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**BRIEF COMMUNICATION****Blood CD9<sup>+</sup> B cell, a biomarker of bronchiolitis obliterans syndrome after lung transplantation**

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Bronchiolitis obliterans syndrome is the main limitation for long-term survival after lung transplantation. Some specific B cell populations are associated with long-term graft acceptance. We aimed to monitor the B cell profile during early development of bronchiolitis obliterans syndrome after lung transplantation. The B cell longitudinal profile was analyzed in peripheral blood mononuclear cells from patients with bronchiolitis obliterans syndrome and patients who remained stable over 3 years of follow-up. CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells were increased in stable patients only, and reached a peak 24 months after transplantation, whereas they remained unchanged in patients

**Abbreviations:** Breg, B cells with regulatory properties; BOS, Bronchiolitis obliterans syndrome; BAL, Broncho-alveolar lavage; CRB, Centre de Ressources Biologiques; CLAD, Chronic lung allograft dysfunction; COLT, Cohort in Lung Transplantation; DSA, Donor-specific antibody; HV, Healthy volunteers; LTR, Lung transplant recipients; LT, Lung transplantation; PBMC, Peripheral blood mononuclear cells; STA, Stable.

Carole Brosseau and Richard Danger contributed equally to this work.

Antoine Magnan and Sophie Brouard contributed equally to this work.

\*A complete list of members of the COLT and SysCLAD Consortia is provided in the Appendix.

who developed a bronchiolitis obliterans syndrome. These CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells specifically secrete IL-10 and express CD9. Thus, patients with a total CD9<sup>+</sup> B cell frequency below 6.6% displayed significantly higher incidence of bronchiolitis obliterans syndrome (AUC = 0.836, PPV = 0.75, NPV = 1). These data are the first to associate IL-10-secreting CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells expressing CD9 with better allograft outcome in lung transplant recipients. CD9-expressing B cells appear as a contributor to a favorable environment essential for the maintenance of long-term stable graft function and as a new predictive biomarker of bronchiolitis obliterans syndrome-free survival.

#### KEY WORDS

B cell biology, basic (laboratory) research/science, biomarker, bronchiolitis obliterans (BOS), lung transplantation/pulmonology, translational research/science

## 1 | INTRODUCTION

Lung transplantation (LT) is the treatment for patients suffering from end-stage pulmonary diseases. Chronic lung allograft dysfunction (CLAD) is responsible for the poor long-term outcome of LT, with an occurrence of 50% within 5 years.<sup>1</sup> The most prevalent morphological feature in CLAD is bronchiolitis obliterans syndrome (BOS), defined as an obstructive pulmonary function defect in the absence of other identifiable causes, mostly not amenable to treatment.<sup>2</sup> Currently, the clinical surrogates used to define BOS do not allow for detecting early stages of the pathology, thus making the recognition of damage prior to its development necessary in designing efficient therapies. In this context, studies have been performed to identify early indicators such as lung biopsy profiling,<sup>3</sup> broncho-alveolar lavage (BAL) neutrophilia,<sup>4</sup> cytokines,<sup>5</sup> blood level of endothelin-1,<sup>6</sup> plasma level of soluble CD59,<sup>7</sup> human leukocyte antigen G (HLA-G),<sup>8</sup> or matrix metalloproteinase-9 (MMP-9)<sup>9</sup> but none have yet demonstrated sufficient robustness to achieve clinical acceptance.

Nowadays, many studies focus on the involvement and correlation between long-term allograft survival and B cells. Recently, we described the down-regulation of three genes associated with B cells able to predict BOS in whole blood.<sup>10</sup> Growing evidence suggests that B cells with regulatory properties (Breg) are associated with maintenance of long-term allograft function in skin, hematopoietic stem cell, and kidney transplantation.<sup>11-14</sup> They are thought to mediate their regulatory capacity predominantly via the secretion of IL-10.<sup>15,16</sup> IL-10-secreting CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup> transitional B cells were thus described to play an important role not only in maintaining long-term allograft function but also in promoting allograft tolerance.<sup>13,17-21</sup> We have previously demonstrated that murine IL-10<sup>+</sup> Bregs were enriched in a CD9<sup>+</sup> B cell subset and that adoptive transfer of CD9<sup>+</sup> B cells alone was sufficient to abrogate asthma in an IL-10-dependent manner.<sup>22</sup> We have also shown that CD9 expression in humans was dramatically increased at the surface of CD24<sup>hi</sup>CD38<sup>hi</sup> B cells, thus defining an important IL-10 Breg subset. This finding has been confirmed by others,<sup>23,24</sup> and CD9 thus

appears as a reliable marker for defining more precisely both mouse and human IL-10-secreting Bregs with suppressive properties.

In this study, we aimed to analyze the modifications of the B lymphocyte subset profile in lung transplant recipients (LTR) and/or to identify a potential B lymphocyte signature predictive of BOS and/or functional stability in LT. We report here a higher level of CD19<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup> transitional B cells expressing the CD9 marker and secreting IL-10 in patients with stable graft function and suggest that CD9 may serve as a potential blood biomarker of BOS.

## 2 | METHODS

### 2.1 | Patients and study design

The local ethical committee (Comité de Protection des Personnes Ouest 1-Tours, 2009-A00036-51) approved this study. Patients who participated in this study gave written informed consent. Blood samples were selected from patients included in the multicenter longitudinal cohort COLT (Cohort in Lung Transplantation, NCT00980967) with 3 years of follow-up. Pulmonary function tests, clinical data, and blood samples were collected at the inclusion visit before transplantation, the day of transplantation, 1 month and every 6 months post-LT. Stable (STA) and BOS phenotypes were confirmed by an adjudication committee of clinicians and researchers based on the values of the pulmonary function test, computerized tomography, and after elimination of confounding factors in line with the charter ISHLT/ERS/ATS.<sup>2</sup> BOS was defined by a decrease of at least 20% of the maximum FEV1 value recorded after transplantation. Donor-specific antibody (DSA) levels were measured for all LTR selected by Luminex High Performance assay. A kinetic was performed up to 36 months post-LT. All data are summarized in Tables 1 and 2 for the cohort of investigation and validation, respectively.

