Ineffectual AEC1 Differentiation from KRT8^{hi} Transitional Cells without Fibrosis Associated with Fatal Acute Respiratory Failure in COVID-19 ARDS

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Abstract

COVID-19 ARDS is associated with prolonged ventilator dependence and high mortality, but the underlying mechanisms are unknown. Critical to the pathogenesis of ARDS is injury to the alveolar epithelial cell (AEC) barrier; clinical recovery requires epithelial regeneration. We previously identified a KRT8^{hi} transitional state that regenerating AEC2s adopt during differentiation into AEC1s, the persistence of which may be pathogenic in pulmonary fibrosis. Here, we hypothesize that ineffectual differentiation of transitional cells into AEC1s without fibrosis may underlie ongoing barrier permeability and poor clinical outcomes in COVID-19 ARDS. To test this hypothesis, we examined postmortem lung tissue of COVID-19 ARDS patients. We observed severe epithelial injury, AEC2 proliferation, and abundant transitional cells but ineffectual AEC1 differentiation. Transitional cells were cuboidal, partially spread, or flat and adherent to alveolar septa that were denuded of AEC1s but structurally normal without fibrosis. We conclude that ineffectual AEC1 differentiation from transitional AECs may underlie ongoing barrier permeability and poor clinical outcomes in COVID-19 ARDS. However, in contrast to fibrosis, transitional cells may retain the capacity for AEC1 differentiation with restoration of normal alveolar architecture and function. Novel therapies that promote differentiation of transitional cells into AEC1s may accelerate barrier restoration and clinical recovery in ARDS.

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 (1-3). 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 (4). The alveolar epithelium is comprised of AEC1s and AEC2. AEC1s cover >95% of the alveolar surface, thus playing a critical role in barrier integrity, and are exquisitely thin, permitting efficient gas exchange (5). AEC2s, are cuboidal, produce surfactant, which prevents alveolar collapse, and serve as progenitors (6). 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 (4). Conversely, regeneration of the alveolar epithelium is critical for the restoration of barrier integrity, resolution of edema, liberation from the ventilator, and survival (7, 8). Therefore, we speculated that ineffectual alveolar regeneration may underlie the prolonged ventilator dependence and high mortality rates observed in COVID-19 ARDS.

Surviving AEC2s are the principal progenitor responsible for alveolar regeneration (6), though other progenitors may contribute (9). AEC2s regenerate the alveolar epithelium in two stages: they proliferate and then differentiate into AEC1s to restore normal alveolar architecture and barrier integrity. Which stage of alveolar regeneration, if either, may be impaired in COVID-19 is unknown. The histology of COVID-19 ARDS is diffuse alveolar damage (DAD) and includes AEC2 hyperplasia (10, 11), suggesting that proliferation may be preserved.

Using mouse models, we made the seminal discovery that after proliferation, AEC2s exit the cell cycle and assume a transitional state before differentiating into AEC1s (12). The transitional state is characterized by markers of cell cycle arrest, 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 etiology of injury (13-16). 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 (5, 12, 13, 17) (Graphic Abstract).

However, as we and others subsequently discovered, transitional cells persist with ineffectual AEC1 differentiation in idiopathic pulmonary fibrosis (IPF), a condition of irreversible scarring leading to chronic respiratory failure (13-19). Pulmonary fibrosis is widely believed to arise from impaired alveolar regeneration after repetitive injury, so persistence of the transitional state with impaired AEC1 differentiation may be the specific regenerative defect underlying the pathogenesis of fibrosis. Transcriptomic analysis suggests the transitional cells in fibrosis may be senescent (13, 18, 19). Similarly, recent case reports identified transitional cells in the fibrosis that develops after months of nonresolving COVID-19, so-called “fibroproliferative ARDS” (20, 21). In these cases, transitional cells existed 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 (20). However, it is unknown whether transitional cells arise in early ARDS and whether ineffectual AEC1 differentiation from transitional cells without fibrosis may be pathogenic in acute respiratory failure.

