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Alveolar fibroblasts in acute lung injury: biological behaviour and clinical relevance

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ABSTRACT (200 words):

Although fibroblasts are key cells in lung repair/fibrosis process, their characteristics are poorly studied in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). The aims of our study were 1) to determine the biological behaviour of alveolar fibroblasts during acute lung injury,2) to evaluate the clinical relevance of positive alveolar fibroblast culture from patients with ALI/ARDS.

Cells were cultured from 68 bronchoalveolar lavage (BAL) of critically ill ventilated patients: 17-ALI, 31-ARDS and 20-ventilated controls. Patients were followed for 28 days and clinical data recorded. We studied proliferation, migration, and collagen-1 synthesis capacities of fibroblasts.

Cells expressing fibroblast markers were cultured from BAL obtained in 6/17 (35%) ALI and 6/31 (19%) ARDS patients respectively but never from ventilated controls. Alveolar fibroblasts exhibited a persistent activated phenotype with enhanced migratory and collagen-1 production capacities, with hyporesponsiveness to PGE(2) compared to normal lung fibroblasts ($p \leq 0.04$). Positive fibroblast culture was associated with both an increased collagen-1 concentration and monocyte/macrophage percentage in BAL fluid ($p \leq 0.01$), and with a reduced duration of mechanical ventilation ($p < 0.001$).

We conclude that activated alveolar fibroblasts can be cultured either in ALI and ARDS and their presence might reflect the initiation of the organizing phase of acute lung injury.

Keywords: acute respiratory distress syndrome, bronchoalveolar lavage fluid, collagen 1, migration, fibrocyte, repairs.

INTRODUCTION

Acute lung injury (ALI) and its extreme form, acute respiratory distress syndrome (ARDS) are important causes of mortality in critically ill patients [1]. ARDS is characterized by an acute pulmonary inflammation that contributes to alveolar-capillary membrane injury with concomitant tissue repair and remodeling which can evolve to lung fibrosis. Histological evidence of lung fibrosis is associated with poor outcome in patients with ARDS during ICU stay [2, 3]. Fibroblasts play a key role in normal and pathologic repair [4]. These mesenchymal cells produce large quantities of extracellular matrix components, cytokines and repair growth factors during wound healing [5, 6]. After lung injury, fibroblasts proliferate, differentiate into myofibroblasts expressing α-smooth muscle actin and migrate in the fibrinous exudate inside the alveolar airspace [7, 8]. In resolutive ALI/ARDS, repair proceeds normally with complete reestablishment of the normal alveolar architecture. In non resolutive ALI/ARDS, normal healing is disrupted with persistent mesenchymal cells in the interstitial/alveolar spaces, chronic myofibroblast activation and excessive extracellular matrix deposits such as collagen 1 [4]. Despite their fundamental role in lung repair, lung fibroblasts obtained either from lung explants or from bronchoalveolar lavage (BAL) fluid have been the subject of a limited number of studies [9, 10]. Many aspects of fibroblast functional properties and the clinical relevance of their detection in alveolar lumen in patients with different stage of lung injury as ALI or ARDS remain unknown. We hypothetized that in critically ill ventilated patients, alveolar fibroblasts with an activated phenotype may be predominantly cultured from BAL of the most severe patients with ARDS and could adversely affect their outcome. Therefore, the aims of our study were: 1) to determine the biological behaviour of alveolar fibroblasts during acute lung injury 2) to evaluate the clinical relevance of positive alveolar fibroblast culture from patients with ALI/ARDS.

METHODS

Study population

The protocol was approved by the ethical committee of Paris-Hotel-Dieu Hospital. Ventilated patients from 3 intensive care units (ICU) were prospectively enrolled if a BAL procedure was performed to confirm a clinically suspected ventilator-associated pneumonia [11]. The patients were classified in 3 groups after inclusion (ALI, ARDS and a ventilated-control group without ALI/ARDS) according to the criteria of the American-European Consensus Conference on ALI/ARDS [12]. All the patients were ventilated and weaned from ventilator according to current guidelines [13]. Patients with pre-existing fibrotic lung disease, corticosteroid medication, HIV infection, end stage cancer, age<18 years or current pregnancy were excluded. The day of inclusion, the following clinical data were recorded: age, sex, reason for ICU admission, presence or absence of sepsis and pulmonary infection, PaO2/FiO2 ratio, PaCO2, Simplified Acute Physiologic Score II on admission to ICU (SAPSII) [14], Sequential Organ Failure Assessment score (SOFA) [15] and Lung Injury Score (LIS) [16]. The length of time between the onset of the mechanical ventilation support and the BAL, as well as the length of ventilatory support, the stay in ICU and mortality 28 days after the BAL were recorded for all patients.

Bronchoalveolar lavage protocol and BALF sample processing

The BAL was performed and processed as previously described [17]. A differential BAL cell count was performed on a cytocentrifuge smear with a Diff-Quik stain kit (Dade International, Miami, USA). BAL fluid (BALF) and plasma protein concentrations were measured with the analyst Hitachi-911 (Roche, Meylan, France), and the protein ratio was determined to evaluate the alveolar permeability induced by lung injury.

Isolation and culture of fibroblasts

The BAL cell viability was determined before initial plating by Trypan blue vital staining (viability always >92% in the different groups tested). The BAL cells (n=68) were cultured at a density of 2.10^5cells/cm^2 in 25cm^2 flask (Corning Costar, Lowell, USA) in 5ml of RPMI-1640 (Gibco, Grand Island, USA) supplemented with 10% heat decomplemented fetal calf serum (FCS), 2mM L-glutamine, and antibiotics (Invitrogen, Cergy Pontoise, France) for at least 28 days. Cells grown from BAL were used for immunocytochemical characterization 24 hours and 21 days after initial plating (n=5), and then at passage 1 and 3 (n=12). Nine BAL cell lines were randomly chosen and their proliferation, migration and collagen 1 production capacities were compared to those of fibroblasts derived from normal lung explants obtained from 5 patients (two women and three men, mean age: 60 years) undergoing lung surgery for removal of a primary lung tumour. Three patients had never smoked, two were ex-smokers. As previously described [18], normal lung from a healthy segment distant from the solidary lesion was obtained. Fibroblasts were cultured with complete medium and 10% FCS supplemented with antibiotics and were used at passage 3.

