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Macrophage Polarization Favors Epithelial Repair During Acute Respiratory Distress Syndrome*

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Dr. Garnier conceived and performed the experiments, analyzed the data, and drafted the article. Dr. Gibelin developed the in vitro models and revised the article. Dr. Mailleux contributed to experiments and revised the article. Ms. Leçon, Drs. Hurtado-Nedelec, and Laschet contributed to ex vivo and in vitro experiments. Drs. Trebbia, Neuville, and Tanaka provided patients' bronchoalveolar lavage samples and revised the article. Drs. Crestani, Dehoux, and Quesnel conceived the experiments, analyzed the data, and revised the article.

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Objectives: Alveolar macrophage polarization and role on alveolar repair during human acute respiratory distress syndrome remain unclear. This study aimed to determine during human acute respiratory distress syndrome: the alveolar macrophage polarization, the effect of alveolar environment on macrophage polarization, and the role of polarized macrophages on epithelial repair.

Design: Experimental ex vivo and in vitro investigations.

Setting: Four ICUs in three teaching hospitals.

Patients: Thirty-three patients with early moderate-to-severe acute respiratory distress syndrome were enrolled for assessment of the polarization of alveolar macrophages.

Interventions: Polarization of acute respiratory distress syndrome macrophages was studied by flow cytometry and quantitative polymerase chain reaction. Modulation of macrophage polarization was studied in vitro using phenotypic and functional readouts. Macrophage effect on repair was studied using alveolar epithelial cells in wound healing models.

Measurements and Main Results: Ex vivo, alveolar macrophages from early acute respiratory distress syndrome patients exhibited anti-inflammatory characteristics with high CD163 expression and interleukin-10 production. Accordingly, early acute respiratory distress syndrome-bronchoalveolar lavage fluid drives an acute respiratory distress syndrome-specific anti-inflammatory macrophage polarization in vitro, close to that induced by recombinant interleukin-10. Culture supernatants from macrophages polarized in vitro with acute respiratory distress syndrome-bronchoalveolar lavage fluid or interleukin-10 and ex vivo acute respiratory distress syndrome alveolar macrophages specifically promoted lung epithelial repair. Inhibition of the hepatocyte growth factor pathway in epithelial cells and hepatocyte growth factor production in macrophages both reversed this effect. Finally, hepatocyte growth factor

and soluble form of CD163 concentrations expressed relatively to macrophage count were higher in bronchoalveolar lavage fluid from acute respiratory distress syndrome survivors.

Conclusions: Early acute respiratory distress syndrome alveolar environment drives an anti-inflammatory macrophage polarization favoring epithelial repair through activation of the hepatocyte growth factor pathway. These results suggest that macrophage polarization may be an important step for epithelial repair and acute respiratory distress syndrome recovery. (*Crit Care Med* 2018; 46:e692–e701)

Key Words: acute respiratory distress syndrome; epithelial repair; hepatocyte growth factor; macrophage; polarization

Acute respiratory distress syndrome (ARDS) remains associated with high mortality in critically ill patients (1). Inadequate control of lung inflammation and alveolar repair lead to perpetuation of lung injury impacting patient's prognosis.

Monocytes/macrophages play a causal role in pathogenesis and resolution of ARDS (2). During the first hours after injury, resident alveolar macrophages promote the inflammatory response. Then, recruited monocytes/macrophages dramatically invade alveoli during the next first days (3–5). Contribution of macrophages to alveolar repair during ARDS has been reported in animal models, notably suggesting the beneficial role of apoptotic cell clearing (6). Furthermore, a switch from an initial inflammatory toward an anti-inflammatory macrophage phenotype may regulate ARDS resolution (5, 7). Indeed, macrophages can endorse various roles depending on whether the microenvironment modulates their polarization. These cells may be polarized across a continuum of spectrum of activation, whose ends are typically represented by macrophages stimulated with interferon (IFN)- γ , interleukin (IL)-4, and IL-10, so named M(IFN- γ), M(IL-4), and M(IL-10) rather than the former “proinflammatory M1,” “proresolutive/profibrotic M2a,” and “anti-inflammatory M2c” designations (8).

To date, human macrophage polarization during ARDS remains poorly investigated, and polarization data are most often reported from murine models, although important differences in phenotypic markers exist between human and mouse lung macrophages (8, 9). Furthermore, the influence of the alveolar environment on this polarization is unknown.

We hypothesized that the alveolar environment modulates human macrophage polarization since the acute phase of ARDS, thus affecting lung reparative processes. In this study, we aimed to determine alveolar macrophage polarization and its modulation by the alveolar environment during human early ARDS.

MATERIAL AND METHODS

Full details are available in the **Supplementary Methods** (Supplemental Digital Content 1, <http://links.lww.com/CCM/D500>).

Population

Bronchoalveolar lavage fluids (BALFs) were sampled between day 1 and day 3 after ARDS onset from moderate-to-severe ARDS patients according to Berlin definition (10) included in the IFRA2 study (NCT01854424) (for patients' characteristics, see **Supplementary Table 1**, Supplemental Digital Content 3, <http://links.lww.com/CCM/D502>). This multicenter prospective study was approved by the ethical committee Paris-Ile-de-France IV.

Peripheral blood monocytes were isolated from residual cell pellets available after blood cytapheresis of healthy platelet donors.

Flow Cytometry

BALF cells were immunostained with fluorochrome-conjugated mAbs to CD14, CD80, CD200R, CD163, and CD16 according to manufacturer's protocol. Preliminary experiments with corresponding isotypes demonstrated the high specificity of monoclonal antibodies (**Supplementary Fig. 1A**, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>). Then, BAL neutrophils were used as internal negative control to rule out nonspecific staining in further experiments (**Supplementary Fig. 1B**, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>) (11).

Results were expressed as mean fluorescence intensity (MFI) after subtraction of macrophage autofluorescence ($MFI = MFI_{\text{labeled tube}} - MFI_{\text{unlabeled tube}}$).

