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► **To cite this version:**

Murielle Gregoire, Fabrice Uhel, Mathieu Lesouhaitier, Arnaud Gacouin, Marion Guirriec, et al.. Impaired efferocytosis and neutrophil extracellular traps clearance by macrophages in ARDS. *European Respiratory Journal*, 2018, 52 (2), pp.1702590. 10.1183/13993003.02590-2017 . hal-01833937

HAL Id: hal-01833937

<https://univ-rennes.hal.science/hal-01833937v1>

Submitted on 14 Sep 2018

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Impaired efferocytosis and neutrophil extracellular traps clearance by macrophages in ARDS

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Take home" message: *Restoration of AMPK activation and specific inhibition of HMGB1 could reduce lung inflammation during human ARDS*

ABSTRACT

Rationale Exaggerated release of neutrophil extracellular traps (NET) along with decreased NET clearance and inability to remove apoptotic cells (efferocytosis) may contribute to sustained inflammation in Acute respiratory distress syndrome (ARDS) . Recent studies in experimental models of ARDS have revealed the crosstalk between AMPK and HMGB1 which may contribute to effectiveness of efferocytosis, therefore reducing inflammation and ARDS severity.

Methods We investigated neutrophil and NET clearance by macrophages from control and ARDS patients and examined how bronchoalveolar lavage (BAL) fluids from control and ARDS patients could affect NET formation and efferocytosis. Metformin, an AMPK activator, and neutralizing antibody against HMGB1 were applied to improve efferocytosis and NET clearance.

Main results Neutrophils from ARDS showed a significantly reduced apoptosis. Conversely, NET formation was significantly enhanced in ARDS. Exposure of neutrophils to ARDS BAL fluid promoted NETs production, while control BALs have no effects. Macrophage engulfment of NETs and apoptotic neutrophils was diminished in ARDS patients. Notably, activation of AMPK in macrophages or neutralization of HMGB1 in BAL fluids improved efferocytosis and NET clearance.

Conclusions Restoration of AMPK activity with metformin or specific neutralization of HMGB1 in BALs fluids represent promising therapeutic strategies to decrease sustained lung inflammation during ARDS.

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is an acute inflammatory lung injury characterized by a hypoxemic respiratory failure following a disruption of the endothelial-epithelial barrier, alveolar damage and pulmonary edema(1, 2). In spite of significant advances in critical care, antibiotics, and lung ventilation strategies, effective therapeutic interventions to diminish the severity of lung injury and mortality among ARDS are not available(3-5). Neutrophils are the first line of innate immune response producing anti-bacterial peptides, reactive oxygen species, cytokines, and other inflammatory mediators(6). Neutrophils are also able to release extracellular traps (NETs), a unique mechanism of DNA deployment into the extracellular milieu (7, 8). Although these functions are important to target microbial agents, neutrophil exaggerated and prolonged activation could contribute to the development of acute lung injury (ALI)(9-11). In particular, prolonged life span of neutrophils occurs during ARDS and several studies have shown a deleterious impact associated with neutrophil delayed apoptosis(9, 12-14). Similarly to substantial production of inflammatory mediators, neutrophil-driven excessive NETs formation can worsen inflammation, in particular in sterile inflammatory conditions (15-17). Therefore, time dependent neutralization of apoptotic cells, especially apoptotic neutrophils and clearance of NETs appeared to be important steps in the resolution phase and recovery from lung injury since an effective removal of dying cells known as efferocytosis plays a crucial role in the maintenance of tissue homeostasis(18). Macrophage phagocytic function is typically associated with engulfment of dying cells, however less is known about the mechanisms involving NET clearance(19-21). Besides recently described benefit of DNase I in experimental sepsis, the role of

macrophages in the clearance of NETs, including in conditions associated with development and resolution from ARDS are not determined(22).

AMP-activated protein kinase (AMPK) is a serine-threonine protein kinase that functions as crucial metabolic sensor and regulates cellular energy production and expenditure (23). More recent studies indicate that AMPK activation has also a potent anti-inflammatory effect. In addition, AMPK activation can stimulate macrophage efferocytosis, along with neutrophil and macrophage capacity to ingest bacteria(24, 25). However, inflammatory conditions are accompanied with a reduced activity of AMPK in macrophages, neutrophils and in lung tissue. Restoration of AMPK activity could be an interesting approach to increase efferocytosis and likely to decrease inflammatory-lung injury in human, as already reported in mouse models of ALI(25, 26). Moreover, High-mobility group box (HMGB)-1, an alarmin that may promote inflammation, has been involved in the development of severe ARDS and has been shown to inhibit efferocytosis(27-29).

We thus designed the present study to investigate the ability of regulation of lung inflammation by neutrophils and macrophages in patients with ARDS. Our objectives were first to evaluate the survival of neutrophils and their ability to produce NETs. Then, we studied macrophage capacity to engulf apoptotic cells and NETs. Finally, two potential therapeutic targets, AMPK and HMGB1, were considered to restore efferocytosis and NET clearance, and thus to reduce persistent inflammation and decrease lung injury in patients with ARDS.

MATERIAL AND METHODS

Patients

This study was conducted in the medical intensive care unit (ICU) of Rennes University Hospital. The study protocol was approved by local ethic committee (n°14.38). Because of the observational nature of the study, a non-opposition form was provided to families and patients. Patients with Berlin criteria for ARDS were consecutively enrolled and compared with patients who underwent bronchoscopy in the department of pulmonary medicine with normal BAL (control patients)(30).

Bronchoalveolar lavages (BAL)

BAL were performed within two days following initiation of mechanical ventilation in ARDS patients, or in an outpatient setting for control participants. BAL fluid was obtained by centrifugation and cell population differentials were determined on cytopsin slides after May-Grunwald Giemsa coloration.

Cytokine quantification

Interleukine (IL)-6, IL-8, chemokine ligand 2 (CCL2), C-X-C motif chemokine 10 (CXCL10), plasminogen activator inhibitor-1 (PAI-1) (R&D System, Abingdon, UK), and HMGB-1 (IBL International GmbH, Hamburg, Germany), were quantified in BAL fluids by ELISA.

