



**HAL**  
open science

# **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 Rosenz wajg, Antoine Monsel

## ► To cite this version:

Sebastien Halter, Lucrèce Aimade, Michèle Barbié, Hélène Brisson, Jean-Jacques Rouby, et al.. T regulatory cells activation and distribution are modified in critically ill patients with acute respiratory distress syndrome: A prospective single-centre observational study. *Anaesthesia Critical Care & Pain Medicine*, 2020, 39, pp.35 - 44. 10.1016/j.acepm.2019.07.014 . hal-03490095

**HAL Id: hal-03490095**

**<https://hal.science/hal-03490095>**

Submitted on 22 Aug 2022

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

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



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Title:**

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

4

5 **Running Title:**

6 Treg phenotype and distribution in ARDS

7

8 **Authors:**

9 Sebastien Halter<sup>a,b,c</sup>, M.D., M.Sc; Lucrece Aimade<sup>b,c</sup>, M.Sc.; Michèle Barbié<sup>b</sup>; Hélène  
10 Brisson<sup>a</sup>, M.D., M.Sc.; Jean-Jacques Rouby<sup>a</sup>, M.D., Ph.D; Olivier Langeron<sup>a</sup>, M.D., Ph.D;  
11 David Klatzmann<sup>b,c</sup>, M.D., Ph.D; Michelle Rosenzweig<sup>b,c</sup>, M.D., Ph.D; Antoine Monse<sup>a,b,c,\*</sup>,  
12 M.D., Ph.D.

13

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

18

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

22

23 <sup>c</sup> From the Sorbonne University, INSERM, UMR\_S 959, Immunology-Immunopathology-  
24 Immunotherapy (i3), F-75651, Paris, France (S.H., L.A., D.K., M.R, A.M.)

25

26 **Author Contributions:** S.H. contributed to overall study design, performance of the  
27 experiments, data analysis and interpretation, and writing of the manuscript. L.A. contributed  
28 to the study design, performance of the experiments, and data analysis and interpretation.

1 M.B. contributed to the study design, and performance of the experiments and data analysis.  
2 J.-J.R., O.L., H.B. and D.K. contributed to the data analysis and interpretation, and editing of  
3 the manuscript. M.R. contributed to the study design, data analysis and interpretation, and  
4 editing the manuscript. A.M. contributed to overall study design, financial support,  
5 performance of the experiments, data analyses and interpretation, writing of the manuscript,  
6 and final approval.

7

8 **\*Corresponding author at:** Antoine Monsel, M.D., Ph.D. Polyvalent Critical Care Unit,  
9 Department of Anaesthesiology and Critical Care Medicine, Pitié–Salpêtrière Hospital Group,  
10 47–83, boulevard de l’Hôpital, 75651 Paris Cedex 13, France. Tel: +33 (0)1 84 82 73 99;  
11 Fax: +33 (0)1 42 16 22 69. E-mail: [antoine.monsel@gmail.com](mailto:antoine.monsel@gmail.com)

12

13 **Sources of funding:** Supported by a National Research Contract Grant from the French  
14 Society of Anaesthesia & Intensive Care Medicine (SFAR) (Paris, France) (A.M.). This work  
15 was also funded by the ANR within the French government Investissement d’Avenir  
16 programme (Labex Transimmunom, ANR-11-IDEX-0004-02).

17

18 **Clinical trial number and registry URL:** NA

19

20 **Abstract** = 250 words, **Introduction** = 333 words, **Methods** = 794, **Results** = 957 words,

21 **Discussion** = 953 words, **total word count** = 3037 words.

22 **Running Title (50 car max):** Treg phenotype and distribution in ARDS

23

24 **Consent for publication:** Not applicable.

25 **Availability of data and material:** The datasets used and/or analysed during the current  
26 study are available from the corresponding author on reasonable request.

27

1 **ABSTRACT (250)**

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

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

12 **Results:** Blood Tregs/CD4+ percentage (median %, 25-75% interquartile) was higher in  
13 ARDS patients than in non-ARDS patients: 12.1% [9.0-16.0] *versus* 9.9% [8.1-12.6],  $p = 0.01$ .  
14 Alveolar Tregs/CD4+ percentage was lower in ARDS patients than in non-ARDS patients:  
15 10.4% [6.3-16.6] *versus* 16.2% [12.4-21.1],  $p = 0.03$ . In ARDS patients, Tregs activation was  
16 reduced in the blood and increased in the alveolus, compared to non-ARDS patients. ROC  
17 analysis revealed a threshold of 10.4% for the Tregs/CD4+ percentage in the blood collected  
18 within the first week of ARDS to discriminate between survivors and non-survivors  
19 (sensitivity: 75%; specificity 76%; area under the curve [95% confidence interval]: 0.72 [0.5-  
20 0.9]).

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

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

26

## 1 **1. Introduction**

2

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

9 Aside from neutrophils, other immune cells encompassing lymphocytes are involved  
10 in the pathophysiology of ARDS. More specifically, T-regulatory cells (Tregs), a subset of  
11 CD4 expressing lymphocytes that highly express CD25 (interleukin (IL)-2 receptor  $\alpha$ ) as well  
12 as the transcription factor Forkhead box protein 3 (FoxP3), have been recently demonstrated  
13 to play a crucial role in maintaining immune homeostasis [5, 6]. They can suppress  
14 inflammatory, allergic, and autoimmune disorders [7] including in pneumonia [8-10], through  
15 contact-dependent suppression or releasing cytokines, *e.g.* IL-10 and transforming growth  
16 factor (TGF)- $\beta$ 1 [6]. Several studies investigated the role of Tregs in either experimental  
17 models of acute lung injury or even more rarely in ARDS patients. Most of them yielded  
18 conflicting results [10-20]. In murine models, Tregs have been shown to contribute to the  
19 resolution of lung injury [11-14]. However, recent clinical studies presented Tregs as either a  
20 risk factor [10] or a protective factor [20] for mortality. Others yielded inconclusive results [16].  
21 Moreover, their distribution over time between blood and lungs has never been studied in the  
22 clinical settings. Similarly, Tregs expression of immunosuppressive markers remains  
23 uncovered in patients with ARDS. Finally, phenotyping of T helper (Th) polarisation and  
24 innate lymphoid cells (ILCs) populations has been under-studied in ARDS patients [15, 20].

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

- 1 investigate both systemic and bronchoalveolar Tregs phenotype over time, and its potential
- 2 impact on the mortality of patients with ARDS.
- 3

1 **2. Patients and Methods**

2 **2.1. Study design**

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

10

11 **2.2. ARDS group**

12 Patients admitted to our ICU were considered eligible if they met the Berlin criteria for ARDS  
13 [21], and had no previous history of ARDS. Patients under 18 years old, with  
14 immunosuppressive therapy or previously characterised immune alterations were excluded.  
15 The duration of patient follow-up was at least 90 days. Clinical and demographic data were  
16 calculated and reported over the 24 hours after ARDS diagnosis. The characterisation of  
17 focal versus non-focal ARDS was based on lung ultrasounds [22]. Primary ARDS was  
18 defined as direct lung injury, such as pneumonia, lung contusion or aspiration.

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

21

22 **2.3. Non-ARDS group**

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

28

1 **2.4. Blood samples**

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

5

6 **2.5. Bronchoalveolar lavage samples**

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

13

14 **2.6. Antibodies**

15 The antibodies used are listed in the supplemental table 1.

16

17 **2.7. Flow cytometric analysis of T regulatory phenotypes from blood and**  
18 **bronchoalveolar lavage samples**

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

24 The first panel was designed to gate on Tregs (defined as CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo/-</sup> cells)  
25 [26-28]. The second and third panels aimed to investigate the functionality of Tregs.  
26 Functional/activation markers chosen in those panels were LAP, LAG-3, GITR, CTLA-4,  
27 CD45RA, and HLA-DR. The Figure 1 represents the gating strategy determining the Tregs



1 phenotypes in blood and BAL samples. Effector CD4<sup>+</sup> T cells were defined as FoxP3<sup>-</sup> cells  
2 among CD4<sup>+</sup> T cells.

