



HAL
open science

Serum Amyloid P Contained in Alveolar Fluid From Patients With Acute Respiratory Distress Syndrome Mediates the Inhibition of Monocyte Differentiation into Fibrocyte

Marc Garnier, Arnaud A Mailleux, Valérie Besnard, Paer S Abback, Véronique Leçon, Mathilde Neuville, Aurélie Gouel, Bruno Crestani, Monique Dehoux, Christophe Quesnel

► **To cite this version:**

Marc Garnier, Arnaud A Mailleux, Valérie Besnard, Paer S Abback, Véronique Leçon, et al.. Serum Amyloid P Contained in Alveolar Fluid From Patients With Acute Respiratory Distress Syndrome Mediates the Inhibition of Monocyte Differentiation into Fibrocyte. *Critical Care Medicine*, 2016, 44, pp.e563 - e573. 10.1097/ccm.0000000000001612 . hal-04412487

HAL Id: hal-04412487

<https://hal.science/hal-04412487>

Submitted on 23 Jan 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

Serum Amyloid P Contained in Alveolar Fluid From Patients With Acute Respiratory Distress Syndrome Mediates the Inhibition of Monocyte Differentiation into Fibrocyte

Marc Garnier, MD, MSc^{1,2,3}; Arnaud A. Mailleux, PhD¹; Valérie Besnard, PhD¹; Paer S. Abback MD, MSc⁴; Véronique Leçon⁵; Mathilde Neuville, MD⁶; Aurélie Gouel, MD⁷; Bruno Crestani, MD, PhD^{1,8}; Monique Dehoux, PharmD, PhD^{1,5}; Christophe Quesnel, MD, PhD^{1,2,3}

Objective: Alveolar fibrocytes are monocyte-derived mesenchymal cells associated with poor prognosis in patients with acute respiratory distress syndrome. Our aims were to determine the following: 1) the ability of monocytes from acute respiratory distress syndrome patients to differentiate into fibrocytes; 2) the influence of the acute respiratory distress syndrome alveolar environment on fibrocyte differentiation; and 3) mediators involved in this modulation, focusing on serum amyloid P.

Design: Experimental in vitro investigation.

¹Unité INSERM 1152, Université Paris Diderot Sorbonne Paris Cité, Paris, France.

²APHP Hôpital Tenon, Département d'Anesthésie et Réanimation, Paris, France.

³Université Pierre et Marie Curie Sorbonne Université, Paris, France.

⁴APHP Hôpital Beaujon, Département d'Anesthésie et Réanimation, Paris, France.

⁵APHP Hôpital Bichat, Service de Biochimie, Paris, France.

⁶APHP Hôpital Bichat, Service de Réanimation médicale, Paris, France.

⁷APHP Hôpital Bichat, Département d'Anesthésie et Réanimation, Paris, France.

⁸APHP Hôpital Bichat, Service de Pneumologie, DHU FIRE, Paris, France.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (<http://journals.lww.com/ccmjournal>).

Supported by research grants from the Fondation pour la Recherche Médicale (FDM20130727579) and from the French Intensive Care and French Pneumology Societies (SRLF/SPLF 2014 research grant).

Dr. Garnier received funding from Fondation pour la Recherche Médicale (FRM) and from Société de Réanimation de Langue Française (SRLF)/Société de Pneumologie de Langue Française (SPLF). Dr. Crestani received funding from boehringer ingelheim, Intermune/Roche, and Sanofi. The remaining authors have disclosed that they do not have any potential conflicts of interest.

For information regarding this article, E-mail: marcgarnier@gmail.com

Copyright © 2016 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

DOI: 10.1097/CCM.0000000000001612

Setting: Two ICUs of a teaching hospital.

Patients: Twenty-five patients (19 mild-to-severe acute respiratory distress syndrome and six matched ventilated controls without acute respiratory distress syndrome) were enrolled. Six healthy volunteers served as non-ventilated controls.

Interventions: Peripheral blood mononuclear cells were isolated from acute respiratory distress syndrome, ventilated controls, and non-ventilated controls blood and cultured in vitro. Fibrocytes were counted at basal condition and after culture with broncho-alveolar lavage fluid. Plasma and broncho-alveolar lavage fluid serum amyloid P contents were determined by western blot and enzyme-linked immunosorbent assay. Serum amyloid P was located in normal and acute respiratory distress syndrome lung by immunohistochemistry.

Measurements and Main Results: Acute respiratory distress syndrome peripheral blood mononuclear cells had a three-fold increased ability to differentiate into fibrocytes compared to ventilated controls or non-ventilated controls. Acute respiratory distress syndrome broncho-alveolar lavage fluid inhibited by 71% (55–94) fibrocyte differentiation compared to saline control. Ventilated controls' broncho-alveolar lavage fluid was a less potent inhibitor (51% [23–66%] of inhibition), whereas non-ventilated controls' broncho-alveolar lavage fluid had no effect on fibrocyte differentiation. Serum amyloid P concentration was decreased in plasma and dramatically increased in broncho-alveolar lavage fluid during acute respiratory distress syndrome. Alveolar serum amyloid P originated, in part, from the release of serum amyloid P associated with lung connective tissue during acute respiratory distress syndrome. Serum amyloid P depletion decreased the inhibitory effect of acute respiratory distress syndrome broncho-alveolar lavage fluid by 60%, whereas serum amyloid P replenishment of serum amyloid P-depleted acute respiratory distress syndrome broncho-alveolar lavage fluid restored their full inhibitory effect.

Conclusions: The presence of fibrocytes in the lung during acute respiratory distress syndrome could result in a balance between

higher ability of monocytes to differentiate into fibrocytes and the inhibitory effect of the alveolar environment, mainly dependent on serum amyloid P. (*Crit Care Med* 2016; 44:e563–e573)

Key Words: acute respiratory distress syndrome; fibrocyte; monocyte; peripheral blood mononuclear cell; serum amyloid P

Acute respiratory distress syndrome (ARDS) remains an important cause of mortality in critically ill patients (1). Prognosis depends on its severity, and on the progression of lung injury in case of inadequate lung inflammatory response and alveolar repair. Excessive fibroproliferation and ongoing inflammation are both associated with prolonged mechanical ventilation and excess mortality (2, 3).

Experimental and clinical studies showed that fibroblasts and their progenitors are key actors of normal and pathologic alveolar repair during ARDS (4). Fibrocytes are one of these progenitors and have been identified as an independent predictor of poor outcome during ARDS (5). These spindle-shaped mesenchymal cells derived from CD14+ monocytes (6–9) co-express leukocyte (CD45+) and fibroblast markers (collagen 1+) (10) and have been involved in many pulmonary and extra-pulmonary repair processes (11–13). Fibrocytes are detected in both blood (14) and alveolar fluid of ARDS patients (5). Alveolar fibrocytes may originate from the recruitment of circulating fibrocytes to the lung (15, 16) and from local differentiation of monocytes in the alveolar spaces (7–9). Understanding the mechanisms that influence this differentiation is essential to develop treatments capable of modulating these cell populations.

Several cytokines or mediators are able to modulate monocyte-to-fibrocyte differentiation *in vitro*. Pro-inflammatory cytokines such as interferon (INF)- γ and interleukin (IL)-12 decrease fibrocyte differentiation, whereas pro-fibrotic mediators, such as IL-4, IL-13, and transforming growth factor (TGF)- β , which are increased in serum and broncho-alveolar lavage fluid (BALF) of ARDS patients (17–19), increase this differentiation (20). The serum amyloid P protein (SAP or pentraxin-2) was identified as the most potent inhibitor of fibrocyte differentiation both *in vitro* and *in vivo* (6, 21). SAP is a 27-kDa member of the short pentraxin family, secreted by the liver and contained in normal serum. This lectin inhibits fibrocyte differentiation during experimental lung fibrosis in mice *in vivo* (13, 22, 23). In humans, SAP is detected in normal lungs on microfibrils of elastic fibers (24). Although data suggest that SAP could participate in the modulation of fibrocyte population, its specific role during ARDS remains unknown.

We hypothesized that the complex alveolar environment may influence the monocyte-to-fibrocyte differentiation during ARDS. The aims of our study were 1) to determine the ability of monocytes from ARDS patients to differentiate into fibrocytes; 2) to determine the influence of the alveolar environment during ARDS on the differentiation process; and 3) to determine the potential role of SAP in this modulation.

MATERIALS AND METHODS

Study Population and Data Collection

Details are available in the **Supplemental methods** (page 1) (Supplemental Digital Content 1, <http://links.lww.com/CCM/B684>).