We hypothesized that ineffectual differentiation of transitional cells into mature AEC1s may underlie ongoing barrier permeability, edema, ventilator dependence, and mortality from acute respiratory failure in COVID-19 ARDS. We further hypothesized that this occurs without fibrosis, suggesting that, as in animal models of physiologic regeneration, transitional cells retain the capacity to differentiate into AEC1s and restore normal alveolar architecture and function. To explore this hypothesis, 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, and fibrosis. The findings fundamentally advance our 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 COVID-19 ARDS may lead to novel therapies to promote physiologic regeneration, thus accelerating restoration of barrier integrity, resolution of edema, liberation from the ventilator, and survival.

Results & Discussion

Clinical Presentation

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

Severe Epithelial Damage in COVID-19 ARDS

Histology revealed acute DAD. There was diffuse airspace filling with edema, fibrin, and hyaline membranes (Figure 1A-C, Supplemental Figures 2,3), all indicative of epithelial permeability leading to the influx of proteinaceous edema fluid into the airspaces. 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 (22) but are thicker and cover less surface area than AEC1s (23), raising doubt as to their identity. Regardless, the radiographic and histologic findings observed in COVID-19 ARDS indicated epithelial permeability resulting in proteinaceous alveolar edema.

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. Staining for 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 interstitial edema and inflammatory cells. Small defects in staining were frequent (Figure 1E2). Complete absence of staining from one or both sides of the alveolar septa were less frequent (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 causing respiratory failure.

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

To assess whether surviving AEC2s mobilized to regenerate the injured epithelium, we examined histologic sections. We observed hyperplastic cuboidal epithelial cells (Figure 2A), suggesting that AEC2s successfully proliferated to replace damaged AECs. Although historically termed “AEC2 hyperplasia” (22), only rare areas of hyperplastic AEC2s were observed amidst vast areas devoid of mature AEC2s, raising doubt as to their identity (Figures 1G, 2B, Supplemental Figures 6,7). The hyperplastic AEC2s were hypertrophic, as previously described (24). 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). [Normal AEC1s and AEC2s express KRT8 at much lower levels (Supplemental Figure 10) (12-19).] Some of the KRT8^{hi} cells observed in COVID-19 were cuboidal; some existed as single cells while others were doublets or hyperplastic suggesting recent cell division (Figure 2C, Supplemental Figure 8). Given the abundance of hyperplastic transitional cells and paucity of mature AEC2s (Figure 1G, Supplemental Figure 6), we conclude that the hyperplastic cells historically designated as “AEC2s” in DAD are actually transitional cells. The morphology of KRT8^{hi} cells was often partially spread and occasionally flat approaching AEC1 morphology, suggesting ongoing differentiation into AEC1s (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 differentiation. However, some KRT8^{hi} cells were hypertrophic, bizarrely-shaped, and disorganized (Figure 2C6, Supplemental Figure 9), interpreted as haphazard regeneration. KRT8^{hi} transitional cells did not express SPC (Figure 2D), consistent with known downregulation of AEC2 markers as cells assume the transitional state (12-16, 18, 19). Moreover, transitional cells were abundant in areas devoid of SPC+ cells (Figure 2D), suggesting that the extensive loss of SPC+ cells (Figure 1G) represents not massive AEC2 death but acquisition of the transitional state. In some areas, KRT8^{hi}

transitional cells coexisted with hyperplastic mature AEC2s (Figure 2D), suggesting that while some AEC2s proliferate, others assume the transitional state. [Although transitional cells may arise from club-like progenitors (9, 14, 18, 19), bronchiolization and honeycomb cysts were not observed (Supplemental Figure 2)]. Taken together, these data support several novel and important conclusions. First, transitional cells arise in early human ARDS. Second, extensive acquisition of the SPC- transitional state likely contributes to surfactant deficiency and atelectasis in ARDS, which exacerbates hypoxemia and leads to alveolar overdistension and ventilator-associated lung injury (4). Most importantly, “acute DAD” represents a significantly more advanced state of regeneration than previously recognized. The progressive edema, prolonged ventilator dependence and high mortality observed in COVID-19 ARDS are not due to an inability of progenitor cells to proliferate or assume the transitional state (Graphic Abstract).

