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# An increased alveolar CD4 + CD25 + Foxp3 + T-regulatory cell ratio in acute respiratory distress syndrome is associated with increased 30-day mortality

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**Abstract** *Purpose:* Cell therapy may become an option for lung injury treatment. However, no data are available on the alveolar presence and time course of CD4+ CD25 + Foxp3 + T-regulatory lymphocyte cells (Tregs) in acute respiratory distress syndrome (ARDS). Accordingly, we (1) measured the ratio of CD4 + CD25 + Foxp3 + Tregs to all (CD4+) lymphocytes in the bronchoalveolar lavage (BAL) of ARDS patients and of control subjects without lung disease and (2) assessed their impact on 30-day mortality. *Methods:* In a prospective study, the ratios of CD4 + CD25 + Foxp3 + T-regulatory cells to all CD4+ cells were measured (FACS) within 24 h of the patients' ICU referral in the BAL and in the blood of 47 patients with ARDS (32 males, 15 females; mean age 44 years ±13) as well as in 8 controls undergoing elective abdominal surgery (5 men, 3 women; mean age 49 years ±4). BAL concentrations of several cytokines were also measured in ARDS patients. *Results:* Tregs were detected in the BAL of control subjects and ARDS patients. However, the mean ratio of Tregs to all CD4+ lymphocytes was threefold greater in ARDS non-survivors (16.5 %; $p = 0.025$) and almost twofold greater in ARDS survivors (9.0 %; $p = 0.015$) compared to controls (5.9 %). Multivariate Cox regression analysis revealed the ratio of CD4 + CD25 + Foxp3 + T-regulatory lymphocytes to all CD4+ lymphocytes in the BAL to be an important and independent prognostic factor for 30-day survival (HR 6.5; 95 % CI, 1.7–25; $p = 0.006$). *Conclusion:* An increased T-regulatory cell ratio in the admission BAL of patients with ARDS is an important and independent risk factor for 30-day mortality.

**Keywords** T-regulatory cell · ARDS · 30-Day mortality · Bronchoalveolar lavage · BAL cytokines · Pneumonia

## Introduction

The acute respiratory distress syndrome (ARDS), characterized by increased capillary and alveolar permeability, hypoxemia, decreased lung compliance, and diffuse bilateral pulmonary infiltrates [1, 2], remains an important cause of death.

However, due to advanced intensive care unit (ICU) treatments, including maintenance of gas exchange by extracorporeal membrane oxygenation (ECMO), many patients do not die during early ARDS but rather because of insufficient lung repair [3] and progression of lung injury to fibrosis, the latter indicating a poor prognosis [3]. Injury resolution is an active process [4]. However,

the mechanisms involved in the resolution of inflammation, avoidance of irreversible fibrosis, and reconstitution of alveolar endothelium and epithelium are still poorly understood. Recently, the potential role of stem cells and of regulatory cells has received attention, with enhancement of ARDS resolution by cell therapy being explored.

Specifically, T-regulatory cells (Tregs), a subset (CD4 + CD25 + Foxp3+) of CD4+ lymphocytes that express CD25 (IL-2 receptor α) as well as the transcription factor Forkhead box protein 3 (Foxp3) on their surface, have received much attention in an animal model of ARDS [5]. Tregs can suppress inflammatory [6], allergic, and autoimmune disorders [7] as well as inflammation in pulmonary aspergillosis [8] or pneumocystis pneumonia [9]; therefore, Tregs might participate in ARDS and/or its resolution. In mice with lung injury induced by intratracheal lipopolysaccharide (LPS), resolution was influenced by the presence or absence of Tregs [5]. While depletion of Tregs in wild-type mice delayed recovery from lung injury and lymphocyte-deficient recombinase activating gene–1–null (Rag-1–/–) mice [10] showed impaired resolution, this could be compensated for by intratracheal administration of isolated Tregs. Since the ratio of effector cells and suppressor cells maintains the response of the immune system [11], endogenous Tregs and the ratio of CD4 + CD25 + Foxp3 + T-regulatory cells (Tregs) to all (CD4+) lymphocytes might play an important role in lung injury and repair in humans. However, no data are available on the alveolar presence of Tregs in human ARDS or their course over time.

Accordingly, we (1) acquired data on the presence of Tregs in the alveolar space of ARDS patients and human volunteers and (2) tested the hypothesis that the ratio of CD4 + CD25 + Foxp3 + T-regulatory cells (Tregs) to all (CD4+) lymphocytes in the bronchoalveolar lavage (BAL) of patients with ARDS has an impact on 30-day mortality.

## Materials and methods

This study was reviewed and approved by the Ethics Committee of the Medical Faculty of University of Duisburg-Essen (protocol no. 01-97-1697). Over a period of 2 years, patients admitted to our ICU were considered eligible if they fulfilled the joint American/European Consensus Committee criteria for ARDS [12], had no previous history of ARDS, and informed consent had been obtained from the patients' guardians. All patients (32 males, 15 females, mean age 44 years ±13) were Germans of Caucasian ethnicity.

