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Royce Calhoun
Krovvidi S. R. SivaSai
Sudhir Sundaresan
Elbert P. Trulock
John P. Lynch
G. Alexander Patterson
Joel D. Cooper
T. Mohanakumar

Development of bronchiolitis obliterans syndrome despite blood chimerism in human lung transplant recipients

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Authors R. Calhoun and K. S. R. SivaSai contributed equally

R. Calhoun · K. S. R. SivaSai · T. Mohanakumar (✉)
Department of Surgery and Pathology, Washington University School of Medicine, Box 8109–3328 CSRB, 660 S. Euclid Avenue, St. Louis, MO 63110, USA
e-mail: kumart@msnotes.wustl.edu
Tel.: + 1–314–362–8463
Fax: + 1–314–747–1560

S. Sundaresan · G. A. Patterson · J. D. Cooper
Division of Cardiothoracic Surgery, Washington University School of Medicine, Box 8234, 660 S. Euclid Avenue, St. Louis, MO 63110, USA

E. P. Trulock · J. P. Lynch
Department of Medicine, Washington University School of Medicine, Box 8052, 660 S. Euclid Avenue, St. Louis, MO 63110, USA

Abstract Bronchiolitis Obliterans Syndrome (BOS) remains the overwhelming obstacle to the success of lung transplantations (LTx). The presence of donor-specific microchimerism (DSM) and its association with lung allograft function is not well defined. To investigate the relationship between chimerism and BOS, blood was obtained from 21 LTx recipients. Genomic DNA was isolated from patient blood, and PCR-based techniques were used to identify recipient and donor HLA-DR. Fifty percent of the LTx recipients with BOS exhibited DSM at "T_1" time post transplant, and 40% at one year follow-up (T_2). However, 54% exhibited DSM in the BOS-free group at T_1, and 44% at T_2. Of the BOS-free, DSM-positive patients at T_1, 29% developed BOS by T_2. In contrast, 50% of BOS-free DSM-negative patients 50% developed BOS ($P > 0.05$). Double LTx had a higher prevalence of DSM (73%) and a lower prevalence of BOS (46%) than single LTx (50% and 80% respectively, $P > 0.05$). One-HLA-DR-antigen-matched LTx recipients show a low prevalence of DSM compared to non-matched ($P < 0.05$). This study demonstrates that the development of BOS in LTx recipients could also occur in the presence of blood chimerism.

Key words Bronchiolitis Obliterans Syndrome · Lung transplantation · Donor specific microchimerism · Peripheral blood · Polymerase chain reaction

Introduction

Chronic rejection remains the number one barrier to long-term allograft success in lung transplant (LTx) recipients. There is approximately a 50% incidence of Bronchiolitis Obliterans Syndrome (BOS), taken to be chronic rejection, by 3 years post-LTx [26]. The detection of donor-specific microchimerism (DSM) largely in peripheral blood analysis, and in some cases in tissues of patients with long-term function of their allograft [20, 22], lead to the concept that the presence of donor-derived cells, notably leukocytes, in the recipient, confers

allograft acceptance and/or tolerance [21, 23, 24]. Solid organ transplant recipients that have received either donor-specific transfusions (DST) or a pre-operative bone-marrow transplant, had beneficial effect on the outcome of allograft survival [4, 15, 18, 28]. Furthermore, some studies have shown that DST or bone marrow transplants facilitate the development and/or establishment of DSM [5, 10, 16]. However, it is not clear from these studies if the chimerism plays an integral role in the increased graft survival or if it is merely an epiphenomenon. With rare exceptions [1], the observed relationship between DSM and allograft acceptance/tolerance has been associative, and thus no cause and effect can be established. Indeed, there have been several reports that have not found an association between DSM and allograft function [6, 7, 19], lack of DSM in well-functioning long-term allografts [2, 25] and presence of chimerism in allografts undergoing rejection [12,19].

