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Development of Antibodies to HLA Precedes Development of Antibodies to MICA and Are Significantly Associated With Development of Chronic Rejection Following Human Lung Transplantation

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Abstract

The development of antibodies (Abs) to major histocompatibility (MHC) class I related chain A (MICA) and human leukocyte antigen (HLA) and their role in the immunopathogenesis of chronic rejection (bronchiolitis obliterans syndrome (BOS)) following human lung transplantation (LTx) was analyzed. Sera from 80 LTx recipients were analyzed for anti-MICA and anti-HLA Abs using Luminex and flow PRA (panel reactive assay). Development of Abs either to MICA alone or MICA and HLA together significantly correlated ($P < 0.01$) with development of BOS. Kinetic analysis in the post-LTx period revealed that development of anti-HLA Abs (7.6 ± 4.7 months) preceded the development of anti-MICA Abs (10.0 ± 3.5 months). Abs to MICA alleles (*001 and *009) developed approximately 6 months following LTx and peak titers were present at the time of clinical diagnosis of BOS (16.3 ± 2.7 months). The development of Abs to both MICA and HLA was strongly associated with the development of BOS thereby suggesting a synergistic effect. Furthermore, immune response to mismatched HLA can lead to development of Abs to other MHC related antigens expressed on the airway epithelial cells. Cumulatively, these immune responses contribute to the pathogenesis of chronic rejection following human LTx.

Keywords

MICA; HLA; antibodies; lung transplantation; BOS; Chronic rejection

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1. Introduction

Long-term patient outcomes following lung transplantation (LTx) are limited by the development of chronic rejection (CR), clinically diagnosed as bronchiolitis obliterans syndrome (BOS). The incidence of BOS approaches 50% at five years and 90% after nine years post-transplant [1,2]. Obliterative bronchiolitis is the histological correlate of BOS and is characterized by the cellular infiltration, fibrosis, collagen deposition and occlusion of small airways in the allograft. The diagnosis of BOS is clinically established based on a decline in pulmonary function in the absence of reversible causes of respiratory failure such as infections and acute rejection (AR) [3,4].

Previous studies from our laboratory [5–7] and others have demonstrated that development of anti-human leukocyte antigen (HLA) antibodies (Abs) precedes the development of chronic rejection (CR) following LTx [8,9]. Studies have also shown that the development of anti-HLA Abs during the post-transplant (Tx) period is a significant risk factor for the development of CR in kidney [10,11], and heart transplantation [12,13]. Recent studies suggest that Abs developed during the post-Tx period against the mismatched non-HLA antigens may also contribute to the pathogenesis of CR in solid-organ transplantation [14–16].

One target antigen that has received considerable attention recently is the major histocompatibility (MHC) class I related chain A (MICA). MICA, a single chain glycoprotein expressed on cellular membrane, is determined by a genetic locus closely linked to the HLA-B. MICA is polymorphic, with 52 protein variants and more than 60 alleles [17,18]. MICA molecules function as signals of cellular stress and trigger a range of immune effector mechanisms including cellular cytotoxicity and cytokine secretion [19,20]. While the MICA protein has not been implicated in antigen presentation, it plays an important role in initiating an immune response by binding to the immune-receptor NKG2D. Upon binding to MICA, NKG2D stimulates cell lysis pathways and hence MICA has an important role in providing a co-stimulatory signal for the activation of NK (natural killer) cells, CD8+ T cells and T cells [21–23]. Recent reports suggest that post-Tx development of Abs to MICA correlate with renal allograft loss and is an independent risk factor for the development of CR [24–26]. Similarly a relationship between the development of anti-MICA Abs and CR has been reported in heart transplant recipients [27,28].

The increased expression of polymorphic MICA genes in the human lung airway epithelial cells during conditions of stress can lead to apoptosis of epithelial cells and pulmonary fibrosis [29]. During donor procurement and LTx, the airway epithelial cells undergo mechanical stress which causes damage to epithelial integrity. Similarly, anti-HLA Abs developed in the post-Tx period can also injure epithelial cells leading to the expression of otherwise cryptic self-antigens and cell surface expression of MICA antigens. The study objective is to determine the significance of MICA in development of CR following LTx and to assess whether sensitization to MICA is an independent risk factor for the development of CR following human LTx.