ARDS in all cases was caused by community-acquired pneumonia, and most patients were referred from other ICUs for possible ECMO therapy (rapid entry criteria) following rapidly progressive ARDS and fulminant pneumonia. Pneumonia was defined as primary lung infection from bacterial (23 patients) or H1N1 viral infection (24 patients). Four patients showed bacterial superinfection, i.e., in two patients with *Staphylococccus aureus*, in one patient with *Klebsiella pneumoniae*, and in another patient with a Bacillus species. Microbiologic diagnosis were established by Gram's stain, cultures, and/or PCR diagnostics of tracheal aspirate or BAL.

Clinical and demographic data, including the Lung Injury Score (LIS), Simplified Acute Physiology Score II (SAPS II) [13], body mass index, necessity for continuous hemofiltration/dialysis, and mean airway pressure were calculated over the first 24 h after the patient had been admitted with ARDS. Patients were treated with a multimodal concept which included analgosedation, fluid administration, protective mechanical ventilation, hemodynamic, antibiotic, and diagnostic management. Continuous hemofiltration/dialysis, if required, was performed according to standardized protocols. All patients were treated with hydrocortisone 100 mg/24 h. Clinical data of the ARDS cohort are presented in Table 1.

Patients were categorized as survivors if they were alive 30 days after the diagnosis of ARDS or when discharged from the hospital without mechanical ventilation.

### Controls without lung disease

Eight patients (5 men, 3 women; mean age, 49 years ±4) without lung disease undergoing elective extrapulmonary surgery (six patients underwent partial hepatectomy due to localized colorectal liver metastasis and two patients underwent ovarectomy due to ovarian cancer) served as controls following informed consent. They were free of lung, cardiac, infectious, and allergic disease, and were non-smokers. In these individuals, BAL for cell isolation and blood sampling was performed immediately following induction of anesthesia.

### Bronchoalveolar lavage

In patients with ARDS, a BAL was performed for diagnostic workup within 24 h of ICU admission during anesthesia/sedation in a lung segment showing consolidation and infiltration on chest X-ray. Additional BALs were performed for routine infectious disease workup on days 7, 14, and 21, unless the patient had died, did not require further diagnostics, or was discharged from the ICU. For BAL, four aliquots of warm (37 °C), sterile saline (40 ml) were instilled via a bronchoscope wedged into a segmental bronchus and gently withdrawn according to the American Thoracic Society Clinical Practice Guidelines [14]. To remove debris, the recovered volume was filtered through cotton gauze and stored in EDTA tubes (1.6 mg EDTA/ml recovered volume). The volume of BAL fluid recovered averaged 98 ± 24 ml in ARDS patients and 90 ± 21 in controls ($p = 0.16$).

**Table 1** Characteristics of patients with severe ARDS grouped for survival and non-survival

| Variable | Survivors ($n = 30$) | Non-survivors ($n = 17$) | $p$ value |
|---|---|---|---|
| Age (years)* | 42 ± 14 | 49 ± 8 | 0.07* |
| Male/female | 19/11 | 13/4 | 0.35* |
| BMI (kg/m$^2$)* | 29 ± 5 | 29 ± 5 | 0.9* |
| CD4 + CD25 + FoxP3 + absolute cell count per µl blood | 13 ± 18 | 9 ± 13 | 0.47 |
| CD4 + CD25+ FoxP3 + absolute cell count per µl BAL | 1.2 ± 2.5 | 1.6 ± 3.4 | 0.65 |
| Leukocytes in bronchoalveolar lavage (cell count per µl) | 537 ± 862 | 1003 ± 1447 | 0.15* |
| Lymphocytes in bronchoalveolar lavage (cell count per µl) | 20.3 ± 9.3 | 18.7 ± 10.8 | 0.5* |
| Bronchoalveolar lavage (cell count per µl) | $63^6 \pm 10^6$ | $87^6 \pm 19^6$ | 0.35* |
| Recovery volume (ml) | 98 ± 24 | 96 ± 31 | 0.16* |
| LDH in bronchoalveolar lavage (U/l) | 51 ± 57 | 272 ± 458 | 0.009° |
| Albumin concentration in bronchoalveolar lavage (mg/ml) | 0.34 ± 0.39 | 1.15 ± 1.87 | 0.012° |
| Protein concentration in bronchoalveolar lavage (mg/ml) | 0.78 ± 0.8 | 2.43 ± 3.8 | 0.06* |
| Transforming growth factor beta (pg/ml) | 42 ± 78 | 20 ± 43 | 0.25° |
| Interleukin 6 (pg/ml) | 845 ± 2642 | 839 ± 1727 | 0.9° |
| Interleukin 2 receptor alpha (pg/ml) | 121 ± 314 | 69 ± 181 | 0.5° |
| Tumor necrosis factor-alpha (pg/ml) | 9.7 ± 17 | 12.8 ± 24 | 0.6° |
| Interleukin 4 (pg/ml) | 2.7 ± 5.6 | 1.5 ± 1.0 | 0.35° |
| Interleukin-12p70 (pg/ml) | 3.5 ± 9.6 | 1.1 ± 0.4 | 0.3° |
| Interleukin 10 (pg/ml) | 20 ± 93 | 40 ± 161 | 0.5° |
| Interferon alfa-2b (pg/ml) | 106 ± 614 | 2 ± 0.5 | 0.47° |
| Interferon beta (pg/ml) | 11.3 ± 26.8 | 5.1 ± 5 | 0.1° |
| Interferon gamma (pg/ml) | 41.3 ± 190 | 70 ± 295 | 0.7° |
| Interleukin-1 beta (pg/ml) | 28.5 ± 70 | 77 ± 167 | 0.1° |
| PO2/FiO2 ratio (mmHg) | 180 ± 29 | 127 ± 29 | 0.09* |
| Mean airway pressure (mbar) | 27 ± 4 | 27 ± 3 | 0.7* |
| ECMO therapy (yes/no) | 9/21 | 7/10 | 0.4# |
| Lung injury score | 3 ± 0.6 | 3.2 ± 0.5 | 0.24* |
| CVVHD (yes) | 30 | 17 | 0.9* |
| C-reactive protein concentration (mg/dl)* | 13 ± 9 | 15 ± 10 | 0.4* |
| Procalcitonin concentration (ng/ml)* | 13 ± 31 | 40 ± 60 | 0.1* |
| SAPS II score* | 44 ± 22 | 51 ± 16 | 0.3* |
| Bacterial isolates in BAL | 13 | 10 | 0.4# |
| H1N1 isolates | 17 | 7 | |

