

Update on acute respiratory distress syndrome's pathology. Recent insights into *in vivo* alveolar regeneration

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Summary

Acute Respiratory Distress Syndrome (ARDS) is a type of acute lung injury characterized on histology by a pattern of Diffuse Alveolar Damage (DAD). Since patients who survive their critical illness generally recover a normal respiratory function, ARDS is regarded as a remarkable example of *in vivo* alveolar regeneration and mice with chemical or viral-induced ARDS represent a useful experimental model for studying these processes. Recent studies opened new insights into the mechanisms of alveolar regeneration, partly challenging the traditional hypothesis that identifies alveolar type II cells as the progenitor of the alveolar epithelium. Particularly, we proposed Krt14 as a robust marker of alveolar regeneration and repair, since its expression in the lung parenchyma is only found in pathological conditions during pneumocyte proliferation after severe damage. A better understanding of the processes that regulate *in vivo* alveolar regeneration would make it possible to revolutionize the therapeutic approach to patients with respiratory chronic disease, paving the way to the so called “regenerative lung medicine”.

KEY WORDS: ARDS, DAD, lung stem cells, alveolar regeneration, Keratin 14.

Acute Respiratory Distress Syndrome (ARDS) is a type of acute diffuse lung injury defined by clinical, radiological and histological criteria. ARDS is associated to a large variety of pathogenic factors, generally divided into direct lung injury factors (such as pneumonia or aspiration of gastric contents) and indirect lung injury factors (such as major trauma or non-pulmonary sepsis). The estimated incidence of ARDS in the United States ranges between 15 and 34 cases per

100,000 inhabitants per year (1), but there is a large incidence variability across countries probably due to ethnical, demographical and economical factors (2). ARDS was first described in 1967 by Ashbaugh et al. (3) in a group of patients who presented with an acute onset of severe hy-

poxemia refractory to oxygen supplementation, but sometimes responsive to the application of positive end-expiratory pressure (PEEP). In 1994 the American European Consensus Conference (AECC) established clinical and radiological criteria for the diagnosis of ARDS, in order to provide a definition that could be widely accepted and used (4). A recent revision of the AECC diagnostic led to the Berlin definition of ARDS (5), that stratifies patients in three mutually exclusive categories of increasingly severe ARDS (mild, moderate and severe) by the degree of arterial hypoxemia, measured as PaO₂/FIO₂ ratio (P/F ratio). The introduction of a severity grading system responds to the emerging need to better categorize patients with different outcomes and potential responses to therapy, since high-risk interventions proved to be beneficial only to those patients who presented with a severe form of ARDS (6, 7). The pathological correlate of ARDS is Diffuse Alveolar Damage (DAD), traditionally divided into three distinct phases according to the pathological findings (8-11):

- *the exudative phase* (develops within hours of the initial injury and lasts approximately one week): injury occurs to both alveolar type I cells (AT1s) and capillary endothelial cells, resulting in intra-alveolar and interstitial oedema, acute inflammatory cell infiltration, and AT1s necrosis. Hyaline membranes, the histological hallmark of DAD, appear by day 2 and reach their peak after days 4-5. The membranes are made up of proteinaceous material, as well as cellular debris, and can be found in the alveolar spaces, especially along the alveolar septa. During the later part of the exudative phase, AT2s hyperplasia can be present (Figure 1a);
- *the proliferative/organizing phase* (from day 7 to day 21 after the initial injury): the acute inflammatory cell infiltration is replaced by a predominantly mononuclear cell infiltration, hyaline membranes disappear (phagocytosed by macrophages or transformed into granulation tissue), and both AT2s and fibroblasts undergo massive proliferation in order to repair the damaged tissue. During

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this phase enlarged AT2s predominate, and may show rather pronounced atypia, which should not be confused with malignancy or viral cytopathic changes. Interstitial spaces appear in this phase as abnormally enlarged, filled by proliferating myofibroblasts, and also gradually incorporate hyaline membranes and granulation tissue (a process known as “fibrosis by ac-

cretion”) (10). Vascular remodeling commonly leads to medial muscular hypertrophy and endarteritis obliterans, occasionally causing ischaemic necrosis (Figure 1b, c);

- *the fibrotic phase* (from day 21 to 4-6 months after the initial injury): characterized by mature collagen deposition, resulting in interstitial fibrosis and architectural remodeling with cyst/microcyst formation. Patients who survive ARDS generally show resolution of the increased fibroblastic proliferation and collagen deposition within 4-6 months of the initial injury (11).

