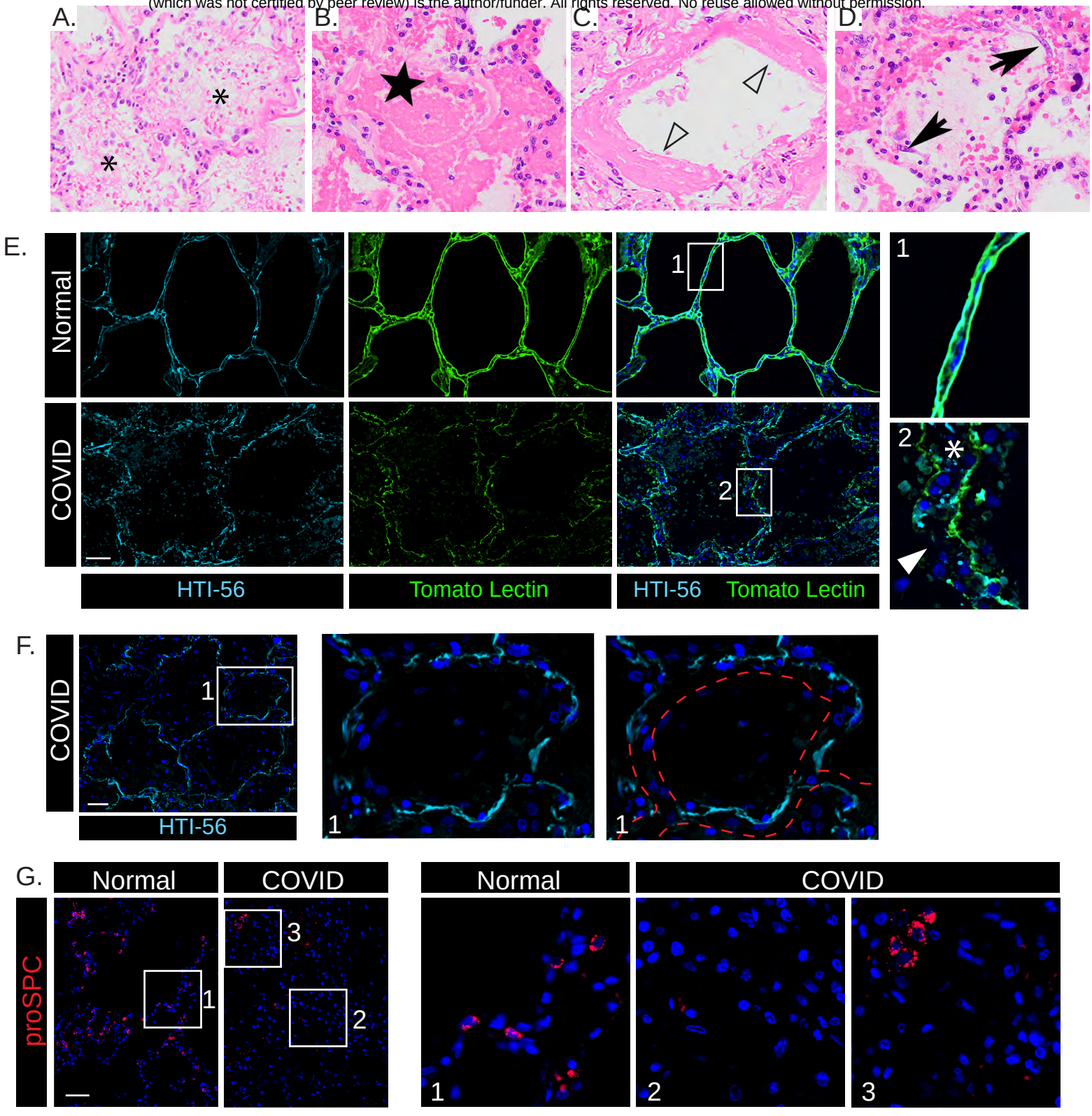

Figure 1. Epithelial Injury and Permeability in COVID-19 ARDS. H&E staining of lungs from COVID-19 patients reveals (A-D) acute DAD with (A) edema (asterisks), (B) fibrin (star), (C) hyaline membranes (arrowheads), and (D) desquamated epithelial cells (arrows) (200x). Immunostaining for the AEC1 markers HTI-56 and tomato lectin revealed a speckled pattern diffusely (E) with frequent small defects in staining (arrowhead) and septal thickening consistent with interstitial edema and/or inflammation (asterisk). Less frequent were areas in which staining was completely absent from one or both sides of the alveolar septa (F), consistent with denudation (indicated by red dashed tracing). Immunostaining for the AEC2 marker proSPC revealed vast areas devoid of mature AEC2s with rare AEC2s remaining (G). Scale bars = 50 μm. n=3.