Data are presented as frequency or mean ± SD
*BAL* bronchoalveolar lavage, *BMI* body mass index, *CVVHD* continuous hemofiltration/dialysis, *ECMO* extracorporal membrane oxygenation, *SAPS II* Simplified Acute Physiology Score, *H1N1* influenza A virus H1N1

* $p$ value based on Student's $t$ test
\# $p$ values based on chi-square tests
° $p$ value based on Mann-Whitney $U$ test

## Blood samples

To detect Tregs in the blood, venous blood was drawn into EDTA tubes from all ARDS patients and controls. To separate supernatant (plasma) from cell pellet blood was centrifuged (500 g, 10 min, 5 °C). BAL and blood samples were performed at the same time.

## Measurements

Albumin concentration in the BAL was measured using an albumin ELISA kit (Dade Behring, Marburg, Germany). The detection limit was 1.18 mg/ml.

The total protein concentration was determined after trichloroacetic acid precipitation (5 %), washing, and resolubilization using an autoanalyzer (Technicon, Oakland, CA) and with bovine serum albumin (BSA) as standard. Total LDH activity (LDH1–LDH5) in the BAL was measured by a kinetic UV test (Diaglobal GmbH, Berlin, Germany) using an optimized standard method (IFCC). Procalcitonin (PCT) and C-reactive protein (CRP) concentrations in blood serum were measured via an immunoassay (*ADVIA Centaur XP-Systeme,* Siemens Healthcare Diagnostics GmbH, Eschborn, Germany).

Cytokine concentration in the BAL was performed with a Procarta Cytokine assay kit (Panomics, Fremont, CA) according to the manufacturer's recommendations. The assay was run with a Luminex 200 instrument using the Luminex IS software (Luminex Corp., Austin, TX).

## FACS analysis of bronchoalveolar and blood lymphocytes

Within 3 h of BAL we performed FACS analysis using a FACS-Calibur flow cytometer (Becton–Dickinson, San Jose, CA) and a Treg Detection Kit human (Miltenyi

Biotec, Bergisch Gladbach, Germany). This kit contains FITC-conjugated CD4 antibody and PE-conjugated CD25 antibody for surface staining and APC-conjugated FoxP3 antibody for intracellular staining.

First, peripheral blood and BAL samples were incubated with CD4-FITC and CD25-PE antibodies for 10 min at 4 °C. Then cells were fixed, permeabilized, and stained with the anti-FOXP3-APC antibody for 30 min at 4 °C.

Gating was performed as follows: autofluorescent debris was excluded using forward and side scatter. Gating of CD25 and FoxP3 positive cells was performed according to CD4-positive cell expression. Isotype controls characterized the position of the quadrants (see Fig. 1), as described [15]. Dot plots showed the percentage of CD25 and FOXP3-coexpressing CD4-positive cells.

## Statistical analysis

Statistical analyses were performed using SPSS (version 11.0, Chicago, IL, USA). Continuous variables are presented as mean ± standard deviation (SD). If not normally distributed, data are presented as median and range.

