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ALLOIMMUNITY INDUCED AUTOIMMUNITY AS A POTENTIAL MECHANISM IN THE PATHOGENESIS OF CHRONIC REJECTION OF HUMAN LUNG ALLOGRAFTS

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Abstract

Background—Bronchiolitis obliterans syndrome (BOS) is a major cause of morbidity and mortality post lung transplantation (LTx). We sought to determine the relationship between alloimmune responses and autoimmunity, and subsequently how autoimmunity leads to chronic rejection.

Methods—We analyzed the development of donor specific antibodies (Abs) in LTx by flow PRA and the development of Abs to K-α1 tubulin (K-α1T) and collagen V (ColV) by ELISA. The frequency of K-α1T and ColV specific T cells that secrete IFN-γ, IL-17 and IL-10 in LTx recipients was measured by ELSIPOT.

Results—In a retrospective analysis of 42 LTx recipients, we demonstrated a strong correlation between development of donor specific anti-HLA Abs, Abs to self-antigens, and BOS ($p<0.05$). To test the hypothesis that alloimmunity is related to an immune response to self-antigens, we analyzed 103 LTx patients prospectively for the development of donor specific Abs (DSA) and Abs to self-antigens. 42.7% of recipients developed DSA and 30.1% developed Abs to K-α1T and ColV. Development of DSA preceded development of Abs to self-antigens. BOS+ patients had higher frequency of T-cells secreting IL-17 ($p<0.01$) and IFNγ ($p<0.05$) with decreased IL-10 ($p<0.05$) compared to BOS- patients.

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Conclusion—Based on these results we propose that alloimmune responses to donor HLA can induce autoimmune responses to airway epithelial self-antigens, characterized by activation of the IL-17 pathway. These immune responses to self-antigens along with alloimmunity contribute to the pathogenesis of BOS. Strategies to prevent development of autoimmunity may be important in preventing the development of chronic rejection.

Introduction

Chronic rejection following human lung transplantation (LTx), bronchiolitis obliterans syndrome (BOS), is the leading cause of morbidity and mortality following transplantation. BOS develops in 50% of LTx patients at 5 years, and in 90% at 10 years after transplantation (1). The pathologic correlate, obliterans bronchiolitis, is histologically characterized by cellular infiltration and fibrosis, resulting in occlusion of small airways (2). Our earlier studies have demonstrated a role for immune responses to mismatched donor HLA in the pathogenesis of BOS (3). Development of antibodies (Abs) to donor HLA (4,5), and increased precursor frequency of CD4+ T-cells to HLA class I and II (3,6,7) are important risk factors for BOS.

Development of Abs to mismatched HLA, donor specific Abs (DSA), has been shown to precede development of BOS (6-8). We recently identified the *de novo* development of Abs to a self-antigen, K-α1Tubulin (K-α1T), in LTx patients with BOS (9). Binding of anti-K-α1T Abs to Airway epithelial cells (AECs) led to up-regulation of transcription factors (TCF5 and c-Myc), which increased expression of fibrogenic growth factors (9), activated cell cycle signaling, and caused fibroproliferation, all central events in the immunopathogenesis of chronic rejection. Immune responses to another self-antigen, Collagen V (ColV), have also been associated with BOS (10).

In this study we demonstrate a correlation between an alloimmune response, donor specific Abs, and generation of an autoimmune response to self-antigens (K-α1T and ColV). Our data demonstrates that the development of DSA during the post-transplant period correlates strongly, not only with BOS, but also to the development of Abs to self-antigens K-α1T and ColV. Additionally, we demonstrate that development of this autoimmune response is likely mediated by IL-17. Abs to self-antigens appear prior to BOS and persist when DSA are undetectable. This, along with our finding that Abs to K-α1T can activate AECs generating a fibroproliferative response, strongly suggests that Abs to self-antigens may play a vital role in BOS pathogenesis.