Our present study evaluates whether there is any relationship between peripheral blood DSM and chronic rejection (BOS) in LTx patients, when measured at a single time point. Furthermore, the relationship between DSM at one time point and the subsequent development of BOS was studied prospectively. DSM was analyzed at one-year follow-up to determine any relationship between chimerism and BOS in long-term LTx patients, and to discern if the serial detection of chimerism improved any association with the presence of BOS or prediction of BOS development. Lastly, double vs. single LTx and HLA-DR match vs mismatch in the recipient and donor were compared with respect to presence of DSM and/or BOS.

Materials and methods

Patient population

Twenty-one LTx recipients with a known HLA-DR mismatch between the donor and recipient, and known BOS status at the time of investigation, were included in this study. Out of 21 patients, 8 were females (age:42.5 ± 13.4 years) and 13 males (age: 52 ± 12.2). Clinical status of each patient is depicted in Table 1. The underlying pulmonary disease necessitating LTx was Chronic Obstructive Pulmonary Disease (8), Cystic Fibrosis (4), Atrial Septal Defect/ Eisenmenger's Syndrome (2), Primary Pulmonary Hypertension (3), α_1-Trypsin Deficiency (2), Idiopathic Pulmonary Fibrosis (1) and Rheumatoid Arthritis/Bronchiolitis Obliterans (1). Standard maintenance immunosuppression therapy for these patients consisted in administering cyclosporine (levels kept at upper limits of normal based on whole blood, TDX assay), azathioprine (2 mg/kg per day adjusted for leukopenia) and prednisone (15 mg every other day). Peripheral blood was obtained from these patients pre- and post Tx (T_1 and T_2, one year apart) after informed consent was obtained in compliance with institutional human studies protocols. The patients of the study are those that survived to time T_1. Lung transplant patients who did not develop BOS were randomly selected, and blood samples were obtained at a different time point post transplant, ranging between 11–63 months (T_1). Clinical status of each patient was followed from time T_1 to T_2; BOS was defined as sustained decrease/fall in forced expiratory volume in one second (FEV1), to less than 80% of the previously established post transplant baseline value. Other conditions such as airway complications, infection, congestive heart failure, reversible airway reactivity, and systemic diseases were excluded [3].

Isolation of genomic DNA

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and DNA were extracted as previously described [19].

Polymerase chain reaction (PCR) and hybridization

Details of the PCR reaction and sequence of primers used for PCR amplification of the HLA-DRB1 region and HLA-DR allele specific oligonucleotide probes used for hybridization have been previously described[19]. Briefly, 40 cycles were used in the DNA amplification process. Amplified DNA was analyzed on 2% agarose gels stained with ethidium bromide and transferred to nitrocellulose membranes by Southern blot method. Blots were probed with DR allele-specific ^{32}P-labeled probes specific for recipient, donor, irrelevant and positive control DR in each patient. Probe binding was visualized by autoradiography.

Results

Detection of DSM

Peripheral blood mononuclear cells (PBMC) were assessed for recipient and donor HLA-DR by PCR and Southern blot analysis in 21 LTx recipients pre and post Tx at two different times (T_1 and T_2, one year apart). To verify the specificity of each HLA-DR specific probe, a Southern blot containing HLA-DR homozygous B-cell line PCR products of all available HLA-DR alleles, was probed with one ^{32}P labeled HLA-DR allele specific probe. Allele specific binding was verified in this fashion for each probe used for patients HLA-DR blots and a representative gel is illustrated for HLA-DR3 in Fig. 1. An agarose gel containing PCR products from the HLA-DR homozygous B cell lines and stained with ethidium bromide is shown (Fig. 1-top). The HLA-DR3 specific ^{32}P labeled probe stained the southern blot HLA-DR3 product as demonstrated by autoradiography (Fig. 1-bottom). None of the other DR allele specific DNA demonstrated a signal indicating the specificity of the DR3 probe (Fig. 1-bottom). Figure 2 shows representative data from 3 LTx recipients (A, B and C) who were positive for the donor HLA-DR allele specific probe at the time point tested. The intensity of the signal by autoradiography is proportional to the amount of template DNA (100, 250, 500 ng). DNA from each LTx recipient stained positive for their own HLA-DR and negative for irrelevant probes (probes specific for HLA-DR of neither recipi-

