ORIGINAL ARTICLE

Increased frequency of $CD4^+CD25^{high}CD127^{low}$ T cells early after lung transplant is associated with improved graft survival – a retrospective study

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SUMMARY

In this retrospective study, we analyzed the presence of any association of three $CD4^+CD25^{high}$ regulatory T-cell subpopulations at 3 weeks after lung transplantation with the later incidence of chronic lung allograft dysfunction and graft survival. Among lung-transplanted patients between January 2009 and April 2018, only patients with sufficient T-cell measurements at 3 weeks after transplantation were included into the study. Putative regulatory T cells were defined as $CD4^+CD25^{high}$ T cells, detected in peripheral blood and further analyzed for $CD127^{low}$, $FoxP3^+$, and $CD152^+$ using fluorescence-activated cell sorting (FACS) analysis. Associations of regulatory T cells with chronic lung allograft dysfunction (CLAD) and graft survival were evaluated using Cox analysis. During the study period, 724 (71%) patients were included into the study. Freedom from chronic lung allograft dysfunction (CLAD) and graft survival amounted to 66% and 68% at 5 years. At the multivariable analysis, increasing frequencies of $CD127^{low}$ were associated with better freedom from CLAD (hazard ratio for each 1% increase of $\%CD127^{low}$, HR = 0.989, 95% CI = 0.981–0.996, $P = 0.003$) and better graft survival (HR = 0.991, 95% CI = 0.984–0.999, $P = 0.026$). A higher frequency of $CD127^{low}$ regulatory T cells in peripheral blood early after lung transplantation estimated a protective effect against chronic lung allograft dysfunction, mortality, and re-transplantation.

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Key words
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Introduction

Long-term survival after lung transplantation is impaired by the development of chronic lung allograft dysfunction (CLAD) [1]. Mechanisms of CLAD development may well involve an imbalance between effector T cells and regulatory T cells (Treg). Treg control the effector response against alloantigens of the graft and thereby improve graft tolerance [2].

Treg constitute 3–10% of the naive peripheral $CD4^+$ T cell pool in humans, are especially enriched in the $CD4^+CD25^{high}$ population, may express the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, i.e., CD152), constitutively express the intracellular transcription factor FoxP3, and express low levels of $CD127^{low}$ (IL-7 receptor) [3–9].

In comparison with effector T cells, the mechanisms of action of Treg have been investigated only recently [10–18]. Moreover, few clinical studies have evaluated the relationship between Treg and outcomes after lung transplantation [19–27].

At our institution, we have recently demonstrated the tolerogenic effects of Treg in a humanized mouse model [16,17] and that, in a smaller cohort of 138 patients transplanted between 2009 and 2011, increasing frequencies of $CD4^+CD25^{high}CD127^{low}$ and $CD4^+CD25^{high}FoxP3^+$ regulatory T cells in peripheral blood as early as 3 weeks after lung transplantation estimated a protective effect against development of CLAD at 2 years [26].

In the present study, we present our complete 9-year results with Treg measurements in adult lung-transplanted patients. Particularly, we analyzed the presence of any association of three putative Treg subpopulations measured at 3 weeks after lung transplantation with the later incidence of CLAD and graft survival and looked for baseline factors influencing Treg frequencies.

Methods

Patients and variable definition

The inpatient and follow-up records of all patients who underwent lung transplantation at our institution between January 2009 and April 2018 were retrospectively reviewed.

Adult (>18 years old) patients in whom Treg measurement was performed at 3 weeks after transplantation were included into the study (Fig. 1). The time point 3 weeks after transplantation was chosen, because a protective effect of increasing Treg frequencies against CLAD had been observed as early as 3 weeks in a previous publication [16,26].

Adult patients who survived beyond three weeks after transplantation, but did not have any Treg measurement at 3 weeks, were excluded from the study (Fig. 1).

Follow-up ended on May 1st, 2018, and was 100% completed. Median (interquartile range, IQR) follow-up time amounted to 36 (18–61) months versus 42 (17–65) months in included versus excluded patients, respectively ($P = 0.19$).

The institutional ethical review board waived the need of patient consent to the study, since all patients had given their consent to handle their personal data for research purposes and to additional blood sampling for Treg measurement at the time of listing for lung transplantation. The study was conducted according to the Declaration of Helsinki.

The definitions of primary graft dysfunction (PGD), postoperative extended extracorporeal membrane oxygenation (ECMO) support, early anti-HLA donor-specific antibodies (eDSA), and of the outcomes infection requiring hospitalization, pulsed steroid therapy for presumed acute rejection and biopsy-confirmed rejection, have been reported elsewhere [28–32]. CLAD was defined as an irreversible and persistent (lasting for 3 weeks) decline in lung function tests (FEV_1 or FVC <80% of baseline), after exclusion of other reversible reasons explaining the fall of lung function tests [33].

Treg measurement protocol

Our protocol for Treg identification is reported as supplemental content and in a previous publication [26]. We defined Treg as frequency of $CD127^{low}$, $FoxP3^+$, and $CD152^+$ cells within the $CD4^+CD25^{high}$ population according to our previous findings showing this frequency to correlate with outcomes after lung transplantation [22,26]. Treg subpopulations $CD4^+CD25^{high}CD127^{low}$, $CD4^+CD25^{high}FoxP3^+$, and $CD4^+CD25^{high}CD152^+$ were measured in adult patients from peripheral whole blood samples immediately before transplantation, and prospectively at 3 weeks, at 3 months, at 6 months, at 1 year, and at 2 years after transplantation, whenever possible.

