ELSEVIER

The Journal of
Heart and Lung
Transplantation

http://www.jhltonline.org

ORIGINAL PRE-CLINICAL SCIENCE

Characterization of immune responses to cardiac self-antigens myosin and vimentin in human cardiac allograft recipients with antibody-mediated rejection and cardiac allograft vasculopathy

Dilip S. Nath, MD,[a] Haseeb Ilias Basha, MD,[b] Venkataswarup Tiriveedhi, MD, PhD,[b] Chiraag Alur, BS,[b] Donna Phelan, BA,[c] Gregory A. Ewald, MD,[d] Nader Moazami, MD,[a] and Thalachallour Mohanakumar, PhD[b,e]

From the [a]Division of Cardiothoracic Surgery, [b]Department of Surgery and [c]HLA Laboratory, Barnes–Jewish Hospital, [d]Division of Cardiology, [e]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri.

KEYWORDS:
myosin;
vimentin;
self-antigens;
antibody mediated rejection;
cardiac allograft vasculopathy

BACKGROUND: Herein we study the role of donor-specific antibodies (DSA) to mismatched human leukocyte antigen (HLA) and antibodies (Abs) to the cardiac self-antigens myosin (MYO) and vimentin (VIM) in the pathogenesis of acute antibody-mediated rejection (AMR) in the early post-transplant period (EP, <12 months) and cardiac allograft vasculopathy (CAV) in the late post-transplant period (LP, >12 months) after heart transplantation (HTx).

METHODS: One hundred forty-eight HTx recipients (65 in EP, 83 in LP) were enrolled in the study. Development of DSA was determined by Luminex. Circulating Abs against MYO and VIM in sera were measured using enzyme-linked immunoassay (ELISA). Frequency of $CD4^+$ T-helper cells ($CD4^+$ Th) secreting interferon (IFN)-γ, interleukin (IL)-17, IL-10 or IL-5 specific to either MYO or VIM were analyzed in vitro using ELISpot assays.

RESULTS: AMR patients were more likely DSA positive (AMR^-: 15%; AMR^+: 70%; $p = 0.03$) and demonstrated increased Abs to MYO (AMR^-: 144 ± 115 μg/ml; AMR^+: 285 ± 70 μg/ml; $p = 0.033$) and VIM (AMR^-: 37 ± 19 μg/ml; AMR^+: 103 ± 43 μg/ml; $p = 0.014$). AMR patients demonstrated increased IL-5 $CD4^+$ Th cells specific to MYO (5.2 ± 0.9 fold, $p = 0.003$) and VIM (7.3 ± 2.9-fold, $p = 0.004$) and decreased IL-10 $CD4^+$ Th cells specific to MYO (2.2 ± 0.4-fold, $p = 0.009$) and VIM (1.7 ± 0.2-fold, $p = 0.03$). CAV patients were more likely DSA positive (CAV^-: 25%; CAV^+: 79%; $p = 0.03$) and demonstrated increased Abs to MYO (CAV^-: 191 ± 120 μg/ml; CAV^+: 550 ± 98 μg/ml; $p = 0.025$) and VIM (CAV^-: 55 ± 25 μg/ml; CAV^+: 255 ± 49 μg/ml; $p = 0.001$). CAV patients demonstrated increased IL-17 $CD4^+$ Th cells specific to MYO (10.5 ± 7.3-fold, $p = 0.002$) and VIM (7.0 ± 3.9-fold, $p = 0.003$).

CONCLUSIONS: The presence of DSA in AMR and CAV is significantly associated with development of Abs to MYO and VIM in post-HTx patients. Induction of high $CD4^+$ Th cells specific to cardiac self-antigens that secrete predominantly IL-5 and IL-17 plays a significant role in the development of Abs to self-antigens leading to AMR and CAV, respectively.

J Heart Lung Transplant 2010;29:1277–85

Reprint requests: Thalachallour Mohanakumar, PhD, Department of Surgery, Washington University School of Medicine, Box 8109-3328 CSRB, 660 South Euclid Avenue, St. Louis, MO 63110. Telephone: 314-362-8463. Fax: 314-747-1560.