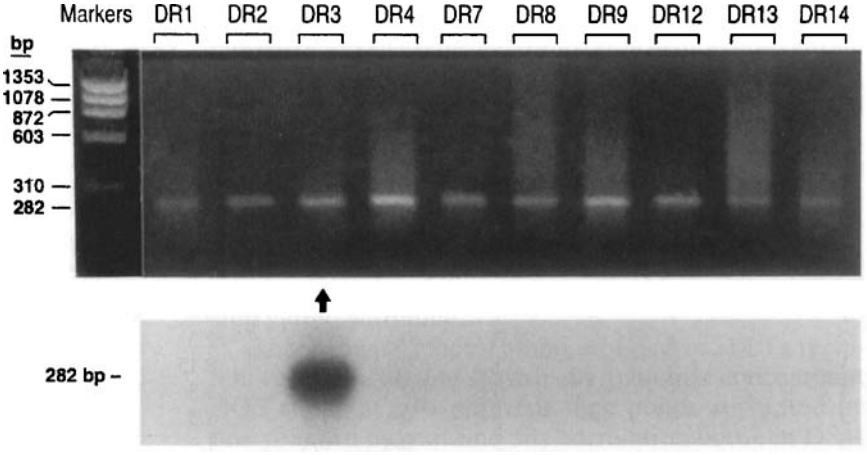

Fig. 1 Representative blot hybridized with HLA-DR3 for determining HLA-DR allele specific oligonucleotide probe binding. *Top* The PCR amplified "DRB1" regions of different HLA-DR from homozygous B cell lines. *Bottom* The above PCR product panel was hybridized with ^{32}P radiolabelled HLA-DR3 oligonucleotide probe. The specificity of all the probes used were assessed as specified above

ent nor donor origin in all cases – data not shown). These patients did not express this HLA-DR phenotype pre-operatively (not shown). The sensitivity of our PCR technique was assessed by combining a constant number of recipient cells with a serial dilution of known donor cells, and was found to be 0.002%, > 1 donor cell in 50,000 recipient cells [19].

Chimerism (DSM) does not correlate with BOS at discrete time points

To assess if the detection of DSM correlates with allograft acceptance at specific time points, we analyzed for DSM in the peripheral blood of LTx recipients at T_1 (11–63 months post Tx) and T_2 1 year later in the same recipients. Table 1 illustrates that the detection of DSM in the patients' blood does not correlate with the absence of BOS. At T_1, 4/8 (50%) of the BOS positive patients had DSM as well as 7/13 (54%) of the BOS free patients. Similarly, at T_2, 4/10 (40%) of BOS positive patients exhibited DSM versus 3/6 (50%) in the BOS free group. Thus, at two discrete time points, one year apart, there was no correlation between peripheral blood DSM and BOS. All the patients were assessed for DSM in pre-operatively blood sample and were negative for chimerism (data not shown).

We were interested to see if the pattern of change or consistency of detectable DSM over time at T_1 and T_2 had any relationship to BOS, either existing or its development. As shown in Table 1, patients 14–16 and 19–21 maintained their DSM status at 1-year follow-up persistently negative and positive, respectively. Further, analysis of the pattern of detectable DSM in BOS-free subgroup relates to subsequent BOS status at T_2 (Table 2). Four patients exhibited a change in detectable DSM when measured at T_1 and T_2. Two patients went from DSM positive to negative, 2 from DSM negative to positive, and all 4 remained free of BOS at 12 month