Materials and Methods

Subjects

Patients' undergoing LTx at Washington University/Barnes-Jewish Hospital were enrolled with informed consent according to protocol approved by the Institutional Review Board. In a retrospective study, sera from 20 BOS+ and 22 BOS− patients, matched for age, race and time post-transplant, was evaluated for the presence of Abs to mismatched HLA and K-α1T/ColV. Pre-transplant Abs to HLA and K-α1T/ColV were absent in this cohort. Age at transplant was 52.0±8.1 years and male-to-female ratio was 1:1. BOS was diagnosed according to International Society for Heart & Lung Transplantation (ISHLT) criteria (11). Serum and Bronchoalveolar Lavage (BAL) samples were collected serially post-transplant and stored at −70°C. BAL samples were preferentially obtained in the right middle lobe in all patients unless they were a single left lung transplant where the BAL was obtained from the lingua. 103 patients who underwent transplantation for COPD, alpha-1-antitrypsin deficiency, cystic fibrosis and idiopathic pulmonary fibrosis were followed and tested for the presence of DSA and Abs to K-α1T/ColV. 97 of the 103 transplants were bilateral. 53.4% of

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the patients were male. Standard immunosuppression consisted of cyclosporine, azathioprine and prednisone. If patients developed Abs to HLA they were treated with either IVIG or Rituximab and IVIG per institutional practice.

HLA testing

Abs to HLA and their specificity were detected in patient serum by solid phase assay (Luminex) (One Lambda, Canoga Park, CA, USA). A sample was considered positive if the ratio of the sample mean fluorescence intensity (MFI) to the control MFI was greater than 0.2. Testing for Abs was done at the time of surveillance bronchoscopy (1, 2, 3, 6 and 12 months) or with evidence of allograft dysfunction.

Flow Panel Reactive Antibody (PRA)

Flow PRA was assayed according to manufacturer's instructions (One Lambda). For HLA class I and II, positivity with a single bead produced a distinct peak of fluorescence containing ~2.4% and 2.9% of total events collected, respectively. Cutoff was determined based on average percentage binding of 20 different normal human sera. Results were positive when ≥2.4% of class I and ≥2.9% of class II beads exhibited fluorescence peak above the fluorescence of controls.

ELISA for anti-K-α1T/ anti-ColV Abs

We developed an ELISA to detect anti-ColV/anti-K-α1T Abs in samples. An ELISA plate was coated with recombinant K-α1T/pure ColV (Chemicon) (1μg/ml) in PBS over night at 4°C. Patient and normal sera were tested (1:500/1:1250) for binding. Detection was done with anti-Human IgG, IgM–HRP (1:10000), developed using TMB substrate and read at 450nm. A sample was positive if values were greater than the mean + 2 SD [218 mcg/ml for K-α1T and 160 mcg/ml for ColV] from sera of normal (HLA-, nonsmoker). The concentration of Abs was calculated using a standard curve of known concentration (microgram/ml) of anti-K-α1T or anti-ColV Abs (Santacruz).

Peripheral Blood Mononuclear Cell (PBMC) isolation

PBMC were isolated by Ficoll gradient centrifugation and frozen at −70°C. For assays cells were thawed, trypan blue was used to determine viability, and only those samples with >90% viability were used. PHA was used as the positive control. Cell samples and purified proteins were negative for endotoxin with LAL assay.

Proliferation assay

PBMCs at 3×10^5cells/well were stimulated with 5μg/ml K-α1T/ColV. Proliferation was measured at day 3 by ^{3}H-thymidine incorporation using a Wallac MicroBeta counter.

ELISPOT assay

ELISPOT using PBMCs at 3×10^5 cells/well, stimulated with 5μg/ml K-α1T/ColV, or BSA (control) was carried out as described earlier (12). Frequency of IL-10, IFN-γ, and IL-17 secreting cells were measured using in ImmunoSpot Image Analyzer (Cellular Technology). The mean number of spots in control wells was subtracted from the mean number of spots in the test wells.

Statistical Analysis

Abs to HLA and Abs to self-antigens K-α1T/ColV were compared for BOS− and BOS+ patients by non-parametric t-tests using Graph Pad Prism and SPSS.

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Results

Autoimmunity directed at K-α1T in LTx patients is associated with BOS

Retrospective analysis of serum for the presence of Abs to self-antigens K-α1T was performed in 12 normal (132±43 mcg/ml), 22 BOS− (203.5±196.54 mcg/ml), and 20 BOS+ (458.86±342.68□cg/ml) LTx patients using ELISA. BOS+ patients have higher levels of Abs to K-α1T in comparison to BOS− patients ($p<0.05$) and control ($p<0.01$) (Figure 1A).