E-mail address: kumart@wustl.edu

1053-2498/$ -see front matter
doi:10.1016/j.healun.2010.05.025

Acute antibody-mediated rejection (AMR) is recognized as a major cause of allograft dysfunction after heart transplantation (HTx) and its overall prevalence during the initial post-operative phase has been reported to exceed 40%.[1–7] The clinical diagnosis of AMR is heralded by the onset of hemodynamic instability in the absence of cellular rejection or graft atherosclerosis after HTx.[3,8] Recent International Society of Heart and Lung Transplantation (ISHLT) guidelines have proposed AMR as a distinct clinicopathologic entity characterized by presence of allograft dysfunction in concert with histologic findings for capillary injury, positive immunofluorescence for C4d in endomyocardial biopsies, and detection of circulating donor-specific antibodies (DSA).[8,9] There is now accumulating evidence suggesting that early post-transplant events promote development of a chronic inflammatory process that subsequently leads to transplant rejection.[2,10] Previous studies from our laboratory have demonstrated that lung transplant recipients with primary graft dysfunction have elevated pro-inflammatory mediators including interferon-inducible protein (IP)-10; monocyte chemotactic protein (MCP)-1; interleukin (IL)-1, IL-2, IL-12, IL-15 and IL-17; and interferon (IFN)-γ during the early post-transplant period.[10,11] The increase in pro-inflammatory mediators is associated with the development of donor-specific human leukocyte antigen (HLA) alloimmunity.[12] Although there is increasing evidence for the role of circulating Abs in mediating allograft rejection post-HTx, the exact mechanisms by which the alloimmune response leads to rejection still remain unclear.[13,14]

A major limitation to long-term survival after cardiac transplantation is the development of chronic cardiac allograft vasculopathy (CAV).[12,15,16] Although recent advances in early post-operative management have significantly improved the 1-year post-HTx survival rate, long-term survival remains low at 43%, at 7 years.[17] Cardiac allograft vasculopathy is characterized by obliterative arteriosclerosis with chronic inflammation, medial thickening and concentric fibrous intimal hyperplasia.[12,16] It likely results from an initial injury to the allograft endothelium, which sets the stage for a chronic inflammatory state.[18] The key determinants contributing to the endothelial cell inflammation include both immunologic and non-immunologic factors. It has been suggested that an alloimmune response to mismatched donor major histocompatibility complex (MHC) antigens can break the tolerance to self-antigens.[19] Recent studies, both from our laboratory and others, have demonstrated that Abs to self-antigens develop after solid-organ transplantation.[20–22] Post-HTx patients have been shown to develop anti-phospholipid antibodies (Abs), anti-ribosomal Abs, anti-muscle protein Abs and anti–intracellular adhesion molecule-1 Abs.[23–26] However, the clinical relevance of Abs to donor-specific mismatched HLA and cardiac self-antigens in the development of AMR and CAV in HTx recipients has yet to be established.

In the present study, we evaluated the role of DSA to mismatched HLA and serum levels of Abs against two important cardiac self-antigens, myosin (MYO) and vimentin (VIM), in post-HTx patients who were diagnosed with AMR and CAV in the early and late post-operative period, respectively. In addition, we identified immune mechanisms that contribute to the induction of Abs to self-antigens in post-HTx patients. Our results highlight a significant association for Abs to both MYO and VIM in patients with AMR and CAV compared with patients without AMR and CAV, respectively. Importantly, serial monitoring of post-operative sera has indicated that detection of DSA precedes the detection of Abs to MYO and VIM by 1.7 $\pm$ 0.3 and 3.0 $\pm$ 0.4 months, respectively, suggesting a temporal relationship between DSA and development of Abs to self-antigens in post-HTx patients. Furthermore, the development of Abs to self-antigens was associated with induction of predominantly IL-5 secreting $CD4^+$ T cells and IL-17 secreting $CD4^+$ T cells against corresponding self-antigens in patients with AMR and CAV, respectively. This was in parallel to a concomitant loss of IL-10 secreting $CD4^+$ T cells in patients with AMR and CAV, indicating a breakdown of peripheral tolerance to self-antigens.

Methods

Study population

One hundred forty-eight HTx patients at Barnes–Jewish Hospital/Washington University were prospectively enrolled in the study in accordance with a protocol approved by the institutional review board. Serum and peripheral blood lymphocytes were isolated from whole blood as described previously[27,28] and stored at -135°C. Concurrent with blood sample collection, endomyocardial biopsies were performed in 65 patients in the early ($<$12 months) post-HTx period and coronary angiograms were performed in 83 patients in the late ($>$12 months) post-HTx period. A diagnosis of AMR was made based on ISHLT recommended guidelines on clinical, histologic and serologic criteria. The features evaluated to reach a clinical diagnosis included patient symptoms (fatigue, palpitations), echocardiographic findings of cardiac dysfunction (decreased ejection fraction, restrictive physiology), need for inotropic support, histologic evidence (acute capillary injury manifest by capillary endothelial swelling, macrophages or neutrophils in capillaries, absence of features consistent with cellular rejection), immunopathologic evidence (C4d capillary staining, CD68 positivity for capillary macrophages) and serologic evidence of donor-specific HLA Ab. Treatment was initiated within 24 hours of reaching a diagnosis of AMR by the attending transplant cardiologist. Our institutional preference for initial treatment of AMR includes plasmapheresis, intravenous immunoglobulin (IVIg) and intravenous methylprednisone. Secondary therapy includes use of plasmapheresis, rituximab, IVIg and methylprednisone. Cardiac allograft vasculopathy was diagnosed based on angiographic criteria. Angiographic evidence of coronary artery stenosis ($>$50% luminal diameter) of one vessel or less was termed none/minimal, whereas coronary artery stenosis ($>$50% luminal diameter) of two vessels or more was termed moderate/severe.