Monocytes/Macrophages Isolation and In Vitro Polarization

Monocytes were sorted from peripheral blood mononuclear cells using magnetic cell sorting. Monocytes were differentiated into macrophages with granulocyte-macrophage colony-stimulating factor (CSF) and macrophage-CSF and subsequently polarized with recombinant human (rh) IFN- γ , rhIL-4, rhIL-10, 25% volume-to-volume ARDS-BALF, or 0.9% saline solution as BALF control. Macrophages left in medium alone were used as basal controls. Alveolar macrophages were isolated from BALF cells as described above for monocytes.

Phagocytosis Assay

Macrophage phagocytic ability was assessed with live *Pseudomonas aeruginosa* as previously described (12). Results were expressed as a phagocytosis index relatively to basal macrophages.

Human Type II Alveolar Cells

Human type II alveolar epithelial cells (AECII) were isolated from lung tissue of surgical pieces histologically deemed normal using a protocol adapted from Dobbs et al (13).

Immunostaining

Lung slices from seven ARDS patients were stained for hepatocyte growth factor (HGF). Macrophage morphology was assessed by actin and β -tubulin staining. ARDS-BALF cells were stained for CD163 and CD45.

Epithelial Wound Healing

Epithelial cell migration was assessed using A549 cells seeded in Ibidi culture-inserts (Ibidi, Planneg, Germany). Free-cell

area was measured at hour 0 and hour 15 in the same two randomly selected fields/well, and epithelial wound healing was expressed as a closure ratio (Supplementary Methods, Supplemental Digital Content 1, <http://links.lww.com/CCM/D500>). Results were confirmed using scratch assay on human AECII monolayers. Eventually, epithelial wound closure was assessed using time-lapse microscopy in the two models. Epithelial cell proliferation was assessed using the CyQUANT NF Cell Proliferation Assay kit (ThermoFisher Scientific Inc, Waltham, MA).

Quantitative Polymerase Chain Reaction

Before quantitative polymerase chain reaction performing, messenger RNA (mRNA) was extracted and reverse transcribed. Primers sequences are shown in **Supplementary Table 2** (Supplemental Digital Content 3, <http://links.lww.com/CCM/D502>).

Enzyme-Linked Immunosorbent Assay

Concentrations of soluble form of CD163 (sCD163) in BALF, phospho-cMet in epithelial cell lysates, and HGF and IL-10 in macrophage supernatants were determined by enzyme-linked immunosorbent assay. IL-6, tumor necrosis factor (TNF)- α , C-C motif chemokine ligand 22, and platelet-derived growth factor-AB/BB were measured with a bead-based multiplex assay using the Luminex technology (Magpix; Luminex Corp., Austin, TX).

Western Blotting

Downstream activation of the HGF/cMet pathway was assessed by Akt/phospho-Akt western blotting.

Statistical Analysis

Results were expressed as median (25–75th percentile). Differences between groups and correlations of sCD163, HGF, and macrophage count in BALF were assessed by nonparametric tests using GraphPad Prism 6 (GraphPad Software, San Diego, CA). A *p* value of less than 0.05 was considered as significant.

RESULTS

Alveolar Macrophages From ARDS Patients Exhibited Several Anti-Inflammatory Characteristics

Macrophage count in BAL from the 33 included patients was 139.000 (70.000–310.000)/mL. Based on their size and granularity characteristics on flow cytometry analysis, two alveolar macrophages subpopulations were identified (**Fig. 1A**). Small and less granular monocytes/macrophages were CD16^{high} CD163^{low}, whereas mature macrophages were CD16^{low} CD163^{high} (**Fig. 1, A and B**). Mature macrophages were also more intensively stained for CD163 than freshly recruited monocytes/macrophages (**Fig. 1C**). The mRNA expression pattern of alveolar macrophages showed high expression of CD64, CD163, and CD16, similar to that observed for anti-inflammatory IL-10–polarized monocyte-derived macrophages [M(IL-10)] (**Fig. 2A**).

Early ARDS Alveolar Environment Specifically Drove an Anti-Inflammatory Macrophage Polarization In Vitro

Morphologically, monocyte-derived macrophages polarized with early ARDS-BALF [M(ARDS-BALF)] appeared rounded with various cell sizes, similar to M(IL-10) aspect, whereas M(IFN- γ) were racket shaped or extended shaped with numerous pseudopodia and M(IL-4) remained homogeneously rounded (**Fig. 2B**).

Considering mRNA expression patterns, early ARDS-BALF induced a M(IL-10)–like polarization with significant increases of CD163 and CD16 expression (5.4 [3.8–6.8] and 3.6 [2–5.9]; *p* < 0.001 vs saline control) (**Fig. 2C**). The ARDS-BALF–induced polarization was independent of the presence of concomitant pneumonia (**Supplementary Fig. 2, A–C**, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>). Moreover, this polarization was specific of ARDS as by contrast BALF from ventilated patients without ARDS (i.e., ventilated controls) [M(VC-BALF)], stable idiopathic pulmonary fibrosis [M(IPF-BALF)] patients, and healthy nonventilated controls [M(NVC-BALF)] did not induce any significant polarization (**Supplementary Fig. 3A**, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>). In a limited number of experiments, we observed that ARDS-BALF–induced CD163 and CD16 overexpression decreased with ARDS recovery (**Supplementary Fig. 2D**, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>). Finally, both ARDS-BALF and rhIL-10 induced a significant increase of macrophage human leukocyte antigen (HLA)-DR expression, at level similar to that observed in alveolar macrophages from ARDS patients (**Supplementary Fig. 4, A and B**, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>).

Phagocytic ability depended on macrophage polarization. M(IL-10) showed the best phagocytic index, whereas M(IFN- γ) showed poor phagocytosis ability (123% [103–141%] vs 16% [14–19%] of basal macrophages phagocytosis; *p* < 0.01). M(ARDS-BALF) showed better phagocytosis ability than M(IFN- γ) (54% [37–73%]; *p* < 0.05) (**Supplementary Fig. 4C**, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>).

M(ARDS-BALF) showed a M(IL-10)–like secretory pattern characterized by increased productions of IL-10 and HGF both at mRNA and protein levels and increased production and release of the sCD163, distinct from those of M(IFN- γ) and M(IL-4) (**Fig. 2C and Supplementary Fig. 2E**, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>). The BALF-induced enhanced production of IL-10 and HGF was specific of ARDS as M(VC-BALF), M(IPF-BALF), and M(NVC-BALF) showed inversely lower IL-10 and HGF mRNA expression than saline control macrophages (**Supplementary Fig. 3B**, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>).