3

#### 4 **2.8. Flow cytometric analysis of T helper polarisation and innate lymphoid cells from** 5 **blood samples**

6 In the T helper (Th) polarisation panel, antibodies directed against chemokine receptors  
7 (CCR6, CCR10, CCR4, and CXCR3) were used to discriminate Th1  
8 (CXCR3<sup>+</sup>CCR4<sup>-</sup>CCR6<sup>-</sup>CCR10<sup>-</sup>), Th2 (CXCR3<sup>-</sup>CCR4<sup>+</sup>CCR6<sup>-</sup>CCR10<sup>-</sup>), Th17  
9 (CXCR3<sup>-</sup>CCR4<sup>+</sup>CCR6<sup>+</sup>CCR10<sup>-</sup>), and Th22 (CCR4<sup>+</sup>CCR6<sup>+</sup>CXCR3<sup>-</sup>CCR10<sup>+</sup>) subsets [26, 27,  
10 29]. For ILCs phenotyping we used, anti-lineage, -NKp44, -CD117, -CD127, and -CD294  
11 antibodies. Samples were then fixed and red blood cells were lysed. Samples were acquired  
12 on a Galios cytometre software (Beckman Coulter, Villepinte, France), and analysed through  
13 Kaluza software (Beckman Coulter, Villepinte, France).

14

#### 15 **2.9. Blood and Bronchoalveolar lavage cytokine measurements**

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

22

#### 23 **2. 10. Statistical analyses**

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

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

4

## 1 **3. Results**

### 2 **3.1. Patients**

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

11

### 12 **3.2. Blood and bronchoalveolar cytokines/chemokines concentrations at the onset of** 13 **ARDS**

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

21

### 22 **3.3. Proportion of blood and alveolar Tregs and their variation over time**

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

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

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

9

#### 10 ***3.4. Comparison of the proportion of blood Tregs expressing functionality/activation*** 11 ***markers between ARDS and non-ARDS patients***

12 Compared to that of non-ARDS patients, the proportion of blood Tregs expressing functional  
13 markers in ARDS patients was significantly reduced by 29% for LAP<sup>+</sup>, 63% for GITR<sup>+</sup>, 76%  
14 for LAG-3<sup>+</sup>, and 24% for HLA-DR<sup>+</sup> (Fig. 4). In contrast, the proportion of blood Tregs  
15 expressing CTLA-4, and CD-45RA did not differ between ARDS and non-ARDS patients (Fig.  
16 4).

17

#### 18 ***3.5. Comparison of the proportion of alveolar Tregs expressing functionality/activation*** 19 ***markers between ARDS and non-ARDS patients***

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

23

#### 24 ***3.6. Distribution of Tregs and activated Tregs according to blood and alveolar*** 25 ***compartments***

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

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

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

8

### 9 **3.7. Teffs expressing activation markers and their relative expression of activation** 10 **markers compared to Tregs**

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

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

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

22

### 23 **3.8. T helper polarisation and innate lymphoid cells phenotypes in blood compartment**

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

27

### 28 **3.9. Blood Tregs/CD4+ percentage, mortality and ARDS phenotypes**

1 Blood Tregs/CD4<sup>+</sup> percentage at T1 differed significantly between ARDS survivors and non-  
2 survivors on day 28 and 90 (Fig. 6, A and B). ROC analysis revealed a cutoff of 10.4% for  
3 Tregs/CD4<sup>+</sup> percentage to discriminate between survivors and non-survivors (sensitivity:  
4 75%; specificity 76 %; area under the curve [95% confidence interval]: 0.72 [0.5-0.9]). Ninety-  
5 day survival was 83% for patients with Tregs/CD4<sup>+</sup> percentage equal to or greater than  
6 10.5%, but only 41% for patients with Tregs/CD4<sup>+</sup> percentage less than 10.5% (p=0.01, Fig.  
7 6C). Among age, SAPS II, SOFA, P<sub>a</sub>O<sub>2</sub>/FiO<sub>2</sub> and the presence of shock, the SOFA score  
8 was higher in non-survivors than in survivors (14 [12.2-17.5] *versus* 10 [9-12]), respectively;  
9 p=0.02).

10 Blood Tregs/CD4<sup>+</sup> percentage did not differ according to the ARDS cause or morphotype  
11 (Fig.6, D—F).

12

#### 1 **4. Discussion**

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

8 We assessed activation and distribution of Tregs in systemic and BAL compartments  
9 in ARDS patients. D'Alessio et al. were the first to set out Tregs-induced resolution of acute  
10 lung injury as a bedrock concept [11-14], and to investigate the presence of Tregs in BAL  
11 and blood samples collected from 3 patients with ARDS [13]. In ARDS patients, Adamzik et  
12 al. identified the BAL Tregs/CD4<sup>+</sup> percentage collected at admission, as an independent risk  
13 factor for 30-day mortality [10]. However, it is important to note the small sample size of their  
14 control group (n=8) made of patients undergoing surgery for progressing cancer, and that all  
15 ARDS patients were treated with hydrocortisone, two factors impacting immunity and Tregs  
16 proportion. Furthermore, we used a more stringent Tregs gating strategy combining four  
17 specific markers including CD127 (CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo/-</sup> cells), which was not the  
18 case in their study. Finally, time variation in BAL and blood Tregs phenotype was not  
19 evaluated. Although our results showed a link between blood Tregs/CD4<sup>+</sup> percentage and  
20 ARDS mortality, the design and sample size of our study do not allow concluding that ARDS  
21 mortality is caused by a Tregs deficit. A second clinical study, more in line with our results,  
22 showed that a higher Th17/Treg percentage in blood was associated with poorer prognosis  
23 [20]. In a third study, Risso et al. did not find any difference in Tregs subsets between ARDS  
24 patients and controls [16]. Aside from a limited sample-size, this study evaluated Tregs  
25 proportion only in BAL samples in the early phase of ARDS. Finally, Ronit et al. evaluated  
26 the change in BAL Tregs proportion over time after lipopolysaccharide intratracheal  
27 instillation in 15 healthy volunteers [17]. Despite a restricted sample size, they showed an  
28 increase in Treg subset after initiation of lung injury. The fact that no blood sampling was

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

3 One original finding of our study is the bidirectional changes in Treg phenotype  
4 depending on the compartment studied, *i.e.* a decrease in BAL Treg subset contrasting with  
5 an increase in systemic Treg subset. These findings are in line with studies reporting  
6 opposite inflammatory status simultaneously occurring in lung and blood [33]. Other acute  
7 inflammation-induced organ injuries were demonstrated to be accompanied with  
8 simultaneous systemic immunosuppression [34-36]. It remains to be clarified which  
9 compartment-related status constitutes a counterbalancing response to the other. Why this  
10 extra- subset of systemic Tregs does not migrate into the alveolar space remains to be  
11 unveiled. Since Treg homing were demonstrated to be crucial in resolving inflammation [37,  
12 38] and even altered in some inflammatory diseases [39-41], we can speculate that there  
13 might be an alteration in the homing capacities of the blood Tregs to the damaged alveolus  
14 of the ARDS. Further studies are warranted to investigate this hypothesis. Our data, obtained  
15 in ARDS patients, suggested an increase in BAL activated Tregs expressing  
16 immunosuppressive markers. Whether this overexpression constitutes a compensatory  
17 response to the uncontrolled lung inflammation aggravated by a shortage of Tregs remains  
18 to be evaluated.