Cell isolation and culture

Human primary bronchial epithelial cells (BEC) were obtained from lung donor trachea or bronchi of the Cohort Of Lung Transplantation (COLT, NCT00980967). Tissues were dissociated overnight at 4°C with collagenase in HEPES-buffered

RPMI medium (Sigma-Aldrich, St Louis, MO, USA). BEC were cultured in cnT17 medium (CELLnTEC Advanced Cell systems AG, Bern, Switzerland) containing penicillin and streptomycin, on human type IV collagen (Sigma-Aldrich) coated plates. Blood samples were obtained from ARDS patients within hours following BAL, or from healthy donors. Neutrophils were purified as previously described (31). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient (Eurobio, Courtaboeuf, France). PBMCs were incubated in RPMI 1640 containing 7% fetal calf serum (FCS) and 1% penicillin-streptomycin at 37°C. After 1 hour, non-adherent cells were removed by washing with complete medium. Human monocyte-derived macrophages (HMDMs) were then derived from adherent monocytes by culture with 20 ng/mL macrophage-colony stimulating factor (M-CSF, R&D Systems, Abingdon, UK) for 5 days. Purity of HMDMs was >80% and evaluated by flow cytometry.

Apoptosis and necrosis Assay

BEC were cultured for 24 hours in 50% cnT17 medium and 50% BAL fluid or normal saline solution (Fresenius Kabi, Sèvres, France). BEC apoptosis and necrosis were assessed by flow cytometry using AnnexinV (Cell Signaling Technology, Danvers, MA) and Dapi (Life Technologies, Gand Island, NY).

Circulating neutrophils purified from ARDS patients or healthy donors were cultured for 24 hours in 50% RPMI-7% FCS and 50% BAL fluid or saline. Neutrophil apoptosis and necrosis were assessed by flow cytometry using phycoerythrin (PE)-conjugated active caspase-3 apoptosis kit (Becton Dickinson, San Jose, CA) and FITC anti-CD66b mAb (Beckman Coulter, Miami, FL) for apoptosis. AnnexinV and Dapi were used to measure necrosis.

NET release quantification

Neutrophils were incubated for 30 minutes in 50% RPMI-7% FCS and 50% BAL fluid or saline. When indicated, BAL fluids were before neutralized with an anti-HMGB1 (IBL International GmbH, Hamburg, Germany) mAb or isotype for 2 hours. Neutrophils were then labeled with 5 $\mu\text{mol/L}$ Sytox blue (Invitrogen, Carlsbad, CA) in RPMI-0.5% FCS with or without DNase I (200UI/mL, Roche, Switzerland), seeded in Costar 96-well black plates (Corning Costar Corporation, Cambridge, MA) and stimulated or not with 10 $\mu\text{mol/L}$ phorbol myristate acetate (PMA, Sigma-Aldrich) for 3 hours at 37°C. The release of NETs was quantified by measuring fluorescence with a microplate fluorescence reader (Varioskan, ThermoFisher Scientific, Waltham, MA).

NET isolation and phagocytosis by macrophages

Neutrophils from ARDS patients or healthy donors were incubated in RPMI with 25 nmol/L PMA for 2 hours at 37°C. After centrifugation, NETs were quantified in the supernatant by measuring fluorescence using Sytox blue (5 $\mu\text{mol/L}$). HMDMs were allowed to attach in Corning 96-well black plates for 3 hours in 50% RPMI-7% FCS and 50% BAL fluid or saline, then Sytox blue-labelled purified NETs were added. After incubation for 2 hours at 37°C, HMDMs were washed and NET phagocytosis was assessed by fluorescence quantification. NET engulfment ratio was determined as the ratio of fluorescence of HMDMs having phagocytized NET to the fluorescence of HMDMs alone. When indicated, HMDMs were incubated with an anti-HMGB1 neutralizing antibody or isotype, or with metformin (500 $\mu\text{mol/L}$, Sigma-Aldrich, St Louis, MO) for 2.5 hours.

Immunofluorescence stainings

For NET imaging, purified neutrophils were immobilized on slides coated with poly-D-lysine (Sigma-Aldrich, St Louis, MO), and incubated with 50% RPMI-7% FCS and 50% BAL fluid from control or ARDS patients for 3 hours. Cells were fixed with 4% paraformaldehyde (PFA, Antigenfix Diapath, Martingo, Italy). Coverslips were mounted with mowiol including Sytox blue (5 $\mu\text{mol/L}$).

For phagocytosis imaging, HMDMs were derived from monocytes on chamber coverslips with M-CSF (20ng/mL) for 5 days. HMDMs were then incubated for 3 hours with RPMI containing neutrophil-isolated NETs or not. Cells were fixed with 4% PFA and labelled with anti-neutrophil elastase mAb (Dako, Carpinteria, CA) followed by Alexa Fluor 488 anti-mouse secondary antibody (Jackson ImmunoResearch, Cambridgeshire, UK), and Texas Red-X Phalloidin (Life Technologies, Gand Island, NY) for actin. Coverslips were mounted with Mowiol including Topro-3 (1 $\mu\text{mol/L}$, Life Technologies, Gand Island, NY).

For efferocytosis assays, HMDMs derived on chamber coverslips were incubated for 3 hours with BAL fluid from control or ARDS patients, with or without 500 $\mu\text{mol/L}$ metformin for 2.5 hours. When indicated, BAL fluid was pre-treated with an anti-HMGB1 neutralizing antibody or isotype. Efferocytosis was evaluated by adding 10^6 carboxyfluorescein succinimidyl ester (CFSE)-labeled apoptotic neutrophils. After incubation at 37°C for 1 hour, cells were washed and fixed with 4% PFA. The efferocytosis index was determined on 300 cells as the percentage of HMDMs containing at least one ingested apoptotic neutrophil.

For all imaging, slides were examined with a SP5 confocal microscope (Leica Microsystem, Wetzlar, Germany). Digital images were processed using ImageJ software.

Western blot

(Phospho)-AMPK Western blotting was performed using mouse anti-AMPK α or rabbit anti-phospho-AMPK α antibodies (Cell Signaling Technology, Danvers, MA), followed by HRP-conjugated anti mouse or anti rabbit secondary IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Actin was blotted as loading control, using mouse anti- α -actin (Sigma-Aldrich, St Louis, MO) and HRP-conjugated anti mouse secondary IgG. Blots were quantified by using ImageJ software (National Institute of Health, Bethesda, MD).