Measurement of C-terminal propeptide of type I procollagen, MCP-1, IL-8 and TGF-β1

C-terminal propeptide of type I procollagen (PICP) was measured by enzyme immunoassay (OSTEOmedical, Paris, France). The detection threshold of the assay was 0.2ng/ml. Transforming Growth Factor (TGF-β1), Monocyte Chemoattractant Protein-1 (MCP-1) and IL-8 concentrations were measured by ELISA (R&D systems, Minneapolis, USA). The detection thresholds of TGF-β1, IL-8 and MCP-1 assays were 5pg/ml, 3.5pg/ml and 5pg/ml, respectively.

Immunocytochemistry

Alveolar fibroblasts cultured on Lab-Tek slides (Nunc, Naperville, USA) were fixed with acetone. Anti-human antibodies (Ab) directed against collagen 1 (Sigma, Saint Louis, USA), vimentin (Immunotech, Marseille, France), prolyl-4-hydroxylase (α-4H), desmin, pancytokeratin, CD31, alpha-smooth muscle actin (α-SMA), CD45 (Dako SA, Trappes, France), smooth muscle myosin 1 (Abcam, Paris, france), CD14, CD34 (BD-pharmingen, Le Pont-De-Claix, France) or isotype-matched control Ab were used. Positive cells were revealed using the Vectastain ABC-alkaline phosphatase kit mouse IgG (Vector-Abcys, Paris, France) and the fast red substrate (Dako SA).

Confocal microscopy analysis

Cells cultured on Lab-Tek slides were fixed after 21 days of culture with acetone and saturated with 50 mM NH4Cl and permeabilised with 0.1% Triton X100. After saturation with normal horse serum, slides were incubated with appropriate dilution of anti-collagen 1 monoclonal Abs (1:290) (Sigma) prior to the addition of fluorescent-labelled Abs (biotinilated horse antimouse (1:300) + Alexa Fluor 568-conjugated streptavidin (1:100) (Invitrogen, Carlsbad, CA)). After washing, the cells were incubated with anti-CD45-Alexa Fluor 488 Abs (1:25) (Santa cruz, San Diego, CA). Cell nuclei were localized by DNA staining with TO-PRO-3 (0.4 µg/ml) (Molecular Probes, Oregon, USA). Slides were mounted with Prolong Gold anti-fade reagent (Invitrogen) and examined by confocal laser scanning microscopy (LSM-510-META microscope; Zeiss, Oberkochen, Germany). To test for the specificity of immunostaining, Abs were replaced by an isotype-matched control Ab.

Cell proliferation assay

After 72h of incubation with 0.1% FCS alone (basal condition) or with PGE(2) 10^{-6} M, rhIL-1β 10ng/ml, rhPDGF-BB 10ng/ml or rhTGF-β1 10ng/ml (R&D systems), proliferation was

measured as bromodeoxyuridine (BrdU) incorporation according to the manufacturer's protocol (Roche).

Cell migration assay

Cell motility was examined using a modified Boyden chamber apparatus (Transwell Costar) fitted with an 8µm pore membrane coated with fibronectin (10µg/ml). Serum deprived fibroblasts (2.10^5/well) were placed into the upper well and allowed to migrate for 24 hours in presence or absence (basal condition) of PGE(2) 10^{-6} M, Oleoyl-L-α-lysophosphatidic acid sodium salt (LPA, 10^{-6} M) (Sigma), or rhPDGF-BB 10ng/ml into the lower well. Non migrated cells were removed and the membrane was fixed and stained with Diff-Quick staining kit. The number of cells that migrated to the lower surface of the membrane was counted under 400× magnification. Five high power random fields (HPF) were counted per sample and run in duplicate. Chemotactic index was determined as the ratio of cells moving in response to a chemoattractant relative to cells moving in response to media control.

Assessment of collagen 1 and TGF-β1 protein secretion

Cells (5.10^4 cells/well) were cultured in serum-free medium (basal condition) or with mediators: PGE(2) 10^{-6} M, rhIL-1β 10 ng/ml or rhTGF-β1 10 ng/ml for 48 hours. The concentration of PICP or TGF-β1 in fibroblast supernatants was divided by the total protein amount of the cell monolayer (Bio-Rad protein assay, Hercules, USA).

Assessment of Collagen 1 or TGF-β1 mRNA expression

After 18 hours of culture, the relative content of mRNA was analyzed after reverse transcription by real-time polymerase chain reaction (RT-PCR) as previously described [17]. The following primer sequences were used: collagen 1-alpha1 (COL1A1) CGAGGTAGTCTTTCAGCAACACAGT and AGCCACCAGCCCCTCACT, collagen 1-alpha2 (COL1A2) CAGTTCTTGGCTGGGATGTTT and

TTGAGACTCAGCCACCCAGAGT, (TGF-β1) TGCTGTCACAGGAGCAGTG and AGGGGAAATTGAGGCTTT, Ubiquitin C (UBC) TTTTTTGGGAATGCAACAACTTT and CACTTGGTCCTGCGCTTGA.

Statistical analysis

Demographic data were expressed as means ± SD and biological data as medians and range values. All proportional values were compared with the Fischer's Exact test or Chi-2 test for multiple comparisons. The continuous variables were compared by the Mann-Whitney test or Wilcoxon paired test when appropriate. Multiple comparisons were tested by the Kruskal-Wallis or Friedman test followed by Dunn's multiple-comparison post-hoc analysis. Durations of ventilation after the BAL procedure were analysed by Kaplan-Meier survival curves and compared by log-rank analysis. Correlations were assessed with the Spearman rank-order test. Statistical significance was accepted as $p \leq 0.05$.

RESULTS

Characteristics of the study population

Thirty-one ARDS, 17 ALI and 20 ventilated patients without ALI/ARDS criteria were enrolled. The patient characteristics are depicted in **Table 1.** ALI/ARDS patients were admitted for septic shock (n=23), multiple organ failure (n=18), cerebral stroke or convulsive state (n=4), severe trauma (n=2) or cardiac arrest (n=1). The ventilated patients without ALI/ARDS were admitted for acute heart failure and postoperative complications following cardiopulmonary bypass (n=14), postoperative localized pneumonia (n=3), acute renal failure (n=1), hemorrhagic shock (n=1) or acute intoxication (n=1).

