T regulatory cells activation and distribution are modified in critically ill patients with acute respiratory distress syndrome: A prospective single-centre observational study

Sebastien Halter, Lucrèce Aimade, Michèle Barbié, Hélène Brisson, Jean-Jacques Rouby, Olivier Langeron, David Klatzmann, Michelle Rosenzwajg, Antoine Monsel

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Running Title:

Treg phenotype and distribution in ARDS

Authors:

Sebastien Halter[a,b,c], M.D., M.Sc; Lucrèce Aimade[b,c], M.Sc.; Michèle Barbié[b]; Hélène Brisson[a], M.D., M.Sc.; Jean-Jacques Rouby[a], M.D., Ph.D; Olivier Langeron[a], M.D., Ph.D; David Klatzmann[b,c], M.D., Ph.D; Michelle Rosenzwajg[b,c], M.D., Ph.D; Antoine Monsel[a,b,c,*], M.D., Ph.D.

[a] From the Multidisciplinary Intensive Care Unit, http://www.reapitie-univparis6.aphp.fr, Department of Anaesthesiology and Critical Care, La Pitié–Salpêtrière Hospital, pUBLIC Hospitals of Paris (APHP), Sorbonne University, Paris, France (S.H., H.B., O.L., J.-J.R., A.M.)

[b] From the Public Hospitals of Paris (APHP), La Pitié–Salpêtrière Hospital, Biotherapy (CIC-BTi) and Inflammation-Immunopathology-Biotherapy Department (i2B), F-75651, Paris, France (S.H., L.A., D.K., M.B., M.R, A.M.)

[c] From the Sorbonne University, INSERM, UMR_S 959, Immunology-Immunopathology-Immunotherapy (i3), F-75651, Paris, France (S.H., L.A., D.K., M.R, A.M.)

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***Corresponding author at:** Antoine Monsel, M.D., Ph.D. Polyvalent Critical Care Unit, Department of Anaesthesiology and Critical Care Medicine, Pitié–Salpêtrière Hospital Group, 47–83, boulevard de l'Hôpital, 75651 Paris Cedex 13, France. Tel: +33 (0)1 84 82 73 99; Fax: +33 (0)1 42 16 22 69. E-mail: antoine.monsel@gmail.com

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ABSTRACT (250)

Backgrounds: Acute respiratory distress syndrome (ARDS) is a common and fatal inflammatory condition. Whether T regulatory cells (Tregs) are beneficial or detrimental remains controversial, and longitudinal studies are lacking. Phenotyping of Tregs activation markers has been poorly reported. We aimed to evaluate quantitative and functional alterations in blood and bronchoalveolar Treg phenotype of ARDS patients.

Methods: We performed a single-centre observational study in a French intensive care unit. The study enrolled 60 ARDS and 45 non-ARDS patients. Patients under 18 years old or with immunosuppression (native or acquired) were excluded. Tregs phenotypes were assessed by flow cytometry, while cytokines were measured by multiplex-based assays in blood and bronchoalveolar samples collected over 3 weeks after the onset of ARDS.

Results: Blood Tregs/CD4+ percentage (median %, 25-75% interquartile) was higher in ARDS patients than in non-ARDS patients: 12.1% [9.0-16.0] *versus* 9.9% [8.1-12.6], p = 0.01. Alveolar Tregs/CD4+ percentage was lower in ARDS patients than in non-ARDS patients: 10.4% [6.3-16.6] *versus* 16.2% [12.4-21.1], p = 0.03. In ARDS patients, Tregs activation was reduced in the blood and increased in the alveolus, compared to non-ARDS patients. ROC analysis revealed a threshold of 10.4% for the Tregs/CD4+ percentage in the blood collected within the first week of ARDS to discriminate between survivors and non-survivors (sensitivity: 75%; specificity 76%; area under the curve [95% confidence interval]: 0.72 [0.5-0.9]).

Conclusions: Quantitative and functional alterations in Treg phenotype were observed in patients with ARDS. Whether rebalancing Tregs phenotype with therapeutic interventions would be beneficial deserves further investigations.

Keywords: acute respiratory distress syndrome; T-regulatory cells; immune phenotype; innate lymphoid cells; T helper polarisation.

1. Introduction

Acute respiratory distress syndrome (ARDS) is a life-threatening syndrome frequently encountered and a major cause of death in intensive care unit (ICU) [1]. ARDS still concerns 5-20% of patients under mechanical ventilation with a mortality rate of 40-60% [2, 3]. This syndrome is characterised by a dysregulated neutrophil infiltration leading to increased capillary and alveolar permeability. Extensive research testing anti-inflammatory therapies [4] has failed to improve ARDS outcomes.

Aside from neutrophils, other immune cells encompassing lymphocytes are involved in the pathophysiology of ARDS. More specifically, T-regulatory cells (Tregs), a subset of CD4 expressing lymphocytes that highly express CD25 (interleukin (IL)-2 receptor α) as well as the transcription factor Forkhead box protein 3 (FoxP3), have been recently demonstrated to play a crucial role in maintaining immune homeostasis [5, 6]. They can suppress inflammatory, allergic, and autoimmune disorders [7] including in pneumonia [8-10], through contact-dependent suppression or releasing cytokines, *e.g.* IL-10 and transforming growth factor (TGF)-β1 [6]. Several studies investigated the role of Tregs in either experimental models of acute lung injury or even more rarely in ARDS patients. Most of them yielded conflicting results [10-20]. In murine models, Tregs have been shown to contribute to the resolution of lung injury [11-14]. However, recent clinical studies presented Tregs as either a risk factor [10] or a protective factor [20] for mortality. Others yielded inconclusive results [16]. Moreover, their distribution over time between blood and lungs has never been studied in the clinical settings. Similarly, Tregs expression of immunosuppressive markers remains uncovered in patients with ARDS. Finally, phenotyping of T helper (Th) polarisation and innate lymphoid cells (ILCs) populations has been under-studied in ARDS patients [15, 20].

In this context, and since ARDS is characterised by a dysregulated immune response, we hypothesised that either quantitative or functional Tregs impairment may occur over time and could alter outcomes. Thus, we designed this observational longitudinal clinical study to

investigate both systemic and bronchoalveolar Tregs phenotype over time, and its potential impact on the mortality of patients with ARDS.

2. Patients and Methods

2.1. Study design

This prospective observational study was conducted in our 26-bed multidisciplinary ICU from April 2014 to July 2018. The study was approved by the Institutional Ethics Committee *Île-de-France VI*. Blood and bronchoalveolar lavage (BAL) samples for flow cytometric and cytokines analyses were collected from residual sampling after completion of routine biological and bacteriological follow-up. Because our protocol did not modify usual patient care, the Institutional Review Board waived written informed consent. Patients and/or their relatives received written information.

2.2. ARDS group

Patients admitted to our ICU were considered eligible if they met the Berlin criteria for ARDS [21], and had no previous history of ARDS. Patients under 18 years old, with immunosuppressive therapy or previously characterised immune alterations were excluded.

The duration of patient follow-up was at least 90 days. Clinical and demographic data were calculated and reported over the 24 hours after ARDS diagnosis. The characterisation of focal versus non-focal ARDS was based on lung ultrasounds [22]. Primary ARDS was defined as direct lung injury, such as pneumonia, lung contusion or aspiration.

Intensive care management of patients included in the study was conducted using available guidelines [23, 24].

2.3. Non-ARDS group

Mechanically ventilated patients requiring BAL sampling in the context of standard care or routine ventilator-associated pneumonia (VAP) screening, were enrolled in the non-ARDS group if they did not meet the Berlin criteria for ARDS and had no previous history of ARDS. Patients under 18 years old, with immunosuppressive therapy or previously characterised immune alterations were excluded.

2.4. Blood samples

Blood samples were collected at T1 during the first week (exudative/inflammatory phase), T2 during the second week (resolving phase), and T3 after the second week (fibroproliferative phase) of ARDS. Serum was obtained after centrifugation and stored at -80°C until used.

2.5. Bronchoalveolar lavage samples

In ARDS patients, BAL was performed according to Guidelines [25] for diagnostic workup at T1, and repeated for routine VAP screening at T2 and T3, unless the patient had died, did not require further infectious diagnostics, was extubated or was discharged from the ICU. After centrifugation, BAL supernatants were stored at -80°C until used (cytokines measurements), and isolated mononuclear cells from BAL samples were washed, counted, and resuspended.

2.6. Antibodies

The antibodies used are listed in the supplemental table 1.

2.7. Flow cytometric analysis of T regulatory phenotypes from blood and bronchoalveolar lavage samples

Immunostaining was performed on either 100 µl of whole blood sample, or on 50 µl of mononuclear cells (2 x 10^5 to 2 x 10^6 cells in 50 µl) derived from BAL sample using the PerFix-nc kit (Beckman Coulter, Villepinte, France) [26, 27]. Samples were acquired on a Navios cytometre software (Beckman Coulter, Villepinte, France), and analysed through Kaluza software (Beckman Coulter, Villepinte, France).