Combined staining of $CD4^+CD25^{high}CD127^{low}CD152^+FoxP3^+$ was not routinely performed in our laboratory. In addition, we kept on using our early Treg definition since, already in Treg measurements performed as early as 2009, we found evidence of an association between the frequency of $CD127^{low}$ cells

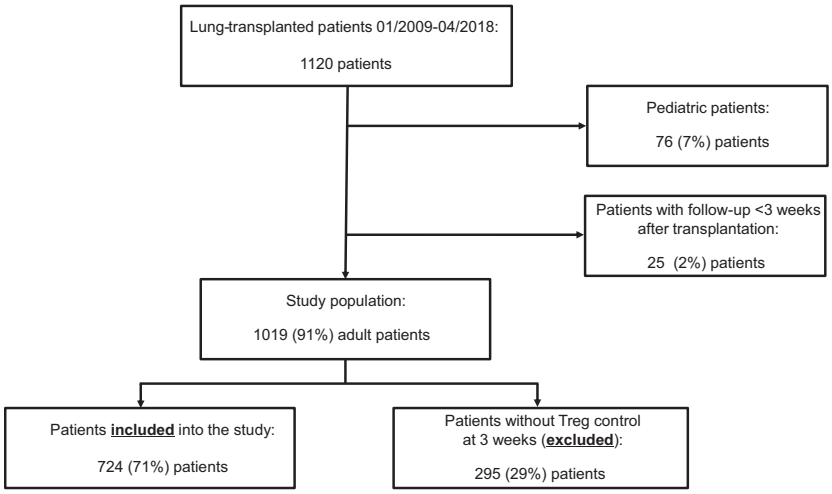

Figure 1 The flowchart shows the distribution of lung transplantation recipients included into the study and of those who were excluded.

within the $CD4^{+}CD25^{high}$ gate and the outcomes after lung transplantation [26].

Moreover, the BD Trucount™ flow cytometry analysis (BD Biosciences, Erembodegem, Belgium) for measurement of Treg cell counts was available at our institution only since 2013. Therefore, we preferred reporting throughout the manuscript the percentage of Treg rather than the Treg cell count. Moreover, we did not measure Treg in samples from bronchoalveolar lavages (BAL) and did not routinely perform the analysis of the methylation of Treg-specific demethylated region of FOXP3.

In late 2011, we changed our FACS staining protocol by including additional antibodies or antibodies with different fluorescent color conjugates accounting for the improved availability of antibodies against markers of interest in our study. In order to reduce the risk of impaired comparability between the "old" [26] and the "new" staining protocol, we did run all samples of 20 patients in an overlap period in duplicate, adjusting the gating strategy of the "new" protocol accordingly, to gain comparable results despite not using exactly the same staining protocol. For analysis, we use fluorescence minus one (FMO) staining controls combined with automated gating. Figure 2 shows a comparison between the "old" and "new" staining, and Fig. 3A shows another example of FACS analysis.

Patient management

Management of lung-transplanted patients at our institution has been widely reported in previous publications [26,29–31]. Immunosuppressive therapy was based on a triple therapy (calcineurin inhibitor, mycophenolate mofetil, and prednisolone). Since January 2013, tacrolimus was used as first-line calcineurin inhibitor in all patients after transplantation, instead of cyclosporine. Patients with assumed low immunological risk or who showed side effects related to tacrolimus therapy were later switched to cyclosporine in the outpatient setting. Patients usually received mycophenolate mofetil as cell cycle inhibitor, which was later switched to everolimus in some patients. No induction therapy was used.

Patients who developed early donor-specific antibodies (eDSA) had usually been treated only with therapeutic plasmapheresis (tPE) and a single dose of anti-CD20 antibody (rituximab) until 2013. Since March 2013, patients with eDSA were treated with a protocol based on successive infusions of IgA- and IgM-enriched human intravenous immunoglobulins (IgGAM, Pentaglobin®, Biotest AG, Dreieich, Germany; starting dose, 2 g/kg, then 0.5 g/kg each 4 week for a maximum of 6 months) with or without rituximab and tPE/immunoabsorption [30,31]. Treatment of eDSA was usually performed pre-emptively, independent of the presence of antibody-mediated rejection [34].

Statistics

IBM SPSS 25.0 (Armonk, New York, NY, USA) was used for the data analysis.

Primary endpoint was CLAD development. Secondary endpoints were graft survival, defined as freedom from mortality and retransplantation, and a composite

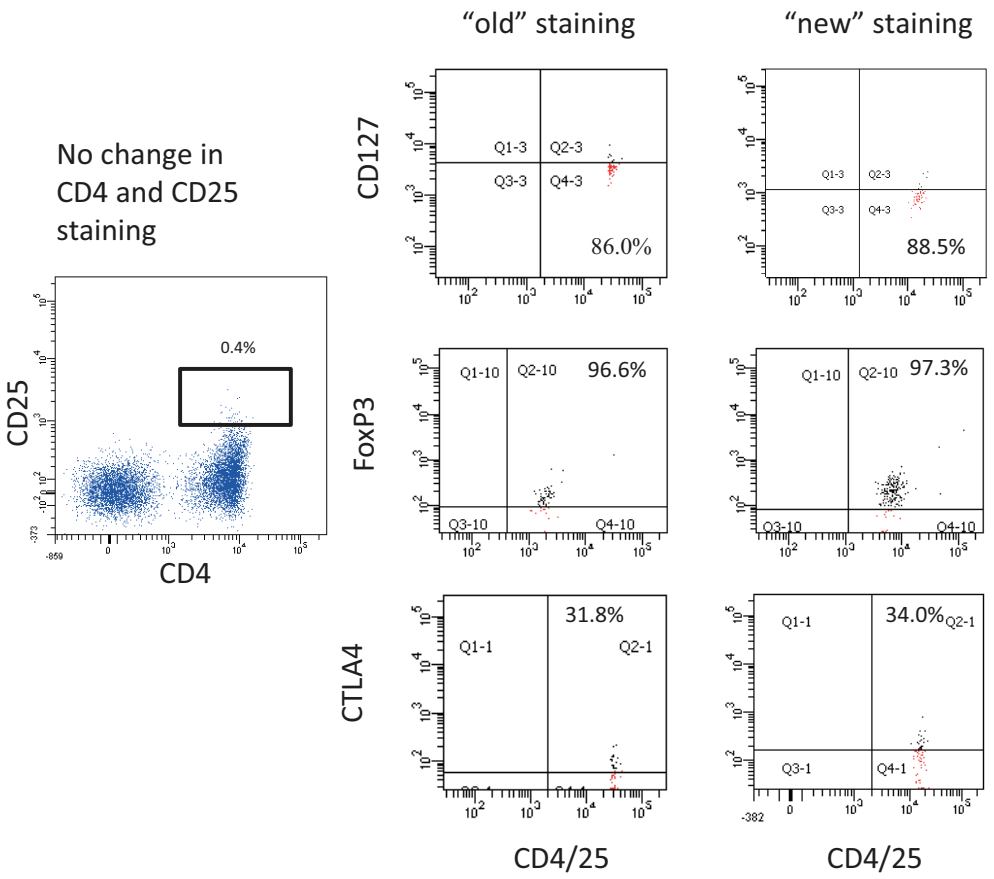

Figure 2 Figure 2 shows representative FACS plots from stained human peripheral blood mononuclear cells (PBMC). The sample was multicolor stained with anti-human CD4, CD25, CD127, FoxP3, and CTLA4 antibodies. The double $CD4^{+}CD25^{high}$ population was marked in the black frame. A comparison of all parameters (CD127, FoxP3, CTLA4) relevant for us between the old and new coloring is shown on the left part of the figure. The ratio of the relevant cell populations did not change significantly after the change of the staining protocol.