Macrophages Accelerate Alveolar Epithelium Wound Repair Depending on Their Polarization

Epithelial Cell Migration and Spreading. Culture supernatants from M(IFN- γ) and M(IL-10) accelerated lung epithelial A549 cells wound closure by 127% (115–139%) and 123% [(110–128%), respectively (*p* < 0.05 vs control), whereas

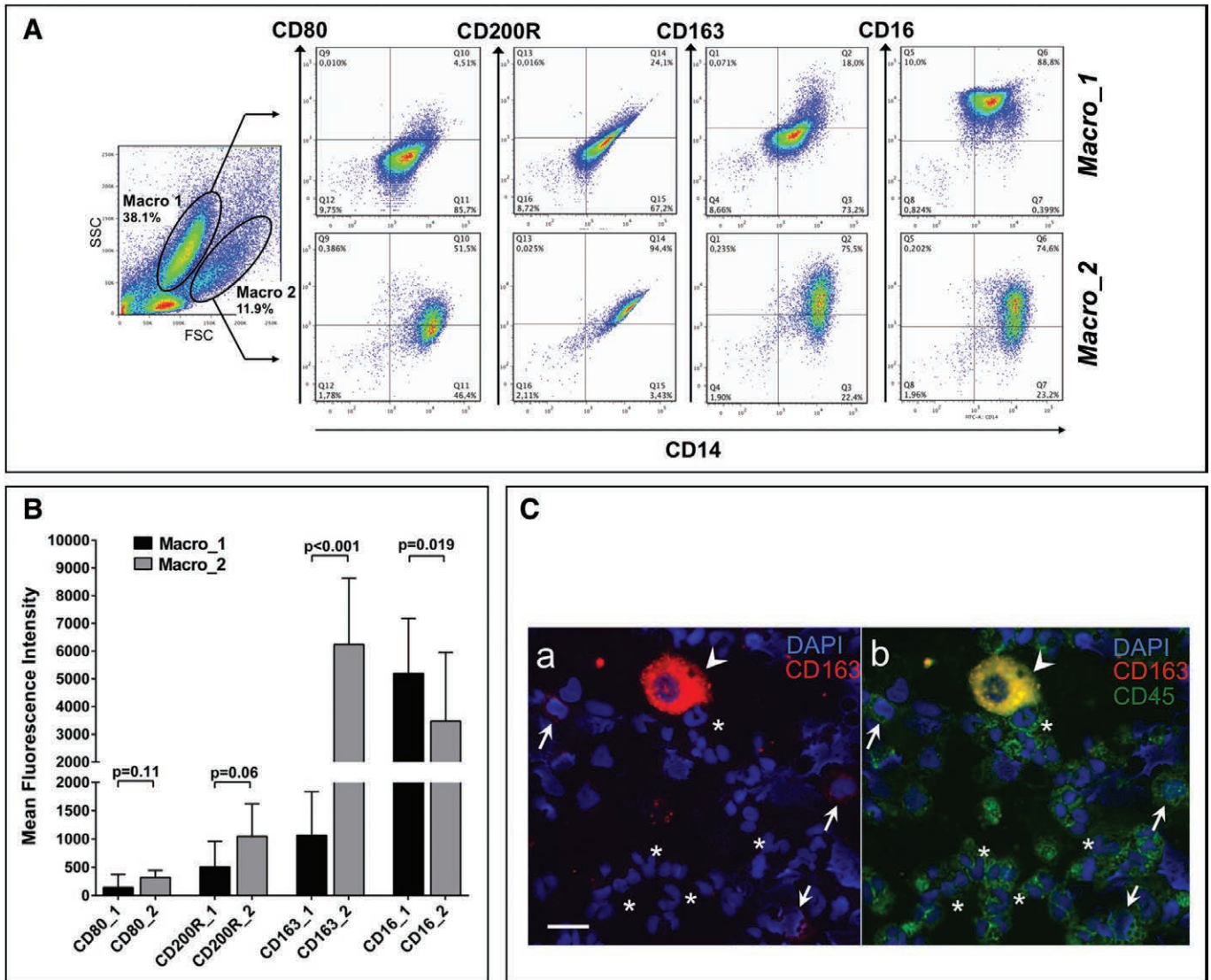


Figure 1. Polarization of alveolar macrophages from early acute respiratory distress syndrome (ARDS) patients. **A**, Representative flow cytometry results for CD14, CD80, CD200R, CD163, and CD16 on alveolar macrophages obtained from an ARDS patient. Two macrophage populations can be individualized: freshly recruited monocytes/macrophages (Macro 1) are CD163^{low} CD16^{high}, whereas mature macrophages (Macro 2) are CD163^{high} CD16^{low}. **B**, Flow cytometry results from 33 early ARDS patients. Markers intensity is expressed as mean fluorescence intensity (MFI), taking into account macrophage autofluorescence (MFI = MFI_{labeled tube} - MFI_{unlabeled tube}). **Black and gray bars** represent Macro_1 and Macro_2 populations, respectively. Results are expressed as median, interquartile range. **C**, Costaining for CD163 and CD45 of alveolar cells from ARDS-bronchoalveolar lavage fluid (×400). The large mature macrophage (*arrowhead*) is intensively stained, whereas freshly recruited macrophages (*arrows*) are positively but moderately stained for CD163 (**a**). By contrast, neutrophils (*) are not stained for CD163. All alveolar cells have hematopoietic origin, as assessed by CD45 staining (**b**). Pictures are representative of results obtained for three ARDS bronchoalveolar lavage cell pellets. *Scale bars* represent 25 μm in (**a–b**). Nuclear staining (4',6-diamidine-2'-phenylindole dihydrochloride [DAPI]) is shown in blue. FSC = forward scatter, SSC = side scatter, Q = quartile.