Detection of Abs to HLA

The presence of DSA in sera was identified using a solid-phase assay by Luminex technology (Biosource International). In brief, primary Ab-coated beads and incubation buffer were placed into 96-well filter plates. Both samples and standards were incubated with the primary Ab beads at room temperature on an orbital shaker. The wells were then washed and biotinylated secondary Abs were added for a further incubation period of 30 minutes. The wells were washed again and strepatividin–R-phyocoerythrin solution was added and incubated for 15 minutes. The wells were then washed and data were read utilizing a dual-laser flow analyzer (Luminex-100 System, v1.7; Biosource International). Data analysis was then performed (MasterPlex QT 1.0 System; MiraiBio) and a 5-parameter regression formula was utilized to allow detection compared with standard curves.

Detection of Abs to self-antigens MYO and VIM

The sera were tested for the development of Abs to MYO and VIM by an enzyme-linked immunosorbent assay (ELISA) developed in our laboratory. In brief, 96-well plates (Nunc) were coated with 1 μg/ml of commercially available porcine MYO or recombinant purified VIM in phosphate-buffered saline (PBS) overnight at 4°C. The antigen-coated wells were blocked for non-specific binding with 1% bovine serum albumin for 2 hours. Sera from post-HTx patients and normal volunteers were tested at two dilutions (1:750 and 1:1,500) for the presence of Abs against MYO and VIM. Commercially available anti-MYO and anti-VIM were used as positive controls. Specific binding was detected with anti-human IgG, IgM bound to horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratory) and developed with tetramethylbenzidine substrate (Millipore). Immunosorbance was detected at 460 nm. Concentration of Abs was calculated based on a standard curve using the binding of a known concentration of commercial anti-MYO and anti-VIM Abs (Santa Cruz Biotechnology). A 2-standard-deviation-from-the-mean concentration of Abs to MYO and VIM in health control subjects was used as cut-off to determine positive titers of auto-Abs in experimental samples.

Antigen stimulation

Freshly isolated peripheral blood mononuclear cells (PBMC) were stimulated with individual antigens (20 μg/ml) in 24-well culture plates (Fisher Scientific) resuspended in complete RPMI-1640 (Gibco) supplemented with 10% heat-inactivated AB negative human sera, 2 mmol/liter L-glutamine, 20 mmol/liter HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin for 3 to 5 days in a humidified 5% CO_2 incubator at 37°C. $CD4^+$ T cells were then purified from stimulated PBMC using immunomagnetic separation cocktails by negative selection (Stem Cell Technologies). The purity of $CD4^+$ T cells obtained by this method was >95% (data not shown).

ELISpot

$CD4^+$ T cells purified from PBMC cultures stimulated with individual antigens were utilized for performing IFN-γ, IL-5, IL-10 and IL-17 ELISpot assays as described in our earlier publications.[10,11,22] In brief, a multi-screen 96-well filtration plate (Millipore) was used to coat with monoclonal Ab to IFN-γ, IL-5, IL-10 and IL-17 (5 μg/ml; BD Biosciences). The plates were blocked with 5% bovine serum albumin (BSA) for 1 hour and washed three times with phosphate-buffered saline (PBS). $CD4^+$ T cells (3×10^5) were cultured in triplicate in the presence of either MYO (20 μg/ml) or VIM (20 μg/ml) and autologous irradiated CD4-depleted PBMC as antigen-presenting cells (APCs, 1:1) in complete RPMI-1640 medium in a humidified 5% CO_2 incubator at 37°C. After 72 hours, the plates were washed three times with PBS and then with 0.05% PBS/Tween-20 three times. We then added 3 μg/ml of biotinylated monoclonal Abs specific for IFN-γ, IL-5, IL-10 and IL-17 (BD Biosciences) resuspended in PBS/BSA/Tween-20. After 12 hours at 4°C, the plates were washed three times and HRP-labeled streptavidin (1:2,000; Pharmigen) was added for 2 hours at room temperature. The spots were developed using amino-9-ethylcarbazole solution (Pharmigen). Spots were then analyzed (ImmunoSpot Series 1; Cellular Technology) and the results reported as spots per million cells (spm). $CD4^+$ T cells cultured alone in medium with antigen-presenting cells were used as a negative control. $CD4^+$ T cells cultured in the presence of phytohemagglutinin (PHA, 5 μg/ml) were used as positive control. The number of spots observed in the negative control was subtracted from the number of spots observed in experimental wells.

Statistical correlation

GraphPad Prism v4.03 software was used to analyze the data. The Mann–Whitney *U*-test was used to determine the differences in $CD4^+$ T-cell responses specific to individual antigens between the two groups. The correlation analysis was done using Spearman's rank test. Two-sided $p < 0.05$ was considered statistically significant.