Cells cultured from BAL expressed fibroblast markers

All BAL cell pellets (n=68) were maintained in culture for 28 days without any bacterial or fungal contamination. At the time of initial plating, BAL cells were mainly neutrophils and mononuclear cells (macrophage/monocyte)**.** In positive cell cultures (n=12), isolated spindle-shaped cells were usually detected after the first week of culture (median time: 10 days [4-23 days]) **(Fig.1A)**. After 3 weeks of culture, spindle-shaped cells formed foci **(Fig.1A)**, while round cells (likely macrophages) were quiescent. After 4 weeks of culture, the cell monolayers were harvested to produce a homogenous cell population in the subsequent cultures **(Fig.1A)**.

We characterized the phenotype of adherent BAL cells from 5 different patients (3 ARDS and 2 ALI) 24 hours after initial plating. At that time point, 95% of adherent cells were mononuclear round cells expressing the common leukocyte marker (CD45+) **(Fig.1B)**. The remaining cells were CD45-, including some endothelial cells (CD31+) and epithelial cells (pancytokeratin+). No mature form of fibroblast (CD45-) with large cytoplasm expressing collagen 1 was detectable 24 hours after plating. By contrast, we observed a side population (<1% of the total cell population) of very typical elongated spindle-shaped cells with a ratio

of cell length/cell width equal to 16. All these cells were CD45+, prolyl-4-hydroxylase+ and weakly CD34+ (**Fig.1B)**. This pattern of expression is consistent with that of fibrocytes [19] and was confirmed by confocal microscopy showing the coexpression of collagen 1 and CD45 on day 21, before the first passage **(Fig.1C)**. At first passage, most of the spindle-shaped cells were positive for prolyl-4-hydroxylase, collagen 1 and vimentin but only a few population were still CD45+ **(Fig.1D)**. A limited population of cells with large cytoplasm expressing the alpha-smooth muscle actin (αSMA) was also observed at this passage. The cells characterized at passage 3 demonstrated a stable mesenchymal phenotype with positive staining for vimentin, collagen 1 and prolyl-4-hydroxylase **(Fig.1E)**. The alpha-smooth muscle actin was expressed in 8 out of 12 cell lines in which 20% of cells were αSMA positive. Markers for vascular smooth muscle cells (desmin, smooth muscle myosin 1), epithelial cells (pancytokeratin), endothelial cells (CD31), monocytes (CD14), leukocytes (CD45) and hematopoieteic progenitor cells (CD34) were negative at this passage. Together, this pattern of markers is consistent with a differentiated fibroblast phenotype. According to these characteristics, the cells are hereby referred to as "alveolar fibroblasts".

Proliferative rate and migration of alveolar fibroblasts

After 72 hours of culture, no significant difference in the proliferative rate was observed between unstimulated alveolar and control fibroblasts studied at passage 3 (BrdU median absorbance 0.58 vs. 0.60, $p>0.05$). IL-1β, TGF-β1 or PDGF-BB induced a similar weak mitogenic effect in both types of fibroblasts (125 % of basal condition, $p>0.05$). PGE(2) induced a weak and similar inhibitory effect on proliferation in both cell types (75% of basal condition, $p>0.05$). We examined the migration of alveolar fibroblasts in the presence or absence of mediators known to modulate mesenchymal cell migration. In basal condition, the migration of alveolar fibroblasts was 3 fold higher than that demonstrated by control fibroblasts (26 vs. 8 cells/high power field (HPF), $p=0.04$) **(Fig.2A)**. PDGF-BB (10ng/ml) and

LPA (10^{-6}M) stimulated control and alveolar fibroblast migration to the same extent **(Fig.2B, 2C)**. By contrast, alveolar fibroblasts were hyporesponsive to the inhibitory effect of PGE(2) as compared to control fibroblasts (73 vs. 23% of basal condition, p=0.01).

Collagen 1 production was elevated in alveolar fibroblasts and correlated with TGF-β1 production

Collagen 1 production was assessed by measuring PICP in cell culture supernatants. PICP secretion by alveolar fibroblasts was four-fold higher than that of control fibroblasts (7.69 vs 1.63 ng/µg/well, p<0.01) **(Fig.3A)**. In addition, COL1A1 and COL1A2 mRNA expression in alveolar fibroblasts was higher than in control fibroblasts (p<0.01, **Fig.3B)** and was positively correlated with collagen 1 protein secretion (rho 0.75, p=0.03). Recombinant TGF-β1, and to a lesser extent rhIL-1β, stimulated collagen 1 production by control and alveolar fibroblasts (p<0.05). After stimulation, collagen 1 production remained higher in alveolar fibroblasts than in control fibroblasts (p≤0.04) **(Fig.3A)**. Interestingly, as compared to fibroblasts obtained from normal lung explants, alveolar fibroblasts were hyporesponsive to the inhibitory effect of PGE(2) (p<0.05). As TGF-β1 is a key factor in collagen 1 production by fibroblasts, its role was specifically studied. The basal levels of TGF-β1 secretion by alveolar and control fibroblasts were similar (5.3 vs. 4.8 pg/µg/well, p=0.24) **(Fig.4A)**. A strong correlation was found between TGF-β1 and collagen 1 production by alveolar fibroblasts at both protein and transcriptional levels **(Fig.4B, 4C)**. Such a correlation was not found in control fibroblasts **(Fig.4D)**.

Clinical determinants of alveolar fibroblast culture from BAL and influence on outcome

Alveolar fibroblasts were grown from BAL of patients with ALI (n=6/17; 35%) or ARDS (n=6/31; 19%) but never from ventilated controls (n=0/20) (p≤0.04, control vs. ALI or ARDS). The proportion of positive fibroblast cultures was higher in ALI than in ARDS

without reaching significance (35% vs 19%, p=0.30). There was a greater proportion of positive cultures (8/25 BAL, 35%) in the early phase (<7 days) of ALI/ARDS when compared to the late phase (≥ 7days) (4/23 BAL, 17%), although this difference was not statistically significant (p=0.32).

