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High circulating CD4⁺CD25^{hi}FOXP3⁺ T-cell sub-population early after lung transplantation is associated with development of bronchiolitis obliterans syndrome

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³A complete listing of members of the COLT and SysCLAD Consortia is presented in the Appendix.

BACKGROUND: Chronic bronchiolitis obliterans syndrome (BOS) remains a major limitation for long-term survival after lung transplantation. The immune mechanisms involved and predictive biomarkers have yet to be identified. The purpose of this study was to determine whether peripheral blood T-lymphocyte profile could predict BOS in lung transplant recipients.

METHODS: An in-depth profiling of CD4⁺ and CD8⁺ T cells was prospectively performed on blood cells from stable (STA) and BOS patients with a longitudinal follow-up. Samples were analyzed at 1 and 6 months after transplantation, at the time of BOS diagnosis, and at an intermediate time-point at 6 to 12 months before BOS diagnosis.

RESULTS: Although no significant difference was found for T-cell compartments at BOS diagnosis or several months beforehand, we identified an increase in the CD4⁺CD25^{hi} FoxP3⁺ T-cell sub-population in BOS patients at 1 and 6 months after transplantation ($3.39 \pm 0.40\%$ vs $1.67 \pm 0.22\%$ in STA, $p < 0.001$). A CD4⁺CD25^{hi} FoxP3⁺ T-cell threshold of 2.4% discriminated BOS and stable patients at 1 month post-transplantation. This was validated on a second set of patients at 6 months post-transplantation. Patients with a proportion of CD4⁺CD25^{hi} FoxP3⁺ T cells up to 2.4% in the 6 months after transplantation had a 2-fold higher risk of developing BOS.

CONCLUSIONS: This study is the first to report an increased proportion of circulating CD4⁺CD25^{hi} FoxP3⁺ T cells early post-transplantation in lung recipients who proceed to develop BOS within 3 years, which supports its use as a BOS predictive biomarker.

Lung transplantation is a validated option for patients with end-stage lung diseases. However, patient survival is compromised by chronic lung allograft dysfunction (CLAD). The obstructive form of CLAD, bronchiolitis obliterans syndrome (BOS), is the most prevalent phenotype and affects up to 50% of lung transplant recipients (LTRs) in the 5 years post-transplantation.¹ BOS development involves irreversible fibrotic processes, which result in lung allograft obstruction, damage, and progressive loss of function.^{1,2} BOS diagnosis is currently based on a pulmonary function test and is defined as a decrease of at least 20% of the forced expiratory volume in 1 second (FEV₁) from baseline.³ This FEV₁ decrease represents a clinical surrogate marker with low clinical interest, as it is evidenced only when irreversible BOS has already developed.⁴ There is therefore a need for early predictive biomarkers for preventing BOS initiation and development, especially within the first months and 1 year post-transplantation, when BOS carries the worst prognosis.^{5,6} In this study, we assessed whether the circulating T-cell profile would allow biomarkers associated with BOS development to be identified in patients from follow-up to 3 years post-transplantation.

We report an increased proportion of circulating CD4⁺CD25^{hi}FoxP3⁺ T cells early post-transplantation in lung recipients who will develop BOS within 3 years, and its possible use as a BOS predictive biomarker.

Methods

Patients

The University Hospital Ethical Committee of Nantes and the Committee for the Protection of Patients from Biologic Risks

approved this study. All patients were informed of the final use of their blood and signed an informed consent document. This study was performed in the strict compliance of the ethics statement of the International Society for Heart and Lung Transplantation (ISHLT). Blood samples were selected from lung transplant recipients enrolled in the multicenter longitudinal COLT study (COhort in Lung Transplantation, NCT00980967). BOS is defined by a decrease of at least 20% of the maximum FEV₁ value recorded after transplantation. Stable graft function (STA) and BOS status were confirmed by repeated sessions of an ad-hoc adjudication multidisciplinary committee using pulmonary function tests, computerized tomography, and after elimination of confounding factors in line with the ISHLT/European Respiratory Society/American Thoracic Society clinical practice guideline,⁷ as described elsewhere.⁸ In this study, 82 LTRs were enrolled and followed up to 3 years after transplantation. Fifty patients remained stable throughout the study, whereas 32 developed BOS. These patients were selected according to the availability of blood samples and to obtain homogeneous groups. Patients were sampled before transplantation, at 1 and 6 months after transplantation, and then every 6 months thereafter. Samples were thus available at 1 and 6 months after transplantation but also 6 months to 1 year before the diagnosis of BOS, depending on the date of occurrence of FEV₁ decline. [Supplementary Material Figure S1](#) (refer to [Supplementary Material](#) available online at www.jhltonline.org/) summarizes the patients chosen for CD8⁺, CD4⁺, and CD4⁺CD25^{hi}FoxP3⁺ analyses. [Table 1](#) and [Table S1](#) ([Supplementary Material](#) online) list the baseline characteristics of the whole cohort and the 3 groups of patients, respectively.

Cell isolation and flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE Healthcare, Marolles-en-Hurepoix, France) gradient centrifugation, then frozen and stored at the Centre de

Table 1 Demographic and Clinical Characteristics of the 82 Lung Transplant Recipients Included in the Study

Demographic characteristics	Groups		p-value
	STA	BOS	
Participants (<i>n</i>)	50	32	
Recipient gender [<i>n</i> (%)]			0.9382
Male	27 (54)	17 (53)	
Female	23 (46)	15 (47)	
Recipient age (years) [median (min to max)]	40.5 (16 to 65)	46.5 (15 to 65)	0.9032
Donor gender [<i>n</i> (%)]			0.4776
Male	29 (58)	16 (50)	
Female	21 (42)	16 (50)	
Donor age (years) [median (min to max)]	38 (16 to 69)	47 (10 to 68)	0.1566
Pretransplant diagnosis [<i>n</i> (%)]			0.1113
Cystic fibrosis	25 (50.0)	9 (28.1)	
Emphysema/COPD	12 (24.0)	7 (22.0)	
Pulmonary arterial hypertension	4 (8.0)	3 (9.3)	
Idiopathic pulmonary fibrosis	5 (10.0)	7 (22.0)	
Bronchiectasis	4 (8.0)	3 (9.3)	
Other	0 (0)	3 (9.3)	
Transplant type [<i>n</i> (%)]			0.9645
Bilateral	39 (78)	25 (78.1)	
Single	7 (14)	4 (12.5)	
Lobar	2 (4)	2 (6.3)	
Heart-lung	2 (4)	1 (3.1)	
Ischemic time (minutes) [median (min to max)]	350 (205 to 480)	347 (176 to 540)	0.5511
Induction agent [<i>n</i> (%)]			0.0553
Basiliximab	17 (34)	4 (12.5)	
Thymoglobulin	17 (34)	18 (56.3)	
None	16 (32)	10 (31.2)	
IS therapy [<i>n</i> (%)]			0.3602
Steroids	49 (98)	31 (97)	
Cyclosporine	26 (52)	26 (81)	
Tacrolimus	39 (78)	20 (63)	
MMF	48 (96)	30 (94)	
HLA mismatch $\geq 4/6$ [<i>n</i> (%)]	39 (78)	25 (78)	0.4571
HLA-A $\geq 1/2$	45 (90)	28 (88)	0.9638
HLA-B $\geq 1/2$	45 (90)	30 (94)	0.6306
HLA-DR $\geq 1/2$	45 (90)	30 (94)	0.8980
Acute cellular rejection [<i>n</i> (%)]			0.2561
Only 1 after TP	13 (26)	9 (28)	
> 1 after TP	17 (34)	9 (28)	
Onset of BOS (months) [median (min to max)]	—	24 (6 to 42)	—

BOS, bronchiolitis obliterans syndrome; COPD, chronic obstructive pulmonary disease ; HLA, human leukocyte antigen; IS, immunosuppression; MMF, mycophenolate mofetil; STA, stable graft function.