M(IL-4) had no effect (103% [99–120%]— $p = 0.10$) (**Fig. 3A**). Similarly, supernatants from M(ARDS-BALF) and alveolar macrophages from ARDS patients promoted wound closure (123% [115–128%] and 120% [116–136%]), whereas ARDS-BALF diluted as in M(ARDS-BALF) supernatants did not provide any significant effect on their own (**Fig. 3, A and B**; and **Supplementary Video 1**, Supplemental Digital Content 4, <http://links.lww.com/CCM/D527>; **legend**, Supplemental Digital Content 6, <http://links.lww.com/CCM/D529>). Furthermore, M(ARDS-BALF)– and M(IL-10)–induced acceleration of wound closure were confirmed in a scratch assay using primary human AECII (**Fig. 3C**; and **Supplementary Video 2**, Supplemental Digital Content 5, <http://links.lww.com/CCM/>

D528; **legend**, Supplemental Digital Content 6, <http://links.lww.com/CCM/D529>).

Epithelial Cell Proliferation. Unlike M(IFN- γ) and M(IL-4) supernatants, culture supernatants from M(ARDS-BALF) and M(IL-10) significantly increased 48-hour proliferation of A549 cells compared with fetal calf serum control (309% [221–337%] and 289% [266–314%] of basal proliferation; $p < 0.05$) (**Fig. 3D**).

M(ARDS-BALF) and M(IL-10) Improved Alveolar Epithelium Repair Through HGF Production
Macrophages Produced HGF Depending on Their Polarization. Among several known inducers of epithelial wound healing (TNF- α , PDGF-A, PDGF-B, transforming growth