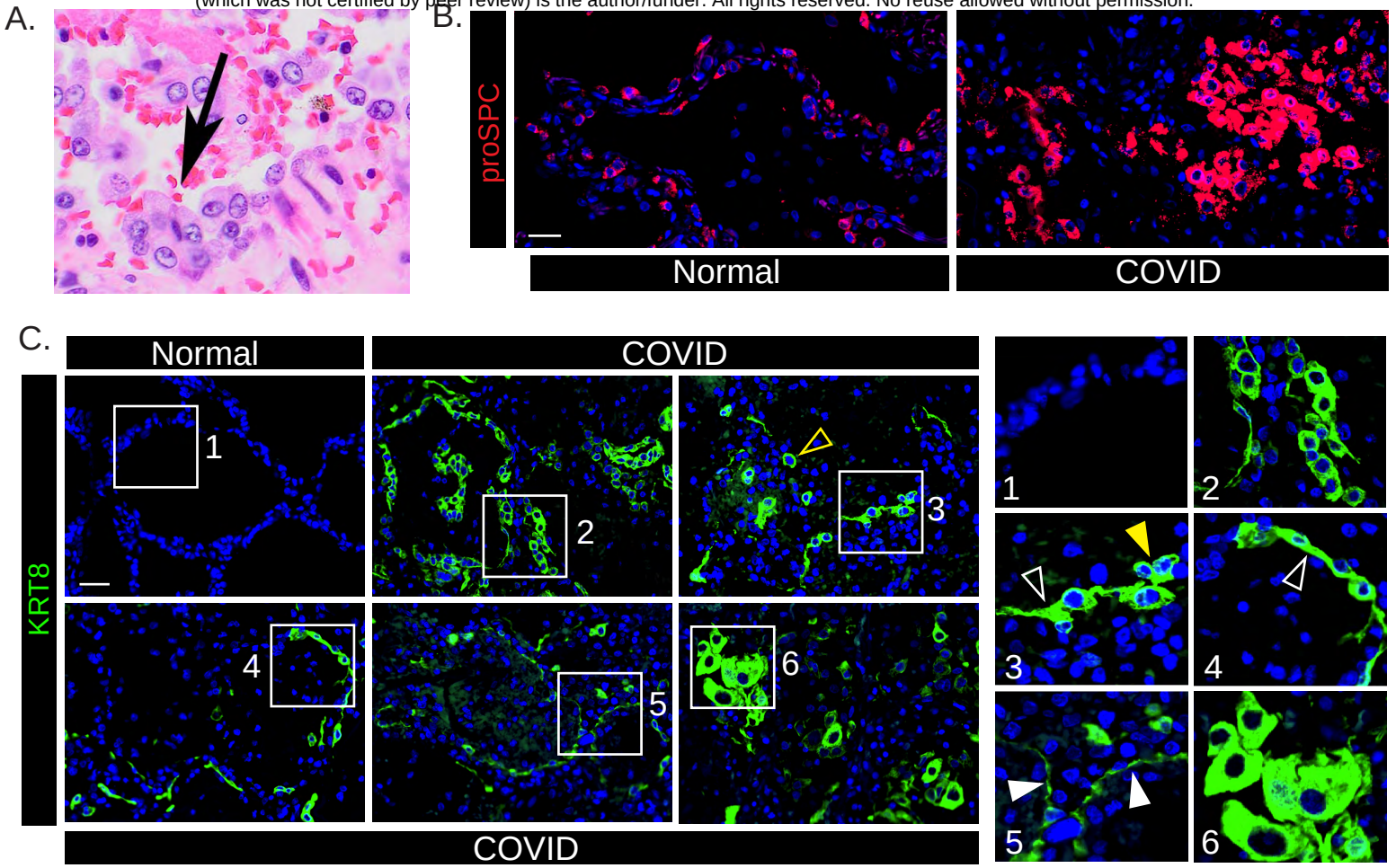

Figure 2. Epithelial Proliferation Yields Abundant Transitional Cells in COVID-19 ARDS. Sections were stained with (A) H&E or (B-D) the AEC2 marker proSPC and/or the transitional cell marker KRT8. Staining revealed (A) hyperplastic cuboidal epithelial cells (arrow). Hyperplastic AECs were rarely mature AEC2s (B); the vast majority were KRT8^{hi} transitional cells (C,D). C) Some KRT8^{hi} transitional cells were cuboidal and isolated (open yellow arrowheads) but many occured in pairs (closed yellow arrowheads) or were hyperplastic (2), suggestive of recent cell division. Transitional cells were most often