Ressources Biologiques (CRB) of Nantes. PBMCs were rapidly thawed by placing cryovials at 37°C, washed, and stained. The antibodies used for staining CD8, CD4, and regulatory T cells are presented in [Table S2 \(Supplementary Material online\)](#). A FoxP3 Fixation/Permeabilization Kit and an Intracellular Fixation and Permeabilization Buffer Set (eBioscience, Santa Clara, CA) were used for intracellular staining of FoxP3 and granzyme B (GZMB), perforin (PERF), and T-box transcription factor (T-bet), respectively. Dead cells were excluded using the Zombie NIR Fixable Viability Kit (BioLegend, San Diego, CA) or Live/Dead Fixable Dead Cell Stain Kit (eBioscience). An analysis of the different T-cell subsets is shown in [Table S3 \(Supplementary Material online\)](#). BD LSRFORTESSA X20, BD LSR II (BD Biosciences, Franklin Lakes, NJ) and FLOWJO (FlowJo Software, Ashland, OR) were used for acquisition and analysis, respectively.

Cytokine secretion assay

For the T-regulatory cell (Treg) secretion assay, peripheral blood mononuclear cells were thawed and rested 1 night in complete medium (RPMI-160, 10% fetal bovine serum, 1% glutamine, 1% penicillin/streptomycin; 5 million/ml). PBMCs were then cultured at 5 million/ml in complete medium and activated for 5 hours with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml), ionomycin (1 µg/ml), and brefeldin A (1×) (Sigma Aldrich, St. Louis, MO) to block intracellular trafficking. The following antibodies and reagents were used for flow cytometry: CD3-BUV395 (UCHT1); CD25-BV786 (M-A251); CD4-BV650 (SK3); CD127-BV450 (HIL-7R-M21); IL-10-APC (JES3-19F1); IL-17A-PE (SCPL1362); IL-13-PE (JES10-5A2); IL-4-APC (MP4-25D2); and IfnG-FITC (B27) (BD Biosciences, Heidelberg, Germany); CD45RA-APCeFluor780 (HI-100); and FoxP3-PerpcCy5.5

(PCH101) (eBioscience). For cytokine secretion assay, the FoxP3 Fixation/Permeabilization Kit was used (eBioscience). The Live/Dead Fixable Aqua Blue Kit was used to exclude dead cells during staining (Life Technologies, Carlsbad, CA). BD LSRFORTESSA X20 was used for analysis.

In-vitro CD8 T-cell stimulation

PBMCs were thawed by placing cryovials at 37°C and washed. After overnight culture, 5 million PBMCs were stimulated with anti-CD3 (OKT3, 1 µg/ml), anti-CD28 (28.2, 1 µg/ml), and interleukin (IL)-15 (10 ng/ml; Miltenyi Biotec, Bergisch Gladbach, Germany) for 2 hours in complete RPMI medium (fetal bovine serum 10%, penicillin streptomycin 100 U/ml, glutamine 2 µmol/liter, sodium pyruvate 10 µmol/liter). Cellular activation was assessed using anti-CD25-FITC (M-A251; BD Biosciences) and anti-CD69-PerCP Cy5.5 (SK7; BD Biosciences) staining. BD LSR II (BD Biosciences) and FLOWJO were used for acquisition and analysis, respectively.

Statistical analysis

Longitudinal analysis was performed using two-way analysis of variance (ANOVA), followed by a Sidak multiple comparisons test. The non-parametric Mann-Whitney *U*-test and Kruskal-Wallis test with Dunn's ad-hoc pairwise comparisons were used to compare BOS and STA, and BOS, STA, and healthy volunteers (HV) T-cell subset proportions at specific time-points. Non-parametric Wilcoxon matched-pairs signed rank tests were used to compare paired BOS and STA CD8⁺ T-cell subset proportions at BOS diagnosis and immediate months beforehand. Correlations were assessed using the Pearson test (for $n > 30$) and non-parametric Spearman test (for $n < 30$). Thresholds were determined using receiver operating characteristic (ROC) curve analysis as the value maximizing sensibility and specificity. BOS-free survival analyses were performed using the Kaplan-Meier log rank test. Cox proportional analysis was used for univariate time-to-event analysis between clinical and biological parameters and time to BOS with the R *survival* package (*Coxph* function in R version 3.3.2, R Foundation for Statistical Computing, Vienna, Austria). Parameters with a $p < 0.2$ by the Wald test were entered into the multivariate analysis. Mann-Whitney *U*-tests and chi-square tests for categorical variables were performed using PRISM version 6 software (GraphPad, Inc., La Jolla, CA). Differences were defined as statistically significant when $p < 0.05$.

Results

Patients' characteristics

Eighty-two LTRs were enrolled in this study. After 3 years of follow-up, 50 patients remained stable, whereas 32 developed BOS. Eight healthy volunteers were also included as controls (see Methods section). None of the already known risk factors of BOS differed significantly between the 2 groups of patients. Most patients received double lung transplantation with the other transplant types (single lung, lobar, and heart-lung) being equally represented among both groups. Recipient ages were not significantly different and no significant difference regarding acute rejection episodes, ischemic time, immunosuppressive treatment regimen, and induction agents was apparent.

CD8 T-cell immune profile analysis in the months before BOS and at BOS event

To evaluate how the T-cell profile could be associated with long-term BOS development, we analyzed CD4⁺ and CD8⁺ T-cell subset frequencies in the blood of STA and BOS patients at 6 and 12 months before the BOS and at BOS diagnosis. We analyzed the distribution of CD8⁺ subsets according to the expression of CD45RA and CCR7. The frequencies of CD45RA⁺CCR7⁺ naive, CD45RA⁻CCR7⁻ effector memory (EM), CD45RA⁻CCR7⁺ central memory (CM), and CD45RA⁺CCR7⁻ effector memory CD45RA⁺ (TEMRA) CD8⁺ T cells were not different between BOS and paired STA patients at diagnosis of BOS or in the months beforehand (Figure 1A and B). Because a modification of the CD8 compartment could also result in the alteration of functional markers related to their activation, such as CD28 or T-bet expression, or to their cytotoxic potential through the secretion of GZMB and PERF, we analyzed the differentiation profile of memory EM and TEMRA CD8⁺ T cells. At the time of diagnosis and in the months beforehand, the activation status of memory CD8⁺ T cells remained stable between the 2 groups of patients with no difference, as was the case for CD28 or T-bet expression or cytotoxic functions (GZMB and PERF expression) (Figure 1A and B). Finally, we tested the ability of CD8⁺ T cells from BOS and STA patients to respond to short-term stimulation with anti-CD3, anti-CD28, and IL-15 with no difference for the regulation of CD25 and CD69 across both groups (Figure 1C).