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

Since progenitors successfully proliferated and assumed the transitional state, we hypothesized that ineffectual differentiation of transitional cells into mature AEC1s may be the specific regenerative defect underlying the persistent barrier permeability and poor clinical outcomes in COVID-19. In support of this hypothesis, the transitional cells were typically partially spread, only occasionally assuming the flat morphology of AEC1s (Figure 2C). To determine 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 11-14). Most were cuboidal or partially spread, but even those with a flat morphology very rarely expressed AEC1 markers. In areas of severe injury, transitional cells occasionally spread on top of damaged AEC1s (Figure 3B). Flat cells expressing AEC1 markers but not KRT8 were interpreted as native AEC1s that withstood injury (although we cannot exclude nascent AEC1s that downregulated KRT8 after differentiation). The organization of transitional cells in a monolayer on alveolar septa filling gaps denuded of AEC1s and displaying increasingly spread morphologies without AEC1 marker expression support the conclusion that differentiation along a linear trajectory into mature AEC1s is ongoing but incomplete. Rarely, transitional cells assumed bizarre morphologies and/or sloughed off

the septa into the airspaces (Figure 3C; Supplemental Figure 14,15). This suggests that the desquamated epithelial cells historically identified in DAD as AEC1s (Figure 1D) may be transitional cells. Although widespread death of progenitors would be catastrophic, rare sloughed transitional cells are unlikely to contribute significantly to poor clinical outcomes. Still, the bizarre morphologies and sloughing do raise doubt about the eventuality of physiologic regeneration in these focal areas. In conclusion, we speculate that the ineffectual, albeit ongoing, differentiation of transitional cells into mature AEC1s is the specific defect underlying the persistent barrier permeability, pulmonary edema, ventilator dependence and mortality associated with COVID-19 ARDS (Graphic Abstract).

Absence of Fibrosis in COVID-19 ARDS

Persistence of the transitional state with ineffectual AEC1 differentiation has previously been observed in humans only in the setting of fibrosis, an irreversible condition causing chronic respiratory failure (13, 14, 16-21). It is unknown whether transitional cells are inevitably associated with and/or cause fibrosis and therefore whether fibrosis may be the cause of acute respiratory failure in COVID-19 ARDS (20, 21). In mouse models of physiologic regeneration (e.g., lipopolysaccharide and pneumonectomy), transitional cells appear transiently then differentiate into AEC1s to restore normal alveolar architecture without fibrosis (13, 15, 16). Therefore, we hypothesized that in early COVID-19 ARDS transitional cells may exist in the absence of fibrosis. Indeed, we observed no excessive collagen deposition, myofibroblast accumulation, or architectural distortion in COVID-19 ARDS, in contrast to IPF (Figure 4A,B, Supplemental Figures 2B, 16-18). Moreover, whereas in IPF the KRT8^{hi} transitional cells overlie fibroblastic foci (Figure 4C) and line honeycomb cysts (16), the transitional cells in COVID-19 ARDS line structurally normal alveolar septa (Figure 3).

Taken together, these data suggest that ineffectual differentiation of transitional cells into AEC1s underlies refractory respiratory failure in acute COVID-19 ARDS via ongoing barrier permeability and pulmonary edema, not by causing fibrosis. Moreover, the appearance of cuboidal, partially spread, and flat transitional cells on structurally normal alveoli in the absence of fibrosis suggests that transitional cells retain the capacity for AEC1

differentiation with restoration of normal alveolar architecture and barrier integrity, as occurs in mouse models of physiologic regeneration. This provides a pathophysiologic rationale for our clinical intuition that recovery is possible and ongoing aggressive supportive care is warranted. Moreover, elucidation of the mechanisms by which transitional cells differentiate into mature AEC1s may ultimately lead to novel therapies to accelerate AEC1 differentiation, barrier restitution, liberation from the ventilator, and survival in ARDS. Signaling pathways differentially activated in the transitional and AEC1 states, including TGFβ, Sox4 and TNF (12-14), are plausible candidates. Although COVID-19 ARDS may be associated with worse outcomes than ARDS from other etiologies (1-3), we suspect that this pathology is common to severe ARDS regardless of etiology.