#### 2.1.1 | Cohort of investigation

Twenty STA and 21 BOS patients were selected based on sample availability (at least seven time points per patient, up to 60 months

**TABLE 1** Demographic and clinical data

	Stable	BOS	HV	P-value
Number of patients	20	21	19	
Recipient age (median, years) [range]	48 [23-71]	59 [27-68]	50 [21-66]	.396
Recipient gender, n (%)				.252
Male	8 (40)	10 (48)	13 (68)	
Female	12 (60)	11 (52)	6 (32)	
Donor age (median, years) [range]	49 [18-71]	44 [16-65]		.640
Donor gender, n (%)				.661
Male	10 (50)	12 (57)		
Female	10 (50)	9 (43)		
Pathology leading to Tx, n (%)	0.030			
Emphysema/COPD	7 (35)	9 (43)		
Cystic fibrosis	10 (50)	3 (14)		
Pulmonary arterial hypertension	2 (10)	6 (29)		
Idiopathic pulmonary fibrosis	1 (5)	2 (9)		
Other	0	1 (5)		
Type of Tx, n (%)	0.550			
Bilateral—heart-lung	15 (75)	14 (67)		
Single	5 (25)	6 (28)		
Lobar	0	1 (5)		
Median ischemic time (min) [range]	315 [215-467]	330 [150-540]		.648
Induction agent, n (%)				.350
Basiliximab	9 (45)	5 (24)		
Thymoglobulin	8 (40)	12 (57)		
None	3 (15)	4 (19)		
Immunosuppressive therapy (follow-up treatment), n (%)				
Steroids (corticosteroids)	18 (90)	18 (86)		.440
MMF (Cellcept)	7 (35)	11 (52)		
Tacrolimus	12 (60)	8 (38)		.148
Cyclosporin	7 (35)	12 (58)		
Infections, n (%)				.588
Bacteria	15 (75)	18 (89)		
Virus	14 (70)	16 (73)		
Fungi	14 (70)	10 (42)		
Mismatch				
CMV, n (%)	10 (50)	9 (43)		.661
Toxoplasmosis, n (%)	7 (35)	7 (37)		.841
EBV, n (%)	2 (10)	9 (9)		.978
HLA-A n ≥ 1/2	20 (100)	17 (89)		.3152
HLA-B n ≥ 1/2	20 (100)	19 (100)		.4506
HLA-DR n ≥ 1/2	20 (100)	19 (100)		.1110
HLA-DQ n ≥ 1/2	20 (100)	18 (95)		.0472
Median occurrence of BOS, month after Tx [range]	-	24 [12-42]		-
Acute cellular rejections, n (%)				.390
Ever A1 grade	8 (40)	9 (43)		
Ever ≥A2 grade	7 (35)	4 (19)		

Abbreviations: BOS, bronchiolitis obliterans syndrome; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; EBV, Epstein-Barr Virus; HLA, human leukocyte antigen; HV, healthy volunteers; MMF, mycophenolate mofetil; Tx, transplantation.

**TABLE 2** Demographic and clinical data of the validation cohort

	Stable	BOS	P-value
Number of patients	18	16	
Recipient age (median, years) [range]	48 [25-61]	36 [15-60]	.275
Recipient gender, n (%)			.505
Male	8 (44)	6 (37)	
Female	10 (56)	10 (63)	
Donor age (median, years) [range]	41 [26-55]	46 [10-66]	.931
Donor gender, n (%)			1.000
Male	11 (61)	6 (37)	
Female	7 (39)	10 (63)	
Pathology leading to Tx, n (%)			.731
Emphysema/COPD	7 (39)	7 (44)	
Cystic fibrosis	5 (28)	5 (31)	
Pulmonary arterial hypertension	1 (6)	1 (6)	
Idiopathic pulmonary fibrosis	4 (22)	1 (6)	
Other	1 (6)	2 (13)	
Type of Tx, n (%)			.882
Bilateral—heart-lung	12 (67)	12 (75)	
Single	4 (22)	3 (19)	
Lobar	2 (11)	1 (6)	
Median ischemic time (min) [range]	285 [120-420]	270 [120-500]	.543
Induction agent, n (%)			.168
Basiliximab	4 (22)	1 (6)	
Thymoglobulin	4 (22)	8 (50)	
None	10 (56)	7 (44)	
Immunosuppressive therapy (follow-up treatment), n (%)			
Steroids (corticosteroids)	17 (94)	16 (100)	.885
MMF (Cellcept)	16 (89)	14 (88)	
Tacrolimus	16 (89)	14 (88)	.654
Cyclosporin	5 (28)	6 (38)	
Infections, n (%)			.953
Bacteria	15 (83)	11 (69)	
Virus	14 (78)	9 (56)	
Fungi	13 (72)	10 (63)	
Mismatch			
CMV, n (%)	3 (17)	2 (13)	1.000
Toxoplasmosis, n (%)	2 (12)	6 (38)	.117
EBV, n (%)	0 (0)	3 (19)	.093
HLA-A n ≥ 1/2	18 (100)	16 (100)	.509
HLA-B n ≥ 1/2	18 (100)	16 (100)	.681
HLA-DR n ≥ 1/2	17 (94)	16 (100)	.290

(Continues)

**TABLE 2** (Continued)

	Stable	BOS	P-value
HLA-DQ n ≥ 1/2	16 (94)	13 (81)	.522
Median occurrence of BOS, month after Tx [range]	-	27 [11-42]	-
Acute cellular rejections, n (%)			1.000
Ever A1 grade	10 (56)	6 (40)	
Ever ≥A2 grade	4 (22)	3 (20)	

Abbreviations: BOS, bronchiolitis obliterans syndrome; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; EBV, Epstein-Barr Virus; HLA, human leukocyte antigen; MMF, mycophenolate mofetil; Tx, transplantation.

for stable patients) and were matched regarding age and sex. Also, 19 healthy volunteers (HV) were used as controls but, as these individuals are not transplanted, they were not used in statistical analyses. Demographic features and clinical data are summarized in Table 3.

### 2.1.2 | Cohort of validation

For the cohort of validation, another 19 STA and 15 BOS patient samples were analyzed at 24 months post-LT and were matched regarding age, sex, and initial disease. Demographic features and clinical data are summarized in Table 4.

### 2.2 | Cell isolation and flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (GE Healthcare, Marolles-en-Hurepoix, France) gradient centrifugation, frozen, and stored at the Centre de Ressources Biologiques (CRB) of Nantes. PBMC were rapidly thawed by placing cryovials at 37°C, washed, and stained according to standard protocols using the following mAbs: CD19-BUV395, CD27-BUV737, CD38-BV605, CD138-APC, CD24-PerCP-Cy5.5, CD9-BV510, CD22-PeCy7, IgD-PE, and IgM-FITC (BD Biosciences, Le Pont de Claix, France). Markers were used to distinguish CD19<sup>+</sup> B lymphocytes, CD19<sup>+</sup>CD27<sup>+</sup> memory cells, CD19<sup>+</sup>CD27<sup>-</sup> naive cells, CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional cells, and CD19<sup>+</sup>CD24<sup>-</sup>CD38<sup>+</sup> plasma cells. Dead cells were excluded using the Zombie NIR Fixable Viability kit (BioLegend, London, UK). Samples were run on a BD LSRFORTESSA X-20 (BD Biosciences, Le Pont de Claix, France), and data were analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR).