Circulating CD4⁺CD25^{hi}FoxP3⁺ T-cell proportion increases in early months post-transplantation in BOS patients

We analyzed the distribution of CD4⁺ T-cell subsets according to the expression of CD45RA, CXCR5, FoxP3, and CD25. Whereas no difference was found for CD45RA⁺CXCR5⁻ naive, CD45RA⁻CXCR5⁻ memory, and CD25^{hi}FoxP3⁺ subsets, we observed a trend for an increased proportion of CD45RA⁻CXCR5⁺ follicular helper CD4⁺ T cells in BOS patients at 6 months before BOS diagnosis compared with STA at 18 months post-transplantation (BOS/STA fold change of 2.54; see Figure 2A, and Supplementary Material Figure S2B and C online). Because of a trend in an increased proportion of CD45RA⁻CXCR5⁺ follicular helper CD4⁺ T cells in the 6 months before the BOS diagnosis, we further analyzed CD4⁺ T-cell phenotypes earlier after lung transplantation (1 and 6 months) to identify early predictive biomarkers of BOS development. Despite a more variable CD4⁺ T-cell profile at 1 month post-transplantation than at BOS diagnosis and the months immediately beforehand (Figure 2A and B), we did not observe a significant difference in total CD4⁺, naive CD4⁺, memory CD4⁺, or follicular helper CD4⁺ T-cell blood frequencies between BOS and STA patients (Supplementary Material Figure S2A online). Interestingly, only circulating CD4⁺CD25^{hi}FoxP3⁺ T-cell proportion was significantly increased at 1 month post-transplantation in

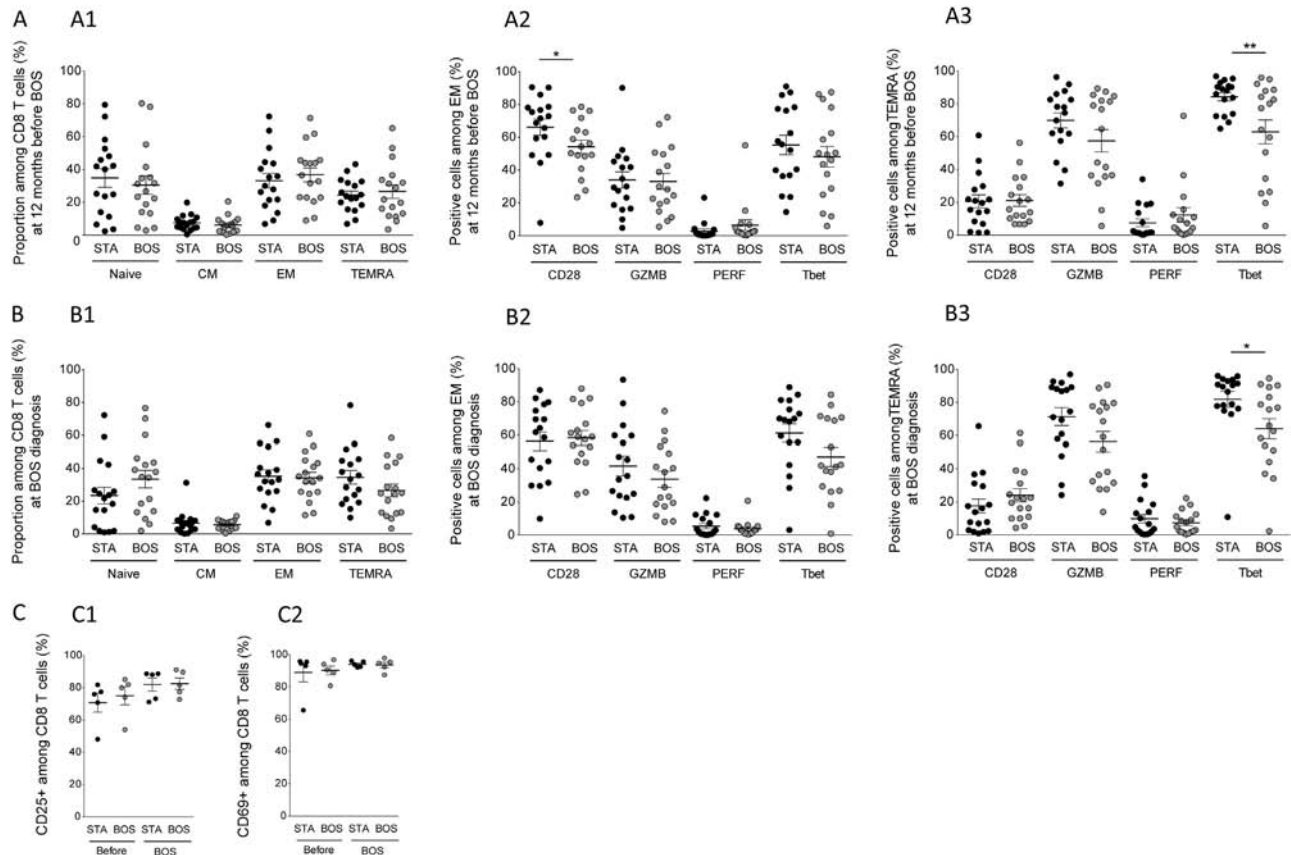


Figure 1 Analysis of the blood CD8 T-cell profile in lung transplant patients at BOS diagnosis and in the months beforehand. (A) Distribution of CD8 subsets; naive (CD45RA⁺CCR7⁺), central memory (CM, CD45RA⁻CCR7⁺), effector memory (EM, CD45RA⁻CCR7⁻), and terminally differentiated effector memory (TEMRA, CD45RA⁺CCR7⁻) (A1); expression of CD28, granzyme B (GZMB), perforin (Perf), and T-bet (T-box transcription factor 21 [TBX21]) by EM (A2) and TEMRA CD8 T cells (A3) at 12 months before BOS for BOS ($n = 17$) matched with STA ($n = 17$). (B) Distribution of CD8 subsets; naive (CD45RA⁺CCR7⁺), central memory (CM, CD45RA⁻CCR7⁺), effector memory (EM, CD45RA⁻CCR7⁻), and terminally differentiated effector memory (TEMRA, CD45RA⁺CCR7⁻) (B1); expression of CD28, GZMB, PERF, and TBX21 by EM (B2) and TEMRA CD8 T cells (B3) at the time of BOS diagnosis for BOS ($n = 17$) matched with STA ($n = 17$). (C) Activation state, that is, CD25 and CD69 expression by EM (C1) and TEMRA CD8 T cells (C2) after aCD3, aCD28, and interleukin-15 stimulation of PBMCs at BOS diagnosis and 12 months before for BOS ($n = 5$) matched with STA ($n = 5$). Results are expressed as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

BOS compared with STA patients ($3.36 \pm 0.75\%$ and $0.85 \pm 0.28\%$, respectively, $p < 0.05$; Figure 2C). Because CD4⁺ T cells expressing FoxP3 and CD25 are mainly known as regulatory T cells (Tregs), we further analyzed this compartment according to the expression of CD45RA, FoxP3, CD15s, and CD39. No specific enrichment was observed for CD45RA⁻FoxP3^{low} non-Tregs, CD45RA⁻FoxP3^{hi} memory Tregs, CD45RA⁺FoxP3^{low} naive Tregs, CD15s⁺FoxP3⁺ activated effector Tregs, or CD39⁺FoxP3⁺ Tregs between BOS, STA, and HV patients (Figure 2D and E, and Supplementary Material Figure S3A–C). These observations suggest that a global increase in the entire CD4⁺CD25^{hi}FoxP3⁺ T-cell compartment at 1 month post-transplantation is associated with BOS development within the 3 years after lung transplantation. This profile remained stable over the first 6 months post-transplantation with an average proportion of $3.39 \pm 0.40\%$ and $1.67 \pm 0.22\%$ of CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4⁺ T cells for BOS and STA, respectively ($p < 0.005$; Figure 3A–C).