Results

Patient demographics

The study cohort consists of 148 patients who underwent HTx and their demographics are given in Table 1. Mean age at transplant was 51.5 ± 3.5. One hundred nineteen patients (81%) were male; 122 patients (82%) were Caucasian, 21 (14%) were African-American and 5 (4%) were classified as "other." The indication for HTx included ischemic cardiomyopathy and dilated cardiomyopathy. Mean (± standard deviation) follow-up after HTx was 3.2 ± 2.7 years and the median follow-up was 3.5 years. Of 148 patients, 65 patients were followed for development of acute AMR in the early post-HTx period (<12 months), whereas 83 patients were followed for development of CAV in the late post-HTx period (>12 months). Ten patients had diagnostic

Table 1 Patients' Demographics

	Early HTx (n = 65)			Late HTx (n = 83)		
	AMR (n = 10)	No AMR (n = 55)	p-value	CAV (n = 14)	No CAV (n = 69)	p-value
Age at HTx (years)	49.9 ± 13.4	52.3 ± 11.8	0.25	57.6 ± 10.3	55.2 ± 9.5	0.32
Gender (M:F)	7:3	44:11	0.18	11:3	57:12	0.29
Ethnicity						
Caucasian	8 (80%)	47 (85%)	0.12	11 (79%)	56 (81%)	0.67
African American	2 (20%)	6 (11%)	0.17	2 (14%)	11 (16%)	0.35
Other	0 (0%)	2 (4%)	1.0	1 (7%)	2 (3%)	0.43
LVAD prior to HTx	2 (20%)	10 (18%)	0.71	3 (21%)	17 (25%)	0.12
Mean follow-up time (years)	1.7 ± 0.9	1.6 ± 0.7	0.55	8.9 ± 4.9	10.1 ± 6.4	0.31

Values presented as mean ± SD or n (%). Total number of patients = 148.

criteria consistent with acute AMR (AMR^+) and 14 patients had angiographic evidence of moderate or severe CAV (CAV^+). Although patients with active infection at time of study enrollment met the exclusion criteria, 8 patients developed systemic infections (4 with bacterial and 2 with viral) after study enrollment.

Abs to self-antigens MYO and VIM are significantly increased in post-HTx recipients with AMR compared with stable cardiac transplant patients

Using ELISA, we determined the development of Abs in the sera to the cardiac self-antigens, MYO and VIM in post-HTx patients during the early post-operative period (≤12 months). As shown in Figure 1A, patients with AMR developed Abs to MYO (control: 87 ± 66 μg/ml; AMR^-: 144 ± 115 μg/ml; AMR^+: 285 ± 70 μg/ml) at significantly higher titers compared with stable cardiac transplant patients without AMR (p = 0.033). Antibodies to MYO were present 3.5 ± 0.0 months prior to the diagnosis of acute AMR. Furthermore, detection of DSA in patients with AMR preceded the detection of anti-MYO Abs by 1.7 ± 0.3 months (see Figure 5). Examination of patient sera for the presence of Abs to VIM, as seen in Figure 1B (control: 26 ± 16 μg/ml; AMR^-: 37 ± 19 μg/ml; AMR^+: 103 ± 43 μg/ml), also revealed a statistically significant increase in Abs to VIM in patients with AMR (p = 0.014). Antibodies to VIM were present 2.1 ± 0.2 months prior to the diagnosis of acute AMR. Furthermore, detection of DSA in patients with AMR preceded the detection of anti-VIM Abs by 3.0 ± 0.4 months (see Figure 5). Thus, DSA followed by Abs to self-antigens, MYO and VIM precedes the diagnosis of AMR in post-HTx patients.

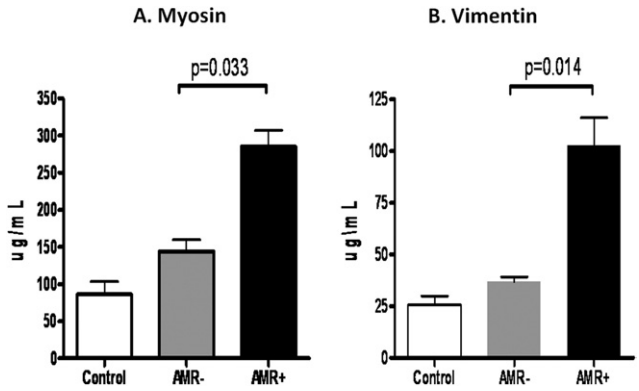

Figure 1 (A) Patients with AMR develop Abs to MYO at significantly higher titers when compared with stable cardiac transplant patients without AMR. (B) Patients with AMR develop Abs to VIM at significantly higher titers compared with stable cardiac transplant patients without AMR.