### 2.3 | Analysis of IL-10 production by stimulated B cells

Cells were stimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL), ionomycin (0.5 mg/mL), and

**TABLE 3** Kinetic of DSA development in stable patients (Median Fluorescence Intensity [MFI])

	Months post-LT	Before LT	Time of LT	1	6	12	18	24	30	36
1	STA	NO	DQ7 (6512)	DQ7 (4045)	DQ7 (1174)	NO	NO	NO	NO	NO
2	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
3	STA	NO	DQA1(1000)	NO	NO	NO	NO	NO	NO	NO
4	STA	NO	NO	NO	NO	DQ7 (1200)	NO	NO	NO	NO
5	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
6	STA	-	-	-	-	-	NO	NO	-	-
7	STA	-	-	-	-	-	-	NO	-	NO
8	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
9	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
10	STA	-	NO	DR7, DR8, DQ2	DQ2, DQ7	NO	NO	NO	NO	NO
11	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
12	STA	NO	NO	A2, DQ6	NO	NO	NO	NO	NO	NO
13	STA	NO	NO	DQ2, DQ4	DQ4	NO	NO	NO	NO	NO
14	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
15	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
16	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
17	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
18	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
19	STA	DR17	-	-	B8, DR4, DR17, DQ7 DR52	NO	NO	NO	NO	NO
20	STA	-	-	-	-	-	-	-	DR4 (609)	-
21	STA	-	NO	NO	-	-	-	-	-	NO
22	STA	NO	NO	DQ6 (532) DQ8 (1381)	NO	NO	-	-	-	-
23	STA	-	NO	NO	NO	NO	-	-	NO	-
24	STA	-	-	-	-	-	-	-	-	-
25	STA	-	-	-	-	-	-	-	DR4(726)	NO
26	STA	NO	NO	NO	NO	-	-	-	-	-
27	STA	-	-	-	-	-	-	-	-	-
28	STA	-	-	-	-	-	-	-	-	-
29	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
30	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
31	STA	NO	NO	NO	-	-	NO	NO	-	-
32	STA	NO	NO		NO	NO	NO	NO	NO	NO
33	STA	-	-	-	-	NO	NO	NO	NO	NO
34	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
35	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
36	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
37	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO
38	STA	NO	NO	NO	NO	NO	NO	NO	NO	NO

Abbreviations: DSA, donor-specific antibody; LT, lung transplant; STA, stable.

brefeldin-A (10 mg/mL) (Sigma-Aldrich, St. Louis, MO). For detection of intracellular IL-10, cells were fixed and permeabilized using the Permeabilization/Fixation Kit (BD Biosciences) and staining was performed using anti-IL-10-PE (BD Biosciences).

## 2.4 | Statistical analysis

Gaussian distribution was assessed with the D'Agostino-Pearson test. For longitudinal analysis, STA and BOS groups were compared

**TABLE 4** Kinetic of DSA development in BOS patients ( Median Fluorescence Intensity [MFI])

	Months post-LT	Before LT	Time of LT	1	6	12	18	24	30	36
1	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
2	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
3	BOS	NO	DR7 DR11 DQ2 DQ7 (301)	-	-	DR7 DR11 DQ2 DQ4 DQ7 DQ8 DQ9 (4852)	DR7 DR11 DQ2 DQ4 DQ7 DQ8 DQ9 (15673)	DR7 DR11 DQ2 DQ4 DQ7 DQ8 DQ9 (17341)	DR7 DR11 DQ2 DQ4 DQ7 DQ8 DQ9	DR7 DR11 DQ2 DQ4 DQ7 DQ8 DQ9
4	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
5	BOS	NO	NO	NO	NO	NO	NO	DQ2 (3384)	DQ2 (3434)	DQ2 (1046)
6	BOS	NO	NO	YES	YES	YES	YES	YES	YES	NO
7	BOS	NO	NO	NO	NO	NO	-	YES	YES	YES
8	BOS	NO	NO	YES	YES	NO	YES	YES	YES	YES
9	BOS	DQ	DQ (1308)	DQ (1851)	NO	NO	NO	NO	NO	NO
10	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
11	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
12	BOS	DR17 (1000)	DQB1 (700)	NO	NO	-	DP14 (512) DQ4 (811)	DQ4 (1635) DQB1(704)	NO	-
13	BOS	-	-	-	NO	NO	-	-	-	DQ2 (15510)
14	BOS	-	-	-	NO	DQ2(3600) DR53 (2529)	-	-	NO	DPB1 (659)
15	BOS	-	-	-	-	-	-	NO	-	NO
16	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
17	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
18	BOS	-	-	-	-	-	-	-	-	-
19	BOS	DR11	DR11	DR11	YES	NO	NO	NO	NO	NO
20	BOS	NO	NO	NO	NO	NO	NO	DQ7	DQ7	NO
21	BOS	NO	NO	DQ7	DQ7	NO	NO	NO	NO	NO
22	BOS	NO	NO	NO	DQ8, DR7	NO	NO	NO	NO	NO
23	BOS	B7	B7	B7	B7	B7	B7	B7	B7	B7
24	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
25	BOS	NO	NO	B38, DR7, DQ2	DQ2	NO	NO	DQ2	NO	DQ2
26	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
27	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
28	BOS	-	-	-	-	-	-	-	-	NO
29	BOS	YES	NO	DQ9 (2571) DQ7 (2850)	DR4 (521)	NO	NO	-	-	-
30	BOS	-	B44 (813)	DQ6 (624)	NO	NO	NO	-	-	-
31	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO
32	BOS	-	-	-	-	-	-	-	-	DR1 (649)

(Continues)

TABLE 4 (Continued)

	Months post-LT	Before LT	Time of LT	1	6	12	18	24	30	36
33	BOS	-	NO	NO	NO	NO	-	NO	NO	DR1 (698), DP04 (699), DP13 (887)
34	BOS	-	-	-	-	-	-	-	-	-
35	BOS	NO	DR18 DR4 DR53 (468) DQ2 (387)	-	NO	NO	NO	NO	NO	DQ2 (1245)
36	BOS	NO	NO	-	NO	-	NO	-	A1 (529)	-
37	BOS	NO	NO	NO	NO	NO	NO	NO	NO	NO

Abbreviation: BOS, bronchiolitis obliterans syndrome.