The first panel was designed to gate on Tregs (defined as $CD4^+FoxP3^+CD25^+CD127^{lo/-}$ cells) [26-28]. The second and third panels aimed to investigate the functionality of Tregs. Functional/activation markers chosen in those panels were LAP, LAG-3, GITR, CTLA-4, CD45RA, and HLA-DR. The Figure 1 represents the gating strategy determining the Tregs

phenotypes in blood and BAL samples. Effector $CD4^+$ T cells were defined as $FoxP3^-$ cells among $CD4^+$ T cells.

2.8. Flow cytometric analysis of T helper polarisation and innate lymphoid cells from blood samples

In the T helper (Th) polarisation panel, antibodies directed against chemokine receptors (CCR6, CCR10, CCR4, and CXCR3) were used to discriminate Th1 ($CXCR3^+CCR4^-CCR6^-CCR10^-$), Th2 ($CXCR3^-CCR4^+CCR6^-CCR10^-$), Th17 ($CXCR3^-CCR4^+CCR6^+CCR10^-$), and Th22 ($CCR4^+CCR6^+CXCR3^-CCR10^+$) subsets [26, 27, 29]**.** For ILCs phenotyping we used, anti-lineage, -NKp44, -CD117, -CD127, and -CD294 antibodies. Samples were then fixed and red blood cells were lysed. Samples were acquired on a Galios cytometre software (Beckman Coulter, Villepinte, France), and analysed through Kaluza software (Beckman Coulter, Villepinte, France).

2.9. Blood and Bronchoalveolar lavage cytokine measurements

Quantitative determination of 25 cytokines/chemokines was performed in blood and BAL samples collected at the time of inclusion in the non-ARDS group, or within the first 48 hours of ARDS using Human Milliplex HCYTOMAG-60 kits-25 (Millipore, Saint-Quentin-en-Yvelines, France) according to the manufacturer's recommendations. As BAL dilution differences may have existed, we normalised the amount of cytokines to the amount of protein measured in each alveolar sample using a bicinchoninic acid assay.

2. 10. Statistical analyses

Normality of the distribution was verified by the Shapiro-Wilk test. Results are expressed as mean ± standard deviation (SD) if the data were normally distributed and median [25-75^{th}] interquartile range (IQR) if not. Categorical variables are described as frequencies (%). Comparisons of variables between 3 groups were made by Kruskal-Wallis analysis followed by pair-wise comparisons using Dunn's method. Comparison between two groups was made

by Student *t*-test or Mann-Whitney *U* test. All *p* values were two-tailed, and a value of $p<0.05$ was considered statistically significant. All statistical analysis was performed using GraphPadPrism software (La Jolla, CA, USA).

3. Results

3.1. Patients

From April 2014 to July 2018, 105 critically ill patients were included, 60 with and 45 without ARDS criteria. Figure 2 represents the flow chart of the study. Clinical characteristics of ARDS and non-ARDS patients are reported in the Table 1. Patients with ARDS exhibited higher rate of sepsis, antibiotic use, pneumonia, shock and SOFA score. However, both groups remained with similar simplified acute physiology score and no difference was found between groups regarding cardiovascular, respiratory comorbidities or immune status. The table 2 reports the respiratory characteristics of ARDS patients, and the supplemental table 2 their evolution over time.

3.2. Blood and bronchoalveolar cytokines/chemokines concentrations at the onset of ARDS

Results of the cytokine assays are provided in the table 3. Plasma concentrations of IL-8 (CXCL8), IL-6, MCP-1 (CCL2), IL-10 and tumour necrosis factor (TNF)-alpha were respectively, 76-, 26-, 5-, 4- and 2.5-fold higher in ARDS patients than in non-ARDS patients, suggesting a specific ARDS signature consistent with that described in the literature [30-32]. Plasma concentrations of interferon (IFN)-gamma, IL-12p70, IL-1a, IL-9 and IL-7 were also lower in ARDS patients compared to non-ARDS patients**.** In BAL, cytokines/chemokines concentrations were not different between ARDS and non-ARDS patients.

3.3. Proportion of blood and alveolar Tregs and their variation over time

In blood, Tregs/$CD4^+$ percentage increased in ARDS patients compared to non-ARDS patients (12.1% [9.0-16.0] *versus* 9.9% [8.1-12.6], for ARDS and control patients respectively, p=0.01, Fig. 3A). In BAL, Tregs/$CD4^+$ percentage decreased in ARDS patients compared to non-ARDS patients (10.4% [6.3-16.6] *versus* 16.2% [12.4-21.1], for ARDS and control patients respectively, p=0.03, Fig. 3A). Although the improvement in respiratory parameters

suggested a recovery of ARDS between T1 and T3 (supplemental table 2), plasma and BAL Tregs/$CD4^+$ percentage did not vary over time (Fig. 3B).

Immunophenotyping data for circulating total lymphocytes, T, B and natural killer (NK) cells populations in ARDS patients are summarised in the tables 4 and 5. The results showed that patients with ARDS had lymphopenia, and that all $CD4^+$ T, $CD8^+$ T, B and NK lymphocyte subpopulations were affected (table 4). Results of absolute count of total circulating blood lymphocytes, $CD4^+$ lymphocytes and Tregs remained similar between ARDS and non-ARDS patients (table 5).

3.4. Comparison of the proportion of blood Tregs expressing functionality/activation markers between ARDS and non-ARDS patients

Compared to that of non-ARDS patients, the proportion of blood Tregs expressing functional markers in ARDS patients was significantly reduced by 29% for LAP+, 63% for GITR+, 76% for LAG-3+, and 24% for HLA-DR+ (Fig. 4). In contrast, the proportion of blood Tregs expressing CTLA-4, and CD-45RA did not differ between ARDS and non-ARDS patients (Fig. 4).

3.5. Comparison of the proportion of alveolar Tregs expressing functionality/activation markers between ARDS and non-ARDS patients

While the proportion of alveolar Tregs expressing LAP was 3.3 times higher in ARDS patients than in non-ARDS patients, the proportion of alveolar Tregs expressing the other activation markers did not differ between the 2 groups (Fig. 4).

3.6. Distribution of Tregs and activated Tregs according to blood and alveolar compartments

In ARDS patients, Tregs/$CD4^+$ percentage was similar in blood and BAL. In non-ARDS patients, Tregs/$CD4^+$ percentage was 60% greater in BAL than in blood (Fig. 3A). In ARDS patients, except for CD45RA and CTLA-4, all immunosuppressive function markers of Tregs

showed a stronger representation in terms of activated Tregs in the alveolus than in blood (Fig. 4). Indeed, the results indicated proportions multiplied by 5.2, 10 and 4.3 for the % of activated alveolar Tregs expressing LAP, GITR, and LAG-3 respectively. Similarly, the proportion of Tregs expressing HLA-DR was increased by 28% in the alveolus compared to blood in ARDS patients.

In contrast, in non-ARDS patients, the only increase in the proportion of activated Tregs in the alveolus compared to blood was that of GITR+ Tregs (Fig. 4).

3.7. Teffs expressing activation markers and their relative expression of activation markers compared to Tregs

Those results are presented in the supplemental tables 3 and 4. We found a significant reduction in the proportions of blood Teff expressing LAP, LAG-3 and HLA-DR in ARDS patients compared to non-ARDS patients. Conversely, in BAL, the proportion of activated Teff expressing LAP was higher in ARDS patients than in non-ARDS patients (supplemental table 3).

As observed with Tregs, the proportion of activated Teffs was generally higher in the alveolar compartment than in the blood, accompanied by a collapse in the proportion of naive CD45RA+ Teffs. This was as true for patients with ARDS as it was for non-ARDS patients (supplemental table 4).

In both ARDS and non-ARDS patients, the proportion of activated blood and alveolar Tregs was from 1.5- to 10-fold higher than that of Teffs (supplemental table 4).

3.8. T helper polarisation and innate lymphoid cells phenotypes in blood compartment

Apart from a more pronounced Th22 polarisation of circulating $CD4^+$ T lymphocytes in ARDS patients *versus* non-ARDS patients, no differences were observed for other types of Th polarizations as well as for ILCs populations (Fig. 5).

3.9. Blood Tregs/$CD4^+$ percentage, mortality and ARDS phenotypes

Blood Tregs/CD4$^+$ percentage at T1 differed significantly between ARDS survivors and non-survivors on day 28 and 90 (Fig. 6, A and B). ROC analysis revealed a cutoff of 10.4% for Tregs/CD4$^+$ percentage to discriminate between survivors and non-survivors (sensitivity: 75%; specificity 76 %; area under the curve [95% confidence interval]: 0.72 [0.5-0.9]). Ninety-day survival was 83% for patients with Tregs/CD4$^+$ percentage equal to or greater than 10.5%, but only 41% for patients with Tregs/CD4$^+$ percentage less than 10.5% (p=0.01, Fig. 6C). Among age, SAPS II, SOFA, P_aO2/FiO_2 and the presence of shock, the SOFA score was higher in non-survivors than in survivors (14 [12.2-17.5] *versus* 10 [9-12]), respectively; p=0.02).