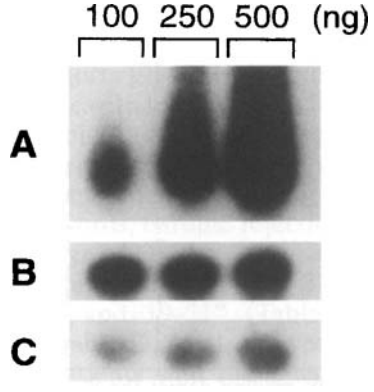

Fig. 2 Detection of DSM in Lung transplant recipients. HLA-DRB1 region from genomic DNA of 3 different transplant recipients (*A,B,C*) was amplified and probed with HLA-DR probes specific for donor origin. The intensity of the bands is dependent upon the concentration of the donor template

follow-up. Furthermore, 3/4 patients who consistently lacked detectable chimerism and 1/2 patients who were persistently chimeric, developed BOS within the year.

To determine if the chimeric status of LTx recipients at T_1 could predict which patients might be more susceptible to development of BOS, we followed the 13 patients who were free of BOS at T_1 over a 12 month period. Of these 13 BOS-free patients, 7 were chimeric and 6 were not, when analyzed at T_1 (Table 1). 2/7 (29%) chimeric patients developed BOS by T_2 versus 3/6 (50%) in the non-chimeric group which was not statistically significant ($P = 0.065$). However, there is a trend suggesting that DSM-positive patients may have a decreased likelihood of developing BOS relative to DSM-negative patients over a 12 month follow-up.

Table 1 Donor-specific chimerism (DSM) and its correlation with development of BOS at two discrete time points (T_1, T_2) in lung transplant recipients. Patient code numbers are universal in all the tables. Statistically significant correlation between the DSM and BOS was not observed at two discrete time points (T_1, T_2), by chi-square test ($P > 0.05$) and Yates correction for small numbers ($P > 0.10$). Numbers in parenthesis ( ) indicate total number of cases. Abbreviations: *SN* sample not available; *PTx* time when the DSM analysis was done at T_1; T_2 time of DSM analysis after one year follow-up; *CMV* cytomegalovirus; *R* recipient; *D* donor; *ARE* number of acute rejection episodes. * Expired

Patient code	CMV			BOS grade					Single or double LTx	Donor specific microchimerism		BOS Status at T_2
	R	D	ARE	T_1	T_2	R-DR	D-DR	PTx		T_1 (21)	T_2 (16)	
BOS negative												
1	+	–	1	0A	0A	3.–	4.6	48	D	–	+	–
2	+	+	3	0A	0A	2.3	7.15	33	D	–	+	–
3	–	–	1	0A	1A	8.15	2.11	47	S	–	–	+
4	+	–	3	0A	0A	1.3	15.–	22	S	–	–	+
5	+	–	5	0A	1A	1.3	1.4	24	D	–	–	+ (E*)
6	–	–	3	0A	0A	1.–	1.12	13	D	–	–	–
7	+	+	5	0A	0A	3.11	4.11	46	S	+	SN	–
8	+	–	0	0A	1A	15.–	4.6	63	S	+	+	+ (E)
9	–	+	5	0A	0A	1.15	3.10	60	S	+	–	–
10	+	+	1	0A	0A	3.6	4.11	54	D	+	–	–
11	+	+	9	0A	0A	15.–	4.–	28	D	+	SN	– (E)
12	–	–	4	0A	1A	3.6	3.9	26	S	–	–	+
13	–	–	1	0A	0A	4.3	7.15	23	D	+	+	–
BOS positive												
14	–	+	11	1A	3B	1.15	4.8	27	D	+	SN	+ (E)
15	+	+	2	1A	2A	1.7	7.17	11	D	–	–	+
16	+	+	4	2B	2B	15.–	14.15	28	S	–	–	+
17	–	+	1	1B	1B	2.3	13.15	28	S	–	SN	+
18	–	+	6	1A	1B	8.15	1.13	24	S	+	SN	+ (E)
19	+	+	1	1B	2B	4.7	7.15	47	S	+	+	+
20	–	+	4	0B	0B	7.11	2.4	45	D	+	+	+
21	+	–	3	3A	3A	11.15	13.–	43	D	+	+	+