using linear mixed-effect models (*lmer* R package) to account for repeated and missing measures using R version 3.4.3. *P*-values were obtained from F tests with Satterthwaite's method (*lmerTest* R package) with Benjamini-Hochberg multiple comparison correction. Total, plasmocyte, transitional and memory B cells data were log transformed (ln) and naive B cells data were transformed with logit to account for right and left skewed distributions, respectively. When group effect was significantly associated with analyzed parameter ( $P < .05$ ), Student's *t*-tests were used to compare BOS and STA groups at each time points with Benjamini-Hochberg correction for multiple comparisons (adjusted *P*-values). Logistic regression (*glm* package) was used to assess association between DSA presence and transitional B cells. Positive and negative predictive values and the Youden index, as the value maximizing specificity and sensitivity discriminating BOS and STA according to a receiver operating characteristic (ROC) curve, were computed using the pROC R package. Pearson correlations, ROC curves, survival analyses with Kaplan-Meier (log-rank test), Student's *t*-tests or chi-squared for qualitative variables were performed using the GraphPad Prism v.5 (GraphPad Software, La Jolla, CA). A  $P < .05$  was considered to be significant. Variations are expressed as standard deviation.

### 3 | RESULTS

#### 3.1 | Baseline characteristics of patient population

CLAD is a heterogeneous disorder that may develop through different clinical phenotypes.<sup>1,2</sup> We focused on BOS, the most common phenotype (Table 1). We compared them with STA and HV. Age and sex are not different between LTR (BOS and STA) and HV. None of the usual risk factors of CLAD significantly differ between STA and BOS. Most of the patients received bilateral LT (75% and 67% of STA and BOS, respectively;  $P = .550$ ). Recipient immunosuppressive treatments, ischemic time, number of acute cellular rejection events, infections, cytomegalovirus, Epstein-Barr virus and human

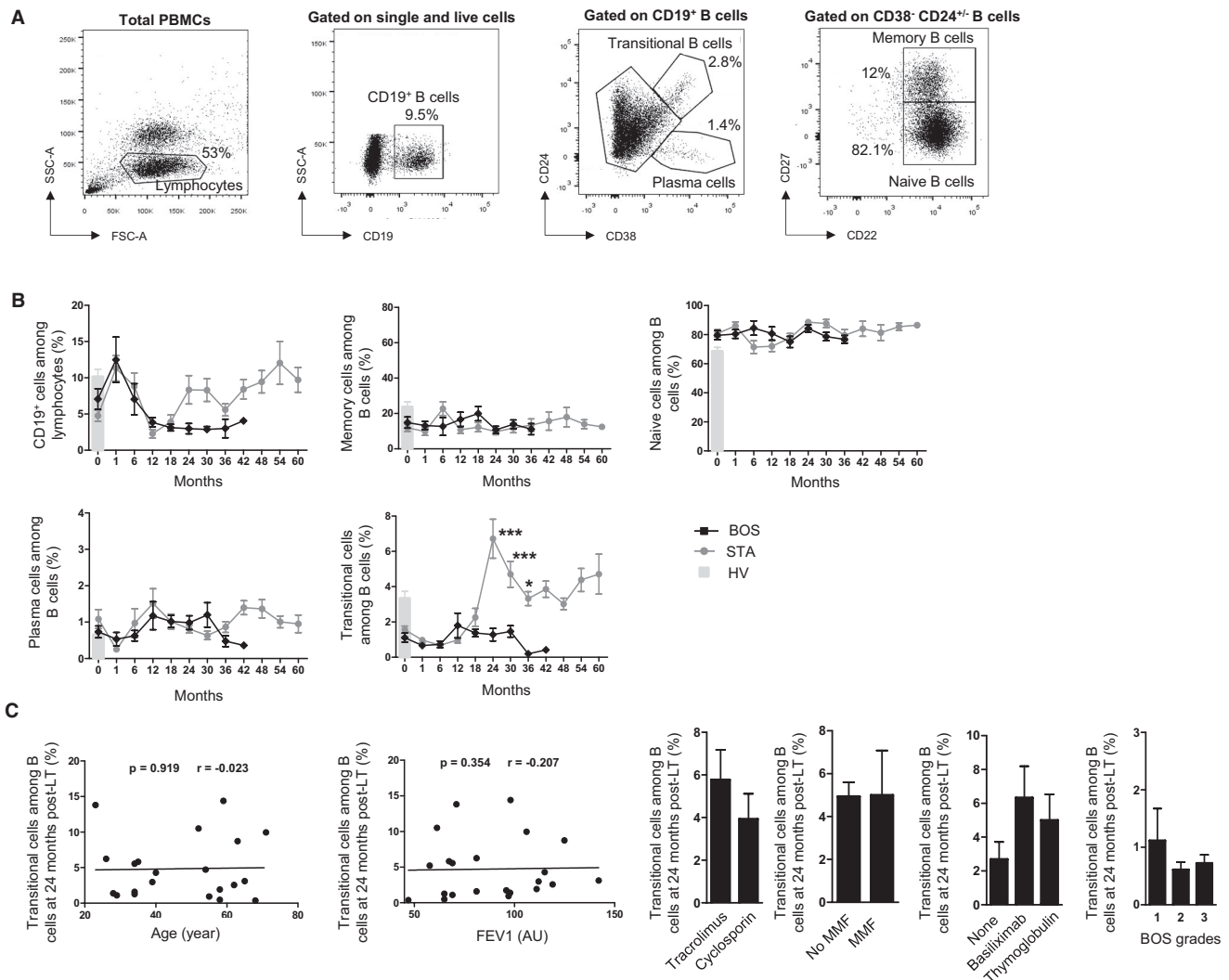
leukocyte antigen (HLA) mismatches were not significantly different between BOS and STA. Concerning the initial disease leading to LT, cystic fibrosis is more prevalent in STA (50% vs 14%, respectively;  $P = .03$ ), which is representative of the COLT cohort. Finally, STA patients present less DSA than BOS patients ( $P = .0001$ ).

#### 3.2 | CD19<sup>+</sup> CD24<sup>hi</sup> CD38<sup>hi</sup> transitional B cells are increased in stable LTR from 24 months after LT

Because B cell homeostasis is reshaped in the context of skin, hematopoietic stem cells, and kidney dysfunction,<sup>11,12,25,26</sup> we assessed whether profile of peripheral blood B lymphocyte subsets may evolve differently over time between BOS and STA (before LT to 60 months post-LT) and HV. We determined the percentage of naive, memory, transitional and plasma cells based on the expression of CD19, CD27, CD38, CD22, and CD24 (Figure 1A). Neither CD19<sup>+</sup> total B cells nor naive, memory, and plasma cell subtype frequencies were significantly different between BOS and STA at any time points (Figure 1B). In contrast, whereas remaining low over time in BOS (1.1% ± 0.1, mean ± standard deviation), the percentage of CD19<sup>+</sup> CD24<sup>hi</sup> CD38<sup>hi</sup> transitional B cells dramatically increased in STA reaching a maximum at 24 months and exceeding the physiological level of CD24<sup>hi</sup> CD38<sup>hi</sup> transitional B cells in HV and that stay high in time (6.3% ± 0.1 vs 3.3% ± 0.4;  $P = .0011$ ). This effect was neither associated with age, lung function, induction, or immunosuppressive treatments nor with BOS grade (Figure 1C).