partially spread (open white arrowheads in 3,4) and occasionally flat (closed white arrowheads in 5). D) Transitional cells did not express proSPC. Transitional cells were abundant in areas devoid of SPC+ cells (left panel); in rare areas of hyperplastic mature AEC2s, KRT8^{hi} SPC- transitional cells coexisted (right panel). Scale bar = 50 μm. n=3.

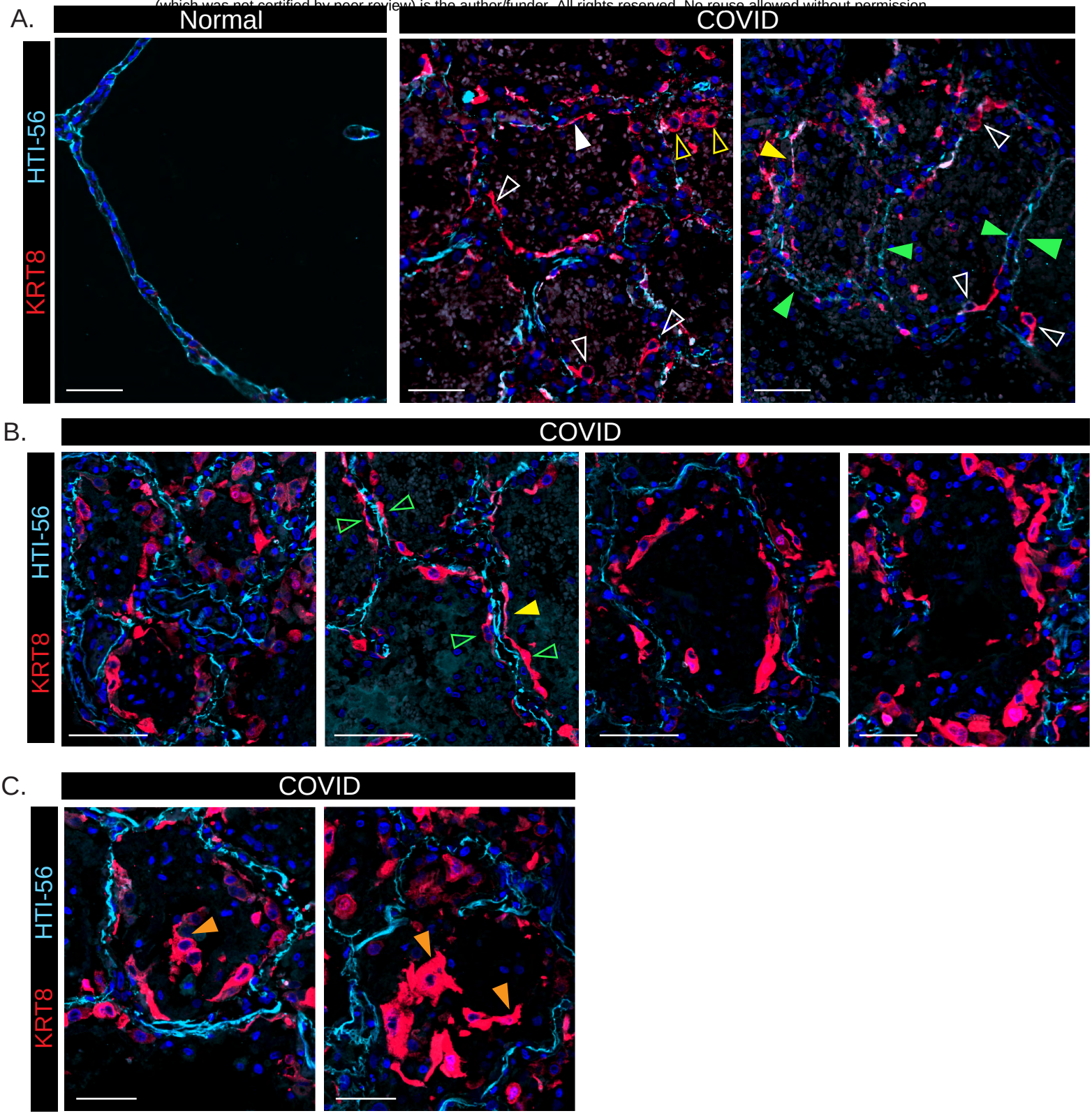

Figure 3. Persistence of KRT8^{hi} Transitional Cells with Ineffectual Differentiation into AEC1s in COVID-19 ARDS. Sections were stained for KRT8 and the AEC1 marker HTI-56. A) KRT8^{hi} transitional cells filled gaps denuded of AEC1s on alveolar septa. Some