Blood Tregs/CD4$^+$ percentage did not differ according to the ARDS cause or morphotype (Fig.6, D—F).

4. Discussion

Our main findings are summarised as follows: (1) ARDS was characterised by an expansion of circulating Tregs whose functional phenotype was altered, an absence of a blood-alveolar gradient in Tregs subset that existed in non-ARDS patients, and a reduction in BAL Tregs, which were paradoxically hyperactivated. (2) The proportions of blood and BAL Tregs remained stable over time. (3) Blood Tregs/$CD4^+$ percentage < 10.5% within the first week of ARDS was associated with a threefold increase in day-90 mortality.

We assessed activation and distribution of Tregs in systemic and BAL compartments in ARDS patients. D'Alessio et al. were the first to set out Tregs-induced resolution of acute lung injury as a bedrock concept [11-14], and to investigate the presence of Tregs in BAL and blood samples collected from 3 patients with ARDS [13]. In ARDS patients, Adamzik et al. identified the BAL Tregs/$CD4^+$ percentage collected at admission, as an independent risk factor for 30-day mortality [10]. However, it is important to note the small sample size of their control group (n=8) made of patients undergoing surgery for progressing cancer, and that all ARDS patients were treated with hydrocortisone, two factors impacting immunity and Tregs proportion. Furthermore, we used a more stringent Tregs gating strategy combining four specific markers including CD127 ($CD4^+FoxP3^+CD25^+CD127^{lo/-}$ cells), which was not the case in their study. Finally, time variation in BAL and blood Tregs phenotype was not evaluated. Although our results showed a link between blood Tregs/$CD4^+$ percentage and ARDS mortality, the design and sample size of our study do not allow concluding that ARDS mortality is caused by a Tregs deficit. A second clinical study, more in line with our results, showed that a higher Th17/Treg percentage in blood was associated with poorer prognosis [20]. In a third study, Risso et al. did not find any difference in Tregs subsets between ARDS patients and controls [16]. Aside from a limited sample-size, this study evaluated Tregs proportion only in BAL samples in the early phase of ARDS. Finally, Ronit et al. evaluated the change in BAL Tregs proportion over time after lipopolysaccharide intratracheal instillation in 15 healthy volunteers [17]. Despite a restricted sample size, they showed an increase in Treg subset after initiation of lung injury. The fact that no blood sampling was

done, that they focused on early time points, and that this model led to a mild lung injury, might have limited the conclusion of the study.

One original finding of our study is the bidirectional changes in Treg phenotype depending on the compartment studied, *i.e.* a decrease in BAL Treg subset contrasting with an increase in systemic Treg subset. These findings are in line with studies reporting opposite inflammatory status simultaneously occurring in lung and blood [33]. Other acute inflammation-induced organ injuries were demonstrated to be accompanied with simultaneous systemic immunosuppression [34-36]. It remains to be clarified which compartment-related status constitutes a counterbalancing response to the other. Why this extra- subset of systemic Tregs does not migrate into the alveolar space remains to be unveiled. Since Treg homing were demonstrated to be crucial in resolving inflammation [37, 38] and even altered in some inflammatory diseases [39-41], we can speculate that there might be an alteration in the homing capacities of the blood Tregs to the damaged alveolus of the ARDS. Further studies are warranted to investigate this hypothesis. Our data, obtained in ARDS patients, suggested an increase in BAL activated Tregs expressing immunosuppressive markers. Whether this overexpression constitutes a compensatory response to the uncontrolled lung inflammation aggravated by a shortage of Tregs remains to be evaluated.

Concerning the absence of difference in alveolar cytokines between patients with and without ARDS, our main hypothesis is that the high rate of pneumonia in the non-ARDS could have explained the presence of alveolar inflammation found in patients without ARDS.

Considering the emergence of an altered Treg phenotype in ARDS, several therapeutic strategies are under development [42]. Numerous clinical trials are underway to either evaluate Treg-based cell therapy, or testing low-dose IL-2 in transplantation or in autoimmune diseases [42].

The current study has some limitations. (1) Tregs and cytokines/chemokines analyses were performed on BAL indicated for clinical follow-up of patients with or without ARDS. Residual sampling remaining after completion of routine bacteriological screening served for

the analyses. Therefore, only a small number of BAL was recovered for Tregs assessment, precluding to draw definitive conclusions concerning their variation over time and the link between BAL Tregs and ARDS-related mortality and morphotypes. (2) Critically ill patients without ARDS criteria served as control group. We therefore did not compare ARDS patients to patients with normal lungs. Although the two groups differed in terms of incidence of shock, sepsis and pneumonia, the cytokine signature of the ARDS group remained consistent with that described in the literature [30-32]. (3) Tregs functional or homing capacity assays are lacking. Tregs impact on macrophages polarisation would be worth investigating. (4) Analyses of Tregs tracking or pulmonary interstitium could not be performed given the clinical context. Those questions need to be further investigated. (5) These results, particularly those linking the Treg phenotype to ARDS mortality, have yet to be corroborated in larger multi-centre studies.

CONCLUSIONS

In conclusion, ARDS was characterised by an increase in the pool of blood Tregs exhibiting an activation defect, which potentially hindered their translation within the bronchoalveolar compartment. This led to a quantitative deficit in the pool of alveolar Tregs, and a compensating hyperactivation of the alveolar Tregs and Teffs. Recognition of the role of the modifications of immune phenotypes in initiating and perpetuating lung inflammation may offer new therapeutic opportunities. Increasing Treg subsets, improving their immunosuppressive or homing capacities to the inflamed lung might allow early and persistent control of the ARDS-related inflammatory dysregulation.

LIST OF ABBREVIATIONS

ARDS: acute respiratory distress syndrome; BAL: bronchoalveolar lavage; CD: cluster of differentiation; CTLA-4: cytotoxic T-Lymphocyte-associated protein-4; FoxP3: Forkhead box protein 3; GITR: glucocorticoid-induced TNFR-related protein; HLA: human leukocytes antigen; ICU: intensive care unit; IL: interleukin; ILCs: innate lymphoid cells; IFN: interferon; IQR: interquartile range; LAG: lymphocyte activation gene-3; LAP: latency-associated peptide; NK: natural killer cells; ROC: receiver operating characteristic; SAPS 2: simplified acute physiology score 2; SD: standard deviation; SOFA: sequential organ failure assessment; Teff: effector CD4+ T cells; TGF-β1: transforming growth factor; Th: T helper cells; TNF: tumour necrosis factor; Tregs: T regulatory cells; VAP: ventilator-associated pneumonia;

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FIGURE LEGENDS

Figure 1. Gating of T regulatory cells strategy. Whole blood sample or mononuclear cells isolated from bronchoalveolar lavage samples were fixed, permeabilised and stained with combined conjugated antibodies. The presented gating strategy allowed us to determine percentage of T regulatory cells (Tregs) subset among $CD4^+$ cells (Tregs/$CD4^+$ percentage) and the percentage of activated Tregs among Tregs subset. CD = cluster of differentiation; CTLA-4 = cytotoxic T-Lymphocyte-associated protein-4; FoxP3 = transcription factor Forkhead box protein 3; GITR = glucocorticoid-induced TNFR-related protein; HLA-DR = human leucocyte antigen D-related; LAG-3 = lymphocyte activation gene-3; LAP = latency-associated peptide; SS = size scatter; Treg = T regulatory cells.

Figure 2. Study flowchart. Among 112 patients assessed for eligibility, 7 were excluded due to the use of immunosuppressive drugs. Finally, 105 patients were included with 60 patients in the ARDS group, and 45 patients in the non-ARDS group.

Figure 3. Spatial and temporal distribution of the proportion of T regulatory cells in ARDS and non-ARDS patients. A, Distribution of blood and bronchoalveolar lavage (BAL) T regulatory cells (Tregs) proportion among CD4+ cells (Tregs/CD4+ percentage) in acute respiratory distress syndrome (ARDS) patients and non-ARDS patients. While the proportion of blood Tregs was significantly increased in patients with ARDS, it was reduced in the BAL of ARDS patients compared to non-ARDS patients. Whereas there was no blood-alveolar gradient for patients with ARDS, the proportion of alveolar Tregs was higher than that in the blood in the non-ARDS group. Blood samples (combined data from all times points: T1, T2 and T3): n = 114 and 29 for ARDS and non-ARDS patients; BAL samples (combined data from all times points: T1, T2 and T3): n = 31 and 16 for ARDS and non-ARDS patients. $^{\#}p$ = 0.01 *versus* blood Tregs/CD4+ percentage in non-ARDS group, $^{\dagger}p$ = 0.03 *versus* BAL

Tregs/CD4+ percentage in non-ARDS patients by Mann-Whitney *U* test. *$p < 10^{-4}$ by Mann-Whitney *U* test. **B**, Tregs/CD4+ percentage change over time in patients with ARDS. T1 (first time point) = during the first week (exudative/inflammatory phase); T2 (second time point) = during the second week (resolving phase) of ARDS; T3 (third time point) = after the second week (fibroproliferative phase) of ARDS. No significant variation over time was detected in the blood and BAL Tregs/CD4+ percentage in ARDS patients. Box plots of Tregs/CD4+ percentage (% of CD4+ cells): internal *horizontal lines* are the medians, *lower* and *upper box limits* are the 25–75th interquartile range, respectively, *T* bars are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are drawn as individual points. *Light grey* is used for ARDS group; *dark grey* is used for non-ARDS group. ARDS = acute respiratory distress syndrome; BAL = bronchoalveolar lavage; CD = cluster of differentiation; Treg = T regulatory cells.