The clinical endpoint was mortality over the first 30 days dependent on the ratio in the BAL of CD4 + CD25 + Foxp3 + Treg cells to all CD4+ cells. Comparison of values of variables between groups (ARDS survivors versus non-survivors and controls) was performed using the Mann-Whitney *U* test. Repeated measurements over time and groups were tested by a two-way ANOVA followed by post hoc testing with the Bonferoni Holm test. Association between the ratio of Treg cells to all CD4+ cells in the BAL and the ratio of Treg cells to all CD4+ cells in the blood and correlation to BAL cytokine concentrations were tested with a linear regression model.

In a second step, a receiver-operating curve analyses was performed and revealed a cutoff ratio of 7.4 % of Treg cells to all CD4+ cells to discriminate between survivors and non-survivors ($p = 0.004$). Afterwards, univariate Kaplan-Meier plots and the log-rank test for trend were used to evaluate the relationship between patients demonstrating ratios of <7.4 vs. ≥7.4 % and 30 day mortality. In a last step, we tested by stepwise binary logistic regression analysis including gender, SAPS II score, C-reactive protein and procalcitonin concentrations, LIS, body mass index, necessity for continuous hemofiltration/dialysis or ECMO therapy, mean airway pressure, and the ratio in the BAL of Treg cells to all CD4+ cells (categorized as <7.4 versus ≥7.4 %) whether the Treg cell ratio is an independent risk

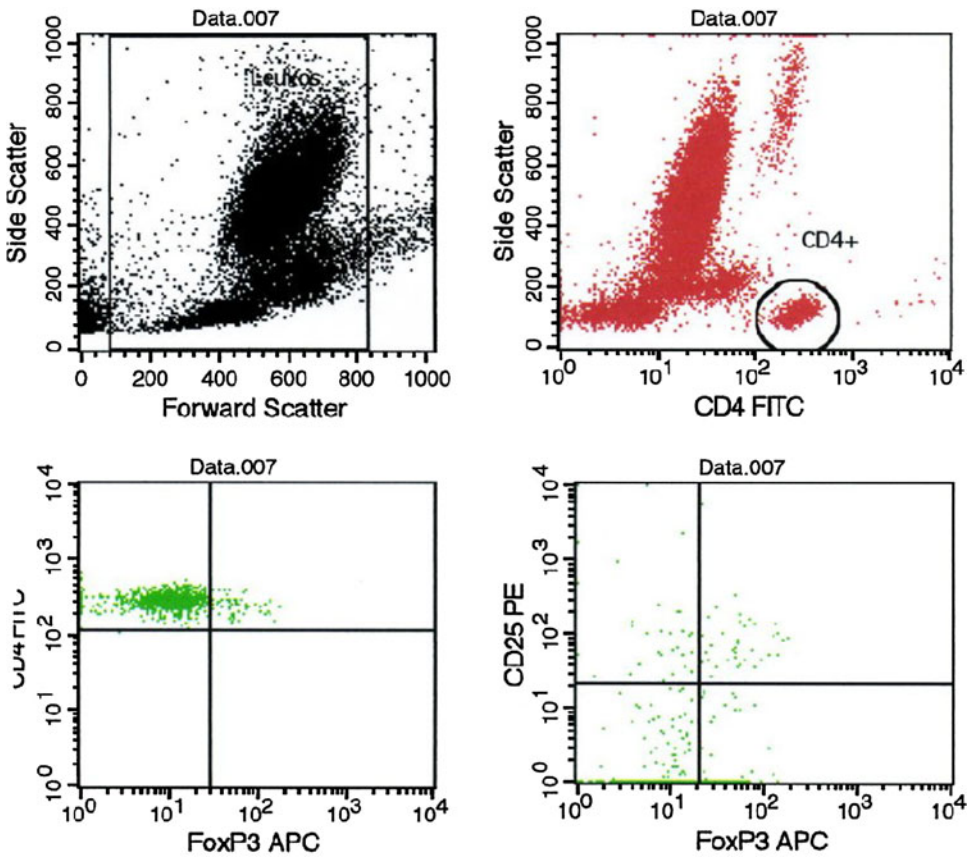


**Fig. 1** Representative FACS analysis with staining of CD25/FoxP3-coexpressing CD4+ T cells. The upper left panel shows a scatter plot where a forward versus a side scatter is used to exclude debris. As shown in the upper right panel, we gated the CD4-positive cells (*circle*) as a subpopulation of leukocytes. As depicted in the lower left panel, we checked whether the gate was set correctly. The upper right quadrant of the latter panel shows the subpopulation of CD4+ lymphocytes coexpressing FoxP3. Eventually, as depicted in the lower right panel we examined only CD4-positive cells (shown in the *circle* of the upper right panel) to detect CD25 and FoxP3 coexpression, as shown in the upper right quadrant of the lower right panel