Increased frequencies of IL-5 and IFN-γ and decreased frequencies of IL-10 secreting $CD4^+$ T cells specific to MYO and VIM in post-HTx recipients with AMR

To identify the immune responses that contribute to the development of Abs to self-antigens, MYO and VIM in post-HTx patients, the frequency of $CD4^+$ T cells secreting IFN-γ, IL-5, IL-17 and IL-10 specific to either MYO and VIM were determined using ELISpot. As presented in Figure 2A and B, patients with AMR demonstrated increased frequencies of IL-5 and IFN-γ against both MYO (IFN-γ: AMR^- 17 ± 6 spm, AMR^+ 46 ± 24 spm, p = 0.008; IL-5: AMR^- 25 ± 12 spm, AMR^+ 120 ± 41 spm, p = 0.003) and VIM (IFN-γ: AMR^- 12 ± 8 spm, AMR^+ 40 ± 25 spm, p = 0.03; IL-5: AMR^- 15 ± 10 spm, AMR^+ 80 ± 29 spm, p = 0.004). Concurrently, patients with AMR demonstrated decreased frequencies of IL-10–secreting $CD4^+$ T cells specific to MYO (AMR^-: 440 ± 105 spm; AMR^+: 220 ± 89 spm; p = 0.009) and VIM (AMR^-: 320 ± 124 spm; AMR^+: 200 ± 100 spm; p = 0.03) compared with patients without AMR. However, no significant difference was noted in the frequencies of $CD4^+$ T cells secreting IL-17 specific to MYO (AMR^-: 35 ± 27 spm; AMR^+: 40 ± 35 spm; p = 0.35) and VIM (AMR^-: 21 ± 15 spm; AMR^+: 25 ± 20 spm; p = 0.29) between AMR^- and AMR^+ post-HTx patients. These results indicate that patients with AMR demonstrate high frequencies of $CD4^+$ T-helper cells specific to self-antigens, which predominantly secrete the Th2 cytokine, IL-5. Therefore, the induction of IL-5 by self-reactive $CD4^+$ T cells in patients with AMR may play an important role in the activation and differentiation of B cells involved in autoantibody production.

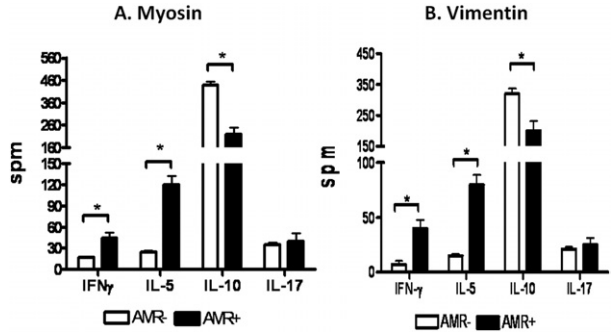

Figure 2 (A) Patients with AMR demonstrated increased frequencies of IL-5 and IFN-γ and decreased frequency of IL-10 secreting $CD4^+$ T cells specific to MYO when compared with patients without AMR. There was no significant difference in the frequency of IL-17 secreting $CD4^+$ T cells specific to MYO when comparing the two cohorts. (B) Patients with AMR demonstrated increased frequencies of IL-5 and IFN-γ and decreased frequency of IL-10 secreting $CD4^+$ T cells specific to VIM when compared with patients without AMR. There was no significant difference in the frequency of IL-17 secreting $CD4^+$ T cells specific to VIM when comparing the two cohorts.

Increased levels of Abs to MYO and VIM are significantly associated with presence of DSA in post-HTx patients with AMR compared with post-HTx patients without AMR

Table 2 depicts the features of AMR and self-antigen profile in all 10 recipients who were AMR-positive in our study. As indicated in Table 3, patients with AMR who tested positive for DSA (DSA^+AMR^+) had increased levels of Abs to MYO (DSA^-AMR^+: 174 ± 65 μg/ml; DSA^+AMR^+: 332 ± 34 μg/ml; $p = 0.03$) and VIM (DSA^-AMR^+: 48 ± 10 μg/ml; DSA^+AMR^+: 126 ± 21 μg/ml; $p = 0.003$) compared with patients diagnosed with AMR without DSA (DSA^-AMR^+). No significant difference in the levels of Abs to MYO (DSA^-AMR^-: 142 ± 121 μg/ml; DSA^+AMR^-: 155 ± 88 μg/ml; $p = 0.92$) and VIM (DSA^-AMR^-: 35 ± 18 μg/ml; DSA^+AMR^-: 45 ± 23 μg/ml; $p = 0.89$) were identified between patients without AMR who tested either positive (DSA^+AMR^-) or negative (DSA^-AMR^-) for DSA. These results strongly suggest that DSA to mismatched HLA are significantly associated with and can play an important role in the development of Abs to self-antigens in patients with AMR after HTx.