outcome that included CLAD, mortality, or retransplantation. Other outcomes, such as incidence of infection requiring hospitalization, of pulsed steroid therapy for presumed acute rejection and of biopsy-confirmed rejection were only analyzed for univariable association with Treg frequency at 3 weeks after transplantation.

Categorical and continuous variables were summarized as percentages and median with interquartile range (IQR), respectively. In order to evaluate any influence of baseline immunosuppressive therapy and eDSA treatment, Treg frequencies at each time point were stratified according to the baseline treatment with cyclosporine versus tacrolimus, and the treatment of eDSA with IgGAM, rituximab, plasmapheresis/immunoabsorption. The nonparametric Mann–Whitney test was used for group comparisons of Treg frequencies.

Survival estimates along with freedom from endpoints were calculated by the product-limit method of Kaplan–Meier.

At the multivariable analysis, CLAD, graft survival, and the composite endpoint that included CLAD, mortality, or retransplantation were considered as time-to-event outcomes. Thus, the Cox regression analysis was used to estimate any effect of the baseline variables on the primary and secondary endpoints [35]. Baseline variables included pretransplant patient characteristics and events (e.g., PGD, therapy for eDSA, immunosuppressive therapy) that happened during or soon after transplantation, before hospital discharge.

For the primary outcome CLAD, the Cox analysis was repeated after inclusion of patients who showed a follow-up longer than 2 years.

Patients with missing data were excluded, which means that, for some few variables, data were not

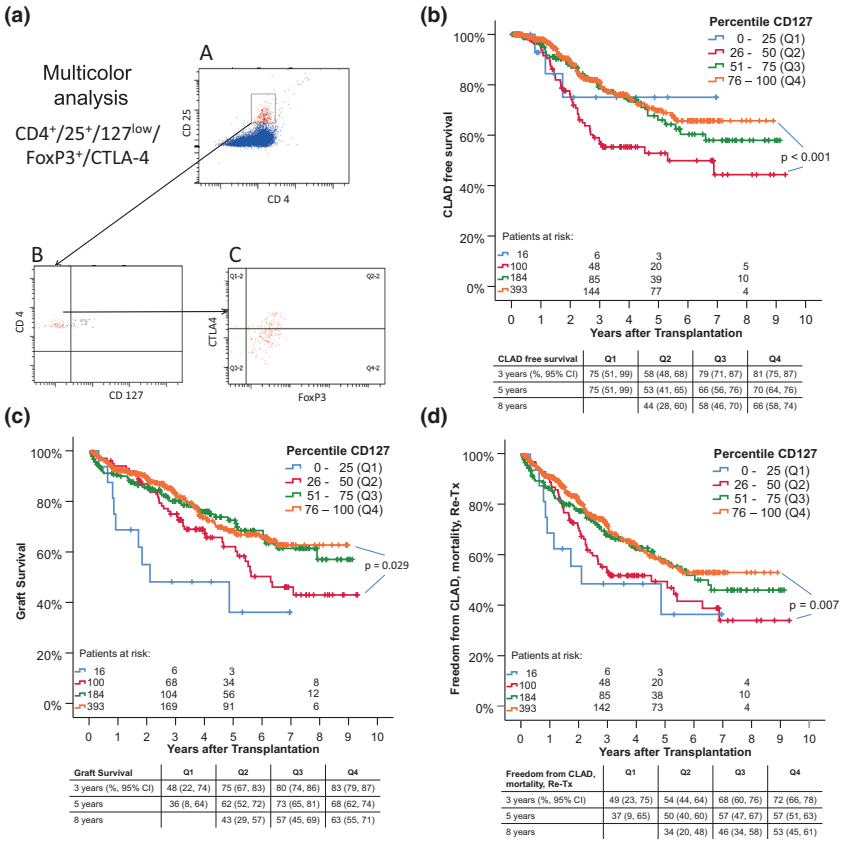

Figure 3 Figure (A) shows representative FACS plots from stained human PBMC. The sample was multi-color stained with anti-human CD4, CD25, CD127, FoxP3, CTLA4 antibodies. The double $CD4^{+}CD25^{high}$ population was marked (red) (a). $CD127^{low}$ cells within the $CD4^{+}CD25^{high}$ gate are shown in plot (b) in red. The vast majority of these $CD4^{+}CD25^{high}CD127^{low}$ cells stained FoxP3 positive (c) with some CTLA4 positives in between. Figure B–D show CLAD-free survival (B), graft survival (C) and freedom from CLAD, mortality, or retransplantation (ReTx) (D) after stratification according to quartiles of $\%CD127^{low}$ Treg at 3 weeks after transplantation. The first quartile included patients with a $\%CD127^{low}$ Treg between 0 and 20% and included only 16 patients; the second quartile included patients with a $\%CD127^{low}$ Treg between 21% and 50%; the third quartile included patients with a $\%CD127^{low}$ Treg between 51% and 75%; and the fourth quartile included patients with a $\%CD127^{low}$ Treg between 76% and 100%. Patients at risk for each quartile are reported above the X-axis. In each figure, survival (% and the respective 95% confidence interval, CI) for each quartile and at 3, 5, and 8 years is reported below the respective Kaplan–Meier plot. Since the measurement of $\%CD127^{low}$ Treg at 3 weeks after transplantation was not successful in 31 patients, $\%CD127^{low}$ Treg of 693 (96%) patients were available for stratification.

available in all patients. Thus, these patients were not considered by SPSS for calculating the Cox multivariable models. For example, a blood sample for Treg analysis at 3 weeks after transplantation was available for all the 724 included patients (study inclusion criteria), but in some few cases, the measurement of one of the three Treg subpopulations was not successful and the Treg value for that subpopulation was missing.