were cuboidal (open yellow arrowheads); most were partially spread (open white arrowheads). Occasional transitional cells approached a flat AEC1 morphology but did not express AEC1 markers (closed white arrowheads) with rare exception (closed yellow arrowheads). Flat cells that expressed AEC1 markers but not KRT8 (closed green arrowheads) were interpreted as native AEC1s that were not damaged during lung injury. Findings suggest ongoing organized, albeit incomplete, AEC1 regeneration. B) In areas of severe injury, some KRT8^{hi} transitional cells spread on top of damaged AEC1s (open green arrowheads). C) Rare cells displayed bizarre morphologies and/or sloughed into the airspaces (orange arrowheads), consistent with haphazard regeneration. Scale bars = 50 μm. n=3.

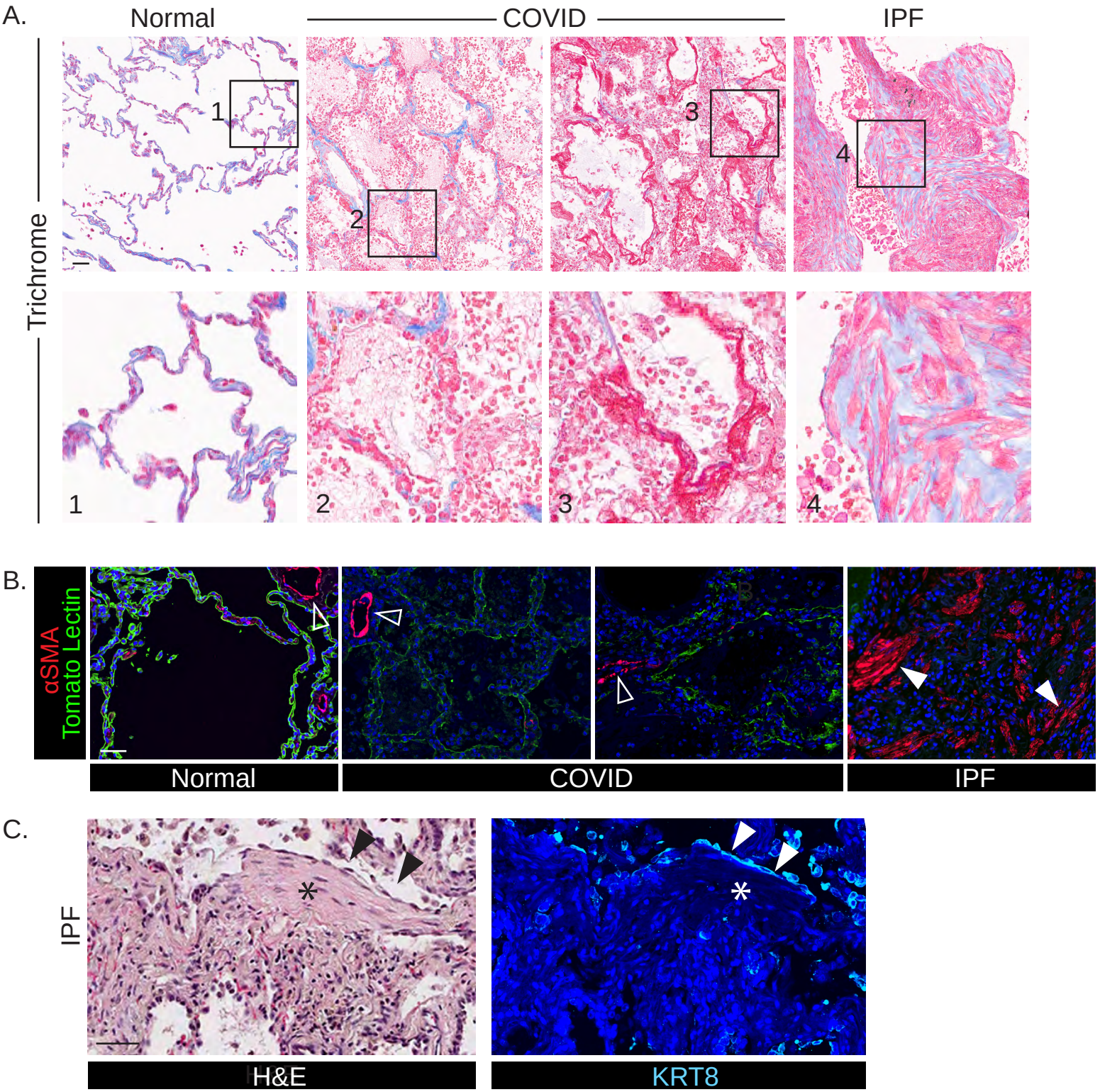

Figure 4. Absence of Fibrosis in Acute COVID-19 ARDS. A) Trichrome highlights (in blue) basement membranes and vascular adventitia in normal and COVID-19 lungs and collagen deposition with marked fibrosis in IPF. B) Immunostaining demonstrates abundant myofibroblasts in IPF but not COVID-19. Open arrowheads indicate smooth muscle cells; closed arrowheads indicate myofibroblasts. C) Transitional cells (arrowheads) overlying fibroblastic focus (asterisks). Scale bars = 50 µm. n=3.