Figure 4. Distribution of activated blood and alveolar T regulatory cells in critically ill patients with or without acute respiratory distress syndrome. The proportion of activated T regulatory cells (Tregs) was found to be reduced in the blood of acute respiratory distress syndrome (ARDS) patients *versus* non-ARDS patients. Conversely, in bronchoalveolar lavage (BAL), the proportion of activated Tregs expressing latency-associated peptide (LAP) was higher in ARDS patients than in non-ARDS patients. In addition, the proportion of activated alveolar Tregs was generally higher than that in the blood of ARDS patients, whereas this was not the case for non-ARDS patients. Blood samples (combined data from all times points: T1, T2 and T3): *n* = 108 — 109 and 28 for ARDS and non-ARDS patients; BAL samples (combined data from all times points: T1, T2 and T3): *n* = 23 and 8 — 9 for ARDS and non-ARDS patients. $^{\#}p < 0.05$ *versus* blood Tregs in non-ARDS group, $^{\dagger}p = 0.02$ *versus* alveolar Tregs in non-ARDS group by Mann-Whitney *U* test. *$p < 0.05$ by Mann-Whitney *U* test. Box plots of Tregs/CD4+ percentage (% of CD4+ cells): internal *horizontal lines* are the medians, *lower* and *upper box limits* are the 25–75th

interquartile range, respectively, *T* bars are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are drawn as individual points. *Light grey* is used for ARDS group; *dark grey* is used for non-ARDS group. ARDS = acute respiratory distress syndrome; BAL = bronchoalveolar lavage; CD = cluster of differentiation; CTLA4 = cytotoxic T-Lymphocyte-associated protein-4; GITR = glucocorticoid-induced TNFR-related protein; HLA-DR = human leucocyte antigen D-related; LAG-3 = lymphocyte activation gene-3; LAP = latency-associated peptide; Treg = T regulatory cells.

Figure 5. T helper polarisation and innate lymphoid cells phenotypes in blood compartment. Apart from a more pronounced T helper (Th) 22 polarisation of circulating CD4+ T lymphocytes in acute respiratory distress syndrome (ARDS) patients *versus* non-ARDS patients, no differences were observed for other types of Th polarisations as well as for innate lymphoid cells (ILCs) populations. Blood samples for Th polarisation and ILCs (combined data from all time points: T1, T2 and T3): n = 114 and 29 for ARDS and non-ARDS patients; *p = 0.04 by Mann-Whitney U test. Box plots of proportion of Th cells or ILCs (% of CD4+ cells or of ILCs): internal *horizontal lines* are the medians, *lower* and *upper box limits* are the 25–75th interquartile range, respectively, *T* bars are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are drawn as individual points. *Light grey* is used for ARDS group; *dark grey* is used for non-ARDS group. ARDS = acute respiratory distress syndrome; CD = cluster of differentiation; ILCs = innate lymphoid cells; Th = T helper cells.

Figure 6. Proportion of blood T regulatory cells, mortality, and phenotype of acute respiratory distress syndrome.

A and B, The proportion of blood T regulatory cells (Tregs) among CD4+ cells (Tregs/CD4+ percentage) collected within the first week of ARDS differed significantly between survivors and non-survivors on day 28 and 90. n = 12 and n = 37 for non-survivors and survivors at day 28; n = 14 and n = 24 for non-survivors and survivors at day 90. *p < 0.05 (Mann-

Whitney U test). **C**, Ninety-day survival was 83% for patients with a blood Tregs/CD4+ percentage ≥ 10.5% and 41% for patients with blood Tregs/CD4+ percentage < 10.5%. p = 0.01 by Log-rank (Mantel-Cox) test. **D — F**, Blood Tregs/CD4+ percentage did not differ according to the cause or the morphotype of ARDS. n = 41 and n = 10 for primary and secondary ARDS; n = 32 and n = 19 for pneumonia (PN) and non-pneumonia (no-PN) ARDS; n = 7 and n = 44 for focal and non-focal ARDS. Box plots of Tregs/CD4+ percentage (% of CD4+ cells): internal *horizontal lines* are the medians, *lower* and *upper box limits* are the 25–75th interquartile range, respectively, *T* bars are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are drawn as individual points. CD = cluster of differentiation; D28 = day 28; D90 = day 90; PN = pneumonia; Treg = T regulatory cells.

Figure 1

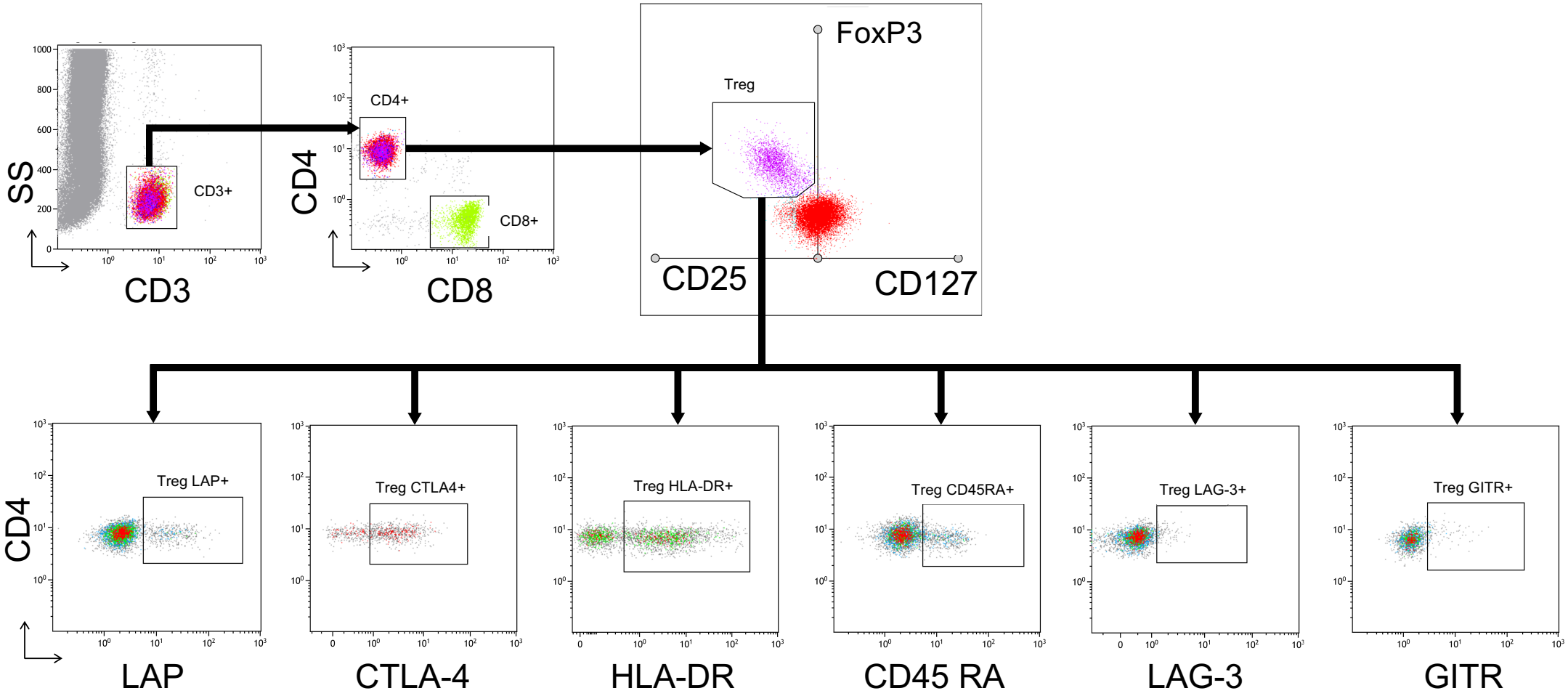

SS
CD3
CD3+
CD4
CD8
CD4+
CD8+
FoxP3
Treg
CD25
CD127
CD4
LAP
Treg LAP+
CTLA-4
Treg CTLA4+
HLA-DR
Treg HLA-DR+
CD45 RA
Treg CD45RA+
LAG-3
Treg LAG-3+
GITR
Treg GITR+