Numbers in the BOS grading depict the % FEV1 compared to baseline value (BV) (0 = FEV1 80% or more of BV; 1 = 66–80% of BV; 2 = 51–65% of BV; 3 = 5% or less of BV) and capital letters next to numbers depict without (A) and with (B) pathological evidence of obliterative bronchiolitis

Double lung transplants may confer a higher prevalence of DSM and less BOS than single lung transplants

It has been suggested that liver allografts may have less rejection problems, relative to other solid organ allografts, in part due to their larger size. To address this possibility in lung transplants, we analyzed our data looking at the percent of DSM and BOS in single lung Tx (SLTx) versus double LTx (DLTx) in our 21 patients. 10 patients underwent SLTx and 11 patients underwent DLTx during the study period with a mean time from transplant to time of analysis of 51 months and 43 months respectively. There was a higher percent of collective DSM (T_1, T_2 or both) in the DLTx cohort versus the SLTx cohort during the study period (Table 3). However, this difference cannot be attributed to the volume of allograft alone because the SLTx cohort had a mean time from transplant to time of analysis of 8 months more than the DLTx cohort (51 vs. 43).

One HLA-DR match LTx recipients confer a lower prevalence of DSM than no HLA-DR match antigens

LTx recipients were divided into two groups based on either one match or no match for HLA-DR antigens. Surprisingly, 8 out of 10 at T_1 and 6 out of 8 at T_2 in one HLA-DR matched recipients group were negative for DSM compared to 2 out of 11 at T_1 and 3 out of 8 at T_2 in no HLA-DR matched antigen group (Table 4). These results suggest that lack of sharing of HLA-DR antigen was associated with a higher incidence of DSM ($P = 0.01$) however, there is no correlation with BOS ($P = 0.09$). No apparent correlation was observed between the DSM vs development of BOS and/or single vs double LTx in both one antigen or no HLA-DR antigen matched recipients (Table 4).

Table 2 DSM as a function of time in BOS free patients at T_1 and its relationship to subsequent BOS status. * All the patients are BOS free at T_1 time of analysis. ** Expired

Patient code*	Donor specific microchimerism		BOS status at T_2
	T_1	T_2	
1	–	+	–
2	–	+	–
9	+	–	–
10	+	–	–
13	+	+	–
8	+	+	+ (E**)
3	–	–	+
4	–	–	+
5	–	–	+ (E)
6	–	–	–

Table 3 Correlation between collective DSM positivity (T_1, T_2 or both) and BOS status on single vs. double lung transplants. Statistically significant correlation between the DSM and BOS in Single vs. double LTx was not observed at two discrete time points (T_1, T_2). P = NS (not significant) by Fischer's Exact Test. * Expired

Patient code	Type of Tx	Donor specific microchimerism		BOS status at T_2
		T_1 (21)	T_2 (16)	
Without BOS at T_1				
3	SLT	–	–	+
4	SLT	–	–	+
7	SLT	+	ND	–
8	SLT	+	+	+ (E*)
9	SLT	+	–	–
12	SLT	–	–	+
BOS at T_1				
16	SLT	–	–	+
17	SLT	–	ND	+
18	SLT	+	ND	+ (E)
19	SLT	+	+	+
Without BOS at T_1				
1	DLT	–	+	–
2	DLT	–	+	–
5	DLT	–	–	+ (E)
6	DLT	–	–	–
10	DLT	+	–	–
11	DLT	+	ND	– (E)
13	DLT	+	+	–
BOS positive at T_1				
14	DLT	+	ND	+ (E)
15	DLT	–	–	+
20	DLT	+	+	+
21	DLT	+	+	+

Discussion

The relationship between allograft acceptance/tolerance and the presence of donor-specific chimerism remains unclear. The majority of reports in the literature have evaluated liver, heart, and kidney transplants in human clinical situations and experimental animal models [23, 27]. To date, there have been few reports [8, 9, 11, 14, 17] on the relationship between chimerism and chronic rejection (BOS) in lung transplant recipients. BOS remains the major obstacle to long-term success of pulmonary allografts with 50% of the recipients developing BOS by 3 years [26]. Therefore, in this study we evaluated if there was any relationship between DSM and BOS in LTx recipients, and how this relationship varied with time.