At the univariable analysis, variables were preselected among the baseline variables ($n = 61$) reported in Tables 1–4 along with Treg frequencies at 3 weeks (Table 9). Preselection was based on the current literature, using information from other data sources and from our own previous experience [1,2,25–28,33,34,36–40]. The number of variables for each primary and secondary outcome was chosen to yield an event per variable (EPV) ratio as close as possible to 15, in order to avoid instability of the multivariable models [41,42]. The EPV ratio was 14 for CLAD, 12 for graft survival and 14 for the composite endpoint.

Then, the models for each outcome were constructed performing a regression analysis using a modified augmented backward analysis [41,42]. Results were reported as hazard ratios (HR) with 95% confidence interval (CI) and corresponding p-value. The proportional hazards assumption was tested for each variable using the complementary log–log Kaplan–Meier plots and including the time-dependent coefficients into the

Table 1. Preoperative recipient data in included patients.

Variable	*n* = 724
Female sex	309 (42.7)
Age (years)	53 (41–59)
Age >60 years	110 (15.2)
Blood group	
A	327 (45.2)
B	82 (11.3)
AB	30 (4.1)
0	285 (39.4)
CMV risk	
Low	163 (22.5)
Intermediate	326 (45.0)
High	235 (32.5)
Transplant indication	
COPD	233 (32.1)
Pulmonary fibrosis	246 (34.0)
Cystic fibrosis	141 (19.5)
Pulmonary hypertension	28 (3.9)
Re-transplant	40 (5.5)
Other	36 (5.0)
Associated pulmonary artery hypertension	291 (40.2)
LAS Score*	35.7 (32.9–40.8)
Preoperative mechanical ventilation	13 (1.8)
Preoperative intensive care unit	45 (6.1)
Preoperative ECMO	34 (4.7)

Values are expressed as median (IQR, interquartile range) or *N* of patients (%).

CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; ECMO, extracorporeal membrane oxygenation; LAS, lung allocating score.

*LAS score was available in 534 patients transplanted since 2012.

Table 2. Donor and intraoperative recipient characteristics in included patients.

Variable	*n* = 724
Donor characteristics	
Female sex	338 (46.8)
Age (years)	49 (37–57)
Age >70 years	39 (5.4)
Ventilation time (days)	4 (2–7)
pO_2 (100%, mmHg)	393 (321–451)
Smoking history	304 (42.1)
Contusion	63 (8.7)
Aspiration	42 (5.8)
Lung Preservation	
Celsior	566 (80.6)
Portable EVLP	44 (6.1)
Intraoperative recipient characteristics	
Thoracotomy	
Sternum sparing	685 (94.6)
Clamshell	39 (5.4)
Single lung	14 (1.9)
Double lung	710 (98.1)
Cardiopulmonary bypass	17 (2.3)
Intraoperative ECMO	150 (20.8)
Postoperative extended ECMO	43 (5.9)
Ischemic time (min)	
First lung	408 (328–504)
Second lung	523 (440–617)
Lung volume reduction	
Atypical	17 (2.3)
Lobar	27 (3.7)

Values are expressed as median (IQR, interquartile range) or *N* of patients (%).

ECMO, extracorporeal membrane oxygenation; EVLP, ex vivo lung perfusion.

regression models. The variables which did not satisfy this assumption were not included in the multivariable models.

Two-tailed *P*-values ≤ 0.05 were considered significant.

Results

Included versus excluded patients

Between January 2009 and April 2018, 1120 patients underwent lung transplantation at our institution (Fig. 1). Seventy-six (7%) pediatric patients and 25 (2%) adult patients with a follow-up shorter than 3 weeks after transplantation, due to in-hospital death ($n = 24$) and retransplantation ($n = 1$), did not undergo any Treg measurements after transplantation as per protocol.

Among the remaining 1019 (91%) adult patients, 724 (71%) patients showed Treg measurements at 3 weeks and were included in the study. The remaining 295 (29%) adult patients did not show any Treg measurement at 3 weeks after transplantation, because they had been transferred to another department ($n = 51$, 17%); they had not given consent to the study ($n = 58$, 20%); they had not been approached ($n = 125$, 42%); or because the Treg control was attempted but the sample quality was insufficient for measuring any Treg subpopulation at 3 weeks ($n = 61$, 21%). Thus, these 295 patients were excluded from the study (Fig. 1).

Baseline characteristics of included patients are reported in Tables 1–4.

Table 3. Anti-HLA antibodies in included patients.

Variable	*n* = 724
Preoperative anti-HLA antibodies	
Anti-HLA I	123 (17)
Anti-HLA II	158 (21.8)
Anti-HLA I + anti-HLA II	44 (6.1)
Cumulative mismatches	
HLA A + B	3 (3–4)
HLA A + B + DR	5 (4–5)
Postoperative anti-HLA antibodies*	
Anti-HLA I	185 (25.6)
Anti-HLA II	273 (37.7)
Anti-HLA I + anti-HLA II	103 (14.2)
Postoperative anti-HLA eDSA	155 (21.4)
Anti-HLA I	38 (5.3)
HLA A	17 (2.4)
HLA B	23 (3.2)
HLA C	2 (0.3)
Anti-HLA II	125 (17.3)
HLA DR	20 (2.8)
HLADQ	117 (16.2)
eDSA therapy	
tPE/immunoabsorption	74 (10.2)
Rituximab	120 (16.6)
IgGAM	93 (12.8)

Values are expressed as median (IQR) or *N* of patients (%).

eDSA, early donor-specific antibodies; IgGAM, IgA- and IgM-enriched human intravenous immunoglobulins; tPE, therapeutic plasmapheresis.

*All patients who developed anti-HLA antibodies after lung transplantation were considered, independently of DSA positivity.

Table 4. Postoperative data in included patients.