While transitional cells may retain the capacity for AEC1 differentiation, restoring normal alveolar architecture and function in mouse models and early human ARDS, these cells may eventually lose the capacity for an AEC1 fate and promote fibrosis in fibroproliferative ARDS and IPF (18-21) (Graphic Abstract). We speculate that the state of cell cycle arrest that emerges transiently as proliferating cells exit the cell cycle in anticipation of AEC1 differentiation after acute lung injury in mice and humans may, after months to years of repetitive injury, evolve into a permanent state of cell cycle arrest, or senescence, in fibrosis. Supporting this notion, interrogation of publicly available datasets revealed that transitional cells in both physiologic regeneration and fibrosis express markers of cell cycle arrest (CDKN2B, TP53, CCND1), but only in fibrosis do they express the highly specific marker of senescence CDKN2A (Supplemental Fig. 20). Accordingly, we did not detect p16 in COVID-19 (data not shown). Based on the spatial proximity between transitional cells and fibroblasts in the fibroblastic foci (Fig.4C), we further speculate that pathologic transitional cells may directly stimulate fibroblasts to become myofibroblasts and deposit matrix. Additional investigation to identify mechanisms by which transitional cells may lose the capacity for an AEC1 fate and activate fibroblasts may yield therapeutic targets to prevent fibrogenesis in fibroproliferative ARDS and IPF.