This study was approved by the ethical committee Paris-Ile de France I, and clinical and biological data collections were approved by the French committee on health research information management (CCTIRS, no. 06.226) and by the French national commission on digital storage of personal data (CNIL, no. 906183). Written information was given to patients or next-of-kin, who could decline inclusion in the study.

Three groups of subjects were defined as follows: 1) “ARDS group,” including 19 patients in the first week of evolution of moderate-to-severe ARDS according to the Berlin definition (25) (among which six patients received hydrocortisone 200 mg/d for an associated septic shock at the time of sampling); patients were recruited consecutively over the study period of 1 year; 2) ventilated controls (VC) group, including, during the same period, six VC patients without ARDS criteria matched with ARDS patients for severity of illness, age, gender, and the length of mechanical ventilation before inclusion (among which none received corticosteroids at the time of sampling); 3) non-ventilated controls (NVC) group including six healthy volunteers. All the ventilated patients were hospitalized in the medical and surgical ICU of Bichat Hospital (Paris, France). Demographic data, general information regarding the ICU hospitalization, results of arterial blood gases, complete blood count, and chest x-ray on the day of inclusion were anonymously and prospectively collected (**Table 1**).

BAL Collection

Details are available in the Supplemental methods (page 2) (Supplemental Digital Content 1, <http://links.lww.com/CCM/B684>). BALF was obtained simultaneously with blood samples in seven patients in the ARDS group (all without corticosteroids). To complete our study, 10 supplemental ARDS BALF, 10 “VC” BALF, and 10 “NVC” BALF were randomly selected among the BALF obtained from patients without steroid treatment and collected in our local bio-bank during the previous 12 months (5). BAL was performed with 5 \times 20 mL of 0.9% saline solution, under fiberoptic control as previously described (4, 5), to assess the microbiological diagnosis of suspected ventilator-associated pneumonia.

Peripheral Blood Mononuclear Cells and Monocyte Isolation, Culture, and Stimulations

Details are available in the Supplemental methods (page 3) (Supplemental Digital Content 1, <http://links.lww.com/CCM/B684>). Three to 5 mL of the remaining total blood sampled in EDTA tubes for routine hematology analysis was collected. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a separation gradient and then seeded in non-coated 24-well plates (Costar; Corning, New York, NY) or in four wells Permanox chamber slides at 2.5 \times 10⁵ cells per well in RPMI-1640 GlutaMAX containing 2 mM of glutamine and 25 mM of HEPES, supplemented with 1% of ITS3+ (ITS3+ Liquid Media Supplement; Sigma-Aldrich, St Louis, MO).

TABLE 1. Characteristics of Acute Respiratory Distress Syndrome Patients, Ventilated Controls and Non-Ventilated Controls

	Non-Ventilated Controls (n = 6)	Ventilated Controls (n = 6)	Acute Respiratory Distress Syndrome (n = 19)
Age (yrs)	31.6 [27.9–50.1]	62.8 [60.9–74.6]	49 [39–66.5]
Sex ratio (female/male) (n)	4/2	1/5	5/14
Simplified Acute Physiology Score 2 (at admission)	–	49 [46.5–60.5]	51.5 [34–61.8]
Lung injury score ^a	–	1.2 [0.5–1.6]	2.8 [2.3–3]
Length of mechanical ventilation before inclusion (d)	–	12 [4.3–20.5]	12 [5.8–22.8]
Concomitant lung infection, n (%)	–	3 (50)	11 (58)
Causes for ICU admission, n (%)			
Pneumonia	–	0	8 (42)
Neurologic disorders	–	0	3 (16)
Suicide attempt	–	0	2 (11)
Acute pancreatitis	–	0	2 (11)
Polytraumatism	–	0	1 (5)
Cardiac arrest	–	2 (33)	0
Cardiogenic shock	–	2 (33)	0
Septic shock	–	2 (33)	1 (5)
Others	–	0	2 (11)
Pao ₂ /Fio ₂ ratio ^a	–	251 [200–295]	122 [81–157]
Diffuse/localized infiltrate on chest x-ray (n)	–	3/3	13/6
WBC count ^a (per mm ³)	–	12,745 [10,158–19,983]	14,640 [10,713–35,755]
Blood monocytes count ^a (per mm ³)	–	1,100 [793–1,288]	750 [485–1,658]
Prognosis			
ICU post-inclusion length of mechanical ventilation (d)	–	3 [2–5]	9 [4–20]
ICU total length of mechanical ventilation (d)	–	21 [9–28]	34 [11–43.5]
ICU length of stay (d)	–	24.5 [10–33]	35 [14.5–48]
Death at day 28, n (%)	0 (0)	2 (33)	7 (37)

Dashes indicate no data for these items.

Results were expressed as median [25th–75th percentile].

^aThe day of blood sampling.

To study the ability of monocytes to differentiate into fibrocytes, PBMCs were cultured in the absence (basal condition), or in the presence of 10 ng/mL recombinant human (rh)-IL-4 (Gentaur, Kampenhout, Belgium), or of 10⁻⁶ M dexamethasone. To study the effect of the alveolar environment, PBMCs from each ARDS patient were stimulated with either 25% of BALF vol/vol, 25% of 0.9% saline solution (saline basal control), or 5 µg/mL of purified human SAP (hSAP; Merck, Darmstadt, Germany), added to RPMI-1640 culture medium. A 25% final concentration of BALF in culture medium was considered optimal in preliminary experiments (**Supplemental Fig. 1**, Supplemental Digital Content 2, <http://links.lww.com/CCM/B685>). Purified hSAP was

centrifuged in Amicon Ultra-0.5 centrifugal filter devices (Merck) to remove azide sodium before use. All the experiments were done in duplicate.

Fibrocyte Characterization and Count

After 5 days of culture, the plates were fixed and stained using a Diff-Quick staining kit (Polysciences, Warrington, PA). Fibrocytes were then identified and counted based on their elongated spindle-shaped morphology as previously described (6, 20, 26, 27), in five different 500-µm-diameter fields of view per well and expressed as a percentage of total cells, or as a percentage of the fibrocyte count in the saline basal control in experiments comparing BALF stimulations.

Evaluation of SAP Involvement in Fibrocyte Differentiation During ARDS

SAP Western Blotting. Details are available in the Supplemental methods (page 4) (Supplemental Digital Content 1, <http://links.lww.com/CCM/B684>). Unconcentrated BALF and one-tenth diluted plasma were resolved by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes, and then probed with 0.5 $\mu\text{g}/\text{mL}$ mouse monoclonal anti-SAP antibody (sc-69796; Santa Cruz Biotechnology, Dallas, TX). Blots were incubated with a sheep anti-mouse IgG antibody coupled with horseradish peroxidase and revealed with the ECL chemiluminescent detection reagent kit (GE Healthcare Life-Sciences). Purified hSAP preparations (Merck-Millipore) were used as standards.

SAP Enzyme-Linked Immunosorbent Assay. SAP concentration in BALF was measured with a commercial human SAP ELISA kit (APCS Human ELISA Kit; Abcam, Cambridge, United Kingdom). ARDS and VC BALF were diluted to 1/50 and 1/20, respectively, whereas NVC BALF was used undiluted. The lower limit of detection was 0.15 ng/mL.

Polymerase Chain Reaction for SAP. mRNA was extracted using the PureLink RNA Mini kit (Ambion; Life Technologies, Waltham, MA) according to the manufacturer's protocol. Then, real-time polymerase chain reaction (PCR) was performed using 500 ng of mRNA and the M-MLV Reverse Transcriptase kit (Promega, Madison, WI), according to the manufacturer's protocol. Finally, quantitative PCRs for SAP were performed with predesigned SYBR green primers (Sigma-Aldrich) for human serum amyloid P (hApcs, NM_001639) and with a probe set for human ubiquitin C as normalization standard.

SAP Depletion From BALF. SAP was depleted from BALF using its linking properties to protein A as previously described (6). Briefly, 1 mL of BALF was incubated twice with 75 μL of washed Protein A-sepharose beads (Protein A-sepharose 4B Fast Flow; Sigma-Aldrich) or with uncoupled sepharose beads as control, for 2 hours at 4°C. Then, BALF was centrifuged at 1,500 rpm for 5 minutes, and the supernatant was collected and frozen at -80°C until utilization. In experiments with SAP replenishment, purified hSAP was added to SAP-depleted BALF, to reach the initial SAP concentration estimated in untreated BALF by western blotting.