We then analyzed the frequency of CD19<sup>+</sup> CD24<sup>hi</sup> CD38<sup>hi</sup> transitional B cell subsets according to their differentiation states in STA, BOS, and HV 24 months post-LT. Surface markers were used to distinguish CD27<sup>+</sup> IgD<sup>+</sup> IgM<sup>+</sup> T1/2, CD27<sup>+</sup> IgD<sup>+</sup> IgM<sup>-</sup> T3, and CD27<sup>-</sup> nonconventional memory CD24<sup>hi</sup> CD38<sup>hi</sup> transitional B cells (Figure 2A). The percentage of all transitional B cell subsets are significantly higher in STA compared to BOS: T1/T2 (2.5% ± 0.5% vs 0.5% ± 0.1% in STA and BOS;  $P = .0001$ ), T3 (2.6% ± 0.6% vs 0.8% ± 0.5%;  $P = .0076$ ), and CD27<sup>-</sup> nonconventional B cells (0.5% ± 0.07% vs 0.15% ± 0.06%;  $P = .0005$ ) (Figure 2B). Altogether, 24 months post-LT, LTR who keep





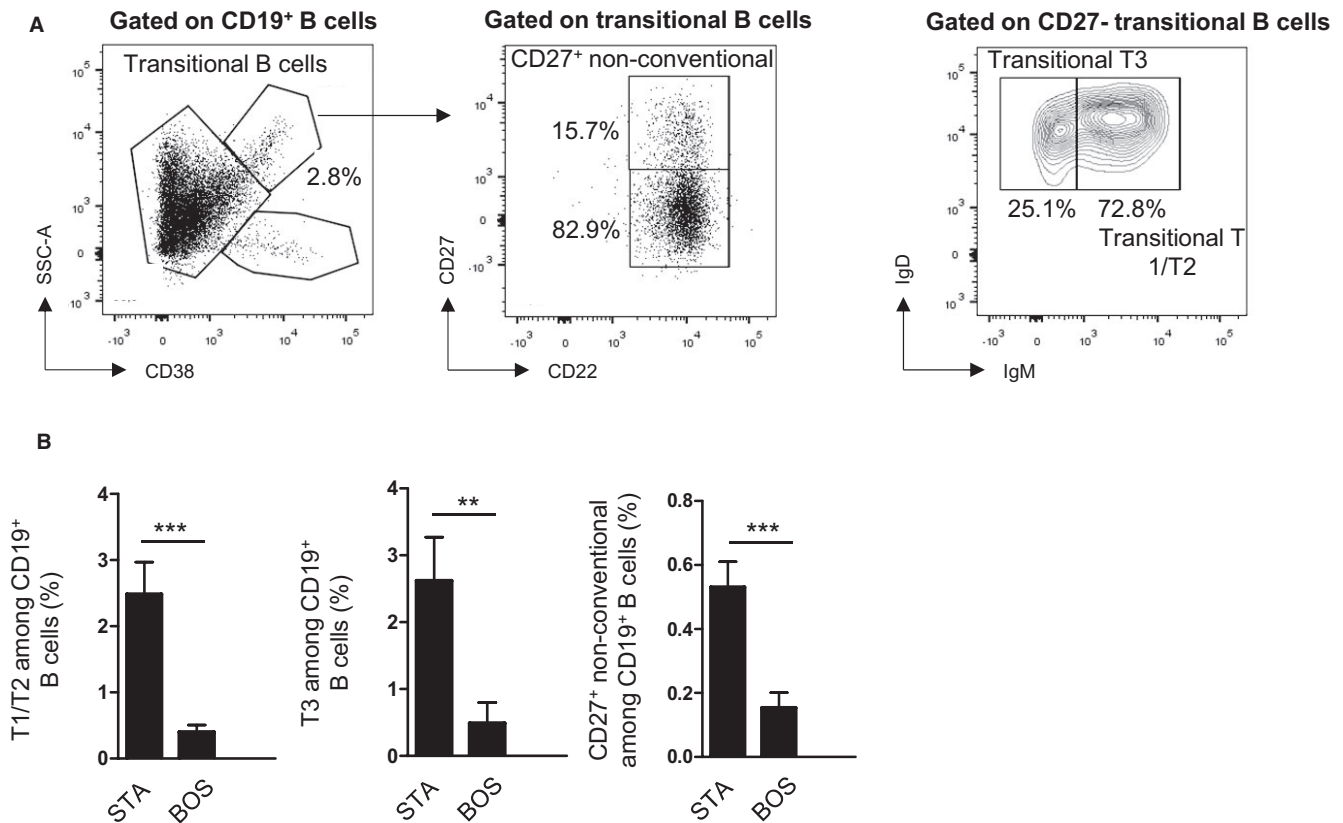
**FIGURE 1** Longitudinal immunomonitoring of B lymphocyte subpopulations. A, Gating strategy of CD19<sup>+</sup> B lymphocytes, CD19<sup>+</sup>CD27<sup>+</sup> memory cells, CD19<sup>+</sup>CD27<sup>-</sup> naive cells, CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional cells, and CD19<sup>+</sup>CD24<sup>-</sup>CD38<sup>+</sup> plasma cells. B, Longitudinal immunomonitoring of CD19<sup>+</sup> B cells and transitional, memory, naive and plasma cells before LT to 60 months post-LT in STA (n = 20), BOS (n = 21), and HV (n = 19) patients. C, Correlation between the percentage of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells and age, forced inspiratory volume in 1s (FEV1), induction and immunosuppressive treatments and BOS grades (\*P < .05, \*\*\*P < .001). BOS, bronchiolitis obliterans syndrome; HV, healthy volunteer; LT, lung transplant; STA, stable

a stable graft function over 60 months harbor a CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells elevation whereas this level remains very low in patients who will develop a BOS.