Patients with BOS have elevated levels of Abs to K-α1T and ColV in BAL when compared to BOS− patients

BAL samples from 20 BOS+ and 22 BOS− patients were analyzed by ELISA for anti-K-α1T Abs. BOS+ patients have higher levels of Abs to K-α1T compared to BOS− patients (367.42±213.4vs.205.74±122.65μg/ml, $p<0.05$) (Figure 1B). Only 12 BOS+ and 12 BOS− patients were analyzed for Abs to ColV, due to availability of BAL fluid. Patients with BOS have higher levels of Abs to ColV when compared to BOS− patients (366±45vs.180±23□g/ml, $p<0.05$) or controls (92.6±34μg/ml) ($p<0.01$) (Figure 1C).

A population of LTx patients who develop alloimmune responses to donor mismatched HLA also develop an autoimmune response

We prospectively analyzed 103 patients, who underwent LTx in the two-year period between June 2006 and May 2008. Results presented in Table 1 show 42.7% of patients developed DSA and 30.09% developed both DSA and Abs to K-α1T and ColV. 31% of LTx patients with Abs to K-α1T were DSA+ whereas 21% of patients with Abs to ColV were DSA+. We found that 12.6% of the DSA+ had no Abs to self-antigens and 12.6% of patients who developed Abs to self-antigens had no DSA. The incidence of BOS and concomitant presence of DSA or Abs to self-antigens is also listed. 50 patients developed BOS (48.5%). Of these, 40% were DSA+ and 26% were DSA+/auto Ab+. 14% of these patients who were DSA+ had no Abs to self-antigens and 16% who developed Abs to self-antigen had no DSA.

The development of Abs to HLA appears to precede the development of Abs to self-antigens, and the Abs to self-antigens persist even in the absence of detectable DSA

We analyzed serial serum from LTx patients who developed DSA for the timing of development of Abs to self-antigens. DSA were detectable on average 3 months (ranging from 1-12 months) post-transplant. Abs to K-α1T and ColV developed following the detection of DSA. DSA developed 1-4 months (average 60 days) prior to the development of Abs to self-antigens. Figure 2 is a representative profile detailing the kinetics of the development of DSA and Abs to K-α1T in the serum of four patients. In patient 1, class I DSA developed 2 years post-transplant in 2003, and persisted for 3 months. Abs to self-antigen K-α1T were detected after DSA and more importantly, Abs to K-α1T persisted even after DSA were undetectable. This was also seen in patient 2. Patient 3 and 4 show similar trends, with the exception that they developed DSA of HLA class II specificity. These results suggest that DSA appear prior to the development of Abs to self-antigens, and that Abs to self-antigens, persist even in the absence of detectable DSA.

LTx recipients who developed BOS and DSA also develop significantly higher levels of Abs to self-antigens compared to recipients who develop BOS but do not have DSA

We analyzed serum from 40 patients (10 BOS+/DSA+, 10 BOS+/DSA−, 10 BOS−/DSA+, and 10 BOS−/DSA−) 3 years post-transplant for Abs to self-antigens. The mean concentrations of the Abs against K-α1T and ColV seen in the above group of patients are presented in Table 2. The levels of anti-K-α1T and anti-ColV were increased by 2 fold in BOS+/DSA+ compared to those who were BOS−/DSA − ($p<0.05$).

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PBMCs from BOS+ LTx recipients show higher proliferation against K-α1T protein

Studies from Wilkes and Burlingham (13) and our previous report (12) demonstrated that circulating lymphocytes from LTx patients respond to self-antigen ColV. We tested 10 BOS + and 10 BOS− LTx recipients' (3 yrs post-transplant) lymphocytes for their ability to proliferate upon stimulation with K-α1T. PBMCs from normal were used as controls. PBMCs from BOS+ patients demonstrated significantly higher proliferation to K-α1T in comparison to BOS− patients (>2 fold, $p<0.01$, Figure 3A).

PBMCs from BOS+ LTx recipients show decreased IL-10 and higher IFN-γ production against K-α1T

During the immediate post-transplant period the ColV specific T cells primarily secrete IL-10 with a reduced number secreting IFN-γ (14). After development of BOS, the cytokine profile favors IFN-γ a break of peripheral tolerance to ColV (12,14). To determine whether this also occurs with K-α1T, we analyzed the cytokine profile of lymphocytes from 10 BOS − and 10 BOS+ LTx patients in response to K-α1T. BOS+ patients showed a significant decrease in frequency of IL-10 secreting cells (64.8vs.212 spm, $p<0.05$) with an increase in IFN-γ (234.6vs.165 spm, $p<0.05$) secreting cells (Figure 3B). BOS− patients demonstrated higher IL-10 with lower IFN-γ producing cells.