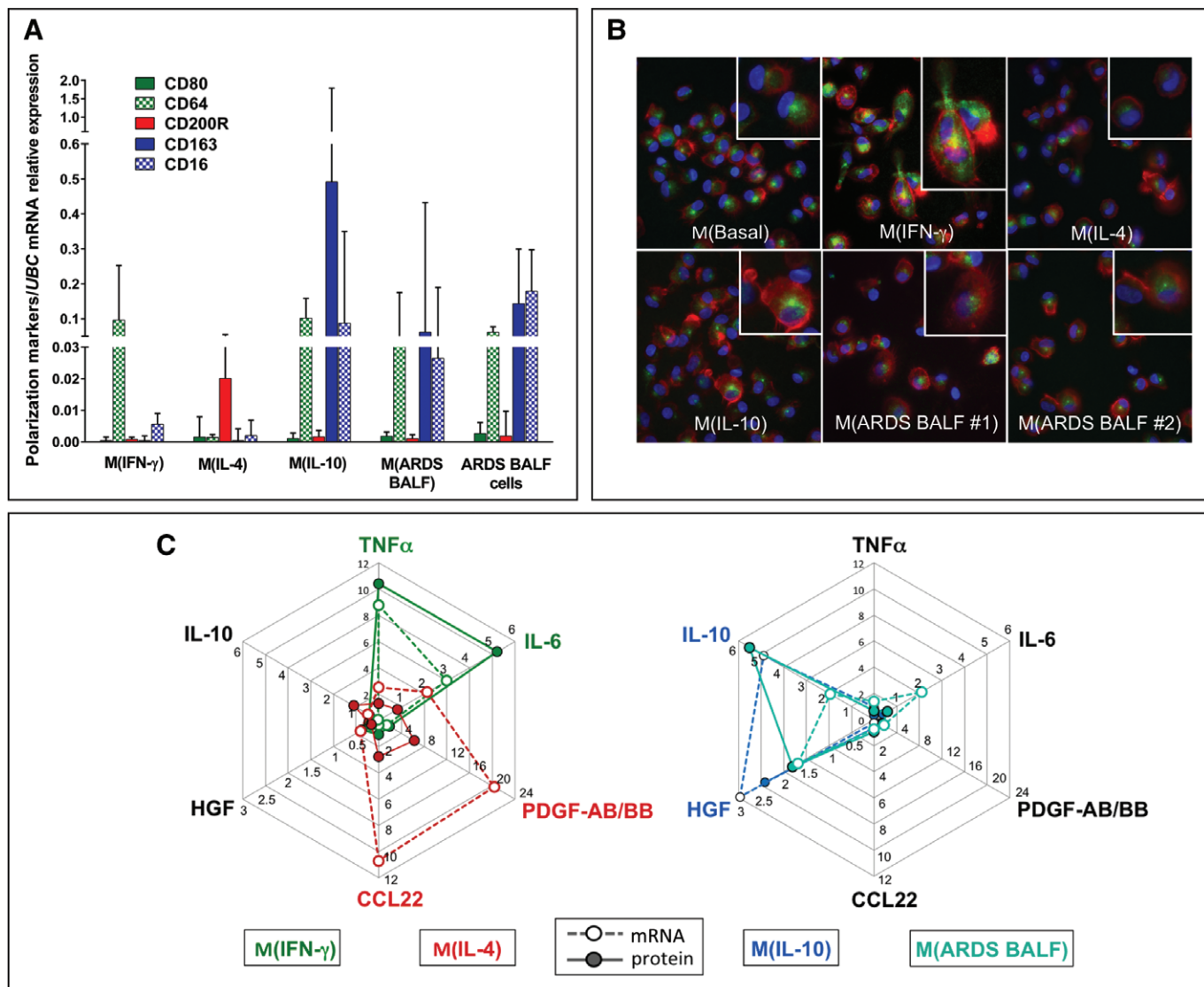


Figure 2. Phenotypic and secretory characteristics of macrophages polarized with acute respiratory distress syndrome (ARDS) bronchoalveolar lavage fluid (BALF) and recombinant human (rh) cytokines. **A**, Relative expression of CD80, CD64, CD200R, CD163, and CD16 messenger RNAs (mRNAs) in monocyte-derived macrophages polarized in vitro with rh interferon (IFN)-γ, rh interleukin (rhIL)-4, rhIL-10, and ARDS-BALF (*n* = 13 monocyte-derived macrophages from healthy donors, each of which stimulated with cytokines and individual BALF from 13 different ARDS patients with pneumonia and 13 ARDS patients without pneumonia) and in ARDS BALF cells (*n* = 9 different ARDS patients). **B**, Costaining of actin (red) and β-tubulin (green) in macrophages polarized with rhIFN-γ, rhIL-4, rhIL-10, and ARDS-BALF. Pictures are representative of results from three independent experiments. Scale bars represent 50 μm. Nuclear staining (4',6-diamidino-2'-phenylindole dihydrochloride [DAPI]) is shown in blue. **C**, Secretomes of in vitro polarized macrophages. Radar charts represent the secretory profiles of macrophages polarized in vitro with rhIFN-γ, rhIL-4, rhIL-10, and ARDS-BALF, both at mRNA (empty circles and dotted lines) and protein (filled circles and solid lines) levels (*n* = 11). Results are expressed relatively to mRNA expression and protein concentration observed for basal macrophages. Interleukin (IL)-10 concentration was not measured in macrophages stimulated with IL-10 [M(IL-10)] supernatants due to interference with rhIL-10 used for macrophage polarization. CCL = C-C motif chemokine ligand 22, HGF = hepatocyte growth factor, M(ARDS-BALF) = macrophages stimulated with ARDS-BALF, M(IFN-γ) = macrophages stimulated with IFN-γ, M(IL-4) = macrophages stimulated with IL-4, PDGF-AB/BB = platelet-derived growth factor-AB/BB, TNFα = tumor necrosis factor-α.

factor [TGF]-β1, TGF-β2, HGF), only HGF showed a significant increase of its mRNA expression specifically in both M(ARDS-BALF) and M(IL-10) (Supplementary Fig. 5, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>). Further, HGF concentrations were higher in M(ARDS-BALF) and M(IL-10) supernatants (211 pg/mL [57–555 pg/mL] and 210 pg/mL [156–321 pg/mL], respectively; *p* < 0.05) versus HGF content in M(IFN-γ) and M(IL-4) supernatants (Fig. 4B). In addition, alveolar macrophages from ARDS patients expressed HGF mRNA at levels close to M(IL-10) and

were intensively stained for HGF on ARDS lung tissue sections (Figs. 4A and 5).

HGF Produced by M(ARDS-BALF) and M(IL-10) Potently Induces Alveolar Epithelial Repair. Similarly to the positive control stimulation with rhHGF, M(ARDS-BALF)- and M(IL-10)-related accelerations of wound closure were inhibited in the presence of 0.5 μM of the HGF receptor (cMet) inhibitor PHA-665752, returning to basal closure level with 1 μM (Fig. 4C). PHA-665752 also inhibited the acceleration of wound closure induced by ex vivo ARDS