Immunohistochemistry and Immunofluorescence

Details are available in the Supplemental methods (pages 5 and 6) (Supplemental Digital Content 1, <http://links.lww.com/CCM/B684>). To assess SAP localization in normal ($n = 2$) and ARDS ($n = 7$) lung, paraffin-embedded sections were incubated with 2.5 $\mu\text{g}/\text{mL}$ mouse monoclonal anti-SAP antibody (sc-69796; Santa Cruz Biotechnology), and then with a labeled polymer anti-mouse coupled with alkaline phosphatase (Histofine; Nichirei Biosciences, Tokyo, Japan), and revealed by an alkaline phosphatase substrate-chromogen (Liquid permanent red; Dako, Glostrup, Denmark).

Ki67 staining was performed on PBMCs from three ARDS patients after 1 and 5 days of culturing with a 1/500^o diluted mouse monoclonal anti-Ki67 antibody (BD Pharmingen, No. 556003).

SAP and cleaved-PARP immunofluorescence were performed on cell pellets from three ARDS BALF with 2.5 $\mu\text{g}/\text{mL}$ mouse monoclonal anti-SAP antibody (sc-69796; Santa Cruz Biotechnology) and 1.25 $\mu\text{g}/\text{mL}$ rabbit polyclonal anti-cleaved PARP (p85) antibody (ab4830; Abcam).

Statistical Analysis

Results were expressed as median [25th–75th percentile]. Differences between the two groups were assessed by Mann-Whitney or Wilcoxon paired test and differences between multiple groups by Kruskal-Wallis or Friedman paired test when appropriate. p value less than 0.05 was considered as significant. Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, San Diego, CA).

RESULTS

The Ability of PBMC to Differentiate Into Fibrocytes Is Increased During ARDS

Cultures of PBMCs from ARDS patients showed an increasing number of fibrocytes from day 1 to day 5, whereas the proliferative Ki67 marker was negative in all cells ($n = 3$; Supplemental Fig. 2, Supplemental Digital Content 3, <http://links.lww.com/CCM/B686>). PBMCs from ARDS patients showed an increased ability to differentiate into fibrocytes on day 5 compared to those obtained from VC or NVC, both at basal condition (1.8% [1.5–2.6%] of fibrocytes among total cells [$n = 13$] vs 0.6% [0.2–1.5%] [$n = 6$] and 0.5% [0.4–0.7%]

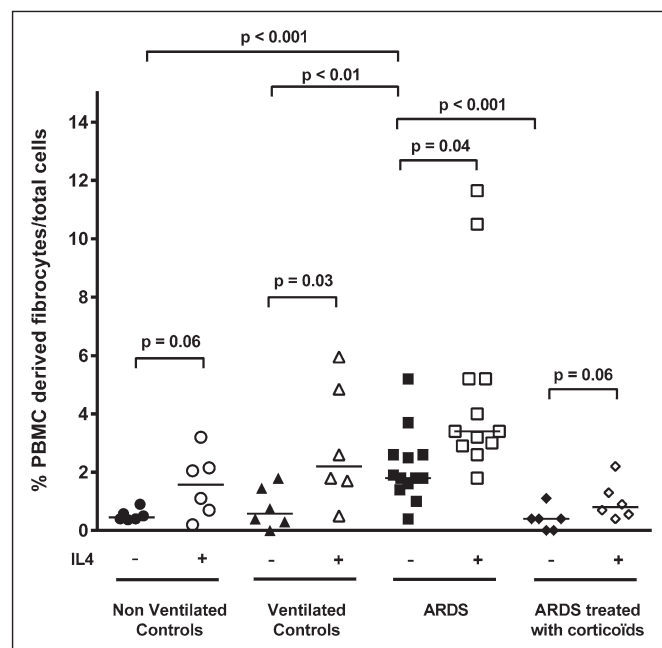


Figure 1. Differentiation of peripheral blood mononuclear cells (PBMCs) from acute respiratory distress syndrome (ARDS) patients and controls into fibrocytes at basal condition (black symbols) and after recombinant human interleukin-4 stimulation (white symbols). PBMCs isolated from non-ventilated controls ($n = 6$; circles), ventilated patients without ARDS ($n = 6$; triangles), ARDS patients ($n = 13$; squares) and ARDS patients treated with corticosteroids ($n = 6$; diamonds) were cultured for 5 d before fibrocyte count. Results are expressed as a percentage of total cells.

[$n = 6$], respectively; $p = 0.002$) and after positive control stimulation with rhIL-4 (3.4% [2.9–5.2%] [$n = 12$] vs 2.2% [1.4–5.1%] [$n = 6$] and 1.6% [0.6–2.4%] [$n = 6$], respectively; $p = 0.02$) (Fig. 1).

PBMCs isolated from ARDS patients treated with corticosteroids demonstrated a decreased capacity to differentiate into fibrocytes compared to those isolated from patients without corticosteroids, both at basal condition (0.4% [0–0.6%] [$n = 6$] vs 1.8% [1.5–2.6%] [$n = 13$]; $p < 0.001$) and after IL-4 stimulation (0.8% [0.5–1.5%] [$n = 6$] vs 3.4% [2.9–5.2%] [$n = 13$]; $p < 0.001$) (Fig. 1). Concordantly, in vitro stimulation of PBMCs from ARDS patients not receiving corticosteroids with 10^{-6} M dexamethasone potently inhibited fibrocyte differentiation both at basal condition ($n = 6$; 1.1% [0.4–3%] vs 0.1% [0–0.9%]; $p = 0.03$) and after rhIL-4 stimulation ($n = 5$; 2.6% [1.1–8.3%] vs 0.4% [0.4–1.3%]; $p = 0.06$) (Supplemental Fig. 3, Supplemental Digital Content 4, <http://links.lww.com/CCM/B687>).

We were able to collect a second blood sample from four ARDS patients, 2 weeks after their inclusion in this study. The three patients who experienced a resolution of their ARDS showed decreased fibrocyte differentiation at basal condition (2.8–0.8%, 1.8–1.1%, and 1.8–0%), whereas the last remaining patient, always ventilated for a refractory ARDS, showed increased fibrocyte differentiation (1.8–3.9%) (Supplemental Fig. 4, Supplemental Digital Content 5, <http://links.lww.com/CCM/B688>).

Alveolar Fluid Strongly Inhibits PBMC-to-Fibrocyte Differentiation During ARDS

Differentiation into fibrocytes of PBMCs from ARDS patients not receiving corticosteroids, cultured with their own BALF (i.e., autologous BALF), was potently inhibited by 68% compared to similar saline dilution or medium alone ($n = 7$; 0.6% [0.4–1.6%] of fibrocytes among total cells vs 1.9% [1.6–3.1%] and 2.3% [1.3–3.7%], respectively; $p < 0.05$) (Fig. 2A). Similarly, differentiation into fibrocytes of PBMCs from ARDS patients not receiving corticosteroids ($n = 3$), cultured with heterologous ARDS BALF, was inhibited by more than 50%, at levels close to those observed with autologous BALF (Fig. 2B). On the other hand, the same ARDS BALF ($n = 3$) tested on four different ARDS PBMC, led to a constant inhibitory effect, relatively close from PBMC from one ARDS patient to each other (Fig. 2C).

BALF from VC also decreased fibrocyte differentiation but to a lesser extent than heterologous BALF from ARDS patients ($n = 10$; 51% [23–66%] of inhibition compared to saline basal control vs 71% [55–94%]; $p = 0.02$); whereas, NVC BALF had no significant effect on fibrocyte differentiation (Fig. 2D).

Concentration of SAP in Plasma Is Reduced, Whereas BALF Contains Large Amounts of SAP During ARDS

Plasma from ARDS patients ($n = 24$) contained lower SAP amounts than NVC ($n = 10$) as measured by enzyme-linked immunosorbent assay (ELISA) (37.5 $\mu\text{g/mL}$ [24.8–42.5 $\mu\text{g/mL}$] vs 58.9 $\mu\text{g/mL}$ [40.9–73.9 $\mu\text{g/mL}$]; $p < 0.01$) (Fig. 3A). SAP concentration in ARDS BALF ($n = 56$) was higher than in VC

BALF ($n = 15$) (87.5 ng/mL [33–217.7 ng/mL] vs 20 ng/mL [5.5–27.6 ng/mL]; $p < 0.001$), or in NVC BALF ($n = 10$) (5 ng/mL [2.5–12.3 ng/mL]; $p = 0.003$) (Fig. 3B).

By using Western Blot in reducing and denaturing conditions, SAP was detected in ARDS BALF ($n = 8$) (Fig. 3C) at concentrations ranging from about 0.1 to 2.5 $\mu\text{g/mL}$. No SAP was detected in VC BALF ($n = 4$) or in NVC BALF ($n = 3$) (Fig. 3C). Eventually, SAP was detected in plasma at concentrations similar to those determined by ELISA ($n = 6$) (Fig. 3D).