2. Materials and Methods

2.1 Patients and samples

From May 1990 to December 2008, all patients undergoing LTx at Washington University/Barnes-Jewish Hospital were enrolled in the study in accordance with a protocol approved by the Institutional Review Board. Serum samples were collected serially in the post-LTx period and were stored at –80°C. Sera from 80 patients were utilized in this study. Of these, 50 recipients developed BOS (BOS+) and 30 were from patients who did not develop BOS (BOS–). The demographic profile of the two cohorts (BOS+ and BOS– patients) including age, sex,

ethnicity, indication for transplantation, and type of Tx (unilateral or bilateral) were tabulated. A clinical diagnosis of BOS was made utilizing the guidelines established by the International Society for Heart and Lung Transplant [30,31]. The maintenance immunotherapy protocol at our center consisted of cyclosporine, azathioprine, and prednisone. After BOS was diagnosed, the immunotherapy protocol was modified to FK506 (tacrolimus), mycophenolate mofetil, and prednisone. Sera from 15 healthy adult volunteers were obtained and served as controls.

2.2 Luminex assay for detection of Abs against MICA

Abs to MICA alleles were determined using LABScreen® assay by Luminex Technology, according to the manufacturer's specifications (One Lambda) using 96-well filter plates. In brief, the assay filter plate was pre-wet by dispensing 300µl of wash buffer (cat. #LSPWABUF) and incubated for 10 minutes on a platform plate shaker at low speed. After aspirating the buffer using a Millipore vacuum manifold with pressure not exceeding 100mmHg, 5µl of LABScreen beads with 20µl of serum was dispensed into test wells. For each test, a negative control serum (OLI Cat. #LS-NC) was used along with a healthy volunteer's serum to establish a cutoff. The above mixture was incubated for 30 minutes with gentle shaking and washed using 275µl of wash buffer. Further, 100µl of 1X PE conjugated anti-human IgG was added to each well and incubated for 30 minutes. Finally, 80µl of 1X PBS was added and the samples were read using LABScan™ 100™ machine.

Serum samples of LTx subjects and healthy donors were tested at 1:3 dilution for Abs against a panel of 10 MICA alleles (MICA *001, *002, *004, *007, *009, *012, *017, *018, *019, and *027). The fluorescent signal was measured using LABScan and analyzed by HLA-Visual™ software (One Lambda). The raw MFI values were normalized utilizing negative control serum (OLI Cat no. LS-NC) by the formula: $[(\text{sample \# N beads} - \text{sample negative control beads}) - (\text{negative control \# N beads} - \text{negative control beads})]$. Luminex reactions were considered positive when the fluorescent signal of each bead was above the normalized mean fluorescence intensity (MFI) of volunteer control sera and normal control sera provided by the company. In addition the MICA positive serum for each of the 10 alleles listed above (obtained from Miyuki Ozawa (One lambda, Inc.) were analyzed and served as positive controls.

2.3 Detection of HLA Abs by FlowPRA assay

FlowPRA assay was performed according to the manufacturer's directions (One Lambda, Canoga Park, CA, USA). For classes I and II FlowPRA assays, positivity with a single bead produced a clear and distinct peak of fluorescence that contained approximately 2.4% for class I and 2.9% for class II of the total events collected (i.e., 1/24th and 1/29th of the bead pool). The cutoff was determined based on the average percentage binding of 15 different normal human sera from healthy volunteers.

2.4 Statistical Analysis

Data are represented as mean value $\pm$ SD. Statistical analysis was performed using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA) and GraphPad Prism version 4.02 (GraphPad Software Inc., San Diego, CA). Two tailed student *t* test was used to compare MICA levels between experimental groups. Uni- and multivariate analysis was performed using SPSS software (SPSS Inc., Chicago, IL). Statistical significance was defined at $P < 0.05$.

3. Results

3.1 Patient demographics

The study cohort consisted of 80 patients; 50 recipients were BOS+ and 30 recipients were BOS-. Table 1 depicts the patient demographics for both cohorts. There were no significant

differences in age, sex, ethnicity, and indication for transplantation between the two groups. The majority of the patients in both cohorts underwent bilateral LTx and there was no statistical difference between the frequency of single LTx and bilateral LTx.

3.2 Abs to HLA and MICA develop in BOS+ patients following LTx

Serum samples from 80 LTx patients were examined for the presence of Abs to HLA and MICA. 72% of BOS+ patients developed Abs to HLA compared to 33% of patients who were BOS- ($p < 0.01$). A greater proportion of patients who were BOS+ (42%) developed Abs to MICA compared to patients who were BOS- (13%); this difference was statistically significant ($p < 0.01$) (Table 2). In summary, BOS+ patients developed Abs to both MICA and HLA (86%) in comparison to only 37% in BOS- patients ($p < 0.01$).