Abs to self-antigens MYO and VIM are significantly increased in post-HTx recipients with CAV compared with stable cardiac transplant patients

Using ELISA, we determined the development of Abs in the sera to cardiac self-antigens, MYO and VIM in post-HTx patients. As shown in Figure 3A and B, patients with CAV develop Abs to MYO (control: 87 ± 66 μg/ml; CAV^-: 191 ± 120 μg/ml; CAV^+: 550 ± 98 μg/ml; $p = 0.025$) and VIM (control: 26 ± 16 μg/ml; CAV^- 55 ± 25 μg/ml; CAV^+: 255 ± 49; $p = 0.001$), significantly higher compared with stable cardiac transplant patients without CAV. These results indicate that patients with CAV develop a significant increase in the levels of Abs to MYO and VIM compared with stable post-HTx recipients without CAV.

Increased frequencies of IL-17 and decreased frequencies of IL-10 secreting $CD4^+$ T cells specific to MYO and VIM in post-HTx recipients with CAV

To identify the immune responses that contribute to the development of Abs to self-antigens MYO and VIM in post-HTx patients with CAV, the frequency of $CD4^+$ T cells secreting IFN-γ, IL-5, IL-17 and IL-10 specific to either MYO and VIM were determined using ELISpot. As presented in Figure 4A and B, patients with CAV demonstrated increased frequencies of IL-17 secreting $CD4^+$ T cells against both MYO (CAV^-: 25 ± 20 spm; CAV^+: 115 ± 26 spm; $p = 0.002$) and VIM (CAV^-: 20 ± 13 spm; CAV^+: 70 ± 34 spm; $p = 0.003$). Concurrently, patients with CAV demonstrated decreased frequencies of IL-10 secreting $CD4^+$ T cells specific to MYO (CAV^-: 420 ± 120 spm; CAV^+: 220 ± 89 spm; $p = 0.008$) and VIM (CAV^-:500 ± 220 spm; CAV^+: 260 ± 130 spm; $p = 0.005$) compared with patients without CAV. However, no significant difference was noted in the frequencies of $CD4^+$ T cells secreting IFN-γ and IL-5 specific to MYO (IFN-γ:

Table 2 Characteristics of AMR^+ Patients

Pt	Symptoms	Abnormal echocardiography	Inotropes	Histology	Immunopathology		DSA	Anti-MYO	Anti-VIM
					CD4	CD68			
1	+	+	+	+	+	−	+	+	+
2	+	+	−	+	+	+	+	+	+
3	−	+	+	+	+	+	−	+	−
4	+	+	+	+	−	+	−	+	+
5	+	+	+	−	+	+	+	−	−
6	−	+	+	−	−	+	+	+	+
7	−	+	+	+	+	+	+	+	+
8	+	+	+	+	+	−	+	+	+
9	+	−	+	+	+	+	−	+	+
10	−	+	+	+	−	+	+	+	+

Pt, patient.

Table 3 Increased Levels of Abs to Myosin and Vimentin are Significantly Associated With Development of DSA in AMR After HTx

	DSA^+	DSA^-	*P*-value
Myosin			
AMR^+	332 ± 34 (n = 7)	174 ± 65 (n = 3)	0.03
AMR^-	155 ± 88 (n = 10)	142 ± 121 (n = 45)	0.92
Vimentin			
AMR^+	126 ± 21 (n = 7)	48 ± 10 (n = 3)	0.003
AMR^-	45 ± 23 (n = 10)	35 ± 18 (n = 45)	0.89

CAV^-:25 ± 15 spm; CAV^+: 45 ± 24 spm; $p = 0.55$; IL-5: CAV^-: 24 ± 19 spm; CAV^+: 34 ± 25 spm; $p = 0.54$) and VIM (IFN-γ: CAV^-: 17 ± 12 spm; CAV^+: 38 ± 17 spm; $p = 0.64$; IL-5: CAV^-: 25 ± 20 spm, CAV^+: 30 ± 18 spm; $p = 0.24$) between CAV^- and CAV^+ post-HTx patients. These results indicate that patients with CAV demonstrate high frequencies of $CD4^+$ T-helper cells specific to self-antigens, which predominantly secrete the Th17 cytokine, IL-17. Therefore, the induction of IL-17 by self-reactive $CD4^+$ T cells in patients with CAV may play an important role in the facilitation of autoantibody development by driving neo-germinal center formation.