SAP Present in BALF During ARDS Results From the Diffuse Alveolar Damage

SAP was stained on lung biopsies obtained from seven ARDS patients (exudative phase, $n = 5$; fibroproliferative phase, $n = 2$) and from two normal lung controls.

In normal lungs, SAP was located in the connective tissue surrounding bronchioles and arterioles and in alveolar septa (Fig. 4, A and B). Although we did not observe any staining in alveolar epithelial cells, alveolar macrophages, endothelial cells, nor in the sparse fibroblast-like cells present in the connective tissue (Fig. 4C), we observed intra-cytoplasmic SAP staining in hepatocytes of liver biopsies used as positive control (Fig. 4J). Amyloid P component serum (APCS) mRNA was not detected in normal lung homogenates (Fig. 4K).

During the exudative phase of ARDS, focal interruptions in SAP staining were observed in damaged alveolar walls, as well as positive staining of hyaline membranes (Fig. 4, D and E) and of phagocytic mononuclear cells (Fig. 4F). Macrophages from the cell pellets of ARDS BALF were almost all positive for SAP in their cytoplasm and for the nuclear apoptotic marker cleaved-PARP p85 (Supplemental Fig. 5, Supplemental Digital Content 6, <http://links.lww.com/CCM/B689>). They did not express APCS mRNA (Fig. 4K).

During the fibroproliferative phase of ARDS, clusters of fibroblasts showed no SAP staining, whereas the remaining inflammatory tissue, with persistent mononuclear cell infiltration and alveoli disorganization, showed focal SAP staining and positive staining of the remaining hyaline membranes (Fig. 4G and H).

SAP Contained in ARDS BALF Inhibits Fibrocyte Differentiation

The addition of 5 $\mu\text{g/mL}$ of purified hSAP inhibited the differentiation of PBMCs from ARDS patients into fibrocytes at a level similar to that observed with autologous ARDS BALF ($n = 8$; 67% [45–77%] of inhibition compared to saline basal control vs 63% [66–93%]) (Fig. 5). Furthermore, the addition of 5 $\mu\text{g/mL}$ of purified hSAP to VC BALF increased its inhibitory effect to a level similar to that observed with autologous ARDS BALF ($n = 5$, from 40% [28–55%] to 73% [61–83%]; $p = 0.03$) (Fig. 5).

SAP was depleted from ARDS BALF, reaching low concentrations similar to those of VC BALF ($n = 6$) (Fig. 6, A and B). SAP depletion reduced the capacity of ARDS BALF to inhibit fibrocyte differentiation by 60% ($n = 6$) (Fig. 6C). Finally, the replenishment of SAP-depleted ARDS BALF with purified

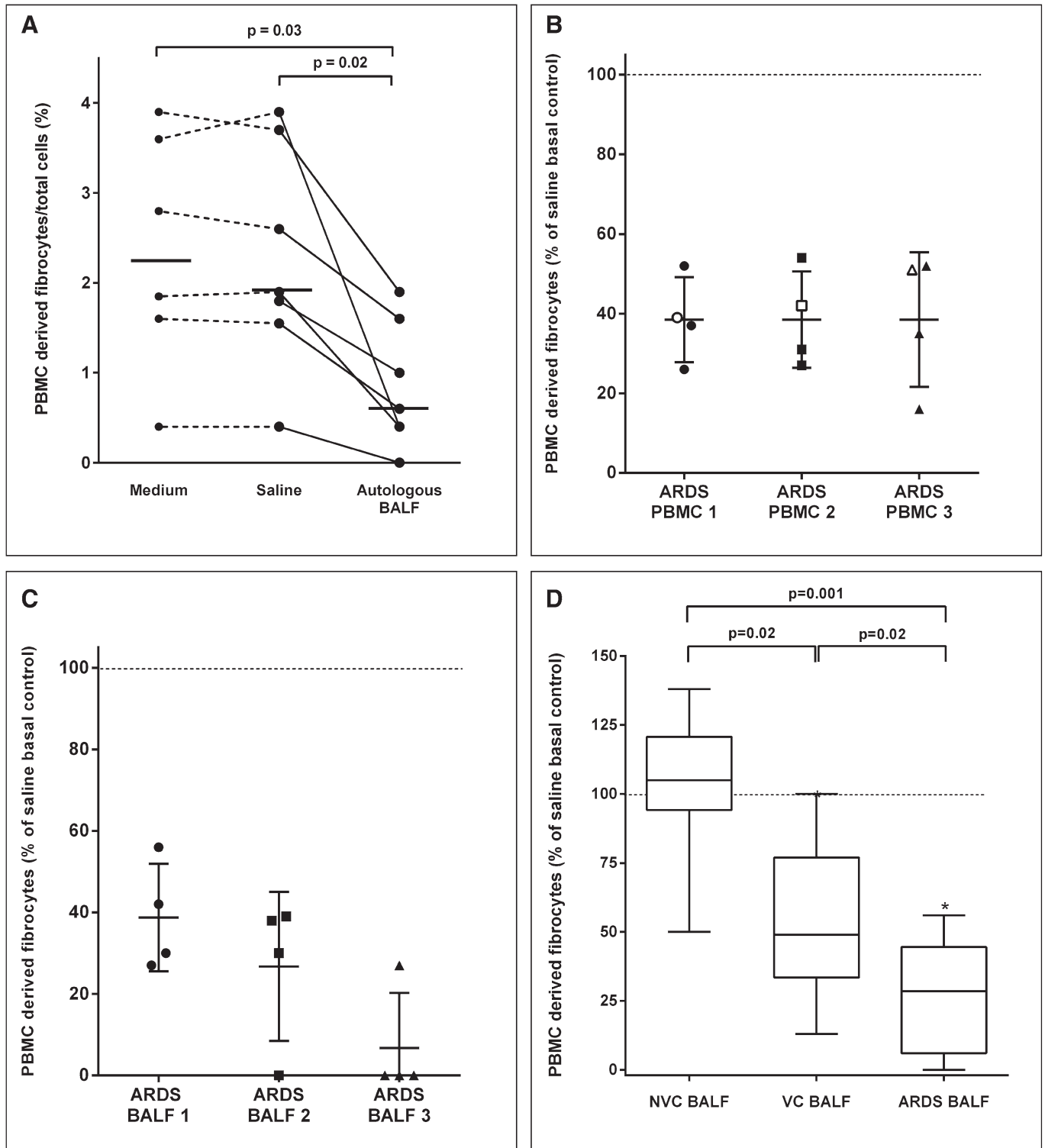


Figure 2. Effects of broncho-alveolar lavage fluid (BALF) on the differentiation into fibrocytes of peripheral blood mononuclear cells (PBMCs) from acute respiratory distress syndrome (ARDS) patients not receiving corticosteroids. **A**, Effect of autologous ARDS BALF on fibrocyte differentiation. Stimulation of PBMC ($n = 7$) with 25% vol/vol of their own BALF significantly decreases fibrocyte differentiation compared with 25% of 0.9% saline solution or medium alone. **B**, Comparative effects of various ARDS BALF on the differentiation of the same PBMCs into fibrocytes. Heterologous ARDS BALF (black symbols) inhibited PBMCs differentiation into fibrocytes at levels close to those observed with autologous BALF (empty symbols, $n = 3$ independent experiments). **C**, Comparative effects of the same ARDS BALF on differentiation of various PBMC into fibrocytes. For each ARDS BALF ($n = 3$ independent experiments), a constant and relatively similar inhibitory effect is observed whatever the considered ARDS PBMCs ($n = 4$). **D**, Comparative effects of non-ventilated controls (NVC) and ventilated controls (VC) and ARDS BALF on fibrocyte differentiation. ARDS BALF ($n = 10$) inhibited ARDS PBMCs differentiation into fibrocytes, more potently than VC BALF ($n = 10$), whereas NVC BALF ($n = 10$) had no effect on fibrocyte differentiation. * $p < 0.05$ vs saline basal control. Results are expressed as percentage of fibrocytes among total cells in **A**, or as percentage of fibrocytes counted in saline basal control, considered as 100% and represented by a dotted line, in **B–D**.