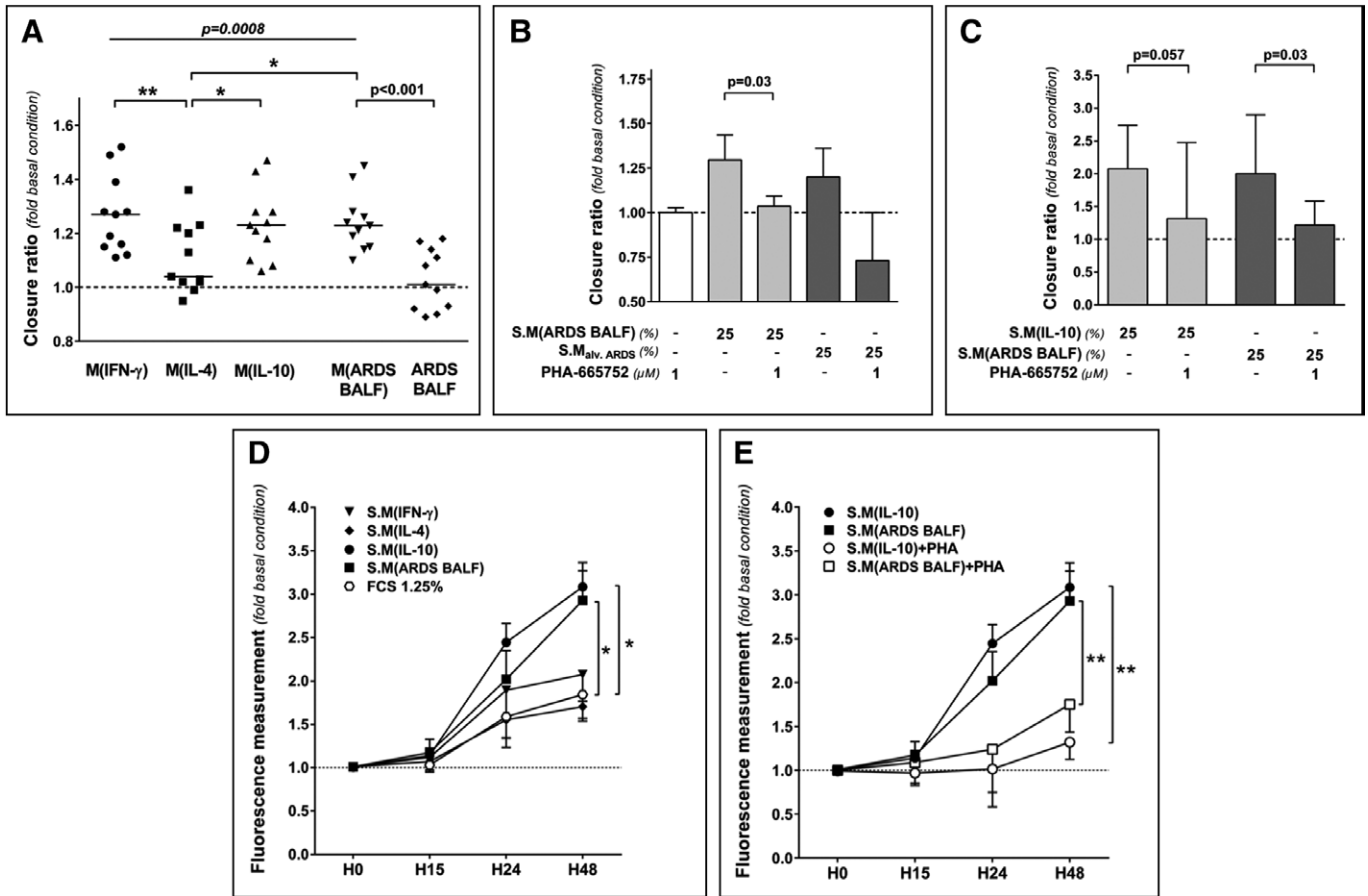


Figure 3. Effect of macrophage supernatants on epithelial wound healing. **A**, Effect of in vitro polarized macrophage supernatants on wound closure of A549 cells ($n = 11$). **B**, Effect of supernatants from macrophages stimulated with acute respiratory distress syndrome (ARDS)-bronchoalveolar lavage fluid (BALF) [S.M(ARDS BALF)] ($n = 6$, light gray) and alveolar macrophages from ARDS patients (S.M_{alv. ARDS}) ($n = 3$, dark gray) on wound closure of A549 cells, with or without the presence of PHA-665752. **C**, Effect of supernatants from macrophages stimulated with interleukin-10 [S.M(IL-10)] ($n = 5$, light gray) and S.M(ARDS-BALF) ($n = 5$, dark gray) on wound closure of primary human type II alveolar cells, with or without the presence of PHA-665752. **D**, Effect of macrophage supernatants on proliferation of A549 cells ($n = 6$). Basal control was 1.25% fetal calf serum (FCS), as diluted in macrophage supernatants. **E**, Inhibition of the epithelial proliferative effect of anti-inflammatory macrophage supernatants in the presence of PHA-665752 ($n = 6$). M(IFN- γ) = macrophages stimulated with interferon- γ , H = hour, M(IL-4) = macrophages stimulated with IL-4.

alveolar macrophage supernatant, whereas it remained ineffective on that induced by M(IFN- γ) (Figs. 3B and 4C). Furthermore, PHA-665752 inhibited M(ARDS-BALF)- and M(IL-10)-induced A549 cells proliferation (Fig. 3E). Eventually, stimulation of A549 cells with M(ARDS-BALF) and M(IL-10) supernatants induced cMet and Akt phosphorylation, which was inhibited by PHA-665752 (Supplementary Fig. 6, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>).

Concordantly to that observed in murine macrophages (14, 15), treatment of M(ARDS-BALF) and M(IL-10) with the selective prostaglandin-endoperoxide synthase (PTGS) 2 inhibitor NS-398 inhibited their HGF production (Supplementary Fig. 7, A and B, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>). Hence, NS-398-treated M(ARDS-BALF) and M(IL-10) supernatants were less potent inducers of epithelial wound closure than those from untreated macrophages (Supplementary Fig. 7, C and D, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>).

Relevance of Anti-Inflammatory Macrophage Polarization and HGF Production in Clinical Setting

sCD163 and HGF were measured in BALF from 85 ARDS patients included in a former prospective cohort (16) sampled 7 days (3–15 d) after their ARDS onset and 30 ventilated controls without ARDS. sCD163 and HGF concentrations were significantly correlated ($r = 0.51$), as with macrophage counts ($r = 0.28$ and $r = 0.71$, respectively; Supplementary Fig. 8, A and B, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>). Although raw sCD163 and HGF concentrations were not associated with ARDS outcome, sCD163/macrophage and HGF/macrophage ratios were significantly higher in ARDS patients than in ventilated controls and in ARDS survivors than in nonsurvivors (Supplementary Fig. 8, C–F, Supplemental Digital Content 2, <http://links.lww.com/CCM/D501>).

DISCUSSION

Our main results may be summarized as follows: 1) mature alveolar macrophages from patients with early ARDS