This schematic model does not entirely match with reality because *in vivo* the three phases largely tend to overlap. Furthermore, not all the members of the Berlin Definition panel agreed on considering DAD as the unique pathological correlate of ARDS, arguing that also acute pneumonia and non-cardiogenic edema could be consistent with the diagnosis of ARDS if the clinical criteria are met (5). This claim seems reasonable if we consider that in a large series of cases (12) only 45% of patients who met the clinical criteria for ARDS were found to have DAD at autopsy. However, in the group of patients with severe ARDS the percentage of DAD raised up to 58%, while in the group of patients with mild ARDS the percentage of DAD dropped to 10-14%. On the contrary, if both DAD or pneumonia (histological criteria for the diagnosis of acute pneumonia included the presence of intense neutrophilic infiltration in the interstitium and in the intra-alveolar spaces, and particularly around terminal bronchioles) were considered as possible correlates of ARDS, the percentage of patients who met clinical and pathological criteria for ARDS raised from 45% to 88% (12). Worth noting, several entities enter the differential diagnosis of DAD histologically, in particular: acute eosinophilic pneumonia (AEP), nonspecific interstitial pneumonia (NSIP), fibrosing type usual interstitial pneumonia, acute fibrinous and organizing pneumonia (AFOP), and diffuse alveolar hemorrhage (8-10).

Even if a declining trend has been recorded since the 1980s (13-15), the current mortality of adult ARDS is still greater than 40% (16). About 80% of deaths occurs in the first 2-3 weeks after the onset of ARDS (17),

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and the most important predictors of mortality seem to be age, the underlying medical condition, the severity of lung damage, extra-pulmonary organ dysfunction and sepsis (18). Recent studies highlighted the potential role of predisposing genetic factors in determining not only susceptibility and clinical manifestations, but also response to therapy and outcomes among ARDS patients (2, 19-21). Interestingly, despite the severity and the extension of the alveolar damage, the lung parenchyma is able to completely regenerate and pulmonary dysfunction is typically considered as a minor morbidity in survivors of ARDS. In effect, patients who survive their critical illness recover a normal lung function within 6 to 12 months, except for a persistently mild reduction in diffusion capacity (22-27). Persistent restrictive disease after ARDS is uncommon and more likely to be secondary to extra-pulmonary respiratory muscle or diaphragmatic weakness, rather than to an underlying pulmonary pathology (27, 28). Our current knowledge about the mechanisms of alveolar regeneration mainly derives from studies conducted on mice with chemical-induced lung injury. The most commonly used chemical agent is represented by bleomycin, a chemotherapeutic drug that is well known for its pulmonary toxicity and is responsible for a variety of pulmonary changes (vascular congestion, interstitial edema, intra-alveolar fibrin deposition, and AT2s hyperplasia) consistent with the diagnosis of DAD/ARDS in both mice (29) and humans (30). However, at variance with ARDS from other causes, bleomycin-induced ARDS invariably evolves into interstitial lung fibrosis (29, 30), generally associated with alveolar wall basement membrane duplication and thickening (31). Oxygen-free derived radicals and intra-cellular ferric ions are thought to be directly involved in the mechanism of bleomycin toxicity on AT2s, since glutathione and iron chelants proved to have a protective role (32). Lately, using a murine model of bleomycin-induced pulmonary fibrosis, Aoshiba et al. (33) provided evidence that persistent DNA damage on alveolar epithelial cells was able to induce a senescence-associated secretory phenotype (SASP), characterized by overexpressions of IL-6, TNF α , MMP-2 and MMP-9. Interestingly, Chilosi et al. (34) described nuclear β -catenin expression in enlarged and/or atypical type II pneumocytes at sites of alveolar damage not only in IPF/UIP (Idiopathic Pulmonary Fibrosis/Usual Interstitial Pneumonia), but also in other forms of interstitial lung disease including DAD, OP/BOOP (Organizing Pneumonia/Bronchiolitis Obliterans Organizing Pneumonia), and NSIP (Non Specific Interstitial Pneumonia). The aberrant activation of Wnt/ β -catenin signaling, a leading pathway of epithelial-mesenchymal-transitions (EMT), would thus be implied in the process of lung remodeling that characterize the reparative phase of