Downloaded from http://journals.lww.com/ccmjournal by BHDMSepHKav1Zecumr1tQIN4akJLHeZgbsIHo4XM10hCy on 01/23/2024

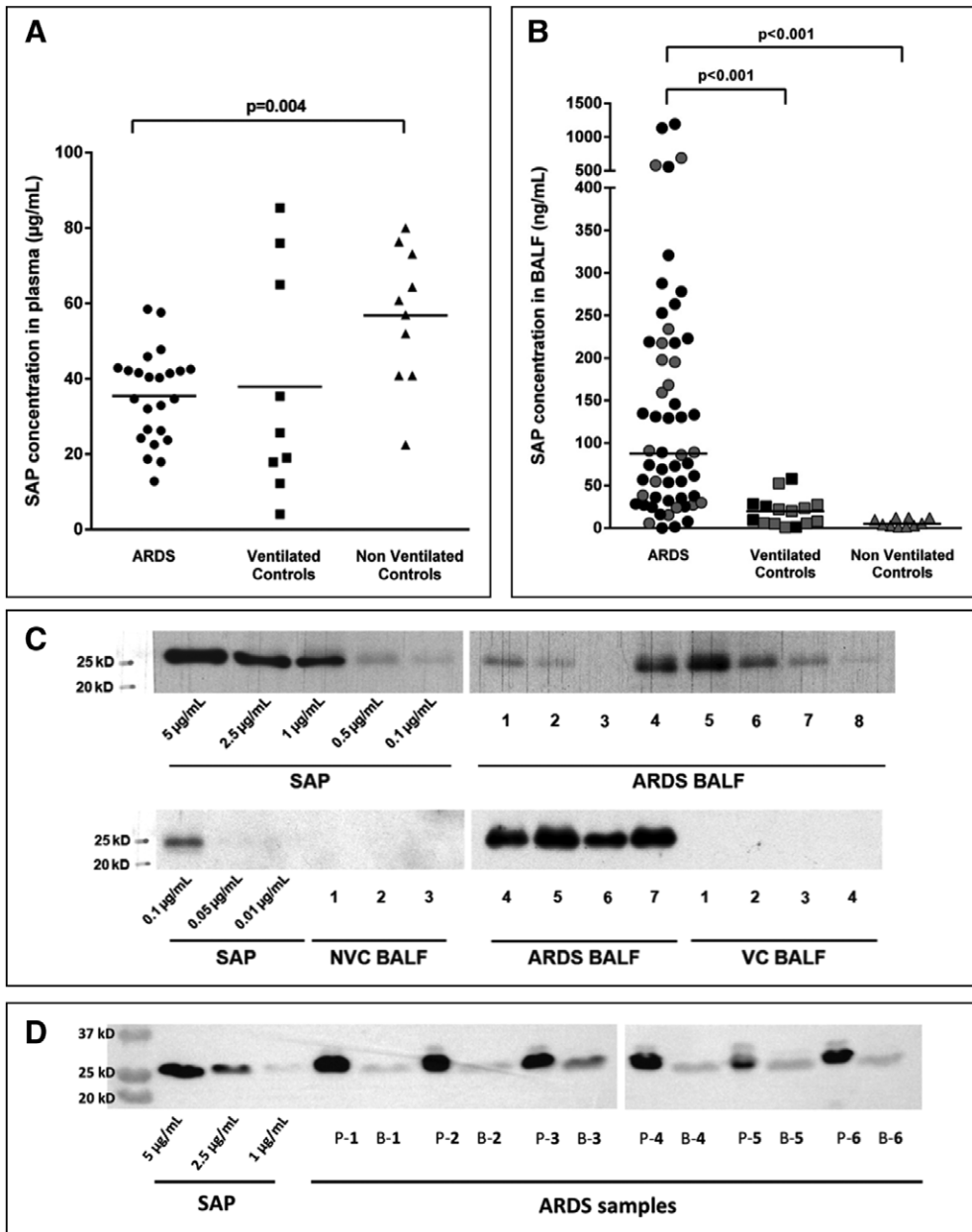


Figure 3. Serum amyloid P (SAP) detection in plasma and broncho-alveolar lavage fluid (BALF). **A**, Plasma SAP concentrations in acute respiratory distress syndrome (ARDS) patients not receiving corticosteroids ($n = 24$), ventilated controls (VC) ($n = 9$) and non-ventilated controls (NVC) ($n = 10$). **B**, BALF SAP concentration in ARDS patients not receiving corticosteroids ($n = 56$), VC ($n = 15$) and NVC ($n = 10$) measured by ELISA. *Gray symbols* represent values for the BALF used in the in vitro study (Fig. 2). **C**, SAP western blotting of BALF from ARDS patients not receiving corticosteroids (*upper*) ($n = 8$) and from VC ($n = 4$) and NVC ($n = 3$) (*lower*). Western Blot for SAP in NVC and VC were revealed with a more sensitive chemiluminescent detection reagent. **D**, SAP western blotting of plasma (P) and BALF (B) from ARDS patients not receiving corticosteroids ($n = 6$). Plasma was one tenth diluted, whereas BALF was left undiluted before resolving.

hSAP to its initial concentration restored its full inhibitory effect ($n = 3$) (Fig. 6, D and E).

DISCUSSION

Our study demonstrated that 1) PBMCs have an increased capacity to differentiate into fibrocytes during ARDS; 2) ARDS alveolar environment, as assessed by broncho-alveolar lavage, strongly inhibits the differentiation of PBMCs from ARDS

patients into fibrocytes; 3) SAP, detected in alveoli during ARDS, is mainly responsible for this inhibition.

Blood Monocytes' Ability to Differentiate Into Fibrocytes Is Increased During ARDS

Consistent with other studies demonstrating that monocytes were precursors of fibrocytes (6–9), we confirmed that fibrocytes observed in vitro after 5 days of culture in serum-free medium were derived from the adherent fraction of the total PBMCs and not from the proliferation of circulating fibrocytes. In these settings, we reported an increased ability of PBMCs from ARDS patients to differentiate in vitro into fibrocytes, consistent with that reported in burn patients (9). In this acute inflammatory state, the monocyte-to-fibrocyte differentiation increased with the severity of damage (i.e., the percentage of burned total body surface), as observed in our study. Several specific factors may modulate fibrocyte differentiation during ARDS, such as composition of monocyte sub-populations (28), interactions between monocytes and lymphocytes (8), and/or exposition to variable amounts of soluble mediators in vivo. The concentration of SAP, which is the main inhibitor of fibrocyte differentiation in plasma (6), was decreased in the plasma of ARDS patients, and may thus participate in part in increased fibrocyte

differentiation. Furthermore, the inflammatory status of the patient may be involved. We observed in several patients that the resolution of ARDS was associated with a decreased ability of PBMCs to differentiate into fibrocytes (Supplemental Fig. 4, Supplemental Digital Content 5, <http://links.lww.com/CCM/B688>). Several studies also suggest a strong link between inflammation and fibrocyte differentiation (13, 29–31). Concordantly, we demonstrated that exposition of monocytes to corticosteroids in vivo or in vitro completely inhibited their