Figure 2

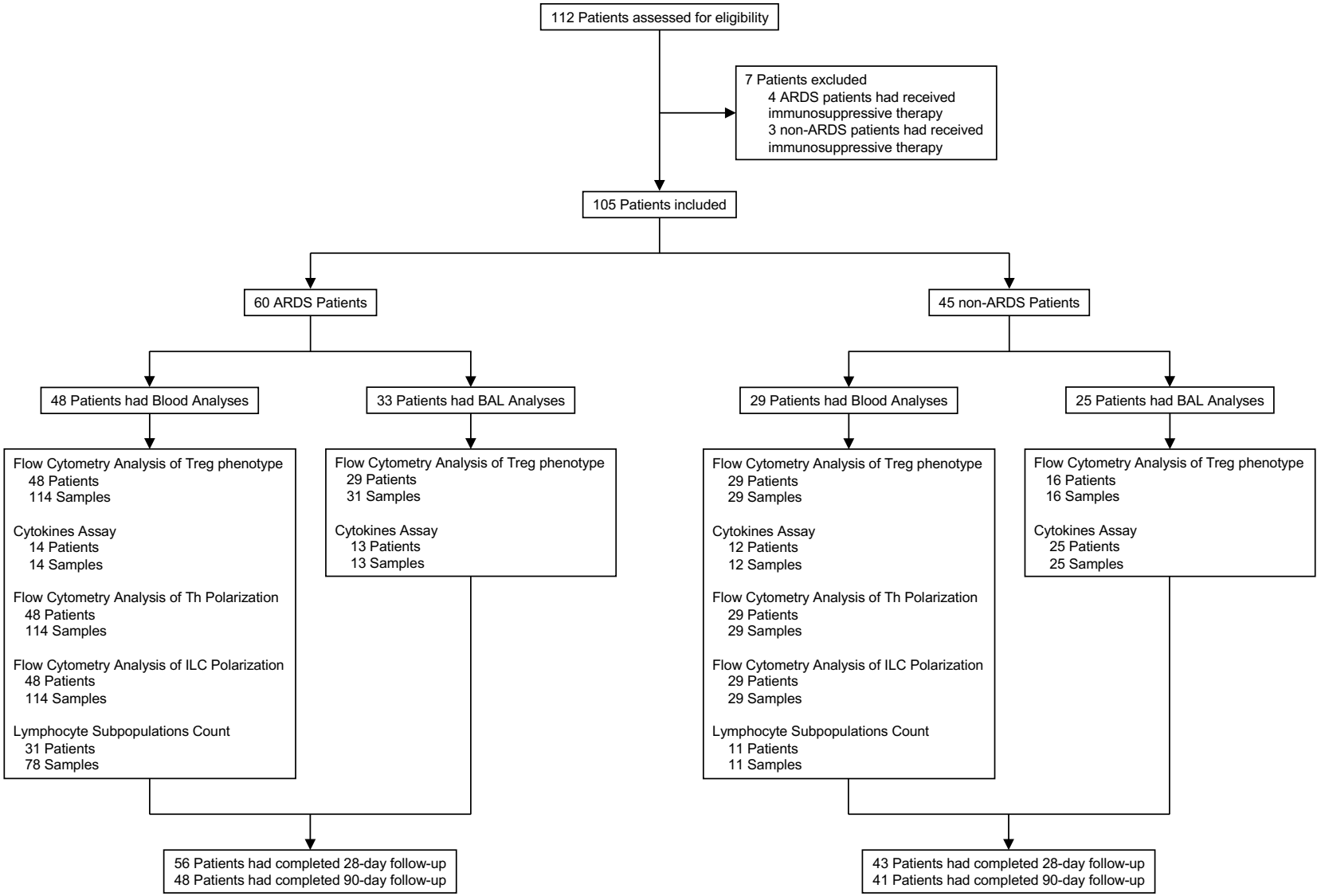
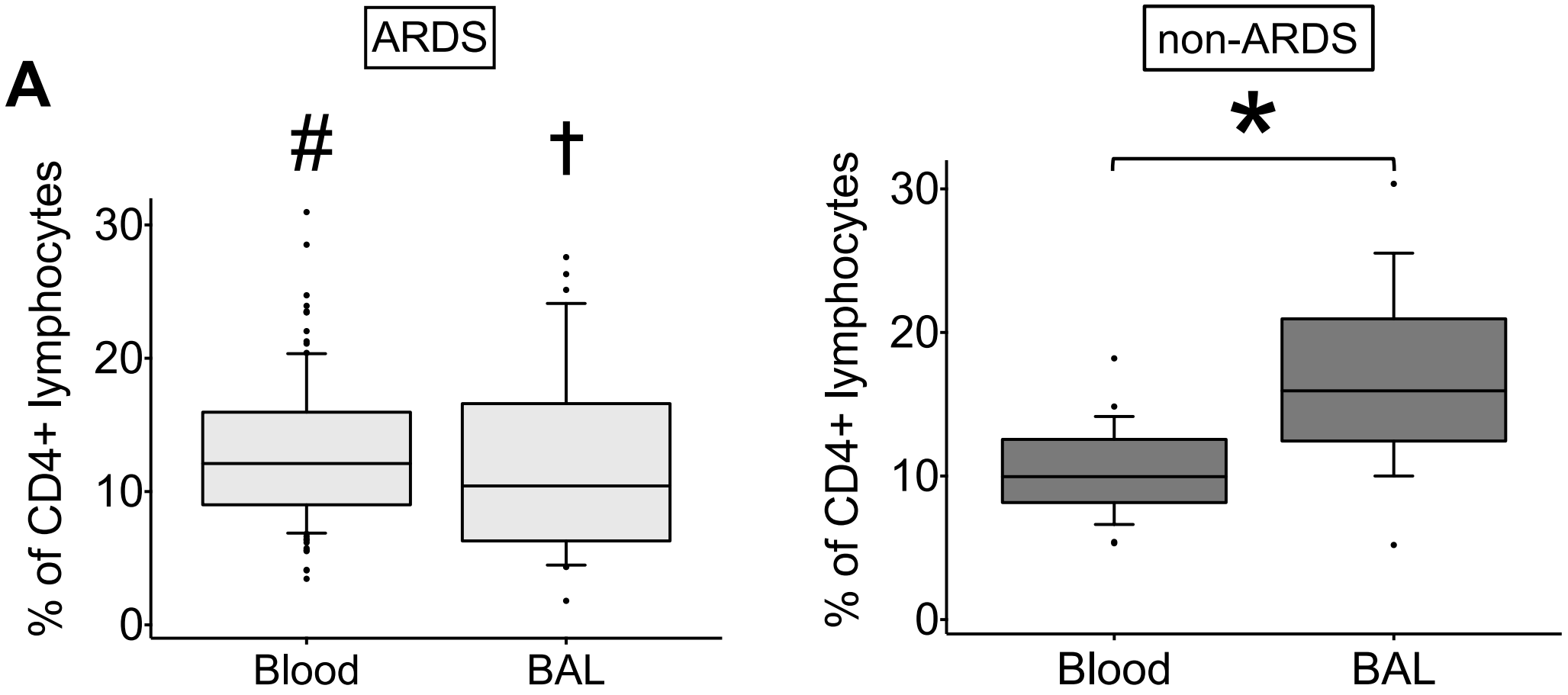

112 Patients assessed for eligibility
7 Patients excluded
4 ARDS patients had received immunosuppressive therapy
3 non-ARDS patients had received immunosuppressive therapy
105 Patients included
60 ARDS Patients
45 non-ARDS Patients
48 Patients had Blood Analyses
33 Patients had BAL Analyses
29 Patients had Blood Analyses
25 Patients had BAL Analyses
Flow Cytometry Analysis of Treg phenotype
48 Patients
114 Samples
Cytokines Assay
14 Patients
14 Samples
Flow Cytometry Analysis of Th Polarization
48 Patients
114 Samples
Flow Cytometry Analysis of ILC Polarization
48 Patients
114 Samples
Lymphocyte Subpopulations Count
31 Patients
78 Samples
Flow Cytometry Analysis of Treg phenotype
29 Patients
31 Samples
Cytokines Assay
13 Patients
13 Samples
Flow Cytometry Analysis of Treg phenotype
29 Patients
29 Samples
Cytokines Assay
12 Patients
12 Samples
Flow Cytometry Analysis of Th Polarization
29 Patients
29 Samples
Flow Cytometry Analysis of ILC Polarization
29 Patients
29 Samples
Lymphocyte Subpopulations Count
11 Patients
11 Samples
Flow Cytometry Analysis of Treg phenotype
16 Patients
16 Samples
Cytokines Assay
25 Patients
25 Samples
56 Patients had completed 28-day follow-up
48 Patients had completed 90-day follow-up
43 Patients had completed 28-day follow-up
41 Patients had completed 90-day follow-up

Figure 3A


A
ARDS
#
†
% of CD4+ lymphocytes
30
20
10
0
Blood
BAL
non-ARDS
*
% of CD4+ lymphocytes
30
20
10
0
Blood
BAL