**Table 2** Correlations between the CD4 + CD25 + Foxp3 + Tregs to CD4+ lymphocyte ratio and the cytokine concentrations in the bronchoalveolar lavage of patients with ARDS

| | Pearson's correlation coefficient | *p* value |
|---|---|---|
| Transforming growth factor beta (pg/ml) | 0.83 | 0.5 |
| Interleukin 2 receptor alpha(pg/ml) | 0.7 | 0.0001 |
| Interleukin-12p70 (pg/ml) | 0.22 | 0.09 |
| Interleukin 4 (pg/ml) | −0.021 | 0.9 |
| Interleukin 10 (pg/ml) | 0.32 | 0.01 |
| Tumor necrosis factor-alpha (pg/ml) | 0.7 | 0.0001 |
| Interleukin 6 (pg/ml) | −0.17 | 0.2 |
| Interferon alfa-2b (pg/ml) | 0.043 | 0.001 |
| Interferon beta (pg/ml) | 0.185 | 0.2 |
| Interferon gamma (pg/ml) | 0.47 | 0.0001 |
| Interleukin-1 beta (pg/ml) | −0.049 | 0.7 |

factor for 30-day mortality. To avoid overfitting, a restricted model with only three variables was assessed afterwards, using only those predictors with a $p$ value of $\leq 0.1$ in the restricted multivariate comparison (Table 2).

Differences were regarded as statistically significant with an a priori alpha error $p$ of $<0.05$.

## Results

Tregs were detected in both the BAL of controls without lung disease and in patients with ARDS. The mean ratio of Treg cells to all CD4+ lymphocytes in patients suffering from ARDS was almost threefold higher in non-survivors (16.5 %; $p = 0.025$) and almost twofold higher in survivors (9 %; $p = 0.015$) in comparison to controls (5.9 %), as depicted in Fig. 2. While the ratio of Treg cells to all CD4+ cells in the BAL proved to be different among survivors, non-survivors, and controls, the ratio of Treg cells to all CD4+ cells in the blood was not associated with 30-day survival in patients with ARDS. Furthermore, it did not differ between patients with ARDS ($2.6 \pm 0.5$ %) and controls ($1.5 \pm 0.4$ %). The ratio of Tregs to all CD4+ cells in the BAL was weakly associated with the ratio of Tregs to all CD4+ cells in the blood ($p = 0.024$, $r^2 = 0.32$) but not with the SAPS score. Of note, the ratio of Tregs to all CD4+ cells in the BAL was not associated with the bronchoalveolar LDH activity or albumin concentration. Furthermore, the ratio of Tregs to all CD4+ cells in the BAL was associated with interleukin 2 receptor alpha ($r = 0.7$, $p = 0.0001$), tumor necrosis factor alpha ($r = 0.7$, $p = 0.0001$), interleukin 10 ($r = 0.32$, $p = 0.01$), interferon alpha-2b