### 3.3 | CD24<sup>hi</sup>CD38<sup>hi</sup> transitional cells secrete IL-10 and express CD9

Previous studies revealed that CD24<sup>hi</sup>CD38<sup>hi</sup> transitional cells are enriched in IL-10-secreting B cells with regulatory properties.<sup>16,27</sup> We analyzed the CD24<sup>hi</sup>CD38<sup>hi</sup> transitional cells IL-10 production after activation with PMA and ionomycin in vitro in BOS and STA patients 24 months post-LT. IL-10-producing B cells are mainly enriched in the CD24<sup>hi</sup>CD38<sup>hi</sup> transitional subset (Figure 3A) and as expected, IL-10-secreting B cells are significantly higher in STA patients compared to BOS (15.9% ± 3.5 vs 6.4% ± 1 respectively, P < .05) and similar to the

rate of IL-10 secreting B cells in HV (P > .05) (Figure 3B). Despite this, CD24<sup>hi</sup>CD38<sup>hi</sup> transitional cells from STA and BOS secrete the same amount of IL-10 (mean fluorescence intensity is similar in both groups, Figure 3B). IL-10-secreting B cells rate is not associated with treatments. We also measured IL-10 level in the sera of 41 STA patients and 30 BOS patients using Bio-plex assay. The level of IL-10 between STA and BOS was not significantly different (P = .780) (mean = 0.7 pg/mL, data not shown). Next, we analyzed the expression of the CD9 surface marker within CD24<sup>hi</sup>CD38<sup>hi</sup> transitional. Transitional subsets are enriched in CD9<sup>+</sup> B cells (Figure 3A) and as expected, CD9<sup>+</sup> B cells are enriched in IL-10-secreting cells (Figure 3C). Moreover, at 24 months post-LT, the percentage of transitional cells correlates with the percentage of CD9<sup>+</sup> B cells (r = .76, P < .001) suggesting that transitional cells and CD9<sup>+</sup> B cells belongs to the same B cell subtype (Figure 3D). Finally, such as CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells,



**FIGURE 2** Immunomonitoring of  $CD19^+CD24^{hi}CD38^{hi}$  transitional B cell subtypes 24 months post-LT. A, Gating strategy of  $CD19^+CD24^{hi}CD38^{hi}$  transitional cells,  $CD27^+IgD^{+/-}IgM^+$  T1/2,  $CD27^+IgD^+IgM^-$  T3, and  $CD27^+$  nonconventional memory transitional B cells. B, Immunomonitoring of T1/T2, T3,  $CD27^+$  nonconventional transitional B cells among  $CD19^+$  B cells, 24 months post-LT in STA ( $n = 20$ ) and BOS ( $n = 21$ ) patients ( $*P < .05$ ,  $**P < .01$ ,  $***P < .001$ ). BOS, bronchiolitis obliterans syndrome; LT, lung transplant; STA, stable

the longitudinal time-course of the  $CD9^+$  B cell expression peaks 24 months post-LT in STA compared to BOS ( $12.2\% \pm 1.3$  vs  $5.9 \pm 1.1$  respectively,  $P = .0053$ ) (Figure 3E) and remains constant among STA LTR. Altogether, we report that  $CD19^+CD24^{hi}CD38^{hi}$  transitional B cells peaking at 24 months after LT in patients with stable graft function also express the CD9 marker and secrete IL-10.

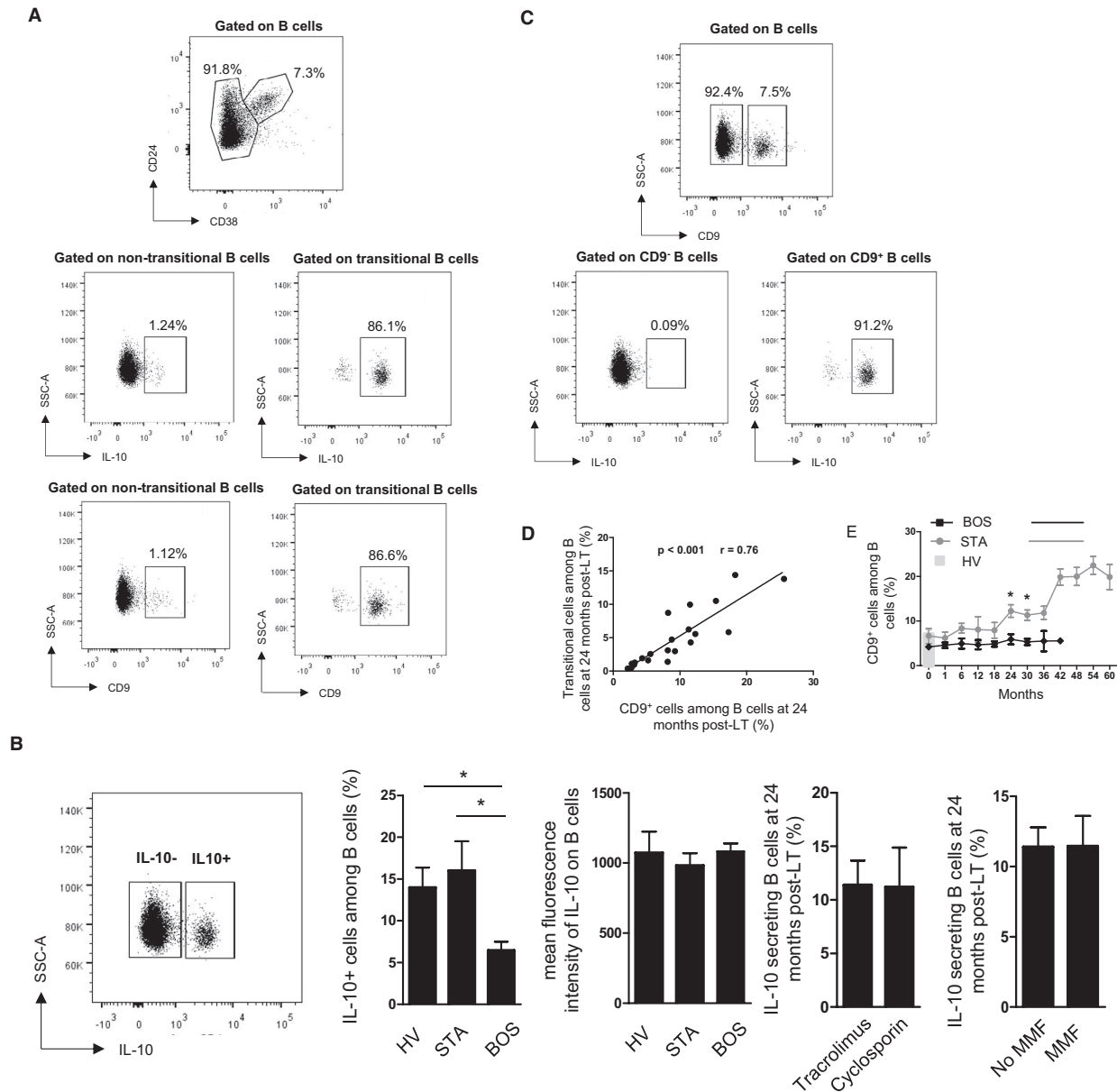
### 3.4 | $CD9^+$ B cell frequency at 24 months is a powerful predictive marker of stability and long-term BOS-free survival after LT