To clarify the determinants of positive fibroblast culture, the ALI/ARDS patients were divided into two groups according to the cell culture results (positive culture (n=12) vs. negative culture (n=36)). As summarized in **Table 2**, the clinical parameters were similar in the two groups. There was no difference in PaO2/FiO2 ratio, severity scores, etiology of ALI, length of ICU stay and 28 days mortality. However, the total duration of mechanical ventilation was notably reduced in patients with positive culture (14 vs. 28 days, p=0.03). This difference was explained by a reduction of mechanical ventilation duration after BAL procedure (8 vs. 21 days, $p < 0.001$) **(Fig.5B)** and was observed in patients with early (8 vs. 22 days, p=0.0024) and late (9 vs 21 days, p= 0.0068) ALI/ARDS.

Patients with positive fibroblast culture exhibited specific BAL fluid characteristics **(Table 3)**. In this group, a higher collagen 1 concentration associated with a reduction of inflammatory response assessed by a decreased neutrophil percentage and a lower IL-8 concentration in BAL was noted. In parallel, an increased percentage of monocytes/macrophages was found, that was positively correlated with BALF MCP-1 concentration (rho= 0.67, p=0.02).

DISCUSSION

We demonstrate for the first time in patients with ALI/ARDS that: 1) A limited population of fibrocytes is detectable during the initial phase of BAL culture 2) Alveolar fibroblasts can be cultured from BAL in 25% of ALI/ARDS patients but never from ventilated patients without ALI/ARDS 3) Alveolar fibroblasts can be isolated either in ALI and ARDS. They exhibit a persistent activated phenotype with enhanced migratory and collagen 1 production capacities and are hyporesponsive to PGE(2) in comparison with control fibroblasts 4) Collagen 1 concentration and monocyte/macrophage percentage are increased in BAL with positive culture whereas neutrophil percentage and IL-8 are decreased 5) The presence of alveolar fibroblasts is associated with a lower duration of ventilatory support.

In this study we have evaluated the ability to culture fibroblasts from a high number of BAL of ventilated critically ill patients (n=68). For the first time, we show that alveolar fibroblast culture is not limited to the most severe form of ARDS as we observed even a higher proportion of positive fibroblast cultures in BAL from ALI. Owing to the lack of positive culture in the control group, our findings argue that BAL fibroblast culture is independent of mechanical ventilation per se. However, a possible selection bias linked to the observational design of our study could occur. As BAL was performed for the clinical suspicion of pneumonia, we may have included the most severe ALI patients with clinical worsening. Otherwise, the equal proportion of fibroblast culture in patients with or without confirmed bacterial pneumonia does not support a significant role of infection in this process.

Alveolar fibroblasts could potentially originate from a local precursor as interstitial fibroblasts, from pneumocytes after epithelial-mesenchymal transition or from a circulating precursor derived from bone-marrow and recruited to the lung [5, 8, 20]. In our study, 24 hours after initial plating we observed a population of spindle-shaped cells expressing fibrocyte markers (CD45+, prolyl-4-hydroxylase + and weakly CD34+) [19]. These cells accounted for less than 1% of total adherent cells. To our knowledge, this is the first

demonstration that cells with characteristics of fibrocytes (plastic adherent, spindle-shaped cells, CD45+, prolyl-4-hydroxylase+) can be detected within 24 hours among adherent cells from BAL fluid during ALI/ARDS in human. In agreement with other studies in human BAL [10, 21, 22], we found that most of the mesenchymal cells that proliferated were CD45- at the first passage. These cells could result from differentiation of fibrocytes after loss of CD45 expression [23], and/or from the proliferation of CD45- progenitors cells, such as mesenchymal stem cells (MSC) [21, 22]. Our data, do not allow us to appreciate the relative contribution of each cellular pathway in this process.

We observed variability in the percentage of cells expressing α-SMA at passage 3. Despite, this phenotypic variability, all the isolated fibroblast lines showed an activated phenotype in comparison with fibroblast derived from normal lung explants. We found a constant upregulation of collagen production, both at baseline and after TGF-β1 stimulation. A similar observation was made in systemic sclerosis and attributed to an upregulation of TGF-β signaling axis [24]. We observed a specific correlation between collagen 1 and TGF-β1 expression in alveolar fibroblasts suggesting the possible implication in ALI/ARDS of such an autocrine TGF-β1 loop in collagen overproduction. However, in vivo, the picture is certainly more complex, as other TGF-β1 producing cells may affect fibroblasts functions as previously described [25, 26]. The alveolar fibroblast activation was also confirmed by the strong enhancement of their migratory capacity which is a crucial step in repair and in fibrotic evolution after acute lung injury [27]. We observed that the alveolar fibroblasts had a relative insensitivity to PGE(2) a major anti-fibrotic mediator, both for migration and collagen 1 production. This finding was in agreement with observations made in lung injury model [28] and in chronic pulmonary fibrosis [29] where a lower PGE(2) susceptibility was explained by EP(2) receptor downregulation.

Our results must be interpreted with caution as the functional differences observed between alveolar fibroblasts cultured from BAL fluid of patients with ALI/ARDS and fibroblast

derived from normal lung explants might represent either an activated state of alveolar fibroblasts, or a difference in phenotype between two distinct populations of fibroblast originating from two distinct areas of the lung. The later hypothesis is consistent with observations made in asthma or systemic sclerosis, where alveolar fibroblasts and interstitial fibroblasts obtained from the same patient exhibited different phenotypes [30-32]. We were not able to perform such a comparison as obtaining fibroblasts from BAL and biopsy in the same patient with ALI/ARDS was not possible in the clinical setting for ethical concerns. Furthermore, alveolar fibroblasts cannot be grown from BAL in healthy subjects [10, 30, 33]. In some experiments (*see online supplement*), we found that ALI/ARDS alveolar fibroblasts had proliferation and collagen 1 secretion capacities very similar to those of IPF fibroblasts cultured form BAL.

Neutrophils were the main cell population in BAL from ALI/ARDS patients included in our study. However, BAL associated with positive fibroblast culture contained a 3-fold higher percentage of monocyte/macrophage and an increased collagen 1 concentration as compared to BAL with negative fibroblast culture. These observations might be interpreted in two non-exclusive ways. Firstly, a cross-talk between fibroblast precursors and monocytes/macrophages may be required for in vitro proliferation of fibroblasts. This hypothesis is supported by in vitro studies showing that 1) monocytes/macrophages stimulated by Th2 cytokines can interact with fibroblasts through CCL18 signaling [34] and 2) monocytes/macrophages produce mediators such as PDGF-BB and TGF-β1, which promote mesenchymal progenitor recruitment, proliferation and fibroblast survival [10]. Secondly, reduced neutrophils and increased macrophages together with increased collagen 1 concentration suggest that BAL was performed at a time where the organizing phase of alveolar repair was engaged [35] with a reduction of inflammatory response. This interpretation is supported by the analysis of consecutive BAL from patients showing that

alveolar macrophages increased in ARDS survivors [36], and by the capacity of macrophages to control neutrophil recruitment to the lung [37].