Increased levels of Abs to MYO and VIM are significantly associated with presence of DSA in post-HTx patients with CAV compared with post-HTx patients without CAV

As indicated in Table 4, patients with CAV who tested positive for DSA (DSA^+CAV^+) had increased levels of Abs to MYO (DSA^-CAV^+: 357 ± 38 μg/ml; DSA^+CAV^+: 603 ± 84 μg/ml; $p = 0.001$) and VIM (DSA^-CAV^+: 173 ± 29 μg/ml; DSA^+CAV^+: 277 ± 48 μg/ml; $p = 0.02$) compared with patients diagnosed with CAV without DSA (DSA^-CAV^+). No significant difference in the levels of Abs to MYO (DSA^-CAV^-: 180 ± 86 μg/ml; DSA^+CAV^-: 224 ± 190 μg/ml; $p = 0.63$) and VIM (DSA^-CAV^-: 51 ± 49 μg/ml; DSA^+CAV^-: 67 ± 56 μg/ml; $p = 0.78$) were identified between patients without CAV who tested either positive (DSA^+CAV^-) or negative (DSA^-CAV^-) for DSA. These results indicate that DSA to mismatched HLA are significantly associated with and can play an important role in the development of Abs to self-antigens in patients with CAV after HTx.

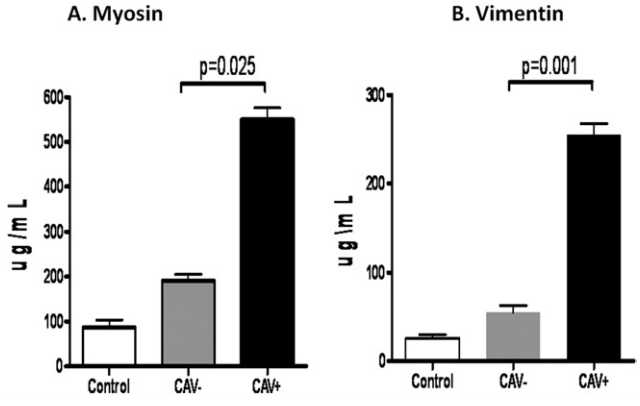

Figure 3 (A) Patients with CAV develop Abs to MYO at significantly higher titers compared with stable cardiac transplant patients without CAV. (B) Patients with CAV develop Abs to VIM at significantly higher titers compared with stable cardiac transplant patients without CAV.

Discussion

Cardiac allograft dysfunction contributes to nearly 25% of the mortality within 10 years after transplantation.[16,29] During the early transplant period (<12 months), acute rejection is a major contributor of allograft dysfunction in HTx recipients and the frequency of acute rejection episodes generally tend to decline after the first year post-HTx.[9,17] Conversely, the incidence of cardiac allograft atherosclerosis in the form of vasculopathy is increased significantly after the first year and accounts for allograft dysfunction during the late HTx period (>12 months).[16,29] It has been

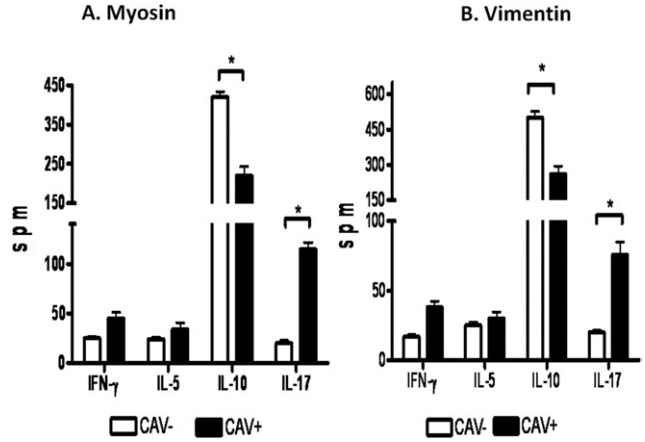

Figure 4 (A) Patients with CAV demonstrated increased frequency of IL-17 and decreased frequency of IL-10 secreting $CD4^+$ T cells specific to MYO when compared with patients without CAV. There was no significant difference in the frequencies of IFN-γ and IL-5 secreting $CD4^+$ T cells specific to MYO when comparing the two cohorts. (B) Patients with CAV demonstrated increased frequency of IL-17 and decreased frequency of IL-10 secreting $CD4^+$ T cells specific to VIM when compared with patients without CAV. There was no significant difference in the frequencies of IFN-γ and IL-5 secreting $CD4^+$ T cells specific to VIM when comparing the two cohorts.

Table 4 Increased Levels of Abs to Myosin and Vimentin Are Significantly Associated With the Development of DSA in Patients With CAV After HTx

	DSA^+	DSA^-	*p*-value
Myosin			
CAV^+	603 ± 84 (n = 11)	357 ± 38 (n = 3)	0.001
CAV^-	224 ± 190 (n = 17)	180 ± 86 (n = 52)	0.63
Vimentin			
CAV^+	277 ± 48 (n = 11)	173 ± 29 (n = 3)	0.02
CAV^-	67 ± 56 (n = 17)	51 ± 49 (n = 52)	0.78

estimated that 30% to 50% of cardiac transplant recipients will demonstrate histologic evidence of cardiac allograft vasculopathy by the end of 5 years post-HTx.[29] The risk factors that promote allograft dysfunction leading to AMR and CAV in HTx recipients include circulating DSA to HLA and non-HLA antigens, such as ABO and cardiac self-antigens.[7,9,12] Studies have demonstrated that recipient T cells recognize cardiac myosin presented in the context of recipient MHC Class II molecules and can mediate rejection of cardiac allografts, in the absence of an alloimmune response.[30] In this study, we sought to address the role of DSA to mismatched HLA and their association with Abs to self-antigens MYO and VIM in the pathogenesis of acute AMR in the early post-HTx period and CAV in late post-HTx period. To determine the mechanism contributing to the development of immune response to self-antigens, we also assessed the frequency of T-cell–mediated responses to self-antigens, MYO and VIM as well as their cytokine profiles.