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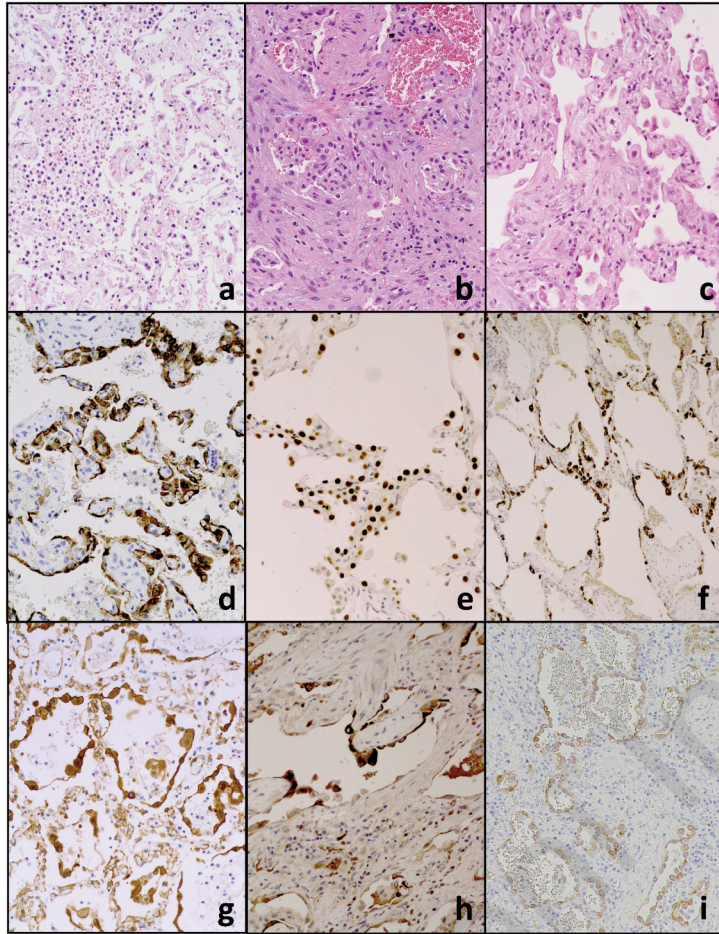


Figure 1 - **a**: the exudative phase of DAD is characterized by intra-alveolar and interstitial oedema, acute inflammatory cell infiltration, and AT1s necrosis. Hyaline membranes, the histologic hallmark of DAD, can be found along the alveolar septa by day 2 of the injury (Ema-toxylin-Eosin, 4x). **b**, **c**: the proliferative/organizing phase of DAD is characterized by mononuclear inflammatory cell infiltration and massive proliferation of both AT2s and fibroblasts. During this phase AT2s may show rather pronounced atypical features (Ema-toxylin-Eosin, 10x and 20x). **d-f**: epithelial alveolar-lining cells (AT1s and AT2s) either in basal or pathologic conditions show immunoreactivity for low molecular weight cytokeratins such as Krt8-18 (**d**), TTF1 (**e**) and specific pneumocyte markers such as CD208 (**f**), ABCA3, surfactant protein-A. **g-h**: aberrant activation of Wnt/ β -catenin signaling, a leading pathway of epithelial-mesenchymal-transitions (EMT), was described in hyperplastic AT2s at sites of alveolar damage in patients with DAD (**g**), IPF and other interstitial lung diseases. Immunoreactivity for other EMT-related markers was then reported in hyperplastic AT2s, in particular the expression of LAM5 γ 2 (laminin-5 gamma-2 chain) (**h**), fascin, HSP27 and phospho-HSP27. **i**: hyperplastic, actively proliferating AT2s were recently found to express Krt14 after severe alveolar damage. Even if its role remains unclear, Krt14 appears as a robust marker of alveolar regeneration and repair, since its expression in the alveoli is only found in pathological conditions.