In our study, the beneficial role of alveolar fibroblasts is suggested by the reduction of mechanical ventilation duration observed in ALI/ARDS survivors with a positive fibroblast culture. This finding could reflect the participation of alveolar fibroblasts in lung repair by production of Keratinocyte Growth Factor and Hepatocyte Growth Factor [6, 17]. Otherwise, the fibroblasts are known to play a specific role in inflammation resolution after an acute injury [38]. In addition, an optimal myofibroblast differentiation seems also necessary to an efficient lung repair [39] and is associated with better outcome during ALI/ARDS [25].

In conclusion, our study demonstrated that fibroblast population could be isolated from alveolar lumen during the early and late phase of ALI and ARDS in 25% of the patients. These cells showed a persistent activated phenotype with enhanced collagen 1 production and migratory capacities. Their presence is associated with a reduction of ventilation duration and with a decrease of inflammatory markers and could reflect an adapted repair process contributing to the resolution of acute lung injury. Our results and other recent works refocus the role of fibroblasts as an essential actor of lung repair during acute lung injury.

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TABLES

Table 1. Characteristics of patients

	w/o ALI or ARDS (n=20)	ALI (n=17)	ARDS (n=31)	*p* (†)
Age (years)*	66 ± 11	61 ± 18	62 ± 16	0.92
Sex ratio (Women/men)	7/13	10/7	11/20	0.23
Simplified acute physiologic score (SAPS II)*	45 ± 19	51 ± 22	50 ± 16	0.67
Sepsis (%)	45	59	58	0.60
Sepsis-related Organ failure Assessment (SOFA)*	7 ± 3	7 ± 3	8 ± 3	0.29
Pulmonary infection (%)	35	41	45	0.77
Etiology of ALI/ARDS (extrapulmonary/pulmonary)	-	5/12	13/18	0.54
Lung Injury Score (LIS)*	-	1.4 ± 0.5	2.0 ± 0.5	< 0.001
PaO2/FiO2 ratio *	262 ± 70	252 ± 51	156 ± 73	< 0.001
PaCO2 (kPa)*	4.9 ± 0.9	5.7 ± 1.2	5.6 ± 1.3	0.04
Length of ICU stay (days)*	25 ± 21	26 ± 16	33 ± 24	0.40
Length of mechanical ventilation before BAL (days)*	7 ± 7	10 ± 11	9 ± 8	0.52
Total time on mechanical ventilation (days)*	21 ± 18	20 ± 15	27 ± 25	0.60
28 days mortality (%)	10	35	39	0.07

ALI: Acute Lung Injury; ARDS: Acute Respiratory Distress Syndrome; w/o ALI or ARDS: ventilated patients without criteria of ALI/ARDS; ICU:Intensive Care unit;* Values are Means ± SD; † Kruskal-Wallis test or Chi-square test when appropriate

Table 2. Clinical characteristics and outcome of ALI/ARDS patients associated to the alveolar fibroblast culture

	Alveolar fibroblast culture		
	negative (n=36)	positive (n=12)	*p* (†)
Age (years)*	65 ± 14	52 ± 20	0.07
Sex ratio (Women/men)	11/25	7/5	0.10
Simplified acute physiologic score (SAPS II)*	51 ± 18	49 ± 21	0.70
Sepsis (%)	58	58	1.00
Pulmonary infection (%)	44	42	0.40
Sepsis-related Organ failure Assessment (SOFA)*	8 ± 3	7 ± 4	0.39
Etiology of ALI/ARDS (extrapulmonary/pulmonary)	14/22	5/7	0.90
Lung injury classification (ALI/ARDS)	11/25	6/6	0.30
Lung Injury Score (LIS)*	1.9 ± 0.5	2.0 ± 0.9	0.92
PaO2/FiO2 ratio *	181 ± 56	217 ± 128	0.44
PaCO2 (torr [kPa])	5.6 ± 1.3	5.9 ± 1.1	0.49
Length of ICU stay (days)*	33 ± 23	23 ± 14	0.22
Length of mechanical ventilation before BAL (days)*	10 ± 9	8 ± 8	0.36
Total time on mechanical ventilation (days)*	28 ± 24	14 ± 9	0.03
28 days mortality (%)	39	33	0.73

ALI: Acute Lung Injury; ARDS: Acute Respiratory Distress Syndrome; ICU:Intensive Care unit;
* Values are Means ± SD; † Mann Whitney test or Fischer exact test when appropriate

Table 3. Characteristics of BAL fluid from ALI/ARDS patients associated to the alveolar fibroblast culture

	Alveolar fibroblast culture		*p* (‡)
	negative (n=36)	positive (n=12)	
BALF protein (g/l)*	0.44 ± 0.32	0.61 ± 0.37	0.24
Protein ratio BALF/serum	0.009	0.011	0.19
BALF total cell count (10.6)*	17 ± 31	20 ± 23	0.51
Neutrophil (%)*	85 ± 17	58 ± 31	< 0.01
Macrophage (%)*	13 ± 15	38 ± 31	< 0.01
IL-8 (pg/ml) †	6344 [177-35988]	2233 [145-9150]	0.03
MCP-1 (pg/ml) †	284 [<5-1060]	337 [85-1100]	0.32
TGFβ-1 (pg/ml) †	46.8 [<5-294]	54.8 [5.4-213]	0.59
proCollagen 1 (ng/ml) †	4.78 [<0.2-51]	11.38 [2.3-25]	0.01

BALF: Bronchoalveolar lavage fluid; IL-8: Interleukin 8;MCP-1: Monocyte chemotactic protein-1;TGFβ-1: Transforming Growth Factor beta-1; * Values are Means ± SD; † Values are Means and extreme values []; ‡ Mann Whitney test

FIGURE LEGENDS

Figure 1. (A) Morphology of adherent BAL cells at different times of culture when a positive culture occured. (Black arrows indicate typical adherent spindle-shaped cells) Original magnification, x40. **(B) Immunocytochemical characteristics of adherent BAL cells after initial plating (H24)** (black arrows indicate typical adherent spindle-shaped cells). Original magnification, x400. **(C) Characterization of fibrocytes (spindle-shaped cells co-expressing CD45 and collagen 1) by confocal microscopy analysis of BAL cell culture at day 21.** Original magnification, x630. **(D) Representative immunocytochemical stain prepared from alveolar fibroblasts at passage 1** (black arrows indicate CD45+ spindle-shaped cells) Original magnification, x100. **(E) Representative immunocytochemical stain prepared from alveolar fibroblasts at passage 3.** Original magnification, x100. (α-4H means prolyl-4-hydroxylase and α-SMA means alpha-smooth muscle actin).