Statistical analysis

Quantitative variables are expressed as mean \pm SD or median (interquartile range, IQR) when indicated, and qualitative variables as number (percentage). Continuous variables were compared using the nonparametric Mann-Whitney *U* test or Wilcoxon test for matched pairs as appropriate. Analyses were performed with GraphPad Prism 6.2 (GraphPad Software, La Jolla, CA).

RESULTS

NET formation in ARDS patients may contribute to lung injury.

Among ARDS BAL leukocytes, neutrophils were the predominant cell population whereas the majority of BAL leukocytes in controls are macrophages (Supplemental Figures 1AB). Specific descriptions of ARDS patients and control subjects are provided in Table 1.

Table 1. Patient characteristics

Characteristics	ARDS	Control
N	25	21
Age, yr	67 (59-75)	59 (50-68)
Gender		
Male (%)	14 (56%)	15 (71%)
Female (%)	11 (44%)	6 (28%)
Mortality		
Dead (%)	7 (28%)	0 (0%)
Alive (%)	18 (72%)	21 (100%)
ARDS Etiology		
Bacterial pneumonia	22 (88%)	NA
Sepsis related	3 (12%)	
Influenza	3	
Mechanical ventilation, days	11 (8-22)	NA
Initial PaO₂/FiO₂ ratio	114 (78-128)	NA

ARDS= acute respiratory distress syndrome, NA= not applicable. Values are reported as median (interquartile range) or numbers (percentage).

Several soluble factors implicated in the development of lung injury, including the pro-inflammatory cytokine IL-6, and CXCL10, CCL2, and IL-8 chemokines were significantly increased in ARDS patients (Supplemental Figure 1C)(13, 32). We also found significant increased levels of PAI-1, implicated in down regulating

efferocytosis in animal models of acute lung injury (ALI) (Figure 1A)(33). Because HMGB1 has been shown to promote NET release in experimental ALI, we also examined this possibility in ARDS patients (34). We found that HMGB1 was significantly increased in BAL fluid of ARDS patients vs controls (Figure 1A). Subsequent analysis revealed substantial amounts of cell free DNA in BAL fluid of ARDS patients, suggesting that HMGB1 accumulation is accompanied by an enhanced NETosis (Figure 1B). Furthermore, BAL fluids from ARDS patients have been found to induce lung epithelial cell injury which could be related to NETs (Figure 1 C-F).

Neutrophils of ARDS patients enhanced capacity to produce NETs.

The cell-free DNA found in BAL fluids could be a result of DNA release from necrotic cell death. However, we found that neutrophils in BALs of ARDS patients had relatively low apoptotic index (data not shown) but also that circulating neutrophils of ARDS patients presented an increased capacity to produce NETs *ex vivo*, as compared to healthy donors (Figure 2A, 2B). In these experiments, NETosis was measured after stimulation of neutrophils with PMA. NETs formation was also used to examine whether BAL fluids from control or ARDS patients influence NETs deployment. When compared to BAL fluid from control patients, BAL fluid from ARDS patients effectively increased spontaneous NET release from either control or ARDS neutrophils (Figure 2C - F).

Neutrophils of ARDS patients show increased viability

In inflammatory conditions, like in ARDS, neutrophils are known to acquire a prolonged viability. To determine neutrophil viability, apoptotic indices were

measured after 24 hours of neutrophil culture. The amounts of apoptotic neutrophils were significantly lower in circulating neutrophils from ARDS patients compared to healthy donors. This result confirmed that viability of neutrophils is increased in ARDS patients (Figure 3A). In subsequent experiments, we examined the effect of BAL fluids on neutrophil viability. As shown in Figure 3C, BAL fluids from ARDS, unlike from control patients, increased the viability of healthy donor circulating neutrophils. Apoptotic percentage was even further decreased after exposure of ARDS circulating neutrophils to ARDS BAL fluids (Figure 3D). However, to ensure that specific constituent(s) of BAL fluids in ARDS patients increased neutrophil viability, we also explored necrosis rate and found a trend toward in diminished neutrophil necrosis when exposed to BAL fluids from ARDS patients (Figure 3E).

Monocyte-derived macrophages from ARDS patients have diminished ability to phagocyte NETs and apoptotic neutrophils.

The ability of macrophages to neutralize apoptotic neutrophils plays a central role in termination and resolution of inflammatory conditions. Recent studies also indicate that macrophages are involved in clearance of NETs(20, 35). As shown in Figures 4A - D, there was a significant reduction in both NET uptake and apoptotic neutrophil efferocytosis by HMDMs from ARDS patients vs healthy donors. Moreover, a similar decrease in phagocytic ability was observed upon exposure of HMDMs from healthy donors to BAL fluids obtained from ARDS patients (Figure 4E). Even further reduction in phagocytic indices were found in ARDS HMDMs treated with ARDS BAL fluids (Figure 4F). In contrast, BAL fluids of control patients had no impact on efferocytosis (Figure 4E - F).. This finding also suggests that reduced efferocytosis was mediated by soluble components in lung fluid of ARDS patients.

The effects of AMPK and HMGB1 on efferocytosis and NET clearance.

To determine factor(s) that are affecting NETosis, efferocytosis and NET engulfment, we first examined the impact of HMGB1 (Figure 5A). We did not observe any significant effect of HMGB1-neutralizing antibody on NET formation (Figure 5B). In contrast, we found that an anti-HMGB1 antibody increased the clearance of apoptotic neutrophils by ARDS HMDMs (Figure 5C) and had no effect on NET uptake (Figures 5D). The ability of HMGB1 to affect efferocytosis is consistent with previous studies in a murine models of inflammatory organ injury, in particular linking HMGB1 release in extracellular milieu with diminished clearance of apoptotic cells.

Besides adverse effects mediated by extracellular HMGB1, inflammatory conditions are associated with metabolic reprogramming of immune and parenchymal cells which are associated with diminished activity of AMPK in macrophages(36). Notably, AMPK activators, including metformin have been shown to promote efferocytosis and also to reduce the severity of ALI. Thus, we examined if AMPK activation can also recover phagocytic capacity of HMDMs from ARDS patients. As shown in Figure 5F, AMPK activation, *i.e.* phosphorylation of Thr172-AMPK was significantly diminished in HMDMs treated with BAL fluid of ARDS patients. Moreover, we indicate that culture of ARDS HMDMs with metformin restored AMPK activation (Figure 5G). This activation was associated with a significant increase in uptake of apoptotic neutrophils and NETs (Figure 5H and I).