To better characterize the suppressive capacities of these CD4⁺CD25^{hi}FoxP3⁺ T cells, we analyzed their cytokine

secretion profile. We identified the same expression of IL-10 by Tregs for BOS, STA, and HV (Figure 3D). Concerning the other cytokines, we found no significant difference in IL-17A, IL-4, IL-13, and interferon-gamma secretion by CD4⁺CD25^{hi}FoxP3⁺ T cells between BOS, STA, and HV (Supplementary Material Figure S4 online). This suggests that CD4⁺CD25^{hi}FoxP3⁺ T cells, shown to be increased in proportion in the blood of BOS patients, are effective regulatory T cells, as in STA and HV, with an equivalent cytokine secretion profile.

Circulating CD4⁺CD25^{hi}FoxP3⁺ T-cell proportion increases in BOS patients unrelated to confounding factors

We analyzed induction and immunosuppressive drug effects, as well as recipient age, HLA mismatches, FEV₁ at blood sampling, primary graft dysfunction, and acute rejection episode association with CD4⁺CD25^{hi}FoxP3⁺ proportion in transplanted patients. We found that this early increase in

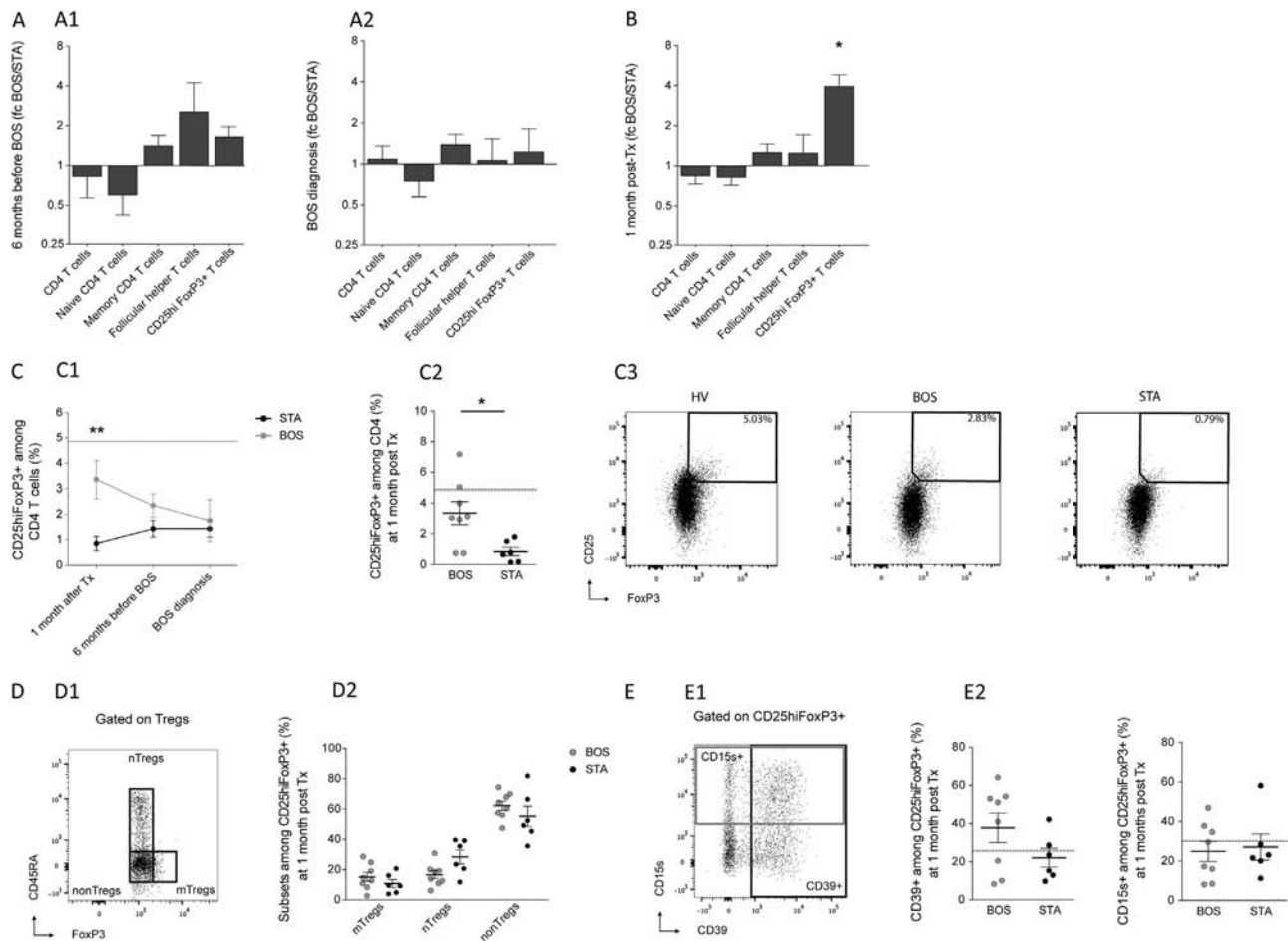


Figure 2 Blood CD4 T-cell profile and phenotypical characterization of CD4⁺CD25^{hi}FoxP3⁺ T cells at 1 month post-transplantation. (A) Proportion of CD4 T-cell subsets at 6 months before BOS (A1) and at BOS diagnosis (A2) represented as fold change BOS/STA. (B) Proportion of CD4 T-cell subsets at 1 month after transplantation represented as fold change BOS/STA. (C) Proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4 T cells over time in the blood of BOS (*n* = 9), STA (*n* = 8), and HV (dotted line) (*n* = 8) (C1); percentage of CD4⁺CD25^{hi}FoxP3⁺ T cells among blood CD4 T cells at 1 month after transplantation in BOS (*n* = 8), STA (*n* = 6), and HV (dotted line) (*n* = 8) (C2); and representative plots for CD4⁺CD25^{hi}FoxP3⁺ T-cell assessment by flow cytometry (C3). (D) Gating strategy for the identification of CD45RA⁺FoxP3^{low} naive Tregs, CD45RA⁻FoxP3^{low} non-Tregs, CD45RA⁻FoxP3^{hi} memory Tregs (D1), and Treg sub-population homeostasis with the proportion of mTregs, nTregs, and non-Tregs among total blood CD4⁺CD25^{hi}FoxP3⁺ T cells for BOS (*n* = 8) and STA (*n* = 6) at 1 month post-transplantation (D2). (E) Gating strategy for the identification of CD15e⁺ Tregs and CD39⁺ Tregs (E1) and percentage of CD39⁺ and CD15e⁺ Tregs among total blood CD4⁺CD25^{hi}FoxP3⁺ T cells for BOS (*n* = 8), STA (*n* = 6), and HV (dotted line) (*n* = 8) at 1 month post-transplantation (E2). Results are expressed as mean ± SEM. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001; **** *p* < 0.0001.