B

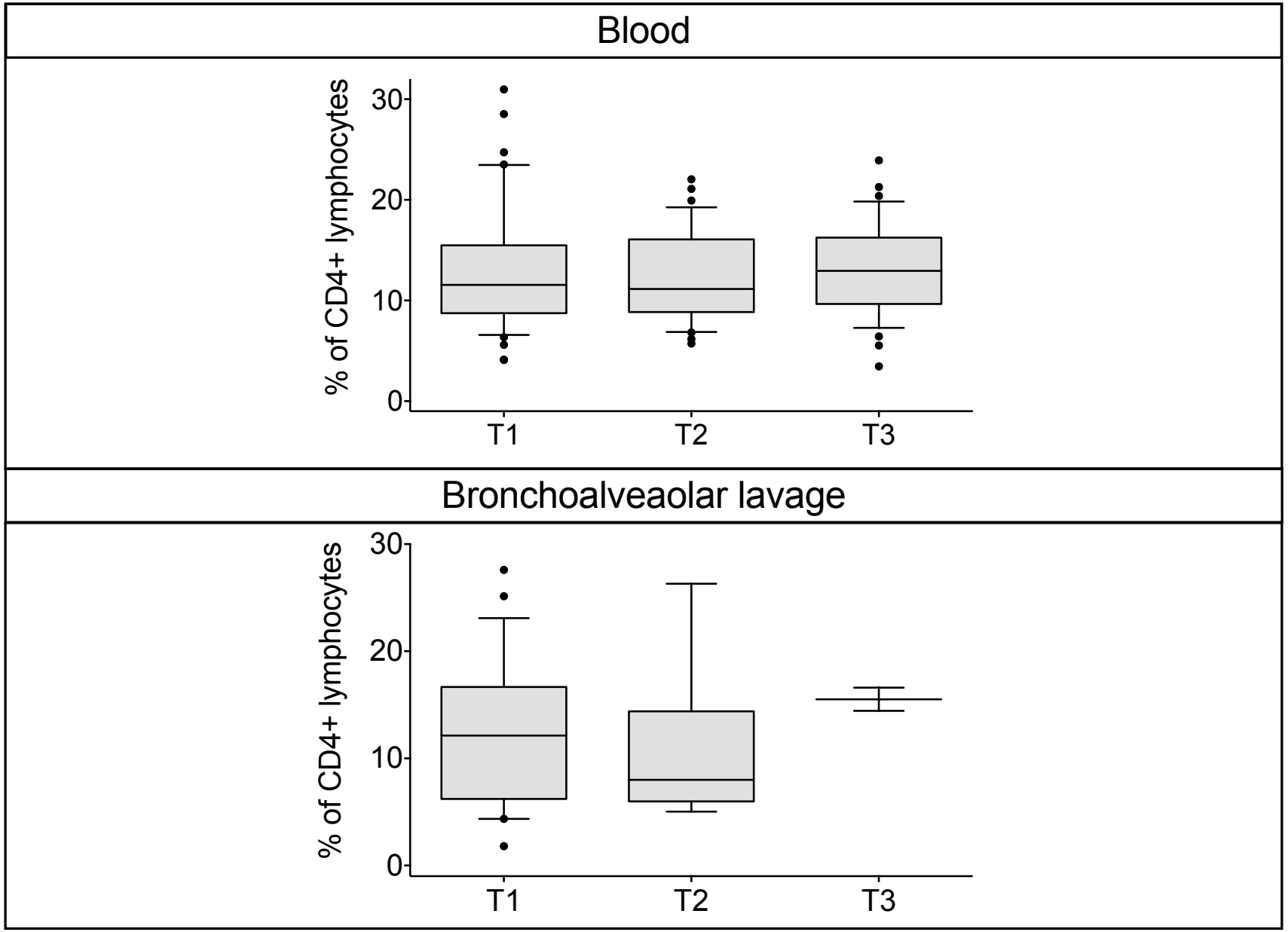

Blood
% of CD4+ lymphocytes
30
20
10
0
T1
T2
T3
Bronchoalveaolar lavage
% of CD4+ lymphocytes
30
20
10
0
T1
T2
T3

Figure 3B

Figure 4

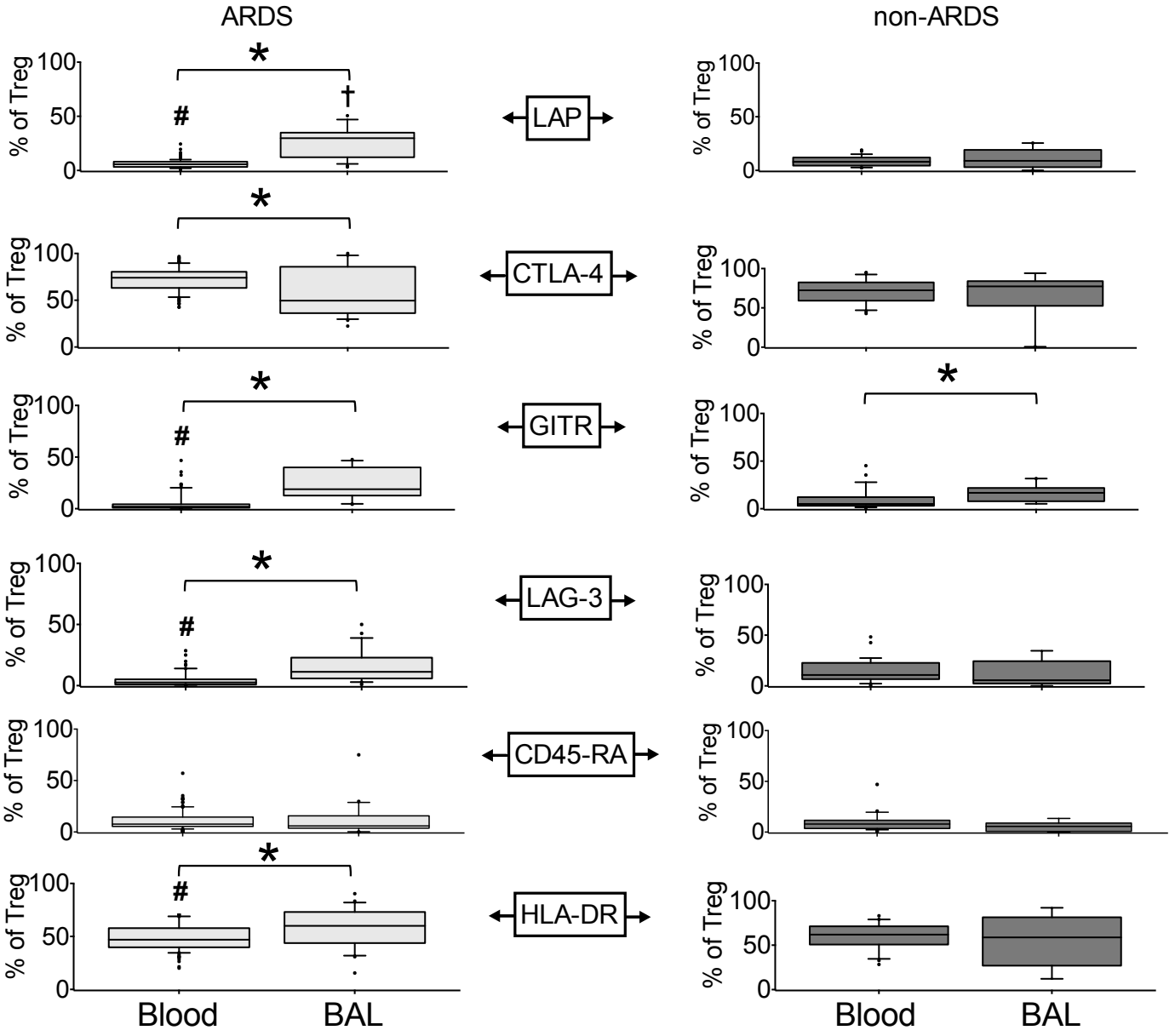

ARDS
non-ARDS
% of Treg
100
50
0
*
#
†
LAP
CTLA-4
GITR
LAG-3
CD45-RA
HLA-DR
Blood
BAL