PBMCs from BOS+ LTx patients show higher IL-17 production against K-α1T/ColV

IL-17 secreting cells can lead to the formation of germinal centers populated by auto-reactive B cells (15) seen in many autoimmune diseases (16). To determine whether the IL-17 is activated in LTx patients with BOS, we measured IL-17 by ELISPOT following stimulation with K-α1T for 10 BOS+ and 10 BOS− LTx recipients, at similar time points post-transplant. BOS+ patients showed more IL-17 producing cells (176vs.63 spm, $p<0.01$) when compared to BOS− patients (Figure 3C). A similar result was seen with ColV (120vs. 50 spm, $p<0.05$).

BOS+ LTx who demonstrate K-α1T and ColV Abs also demonstrate higher IL-17 production

To determine whether LTx patients who developed Abs to self-antigens also show high IL-17 production, we analyzed the LTx patients for both production of Abs to K-α1T and ColV as well as the production of IL-17 following stimulation with self-antigen. Results in Table 3 show the correlation between Abs to self-antigens with the frequency of IL-17 cells in 10 BOS− and 10 BOS+ LTx patients. The BOS+ group showed higher Abs to K-α1T (456±34vs.167±23 μg/ml) as well as to ColV (300±45vs.136±56μg/ml, $p<0.05$) along with higher IL-17 compared to BOS− group (Figure 3C).

Discussion

Several lines of evidence suggest a role for donor specific Abs in the pathogenesis of both acute and chronic allograft rejection following heart, kidney and LTx (6,17). Our studies have demonstrated that the frequency of these alloreactive T cells are increased in LTx patients with BOS (5,18). We demonstrated that the development of DSA (class I) correlates strongly with BOS, and that the development of DSA precedes BOS (7)

In this communication, we provide evidence that immune response to self-antigens may be induced by an immune response to donor HLA. Alloimmune responses to HLA are considered to be one of the insults which leads to chronic rejection following human LTx (19,20) and precedes clinical BOS (9). Our results presented here demonstrate that a subset of LTx patients who developed DSA, developed immune responses to self-antigens, leading to cellular (Figure 3) as well as humoral (Figure 1A, 1B and 1C) immune responses.

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Detection of complement deposition and its activation using C4d staining has increased the awareness of humoral immune mechanisms in pathogenesis of acute and chronic rejection (21). Complement deposition in the allograft in the absence of detectable DSA has been reported (22), suggesting that there are Abs to non-HLA which may play a role in the pathogenesis of chronic rejection. An increasing number of studies have emphasized the clinical importance of these Abs in chronic rejection in kidney, heart and liver allografts (23). Immune responses to myosin and vimentin (24) have been identified as potential autoimmune targets in cardiac allograft vasculopathy. and Abs to angiotensin II type 1 receptor in renal transplant recipients (25). Abs to heat shock protein and epithelial surface gap junction cytoskeletal protein, K-α1T, has been demonstrated to be an immune target in the pathogenesis of BOS (9) (26).

Studies from Wilkes and Burlingham have shown autoimmunity to ColV in BOS (13,27). Our studies have also shown the presence of ColV reactive T cells in human LTx patients (14). Further, we demonstrated that BOS is associated with expansion of IFN-γ producing ColV specific Th-1 cells with reduction in IL-10 producing cells suggesting a break in the peripheral regulatory mechanisms. These results support the hypothesis that an immune response to self-antigens plays a role in the pathogenesis of BOS.

In this report, using a more sensitive solid phase assay system to detect DSA, we demonstrate that a significant proportion of LTx patients (42.7%) develop DSA (Table 1). Studies have shown that MHC class I Ab binding to the endothelial or epithelial surface can activate signaling cascades resulting in production of growth factors, leading to fibrosis and occlusion leading to chronic rejection (8,20). However, this view is often challenged due to the fact that a substantial proportion of LTx patients who have chronic rejection have no detectable DSA (22,28) and has been proposed that the anti-HLA are bound to the graft and thus not in circulation. Our results demonstrate that DSA in many instances was transient, but Abs to self-antigens, once developed, persisted (Figure 2). Additionally, it was noted that these Abs to self-antigens were detected prior to BOS and as such may explain why some patients who are not diagnosed with BOS have detectable levels of auto-Abs. This finding may signify a population “at risk” for the development of BOS.