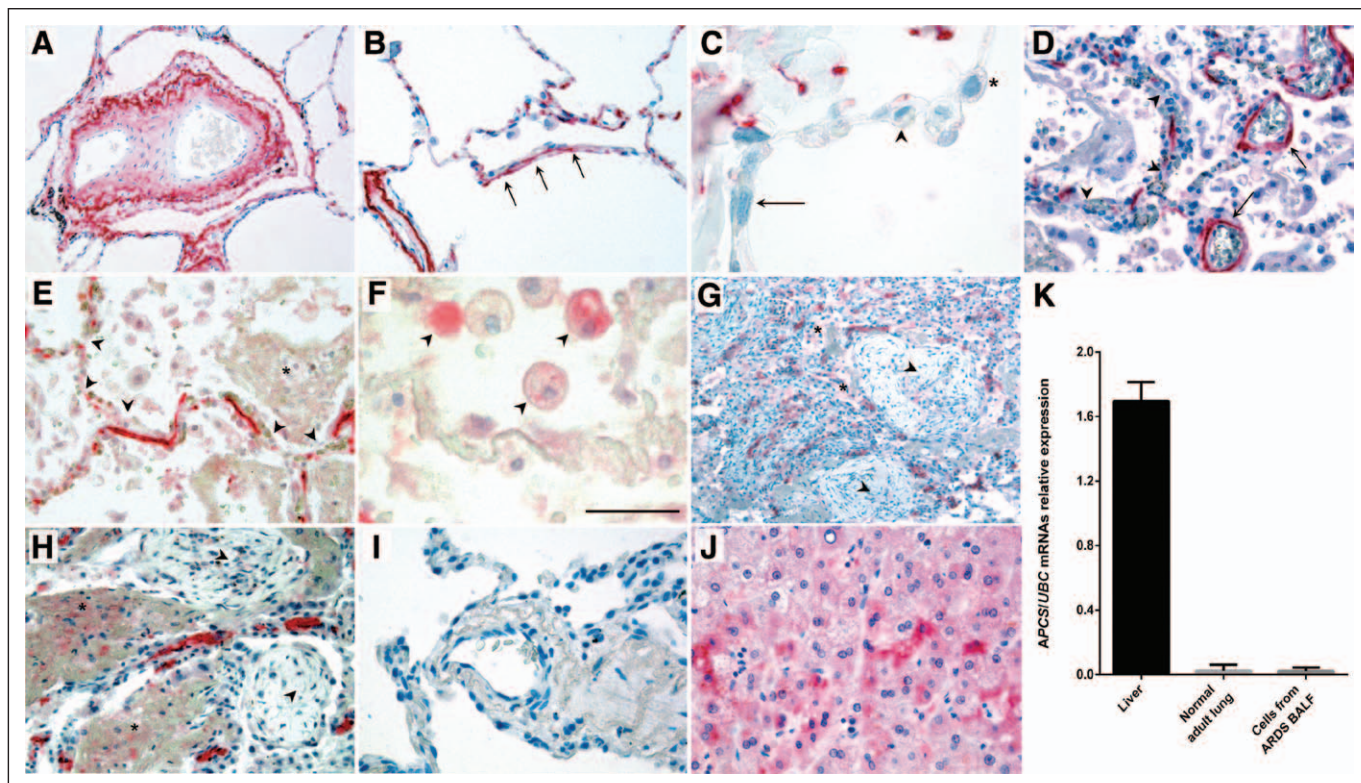


Figure 4. Serum amyloid P (SAP) localization in normal and acute respiratory distress syndrome (ARDS) human lung. **A**, SAP staining in a peri-vascular space and in alveoli ($\times 100$). **B**, SAP staining in normal alveoli ($\times 200$). SAP staining forms a nearly continuous line overlapping the thin connective wall of alveoli (arrows). **C**, SAP staining in normal alveolar cells ($\times 1,000$). Type 1 (arrow) and type 2 (arrowhead) alveolar epithelial cells, and alveolar macrophages (*) are unstained for SAP in normal lung. **D** and **E**, SAP staining during the exudative phase of ARDS ($\times 200$). The alveolar connective wall present breakages, leading to focal interruptions of the normal linear SAP staining (arrowheads), whereas larger structures such as arterioles keep the classical SAP staining (arrows). Alveolar exudate (in pink, **D**) and hyaline membranes (*, **E**) are positively stained for SAP. **F**, SAP positive staining in macrophages ($\times 1,000$). During the exudative phase of ARDS, many alveolar macrophages are positively stained for SAP (arrowheads). SAP staining appears cytoplasmic and evokes a vesicular staining. **G** and **H**, SAP staining during the fibroproliferative phase of ARDS ($\times 100$ and $\times 200$). Clusters of fibroblasts are unstained for SAP (arrowheads), whereas the remaining exudates and hyaline membranes are positively stained (*). **I**, Control isotype, showing no non-specific staining ($\times 400$). **J**, Positive control of SAP staining in human normal liver ($\times 400$). **K**, Relative expression of APCS mRNA in normal adult lung ($n = 6$) and in cell pellets of ARDS broncho-alveolar lavage fluids (BALFs) ($n = 5$). Normal human liver ($n = 3$) was used as positive control. APCS = amyloid P component serum (SAP gene), UBIC = ubiquitin C. Pictures are representative of results obtained in two normal and seven ARDS lungs. Scale bar = 500 μm in **A–G**, 250 μm in **B, D, E**, and **H**, 125 μm in **I** and **J**, and 50 μm in **C–F**.

differentiation into fibrocytes. This is concordant with a previous observation made by Pilling et al (32). Finally, the lack of SAP in an APCS knock-out mice model is not only associated with more fibrocytes and fibrosis deposition during the lung injury induced in vivo by bleomycin but also with increased lung inflammation (23).

Alveolar Fluid Strongly Inhibits Monocyte-to-Fibrocyte Differentiation During ARDS

The increased ability of monocytes to differentiate into fibrocytes demonstrated in vitro may participate in the elevation of fibrocyte count observed in both blood and alveolar space during ARDS (5, 14). Unexpectedly, we observed that ARDS BALF dramatically and reproducibly inhibited monocyte-to-fibrocyte differentiation in vitro. As sodium chloride could modulate fibrocyte differentiation (27), we were particularly careful to compare the effect of ARDS BALF to that provided by the same volume of 0.9% saline solution as a control condition, thus excluding a role of the vehicle and suggesting a specific role for alveolar fluid components.

Several studies reported high levels of TGF- β (33) thrombin (34), pentraxin-3 (35), and IL-4 (Th2 cytokine) (17) in alveolar fluid during ARDS, known to enhance fibrocyte differentiation in vitro (20, 36, 37). Inhibitors of fibrocyte differentiation, such as Th1 cytokines (IL-12 or INF- γ), have been poorly studied in BALF, but their inhibitory effect is considered negligible in the presence of stimulating Th2 cytokines (IL-4, IL-13) (20). Other studies reported the presence in ARDS BALF of mediators with a moderate inhibitory effect, such as INF- α or TLRs agonists, notably bacterial debris (26, 38). By contrast, SAP was reported to be the main inhibitor of fibrocyte differentiation in serum (6), and unlike Th1 cytokines, SAP concentrations above 0.5 $\mu\text{g}/\text{mL}$ were able to counterbalance the stimulatory effect induced both by Th2 cytokines (20) and pentraxin-3 (37). Therefore, we investigated the potential role played by SAP in the BALF inhibitory effect during ARDS.

Detection and Specific Role of SAP in Lung During ARDS

To our knowledge, our study is the first to evaluate SAP both in BALF and plasma during ARDS. The differences between SAP

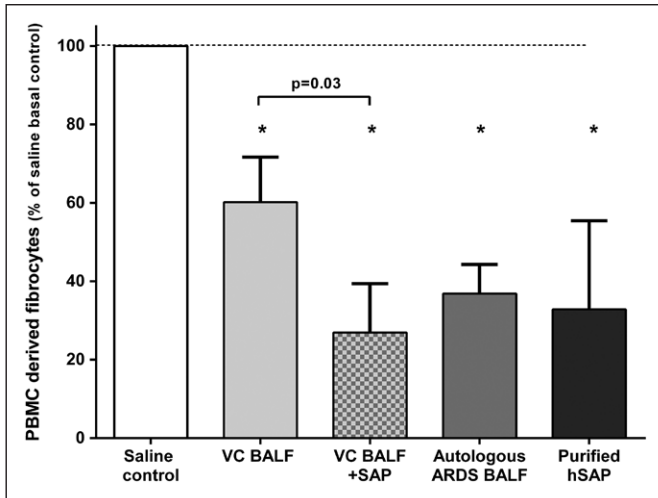


Figure 5. Comparative effects of ventilated controls (VC) broncho-alveolar lavage fluid (BALF) ($n = 4$), VC BALF supplemented with purified human serum amyloid P (SAP) ($n = 4$), autologous acute respiratory distress syndrome (ARDS) BALF ($n = 8$) or purified human SAP (hSAP) alone ($n = 8$) on differentiation into fibrocytes of peripheral blood mononuclear cell (PBMC) from ARDS patients not receiving corticosteroids. * $p < 0.05$ vs saline basal control. Results are expressed in percentage of fibrocytes obtained in saline basal control, considered as 100% and represented by a dotted line.

BALF levels obtained by ELISA and western blotting could be explained at least by 1) the use of different anti-SAP antibodies between ELISA and western blotting, which may lead to differences in SAP recognition and 2) SAP linking to apoptotic and necrotic cellular debris, extra-cellular matrix components, and microbial polysaccharide antigens, due to its lectin properties

and to calcium-dependent non-covalent bonds (39, 40). Thus, it is likely that actual ARDS BALF SAP content is underestimated by ELISA, since alveolar SAP linked large amounts of alveolar debris, as suggested by the positive SAP staining of hyaline membranes and inflammatory exudates. By contrast, BALF resolved under reducing and denaturing conditions allowed for a more complete estimation of SAP concentration.