Variable	*n* = 724
PGD score grade 2 or 3	
24 h	91 (12.6)
48 h	90 (12.4)
72 h	61 (8.4)
Rethoracotomy for bleeding	50 (6.9)
New dialysis	41 (5.7)
Postoperative pulsed steroid therapy	193 (26.7)
Blood products, overall	
PRBCs (units)	6 (4–10)
PC (units)	0 (0–2)
FFP (units)	4 (3–8)
Secondary ECMO	9 (1.2)
Tracheostomy	65 (9.0)
Ventilation time, days	1 (1–1)
ICU stay, days	2 (1–4)
Hospital stay, days	23 (21–28)
In-hospital mortality	19 (2.6)
Initial immunosuppressive therapy before hospital discharge*	
Cyclosporine	225 (31.9)
Tacrolimus	480 (68.1)
Mycophenolate mofetil	698 (99.1)
Immunosuppressive therapy at last outpatient control*	
Cyclosporine	153 (21.7)
Tacrolimus	541 (76.7)
Mycophenolate mofetil	637 (90.4)
Everolimus	59 (8.4)

Values are expressed as median (IQR, interquartile range) or *N* of patients (%).

ECMO, extracorporeal membrane oxygenation; FFP, fresh frozen plasma; ICU, intensive care unit; PC, platelet concentrate; PRBCs, packed red blood cells.

*n-hospital deaths (*n* = 19) are censored.

Multivariable Cox analysis for endpoints

Primary and secondary outcomes of included patients are reported in Table 5. Among the 724 included patients, by the end of follow-up, 247 (34%) patients developed CLAD and either died or were retransplanted before CLAD development; 185 (25%) patients died or were retransplanted; and 162 (22%) patients developed the primary endpoint CLAD (BOS, $n = 134$; RAS, $n = 28$).

Univariable and multivariable Cox analyses for primary and secondary endpoints are reported in Tables 6–8. Increasing frequencies of $CD127^{low}$ cells within the $CD4^{+}CD25^{high}$ population ($CD4^{+}CD25^{high}CD127^{low}$) but not of $CD4^{+}CD25^{high}FoxP3^{+}$ and $CD4^{+}CD25^{high}CD152^{+}$ at 3 weeks estimated a protective effect against CLAD (HR = 0.989, 95% CI = 0.981–0.996, $P = 0.003$, Table 6); against mortality or retransplantation (HR = 0.991, 95% CI = 0.984–0.999, $P = 0.026$, Table 7); and against CLAD, mortality, or retransplantation (HR = 0.992, 95% CI = 0.986–0.999, $P = 0.019$, Table 8). The estimated effect of increasing frequencies of $CD4^{+}CD25^{high}CD127^{low}$ at 3 weeks against CLAD persisted, if only the 490 patients who showed a follow-up of at least 2 years were considered in the Cox analysis (HR = 0.991, 95% CI = 0.983–0.999, $P = 0.035$, Table S1).

The effect of increasing $\%CD4^{+}CD25^{high}CD127^{low}$ at 3 weeks on primary and secondary outcome-free survival is shown in Fig. 3B–D. After outcome stratification, according to quartiles of $\%CD4^{+}CD25^{high}CD127^{low}$ at 3 weeks, patients with $\%CD4^{+}CD25^{high}CD127^{low}$ >76% (4^{th} quartile) showed better outcome-free survival than patients with $\%CD4^{+}CD25^{high}CD127^{low}$ lower than 51% (1st and 2nd quartiles). The improvement ranged variably from 6% to 23%.

Table 5. Outcomes in included patients.

Variable	*n* = 724
Freedom from CLAD (%)	
3 years	77 (73, 81)
5 years	66 (62, 70)
Graft survival (%)	
3 years	81 (77, 85)
5 years	68 (64, 72)
Freedom from CLAD + mortality + retransplantation (%)	
3 years	68 (64, 72)
5 years	56 (52, 60)
Causes of death after hospital discharge*	
CLAD	67 (9.5)
Infection	21 (3)
Malignancy	19 (2.7)
Cardiac	17 (2.4)
Other	17 (2.4)
Freedom from biopsy-confirmed rejection (%)	
1 year	61 (57, 65)
3 years	53 (49, 57)
5 years	49 (45, 53)
ISHLT biopsy grade	
A1	246 (37.3)
A2	82 (12.4)
A3	3 (0.5)
Freedom from pulsed steroid therapy (%)	
1 year	51 (49, 53)
3 years	38 (34, 42)
5 years	34 (30, 38)
Freedom from infection requiring hospitalization (%)	
3 years	63 (59, 67)
5 years	57 (53, 61)

Values are expressed as mean (95% confidence interval, CI) or *N* of patients (%).

CLAD, chronic lung allograft dysfunction; ISHLT, International Society of Heart and Lung Transplantation.

*patients who died before hospital discharge (*n* = 19) were not considered.

Finally, increasing frequencies of $CD4^{+}CD25^{high}CD127^{low}$ at 3 weeks were associated with better freedom from infection requiring hospitalization at follow-up (HR = 0.992; 95% CI = 0.986–0.997, $P = 0.004$), but not with freedom from pulsed steroid therapy or biopsy-confirmed rejection (Table S2).

Treg frequencies in peripheral blood

Frequencies of Treg subpopulations at each time point and for the primary and secondary outcomes are reported in Table 9.

Treg frequencies stratified according to IgGAM, rituximab and tPE/immunoabsorption therapy for eDSA, and according to baseline therapy with tacrolimus versus cyclosporine are reported in Tables S3–S7. Frequencies of $CD4^{+}CD25^{high}CD127^{low}$ were significantly higher throughout all time points in patients who were treated with tacrolimus (Table S6) and with IgGAM (Table S3). Conversely, frequencies of $CD4^{+}CD25^{high}FoxP3^{+}$ were higher in those patients who received initially cyclosporine (Table S6).

Discussion

This retrospective study presented our 9-year experience with measurement of putative regulatory T cells in vivo from peripheral blood samples in lung transplantation and showed that patients with frequencies of $CD127^{low}$ cells within the $CD4^{+}CD25^{high}$ population greater than 76% at 3 weeks after transplantation showed better freedom from CLAD, mortality, or retransplantation than patients with frequencies of $CD4^{+}CD25^{high}CD127^{low}$ lower than 51%. At the Cox analysis, increasing frequencies of $CD4^{+}CD25^{high}CD127^{low}$ at 3 weeks after transplantation estimated a protective effect against death, CLAD, or retransplantation.