To elucidate the mechanism by which Abs to self-antigens are developed, cellular responses to self-antigens in BOS+ and BOS− LTx patients were analyzed. There was an expansion of K-α1T and ColV reactive T cells in LTx patients with BOS (Figure 3A), which was associated with a loss of IL–10 producing cells (Figure 3B). We propose the decline in self-antigen specific IL–10 is related to the loss of functional T-regs since our studies have shown that the IL-10 can down regulate not only alloreactivity but also autoreactivity (12).

Our analysis also demonstrates a significant increase in IL-17 and IFN-γ secreting T cells (Figure 3B&C) to K-α1T as well as ColV. IL-17 is a pro-inflammatory cytokine that acts on epithelial cells, endothelial cells, fibroblasts, and stromal cells (29). IL-17 has also been shown to play a crucial role in the induction of humoral autoimmune responses through chemokines CXCL12 and CXCL13, which are involved in germinal center formation (15,30). This suggests that T cells stimulated by self-antigen can provide signals toward the activation of B cells resulting in Abs to self-antigens (Table 3). Additionally, we have shown that neutralizing IL-17 completely abrogates the development of OAD in a murine model of OAD induced by Abs to MHC (20). Thus, there is indirect evidence that IL-17 plays a central role in the induction of autoimmunity as well as chronic rejection during the post- transplant period.

The prospective analysis shows that 12.6% of patients who developed Abs to self-antigens had no detectable DSA (Table 1). In a previous study we defined K-α1T as a self-antigen to

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which an immune response takes place following LTx even when DSA was not evident. The development of Abs to K-α1T was correlated significantly with BOS, approximately 30% of BOS+ patients who were negative for Abs to HLA demonstrated Abs to K-α1T (9). Additionally, it is shown in Table 1 that of the group of patients that developed BOS (48.5% of prospective cohort) 16% had evidence of Abs to self-antigens but no detectable DSA. These findings suggest that mechanisms other than DSA, such as ischemia-reperfusion injury, gastroesophageal reflux (31), viral infections (32,33) and acute rejections (34) can induce immune response to self-antigens. A common theme is the generation of an inflammatory milieu which would be conducive for the expansion of self-reactive lymphocytes capable of priming T cells with 'low-affinity' TCRs (35).

In conclusion, alloimmunity can expose self-antigens or their determinants to the immune system, which is occurring following transplantation and calcineurin inhibitor based immunosuppression, providing an environment conducive to the generation of an immune response against the newly exposed self-antigens. Early screening and detection of DSA coupled with intervention as soon as DSA is detected, such as treatment with intravenous immune globulin and B-cell directed therapy, have the potential to prevent the development of autoimmunity, thus delaying or preventing chronic rejection.

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Figure 1A.

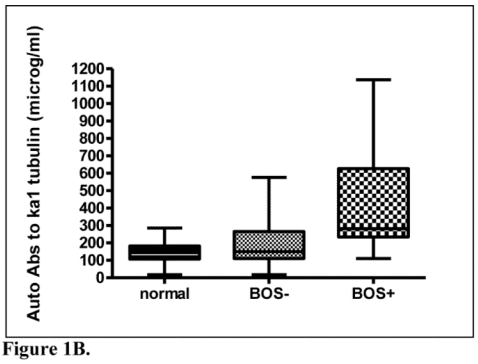

Figure 1B.

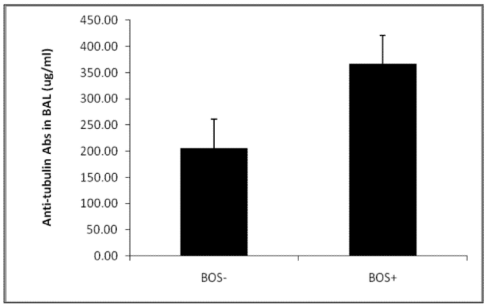

Figure 1C.

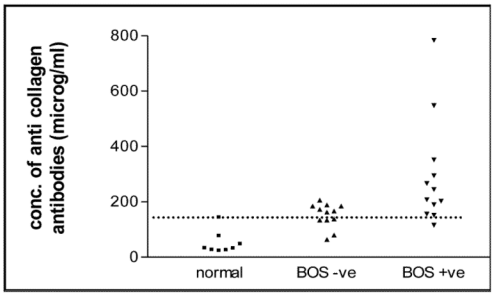

Figure 1.