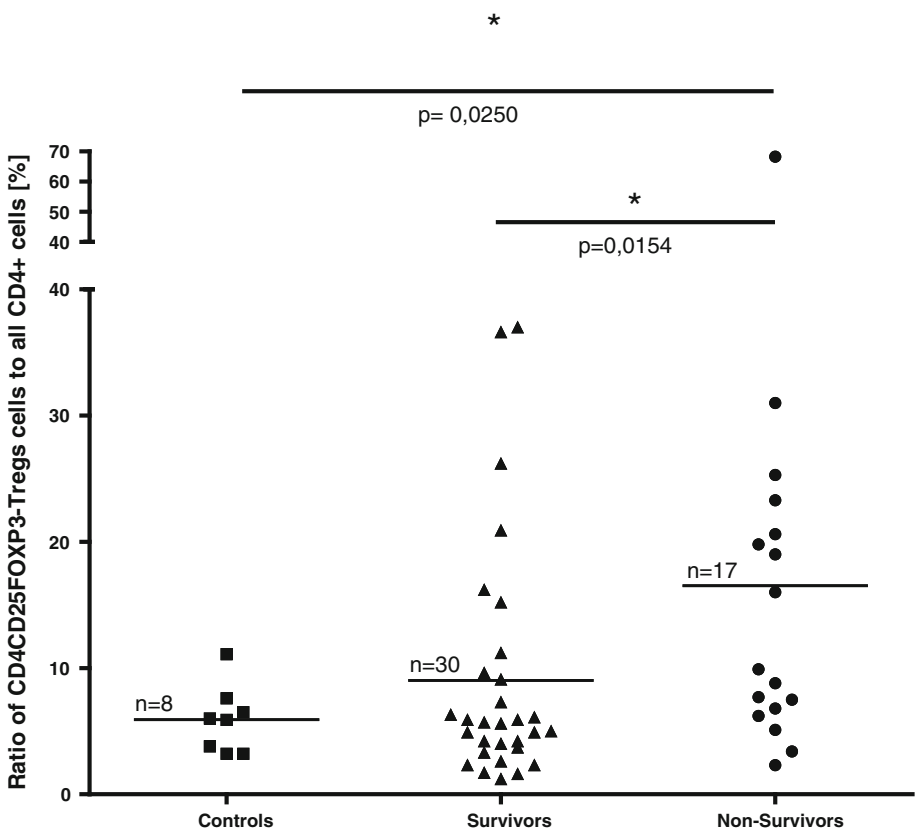


**Fig. 2** CD4 + CD25 + Foxp3 + Tregs to CD4+ lymphocyte ratio in the bronchoalveolar lavage (BAL) of controls and of patients surviving or dying from the acute respiratory distress syndrome (ARDS). BALs were obtained within 24 h of the patient admission. Tregs were detected in both controls and ARDS patients. Although the ratios show substantial overlap, the mean ratio of Tregs to all CD4+ cells was greater in ARDS non-survivors than in ARDS survivors or in controls

($r = 0.43$, $p = 0.001$), and interferon gamma ($r = 0.47$, $p = 0.0001$, Table 2).

The SAPS II score, CRP, PCT, LIS, mean airway pressure, body mass index, and necessity for continuous hemofiltration/dialysis or ECMO therapy, BAL cell count or microbiological etiology was not different between survivors and non-survivors. Furthermore, the absolute Treg cell count in the blood ($p = 0.47$) and in the bronchoalveolar lavage ($p = 0.65$) of patients with ARDS was not different between survivors and non-survivors, as depicted in Table 1.

ROC analysis revealed a cutoff ratio of 7.4 % for the Treg cell to all CD4+ cell ratio in the initial BAL to discriminate between survivors and non-survivors (sensitivity: 69 %; specificity 75 %; area under the curve: 0.72). Thirty-day survival was 81 % for patients with a ratio of less than 7.4 % but only 43 % for patients with a ratio of 7.4 % or greater ($p = 0.005$, Fig. 3).

Multivariate Cox regression analysis including as covariates gender, SAPS II score, CRP, LIS, body mass index, necessity for continuous hemofiltration/dialysis or ECMO therapy, and mean airway pressure revealed the ratio of Treg cells to all CD4+ cells in the initial BAL as a major, important, and independent prognostic factor for 30-day survival (HR 6.5; 95 % CI, 1.7–25; $p = 0.006$, Table 3).

The microbiological etiology of pneumonia was not associated with the ratio of Treg cells to all CD4+ cells in the BAL of patients with ARDS. Furthermore, there was no difference in the mean ratio of Treg cells to all CD4+ cells in survivors with bacterial lung infection (9 ± 10 %) compared to survivors with H1N1 infection (9 ± 10 %) or the mean ratio of Treg cells to all CD4+ cells in non-survivors with bacterial lung infection (14 ± 8 %) compared to non-survivors with H1N1 lung infection (20 ± 23 %).

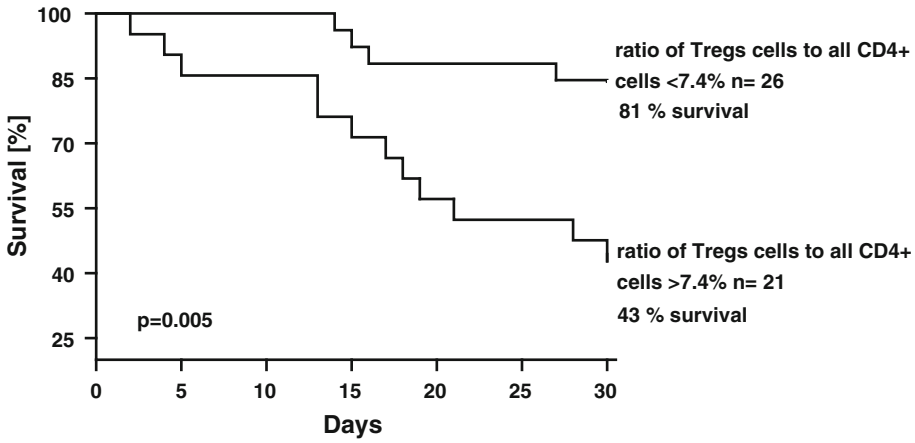


**Fig. 3** Kaplan-Meier analysis of patients with acute respiratory distress syndrome (ARDS) depending on the initial CD4 + CD25 + Foxp3 + (Treg) cell to CD4+ lymphocyte ratio in their bronchoalveolar lavage (BAL). Survival was 81 % in ARDS patients with a ratio of less than 7.4 % but only 43 % in those with a ratio of 7.4 % or greater ($p = 0.005$)