CD4⁺CD25^{hi}FoxP3⁺ T cells is not correlated with age, FEV₁ at blood sampling or HLA-A, -B, and -DR mismatch (Figure 3E). Among the whole cohort of lung transplant patients, there was no significant difference in CD4⁺CD25^{hi}FoxP3⁺ proportion in the 6 months post-transplantation according to the induction treatment (no induction, anti-thymocyte globulin [ATG], or basiliximab), with the exception of ATG-treated patients vs no induction (*p* < 0.05) (Figure 3F). Because more BOS patients are treated with ATG compared with STA, we did the same analysis in BOS and STA independently. We found no significant difference in CD4⁺CD25^{hi}FoxP3⁺ proportion in the 6 months post-transplantation according to induction treatment (no induction, ATG, or basiliximab) (Figure 3F). Immunosuppressive therapies at the blood sampling (tacrolimus, cyclosporine, mycophenolate mofetil [MMF], and corticotherapy) do not impact CD4⁺CD25^{hi}FoxP3⁺ proportion in the 6 months

post-transplantation in lung transplant patients (Figure 3G). Finally, we found that proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells early post-transplantation was not significantly different between patients who had primary graft dysfunction or acute rejection episodes and those who did not (Figure 3H and I). These results suggest that the increased proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells in the 6 months post-transplantation in the blood of BOS patients compared with STA was not due to clinical confounding factors.

Greater risk of BOS in lung recipients with CD4⁺CD25^{hi}FoxP3⁺ T cells higher than the 2.4% threshold in the first 6 months post-transplantation

Because circulating CD4⁺CD25^{hi}FoxP3⁺ T cells are elevated in the 6 months after lung transplantation in

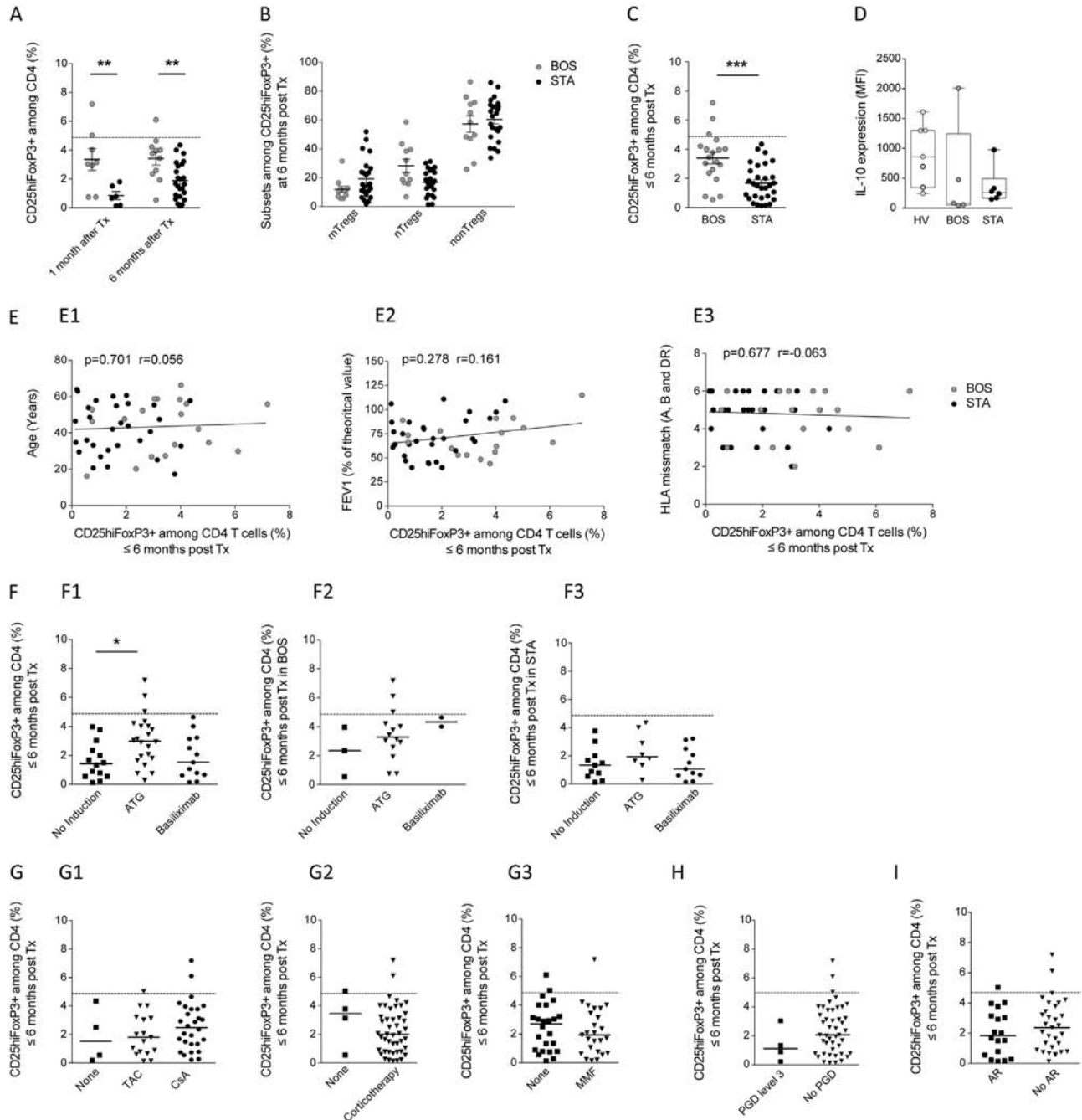


Figure 3 Conserved CD4⁺CD25^{hi}FoxP3⁺ T-cell profile at 1 and 6 months post-transplantation in the blood of lung recipients. (A) Proportion of circulating CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4 T cells at 1 and 6 months post-transplantation for BOS, STA, and HV (dotted line). (B) CD4⁺CD25^{hi}FoxP3⁺ T-cell sub-population homeostasis with the proportion of mTregs, nTregs, and non-Tregs among CD4⁺CD25^{hi}FoxP3⁺ T cells for BOS (*n* = 11) and STA (*n* = 24) at 6 months post-transplantation. (C) Proportion of circulating CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4 T cells in the first 6 months after lung transplantation for BOS (*n* = 19), STA (*n* = 30), and HV (dotted line) (*n* = 8). (D) IL-10 expression by CD4⁺CD25^{hi}FoxP3⁺ T cells in HV and lung transplant patients at 6 months post-transplantation. (E) Correlation between the proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells in first 6 months post-transplantation and age at the sample collection (E1), FEV₁ at sample collection (E2) and HLA-A, -B, and -DR at transplantation (E3) for BOS (*n* = 19) and STA (*n* = 30). (F) Proportion of circulating CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4 T cells in the first 6 months post-transplantation for BOS and STA according to the induction treatment of the patients. (F1) Analysis of the whole group of lungs transplanted in the study. (F2) Analysis of BOS patients. (F3) Analysis of STA patients. (G) Proportion of circulating CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4 T cells in the first 6 months post-transplantation for BOS and STA according to the immunosuppressive treatments. (G1) Comparison of tacrolimus (TAC), cyclosporine (CsA), and non-induction. (G2) Comparison of corticotherapy vs no corticotherapy. (G3) Comparison of MMF vs no MMF. (H) Proportion of circulating CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4 T cells in the first 6 months post-transplantation for BOS and STA, according to the development of primary graft dysfunction (PGD). (I) Proportion of circulating CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4 T cells in the first 6 months post-transplantation for BOS and STA according to the development of acute rejection (AR) episodes. Results are expressed as mean ± SEM. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