To our knowledge, our study included the largest population of lung-transplanted patients having undergone Treg measurements from peripheral blood samples so far. In comparison with the study previously published by our group [26], graft survival and a composite endpoint including CLAD, death, or retransplantation were additionally included for analysis. Moreover, the impact of eDSA treatment on Treg frequencies after lung transplantation was evaluated for the first time.

The results of our study are important, because, in comparison with the experimental in vitro evidence in animal models [10–18], the clinical in vivo evidence for a role of Treg in lung transplantation is scarce, contradictory and often influenced by the limited number of patients evaluated and concomitant confounding factors, such as the immunosuppressive treatment with calcineurin inhibitors and mTOR inhibitors [19–27]. Piloni et al have recently shown that patients with higher mean $CD4^{+}CD25^{high}CD127^{low}$ Treg counts from peripheral blood samples had a significantly lower risk of presenting CLAD or progress of allograft dysfunction [25]. On the contrary, Durand et al, in a prospective study that included 82 patients, showed that patients with an increasing proportion of $CD4^{+}CD25^{high}FoxP3^{+}$ T cells up to 2.4% in the 6 months after transplantation had twofold higher risk of developing BOS. Of note, most of the BOS patients had been treated with

Table 6. Univariable and multivariable Cox analysis for CLAD.

	Univariable			Multivariable		
Variable	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Categorical variables						
Re-transplant	1.210	0.618–2.372	0.578			
Preoperative anti-HLA antibodies	0.804	0.575–1.124	0.201			
PGD score grade 2 or 3, at 72 h	1.184	0.684–2.048	0.547			
Postoperative pulsed steroid therapy	0.767	0.518–1.135	0.184			
Postoperative anti-HLA eDSA	1.030	0.699–1.517	0.881			
Tacrolimus versus cyclosporine at discharge	0.506	0.366–0.700	<0.001	0.514	0.366–0.720	<0.001
Continuous variables						
Cold Ischemic time, first lung (min)	0.999	0.998–1.001	0.237			
Cold ischemic time, second lung (min)	1.000	0.999–1.001	0.929			
Recipient ventilation time (days)	1.033	1.013–1.053	0.001	1.029	1.008–1.050	0.006
%$CD4^+/CD25^{high}/CD127^{low}$ at 3 weeks	0.988	0.981–0.995	0.001	0.989	0.981–0.996	0.003
%$CD4^+/CD25^{high}/FoxP3^+$ at 3 weeks	0.992	0.981–1.003	0.173			
%$CD4^+/CD25^{high}/CD152^+$ at 3 weeks	0.996	0.988–1.005	0.406			

CD, cluster of differentiation; CI, confidence interval; CLAD, chronic lung allograft dysfunction; eDSA, early donor-specific antibodies; HLA, human leukocyte antigen; PGD, primary graft dysfunction.

Table 7. Univariable and multivariable Cox analysis for graft survival.

	Univariable			Multivariable		
Variable	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Categorical variables						
CMV risk, high	1.303	0.972–1.748	0.077			
Pulmonary fibrosis	1.359	1.006–1.835	0.046			
Pulmonary hypertension	0.912	0.428–1.942	0.811			
Re-transplant	1.179	0.623–2.231	0.614			
Preoperative mechanical ventilation	0.888	0.284–2.781	0.839			
Preoperative ECMO	1.522	0.866–2.675	0.144			
Preoperative anti-HLA antibodies	0.804	0.585–1.105	0.179	0.697	0.490–0.992	0.045
PGD score grade 2 or 3, at 72 h	1.809	1.187–2.759	0.006			
Postoperative pulsed steroid therapy	1.383	1.004–1.906	0.048			
Postoperative anti-HLA eDSA	1.453	1.045–2.020	0.026	1.888	1.272–2.803	0.002
Tacrolimus versus cyclosporine at discharge	0.694	0.508–0.948	0.022	0.630	0.444–0.893	0.010
Continuous variables						
Recipient age (years)	1.010	0.998–1.023	0.102			
Recipient BMI	1.039	1.000–1.079	0.052	1.049	1.007–1.049	0.023
Cold Ischemic time, first lung (min)	1.001	1.000–1.002	0.127			
Cold ischemic time, second lung (min)	1.001	1.000–1.002	0.257			
% $CD4^+/CD25^{high}/CD127^{low}$ at 3 weeks*	0.992	0.985–0.999	0.029	0.991	0.984–0.999	0.026

BMI, body mass index; CD, cluster of differentiation; CI, confidence interval; CMV, cytomegalovirus; ECMO, extracorporeal membrane oxygenation; eDSA, early donor-specific antibodies; PGD, primary graft dysfunction.

*% $CD4^+/CD25^{high}/FoxP3^+$ and % $CD4^+/CD25^{high}/CD152^+$ did not satisfy the proportional hazard function and therefore were not included in the analysis.

cyclosporine before [27]. Greenland et al demonstrated that, during episodes of biopsy-confirmed rejection, there was an increase of Treg in peripheral blood [24]. Other authors found an association of decreasing Treg levels and the development of acute rejection and BOS in BAL but not in peripheral blood [19–21]. Moreover, Krustrup et al showed, in their experience with 58 patients, that the number of $FoxP3^+$ cells in the lung

Table 8. Univariable and multivariable Cox analysis for CLAD, mortality, or retransplantation.