DISCUSSION

Our study reveals major findings that could enhance or sustain lung inflammation during ARDS. Firstly, although neutrophil lifespan is significantly increased, intraalveolar neutrophils are releasing NETs through a pathway termed vital NETosis and BAL fluid of ARDS patients can increase the release of NETs which could induce lung injury. Secondly, in ARDS conditions, ability of macrophages to engulf NETs and apoptotic cells is significantly decreased. We also found that blocking HMGB1 and activating AMPK could enhance clearance of NETs and apoptotic cells.

Clinical and histological studies have suggested that the severity and outcome of ARDS were associated with the inflammatory process reflected in bronchoalveolar fluid(13). A large number of clinical and animal studies brought evidences that neutrophils have a direct influence on the onset and the persistence of ARDS. For instance, Steinberg et al. found that alveolar macrophages increased in ARDS survivors compared to non-survivors reaching the conclusions that sustained alveolar inflammation was associated with high mortality (12) . Among factors which could sustain inflammatory conditions, increased NETosis, decreased ability of macrophages to engulf apoptotic cells and NETs, appear to be critical.

We found that neutrophils could produce large amounts of NETs, spontaneously or when exposed to ARDS BAL fluids. NETs are composed of decondensed chromatin fibers coated with antimicrobial proteins. NETosis could require membrane rupture and the loss of neutrophil functions (so called *suicidal NETosis*)(17). However Yipp et al. have demonstrated that, during the early phase of infection, NETosis involved neutrophils that do not undergo lysis and retained the ability to perform recruitment, chemotaxis, and phagocytosis (so called *vital NETosis*)(37). In our study, and

probably in ARDS setting, NETosis does not result in cell death since we found that neutrophils life span was increased. Although the primary role of NETs is to avoid bacterial diffusion, NETs have been found to play deleterious effects on lung injury during ARDS and several studies pointed out that NET formation during bacterial pneumonia was only worsening lung injury without any bactericidal activity(17, 38). Moreover, Narasaraju et al. demonstrated that, in mice challenged with influenza, NETs contributed to acute lung injury by instigating alveolar-capillary damage(15). Therefore, NETosis and clearance of NETs should be adequately regulated *in vivo* and defect in mechanisms responsible for NET clearance may contribute to perpetuate inflammation and worsen tissue injury(15, 39). Two mechanisms have been described in NET clearance: DNase I-dependent digestion and phagocytosis by macrophages which has been found diminished in our study(17). We also demonstrated that efferocytosis was decreased in ARDS. Clearance of cells undergoing apoptosis protects surrounding tissue from exposure to proinflammatory intracellular contents released from necrotic cells. Although there is convincing animal data showing that neutrophils secrete anti-inflammatory peptide while dying, failure to effectively remove apoptotic cells and particularly apoptotic neutrophils perpetuate inflammation, exposing lung to sustained inflammatory conditions that could increase neutrophils influx, NETosis and worsen lung injury(40, 41). Thus, macrophages have a key position in the resolution of inflammation and initiation of tissue repair. Restoring ability of macrophages to engulf both NETs and apoptotic cells could be of interest to decrease lung damages and pulmonary sequelae such as fibrosis.

We found that BAL fluid from ARDS patients could induce a decrease in efferocytosis in both healthy and ARDS macrophages, allowing therapeutic intervention to

enhance efferocytosis and engulfment of NETs. Along these lines, AMPK pathway appears to be a potential therapeutic target. We found in our study that ability of macrophages to activate AMPK was decreased in inflammatory conditions which could be associated to a defect in efferocytosis activity (24). Of note, the ability of AMPK activation to enhance phagocytosis appears to be related to interaction with cytoskeletal organization, (24). Lastly, we found that inhibition of HMGB1 could increase efferocytosis as already reported in animal model of lung injury. HMGB1, originally described as a nuclear non-histone DNA-binding protein, has subsequently been shown to be an alarmin involved in the inflammatory response playing a critical role in the recruitment of neutrophils, lung injury and suppressing bacterial clearance in the lung(31, 34, 42). It is worth noting that metformin has been found to bind and inhibit the action of HMGB1, suggesting that the effects of metformin could be similar to those of HMGB1 inhibition (43) High levels of HMGB1 in BAL fluid from ARDS patients can decrease efferocytosis, suggesting that inhibition of HMGB1 as activation of AMPK, could be of interest to restore efferocytosis diminish lung injuries, and ultimately improve lung function after ICU discharge.

CONCLUSION

Altogether, our results show that efferocytosis and NET engulfment which could contribute to the persistence of lung inflammation are diminished during ARDS. Restoration of AMPK activation with metformin and specific inhibition of HMGB1 appear to be promising targets to decrease lung inflammation, and to limit alveolar damage and progression to lung fibrosis in patients with ARDS.

FUNDING

This work was supported by grants from CORECT 2015 (Comité de la Recherche Clinique et Translationnelle), CHU Rennes, France.

ACKNOWLEDGMENTS

Immunofluorescence study was performed on the Microscopy Rennes Imaging Center (MRic-ALMF; SFR UMS CNRS 3480 – INSERM 018 Biosit, Rennes, France).

Healthy donor monocytes were provided from DTC Center (CIC Biothérapie 0503, Nantes, France).

Doctor Mohinder Pal for the careful reading of the manuscript.

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FIGURE LEGENDS

Figure 1. Characteristics of the bronchoalveolar lavages (BAL) from acute respiratory distress syndrome (ARDS) patients may contribute to lung epithelial cell injury.

A. Quantification of Plasminogen activator inhibitor (PAI)-1, (n=19) and High mobility group box (HMGB)1 (n=6) by ELISA in BAL fluid from Ctrl or ARDS patients. **B. BAL fluids from ARDS patients contain high levels of neutrophil extracellular traps (NETs).** Quantification of NETs by fluorescence measurement after Sytox blue staining in BAL fluids from Ctrl (n=8) and ARDS patients (n=8). Horizontal bars represent medians. Histograms represent mean (\pm standard deviation). **C-D. BAL fluids from ARDS patients induce lung epithelial cell apoptosis and necrosis.** **C.** Human primary bronchial epithelial cells (BEC) were treated with 50% of normal saline solution (NaCl), BAL fluid from control (Ctrl BAL) or ARDS patients (ARDS BAL) for 24 hours. **D.** Apoptosis and necrosis were measured using flow cytometry. **E-F. NETs induce lung epithelial cell apoptosis and necrosis.** **E.** BEC were treated with 50% of RPMI-0.5% FCS or NET for 24 hours. **F.** Apoptosis and necrosis were measured using flow cytometry.