A. Elevated levels of K-α1T Abs in serum of BOS+ patients compared to BOS− patients. Sera from 20 BOS+ patients and 22 BOS− patients and 12 normal volunteers were analyzed by ELISA for anti-K-α1T Abs as described in materials and methods section. BOS+ patients have higher levels of Abs to K-α1T in comparison to BOS− patients ($p<0.05$) and control ($p<0.01$).

B. Elevated levels of K-α1T Abs in BAL of BOS+ patients compared to BOS− patients. BAL from 20 BOS+ patients and 22 BOS− patients were analyzed by ELISA for anti-K-α1T Abs. BOS+ patients have higher levels of Abs to K-α1T in comparison to BOS− patients ($p<0.05$)

C. Elevated levels of ColV Abs in BOS+ patients compared to BOS− patients. Sera from 12 BOS+ patients, 12 BOS− patients and 8 normal volunteers were analyzed by ELISA for Abs to ColV as described in materials and methods. Patients with BOS have significantly higher levels of Abs to ColV compared to BOS– patients ($p<0.05$) as well as controls ($p<0.01$)

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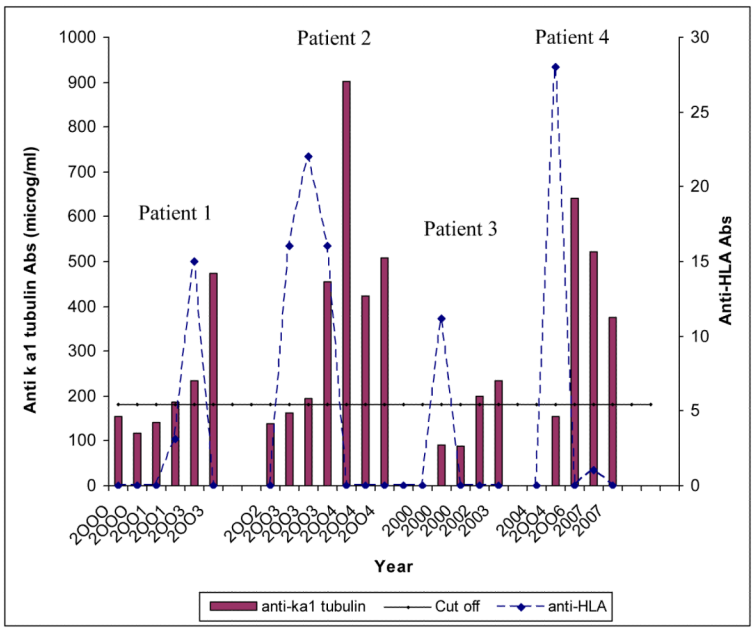

Figure 2. Persistence of Auto-Abs in the serum of LTx patients though anti-HLA Abs are not detected

Development of Anti-HLA Abs and Abs to K-α1T in the serum of four patients with respect to time is plotted. Anti-HLA Abs are shown in dotted lines and anti-K-α1T Abs as bars. Values above the cutoff line are considered positive. These results demonstrate that Abs to mismatched donor HLA (DSA) appear prior to the development of Abs to self-antigens. DSA appears transiently during the post-transplant period. Abs to self-antigens, once developed, persists even in the absence of detectable DSA.

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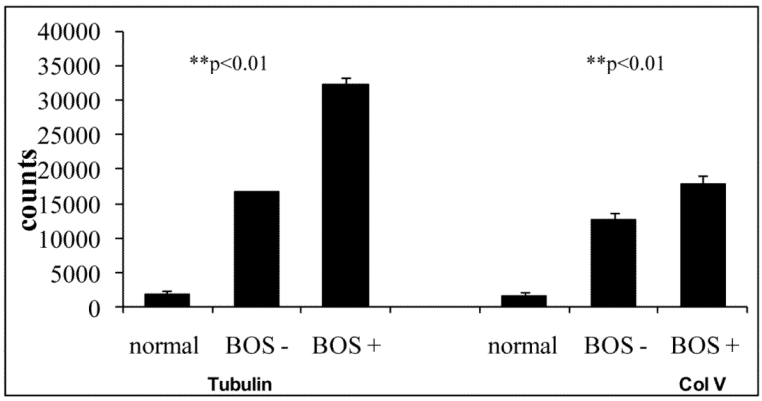

Figure 3A. Higher proliferation in BOS+ LTx patients after PBMCs are stimulated with K-α1T/ ColV

After stimulation with 5μg/ml K-α1T/ColV PBMCs from BOS+ patients were tested for proliferation against these self-antigens by Thymidine incorporation and compared to BOS− patients (n=10 in each group). PBMCs from all BOS+ patients demonstrated significantly higher proliferation to K-α1T compared to BOS− patients (>2 folds, $p<0.01$).