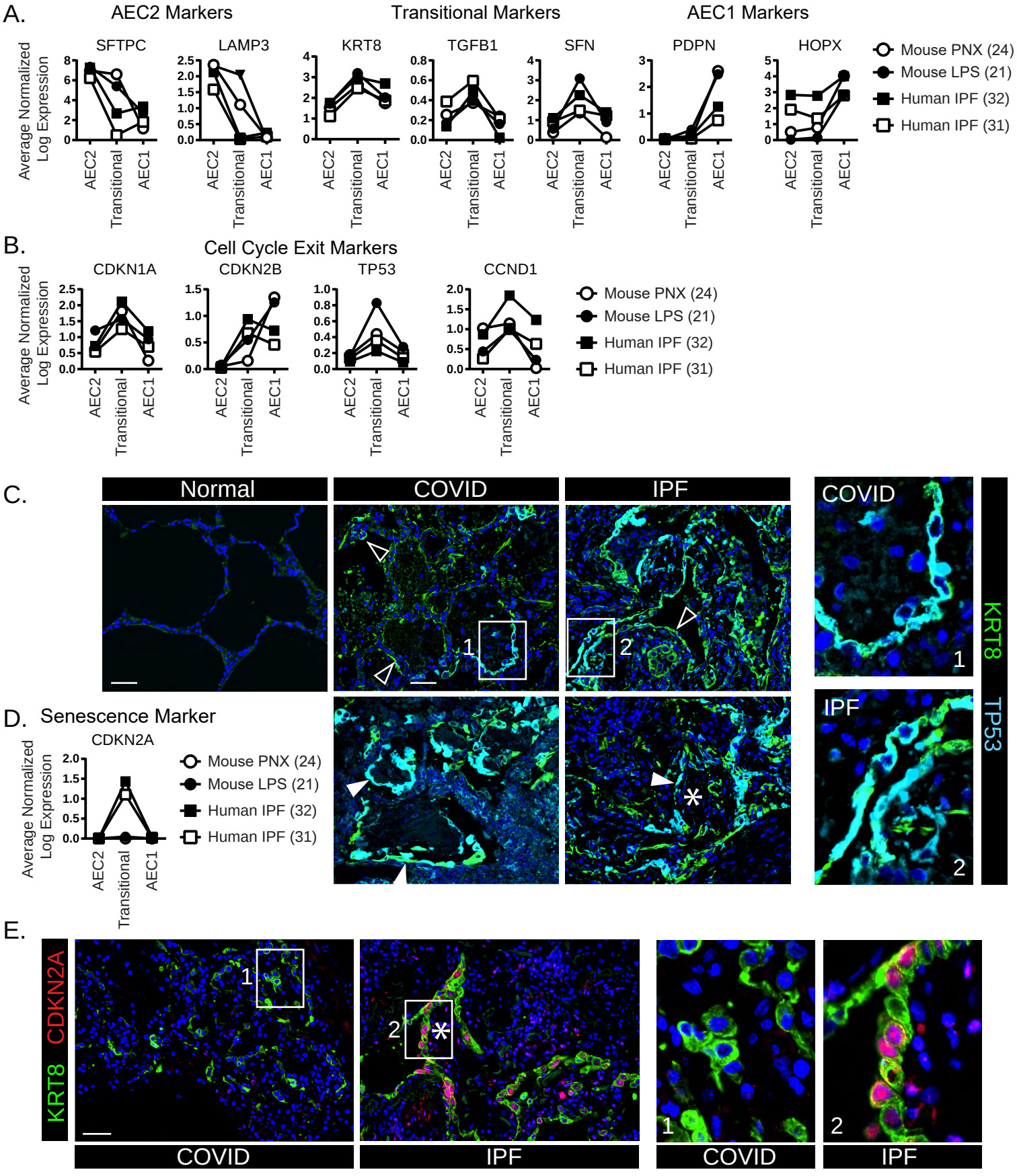

Figure 5. Transitional Cells in Mouse Models of Physiologic Regeneration, COVID-19 ARDS, and IPF Exist in a State of Cell Cycle Exit but only in IPF are Senescent. A,B,D) Single cell RNA sequencing datasets from two mouse model of physiologic regeneration, LPS (21) and pneumonectomy (PNX) (24) and human IPF (31, 32) were interrogated. C,E) Lung sections were immunostained. A) As AEC2s assume the transitional state, they downregulate AEC2 markers and upregulate transitional markers, which are conserved in mouse models of physiologic regeneration and human IPF. AEC1s express low levels of transitional markers and high levels of AEC1 markers. In mouse models of of physiologic regeneration, COVID-19 ARDS, and IPF, transitional cells express general markers of cell cycle exit (B,C), but only in IPF do they express the highly specific marker of senescence CDKN2A/p16 (D,E). Scale bars = 50 μm. n=3.