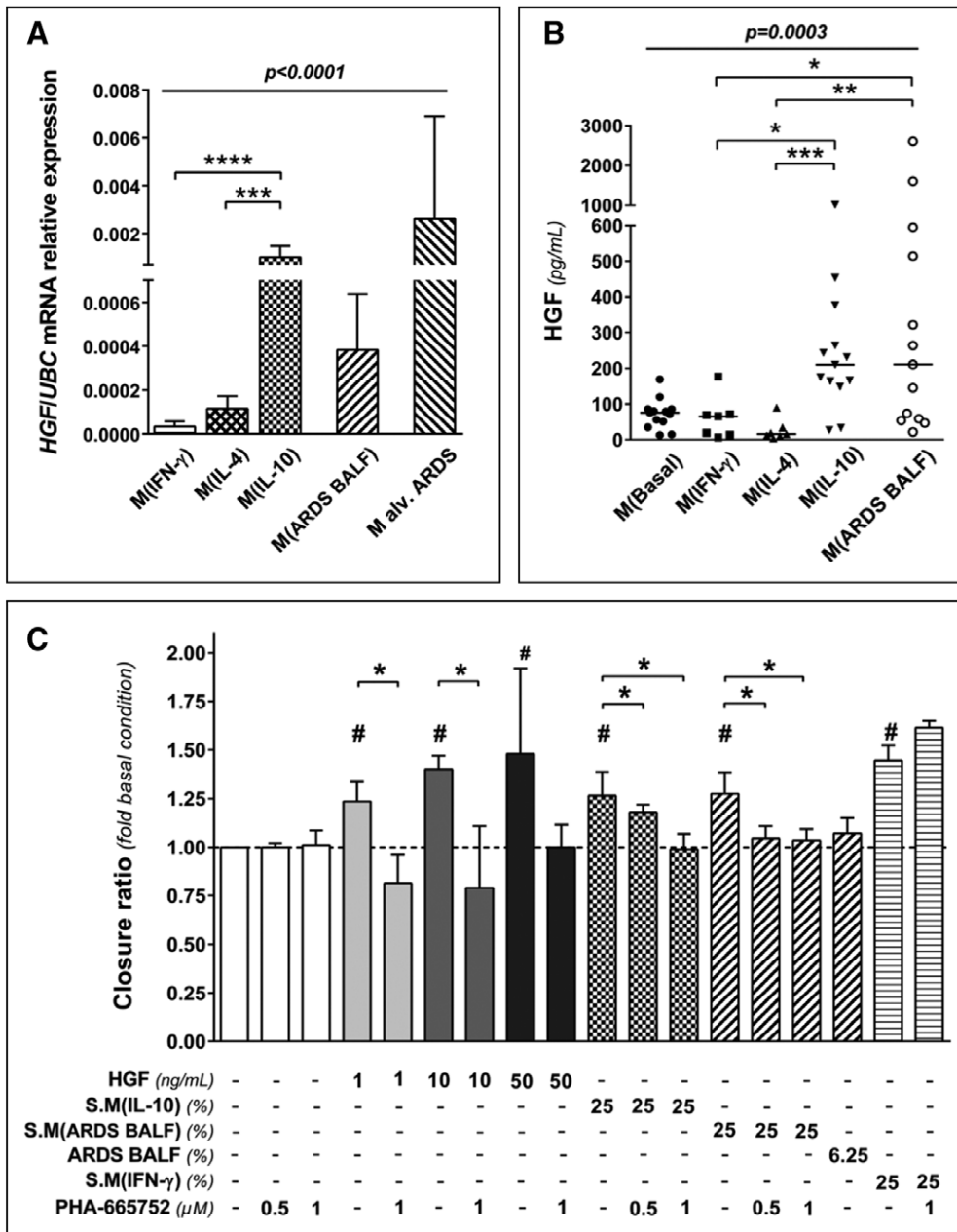


Figure 4. Production of hepatocyte growth factor (HGF) by polarized macrophages and role in epithelial wound closure. **A**, HGF messenger RNA (mRNA) expression in macrophages stimulated with IFN- γ M(IFN- γ), macrophages stimulated with interleukin-4 [M(IL-4)], macrophages stimulated with IL-10 [M(IL-10)], macrophages stimulated with acute respiratory distress syndrome (ARDS)-bronchoalveolar lavage fluid (BALF) [M(ARDS BALF)] ($n = 13$) and in alveolar macrophages from ARDS patients (Malv. ARDS) ($n = 3$). **B**, HGF concentration in macrophage supernatants measured by enzyme-linked immunosorbent assay ($n = 7-13$). Note: HGF concentration in the corresponding ARDS BALF was taken into account when determining HGF concentration in M(ARDS BALF) supernatants. **C**, Effect of recombinant human HGF and supernatants from M(interferon [IFN]- γ) (S.M[IFN- γ]), M(IL-10) (S.M[IL-10]), and M(ARDS BALF) (S.M[ARDS BALF]) on wound closure of A549 cells, with or without the presence of the cMet inhibitor PHA-665752 ($n = 6-12$). # $p < 0.05$ versus basal condition and * $p < 0.05$ between conditions with and without PHA-665752. M(Basal) = basal macrophages.

ARDS Alveolar Environment Drives an Anti-Inflammatory Macrophage Polarization

To our knowledge, this is the first demonstration of the anti-inflammatory driving effect of early ARDS alveolar environment in human. Indeed, macrophage maturation and polarization in the presence of ARDS alveolar environment led to overexpression of anti-inflammatory markers, in particular, membrane and sCD163. This anti-inflammatory polarization was confirmed by other morphologic, phenotypic, and functional characteristics, including IL-10 and HGF productions. Accordingly, we observed in vivo a subpopulation of ARDS alveolar macrophages expressing high CD163 levels. It is likely that this subpopulation contained in part alveolar resident macrophages, known to express CD163 and to be morphologically closer to mature than freshly recruited macrophages (17). Nevertheless, it has been shown that resident macrophages represent a minor pool of alveolar macrophages, following both infectious (4, 18) and noninfectious (19) ARDS. Furthermore, resident macrophages do not proliferate during human ARDS, suggesting that freshly recruited monocyte-derived macrophages contribute to the increase in alveolar macrophage count observed through ARDS evolution in our study as in others (3). Thus, the CD163^{high} fraction of alveolar macrophages probably also comes from maturation of recruited monocytes in alveoli. This was supported by the

exhibited an anti-inflammatory phenotype; 2) the ARDS alveolar environment drives, at least in part, this anti-inflammatory polarization; 3) anti-inflammatory macrophages specifically promote epithelial wound repair through HGF production.

resemblance between the anti-inflammatory pattern driven in vitro by ARDS-BALF on monocyte-derived macrophages and that observed ex vivo in ARDS-BALF cells.

BALF mediators driving the anti-inflammatory macrophage polarization remain unknown. ARDS alveolar environment is a

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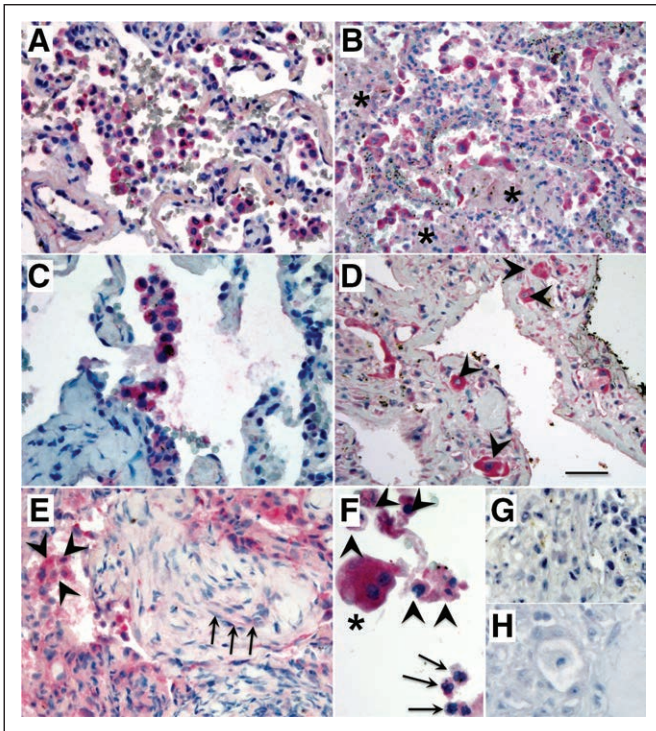


Figure 5. Hepatocyte growth factor (HGF) localization in acute respiratory distress syndrome (ARDS) human lung. **A–C**, Large alveolar macrophages are intensively stained for HGF during the exudative phase of ARDS. Alveolar exudates and hyaline membranes (* in **B**) are moderately stained for HGF. **D–E**, Alveolar (arrowheads in **E**) and interstitial (arrowheads in **D**) macrophages are intensively stained for HGF during the fibroproliferative phase of ARDS. Some fibroblasts inside clusters of fibroblasts are also moderately stained for HGF (arrows in **E**). **F**, Large alveolar macrophages from ARDS bronchoalveolar lavage fluid (BALF) (*) are more intensively stained for HGF than freshly recruited monocytes/macrophages (arrowheads) or neutrophils (arrows). **G–H**, Control isotype showing no nonspecific staining. Pictures are representative of results obtained in seven ARDS lungs. Scale bar represent 100 μm in (**A**, **B**, **D**, **E**, **G**); 50 μm in (**C**), 30 μm in (**F**, **H**).