Further, patients diagnosed with BOS had a greater level of sensitization to MICA as noted by a nearly two-fold higher MFI when compared to stable LTx recipients (Fig. 1 and Fig. 3). We tested pre-LTx or very early post-LTx (within the first month) sera samples from patients who developed Abs to MICA to determine whether there is pre-existing sensitization to MICA antigens in the LTx population. These sera did not demonstrate any Abs to MICA alleles (Table 2) indicating that Abs to MICA developed post-LTx.

3.3 Abs to MICA alleles *002, *004, *009 and *017 develop in BOS+ patients but are not present in BOS- patients following LTx

8% of BOS+ patients had sera reactive to MICA *002 and *004 alleles. In addition Abs to MICA *009 and *017 were seen in 4% and 6% of the samples respectively in BOS+ patients (Table 4). None of the BOS- patients developed Abs to MICA *002, *004, *009 and *017. Overall, 26% of BOS+ patients developed Abs to these specific MICA alleles which correlated significantly with the development of BOS ($p < 0.01$).

3.4 Abs to MICA alleles *001, *007, *012, *018, *019, and *027 develop more frequently in BOS+ patients compared to BOS- patients following LTx

As shown in Table 4, 22% and 32% of BOS+ patients developed Abs against MICA*001 and *019 compared to 6.6% and 10% respectively in BOS- patients ($p < 0.001$). MICA*007, *012, *018, and *027 were represented by 6%, 8%, 6% and 10% in BOS+ patients compared to 3%, 7%, 7% and 3% in BOS- patients respectively. In BOS+ patients, the total frequency of Abs to MICA*001 and *019 was 54% which was significantly higher compared to the frequency of Abs to MICA*007, *012, *018, and *027 which was 30% ($p < 0.05$).

3.5 Abs to multiple MICA alleles develop in BOS+ patients compared to BOS- patients following LTx

Patient sera were analyzed using Luminex in order to assess the presence of Abs to multiple MICA alleles simultaneously (in our case 10 alleles). 16% of BOS+ patients had Abs to any single MICA allele compared to 0% in BOS- group ($p < 0.01$). 10% of BOS+ patients developed Abs to ≥ 5 MICA alleles compared to 0% in BOS- group ($p < 0.05$). Less than 8% of patients from both cohorts had Abs for 2–4 MICA alleles in common (Table 5).

3.6 Abs to HLA precede the development of Abs to MICA

We assessed the temporal sequence of Abs production by determining the presence of anti-HLA Abs and MICA Abs in serial samples. Among the patients we tested for Abs to MICA and HLA (8 patients), we determined that anti-HLA Abs appeared first (7.6 ± 4.7 months) followed by MICA Abs (10.0 ± 3.5 months). Additionally, patients who developed Abs to both HLA and MICA develop BOS earlier, namely 16 ± 9.6 months from the time of LTx (Fig. 2)

in contrast to patients with Abs just to MICA (23 ± 14.3 months) or Abs just to HLA (19 ± 12.3 months).

3.7 Abs titers to MICA peak before development of BOS and persist thereafter

As previously stated, Abs to MICA *001, *019, *027, *002, and *004 were frequently noted in the BOS+ patient cohort. We analyzed serial samples from 8 patients with these alleles and correlated the kinetics of Abs development to MICA to the subsequent development of BOS. The mean time for development of BOS in this patient subgroup was 16.3 ± 2.7 months from the time of LTx. To compare with a similar BOS- cohort, we selected serum samples from 2 patients in the same time frame that had MICA*001 and *019 Abs in common. As shown in Fig. 3A and Fig. 3B, Abs to MICA*001, *002, *004, *019 and *027 initially appeared 6 months post-LTx and were found in higher titers 14–17 months post-transplant. Abs to HLA also developed gradually with a peak Abs titer during the same time frame as that of MICA (Fig. 3D). In contrast, serum from BOS- patients developed Abs to MICA more gradually and did not express a peak Abs titer (Fig. 3C).

4. Discussion

Antibodies directed against mismatched donor HLA, blood group or endothelial cell antigens can lead to Ab mediated graft injury and rejection [14,32,33]. The detection of Ab-mediated rejection has been difficult possibly due to binding of Abs to the allograft and its transient nature in circulation. The development of highly sensitive assays for detecting Abs such as Luminex or flow cytometry, has enabled investigators to assess the presence of Abs during the post-Tx period with greater sensitivity. The most common Abs which are developed following Tx and associated with rejection are Abs to mismatched MHC molecules of the donor. Like HLA, MICA is highly polymorphic and this property makes these antigens ideal candidates for sensitization and development of allo-Abs during the post-Tx period [34].