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

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

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



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

13

## 14 **CONCLUSIONS**

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

23

1 **LIST OF ABBREVIATIONS**

2 ARDS: acute respiratory distress syndrome; BAL: bronchoalveolar lavage; CD: cluster of  
3 differentiation; CTLA-4: cytotoxic T-Lymphocyte-associated protein-4; FoxP3: Forkhead box  
4 protein 3; GITR: glucocorticoid-induced TNFR-related protein; HLA: human leukocytes  
5 antigen; ICU: intensive care unit; IL: interleukin; ILCs: innate lymphoid cells; IFN: interferon;  
6 IQR: interquartile range; LAG: lymphocyte activation gene-3; LAP: latency-associated  
7 peptide; NK: natural killer cells; ROC: receiver operating characteristic; SAPS 2: simplified  
8 acute physiology score 2; SD: standard deviation; SOFA: sequential organ failure  
9 assessment; Teff: effector CD4+ T cells; TGF- $\beta$ 1: transforming growth factor; Th: T helper  
10 cells; TNF: tumour necrosis factor; Tregs: T regulatory cells; VAP: ventilator-associated  
11 pneumonia;

12

13 **Acknowledgements**

14 Not applicable.

15

## 1 REFERENCES

- 2 [1] Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, et al., Incidence  
3 and outcomes of acute lung injury. *N Engl J Med* 2005; 353: 1685-1693;  
4 10.1056/NEJMoa050333
- 5 [2] Herridge MS, Moss M, Hough CL, Hopkins RO, Rice TW, Bienvenu OJ, et al.,  
6 Recovery and outcomes after the acute respiratory distress syndrome (ARDS) in  
7 patients and their family caregivers. *Intensive Care Med* 2016; 42: 725-738;  
8 10.1007/s00134-016-4321-8
- 9 [3] Herridge MS, Tansey CM, Matte A, Tomlinson G, Diaz-Granados N, Cooper A, et al.,  
10 Functional disability 5 years after acute respiratory distress syndrome. *N Engl J Med*  
11 2011; 364: 1293-1304; 10.1056/NEJMoa1011802
- 12 [4] Han S, Mallampalli RK, The acute respiratory distress syndrome: from mechanism to  
13 translation. *J Immunol* 2015; 194: 855-860; 10.4049/jimmunol.1402513
- 14 [5] Burzyn D, Benoist C, Mathis D, Regulatory T cells in nonlymphoid tissues. *Nat*  
15 *Immunol* 2013; 14: 1007-1013; 10.1038/ni.2683
- 16 [6] Zhao H, Liao X, Kang Y, Tregs: Where We Are and What Comes Next? *Front*  
17 *Immunol* 2017; 8: 1578; 10.3389/fimmu.2017.01578
- 18 [7] Singer BD, King LS, D'Alessio FR, Regulatory T cells as immunotherapy. *Front*  
19 *Immunol* 2014; 5: 46; 10.3389/fimmu.2014.00046
- 20 [8] McKinley L, Logar AJ, McAllister F, Zheng M, Steele C, Kolls JK, Regulatory T cells  
21 dampen pulmonary inflammation and lung injury in an animal model of pneumocystis  
22 pneumonia. *J Immunol* 2006; 177: 6215-6226;
- 23 [9] Montagnoli C, Fallarino F, Gaziano R, Bozza S, Bellocchio S, Zelante T, et al.,  
24 Immunity and tolerance to *Aspergillus* involve functionally distinct regulatory T cells  
25 and tryptophan catabolism. *J Immunol* 2006; 176: 1712-1723;
- 26 [10] Adamzik M, Broll J, Steinmann J, Westendorf AM, Rehfeld I, Kreissig C, et al., An  
27 increased alveolar CD4 + CD25 + Foxp3 + T-regulatory cell ratio in acute respiratory

- 1 distress syndrome is associated with increased 30-day mortality. *Intensive Care Med*  
2 2013; 39: 1743-1751; 10.1007/s00134-013-3036-3
- 3 [11] Aggarwal NR, D'Alessio FR, Tsushima K, Sidhaye VK, Cheadle C, Grigoryev DN, et  
4 al., Regulatory T cell-mediated resolution of lung injury: identification of potential  
5 target genes via expression profiling. *Physiol Genomics* 2010; 41: 109-119;  
6 10.1152/physiolgenomics.00131.2009
- 7 [12] Aggarwal NR, Tsushima K, Eto Y, Tripathi A, Mandke P, Mock JR, et al.,  
8 Immunological priming requires regulatory T cells and IL-10-producing macrophages  
9 to accelerate resolution from severe lung inflammation. *J Immunol* 2014; 192: 4453-  
10 4464; 10.4049/jimmunol.1400146
- 11 [13] D'Alessio FR, Tsushima K, Aggarwal NR, West EE, Willett MH, Britos MF, et al.,  
12 CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present  
13 in humans with acute lung injury. *J Clin Invest* 2009; 119: 2898-2913;  
14 10.1172/JCI36498
- 15 [14] Garibaldi BT, D'Alessio FR, Mock JR, Files DC, Chau E, Eto Y, et al., Regulatory T  
16 cells reduce acute lung injury fibroproliferation by decreasing fibrocyte recruitment.  
17 *Am J Respir Cell Mol Biol* 2013; 48: 35-43; 10.1165/rcmb.2012-0198OC
- 18 [15] Muir R, Osbourn M, Dubois AV, Doran E, Small DM, Monahan A, et al., Innate  
19 Lymphoid Cells Are the Predominant Source of IL-17A during the Early Pathogenesis  
20 of Acute Respiratory Distress Syndrome. *Am J Respir Crit Care Med* 2016; 193: 407-  
21 416; 10.1164/rccm.201410-1782OC
- 22 [16] Risso K, Kumar G, Ticchioni M, Sanfiorenzo C, Dellamonica J, Guillouet-de Salvador  
23 F, et al., Early infectious acute respiratory distress syndrome is characterized by  
24 activation and proliferation of alveolar T-cells. *Eur J Clin Microbiol Infect Dis* 2015; 34:  
25 1111-1118; 10.1007/s10096-015-2333-x
- 26 [17] Ronit A, Plovsing RR, Gaardbo JC, Berg RM, Hartling HJ, Konge L, et al., T cell  
27 subsets in human airways prior to and following endobronchial administration of  
28 endotoxin. *Respirology* 2015; 20: 579-586; 10.1111/resp.12497

- 1 [18] Venet F, Chung CS, Huang X, Lomas-Neira J, Chen Y, Ayala A, Lymphocytes in the  
2 development of lung inflammation: a role for regulatory CD4+ T cells in indirect  
3 pulmonary lung injury. *J Immunol* 2009; 183: 3472-3480; 10.4049/jimmunol.0804119
- 4 [19] Wang L, Zhao L, Lv J, Yin Q, Liang X, Chu Y, et al., BLT1-dependent alveolar  
5 recruitment of CD4(+)CD25(+) Foxp3(+) regulatory T cells is important for resolution  
6 of acute lung injury. *Am J Respir Crit Care Med* 2012; 186: 989-998;  
7 10.1164/rccm.201202-0261OC
- 8 [20] Yu ZX, Ji MS, Yan J, Cai Y, Liu J, Yang HF, et al., The ratio of Th17/Treg cells as a  
9 risk indicator in early acute respiratory distress syndrome. *Crit Care* 2015; 19: 82;  
10 10.1186/s13054-015-0811-2
- 11 [21] Force ADT, Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, et  
12 al., Acute respiratory distress syndrome: the Berlin Definition. *JAMA* 2012; 307: 2526-  
13 2533; 10.1001/jama.2012.5669
- 14 [22] Puybasset L, Cluzel P, Gusman P, Grenier P, Preteux F, Rouby JJ, Regional  
15 distribution of gas and tissue in acute respiratory distress syndrome. I. Consequences  
16 for lung morphology. CT Scan ARDS Study Group. *Intensive Care Med* 2000; 26:  
17 857-869;
- 18 [23] Ventilation with lower tidal volumes as compared with traditional tidal volumes for  
19 acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory  
20 Distress Syndrome Network. *N Engl J Med* 2000; 342: 1301-1308;  
21 10.1056/NEJM200005043421801
- 22 [24] Fan E, Del Sorbo L, Goligher EC, Hodgson CL, Munshi L, Walkey AJ, et al., An  
23 Official American Thoracic Society/European Society of Intensive Care  
24 Medicine/Society of Critical Care Medicine Clinical Practice Guideline: Mechanical  
25 Ventilation in Adult Patients with Acute Respiratory Distress Syndrome. *Am J Respir  
26 Crit Care Med* 2017; 195: 1253-1263; 10.1164/rccm.201703-0548ST
- 27 [25] Meyer KC, Raghu G, Baughman RP, Brown KK, Costabel U, du Bois RM, et al., An  
28 official American Thoracic Society clinical practice guideline: the clinical utility of