Methods

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

Author Contributions

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

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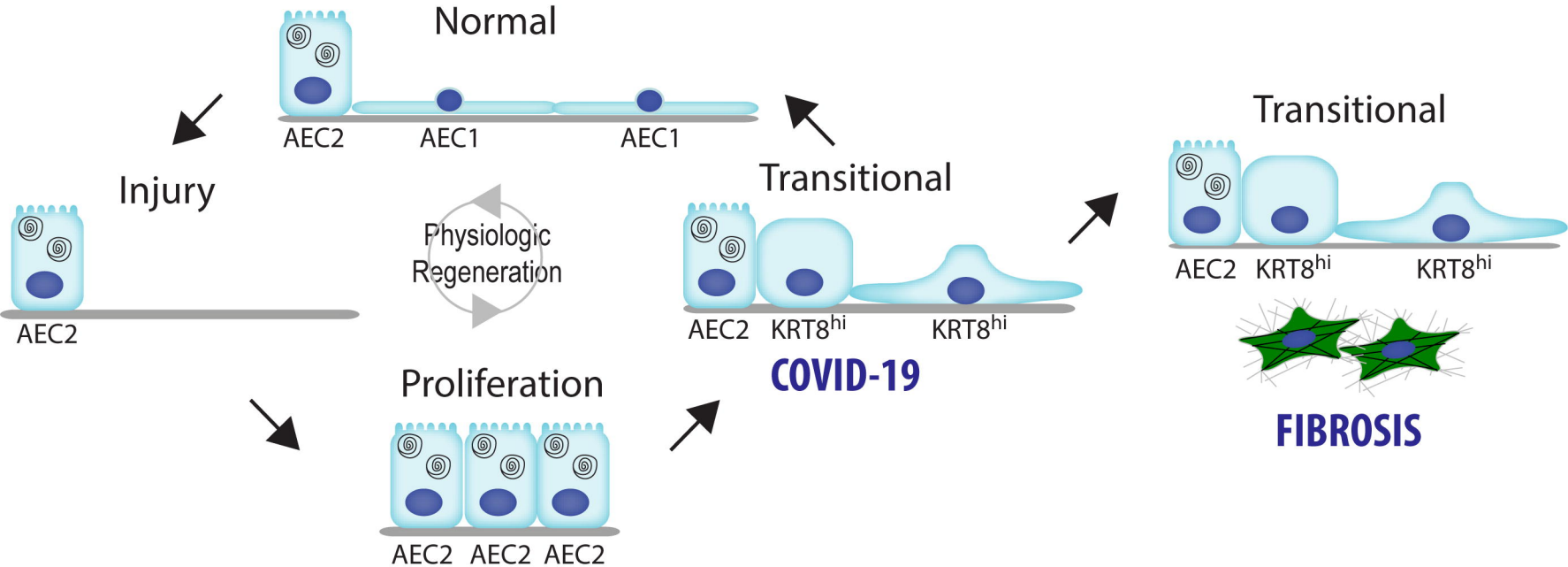
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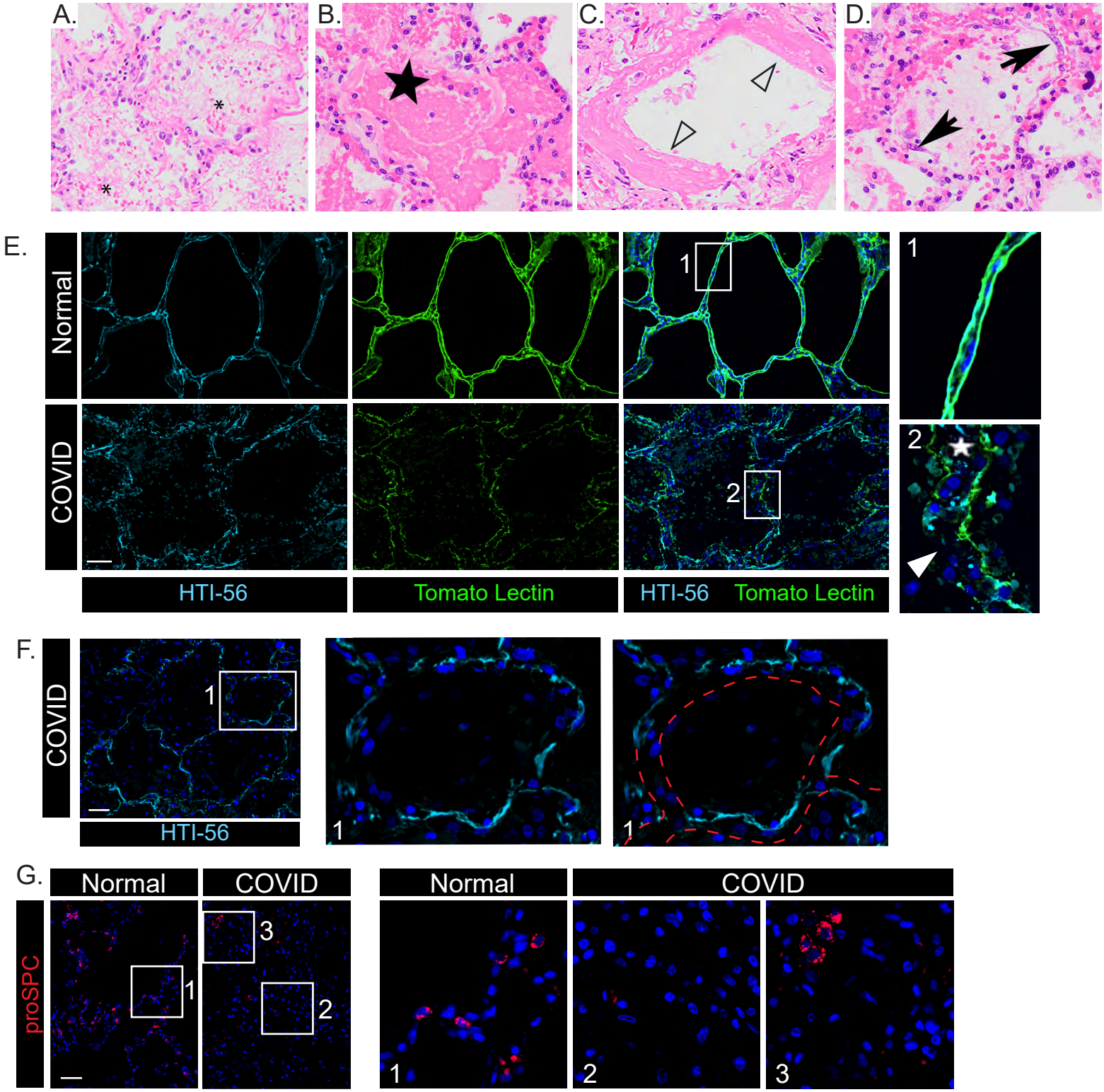

Figure 1. Epithelial Injury and Permeability in COVID-19 ARDS. H&E staining of lungs from COVID-19 patients reveals (A-D) acute DAD with (A) edema (asterisks), (B) fibrin (star), (C) hyaline membranes (arrowheads), and (D) desquamated epithelial cells (arrows) (200x). Immunostaining for the AEC1 markers HTI-56 and tomato lectin revealed a speckled pattern diffusely (E) with frequent small defects in staining (arrowhead) and septal thickening consistent with interstial edema and/or inflammation (star). 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.