**Table 3** Cox regression analysis in patients with ARDS

| (Co) variable | Multivariate | | | |
|---|---|---|---|---|
| | Initial | | Restricted | |
| | Hazard ratio (95 % CI) | *p* value | Hazard ratio (95 % CI) | *p* value |
| Treg/CD4+ cell ratio <7.4 % | 1 | – | 1 | – |
| Treg/CD4+ cell ratio ≥7.4 % | 15 (2–103) | 0.005 | 6.5 (1.7–25) | 0.006 |
| Sex | 4.33 (0.7–24) | 0.095 | 2.2 (0.5–8.7) | 0.26 |
| SAPS II score (per unit) | 1.0 (0.8–1.01) | 0.8 | – | – |
| Lung injury score | 2.5 (0.3–20) | 0.38 | – | – |
| ECMO (no) | 1 | – | – | – |
| ECMO (yes) | 1.01 (0.025–1.2) | 0.069 | 0.3 (0.1–1.1) | 0.053 |
| CVVHD | 0.9 (0.2–3.7) | 0.9 | – | – |
| BMI | 1.01 (0.9–1.2) | 0.6 | – | – |
| C-reactive protein concentration (per $\log_{10}$(mg/dl)) | 1.01 (0.9–1.1) | 0.49 | – | – |
| Procalcitonin concentration (per $\log_{10}$(ng/ml)) | 1.01 (0.025–1.2) | 0.8 | – | – |

Hazard ratio point estimates, 95 % CI, and *p* values (two-sided) from Wald tests are reported
*BMI* body mass index, *CVVHD* continuous hemofiltration/dialysis (), ECMO extracorporeal membrane oxygenation, *SAPS II* Simplified Acute Physiology Score

## Discussion

This study is the first, to our knowledge, to test whether the Treg cell ratio in the BAL is associated with altered survival in patients with severe ARDS. We demonstrated that the mean ratio of Tregs to all CD4+ lymphocytes is threefold higher in ARDS non-survivors and almost twofold higher in survivors when compared to controls. In contrast to these findings in the alveolar space, the ratio of Tregs to all CD4+ cells in the blood was not associated with 30-day survival in patients with ARDS and did not differ between patients with ARDS and controls.

While individual values showed a wide scatter, the ratio of Treg cells to all CD4+ cells in the BAL of ARDS patients was an important and independent risk factor for 30-day mortality. Patients with a ratio of Treg cells to all CD4+ cells above 7.4 % showed a twofold greater 30-day mortality when compared to patients with a ratio of Treg cells to all CD4+ cells with less than 7.4 %. Accordingly, our data show that an initial high ratio of Treg cells to all CD4+ cells in the bronchoalveolar lavage of patients with ARDS is associated with a much worse outcome.

During the past decade, there have been many investigations addressing the potential function of T-regulatory cells in human disease [7]. First recognized for their ability to suppress autoimmunity, T-regulatory cells mediate effector CD4+ and CD8+ T-lymphocytes and impact on the innate immune system by several mechanisms like cell-to-cell contact, secretion of inhibitory cytokines, and cytolysis [16]. However, the suppressive function of T-regulatory cells might also contribute to the immune dysfunction often seen in later stages of sepsis or ARDS. This in turn may promote repetitive nosocomial infections and could contribute to insufficient lung repair and fibrosis. This speculation is supported by the correlation of the concentration of the anti-inflammatory cytokine IL10 with the ratio of Tregs to all CD4+ cells in the BAL of the ARDS patients. The fact that this ratio also correlates with the tumor necrosis factor alpha concentration in the BAL suggests that the level of the inflammatory stimulus also regulates the strength of the ensuing anti-inflammatory reaction and hence the ratio of Tregs to all CD4+ cells in the BAL. Thus, we speculate that the stronger the inflammatory reaction is, the greater the proliferation of the anti-inflammatory CD4 + CD25 + Foxp3 + T-regulatory lymphocytes cells, which promotes repetitive nosocomial infections and therefore increased mortality.

On first sight, our data appear to contradict the observations of D'Alessio et al. [5] showing that resolution of lung injury is accelerated by the presence of Tregs in mice undergoing intratracheal LPS application. Lymphocyte-deficient recombinase-activating gene-1-null mice [10] showed considerable impairment of injury resolution, and this was compensated for by intratracheal administration of isolated Tregs. In addition, depletion of Tregs in wild-type mice delayed recovery from lung injury. Also, in chronic infection models of mice with pulmonary aspergillosis [8] or pneumocystis pneumonia [9], an increase to 4 % of the ratio of Tregs to all CD4+ cells in the bronchoalveolar lavage improved pulmonary inflammation and lung injury and diminished reinfection. Thus, an increased Treg ratio appears to be beneficial in animal models using mice.

Our clinical data in humans may suggest that investigations in knockout mouse LPS models may not be that relevant for humans with infectious disease and ARDS derived from pneumonia [17–20]. In fact, studies support the speculation of a dose-dependent dual role of Tregs in lung immunity. Thus, a decreased percentage of Treg cells by <3.2 % in the BAL obtained from lung transplant recipients correlates with the development of bronchiolitis obliterans syndrome [21]. On the other hand, Sharma et al. [22] found an enrichment of Treg cells, with a ratio of Treg cells to all CD4+ cells as great as 20–95 %, in the BAL of patients with miliary tuberculosis. Additionally, they noted that the increased ratio of Treg cells led to suppressed function of T-effector cells via IL 10 and that this was associated with increased disease severity and worse outcome [22].