- 1 bronchoalveolar lavage cellular analysis in interstitial lung disease. *Am J Respir Crit*  
2 *Care Med* 2012; 185: 1004-1014; 10.1164/rccm.201202-0320ST
- 3 [26] Pitoiset F, Barbie M, Monneret G, Braudeau C, Pochard P, Pellegrin I, et al., A  
4 standardized flow cytometry procedure for the monitoring of regulatory T cells in  
5 clinical trials. *Cytometry B Clin Cytom* 2018; 94: 621-626; 10.1002/cyto.b.21622
- 6 [27] Pitoiset F, Cassard L, El Soufi K, Boselli L, Grivel J, Roux A, et al., Deep phenotyping  
7 of immune cell populations by optimized and standardized flow cytometry analyses.  
8 *Cytometry A* 2018; 93: 793-802; 10.1002/cyto.a.23570
- 9 [28] Lecendreux M, Churlaud G, Pitoiset F, Regnault A, Tran TA, Liblau R, et al.,  
10 Narcolepsy Type 1 Is Associated with a Systemic Increase and Activation of  
11 Regulatory T Cells and with a Systemic Activation of Global T Cells. *PLoS One* 2017;  
12 12: e0169836; 10.1371/journal.pone.0169836
- 13 [29] Mahnke YD, Beddall MH, Roederer M, OMIP-017: human CD4(+) helper T-cell  
14 subsets including follicular helper cells. *Cytometry A* 2013; 83: 439-440;  
15 10.1002/cyto.a.22269
- 16 [30] Fremont RD, Koyama T, Calfee CS, Wu W, Dossett LA, Bossert FR, et al., Acute  
17 lung injury in patients with traumatic injuries: utility of a panel of biomarkers for  
18 diagnosis and pathogenesis. *J Trauma* 2010; 68: 1121-1127;  
19 10.1097/TA.0b013e3181c40728
- 20 [31] Terpstra ML, Aman J, van Nieuw Amerongen GP, Groeneveld AB, Plasma  
21 biomarkers for acute respiratory distress syndrome: a systematic review and meta-  
22 analysis\*. *Crit Care Med* 2014; 42: 691-700; 10.1097/01.ccm.0000435669.60811.24
- 23 [32] Ware LB, Koyama T, Zhao Z, Janz DR, Wickersham N, Bernard GR, et al.,  
24 Biomarkers of lung epithelial injury and inflammation distinguish severe sepsis  
25 patients with acute respiratory distress syndrome. *Crit Care* 2013; 17: R253;  
26 10.1186/cc13080

- 1 [33] Buttenschoen K, Kornmann M, Berger D, Leder G, Beger HG, Vasilescu C,  
2 Endotoxemia and endotoxin tolerance in patients with ARDS. *Langenbecks Arch*  
3 *Surg* 2008; 393: 473-478; 10.1007/s00423-008-0317-3
- 4 [34] Bird MD, Kovacs EJ, Organ-specific inflammation following acute ethanol and burn  
5 injury. *J Leukoc Biol* 2008; 84: 607-613; 10.1189/jlb.1107766
- 6 [35] Lechner AJ, Tredway TL, Brink DS, Klein CA, Matuschak GM, Differential systemic  
7 and intrapulmonary TNF-alpha production in *Candida* sepsis during  
8 immunosuppression. *Am J Physiol* 1992; 263: L526-535;
- 9 [36] Vermeij JD, Aslami H, Fluiter K, Roelofs JJ, van den Bergh WM, Juffermans NP, et  
10 al., Traumatic brain injury in rats induces lung injury and systemic immune  
11 suppression. *J Neurotrauma* 2013; 30: 2073-2079; 10.1089/neu.2013.3060
- 12 [37] Ding Y, Xu J, Bromberg JS, Regulatory T cell migration during an immune response.  
13 *Trends Immunol* 2012; 33: 174-180; 10.1016/j.it.2012.01.002
- 14 [38] Wei S, Kryczek I, Zou W, Regulatory T-cell compartmentalization and trafficking.  
15 *Blood* 2006; 108: 426-431; 10.1182/blood-2006-01-0177
- 16 [39] Griffith JW, Sokol CL, Luster AD, Chemokines and chemokine receptors: positioning  
17 cells for host defense and immunity. *Annu Rev Immunol* 2014; 32: 659-702;  
18 10.1146/annurev-immunol-032713-120145
- 19 [40] Sather BD, Treuting P, Perdue N, Miazgowicz M, Fontenot JD, Rudensky AY, et al.,  
20 Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific  
21 inflammatory disease. *J Exp Med* 2007; 204: 1335-1347; 10.1084/jem.20070081
- 22 [41] Schneider-Hohendorf T, Stenner MP, Weidenfeller C, Zozulya AL, Simon OJ,  
23 Schwab N, et al., Regulatory T cells exhibit enhanced migratory characteristics, a  
24 feature impaired in patients with multiple sclerosis. *Eur J Immunol* 2010; 40: 3581-  
25 3590; 10.1002/eji.201040558
- 26 [42] Sharabi A, Tsokos MG, Ding Y, Malek TR, Klatzmann D, Tsokos GC, Regulatory T  
27 cells in the treatment of disease. *Nat Rev Drug Discov* 2018; 17: 823-844;  
28 10.1038/nrd.2018.148

1

## 2 **FIGURE LEGENDS**

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

12

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

16

17 **Figure 3. Spatial and temporal distribution of the proportion of T regulatory cells in**  
18 **ARDS and non-ARDS patients. A,** Distribution of blood and bronchoalveolar lavage (BAL)  
19 T regulatory cells (Tregs) proportion among CD4<sup>+</sup> cells (Tregs/CD4<sup>+</sup> percentage) in acute  
20 respiratory distress syndrome (ARDS) patients and non-ARDS patients. While the proportion  
21 of blood Tregs was significantly increased in patients with ARDS, it was reduced in the BAL  
22 of ARDS patients compared to non-ARDS patients. Whereas there was no blood-alveolar  
23 gradient for patients with ARDS, the proportion of alveolar Tregs was higher than that in the  
24 blood in the non-ARDS group. Blood samples (combined data from all times points: T1, T2  
25 and T3):  $n = 114$  and  $29$  for ARDS and non-ARDS patients; BAL samples (combined data  
26 from all times points: T1, T2 and T3):  $n = 31$  and  $16$  for ARDS and non-ARDS patients. <sup>#</sup> $p =$   
27  $0.01$  versus blood Tregs/CD4<sup>+</sup> percentage in non-ARDS group, <sup>†</sup> $p = 0.03$  versus BAL



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

13

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

1 interquartile range, respectively, *T* bars are drawn down to the 10<sup>th</sup> percentile and up to the  
2 90<sup>th</sup>. Points below and above the whiskers are drawn as individual points. *Light grey* is used  
3 for ARDS group; *dark grey* is used for non-ARDS group. ARDS = acute respiratory distress  
4 syndrome; BAL = bronchoalveolar lavage; CD = cluster of differentiation; CTLA4 = cytotoxic  
5 T-Lymphocyte-associated protein-4; GITR = glucocorticoid-induced TNFR-related protein;  
6 HLA-DR = human leucocyte antigen D-related; LAG-3 = lymphocyte activation gene-3; LAP  
7 = latency-associated peptide; Treg = T regulatory cells.