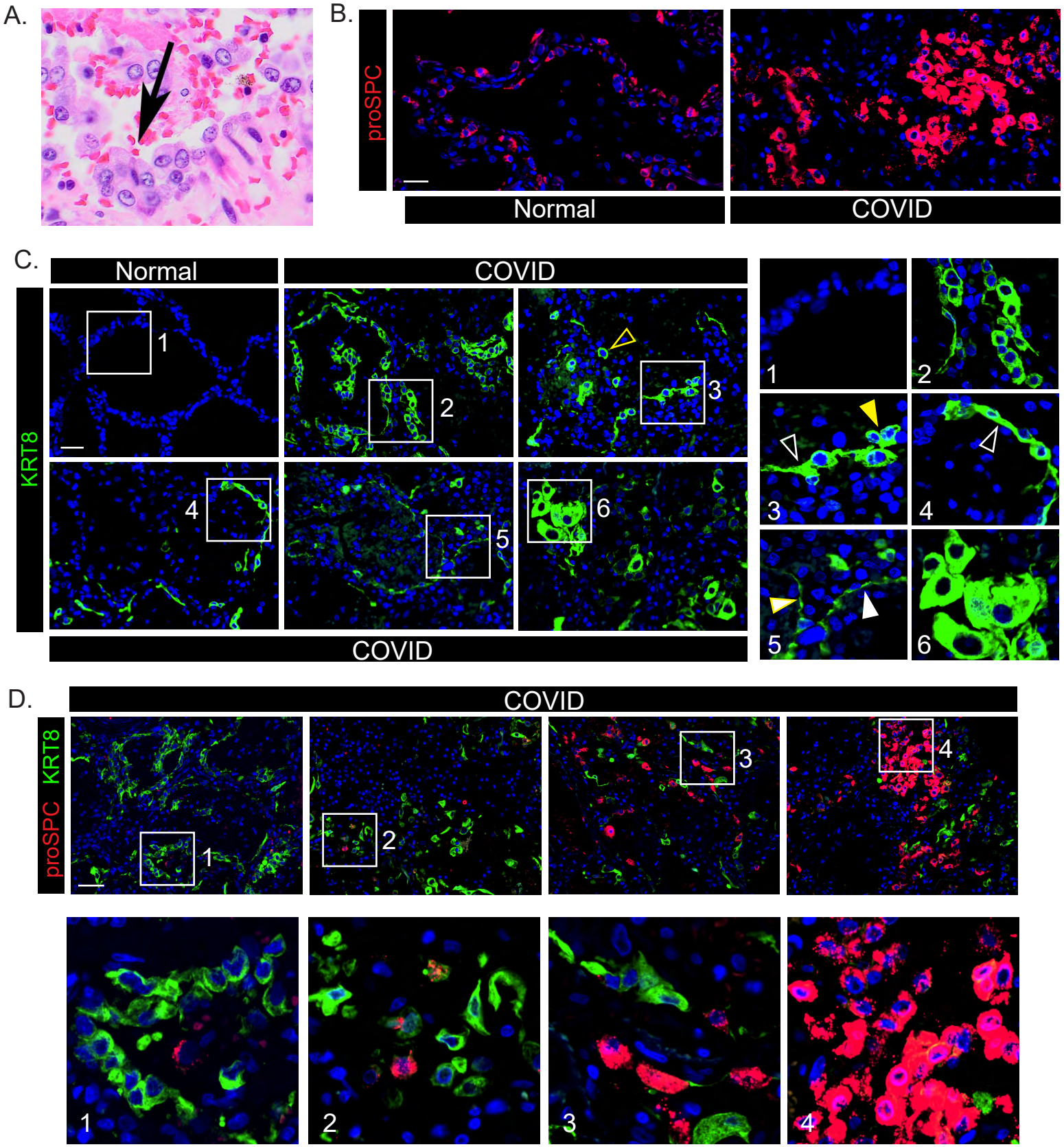

Figure 2. Epithelial Proliferation Yields Abundant Transitional Cells in COVID-19 ARDS. Sections were stained with (A) H&E or (B-D) the AEC2 marker proSPC and/or the transitional cell marker KRT8. Staining revealed (A) hyperplastic cuboidal epithelial cells, some of which were mature AEC2s (B) but the vast majority of which 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 (D). Scale bar = 50 µm. n=3.