However, we showed that unconcentrated BALF from ARDS patients contained five-fold and 30-fold more SAP than BALF from VC and NVC, respectively, reaching concentrations ranging from 0.1 to 2.5 $\mu\text{g}/\text{mL}$. Considering that alveolar fluid is 1/100 diluted by the BAL procedure, comparison of SAP concentrations between plasma and BALF suggested that SAP concentration may be higher in the alveolar fluid than in plasma. Thus, SAP present in alveolar spaces may originate, at least in part, from the lung. Our results confirmed a preliminary report of SAP location in the elastic fibers of human normal lung (24). As we did not observe APCS mRNA expression in normal human lung, concordantly to data available in human tissue mRNA sequencing databases (41, 42), pulmonary SAP probably originates from the accumulation of SAP produced by the liver and transuding from plasma. As the histopathological key feature of ARDS is diffuse alveolar damage, we hypothesized that SAP is released and scattered from the interstitial alveolar wall to alveolar spaces. In addition, SAP exudated from plasma through the damaged alveolo-capillary barrier likely participates in alveolar SAP contents. SAP may then participate in debris removal and phagocytosis, as suggested by the positive cytoplasmic co-staining of alveolar macrophages for SAP and cleaved-PARP, and by the absence of the expression of APCS

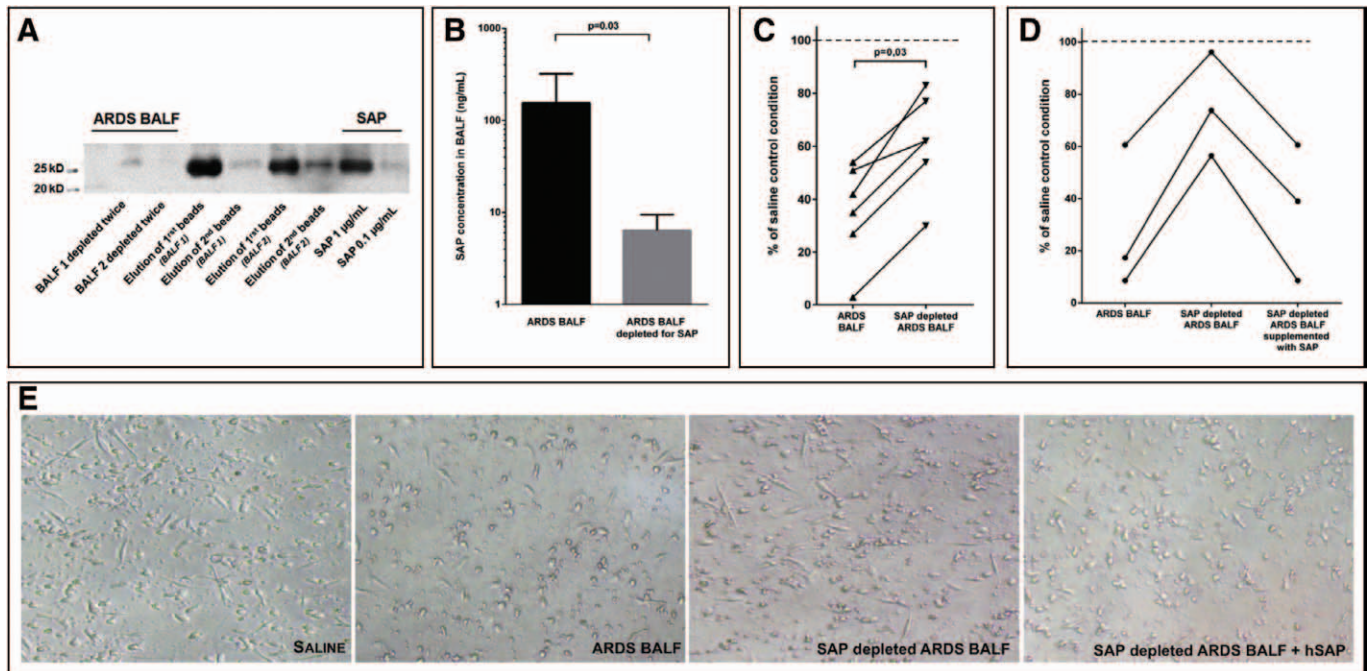


Figure 6. Effects of serum amyloid P (SAP) depletion of acute respiratory distress syndrome (ARDS) broncho-alveolar lavage fluid (BALF). **A** and **B**, Assessment of SAP depletion in ARDS BALF using western blot (**A**, $n = 2$, representative of six different ARDS BALF) and ELISA (**B**, $n = 6$). **C**, Reversion of the inhibitory effect of ARDS BALF with SAP depletion ($n = 6$ independent experiments). **D** and **E**, Reversion of the inhibitory effect of ARDS BALF with SAP depletion and restoration of a full inhibitory effect with purified human SAP (hSAP) replenishment to the initial content ($n = 3$ independent experiments). **C** and **D**, Results are expressed in percentage of fibrocytes obtained in saline basal control, considered as 100% and represented by a dotted line. Scale bar = 100 μm in **E**.

mRNA by the inflammatory cells infiltrating the damaged lung. This hypothesis is supported by several reports demonstrating SAP ability to enhance macrophage phagocytosis of apoptotic cells (40, 43, 44).

We therefore investigated the specific role of BALF SAP on fibrocyte differentiation. We observed a gradual inhibitory effect from NVC BALF to VC BALF, and to ARDS BALF, matching their SAP contents. Consistently, SAP depletion from ARDS BALF reversed the ARDS BALF inhibitory effect. The remaining inhibitory effect, similar to that observed with VC BALF, could be due to residual SAP content and/or to the presence of other inhibitors of fibrocyte differentiation such as Th1 cytokines, TLRs agonists, or INF- α . Conversely, the addition of purified hSAP in VC BALF led to an inhibition similar to that provided by ARDS BALF, whereas depleted ARDS BALF replenishment with original SAP content restored their full inhibitory effect. Taken together, these results indicate that the inhibitory effect of ARDS BALF on fibrocyte differentiation is mainly due to its SAP content. As SAP decreased bleomycin-induced and TGF- β -induced lung fibrosis in murine models (13, 22, 23, 45), it remains to determine if BALF SAP content also impacts the lung fibrotic changes that could be observed during late ARDS in humans.

Limitation and Perspectives

Our work contains some limitations. First, due to the design of our study, we had to cross PBMCs from ARDS patients with heterologous ARDS BALF in some experiments. Nevertheless, we preliminarily confirmed that the inhibitory effect of heterologous BALF was concordant with that provided by autologous BALF. The observational design of our study may have introduced biases that we tried to minimize. Indeed, as we reported a specific inhibitory effect of corticosteroids on the ability of monocytes to differentiate into fibrocytes, we performed all in vitro experiments assessing the role of ARDS BALF and SAP by using PBMCs and BALF obtained from patients without corticosteroid exposure. Further, heterologous ARDS BALF from patients without corticosteroid exposure, VC and NVC BALF, was randomly selected in our biobank. Thus, concomitant lung infections were well balanced between ARDS and VC groups (affecting about 50% of patients), whereas ARDS BALF acted constantly as an inhibitor to a larger extent than VC BALF. This excluded a bias due to lung infection in the constant inhibitory effect of ARDS BALF. Eventually, although our study included a relatively small number of patients, it was powerful enough to obtain significant results, allowing it to be considered as a proof of concept.

The potent inhibition of monocyte-to-fibrocyte differentiation exerted by the ARDS alveolar environment in vitro may appear at first glance to contradict our previous report of increased alveolar fibrocyte counts in vivo (5). Differences between in vitro and in vivo processes may contribute to explain this apparent paradox. Indeed, Haudek et al (46) showed that the inhibitory effect of SAP was lost if monocytes first migrated through an endothelium modeled with human umbilical vein endothelial cells. This suggests that SAP present in alveolar spaces during ARDS could be less efficient in

inhibiting fibrocyte differentiation from the time monocytes migrated to alveoli. Nevertheless, it is probable that the damaged and permeable pulmonary endothelium observed during ARDS does not behave as HUVECs.

Alveolar fibrocytes are most likely the result of a complex balance between an increased ability of monocytes to differentiate into fibrocytes on one hand and the resultant effect of the whole ARDS alveolar fluid on the other. Consequently, alveolar fibrocyte count is likely influenced at least by 1) the evolutionary phases of the ARDS (36); 2) the co-existence of a lung infection (26); 3) the immune cellular response and polarization (20); and 4) the severity of injury (35). Eventually, direct recruitment of circulating fibrocytes to the lung may also be a source of alveolar fibrocytes.

In conclusion, our results bring insight to the role of the alveolar environment on the modulation of the lung fibrocyte population during ARDS and give new translational data on the role of SAP in human diseases. Further studies are needed to determine the potential therapeutic role of SAP in acute respiratory distress syndrome.

ACKNOWLEDGMENTS

We thank Sandy Peltier for her technical support, Dr. D. Cazals-Hatem for having provided liver biopsies, and Dr. A. Cazes for having provided supplemental acute respiratory distress syndrome lung biopsies. The authors thank Joanna Shore for English proofreading.