8

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

22

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

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

1 Whitney U test). **C**, Ninety-day survival was 83% for patients with a blood Tregs/CD4+  
2 percentage  $\geq 10.5\%$  and 41% for patients with blood Tregs/CD4+ percentage  $< 10.5\%$ .  $p =$   
3 0.01 by Log-rank (Mantel-Cox) test. **D — F**, Blood Tregs/CD4+ percentage did not differ  
4 according to the cause or the morphotype of ARDS.  $n = 41$  and  $n = 10$  for primary and  
5 secondary ARDS;  $n = 32$  and  $n = 19$  for pneumonia (PN) and non-pneumonia (no-PN)  
6 ARDS;  $n = 7$  and  $n = 44$  for focal and non-focal ARDS. Box plots of Tregs/CD4+ percentage  
7 (% of CD4+ cells): internal *horizontal lines* are the medians, *lower and upper box limits* are  
8 the 25–75<sup>th</sup> interquartile range, respectively, *T bars* are drawn down to the 10<sup>th</sup> percentile and  
9 up to the 90<sup>th</sup>. Points below and above the whiskers are drawn as individual points. CD =  
10 cluster of differentiation; D28 = day 28; D90 = day 90; PN = pneumonia; Treg = T regulatory  
11 cells.

12

Figure 1

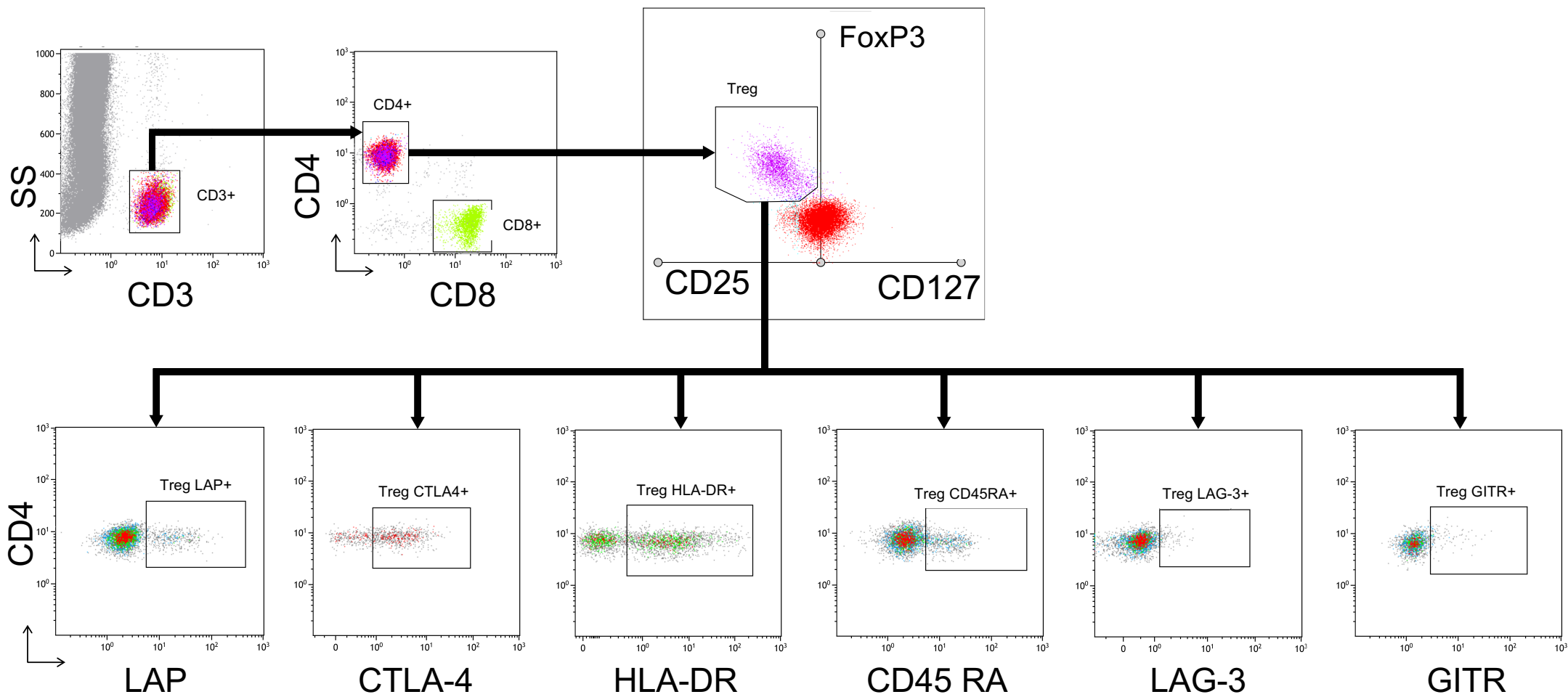


Figure 2

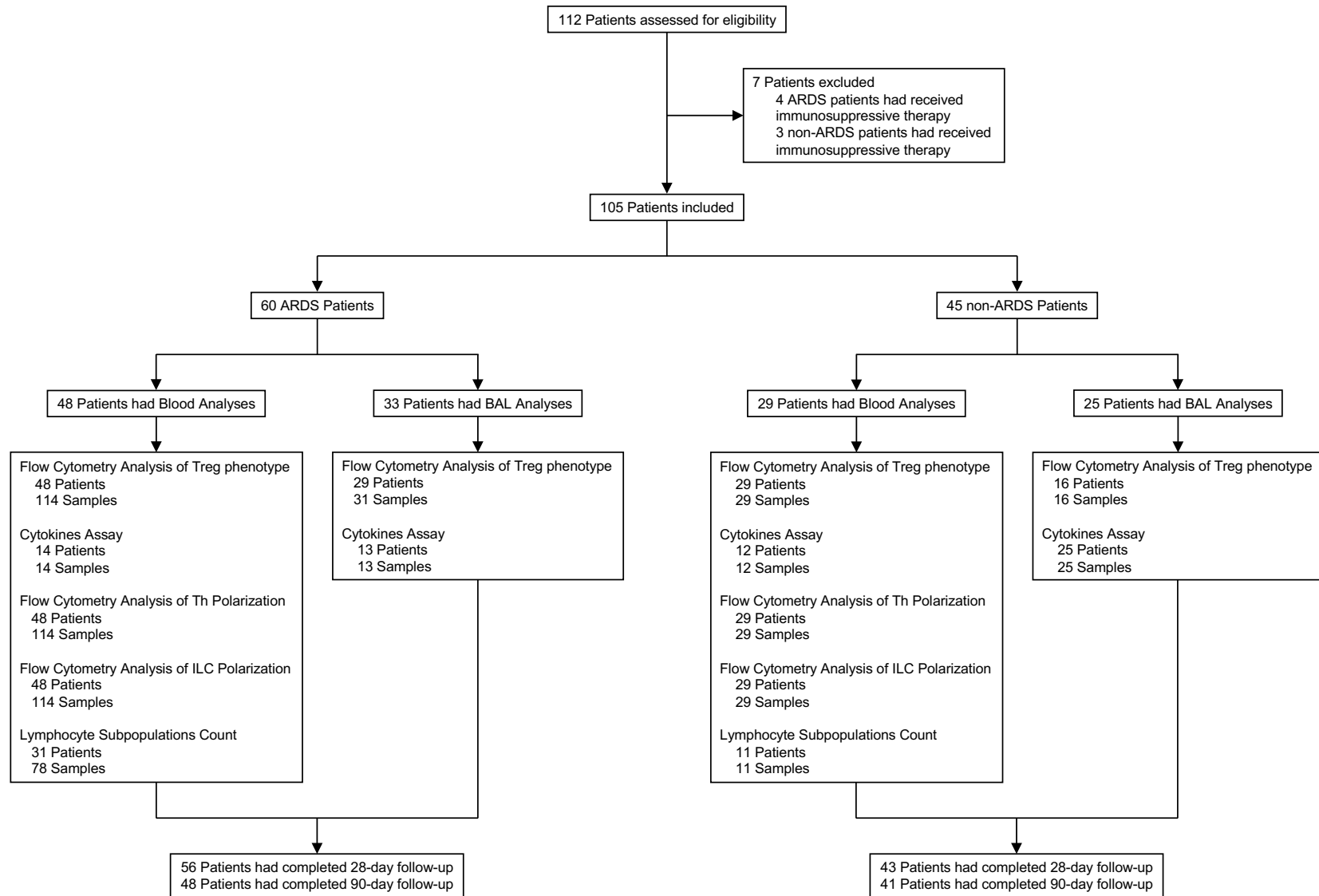
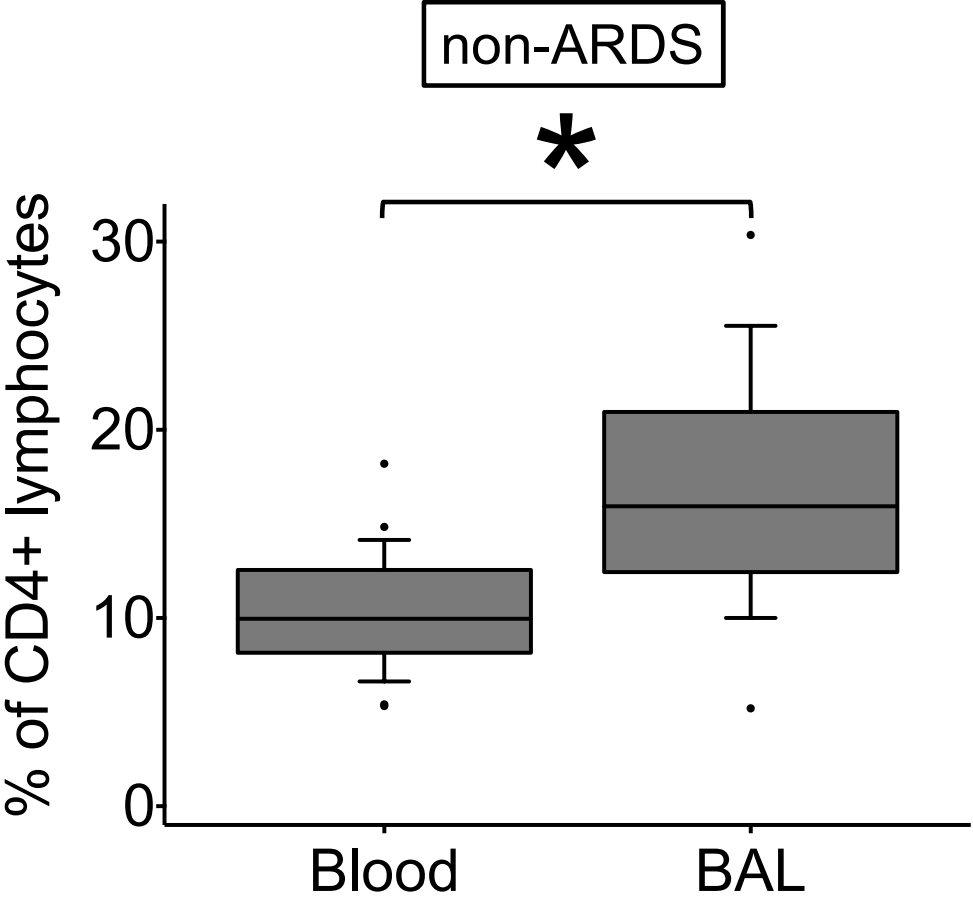
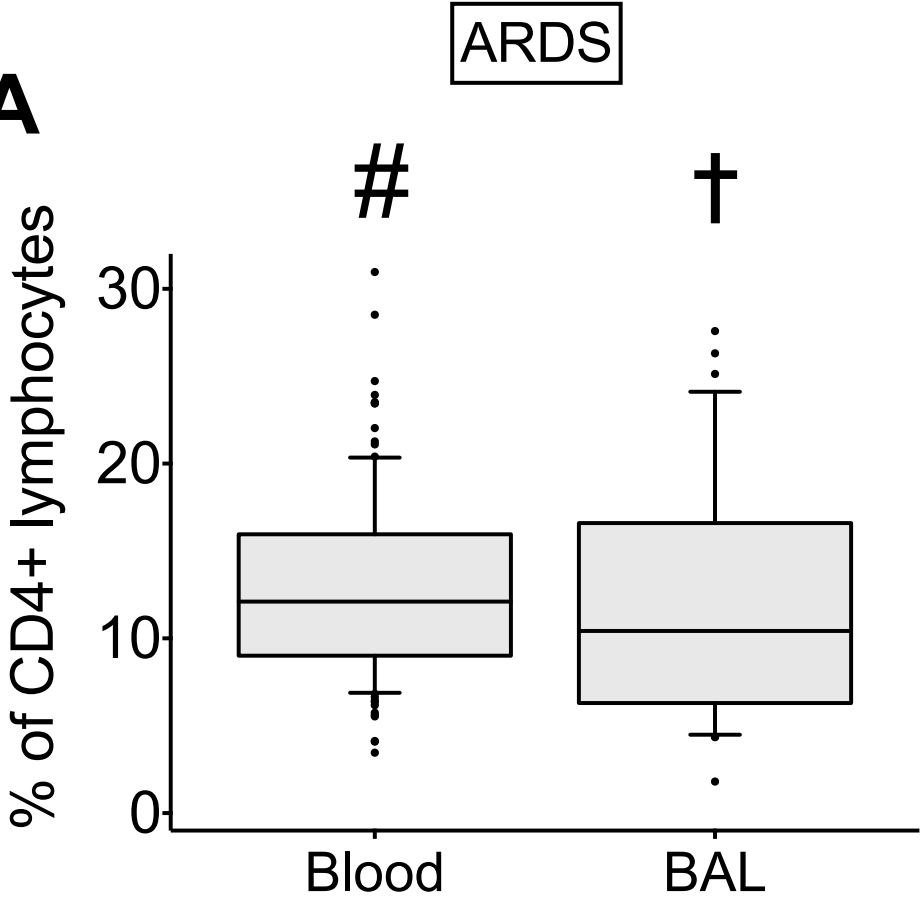