The Mann-Whitney test was used to compare protein and NET quantification and the Wilcoxon test was used for BEC apoptosis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, non-significant.

Figure 2. NETosis is enhanced in peripheral blood-derived neutrophils from acute respiratory distress syndrome (ARDS) patients, and increased by bronchoalveolar lavage (BAL) fluid mediators.

A-B. Neutrophils from healthy donors (HD) or ARDS patients were treated with 10 $\mu\text{mol/L}$ phorbol myristate acetate (PMA) for 3 hours before NET quantification by Sytox blue fluorescence (5 $\mu\text{mol/L}$). **B.** Free DNA (Neutrophil extracellular traps, NETs) production were compared in neutrophils from HD (HD PMN, n=5) and ARDS patients (ARDS PMN, n=5). **C-E.** Neutrophils from HD or ARDS patients were treated with BAL fluid from control (Ctrl BAL) or ARDS patients (ARDS BAL) for 3 hours before NET quantification by Sytox blue fluorescence. **D.** Quantification of NET production by HD neutrophils after incubation with Ctrl BAL or ARDS BAL (n=6). **E.** NET production by ARDS neutrophils were quantified after incubation with Ctrl BAL or ARDS BAL (n=5). **F.** Fluorescence microscopy images showing NET formation from a representative ARDS patient after 3 hours incubation with control or ARDS BAL fluid. Neutrophil DNA was stained with Sytox blue. The Mann-Whitney test was used to compare NET quantification. *P < 0.05.

Figure 3. The proportion of apoptotic peripheral blood-derived neutrophils is decreased in acute respiratory distress syndrome (ARDS) patients, spontaneously and after incubation with bronchoalveolar lavage (BAL) fluids.

A. Proportion of active caspase 3⁺ apoptotic cells among CD66b⁺ neutrophils from healthy donors (HD PMN, n=9) or ARDS patients (ARDS PMN, n=10), assessed by flow cytometry after 24h *ex vivo* culture. **B.** Neutrophils from HD or ARDS patients were treated with 50% of normal saline solution (NaCl), BAL fluid from control (Ctrl BAL) or ARDS patients (ARDS BAL) for 24 hours before neutrophil apoptosis or necrosis measurement. **C.** Proportion of active caspase 3⁺ apoptotic cells among CD66b⁺ neutrophils from HD, assessed by flow cytometry after 24h *ex vivo* culture

with normal saline solution (NaCl, n=8), Ctrl BAL (n=10) or ARDS BAL (n=10). **D.** Proportion of active caspase 3⁺ apoptotic cells among CD66b⁺ neutrophils from ARDS patients assessed by flow cytometry after 24h *ex vivo* culture with saline solution (NaCl, n=10), Ctrl BAL (n=10) or ARDS BAL (n=13). **E.** Proportion of AnnexinV⁺/Dapi⁺ necrotic neutrophils from ARDS patients assessed by flow cytometry after 24h *ex vivo* culture with Ctrl BAL (n=7) or ARDS BAL (n=7). The Mann-Whitney test was used to compare neutrophil apoptosis or necrosis. *P < 0.05; ** P < 0.01; ns, non-significant.

Figure 4. The ability of human monocyte-derived macrophages (HMDMs) to engulf neutrophil extracellular traps (NETs) and apoptotic neutrophils is reduced during acute respiratory distress syndrome (ARDS).

A. Engulfment of Sytox blue-labeled NETs by human monocyte-derived macrophages (HMDMs) from healthy donors (HD macro ϕ , n=6) and ARDS patients (ARDS macro ϕ , n=6). NET engulfment ratio was defined as the ratio of fluorescence emitted by HMDMs which have phagocytized NETs to the fluorescent emitted by HMDMs alone. **B.** Immunofluorescence images showing HMDMs in the process of engulfing NETs. HMDMs were incubated with NETs purified from neutrophils and are internalized by HMDMs. HMDMs actin (phalloidin, red), HMDMs DNA (Sytox blue, blue), NET (Neutrophil Elastase, green). **C-F – The efferocytosis capacities of HMDMs are decreased during ARDS, spontaneously and furthermore after incubation with BAL fluid.** **C.** HMDMs were incubated with RPMI-FCS or BAL fluid for 3 hours before adding CFSE-labeled apoptotic neutrophils for 1 hour. Efferocytosis index was defined as the number of HMDMs which phagocytized

apoptotic neutrophils to the number of HMDMs which did not. **D.** Efferocytosis index of HMDMs from healthy donors (HD macrophages, n=5) or ARDS patients (ARDS macrophages, n=10). **E.** Efferocytosis index of HD macrophages cultured with BAL fluid from control (n=8) or ARDS patients (n=8). **F.** Efferocytosis index of ARDS macrophages cultured with BAL fluid from control (n=8) or ARDS patients (n=8). The Mann-Whitney test was used to compare NET engulfment and efferocytosis. *P < 0.05.

Figure 5. Inhibition of high-mobility group box (HMGB)1 and activation of adenosine 5'-monophosphate-activated protein kinase (AMPK) increase neutrophil extracellular trap (NET) engulfment and efferocytosis by human monocyte-derived macrophages (HMDMs).

A. BAL fluids from ARDS patients were treated for 2 hours with an anti-HMGB1 (α -HMGB1) or isotype antibody (α -IgY) before incubation with neutrophils from healthy donors (HD) or HMDMs from ARDS patients for 3 hours. **B.** NET production by HD neutrophils (n=8). **C.** HMDMs efferocytosis index has been determined after 1 hour contact with apoptotic neutrophils (n=6). Efferocytosis index was defined as the number of HMDMs which phagocytized apoptotic neutrophils to the number of HMDMs which did not. **D.** NET engulfment by HMDMs from ARDS patients (n=6) has been determined after 2 hours contact with neutrophil-derived NETs. NET engulfment ratio was defined as the ratio of fluorescence emitted by HMDMs which have phagocytized NETs to the fluorescent emitted by HMDMs alone. **E.** HMDMs from ARDS patients were incubated with BAL fluids from control (Ctrl BAL) or ARDS patients (ARDS BAL) for 3 hours before treated with or without metformin for 2 hours.