patients who will develop BOS, we investigated whether the proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells could be associated with BOS severity and/or BOS-free survival. First, we analyzed the correlation between the proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells in the 6 months after lung transplantation and FEV₁% decrease at BOS diagnosis, which is the first clinical marker to stratify mild, moderate, or severe BOS. We found no association between BOS severity and proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells early post-transplantation ($r = 0.076$, $p = 0.756$) (Figure 4A). Next, we analyzed how CD4⁺CD25^{hi}FoxP3⁺ T-cell proportions early post-transplantation could discriminate between patients who will maintain stable graft function and those who will develop BOS. We identified that, at 1 month post-transplantation (8 BOS and 6 STA), a threshold of 2.4% of CD4⁺CD25^{hi}FoxP3⁺ T cells allowed for identification of patients who would develop BOS or not in the 3 years of follow-up (ROC curve analysis, area under the curve = 0.875, standard error = 0.097, and $p = 0.020$) with a sensitivity of 100% and a specificity of 75% (Figure 4B). Lung recipients with proportion of

CD4⁺CD25^{hi}FoxP3⁺ T cells at $\geq 2.4\%$ at 1 month after transplantation had a higher risk of developing BOS within 3 years (log rank, $p = 0.0073$; Figure 4C). Because assessment at 1 month after lung transplantation may be too early to draw definitive conclusions due to the effects of surgery or concomitant inflammatory events, we validated this threshold at 6 months post-transplantation in a prospective study on 24 STA and 11 BOS independent patients. Proportions of CD4⁺CD25^{hi}FoxP3⁺ T cells at 6 months post-transplantation still allowed sufficient discrimination between BOS and STA patients (area under the curve = 0.786, standard error = 0.087, and $p = 0.007$; Figure 4D), and lung recipients with CD4⁺CD25^{hi}FoxP3⁺ T cells $\geq 2.4\%$ at 6 months after transplantation had a higher risk of developing BOS during the 3 years (specificity: 62%; sensitivity: 78%; log-rank, $p = 0.0418$; Figure 4E). Altogether, the data show that this threshold allows to identify as early as 1 and 6 months post-transplantation those patients who will maintain stable graft function and those who will develop BOS within 3 years, with good specificity (69%) and sensitivity (76%) and

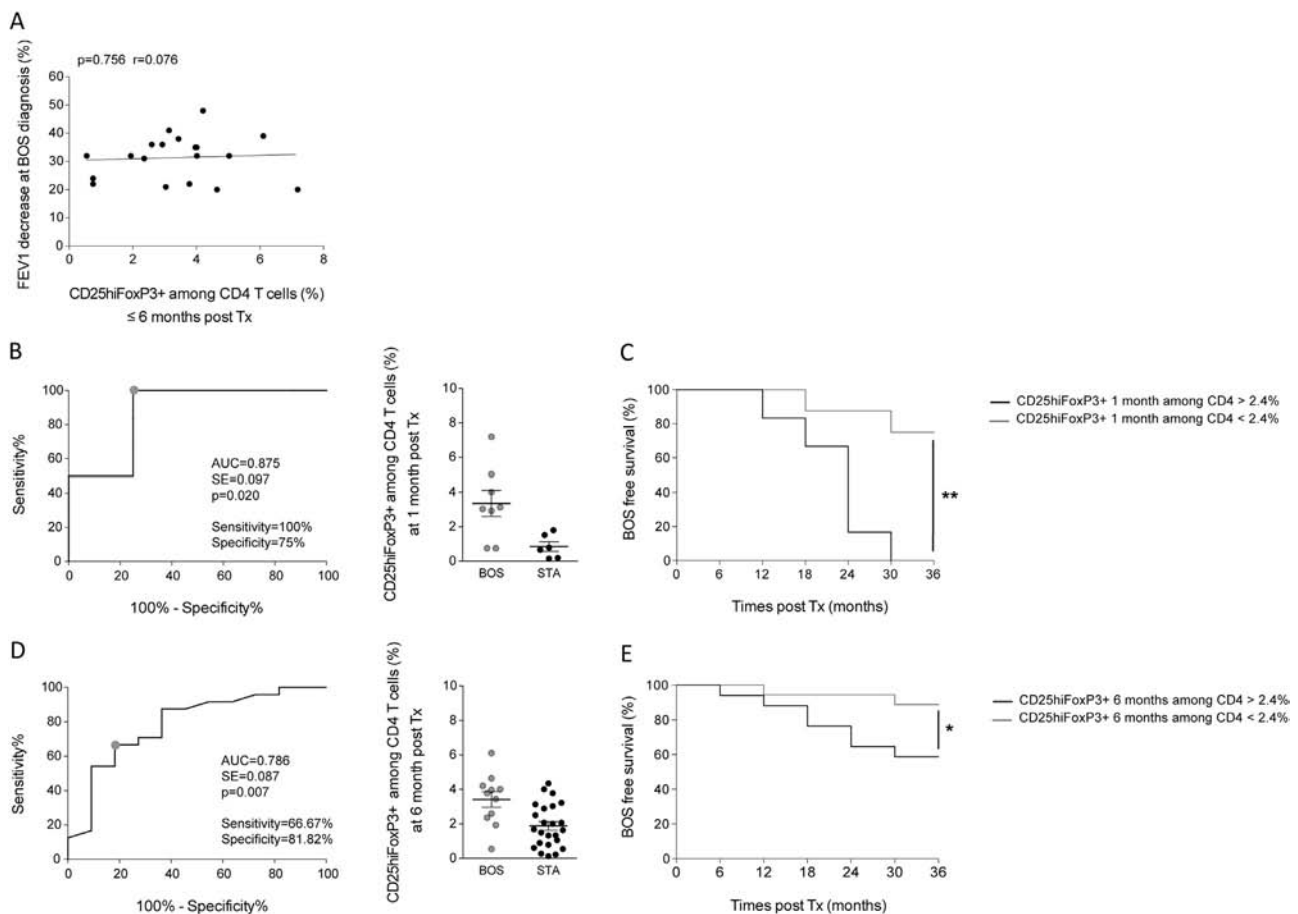


Figure 4 Analysis of CD4⁺CD25^{hi}FoxP3⁺ T-cell proportions early post-transplantation as a predictive biomarker of BOS development. (A) Correlation between the proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells in the 6 months post-transplantation and BOS severity assessed by FEV₁ decrease at BOS diagnosis. (B) ROC curve of the proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4 T cells at 1 month after transplantation for BOS ($n = 8$) and STA ($n = 6$). (C) Kaplan–Meier analysis of BOS-free survival according to CD4⁺CD25^{hi}FoxP3⁺ T-cell proportions among CD4 T cells at 1 month post-transplantation with a cut-off at 2.4% ($n = 14$). (D) ROC curve of the proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells among CD4 T cells at 6 months post-transplantation for BOS ($n = 11$) and STA ($n = 24$). (E) Kaplan–Meier analysis of BOS-free survival according to CD4⁺CD25^{hi}FoxP3⁺ T-cell proportions among CD4 T cells at 6 months post-transplantation with a cut-off at 2.4% ($n = 35$). Results are expressed as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 2 Multivariate Analysis of Parameters Selected as Significantly Modified in the Univariate Model Using a Cox Model

Parameters	Multivariate analysis	
	Wald test <i>p</i> -value	Hazard ratio
CD4 ⁺ CD25 ^{hi} FoxP3 ⁺ T-cell proportion up to 2.4%	0.0012	0.17
Time post-transplantation	0.016	0.99

positive and negative predictive values of 57% and 85%, respectively.