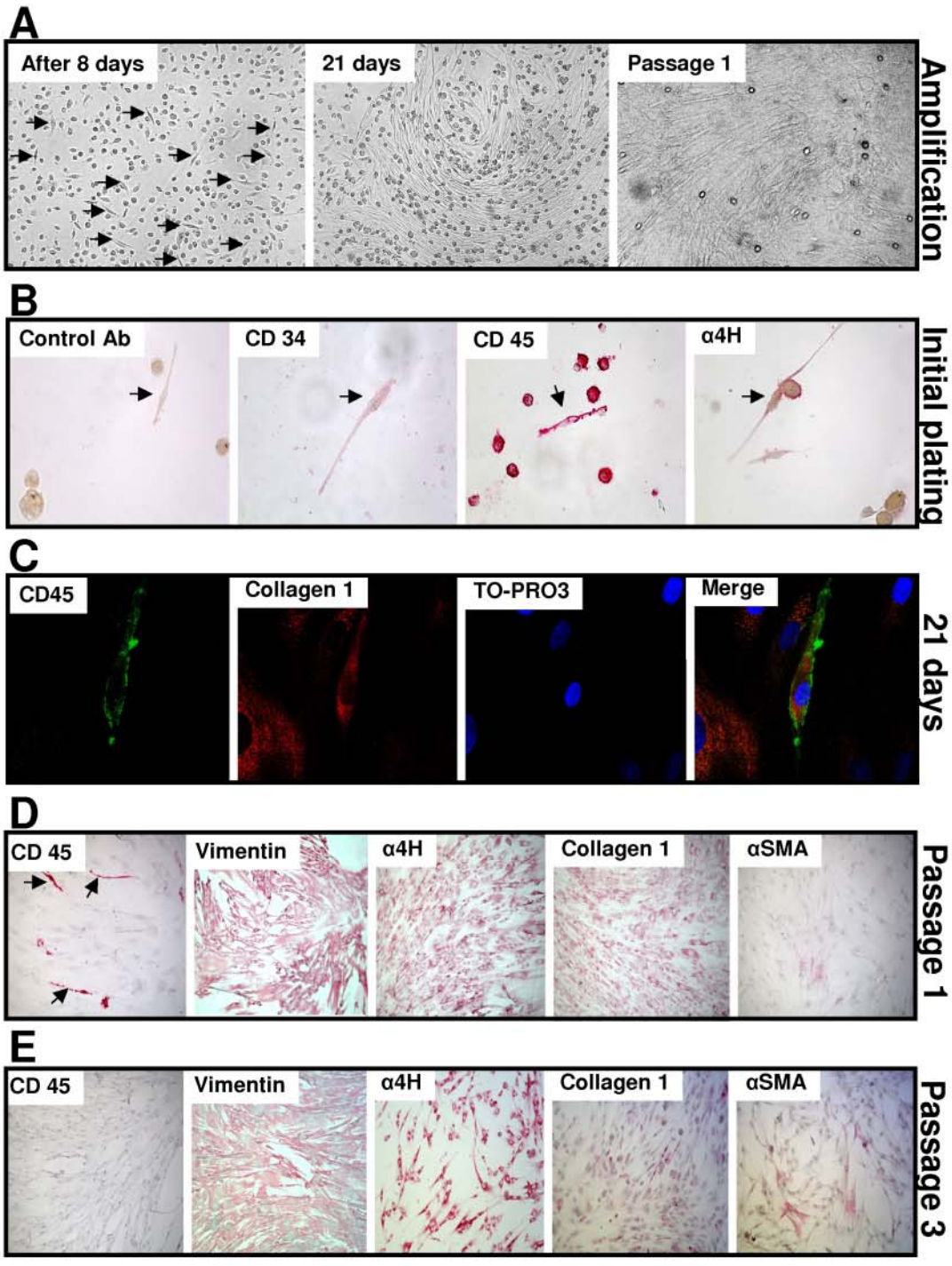

A
After 8 days
21 days
Passage 1
Amplification
B
Control Ab
CD 34
CD 45
α4H
Initial plating
C
CD45
Collagen 1
TO-PRO3
Merge
21 days
D
CD 45
Vimentin
α4H
Collagen 1
αSMA
Passage 1
E
CD 45
Vimentin
α4H
Collagen 1
αSMA
Passage 3

Figure 2. Alveolar fibroblast migration capacities (A) After 24-hours, basal migratory capacity of alveolar fibroblasts (n=9) was assessed in comparison with control fibroblasts (n=5) by cell count in 5 random high power fields (HPF) run in duplicate. Horizontal black bars indicate the median of individual values. **(B)** Cell migration in the presence of Prostaglandin E(2) (PGE(2)) 10^{-6} M; Lysophosphatidic acid (LPA)10^{-6} M and Platelet derived growth factor (rhPDGF-BB) 10 ng/ml. Results are expressed on a logarithmic scale as chemotactic index (ratio of the basal condition performed in the same assay) **(C)** Representative observations of alveolar fibroblasts traversing the chamber membrane. Original magnification, x400.