F. Representative Western blot and quantitative analysis of phosphor (p)-AMPK, total AMPK and actin from ARDS patient HMDMs incubated with BAL fluids. **G.** Representative Western blot and quantitative analysis of phosphor (p)-AMPK, total AMPK and actin from ARDS patient HMDMs incubated with ARDS BAL and metformin or medium alone (NT). **H.** HMDMs efferocytosis index determined after 1 hour contact with apoptotic neutrophils (n=7). **I.** Engulfment by HMDMs of Sytox blue-stained NETs, determined after 2 hours (n =8). The Wilcoxon test was used to compare effects of different HMGB1 or metformin treatment on NET engulfment and efferocytosis. * P < 0.05;; ns, non-significant.

Figure 1

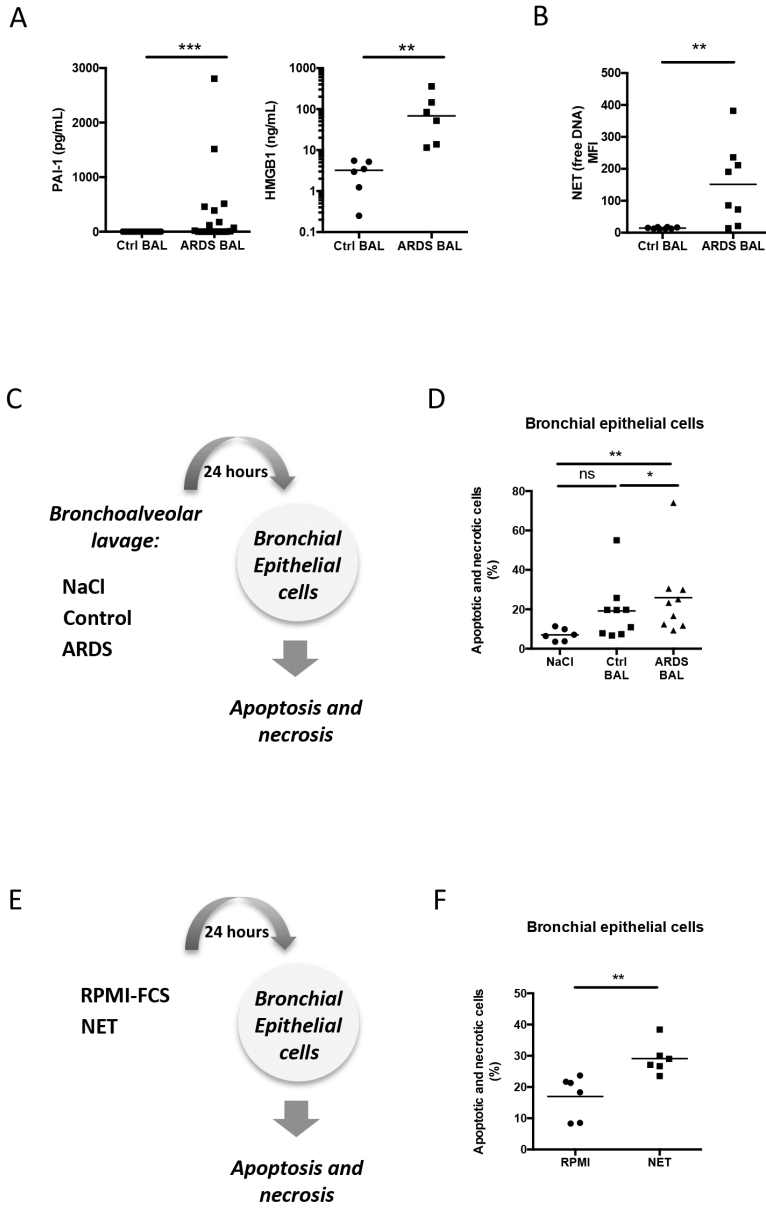


Figure 2

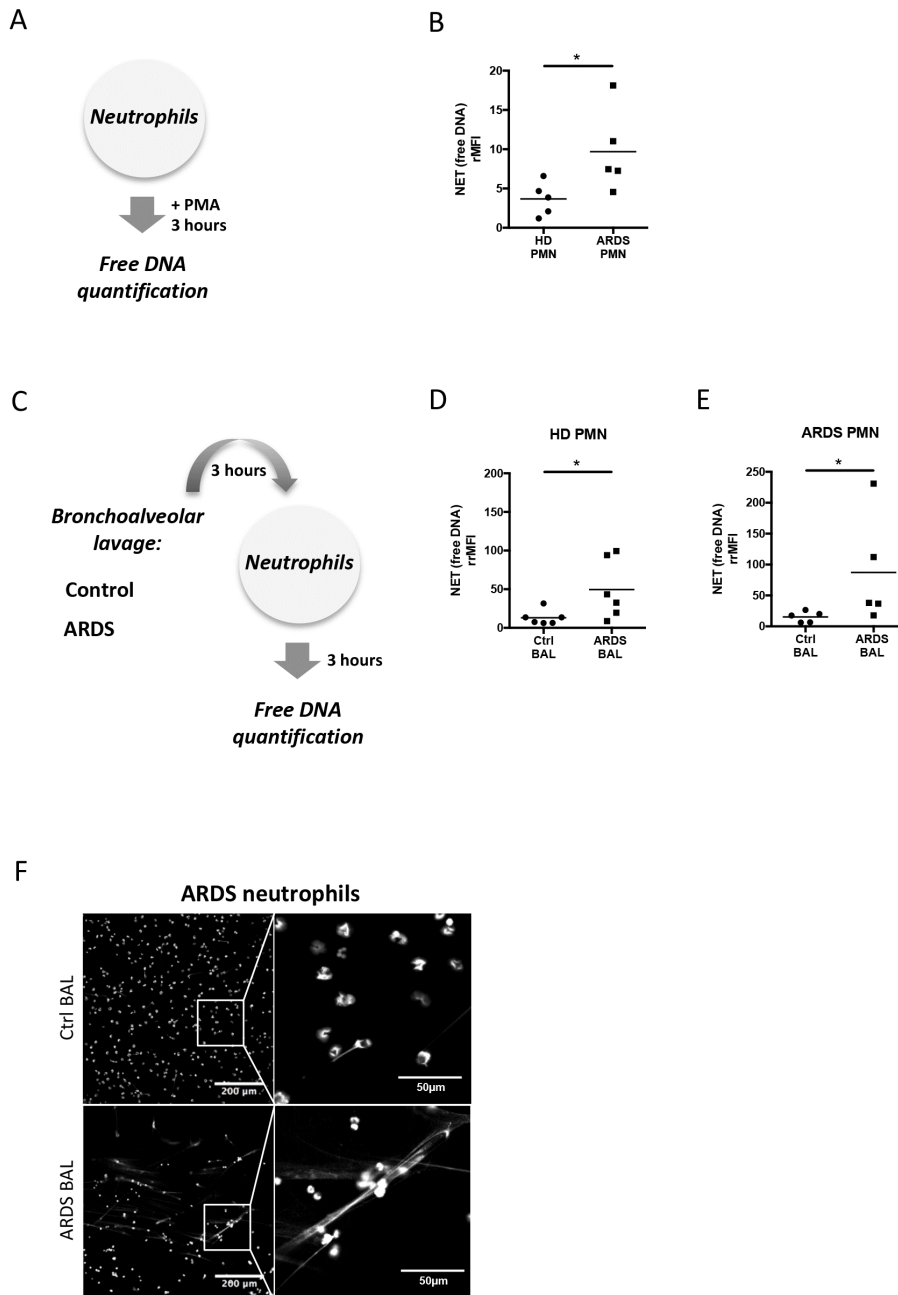
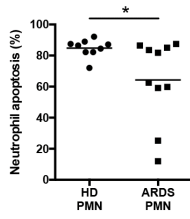
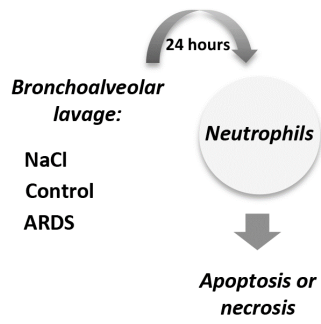