Figure 3A

**A**



**B**

Figure 3B

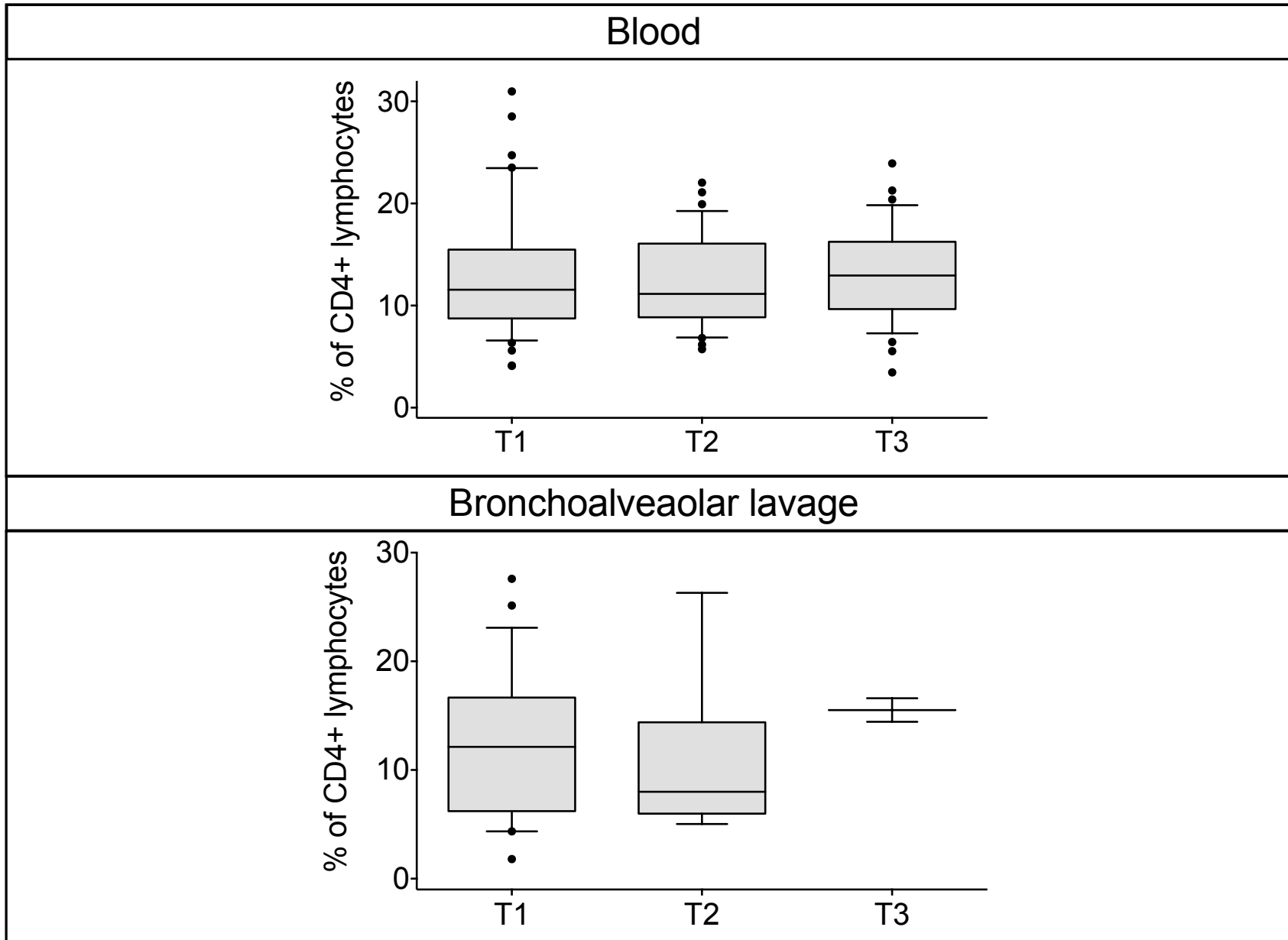


Figure 4

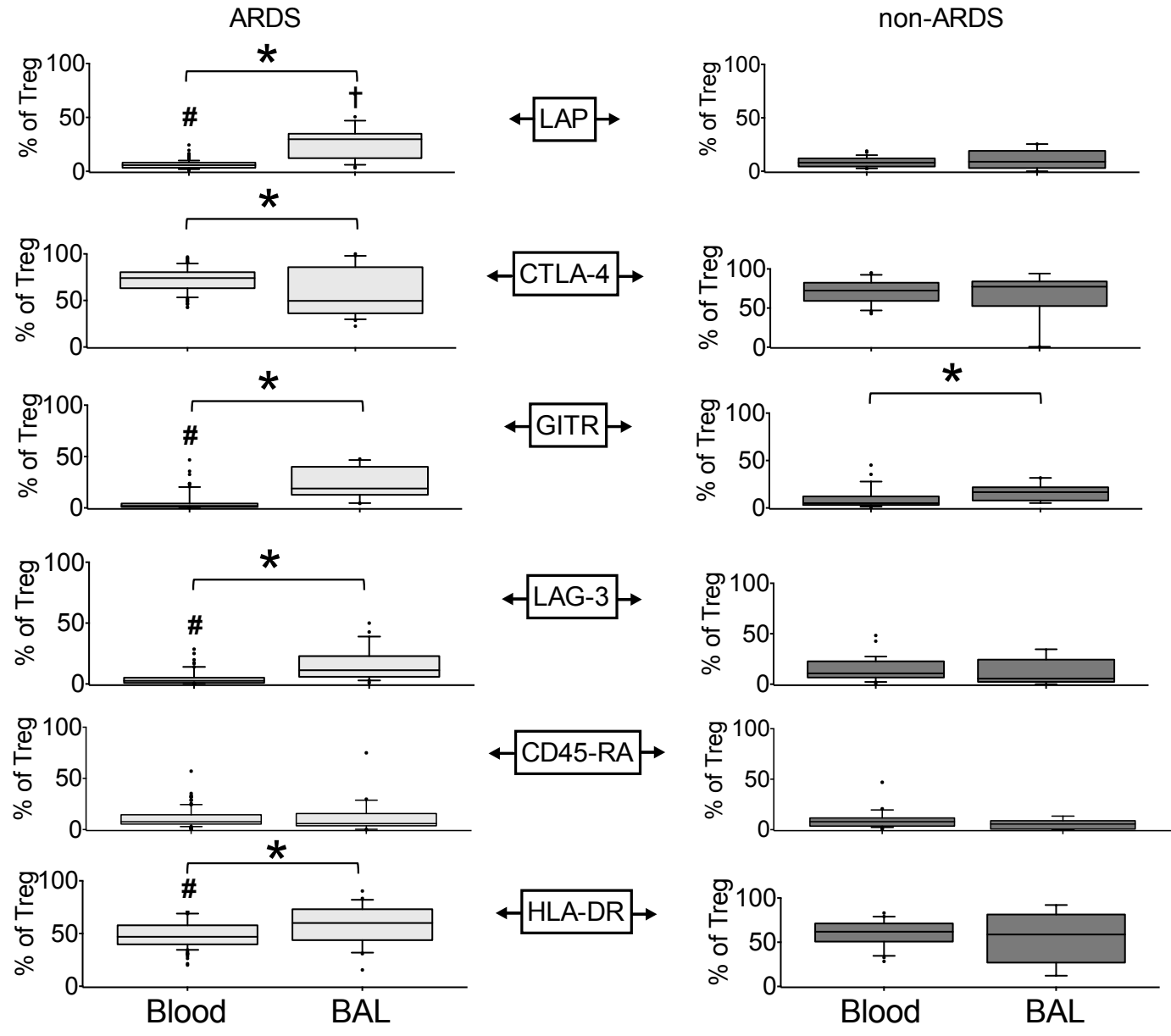




Figure 5

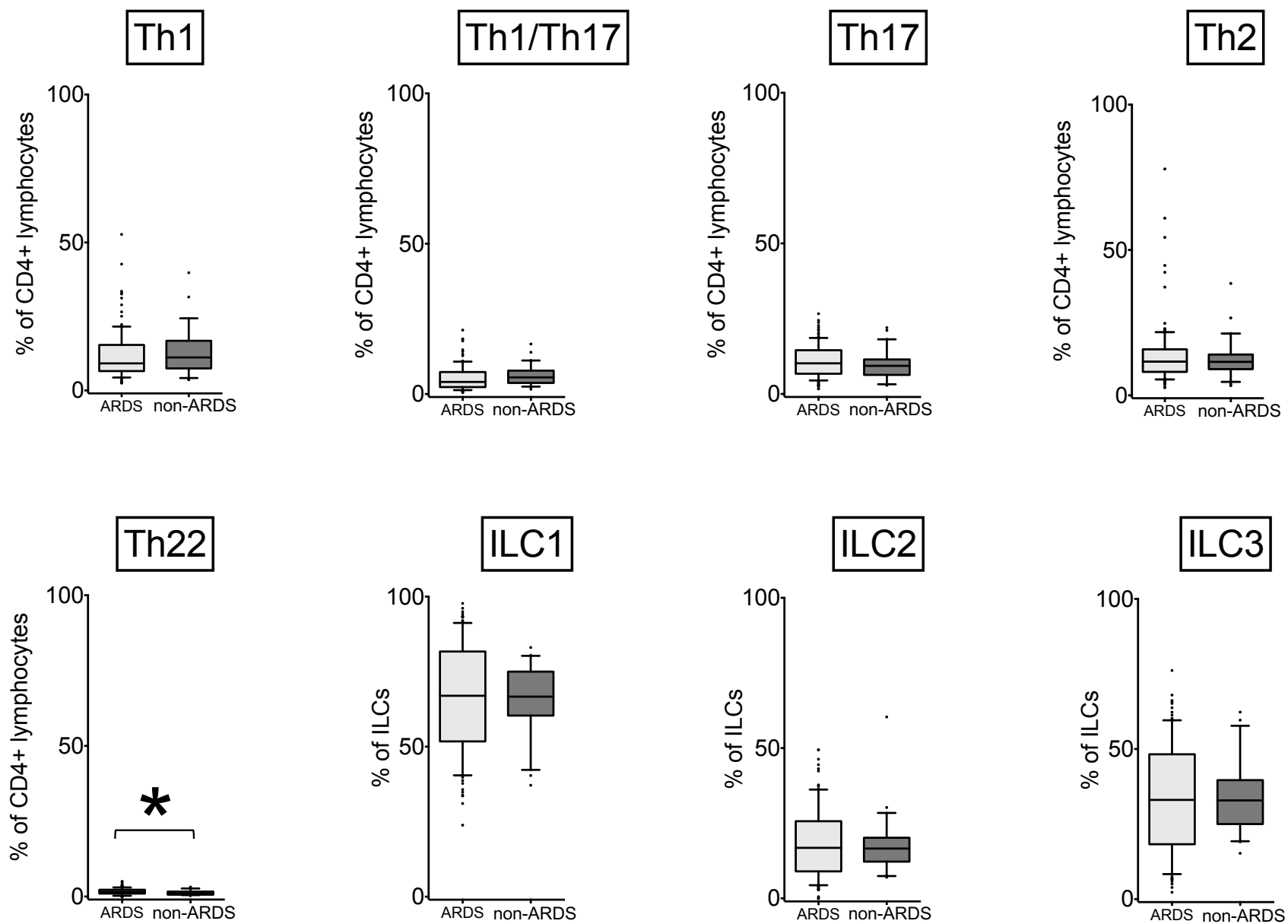


Figure 6

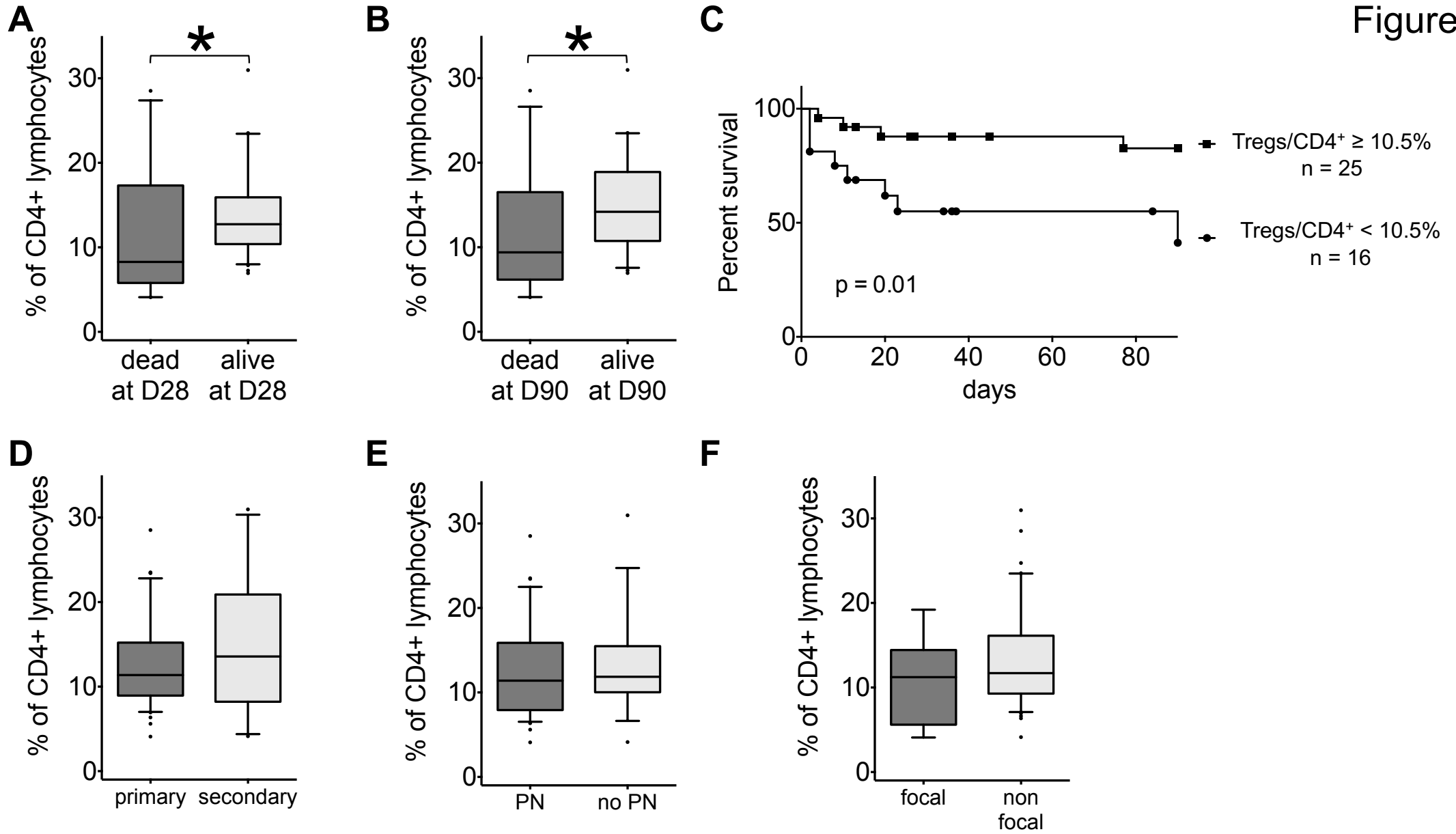


Table 1. Patient characteristics at ICU admission.

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
Corticosteroids	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 <sup>-3</sup> *
PaO <sub>2</sub> /FiO <sub>2</sub>	101.2 ± 60.1	295.1 ± 78.5	<10 <sup>-4</sup> *
Lung ultrasound score	18.0 ± 6.7	12.8 ± 5.4	<10 <sup>-4</sup> *
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<sub>2</sub> = partial arterial pressure in Oxygen ; FiO<sub>2</sub> = 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 <sub>2</sub> O)	8 [8–10]	
Driving pressure (cmH <sub>2</sub> O)	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<sub>2</sub> = partial arterial pressure in Oxygen ; FiO<sub>2</sub> = 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
<b>Th1 /Th17 / inflammation-related cytokines</b>						