Because the rate of  $CD19^+CD24^{hi}CD38^{hi}$  transitional cells is correlated with the rate of  $CD9^+$  B cells and because a biomarker with a single extracellular and dichotomistic molecule would be more appropriate for clinical use (easier gating strategy), we further explored whether the level of  $CD9^+$  B cells could be sufficient to discriminate properly between patients who will maintain stable graft function and those who will develop BOS in the 3 years after LT. Accordingly, using ROC curve analysis,  $CD9^+$  B cell frequencies allow excellent discrimination between STA and patients who will develop BOS at 24 months post-LT ( $AUC = 1$ ) (Figure 4A). The FEV1 at 24 months post-LT is similar between STA and patients that will develop BOS latter one while,

and as expected, the FEV1 is significantly lower in BOS at the time of diagnosis (Figure 4B). ROC curve of  $CD9^+$  B cell frequencies displays a significant higher area than FEV1 one ( $AUC = 1.00$  vs  $0.62$ ,  $P = .0132$ ). At 24 months posttransplantation, the level of CD9 was significantly higher in patient that present stable graft function compared to the patients who will develop BOS, before the FEV1 decline.

### 3.5 | Validation of $CD9^+$ B cell frequency at 24 months as predictive marker of stability and long-term BOS-free survival

In order to validate CD9 as biomarker of stability of graft function and long-term BOS-free survival, independent patient samples taken at 24 months post-LT were analyzed from 19 STA and 15 patients that will develop BOS after 24 months post-LT. 10 HV were also analyzed as reference. All baseline characteristics of LTR are provided in Table 2. None of the clinic or demographic features is significantly different between BOS and STA, neither the percentage of naive, memory, and plasma cells at 24 months post-LT (Figure 4C). At that time, the percentage of  $CD24^{hi}CD38^{hi}$  transitional B cells and  $CD9^+$  B cells are significantly higher in STA compared to BOS ( $4.8\% \pm 0.7$  vs  $2.4\% \pm 0.5$ ;  $P = .0079$  for  $CD24^{hi}CD38^{hi}$  transitional B cells,  $12.3\% \pm 1.7$  vs  $7.5\% \pm 0.51$  for  $CD9^+$



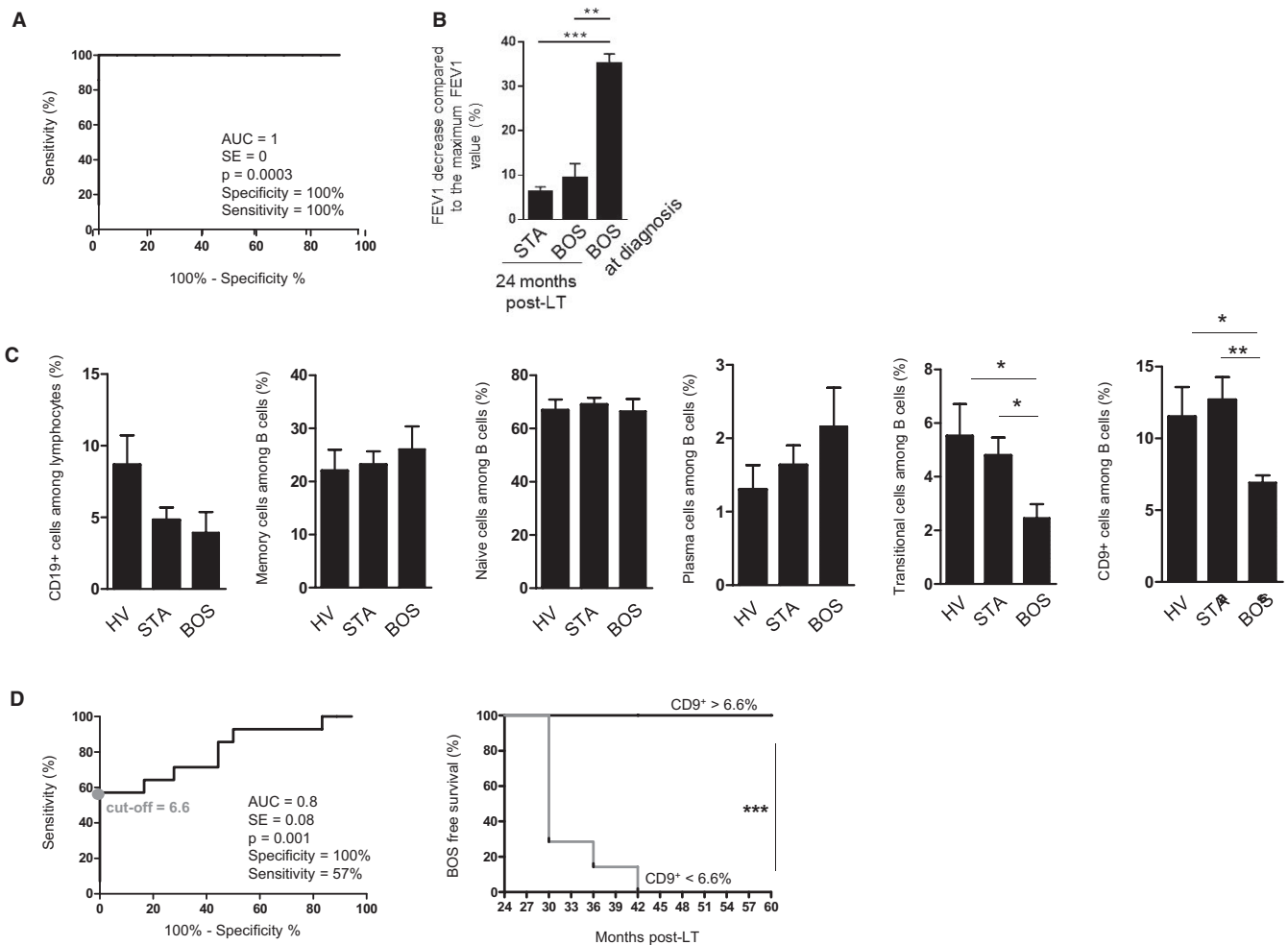
**FIGURE 3** CD24<sup>hi</sup>CD38<sup>hi</sup> transitional cells secrete IL-10 and express the CD9 marker. A, Gating strategy of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells secreting IL-10 and expressing CD9. B, Percentage of IL-10 secreting B cells and mean fluorescence intensity of IL-10 on B cells in HV (n = 10) and STA (n = 20) and BOS (n = 21) patients, 24 months post-LT. Correlation between the percentage of IL-10 secreting B cells and immunosuppressive treatments. C, Gating strategy of CD9<sup>+</sup> B cells secreting IL-10. D, Correlation between the percentage of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells and CD9<sup>+</sup> B cells at 24 months post-LT. E, Longitudinal immunomonitoring of CD9<sup>+</sup> B cells before LT to 60 months post-LT in STA (n = 20), BOS (n = 21), and HV (n = 19) patients. BOS, bronchiolitis obliterans syndrome; HV, healthy volunteer; LT, lung transplant; STA, stable