Analyzing peripheral blood for DSM in 21 LTx recipients and correlating it with the patient's concomitant BOS status at two different time points separated by one year, we did not find any correlation between DSM and BOS (Table 1). These data are in parallel with the lack of association between peripheral blood DSM and allograft acceptance we have found in kidney and liver transplant recipients [19]. Our data supports the findings of other reports [9] which demonstrate that DSM does not correlate with lung allograft function in LTx recipients. Thus, this study suggests the lack of relationship between peripheral blood DSM and BOS in long-term LTx recipients when both are measured at discrete times. In other words, chronic rejection/BOS could occur in the presence of DSM.

Analysis of chimerism at T_1 and T_2 in BOS positive patients "14–16 and 19–21" (Table 1) exhibited no change in detectable DSM at one year follow-up. Furthermore, 4/6 patients who remained free of BOS at 12 month follow-up, exhibited a change in their DSM status (Table 2) illustrating that one does not need a persistent chimeric state to remain BOS free. Patients 9 and 10, who lost the DSM during the follow up time, did not show any sign of development of BOS, compared to patients 3 and 13, who maintained DSM, one of whom developed BOS, and the other not (Table 2). Thus, our data also suggests that peripheral blood DSM could be useful within individuals over a long time interval (1 year) despite an unchanged clinical status. Clinical outcome of BOS was not because of any other factors such as viral infection etc. The number of recipients in each group, though a small trend, looks promising.

Contrary to the lack of association between the presence of DSM and a BOS free state at a discrete point in time, only 1/6 patients who exhibited DSM at T_1 and/or T_2 developed BOS, while 3/4 of patients who persistently lacked DSM, BOS developed (Table 2). The number of observations may be low, but there is an indication that the risk for development of BOS was higher in patients without DSM. These findings are consistent with other reports suggesting that microchimerism may be associated with a lower incidence of persistent acute or chronic rejection [14, 17]. Reinsmoen et al [17] and O'Connell et al [13] using semi-quantitative PCR (> 1

Table 4 Comparison of donor-specific chimerism with development of BOS at two discrete time points (T_1, T_2) in one HLA-DR antigen matched vs. no HLA-DR antigen matched lung transplant recipients. *Bold numbers* indicate the matched HLA-DR antigens between the recipient and the donor. Statistically significant correlation was observed between not sharing a DR antigen and its association with a higher incidence of DSM (chi-square – $P < 0.01$, Yate's correction for small numbers $P < 0.05$).
* Expired

Patient code	R-DR	D-DR	Ptx	Single or double LTx	Donor specific microchimerism		BOS status at T_2
					T_1 (21)	T_2 (16)	
One HLA-DR Match							
2	**2**.3	7.**15**	33	D	–	+	–
3	8.**15**	**2**.11	47	S	–	–	+
5	**1**.3	**1**.4	24	D	–	–	+ (E*)
6	**1**.–	**1**.12	13	D	–	–	–
7	3.**11**	4.**11**	46	S	+	SN	–
12	**3**.6	**3**.9	26	S	–	–	+
15	1.7	7.17	11	D	–	–	+
16	**15**.–	14.**15**	28	S	–	–	+
17	**2**.3	13.**15**	28	S	–	SN	+
19	4.7	7.15	47	S	+	+	+
HLA-DR Mismatch							
1	3.–	4.6	48	D	–	+	+
4	1.3	15.–	22	S	–	–	+
8	15.–	4.6	63	S	+	+	+ (E)
9	1.15	3.10	60	S	+	–	–
10	3.6	4.11	54	D	+	–	–
11	15.–	4.–	28	D	+	SN	– (E)
14	1.15	4.8	27	D	+	SN	+ (E)
13	4.3	7.15	23	D	+	+	–
18	8.15	1.13	24	S	+	SN	+ (E)
20	7.11	2.4	45	D	+	+	+
21	11.15	13.–	43	D	+	+	+