DAD (Figure 1g). Consistently, the same group (35) later reported the immunophenotypical expression of EMT-related markers in regenerating pneumocytes at sites of alveolar damage in the same set of interstitial lung diseases. In particular, abnormal/hypertrophic type II pneumocytes showed immunoreactivity for LAM5 γ 2 (laminin-5 gamma-2 chain) (Figure 1h), fascin, HSP27 (heat shock protein 27) and phospho-HSP27 (phosphorylated heat shock protein 27) (35). Worth noting, fascin can associate with β -catenin using the same binding site as E-cadherin and co-localising at cell-cell borders and leading edges (36). Studies on mice with chemical-induced lung damage contributed to establish the widely accepted hypothesis that the regeneration of the alveolar epithelium was due to the proliferation of pre-existing AT2s and their subsequent differentiation into alveolar AT1s (37-39). This hypothesis has been recently challenged by a number of studies, where different precursors have been proposed for alveolar epithelium. Chapman et al. (40), using a lineage tracing experiment observed that newly generated AT2s are not derived from pre-existing AT2s, thus suggesting that other progenitors could be involved in the process of alveolar regeneration. Kim et al. proposed as possible precursor candidate

the Bronchioalveolar Stem Cell (BASC) (41). BASCs reside at the bronchiole-alveolar junction, express both Clara cells and AT2s markers, and are able to differentiate *in vitro* into both Clara and AT2s. Anyway, their function has not been proved *in vivo*, and their human counterpart – if any – has yet to be identified. Rock et al. (39) showed that Scgb1a1-expressing cells can give rise to AT1s and AT2s in mouse regenerating alveoli after bleomycin exposure. Consistently, Zheng et al. (42) confirmed that Scgb1a1-expressing cells (most likely Clara cells) give rise to alveolar type I and alveolar type II cells in mice after severe pulmonary damage whether chemically (bleomycin) or virally (Influenza Virus) induced. The same group also identified as intermediate element a bronchiolar epithelial cell that expresses both Clara cell (Scgb1a1) and alveolar type II cell (pro-surfactant protein C)

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We found over-expression of Keratin14 in proliferating pneumocytes after diffuse severe alveolar damage, so we proposed this cytoskeleton's protein as a robust marker of alveolar regeneration and repair.

markers, and that gradually loses Scgb1a1 expression while giving rise to alveolar type II cells (43). More recently Kajstura et al. (44) claimed to have identified a c-kit positive human multipotent stem cell that can be cultured from human lung tissue and would be able to differentiate into bronchioles, alveoli, smooth muscle and pulmonary vessels after injection into a mouse model of lung injury. As might be expected, a strong debate followed the publication of this revolutionary hypothesis (45, 46). Shortly later, in the damaged lung of mice infected with a sub-lethal dose of H1N1 Influenza Virus, Kumar et al. (47) identified a population of CK5/p63-positive cells that would migrate from the bronchioles to the surrounding damaged parenchyma, giving rise to new alveoli. According to the Authors (47), mice infected with H1N1 Influenza Virus would represent a more reliable model of ARDS because, at variance with bleomycin-induced ARDS, in this case the alveolar damage is followed by complete regeneration, similarly to what is generally observed in patients who survive ARDS. The introduction of a new and more reliable murine model of ARDS could therefore be the reason why CK5/p63-positive cells would not have been identified as alveolar progenitors in previous studies. Our group (48) recently tried to verify the hypothesis of Kumar et al. (47) on a series of 15 human lung sample of patients with ARDS, but in none of the 15 cases CK5/p63-positive cells were present in the damaged lung parenchyma. In our opinion, the discrepancy with their findings could be related to the differences existing between the human and the murine lung and/or to the overlapping presence of both alveolar and bronchiolar damage after H1N1 Influenza Virus infection (49-51). In effect, since both kinds of damage are followed by regeneration, Krt5/p63-positive cells might be mostly implied in the bronchiolar regeneration rather than in the alveolar one. In addition, examining the cytokeratin expression profile of pneumocytes during ARDS, we described for the first time the expression of Krt14 in the regenerating human alveoli of patients with ARDS-related DAD (48) (Figure 1i). Krt14-expressing cells did not express Krt5/6 (as usually observed in bronchiolar basal cells), and were clearly recognized as AT2s on the basis of immunophenotypical investigation, using low molecular weight cytokeratins (Figure 1d) and robust and specific pneumocyte markers such as TTF1 (Figure 1e), CD208 (Figure 1f), ABCA-3 and surfactant protein-A. As keratins are involved in controlling cell shape and motility, the transient expression of Krt14 may play a role in the dynamic changes in epithelial morphology that occur during the repair process and also be involved in the modulation of cell proliferation and differentiation (52). We thus suggested a possible role for Krt14 as a robust marker of alveolar regeneration and

repair, since its expression in the lung parenchyma is only found in pathological conditions during pneumocyte proliferation after severe damage.

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