CD4⁺CD25^{hi}FoxP3⁺ T-cell proportion > 2.4% in the first 6 months post-transplantation as an independent risk factor for development of BOS

Because we identified CD4⁺CD25^{hi}FoxP3⁺ T-cell proportion as a possible predictive biomarker of BOS development, we investigated how this parameter could be helpful to risk-stratify patients as early as 6 months post-transplantation independently of other risk factors. Toward this end, univariate time-to-event analysis was performed between clinical, demographic, and biological parameters and time to BOS (Supplementary Material Table S4 online). Multivariate analysis highlighted that only CD4⁺CD25^{hi}FoxP3⁺ T-cell proportion > 2.4% in the first 6 months post-transplantation (Wald test, *p*-value = 0.0012; hazard ratio [HR] = 0.17) and time post-transplantation at time of test (Wald test, *p*-value = 0.016; HR = 0.99) were significantly associated with BOS occurrence (likelihood ratio test, *p* < 0.0001) (Table 2).

Discussion

The pathogenesis of BOS is still unknown and no satisfying biomarker is available so far. The recent identification of specific cellular subsets among the main immune cell populations offers the opportunity to deeply decipher the immune system at a time close to both transplantation and BOS development, and propose new potential biomarkers. We identified that the CD8⁺ and CD4⁺ compartments are not biased in BOS patients, neither at the time of BOS diagnosis nor during the 6 or 12 months beforehand. However, a proportion of the CD4⁺CD25^{hi}FoxP3⁺ T-cell subset was significantly increased as early as 1 month post-transplantation in those patients who proceeded to develop BOS within 3 years.

Although early infiltration of CD8⁺ memory T cells into heart, kidney, or liver grafts has been shown to accelerate rejection, and therefore represents a major barrier to long-term allograft survival,^{9–11} the absence of modification in the CD8⁺ T-cell compartment may be surprising. However, results obtained in animal models of lung transplantation have shown that immune responses differ from those observed for other organs, and lung infiltration by central

memory CD8⁺ T cells is critical for graft acceptance.¹² In humans, there have been contradictory reports regarding modification of the CD8⁺ T-cell compartment in BOS. Cytotoxic and pro-inflammatory CD8⁺ T cells may be detected in bronchoalveolar lavage (BAL) during chronic rejection,^{13–15} whereas Budding et al reported a similar proportion of naive, EM, and terminally differentiated effector CD8⁺ T cells at 5 months post-transplantation in BOS vs STA patients.¹⁶ In agreement with this report, we found no modification in the distribution of CD8⁺ subsets or characteristics (differentiation profile, cytotoxic functions), neither at the time of diagnosis nor beforehand. Accordingly, a comparison of the CD8⁺ profile in blood and BAL would be of interest to decipher where active immune responses take place during the pathophysiologic development of BOS.

Although the global T-cell profile was not different at the time of BOS or months beforehand, we detected a modification of this profile at 1 month post-transplantation in patients who would develop BOS compared with STA. This observation, supported by the identification of early risk factors of BOS development¹⁷ suggests that the immune mechanisms leading to BOS development are already established immediately post-transplantation and that biomarkers may be identified earlier. We found an increased proportion of circulating CD4⁺CD25^{hi}FoxP3⁺ T cells in BOS patients as early as 1 month after transplantation, which remained stable over the first 6 months post-transplantation. We also found that this increased proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells in BOS patients is not due to confounding factors such as induction treatments, immunosuppressive drugs, primary graft dysfunction, or acute rejection episodes, and is stable regardless of BOS severity at the diagnosis. Interestingly, CD4⁺CD25^{hi}FoxP3⁺ T cells were mainly characterized as Tregs. Different research groups have reported Treg subsets with distinct regulatory/non-regulatory functions and stability in inflammatory contexts. Memory CD45RA⁺FoxP3^{hi} Tregs, as opposed to naive CD45RA⁺FoxP3^{lo} Tregs and CD45RA⁺FoxP3^{lo} non-Tregs, have been described as a subset with high suppressive capacities.¹⁸ CD39⁺ Tregs have been characterized as memory Tregs,^{19,20} with a more stable and functional state under inflammatory conditions.²¹ Finally, CD15 expression identified highly differentiated and suppressive Tregs in humans.²² We found no bias toward a particular Treg subset between BOS and STA patients. Moreover, we assessed the CD4⁺CD25^{hi}FoxP3⁺ T cell cytokine profile at 6 months post-transplantation in BOS, STA, and HV and found equivalent secretion of the pro-tolerogenic cytokine IL-10 and, more generally, the same cytokine secretion profile between the 3 groups of patients. The fact that highly suppressive Treg subsets (i.e., memory and CD39⁺ Tregs) were increased, and that BOS CD4⁺CD25^{hi}FoxP3⁺ T cells exhibited an equivalent cytokine profile compared with STA and HV may suggest a regulatory phenotype and function.

It is not entirely clear why the concomitant CD4⁺CD25^{hi}FoxP3⁺ T-cell increase was only detected in blood from patients who would develop a BOS years later

and not in stable patients. Are these specific Tregs counteracting sub-clinical early inflammation and rejection processes that are not controlled effectively by immunosuppressive treatments, leading to an imprinted phenotype resulting in the development of BOS? The fact that these elevated CD4⁺CD25^{hi}FoxP3⁺ T cells in BOS patients could be Treg cells may appear challenging, because Tregs have been described as potential regulators in an operational tolerance state,^{23,24} and were reported to favor good outcomes after solid-organ transplantation.²⁵ However, they are also present in acute rejection and increased in response to immune activation.^{26–30} In accordance with this hypothesis, studies reported that inflammatory episodes after transplantation, such as acute rejection,^{31–36} ischemia–reperfusion injury,³⁷ or primary graft dysfunction,³⁸ are risk factors for BOS development. Furthermore, early post-transplant increases in pro-inflammatory serum chemokines and cytokines were found in the blood of BOS compared with non-BOS patients.³⁹ Interestingly, in the context of lung transplantation, Greenland et al reported that the proportion of CD4⁺CD25⁺ Tregs was significantly increased in the BAL of patients with acute rejection of Grade >A1 who would develop BOS,⁴⁰ suggesting that this increase in Tregs could also be found in the target graft long before BOS development. This point nevertheless remains to be clarified, and contradictory reports have been published. Although blood CD4⁺CD25^{hi} Tregs were found to be significantly higher in STA compared with BOS patients at the time of diagnosis,⁴¹ others found no difference in blood, or low BAL Treg proportions being associated with BOS development in several independent studies.^{16,42–44} Then, given the literature and our data showing this increase in circulating CD4⁺CD25^{hi}FoxP3⁺ with a regulatory phenotype in BOS patients only early post-transplantation, we hypothesize that these cells act as negative regulators of chronic dysfunction, which are present to counter sub-clinical inflammation events directed toward the graft and prevent rejection. However, they could be not sufficient in number to be sufficiently effective, or may even display defective suppressive function a long time after transplantation. This hypothesis is reinforced by the fact that this proportion of regulatory CD4⁺CD25^{hi}FoxP3⁺ cells is even more decreased as we get closer to the BOS event. By that time, CD4⁺CD25^{hi}FoxP3⁺ cells are likely not deleterious and do not contribute to BOS development, and thus it appears their proportion in the 6 months after transplantation can be used as an early marker of BOS development.