	Univariable			Multivariable		
Variable	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Categorical variables						
CMV risk, high	1.253	0.970–1.619	0.084			
Pulmonary fibrosis	1.196	0.917–1.558	0.186			
Pulmonary hypertension	0.851	0.437–1.656	0.635			
Re-transplant	0.922	0.504–1.689	0.793			
Preoperative mechanical ventilation	1.312	0.541–3.183	0.548			
Preoperative ECMO	1.558	0.938–2.586	0.087			
Preoperative anti-HLA antibodies	0.767	0.583–1.010	0.058	0.720	0.534–0.970	0.031
PGD score grade 2 or 3, at 72 h	1.678	1.145–2.459	0.008			
Postoperative pulsed steroid therapy	1.167	0.878–1.552	0.286			
Tacrolimus versus cyclosporine at discharge	0.584	0.448–0.760	<0.001	0.579	0.437–0.766	<0.001
Continuous variables						
Recipient age (years)	1.004	0.994–1.015	0.413			
BMI	1.025	0.992–1.060	0.143			
Cold ischemic time, second lung (min)	1.000	0.999–1.001	0.489			
Cold ischemic time, second lung (min)	1.000	0.999–1.001	0.464			
Hospital stay, days	1.006	1.003–1.008	<0.001	1.006	1.003–1.009	<0.001
% $CD4^+/CD25^{high}/CD127^{low}$ at 3 weeks	0.992	0.986–0.998	0.010	0.992	0.986–0.999	0.019
%$CD4^+/CD25^{high}/FoxP3^+$ at 3 weeks	0.996	0.986–1.005	0.373			
%$CD4^+/CD25^{high}/CD152^+$ at 3 weeks	0.998	0.991–1.005	0.565			

BMI, body mass index; CD, cluster of differentiation; CI, confidence interval; CLAD, chronic lung allograft dysfunction; ECMO, extracorporeal membrane oxygenation; PGD, primary graft dysfunction.

allograft did not correlate with BOS-free survival time [23].

In comparison with these experiences, we had previously validated our clinical results in mouse and swine experimental models [14–17]. Siemeni et al showed that recipient Treg, that were generated in the presence of donor antigen even as early as 3 weeks after lung transplantation, identified with same markers used in the present study, and infused in a humanized mouse model of transplant arteriosclerosis (TA), were able to inhibit arteriosclerosis in small arterial grafts previously harvested from the respective human donor. The inhibition of arteriosclerosis was greater in mice reconstituted with peripheral mononuclear blood cells (PBMCs) from lung-transplanted recipients with higher Treg (>1% of all lymphocytes) percentages at 3 weeks after transplantation than in mice reconstituted with PBMCs from recipients with lower Treg percentages at 3 weeks [16]. While TA may be criticized as experimental model for CLAD, we have recently demonstrated in an experimental study using the same humanized mouse model that TA was more severe in mice reconstituted with PMBCs from lung recipients who later developed CLAD (n = 8) than in mice reconstituted with PMBCs from lung recipients who did not develop CLAD (n = 14). Enrichment of the transferred PBMCs with Treg significantly suppressed the TA development in humanized mice of both the corresponding lung-transplanted recipients with and without later CLAD development. Of note, lung-transplanted recipients without CLAD showed higher percentages of $CD4^+CD25^{high}CD127^{low}$ cells at 3 weeks after transplantation than lung-transplanted recipients who later developed CLAD [17]. These results supported further our decision to use the frequencies of Treg measured at 3 weeks after transplantation for correlation analyses in the present study.

The constant finding that, among the in vivo evaluated Treg subpopulations, only $CD4^+CD25^{high}CD127^{low}$ cells were protective against CLAD and mortality, underlined their role for future cell-based therapies against CLAD. The CD127 (IL-7 receptor) antigen is considered the most reliable marker for Treg isolation [43–45] and the majority of these cells are FoxP3+ too [46]. Instead, the FoxP3 antigen alone cannot be used for Treg isolation for cell therapy, technically, because it is expressed intracellularly. Moreover, in comparison with CD127 antigen, the expression of FOXP3 is directly downregulated by the calcineurin inhibitors

Table 9. Treg as % of $CD4^+/CD25^{high}$ T cells at different time points before and after transplantation.

Variable	
% $CD4^+/CD25^{high}$	
Before transplantation	0.6 (0.3, 2.3)
At 3 weeks	0.8 (0.3, 2.2)
At 3 months	0.7 (0.3, 2.2)
At 6 months	0.7 (0.2, 2.7)
At 1 year	0.4 (0.2, 1.5)
At 2 years	0.3 (0.1, 1.12)
% $CD4^+/CD25^{high}/CD127^{low}$	
Before transplantation	79.8 (51.3, 89.5)
At 3 weeks	78.8 (59.3, 87.8)
At 3 months	76.9 (59.3, 88.9)
At 6 months	76.4 (59.8, 87.9)
At 1 year	77.8 (65.0, 87.2)
At 2 years	81.7 (65.6, 88.9)
% $CD4^+/CD25^{high}/FoxP3^+$	
Before transplantation	79.1 (68.5, 84.8)
At 3 weeks	77.8 (69.0, 87.3)
At 3 months	78.2 (69.3, 87.3)
At 6 months	78.3 (69.6, 88.1)
At 1 year	81.6 (73.2, 90.6)
At 2 years	81.8 (73.0, 89.5)
% $CD4^+/CD25^{high}/CD152^+$	
Before transplantation	10.0 (2.9, 25.0)
At 3 weeks	12.5 (6.2, 25.0)
At 3 months	11.3 (5.3, 20.7)
At 6 months	11.3 (6.3, 20.8)
At 1 year	12.5 (6.7, 25.0)
At 2 years	13.1 (6.6, 24.8)

Values are expressed as median (IQR, interquartile range).
CD, cluster of differentiation.

through inhibition of the transcriptor factor NFAT [47]. Since tacrolimus is a more potent immunosuppressive drug than cyclosporine, the suppression of FOXP3 antigen may be greater in patients treated with tacrolimus than cyclosporine. This relationship might explain the greater frequency of $CD4^+CD25^{high}FOXP3^+$ cells in patients treated with cyclosporine (Table S7) and might question about the reliability of FOXP3 as an in vivo marker for Treg in transplanted patients under immunosuppressive therapy.

In contrast to other experiences [19–21], we did not find any significant relationship between Treg and acute rejection. The prespecified sampling times of Treg from the peripheral blood and not from the BAL or from transbronchial biopsies (intragraft Treg), especially not at the same time of acute rejection occurrence, may explain this finding. During acute rejection, Treg are lower at the beginning of the rejection episode than later, when they have undergone expansion to counteract the action of effector T cells [19–21,24]. In the case of CLAD, it can be postulated that a higher Treg frequency after transplantation protects against the future CLAD development, since it moves the balance between effector and regulatory T cells toward a pro-tolerogenic state.