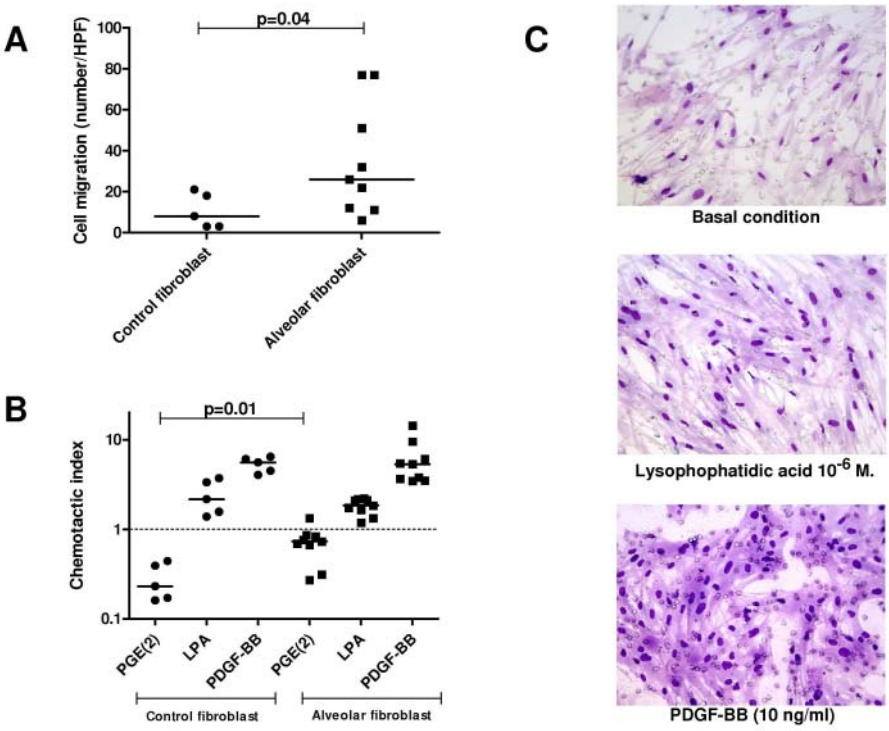
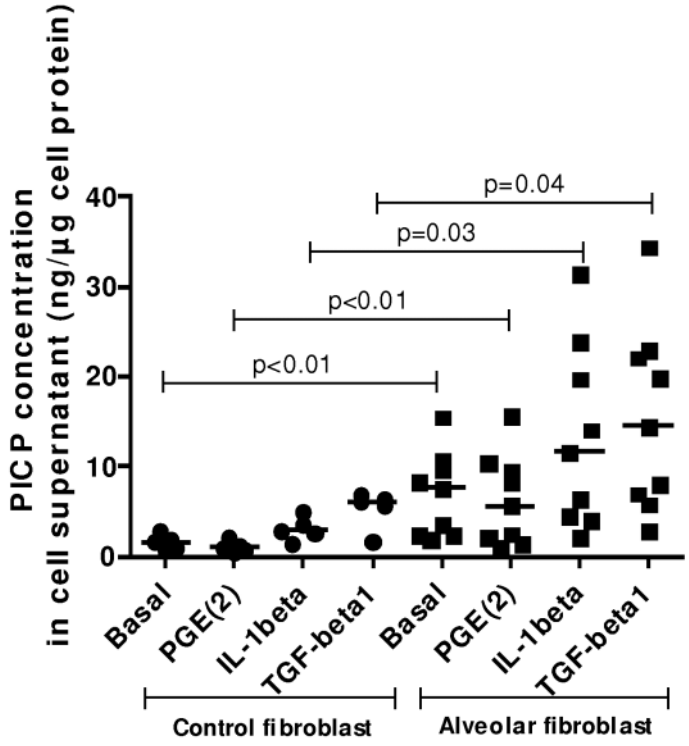


Figure 3. Alveolar fibroblast type I collagen production capacities (A) Type I collagen synthesis of alveolar fibroblasts (n=9, black squares), in comparison with control fibroblasts (n=5, black circles). Fibroblasts were cultured in duplicate for 48h in serum-free medium alone (basal condition) or with Prostaglandin E(2) (PGE(2)) 10^{-6} M, rhIL-1β 10 ng/ml or

rhTGF-β1 10 ng/ml. Horizontal black bars indicate the median of individual values. **(B)** Unstimulated alveolar and control fibroblast type I collagen alpha-1 (COL1A1) and alpha-2 (COL1A2) mRNA relative content analyzed at 18H by quantitative real time RT-PCR and expressed as a ratio to the Ubiquitin C mRNA (UBC). Horizontal black bars indicate the median of individual values.

A


PICP concentration
in cell supernatant (ng/µg cell protein)
40
30
20
10
0
p=0.04
p=0.03
p<0.01
p<0.01
Basal
PGE(2)
IL-1beta
TGF-beta1
Basal
PGE(2)
IL-1beta
TGF-beta1
Control fibroblast
Alveolar fibroblast

B

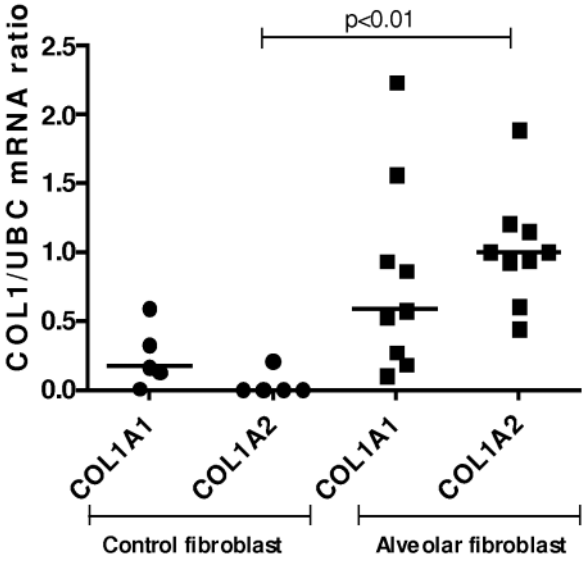

COL1/UBC mRNA ratio
2.5
2.0
1.5
1.0
0.5
0.0
p<0.01
COL1A1
COL1A2
COL1A1
COL1A2
Control fibroblast
Alveolar fibroblast

Figure 4. Correlation between TGF-β1 and type I collagen production in alveolar fibroblasts (A) Basal TGF-β1 synthesis of alveolar fibroblasts (n=9, black squares) in comparison with control fibroblasts (n=5, black circles). Fibroblasts were cultured in duplicate for 48h in serum-free medium alone (basal condition). Horizontal black bars indicate the median of individual values. **(B)** Correlation between TGF-β1 and type I collagen concentration in unstimulated alveolar fibroblast supernatants **(C)** Correlation between both type I collagen alpha-1 (COL1A1) and alpha-2 (COL1A2) mRNA and TGF-β1 mRNA expression in unstimulated alveolar fibroblasts (n=9, rho=0.87 and 0.72 respectively, $p<0.04$) **(D)** Lack of correlation between both type I collagen alpha-1 (COL1A1) and alpha-2 (COL1A2) mRNA and TGF-β1 mRNA expression in unstimulated control fibroblasts (n=5, $p>0.05$). Results are represented on a logarithmic scale.