Recently, several studies have demonstrated the importance of HLA Abs in organ rejection, most notably in renal, heart and lung transplantation [12,14,35–37]. There is evidence that ligation of anti-HLA Abs to endothelial cells results in phosphorylation of a wide array of intracellular proteins which stimulate proliferation of endothelial cells and lead to CR [38–40]. Similarly, a few reports have demonstrated a role for immune responses to MICA in rejection following heart and kidney Tx [25,41]. To our knowledge, there have been no previous studies that have examined the role of MICA in the development of CR (BOS) in post-LTx recipients.

Our results indicate that in patients without Abs to HLA and MICA in the pre-LTx period, a significant portion of (42%) patients in BOS+ group developed Abs to MICA ($p < 0.01$, Table 2) post-LTx. There was also a strong correlation between the development of Abs to HLA and a clinical diagnosis of BOS following LTx (72%, $p < 0.01$, Table 3). We confirmed the absence of HLA antibodies in the MICA positive patients with single antigen bead Luminex assay. This indicates that Abs to MICA like Abs to HLA develop during the post-LTx period in patients that develop BOS. In BOS+ patients, 86% of samples were both MICA+/HLA+ compared to 37% in BOS- patients ($p < 0.01$, Table 3). These results suggest that a cumulative damage of the organ can take place when immune response to both polymorphic antigens occurs. Furthermore, the frequency of Abs to MICA*002, *004, *009 and *017 allele were present only in BOS+ patients (26%, Table 4). In BOS+ patients, the frequency of Ab development to MICA *019 and *001 was 54% which was significantly higher in comparison to BOS- patients (17%, $p < 0.05$). These findings are consistent with report from Alvarez et al. where in heart transplant patients who developed Abs to MICA, the most frequent Ab production was to allele *019 followed by MICA*001 [27,28].

We analyzed the kinetics of Abs production to HLA and MICA in the sera of LTx recipients. The results indicated that Abs to MICA develop after the development of anti-HLA Abs but before development of BOS (Fig. 2). In BOS+ patients, anti-HLA Abs were detectable as early as 7.6 ± 4.7 months, followed by MICA at 10.0 ± 3.5 months. It is likely that the anti-HLA Abs are formed initially and are involved in inducing stress and damage to epithelial and endothelial cells. These stimulatory processes are ideal for the cell surface expression of MICA on graft endothelia, epithelia, fibroblasts and activated monocytes. In this context, we recently reported that exposure of lungs to MHC class I Abs can result in increased expression of chemokines, growth factors and induces IL-17 along with de novo development of Abs to self-antigens, K-alpha1 tubulin, and Collagen V [42]. Therefore, it is possible that Abs to MICA may also lead to similar changes. Hence, anti-HLA may play an important role in eliciting Abs to MICA antigens expressed on airway epithelial cells and these Abs developed during the post-LTx period may contribute to the pathogenesis of BOS.

In the current report, the mean time for development of BOS was 27.1 ± 16.3 months from the time of LTx. Since the majority of BOS patients had Abs to MICA*001 and *019 we tested in serial serum samples obtained during the post-Tx periods for the development of Abs to these alleles and correlated the development Abs to MICA to development of BOS. The mean time for development of BOS in this subgroup of patients was 16.3 ± 2.7 months post-LTx. As shown in Fig. 3A, Abs to MICA*001 and *019 appeared 6 months post-LTx and were significantly higher during time of BOS development. LTx recipients who developed both anti-HLA and anti-MICA developed BOS earlier. Genotyping donors and recipients for MICA alleles would provide useful information in assessing whether the observed MICA Abs constitute an allo or an autoimmune response. However, given that we have only four MICA + patients in the BOS- group, the data would not have sufficient statistical power to reach a meaningful clinical conclusion.

In summary, we demonstrate that the development of Abs to MICA and HLA following human LTx are significantly associated with the onset of BOS. Patients who developed both HLA and MICA Abs developed BOS earlier than those had only anti-HLA or anti-MICA Abs alone. While a minority of patients developed Abs to MICA alone, the majority of patients developed Abs to HLA which preceded the development of Abs to MICA. This suggests that binding of HLA Abs to the graft may increase the expression of MICA resulting in an immune response against this antigen. Therefore, identification of MICA and HLA Abs early during the post-LTx period followed by removal of Abs directed to these alloantigens may be beneficial in preventing CR.

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Abbreviations

Abs	Antibodies
AR	Acute Rejection
BOS	Bronchiolitis Obliterans Syndrome
CR	Chronic Rejection
HLA	Human Leukocyte Antigens

LTx	Lung Transplantation
MHC	Major Histocompatibility Complex
MICA	MHC class I chain-related molecule A
Tx	Transplantation

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Figure 1.