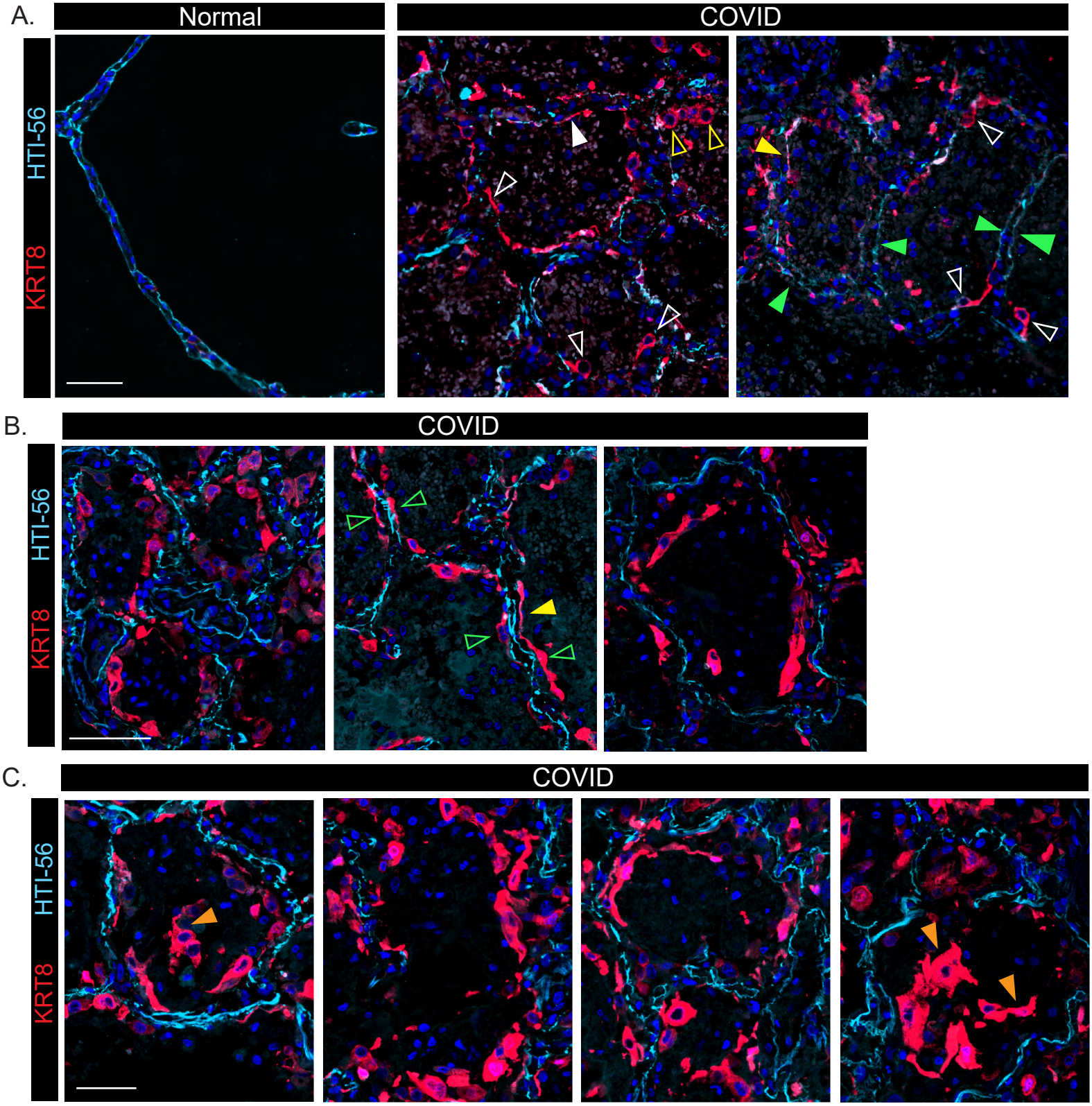

Figure 3. Persistence of KRT8^{hi} Transitional Cells with Ineffectual Differentiation into AEC1s in COVID-ARDS. Sections were stained for KRT8 and the AEC1 marker HTI-56. A) KRT8^{hi} transitional cells filled gaps denuded of AEC1s on alveolar septa. Some were cuboidal (open yellow arrowheads); most 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.