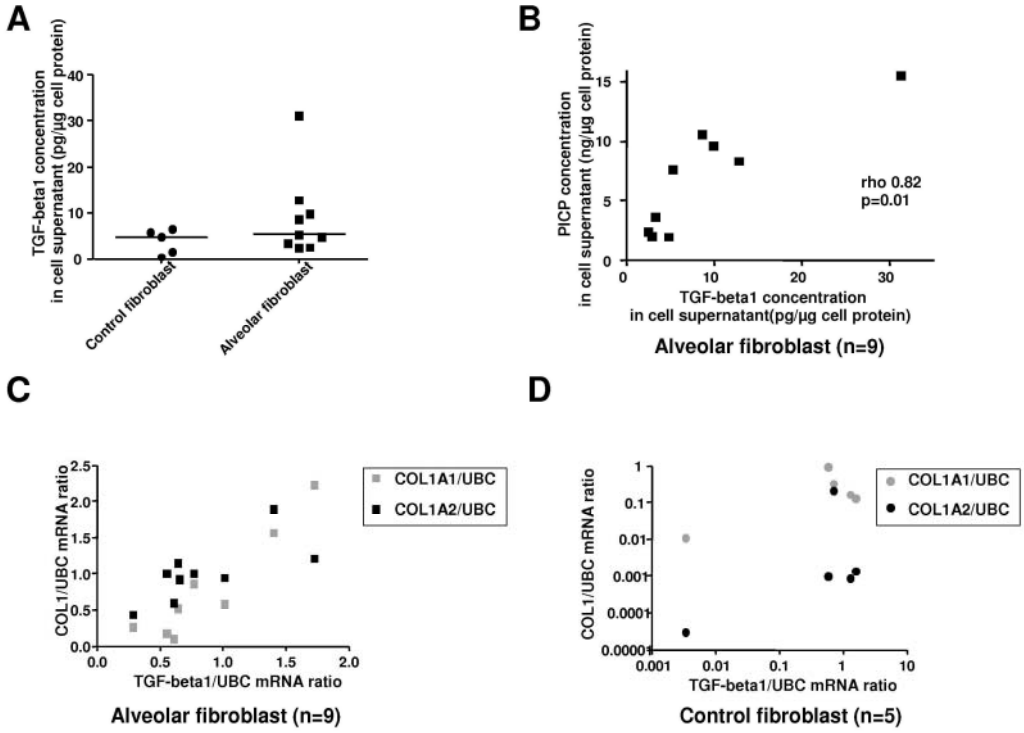
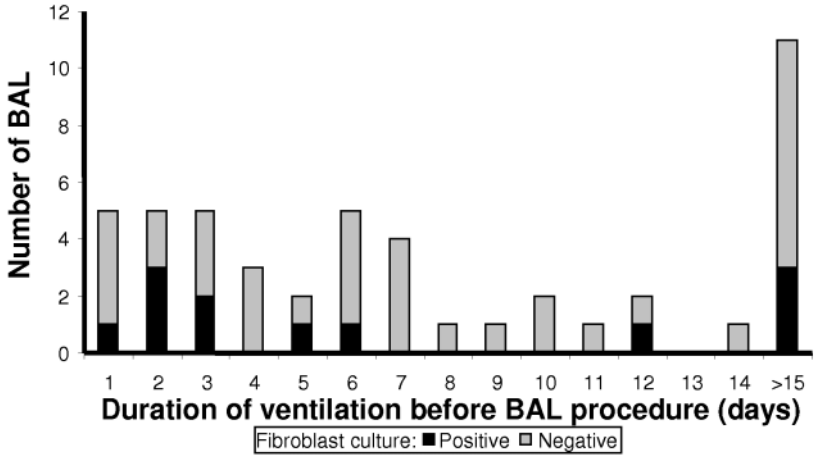


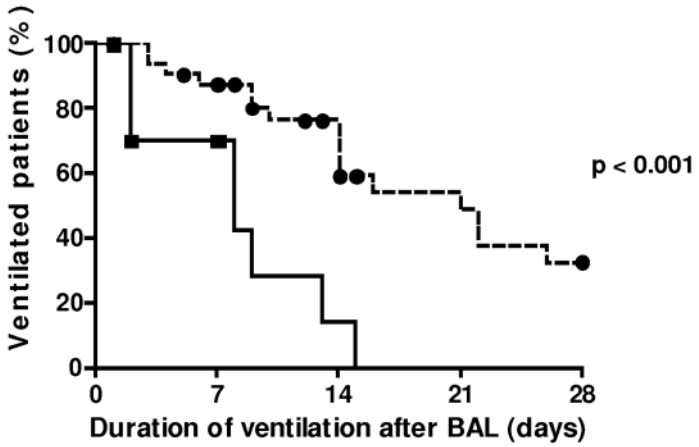
Figure 5. (A) Kinetics of fibroblast isolation in the acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) groups. Positive and negative fibroblast cultures

were plotted as a function of mechanical ventilation duration before BAL. **(B) Duration of mechanical ventilation after BAL procedure according to the growth of alveolar fibroblasts.** Kaplan-Meier survival curves comparing duration of mechanical ventilation after BAL procedure for patients with positive (n=12) and negative fibroblast culture (n=36) ($p < 0.001$; Log-rank test). Continuous line and dotted line represent patients with positive and negative fibroblast culture, respectively. Solid circles and squares represent censored data (death during ventilation or ventilation continuation more than 28 days after BAL procedure)

A


Number of BAL
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 >15
Duration of ventilation before BAL procedure (days)
Fibroblast culture: Positive Negative

B


Ventilated patients (%)
100
80
60
40
20
0
0 7 14 21 28
Duration of ventilation after BAL (days)
p < 0.001

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