B cells; respectively,  $P = .0013$ ). The rate of CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells and CD9<sup>+</sup> B cells in STA at 24 months post-LT is similar to HV ( $P > .05$ ). Using ROC curve analysis, CD9<sup>+</sup> B cell frequencies allow excellent discrimination between STA and patients who will develop BOS after 24 months post-LT (AUC = 0.836, SE = 0.08) and 6.6% appears as the value maximizing specificity and sensitivity discriminating BOS and STA (sensitivity of 57% and specificity of 100%) (Supplementary Table S1). Patients with CD9<sup>+</sup> B cell frequency below these cut-off points display significantly higher incidence of BOS (log-rank  $P = .001$ , positive predictive value = 0.75 and negative predictive values = 1) (Figure 4D).

These data validate the use of CD9 as biomarker of long-term BOS-free survival at 24 months post-LT.

## 4 | DISCUSSION

The understanding of cellular and molecular mechanisms of graft failure and identification of robust predictive biomarkers for allograft outcome are major challenges facing researchers involved in lung transplantation. The correlation we made recently between BOS



**FIGURE 4** CD9<sup>+</sup> B cell frequency is a predictive marker of long-term BOS-free survival at 24 months post-LT. A, ROC curve analysis of the level of CD9<sup>+</sup> B cells 24 months post-LT in BOS and STA patients of the investigation cohort (AUC 1). B, FEV1 decline compared to the maximum FEV1 value recorded after transplantation in patients of the investigation cohort at 24 months posttransplantation in BOS (n = 7) and STA (n = 20) patients and at diagnosis for BOS patients (n = 19). C, Immunomonitoring of CD19<sup>+</sup> B cells, memory, naïve, plasma cells, transitional and CD9<sup>+</sup> B cells at 24 months post-LT in STA (n = 18) and BOS (n = 16) patients and 10 HV as reference. D, ROC curve and Kaplan-Meier analysis of the level of CD9<sup>+</sup> B cells 24 months post-LT in BOS and STA patients of the validation cohort (AUC = 0.8, SE = 0.08, cut-off = 6.6 at 57% sensitivity and 100% specificity (\*\*P < .005, \*\*\*P < .001). AUC, area under the ROC curve; BOS, bronchiolitis obliterans syndrome; HV, healthy volunteer; LT, lung transplant; ROC, receiver operator characteristic; SE, standard error; STA, stable

occurrence and three B cell-related genes reinforced the overriding role of B cells in allograft recipient immunity.<sup>10</sup> This study reports for the first time the longitudinal profile of circulating B cell subsets in LTR.

B cells are mainly known to be involved in graft dysfunction due to their ability to produce DSA. Antibody-mediated rejection is an important cause of graft failure after LT and it is associated with high incidence of CLAD.<sup>28</sup> In accordance with the literature, DSA were higher in BOS than STA patients. At 24 months post-LT, LTR with a stable graft function harbor an increase of CD9<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells whereas this subset remains low in patients who will develop BOS. Logistic regression between DSA and transitional B cells did not highlight a significant association, that make sense since such transitional B cells and CD9<sup>+</sup> B cells do not produce “deleterious” antibodies or even may

counteract antibody production.<sup>29</sup> Interestingly, B cell subsets and especially plasma cell levels remain low in the BOS cohort, whatever their HLA-DSA status, whereas the transitional B cell subset increases only in patients who keep a good function in time, suggesting another role of B cells.

B cells with regulatory properties are associated with maintenance of long-term allograft function in several transplanted organs<sup>11-14</sup> and we and others reported on a significant increase in B cells, particularly CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells, in blood from kidney transplanted patients with long-term graft survival in absence of immunosuppression in contrast with patients who reject their graft.<sup>13,14,19,20,30</sup> Such variation in transitional B cells were thus found in different situations,<sup>11</sup> suggesting that a shift toward a transitional B cell phenotype might be a prerequisite for the development of long-term graft outcome.<sup>31</sup> Our data clearly corroborate this

with stable LTR harboring an increase of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells at 24 months post-LT compared with patients who will develop BOS.

Furthermore, we report here that CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells was correlated with CD9 expression and that the frequency of CD9<sup>+</sup> B cells > 6.6% at 24 months post-LT allows distinguishing between patients who will develop BOS within 3 years post-LT and those who keep a stable graft function evidencing that CD9<sup>+</sup> B cell frequency may serve as a predictive biomarker of BOS. CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> gating is more likely to vary depending on the manipulator as it is not +/- but high/low/neg. Gating on CD9<sup>+</sup> B cells is thus much easiest and transferable to the clinic. Our data evidence CD9<sup>+</sup> B cell at 24 months post-LT as a biomarker able to predict BOS at 3 years post-LT but does not preclude on the outcome of patients later.

We previously identified CD9 as a marker of CD24<sup>hi</sup>CD38<sup>hi</sup> transitional cells with regulatory properties.<sup>22</sup> CD9 expression has been associated in mouse with enhancement and maintenance of IL-10 secretion and in human with plasma cell lineage with potent immunosuppressive properties.<sup>22,23</sup> In parallel with the fact that CD9 may serve as a biomarker of BOS, we hypothesize that CD9<sup>+</sup> B cells and possibly their production of IL-10 may contribute to create a favorable environment that might be essential for the maintenance of long-term stable graft function.

In conclusion, we report on a population of CD9-expressing B cells, not influenced by the treatment that may serve as a new predictive biomarker of long-term BOS-free survival to allow reducing or adjusting treatments according to its circulating level. These data highlight the importance of B cells with potential regulatory properties as well as a new and easy applicable CD9<sup>+</sup> marker in transplantation validated in an independent set of LTR. If validated in a larger and prospective follow-up, our findings could help early prediction of BOS and the development of personalized medicine in lung transplantation.

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## DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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## SUPPORTING INFORMATION

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

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## APPENDIX

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