Comparison of Abs to various MICA alleles in BOS+ and BOS- patient sera. Luminex reactions were carried in duplicates using 1:3 diluted sera. Data are representative of mean $\pm$ SD of all the positive values obtained for each allele from various patients sera of both BOS+ and BOS- cohorts.

Figure 2.

Development of anti-MICA, anti-HLA Abs and development of BOS after LTx. Anti-MICA and anti-HLA Abs were detected by LabScreen Luminex and flowPRA assay systems respectively. The MICA and HLA bars represent the time (mean $\pm$ SD) at which each Abs was first detected in serum samples of study patients. The remaining three bars represent the average time at which the patients developed BOS if they were HLA+, MICA+ or both MICA+ and HLA+.

Figure 3.

Sequential measurements of anti-MICA and anti-HLA Abs in study patients. For specific MICA alleles (*001 and *019 in Panel A; * 027, *002 and *004 in Panel B), the figure represents the change in normalized MFI over time post-LTx. Panels A and B depicts BOS+ patients for alleles specified above while Panel C depicts BOS- patients for alleles *001 and *019. Panel D depicts the frequency of HLA positive recipients who were present in BOS + and BOS- cohorts over time post-LTx.

Table 1

Clinical and demographic profile of lung transplant patients

	All	BOS+	BOS-	P value
Age (years)	48.8 ± 12.6	49.8 ± 11.7	47.7 ± 12.2	0.62
Sex - Female	42	28	14	0.41
Male	38	22	16	
Race - Caucasian	71	44	27	0.78
African American	9	6	3	
Pathology- COPD	40	25	15	1.00
A1AD	12	8	4	0.07
CF	11	7	4	0.07
IPF	8	4	4	0.42
PPH	6	4	2	0.06
Bronchiectasis	3	2	1	0.89
Type of transplant				
Bilateral	70	43	27	0.06
Single	10	7	3	

COPD, chronic obstructive pulmonary disease; A1AD, alpha-1 antitrypsin deficiency; CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis; PPH, Primary Pulmonary Hypertension.

Table 2
Comparison of MICA antibodies in serum samples of patients before and after lung transplant.

Pre-transplant	Post-transplant	
	BOS+	BOS-
00/20 (00.0%)	21/50 (42.0%) **	4/30 (13.3%)

** = P < 0.01

Table 3

Frequency of anti-MICA and anti-HLA antibodies in lung transplant patients

	MICA ⁺	HLA ⁺	MICA ⁺ and/or HLA ⁺	MICA ⁺ + HLA ⁺	MICA ⁻ + HLA ⁺	MICA ⁻ + HLA ⁻
BOS +	21/50 (42%)*	36/50 (72%)*	43/50 (86%)*	7/50 (14%)*	15/50 (30%)	7/50 (14%)
BOS -	4/30 (13.3%)	10/30 (33.3%)	11/30 (36.6%)	1/30 (3.3%)	6/30 (12%)	19/30 (63.3%)*
P value	< 0.01	< 0.01	< 0.01	< 0.05	0.32	< 0.01

Table 4
Frequency of antibodies to MICA alleles in BOS+ and BOS– lung transplanted patients sera

n		Positive percentage for MICA Alleles									
		*001	*002	*004	*007	*009	*012	*017	*018	*019	*027
BOS+	50 (100%)	11 (22%)	04 (8%)	04 (8%)	03 (6%)	02 (4%)	04 (8%)	03 (6%)	03 (6%)	16 (32%)	05 (10%)
BOS –	30 (100%)	02 (6.6%)	00 (0.0%)	00 (0.0%)	01 (3.3%)	00 (0.0%)	02 (6.6%)	00 (0.0%)	02 (6.6%)	03 (10%)	01 (3.3%)

Table 5

Frequency of antibodies to MICA alleles in BOS+ and BOS- lung transplant patients sera

No. of alleles	BOS+ (MICA Alleles) N=50 (100%)	BOS- (MICA Alleles) N=30 (100%)	P value
01	08 - *001,*012,*018,*019,*027 (16.0%)	00- (0 %)	0.01
02	04 - *001,*002,*007,*017 (8.0%)	01 - *001,*007 (3.3%)	0.61
03	02 - *001,*002,*004,*009,*012,*019,*027 (4.0%)	02 - *012,*018,*019 (6.6%)	0.89
04	02 - *001,*004,*019,*027 (4.0%)	02 - *001,*012,*018,*019,*027 (6.6%)	0.37
≥ 05	05 - *001,*002,*004,*007,*009,*012,*017,*018,*019,*027 (10.0%)	00- (0%)	0.05