REFERENCES

- Villar J, Blanco J, Añón JM, et al: ALIEN Network: The ALIEN study: Incidence and outcome of acute respiratory distress syndrome in the era of lung protective ventilation. *Intensive Care Med* 2011; 37:1932–1941
- Martin C, Papazian L, Payan MJ, et al: Pulmonary fibrosis correlates with outcome in adult respiratory distress syndrome. A study in mechanically ventilated patients. *Chest* 1995; 107:196–200
- Forel JM, Guervilly C, Hraiech S, et al: Type III procollagen is a reliable marker of ARDS-associated lung fibroproliferation. *Intensive Care Med* 2015; 41:1–11
- Quesnel C, Nardelli L, Piednoir P, et al: Alveolar fibroblasts in acute lung injury: Biological behaviour and clinical relevance. *Eur Respir J* 2010; 35:1312–1321
- Quesnel C, Piednoir P, Gelly J, et al: Alveolar fibrocyte percentage is an independent predictor of poor outcome in patients with acute lung injury. *Crit Care Med* 2012; 40:21–28
- Pilling D, Buckley CD, Salmon M, et al: Inhibition of fibrocyte differentiation by serum amyloid P. *J Immunol* 2003; 171:5537–5546
- Varcoe RL, Mikhail M, Guiffre AK, et al: The role of the fibrocyte in intimal hyperplasia. *J Thromb Haemost* 2006; 4:1125–1133
- Abe R, Donnelly SC, Peng T, et al: Peripheral blood fibrocytes: Differentiation pathway and migration to wound sites. *J Immunol* 2001; 166:7556–7562
- Yang L, Scott PG, Giuffre J, et al: Peripheral blood fibrocytes from burn patients: Identification and quantification of fibrocytes in adherent cells cultured from peripheral blood mononuclear cells. *Lab Invest* 2002; 82:1183–1192
- Bucala R, Spiegel LA, Chesney J, et al: Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med* 1994; 1:71–81
- Metz CN: Fibrocytes: A unique cell population implicated in wound healing. *Cell Mol Life Sci* 2003; 60:1342–1350

12. Hashimoto N, Jin H, Liu T, et al: Bone marrow-derived progenitor cells in pulmonary fibrosis. *J Clin Invest* 2004; 113:243–252
13. Pilling D, Roife D, Wang M, et al: Reduction of bleomycin-induced pulmonary fibrosis by serum amyloid P. *J Immunol* 2007; 179:4035–4044
14. Moeller A, Gilpin SE, Ask K, et al: Circulating fibrocytes are an indicator of poor prognosis in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2009; 179:588–594
15. Ishida Y, Kimura A, Kondo T, et al: Essential roles of the CC chemokine ligand 3-CC chemokine receptor 5 axis in bleomycin-induced pulmonary fibrosis through regulation of macrophage and fibrocyte infiltration. *Am J Pathol* 2007; 170:843–854
16. Phillips RJ, Burdick MD, Hong K, et al: Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest* 2004; 114:438–446
17. Meduri GU, Kohler G, Headley S, et al: Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. *Chest* 1995; 108:1303–1314
18. Li Q, Qian G, Zhang Q, et al: Changes of plasma interleukin-4, interleukin-10 and interleukin-13 in patients with acute respiratory distress syndrome. *Zhonghua Jie He He Hu Xi Za Zhi* 2002; 25:661–664
19. Synenki L, Chandel NS, Budinger GR, et al: Bronchoalveolar lavage fluid from patients with acute lung injury/acute respiratory distress syndrome induces myofibroblast differentiation. *Crit Care Med* 2007; 35:842–848
20. Shao DD, Suresh R, Vakil V, et al: Pivotal Advance: Th-1 cytokines inhibit, and Th-2 cytokines promote fibrocyte differentiation. *J Leukoc Biol* 2008; 83:1323–1333
21. Naik-Mathuria B, Pilling D, Crawford JR, et al: Serum amyloid P inhibits dermal wound healing. *Wound Repair Regen* 2008; 16:266–273
22. Murray LA, Chen Q, Kramer MS, et al: TGF-beta driven lung fibrosis is macrophage dependent and blocked by Serum amyloid P. *Int J Biochem Cell Biol* 2011; 43:154–162
23. Pilling D, Gomer RH: Persistent lung inflammation and fibrosis in serum amyloid P component (APCs^{-/-}) knockout mice. *PLoS One* 2014; 9:e93730
24. Breathnach SM, Melrose SM, Bhogal B, et al: Amyloid P component is located on elastic fibre microfibrils in normal human tissue. *Nature* 1981; 293:652–654
25. Ranieri VM, Rubenfeld GD, Thompson BT, et al: ARDS Definition Task Force: Acute respiratory distress syndrome: The Berlin Definition. *JAMA* 2012; 307:2526–2533
26. Maharjan AS, Pilling D, Gomer RH: Toll-like receptor 2 agonists inhibit human fibrocyte differentiation. *Fibrogenesis Tissue Repair* 2010; 3:23
27. Cox N, Pilling D, Gomer RH: NaCl potentiates human fibrocyte differentiation. *PLoS One* 2012; 7:e45674
28. Curnow SJ, Fairclough M, Schmutz C, et al: Distinct types of fibrocyte can differentiate from mononuclear cells in the presence and absence of serum. *PLoS One* 2010; 5:e9730
29. Sazuka S, Katsuno T, Nakagawa T, et al: Fibrocytes are involved in inflammation as well as fibrosis in the pathogenesis of Crohn's disease. *Dig Dis Sci* 2014; 59:760–768
30. Galligan CL, Fish EN: Circulating fibrocytes contribute to the pathogenesis of collagen antibody-induced arthritis. *Arthritis Rheum* 2012; 64:3583–3593
31. Garcia de Alba C, Buendia-Roldán I, Salgado A, et al.: Fibrocytes contribute to inflammation and fibrosis in chronic hypersensitivity pneumonitis through paracrine effects. *Am J Respir Crit Care Med* 2015; 191:427–436.
32. Pilling D, Vakil V, Gomer RH: Improved serum-free culture conditions for the differentiation of human and murine fibrocytes. *J Immunol Methods* 2009; 351:62–70
33. Fahy RJ, Lichtenberger F, McKeegan CB, et al: The acute respiratory distress syndrome: A role for transforming growth factor-beta 1. *Am J Respir Cell Mol Biol* 2003; 28:499–503
34. Glas GJ, Van Der Sluijs KF, Schultz MJ, et al: Bronchoalveolar hemostasis in lung injury and acute respiratory distress syndrome. *J Thromb Haemost* 2013; 11:17–25
35. Mauri T, Coppadoro A, Bellani G, et al: Pentraxin 3 in acute respiratory distress syndrome: An early marker of severity. *Crit Care Med* 2008; 36:2302–2308
36. White MJ, Galvis-Carvajal E, Gomer RH: A brief exposure to tryptase or thrombin potentiates fibrocyte differentiation in the presence of serum or serum amyloid p. *J Immunol* 2015; 194:142–150
37. Pilling D, Cox N, Vakil V, et al: The long pentraxin PTX3 promotes fibrocyte differentiation. *PLoS One* 2015; 10:e0119709
38. Suter PM, Suter S, Girardin E, et al: High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon, and elastase, in patients with adult respiratory distress syndrome after trauma, shock, or sepsis. *Am Rev Respir Dis* 1992; 145:1016–1022
39. Manfredi AA, Rovere-Querini P, Bottazzi B, et al: Pentraxins, humoral innate immunity and tissue injury. *Curr Opin Immunol* 2008; 20:538–544
40. Bijl M, Horst G, Bijzet J, et al: Serum amyloid P component binds to late apoptotic cells and mediates their uptake by monocyte-derived macrophages. *Arthritis Rheum* 2003; 48:248–254
41. The Human Protein Atlas: APCS mRNA and SAP protein distribution in human tissues. Available at: <http://www.proteinatlas.org/ENSG00000132703-APCS/tissue>. Accessed September 1, 2015
42. BioGPS: APCS Gene expression in human tissues. Available at: <http://biogps.org/#goto=genereport&id=325>. Accessed September 1, 2015
43. Mold C, Baca R, Du Clos TW: Serum amyloid P component and C-reactive protein opsonize apoptotic cells for phagocytosis through Fc gamma receptors. *J Autoimmun* 2002; 19:147–154
44. van Rossum AP, Fazzini F, Limburg PC, et al: The prototypic tissue pentraxin PTX3, in contrast to the short pentraxin serum amyloid P, inhibits phagocytosis of late apoptotic neutrophils by macrophages. *Arthritis Rheum* 2004; 50:2667–2674
45. Murray LA, Rosada R, Moreira AP, et al: Serum amyloid P therapeutically attenuates murine bleomycin-induced pulmonary fibrosis via its effects on macrophages. *PLoS One* 2010; 5:e9683
46. Haudek SB, Trial J, Xia Y, et al: Fc receptor engagement mediates differentiation of cardiac fibroblast precursor cells. *Proc Natl Acad Sci U S A* 2008; 105:10179–10184