However, what the optimum ratio of Tregs to all CD4+ cells in the BAL of patients may be is unclear. Our data show a wide range of the ratio in controls as well as patients with ARDS. This is similar to data obtained in patients with COPD [23] and in healthy controls showing a wide range (2–45 %) of the ratio of Treg cells to all CD4 cells. This marked overlap of the Treg ratios in controls, ARDS, and COPD patients may suggest high interindividual variability. Accordingly, quantification of Treg cells in the BAL to discriminate between pulmonary health and the degree of disease in general may prove difficult. Nevertheless, in patients with ARDS, our study shows for the first time that patients with a Treg ratio of more than 7.4 % have a greater 30-day mortality. Furthermore, in a multivariate analysis an increased ratio of Treg cells to all CD4+ in the BAL of patients with pneumonia-evoked ARDS was an independent risk factor for 30-day mortality. While our findings should be confirmed in other cohorts, these data should stimulate research on alveolar cell populations and the development of innovative cell therapies targeted at rebalancing the lung's immune response in ARDS.

Studies in patients with severe sepsis support the hypothesis that increased Treg concentrations in the blood may have deleterious consequences in severe inflammatory disease [24]. Hein et al. [25] in patients with shock but without sepsis found a mean ratio of Tregs to all CD4+ cells of 8 %, but a ratio of 12.5 % in patients with septic shock. Of note, in healthy controls the ratio of Tregs to all CD4+ cells was also 8 %. Additionally, the ratio of Tregs to all CD4+ cells in the blood of patients

with septic shock was inversely correlated with the SOFA score ($r^2 = -0.66$) and non-survivors showed a twofold higher ratio (10 %) in comparison to survivors (5 %). Furthermore, Raftogiannis et al. [26] reported that H1N1-related pneumonia is associated with an early increase of circulating Treg cells in the blood.

In our patients with ARDS, however, the ratio of Tregs to all CD4+ cells in the blood overlapped among controls, survivors, and non-survivors and were not statistically different between groups. Furthermore, the mean ratio of Tregs to all CD4+ cells in the blood of our patients was twofold lower than in the cohort of Hein et al. [25] and did not correlate with the SAPS score. Thus, ratios of Tregs to all CD4+ cells in patients with ARDS, even when evoked by pneumonia, appear to differ from those with sepsis. Furthermore, our results indicate that in patients with ARDS Treg cell measurements in the blood are not representative for those in the alveolar space.

Our investigation also demonstrates that the microbiologic etiology of pneumonia-evoked ARDS did not influence the Tregs to CD4+ ratio since the mean ratio in patients with bacterial and H1N1-evoked pneumonia was not different. This is consistent with the hypothesis that the severity of inflammation affects the production of anti-inflammatory cytokines [27] such as IL-10 and TGF-beta and therefore the Treg cell ratio [28], irrespective of microbiologic etiology.

Limitations of this report should be mentioned. Unrecognized selection bias, inherent even to prospective studies, cannot be excluded. Moreover, although all ARDS patients were treated with a rather standardized multimodal regimen, due to the multifactor nature of this disorder, we cannot exclude that unknown potentially confounding factors exist. Furthermore, enrolled patients represent a selected cohort of ARDS patients since only those with ARDS evoked by CAP were included. Thus, results may not be representative for ARDS of other origin. In addition, the fact that patients taken as controls suffered from solid tumors may be problematic and could have altered the ratios of Tregs to all CD4+ cells in some lung healthy controls. However, the controls were free of lung, cardiac, infectious, or allergic disease, and BAL for cell isolation was performed immediately following induction of anesthesia assuming that these BALs approximate the BAL of healthy subjects. Furthermore, using a control group with normal lungs may not take into account potential alveolar cell responses to mechanical stress independent of those resulting from CAP-evoked ARDS alone. On the other hand, mechanical ventilation in patients without pneumonia and/or ARDS is unlikely to evoke alveolar cell stress and damage unless deliberately ventilated with large tidal volumes. Thus, a control group looking at effects of cellular stress evoked by mechanical ventilation only is hard to find.

Nevertheless, although the study cohort may be considered small and the control group may not be ideal, multivariate Cox regression analyses revealed the ratio of Tregs to all CD4+ cells to be an important and strong independent risk factor for 30-day mortality. This underscores the potential relevance of Treg cells in severe ARDS, regardless of the potential mechanisms involved.

In summary, this study shows that an increased Tregs to CD4+ ratio in the BAL of patients with ARDS is associated with 30-day mortality. These data highlight the potentially profound impact of T-regulatory cells on ARDS and its resolution and should, therefore, stimulate further research on alveolar cellular mechanisms in human ARDS.

**Conflicts of interest** None.

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