Figure 3

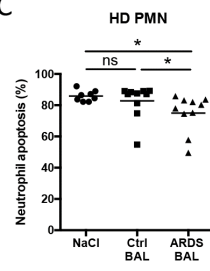
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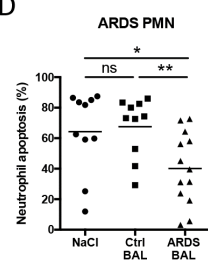
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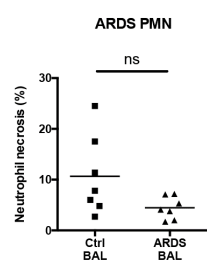


Figure 4

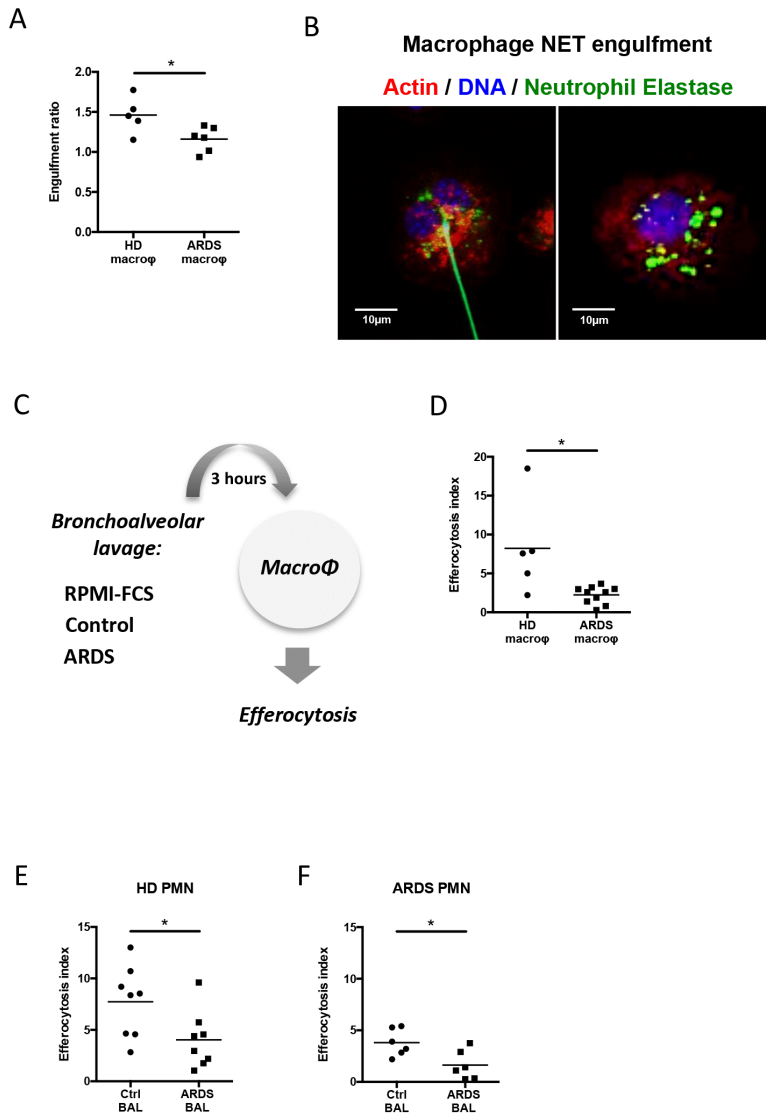
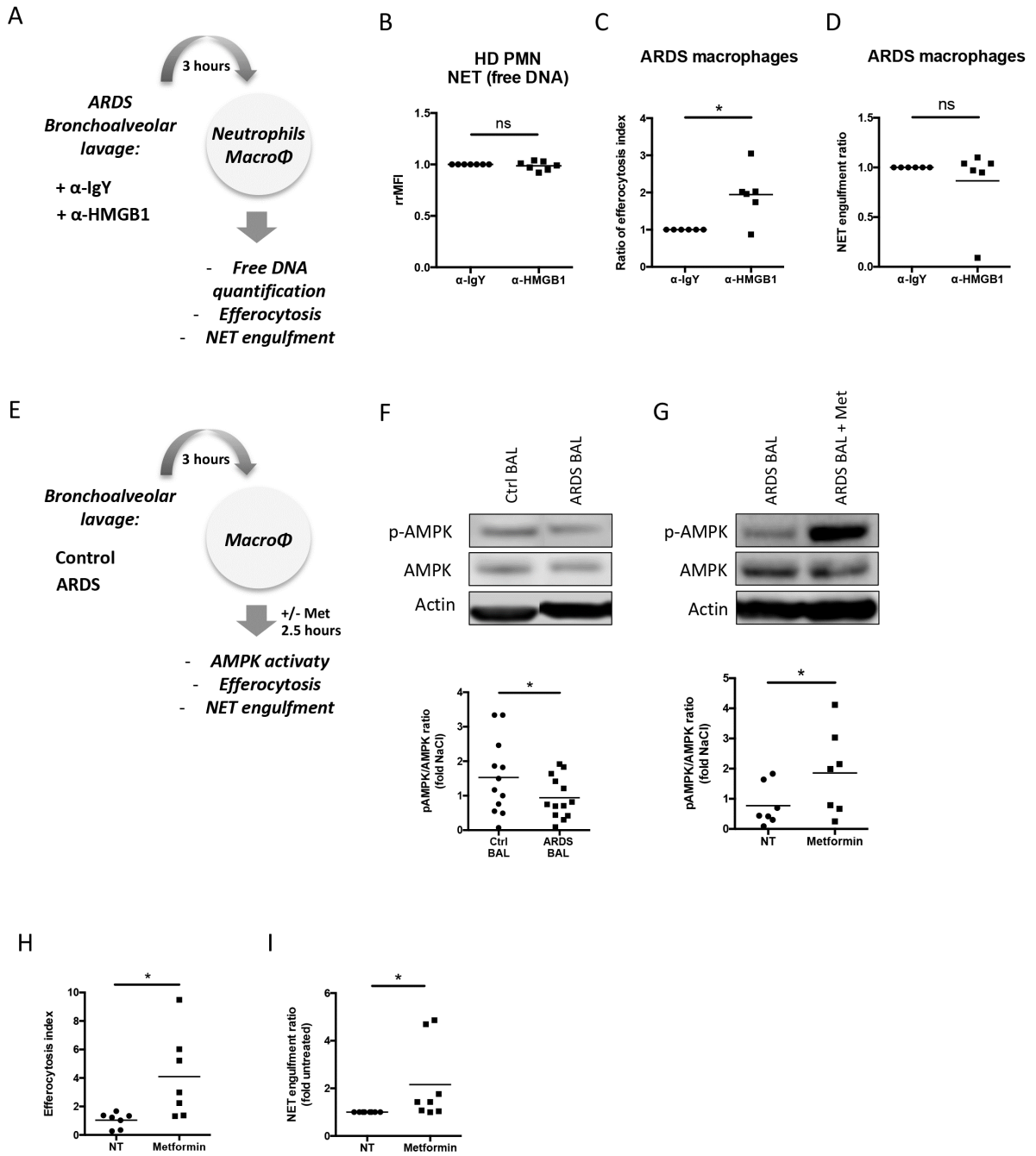
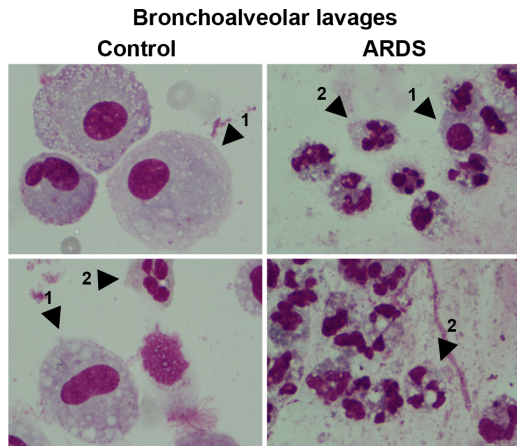


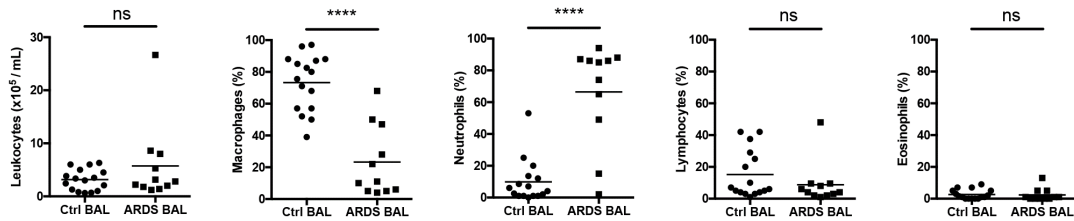
Figure 5



A



B



C

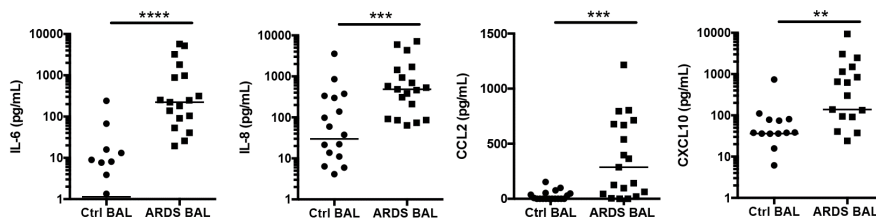


Figure S1. Characteristics of the bronchoalveolar lavages (BAL) from acute respiratory distress syndrome (ARDS) and control (Ctrl) patients.

A-C. BAL fluids from ARDS patients are characterized by a cellular and humoral inflammatory response. **A.** May-Grünwald-Giemsa-stained cytopsin slides of representative BAL from control (Ctrl) and ARDS patients. Arrow 1: macrophage; Arrow 2: neutrophil. **B.** Absolute cell count and differentials of BAL fluids from Ctrl (n=16) or ARDS (n=11). Analysis of bronchoalveolar lavage (BAL) fluids showed 5.74×10^5 cells/mL (IQR 1.24×10^5 /mL – 23.06×10^5 /mL) in BAL fluids of ARDS patients vs 3.15×10^5 cells/mL (IQR 0.68×10^5 /mL – 6.09×10^5 /mL) in BAL fluids of control patients. **C.** Quantification of IL-6, IL-8, CCL2 and CXCL10 (n=19) by ELISA in BAL fluid from Ctrl or ARDS patients.