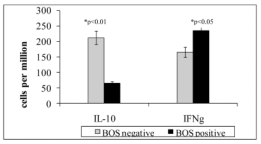

Figure 3B. Decreased IL-10 and higher IFN-γ production against K-α1T in BOS+ LTx patients

PBMCs from BOS+ and BOS− patients (n=10 in each group) were stimulated with 5μg/ml K-α1T, followed by detection of IL-10 and IFN-γ production by ELISPOT. BOS+ patients showed significantly decreased production of IL-10 (64.8 vs. 212 spm, $p<0.05$) and increased IFN-γ (234.6 vs. 165 spm, $p<0.05$) secreting cells against K-α1T.

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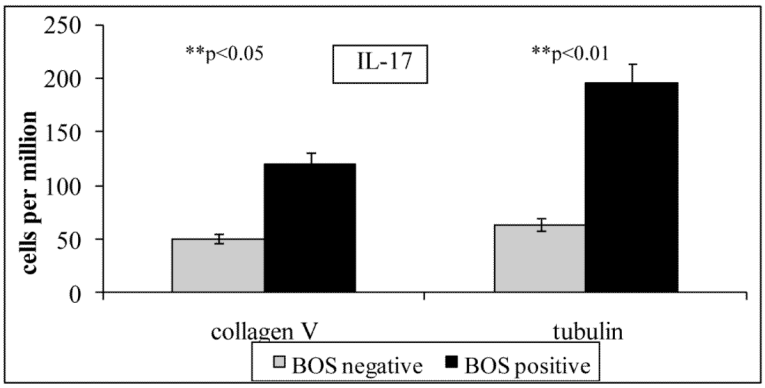

Figure 3C. Higher frequency of IL-17 producing cells in BOS+ LTx patients against K-α1T

PBMCs isolated from BOS+ and BOS− patients (n=10 in each group) were stimulated with 5μg/ml K-α1T or ColV, followed by detection of IL-17 production by ELISPOT. BOS+ patients showed significantly higher IL-17 producing cells against K-α1T (176 vs. 63 spm, $p<0.01$) compared to BOS− patients. ColV stimulated BOS+ cells also produce higher IL-17 compared to BOS− cells (120 vs. 50 spm, $p<0.05$)

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Table 1

Prevalence of DSA, auto-Abs, and BOS in our patient population. n=103

	DSA (%)	DSA + Auto Abs (%)	DSA + Anti-K□1 Abs	DSA + Anti-ColV Abs	No DSA + Auto Abs (%)	DSA + No Auto Abs (%)	No DSA + No Auto Abs
	42.7	30.09	31.06	21.3	12.6	12.6	55.3
Development of BOS (%) n = 50	40	26	26	18	16	14	44

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Table 2

Development of Abs to K-α1T and ColV in BOS+ LTx patients developing anti-HLA allo-Abs.

Patients	Anti-Kα1T Abs (μg/ml)	Anti-ColV Abs (μg/ml)
Normal n=12	132±43	92.57 ± 34.25
BOS− HLA− n=10	147.5 ± 94.54	190.35 ± 113.9
BOS+ HLA+ n=10	458.86 ± 342.68	365.71 ± 219.81
BOS+ HLA− (n=10)	140.1±96.29	185.5 ±91.0
BOS− HLA+ (n=10)	112.6±40.58	137.8±69.46

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Table 3

Correlation of higher IL-17 producing T-cells and BAL IL-17 levels with Auto-Ab production.

IL-17 (cpm) Against K-α1T		Anti-K-α1T Abs (μg/ml)	IL-17(cpm) against ColV	Anti-ColV Abs(μg/ml)	IL-17 in BAL (pg/ml)
BOS+	176	456±34	120	300±45	123.9±54
BOS−	63	167±23	51	136±56	88.7±55