Figure 5

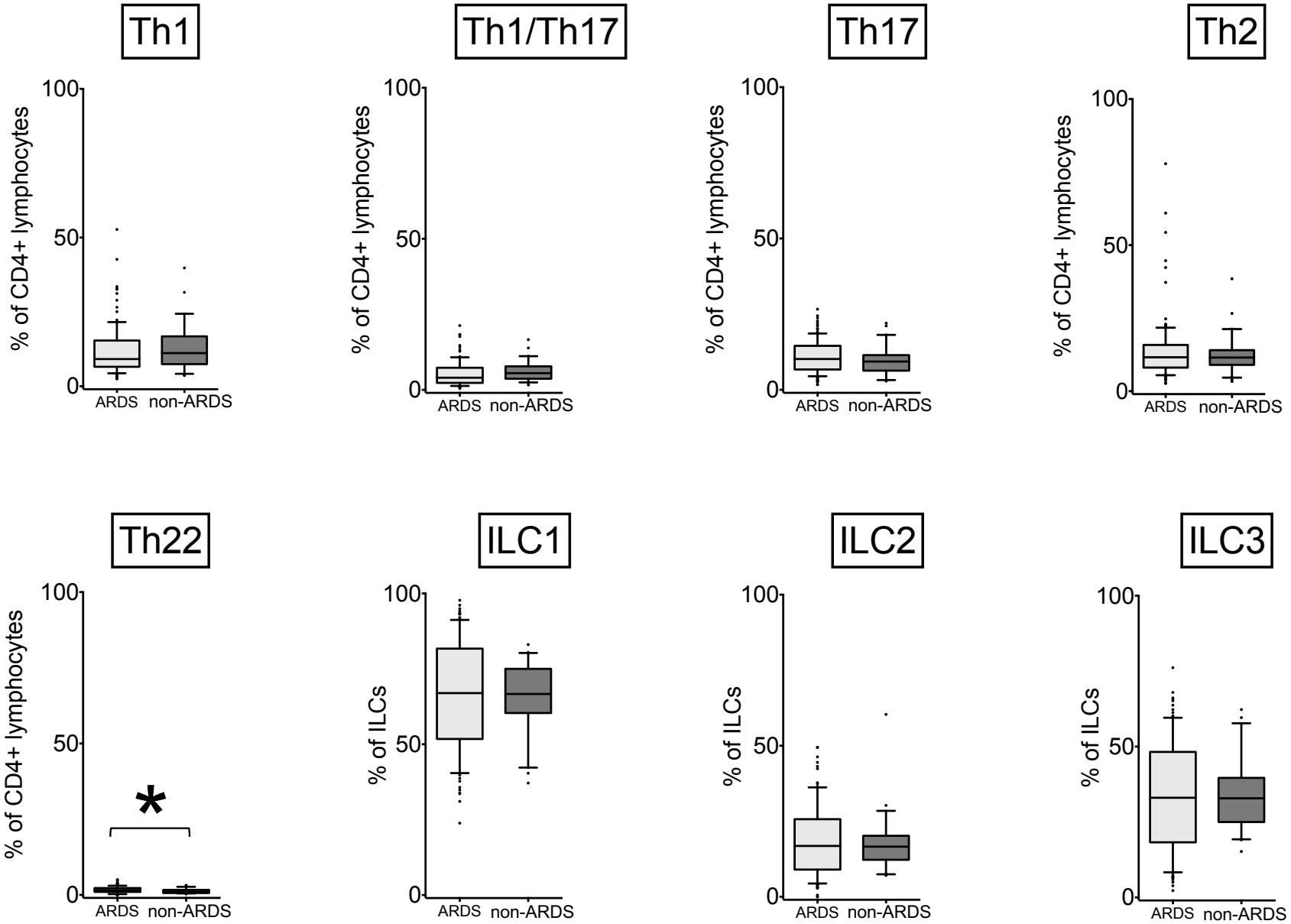

Th1
Th1/Th17
Th17
Th2
Th22
ILC1
ILC2
ILC3
% of CD4+ lymphocytes
% of ILCs
100
50
0
ARDS
non-ARDS
*

Figure 6

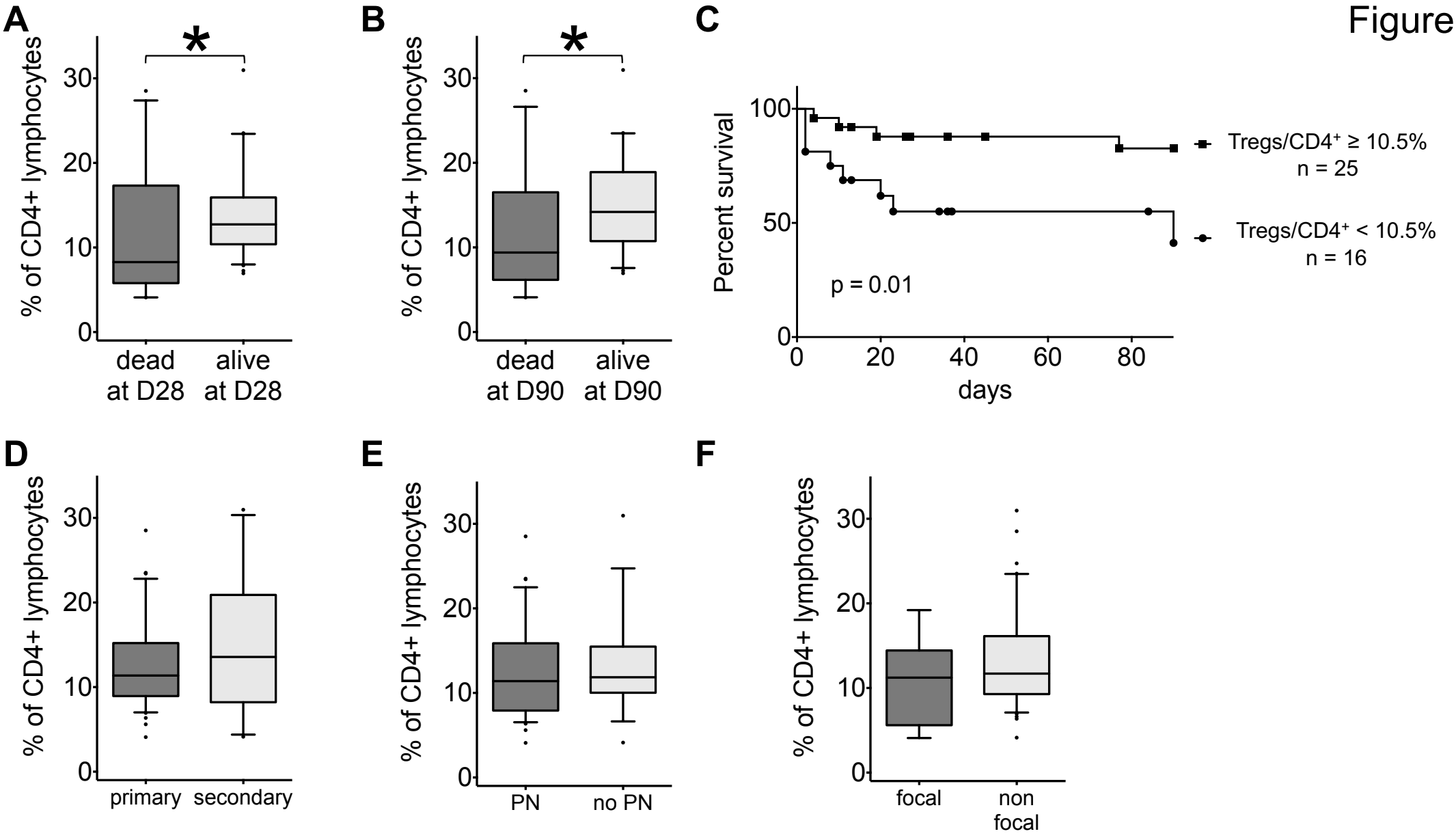

A
% of CD4+ lymphocytes
30
20
10
0
*
dead at D28
alive at D28
B
% of CD4+ lymphocytes
30
20
10
0
*
dead at D90
alive at D90
C
Percent survival
100
50
0
p = 0.01
0
20
40
60
80
days
Tregs/CD4+ ≥ 10.5%
n = 25
Tregs/CD4+ < 10.5%
n = 16
D
% of CD4+ lymphocytes
30
20
10
0
primary
secondary
E
% of CD4+ lymphocytes
30
20
10
0
PN
no PN
F
% of CD4+ lymphocytes
30
20
10
0
focal
non focal

Table 1. Patient characteristics at ICU admission.

Characteristics		ARDS n = 60	Non-ARDS n = 45	p
Age, yr		56.8 ± 16.9	60.4 ± 17.9	0.301
Male sex, n (%)		47 (78.3)	33 (73.3)	0.552
Weight (kg)		82.4 ± 22.3	73.6 ± 22.9	0.055
Height (m)		1.72 ± 0.1	1.73 ± 0.1	0.416
Body mass index		27.8 ± 6.5	25.0 ± 7.2	0.044*
Comorbidities, n (%)				
	Chronic obstructive pulmonary disease	5 (8.3)	6 (13.3)	0.524
	Congestive heart failure	8 (13.3)	4 (8.9)	0.479
	Cancer	7 (11.7)	6 (13.3)	0.797
	Immunodeficiency	0	0	1.0
	Corticosteroïds	3 (5.0)	1 (2.2)	0.633
Type of admission, n (%)				0.255
	Medical	13 (21.7)	16 (36.4)	
	Surgical	35 (58.3)	21 (47.7)	
	Trauma	12 (20.0)	7 (15.9)	
SAPS II		54.1 ± 24.1	51.3 ± 19.0	0.532
SOFA		10.8 ± 5.2	8.9 ± 3.8	0.048*
Shock, n (%)		45 (75.0)	18 (40.9)	$<10^{-3}$*
PaO_2/FiO_2		101.2 ± 60.1	295.1 ± 78.5	$<10^{-4}$*
Lung ultrasound score		18.0 ± 6.7	12.8 ± 5.4	$<10^{-4}$*
Pneumonia, n (%)		47 (78.3)	24 (53.3)	0.007*
Sepsis, n (%)		54 (90.0)	31 (68.9)	0.006*
Antibiotics, n (%)		59 (98.3)	36 (80.0)	0.002*
Mechanical ventilation, n (%)		56 (93.3)	34 (75.6)	0.010*
Mortality, n (%)				
	Overall	22 (36.7)	13 (28.9)	0.403
	At day 28	17 (28.8)	10 (23.3)	0.530
	At day 90	21 (42.9)	12 (29.3)	0.183

Values are expressed as mean ± standard deviation, or number (%). * $p < 0.05$.
ICU = intensive care unit ; SAPSII = simplified acute physiology score; SOFA = sequential organ failure assessment score ; PaO_2 = partial arterial pressure in Oxygen ; FiO_2 = inspiratory fraction in Oxygen.