The finding that increased frequencies of $CD4^+CD25^{high}CD127^{low}$ estimated a protective effect against infective episodes requiring hospitalization at follow-up deserves further investigation. A decrease in relative Treg frequencies in patients with infection would be expected due to the increase of effector T cells in response to infection.

Finally, this study investigated the frequency of peripheral Tregs and their correlation with outcomes in vivo and not in vitro, where any interaction with other variables such as immunosuppressive therapies could be controlled a priori. Thus, the interactions between Treg and immunosuppressive drugs, such as calcineurin inhibitors and IgGAM, that emerged from this study, should not be regarded as just a time-dependent bias, but an additional information on the drug mechanisms of action [47]. Patients developing new eDSA during the first four weeks after lung transplantation and consecutively treated with successive infusions of IgGAM showed an increase of $CD4^+CD25^{high}CD127^{low}$ frequencies during treatment. This increase became statistically significant at 3 months after transplantation, during IgGAM treatment, whose median time amounted to 3 months [30,31]. IgGAM have pleiotropic immunomodulatory effects and, among others, activate Treg through their IgG component [48]. The observation of increased $CD4^+CD25^{high}CD127^{low}$ Treg frequencies in patients treated with tacrolimus instead of cyclosporine is intriguing. Therapy with tacrolimus instead of cyclosporine has been recently associated with a better freedom from acute rejection and BOS [36]. Tacrolimus depresses both effector and regulatory T cells at the same time. However, both T-cell subtypes depend on IL-2 for proliferation, but regulatory T cells produce very little amount of IL-2 and bind IL-2 more avidly through their high-affinity IL-2 receptor (CD25) than effector T cells [49]. It can be speculated that tacrolimus, through greater inhibition of the effector T cells, might expand the regulatory T cells even in the presence of low levels of IL-2 in vivo, since the competition between effector and regulatory T cells for IL-2 is reduced [47].

We are aware that our explanation of the relationship between immunosuppressive drugs and Treg that emerged in this study is only a hypothesis, which we

have not experimentally demonstrated. However, we think that our findings are an interesting starting point for future research, which should aim at explaining the better results of immunosuppressive therapy with tacrolimus than cyclosporine, and help refining the more appropriate immunosuppressive therapy for patients at risk for later CLAD development, as patients with lower Treg percentage early after transplantation are.

Study limitations

We stratified Treg frequencies according to several variables, but we could not exclude that other variables, which were not considered in this study, additionally influenced the fluctuations of Treg frequencies. In order to reduce this confounding effect in the multivariable analysis to a minimum, we considered only Treg values at 3 weeks. Thus, confounding effects of later treatments and procedures, such as changes in immunosuppressive medications or photopheresis, were excluded.

While we attempted to collect clinical biological samples from all lung transplant recipients, in the current study, 29% of the adult lung-transplanted patients at our institution with a follow-up longer than 3 weeks did not have a Treg control at 3 weeks in our database. These were usually patients at higher surgical risk than included patients tending toward worse outcomes. Therefore, it cannot be excluded that results of statistical analysis could have been different, if these patients had been included into the study. During the last five years, Treg sampling has improved up to 91% of adult transplanted patients in 2015.

We did not use the immunosuppressive drug levels to look for any influence of immunosuppressive therapy on Treg frequency, because these levels might have been influenced by many uncontrollable confounding factors.

The T cells investigated in the present study might have only a putative regulatory function. However, our previously published experimental studies, where the Treg were isolated using the same protocol as in the present study, showed that these T cells are indeed regulatory and not effector T cells [16,17].

Finally, the EPV ratios were slightly lower than 15 [41]. However, further reduction of covariate number might have implied the exclusion of covariates which had been previously demonstrated as risk factors for the outcomes considered in this study [1,2,25–28,33,34,36–40].

Conclusions

This study confirms that putative Treg play in vivo an important role in lung transplantation. In particular, increased frequencies of $CD4^{+}CD25^{high}CD127^{low}$ putative regulatory T cells in peripheral blood early after lung transplantation estimate a protective effect against CLAD and mortality. Moreover, we identify a possible role of IgGAM and tacrolimus in expanding Treg subpopulations. Further experimental evidence is required to demonstrate that such $CD4^{+}CD25^{high}CD127^{low}$ T cells have an unequivocal regulatory function.

Authorship

FI: research design, performance of the research, writing of the paper, and data analysis. JS: performance of the research, data analysis, and writing of the paper. AKN: performance of the research. WS: research design and performance of the research. TN: performance of the research. MV: performance of the research. TS: performance of the research. RP: performance of the research. DB: performance of the research. CK: performance of the research. MA: performance of the research. CE: performance of the research. MH: performance of the research. DB: performance of the research and data analysis. HH: statistical revision. NS: performance of the research. CM: performance of the research. TW: critical revision of the manuscript. CF: critical revision of the manuscript. GP: critical revision of the manuscript. AH: research design and critical revision of the manuscript. IT: research design, performance of the research, and critical revision of the manuscript. GW: research design, data analysis, writing of the paper, and critical revision of the manuscript.

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Conflict of interest

FI and GW report personal fees from Biotest, outside the submitted work. TW reports fees from Boehringer and from Roche, outside the submitted work. The remaining authors have no conflict of interest to disclose.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Univariable and multivariable Cox analysis for CLAD performed including only the 490 patients with at least 2-year follow-up.

Table S2. Univariable association between Treg frequency at 3 weeks and infection requiring hospitalization, pulsed steroid therapy and biopsy-confirmed rejection at follow-up.

Table S3. Treg as % of CD4+/CD25high T cells and stratified according to IgGAM therapy for eDSA.

Table S4. Treg as % of CD4+/CD25high T cells and stratified according to therapy with Rituximab for eDSA.

Table S5. Treg as % of CD4+/CD25high T cells and stratified according to therapy with tPE/immunoabsorption for eDSA.

Table S6. Treg as % of CD4+/CD25high T cells and stratified according to initial therapy with tacrolimus versus cyclosporine, before hospital discharge.

Table S7. Frequency of CD4+/CD25high cells stratified according to IgGAM, tPE/immunoabsorption and Rituximab therapy for eDSA, and initial therapy with tacrolimus versus cyclosporine, before hospital discharge.

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