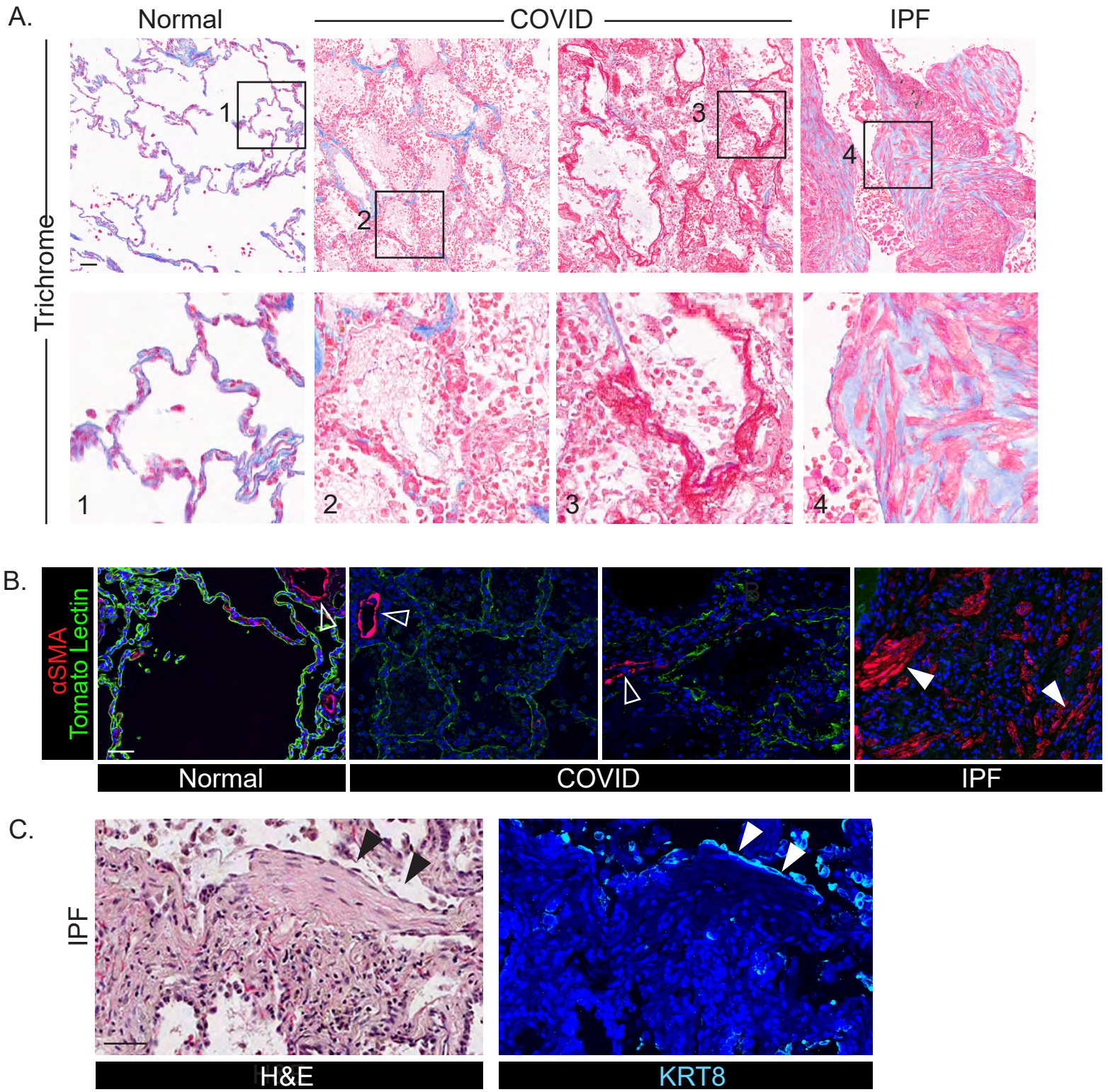

Figure 4. Absence of Fibrosis in Acute COVID-19 ARDS. A) Trichrome highlights (in blue) basement membranes and vascular adventitia in normal and COVID-19 lungs and collagen deposition with marked fibrosis in IPF. B) Immunostaining demonstrates abundant myofibroblasts in IPF but not COVID-19. Open arrowheads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