We wondered whether the CD4⁺CD25^{hi}FoxP3⁺ profile could be predictive of the occurrence of BOS and/or BOS-free survival for helping to risk-stratify lung recipients. Previous studies have already identified an association with CLAD markers,^{17,45–47} but none of them demonstrated sufficient robustness to achieve clinical acceptance, mainly because of limitations or confounding variables that did not allow their link to BOS or other immune events to be conclusive. Herein we determined a CD4⁺CD25^{hi}FoxP3⁺ T-cell threshold of 2.4% at 1 month post-transplantation, allowing for discrimination of BOS and STA patients with

high sensitivity and specificity. Patients with a high proportion of CD4⁺CD25^{hi}FoxP3⁺ T cells (up to 2.4%) presented a significantly higher risk of BOS development. This threshold was determined on a first set of 14 lung recipients at 1 month post-transplantation, and subsequently validated on another independent set of 35 lung recipients at 6 months post-transplantation. This threshold is sufficient to stratify patients with high or low risk of BOS development. Patients with a threshold up to 2.4% of CD4⁺CD25^{hi}FoxP3⁺ cells among CD4 T cells at 1 or 6 months present a significantly higher risk (2-fold) of developing BOS within 3 years post-transplantation. We also found that, contrary to the other known risk factors for BOS development, a proportion of CD4⁺CD25^{hi}FoxP3⁺ cells up to 2.4% is an independent predictive risk factor for a BOS event over time, from the first 6 months after transplantation.

In conclusion, although regulatory CD4⁺CD25^{hi}FoxP3⁺ T cells may act as negative regulator of BOS development not efficient enough to inhibit alloimmune response and in the time to prevent chronic dysfunction, the use of their proportion in the first 6 months after transplantation as predictive biomarker is very encouraging. Although it was validated in 2 independent groups of lung transplant patients, further studies and validation on larger cohorts are now necessary to determine whether this CD4⁺CD25^{hi}FoxP3⁺ T-cell profile, identified on frozen blood cells, may be used as a predictive biomarker of risk of BOS development using routinely collected fresh blood samples. Moreover, the underlying mechanisms leading to this increased proportion of regulatory CD4⁺CD25^{hi}FoxP3⁺ T cells in BOS patients remain to be identified. In this context, investigation of antigen specificity and/or alloreactivity of this cell subset would be of great interest. Unfortunately, this requires allograft-derived donor cells from lung biopsies of BOS patients, which we do not have through this protocol. Also, although it cannot be performed for the same reason, the analysis of what is occurring in the target tissue could help to better understand the distribution and the contribution of these CD4⁺CD25^{hi}FoxP3⁺ T cells in regulation of BOS development.

Disclosure statement

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Appendix

COLT Consortium

The COhort of Lung Transplantation (COLT) Consortium consists of associated surgeons, anesthesiologists/intensivists, physicians, and research staff from the following cities/regions: Bordeaux: J. Jougon, J.-F. Velly, H. Rozé, E. Blanchard, C. Dromer; Bruxelles: M. Antoine, M. Cappello, M. Ruiz, Y. Sokolow, F. Vanden Eynden, G. van Nooten, L. Barvais, J. Berré, S. Brimiouille, D. De Backer, J. Créteur, E. Engelman, I. Huybrechts, B. Ickx, T.J.C. Preiser, T. Tuna, L. Van Obberghé, N. Vancutsem, J.-L. Vincent, P. De Vuyst, I. Etienne, F. Féry, F. Jacobs, C. Knoop, J.L. Vachiéry, P. van den Borne, I. Wellemans, G. Amand, L. Collignon, M. Giroux; Grenoble: D. Angelescu, O. Chavanon, R. Hacini, C. Martin, A. Pirvu, P. Porcu; P. Albaladejo, C. Allègre, A. Bataillard, D. Bedague, E. Briot, M. Casez-Brasseur, D. Colas, G. Dessertaine, M. Durand, G. Francony, A. Hebrard, M.R. Marino, D. Protar, D. Rehm, S. Robin, M. Rossi-Blancher; C. Augier, P. Bedouch, A. Boignard, H. Bouvaist, A. Briault, B. Camara, J. Claustre, S. Chanoine, M. Dubuc, S. Quétant, J. Maurizi, P. Pavèse, C. Pison, C. Saint-Raymond, N. Wion; C. Chérion; Lyon: R. Grima, O. Jegaden, J.-M. Maury, F. Tronc, C. Flamens, S. Paulus, J.-F. Mornex, F. Philit, A. Senechal, J.-C. Glérant, S. Turquier, D. Gamondes, L. Chalabresse, F. Thivolet-Bejui, C. Barnel, C. Dubois, A. Tiberghien; Paris, Hôpital Européen Georges Pompidou: F. Le Pimpec-Barthes, A. Bel, P. Mordant, P. Achouh, V. Boussaud, R. Guillemain, D. Méléard, M.O. Bricourt, B. Cholley, V. Pezella; Marseille: G. Brioude, X.B. D'Journo, C. Doddoli, P. Thomas, D. Trousse, S. Dizier, M. Leone, L. Papazian, F. Bregeon, B. Coltey, N. Dufeu, H. Dutau, S. Garcia, J.Y. Gaubert, C. Gomez, S. Laroumagne, G. Mouton, A. Nieves, Ch. Picard, M. Reynaud-Gaubert, J.M. Rolain, E. Sampol, V. Secq; Nantes: P. Lacoste, C. Perigaud, J.C. Roussel, T. Senage, A. Mugniot, I. Danner, A. Haloun, A. Magnan, A. Tissot, S. Abbes, C. Bry, F.X. Blanc, T. Lepoivre, K. Botturi-Cavaillès, S. Brouard, R. Danger, J. Loy, M. Bernard, E. Godard, P.-J. Royer, E. Durand, K. Henrio, M. Durand, C. Brosseau, A. Foureau; Le Plessis Robinson, Hôpital Marie Lannelongue: Ph. Dartevelle, D. Fabre, E. Fadel, O. Mercier, S. Mussot, F. Stephan, P. Viard, J. Cerrina, P. Dorfmueller, S. Feuillet M. Ghigna, Ph. Hervén F. Le Roy Ladurie, J. Le Pavec,

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at www.jhltonline.org/.

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