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 [1.1–2.5]	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
<b>Th2-related cytokines</b>						
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 <sup>-4</sup> *	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
<b>Treg / anti-inflammatory cytokines</b>						
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 $\pm$ SD
Lymphocytes (cells/ $\mu$ L)	1176 $\pm$ 610.1
CD3+ lymphocytes (cells/ $\mu$ L)	876 $\pm$ 449.5
CD3+ lymphocytes (%)	74.6 $\pm$ 10.1
CD4+ lymphocytes (cells/ $\mu$ L)	583.3 $\pm$ 338.1
CD4+ lymphocytes (%)	49.1 $\pm$ 10.3
CD8+ lymphocytes (cells/ $\mu$ L)	299.1 $\pm$ 188.2
CD8+ lymphocytes (%)	25.7 $\pm$ 9.9
CD19+ lymphocytes (cells/ $\mu$ L)	162.9 $\pm$ 149.3
CD19+ lymphocytes (%)	14.2 $\pm$ 9.9
NK cells (cells/ $\mu$ L)	103.3 $\pm$ 95.1
NK cells (%)	8.2 $\pm$ 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/ $\mu$ L)	1136 [706.3–1521]	734.0 [590.0–1537]	0.5268
CD4+ lymphocytes (cells/ $\mu$ L)	512.5 [299.0–723.0]	416.0 [273.0–791.0]	0.4702
Regulatory T cells lymphocytes (cells/ $\mu$ L)	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.