Table 2. Characteristics of ARDS patients at ICU admission.

		ARDS n = 60
Cause, n (%)		
	Pneumonia	42 (70.0)
	Contusion	2 (3.3)
	Aspiration	4 (6.7)
	Sepsis	6 (10.0)
	Cardiopulmonary bypass	1 (1.7)
	Other	5 (8.3)
Onset of ARDS (days)		1.5 [0–6.0]
Morphology, n (%)		
	Focal	11 (18.3)
	Non focal	49 (81.7)
Type, n (%)		
	Primary	49 (81.7)
	Secondary	11 (18.3)
Lung injury score		3.0 [2.5–3.3]
Positive-end expiratory pressure (cmH_2O)		8 [8–10]
Driving pressure (cmH_2O)		15 [13–18]
Vt/weight (ml/kg)		6 [6–7]

Values are expressed as median (25–75% interquartile range), or number (%).
ICU = intensive care unit ; ARDS = acute respiratory distress syndrome ; PaO_2 = partial arterial pressure in Oxygen ; FiO_2 = inspiratory fraction in Oxygen ; Vt = tidal volume.

Table 3. Expression levels of blood and alveolar cytokines and chemokines.

	Blood			Bronchoalveolar lavage		
	ARDS (n = 14)	Non-ARDS (n = 12)	p	ARDS (n = 13)	Non-ARDS (n = 25)	p
Th1 /Th17 / inflammation-related cytokines						
IFN-g	3.6 [1.9–6.4]	11.1 [7.4–32.2]	0.0016*	0.0 [0.0–1.6]	0.1 [0.0–2.6]	0.2965
IFN-a2	2.5 [0.2–22.6]	18.9 [8.3–63.2]	0.0863	1.7 [0.0–10.3]	4.2 [0.1–9.7]	0.6444
IL-17a	2.8 [1.9–6.3]	4.9 [2.0–14.2]	0.2902	0.1 [0.0–2.2]	0.0 [0.0–0.0	0.6727
TNF-a	46.1 [21.9–68.1]	18.5 [11.7–27.6]	0.0420*	14.7 [1.8–87.3]	7.5 [3.1–42.9]	0.8999
GM-CSF	4.5 [3.0–11.3]	7.9 [5.0–46.9]	0.2456	2.7 [1.5–4.5]	4.0 [1.7–5.2]	0.2833
IL1-a	0.0 [0.0–0.0]	4.0 [0–16.4]	0.0106*	18.4 [0.0–60.4]	13.6 [0.1–41.0]	0.7584
IL1-b	0.0 [0.0–0.0]	0.0 [0.0–9.2]	0.5736	9.7 [3.7–293.3]	36.6 [6.7–231.6]	0.8186
IL-8	247.9 [67.9–1504]	32.6 [24.6–62.3]	0.0003*	1553 [1069–2558]	1778 [503.9–2913]	0.9615
IL-15	15.9 [11.6–33.7]	11.7 [7.8–21.2]	0.2067	1.0 [0.5–3.3]	1.3 [0.1–2.5]	0.8312
IL-12p70	3.2 [2.4–6.5]	8.0 [4.0–30.5]	0.0270*	2.8 [1.7–6.0]	3.8 [0.4–6.6]	0.8610
MCP-1	3642 [1272–7424]	689.3 [377.1–1060]	0.0001*	690.7 [429.9–2877]	943.8 [306.2–3308]	0.7720
MIP-1a	11.4 [5.2–15.4]	9.2 [7.1–13.0]	0.9235	78.7 [16.4–205.1]	63.7 [20.1–251.9]	0.8856
MIP-1b	34.6 [18.4–46.6]	29.5 [16.7–47.8]	0.8498	66.2 [21.2–160.5]	62.4 [20.3–154.6]	0.9576
IP-10	644.3 [336.2–1723]	798.3 [463.2–2788]	0.6594	108.1 [57.6–235.7]	327.8 [86.6–1202]	0.0896
Th2-related cytokines						
IL-4	0.0 [0.0–0.4]	0.0 [0.0–0.0]	0.1226	2.1 [0.0–5.8]	1.9 [0.0–5.3]	0.9812
IL-5	1.1 [0.4–3.2]	1.4 [0.7–2.9]	0.2454	1.2 [0.9–2.1]	1.8 [1.1–4.5]	0.1470
IL-6	1108 [181.2–10000]	42.1 [22.9–52.0]	$< 10^{-4}$*	133.7 [80.3–947.1]	167.2 [77.8–429.3]	0.6463
IL-7	3.2 [1.6–3.8]	6.2 [3.2–11.2]	0.0285*	0.7 [0.0–1.9]	1.9 [0.5–3.3]	0.0779
IL-9	0.7 [0.4–1.3]	1.4 [0.9–8.6]	0.0085*	1.8 [1.3–2.8]	2.0 [0.8–4.1]	0.5741
IL-13	0.0 [0.0–0.0]	0.0 [0.0–2.6]	0.1516	1.7 [0.9–2.4]	2.8 [1.0–4.3]	0.1227
Treg / anti-inflammatory cytokines						
IL-2	0.1 [0.0–2.0]	1.0 [0.1–8.0]	0.1204	0.8 [0.4–1.3]	0.8 [0.4–1.5]	0.8421
IL1-RA	282.2 [102.0–649.6]	136.9 [68.1–388.1]	0.1977	940.9 [501.7–2711]	1021 [539.5–2075]	0.9967
IL-10	149.8 [52.4–617.5]	31.0 [8.5–66.1]	0.0046*	18.1 [6.0–55.9]	8.9 [1.6–36.8]	0.4837
IL-12p40	0.0 [0.0–0.0]	0.0 [0.0–45.7]	0.0638	0.7 [0.0–4.4]	1.2 [0.1–4.1]	0.6909
MDC	423.8 [245.3–905.0]	586.8 [350.7–1057]	0.2099	14.9 [2.3–33.5]	15.4 [3.4–33.0]	0.9756

Values are expressed as median (25–75% interquartile range). *p < 0.05.

ARDS = acute respiratory distress syndrome; GM-CSF = Granulocyte Colony Stimulating Factor; IFN = interferon; IL = interleukin; IP-10 = Interferon gamma-induced protein 10; MCP-1 = monocyte chemoattractant protein 1; MDC = Macrophage-derived chemokine; MIP = macrophage inflammatory protein; Th = T helper lymphocytes; TNF = tumor necrosis factor.

Table 4. Phenotyping of circulating blood lymphocytes in patients with acute respiratory distress syndrome.

Lymphocytes populations	Mean ± SD
Lymphocytes (cells/μL)	1176 ± 610.1
CD3+ lymphocytes (cells/μL)	876 ± 449.5
CD3+ lymphocytes (%)	74.6 ± 10.1
CD4+ lymphocytes (cells/μL)	583.3 ± 338.1
CD4+ lymphocytes (%)	49.1 ± 10.3
CD8+ lymphocytes (cells/μL)	299.1 ± 188.2
CD8+ lymphocytes (%)	25.7 ± 9.9
CD19+ lymphocytes (cells/μL)	162.9 ± 149.3
CD19+ lymphocytes (%)	14.2 ± 9.9
NK cells (cells/μL)	103.3 ± 95.1
NK cells (%)	8.2 ± 5.2

SD = standard deviation ; CD = cluster of differentiation; NK = natural killer cells.

Table 5. Absolute count of total circulating blood lymphocytes CD4+ and T regulatory cells.

	ARDS (n = 78)	Control (n = 11)	p
Lymphocytes (cells/μL)	1136 [706.3–1521]	734.0 [590.0–1537]	0.5268
CD4+ lymphocytes (cells/μL)	512.5 [299.0–723.0]	416.0 [273.0–791.0]	0.4702
Regulatory T cells lymphocytes (cells/μL)	57.0 [33.9–93.0]	36.7 [28.2–66.9]	0.1154

Values are expressed as median [25-75% interquartile].
CD = cluster of differentiation; ARDS = acute respiratory distress syndrome.