complex fluid containing many inducers of macrophage polarization, such as $\text{IFN-}\gamma$, $\text{TNF-}\alpha$, and lipopolysaccharide (LPS) promoting M1, IL-4 promoting M2a, and IL-10 and serum amyloid P (SAP) promoting M2c polarizations (20–23). Despite IL-10 and SAP relevant concentrations in early ARDS-BALF (20), we did not succeed to fully reverse ARDS-BALF-induced macrophage polarization using anti-IL-10 blocking antibodies or SAP-depleted ARDS-BALF (data not shown). LPS is also able to induce an anti-inflammatory counter-regulation in monocyte through up-regulation of IL-10 and cortisol (24). Nevertheless, polarization patterns induced by ARDS-BALF were not influenced by the presence or the absence of concomitant pneumonia. Apoptotic cell clearing both depends on and induces an anti-inflammatory macrophage polarization, notably through the IL-10/*Gas6*/*Mer* tyrosine kinase pathway (25, 26). Furthermore, considering that CD163 is a scavenger receptor and that M(IL-10) exhibited the best bacterial phagocytosis ability, anti-inflammatory macrophage polarization seems linked to injured alveoli clearing and debris removal. Thus, macrophage polarization is most likely the result of a dynamic complex balance involving both inflammatory and anti-inflammatory stimuli, likely contributing to the

heterogeneity of the macrophage population observed in vivo. This hypothesis is in line with current concepts on inflammation and sepsis highlighting that an anti-inflammatory response appears concomitantly to the initiation of the inflammatory activation cascade (27). Accordingly, M2 markers are early expressed concomitantly to M1 markers during murine experimental ARDS (5). Thus, induction of an anti-inflammatory macrophage polarization may be an adapted and protective reaction to the ARDS inflammatory storm. Along these lines, previous studies reported better outcome in IL-10 high-producer ARDS patients carrying the -1082G/G genotype both in Caucasian (28) and Asian (29) populations. Accordingly, we observed that the alveolar sCD163/macrophage ratio was higher in ARDS survivors, suggesting that the anti-inflammatory macrophage polarization may contribute to better prognosis. Besides, while contributing to counter-regulation of the acute inflammatory state, the macrophage polarization induced by the ARDS alveolar environment is associated with high phagocytosis ability and increased HLA-DR expression. Thus, this early anti-inflammatory macrophage polarization should be distinguished from the deleterious late ICU-acquired immunosuppression syndrome induced by persistent or repeated inflammatory injuries, notably mediated by immature myeloid-derived suppressor cells (30).

ARDS-BALF–Polarized Macrophages Promote Epithelial Repair Through Up-Regulation of HGF Synthesis

We demonstrated for the first time that human anti-inflammatory macrophages, polarized either with rhIL-10 or ARDS-BALF, promoted migration and proliferation of alveolar epithelial cells. This confirms in human the concordant results previously obtained in murine models (6, 26, 31). Further, Tang et al (31) demonstrated that adoptive transfer of in vitro IL-10-polarized macrophages into LPS-injured mice provided the best protection against acute lung injury compared with nonpolarized or M2a-polarized macrophages. Taken together, these results highlight the important role of anti-inflammatory macrophages for alveolar epithelial recovery during ARDS.

During human ARDS, HGF is up-regulated in the lung (32, 33), and its recognized cellular sources are neutrophils (34) and fibroblasts (35). To our knowledge, we demonstrated for the first time that human macrophages were an alternative source of HGF in relation with their anti-inflammatory polarization during ARDS. Our results are in agreement with previous studies reporting that the anti-inflammatory polarization induced by apoptotic cells resulted in induction of HGF production by murine macrophages both in vitro (14, 26, 36, 37) and in vivo (6). Furthermore, our results confirmed in human anti-inflammatory macrophages the role of the prostaglandin endoperoxide synthase 2/prostaglandin E2 pathway in HGF up-regulation, previously reported in murine peritoneal macrophages (14). We found HGF concentrations averaging 200 pg/mL in supernatants from macrophages polarized in vitro with ARDS-BALF and IL-10, which have been deemed able to induce epithelial restitution (38). Further, we confirmed that supernatants from alveolar macrophages isolated from ARDS-BALF cell pellets also

promoted epithelial repair through the HGF/cMet pathway. Our results also demonstrated that human primary AECII respond to HGF produced by macrophage, confirming a previous report in which human lung fibroblasts could enhance AECII wound healing through HGF secretion (39). Taken together, our results demonstrated that macrophages contributed to alveolar epithelial repair through HGF production during ARDS resolution.

At first glance, this result could appear contradictory with the association between increased HGF contents in early ARDS-BALF and poor ARDS prognosis (33). However, during early ARDS, alveolar HGF concentrations mainly reflect the amount of recruited neutrophils known to release preformed HGF contents (33, 34), thus attesting of more alveolar damage and inflammation. During the latter reparative phase, macrophages become the predominant alveolar cell type and, as we demonstrated, are able to enhance alveolar repair notably through HGF secretion. Thus, we hypothesize that a biphasic alveolar HGF release may also occur in human alveoli similarly to that documented in a murine model of pneumonia, in which the delayed HGF spike beginning 48–72 hours after the injury was due to recruited macrophages having phagocytized apoptotic cells (36). Hence, unlike the early neutrophil-related HGF release, delayed macrophage-related HGF production might be associated with better ARDS resolution. The higher HGF/macrophage ratio in BALF sampled from ARDS survivors beyond their first 72 hours of evolution tends to support this hypothesis, although further studies are needed to confirm it.

In conclusion, our human results suggest that macrophage polarization is associated with epithelial repair and inflammation resolution during ARDS. In future research, personalized monitoring and modulation of lung macrophage polarization could be a promising target in nonresolving ARDS.

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