donor cell per 6,000 recipient cells) and flow cytometry (> 3 % donor cells in a recipient) respectively, demonstrated that the high levels of DSM correlate with lung allograft function and number of donor cells less than 1 in 600 cells of a recipient correlate with persistent acute or chronic rejection. They did not find a significant correlation between the mere presence of DSM, independent of quantity, measured at a single time point, and a subsequent OB free state. Immunosuppression protocols (i.e. no bone marrow or donor specific transfusions) used in their and our study were similar [17]. The investigation by Reinsmoen et al [17] evaluated LTx recipients at 12–18 months following LTx, and was related to patients who were OB free at the time of initial analysis. Our analysis began at a mean time from LTx of 34 months (range 11–63), a point at which approximately 50 % of the LTx recipients would be expected to have BOS [26]. Our initial time point (T_1) of analysis of LTx recipients is approximately 16–22 months after LTx, and this 1–2 year difference could account for both our lower detection of peripheral blood DSM compared to the patients in Reinsmoen et al's study (52 % vs. 77 %), and explain the higher levels of chronic rejection in our patient population. Secondly, what is deemed chronic rejection is not the same in the two studies. BOS is a clinical diagnosis characterized by > 20 % decrement of FEV, relative to the best post-operative FEV1 and after excluding other potential etiologies as specified in Materials and Methods [26]. We find this more useful than strictly using the pathologic results of transbronchial biopsy because of the latter's relatively low sensitivity [26]. For this reason, our working definition of chronic rejection may be more inclusive and thus we are more likely to characterize a patient as having BOS on clinical grounds in spite of no evidence of Bronchiolitis Obliterans on histopathology. Thirdly, [17] their study did find a statistically significant correlation between the quantity of DSM (> 1 donor cell per 6,000 recipient cells) and a subsequent OB free state. However, we could not correlate the donor cell number with development of BOS as we have not assessed in our group of patients. It is plausible that a LTx recipient with a "high" level of DSM would be more likely to be persistently chimeric when measured at two different time points, and yet 4/6 patients who remained BOS-free at the 12 month follow-up in our group of patients, exhibited a change in their DSM-status at one of the time points (Table 2). Thus, Reinsmoen et al and our group are measuring independent phenomena regarding DSM, but both are observing a similar correlation.

We also assessed whether the single vs double LTx and one-HLA-DR-match vs no-match recipients against the donor has any apparent impact on establishment of DSM and development of BOS. Interestingly, our data suggests a trend that the prevalence of DSM is higher in double LTx compared to single, whether this is due to volume of the lung or to the difference in migration/engraftment of donor cells, is not clear. The

prevalence of DSM is low in one-HLA-DR-matched antigen recipients compared to no HLA-DR matched LTx recipients. No correlation was observed between the DSM and development of BOS, either within and/or between the one-HLA-DR-matched and the non-matched groups.

Our results suggest that the presence of DSM in the blood at discrete time points does not correlate with the development of BOS or tolerance in lung transplant recipients. However, the presence of peripheral blood DSM at one or both of two different time points (T_1 and T_2) show that a trend towards lack of DSM might associate with development of BOS. The cause of DSM in LTx recipients studied is most likely due to migration of passenger leukocytes after organ transplantation, as none of our patients received blood transfusions prior to transplantation. If one has to assess the role of DSM in transplantation, we consider it important to characterize the type of chimeric cells and their functional status in a transplant recipient. This could be more relevant for allograft survival outcome, rather than mere presence of donor specific chimerism.

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