We enrolled 148 patients who underwent HTx. Sixty-five of the patients were followed in the early (<12 months) period for development of acute AMR and 83 patients were followed in the late (>12 months) period for development of CAV. Of these, 10 patients fulfilled the ISHLT criteria for diagnosis of acute AMR. The mean time to diagnosis of acute AMR in these patients was 7.5 ± 0.9 months. There was no significant difference in the clinical and demographic characteristics of patients with AMR and without AMR, including age, gender, ethnicity, mean follow-up time, number of acute rejection episodes, dose of pulse steroids used for treating acute rejection, and maintenance immunosuppressive regimen used. Of the 10 patients who were diagnosed with AMR, 7 (70%) tested positive for DSA to mismatched HLA.

It has been suggested that Abs to donor-specific HLA antigens mediate allograft injury during episodes of acute rejection. Specifically, the binding of anti-HLA Abs to the graft results in activation of complement cascade and endothelial cell activation accompanied by neutrophil and CD68 macrophage infiltration.[31–33] Such an inflammatory milieu is conducive for autoimmunity to self-antigens. Furthermore, anti–MHC Class I Abs have been demonstrated to directly induce the development of autoimmunity to self-antigens such as Col-V and K-alpha-1-tubulin after human lung transplantation.[21,22,34,35] In this context, we monitored the levels of Abs to cardiac antigens, MYO and VIM in post-HTx patients with and without evidence of AMR. In contrast to control subjects and post-HTx patients without AMR, there was a significant increase in the serum levels of Abs to MYO and VIM (Figure 1A and B). Interestingly, patients with AMR who tested positive for DSA (DSA^+AMR^+) had increased levels of Abs to MYO and VIM compared with patients diagnosed with AMR without DSA (DSA^-AMR^+) (Table 3). This indicates that DSA is significantly associated with the development of Abs to self-antigens. Serial monitoring of DSA Abs to MYO and VIM in the early post-HTx period in patients who were DSA^+ indicated that DSA was detectable by 2.8 ± 1.0 months, followed by MYO by 4.5 ± 1.3 months, and VIM by 5.8 ± 1.5 months in patients diagnosed with AMR at 8.0 ± 1.3 months (Figure 5).

To determine the immune mechanisms leading to the development of Abs to cardiac self-antigens, we determined the frequencies of $CD4^+$ T-helper cells specific to MYO and VIM secreting IFN-γ, IL-5, IL-17 and IL-10. An increased frequency of $CD4^+$ T cells that predominantly secrete IL-5 followed by IFN-γ specifically against MYO and VIM were noted in patients with AMR. A significant decline in the frequency of IL-10 secreting $CD4^+$ T cells was also noted in patients with AMR. This strongly suggests that the loss of IL-10 secreting $CD4^+$ T cells specific to cardiac self-antigens may result in the breakdown of peripheral tolerance accompanied by the development of both IL-5 and IFN-γ–dependent $CD4^+$ T-cell–dependent immune responses specific to the individual antigens in post-HTx patients

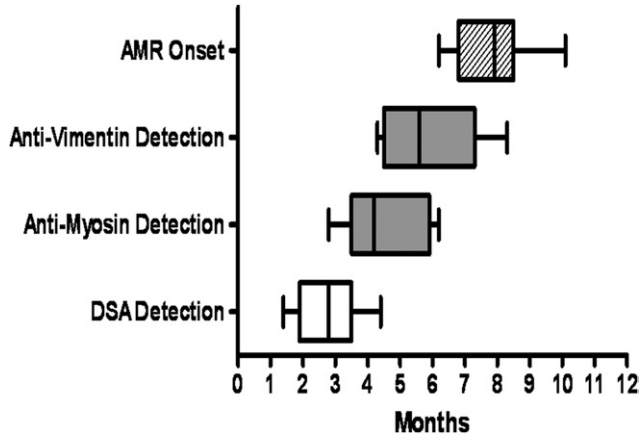

Figure 5 Serial monitoring of DSA Abs to MYO and VIM in the early post-HTx period in 7 DSA^+ patients indicates that DSA was detectable at 2.8 ± 1.0 months, followed by MYO at 4.5 ± 1.3 months and VIM at 5.8 ± 1.5 months in patients diagnosed with AMR at 8.0 ± 1.3 months.

with AMR. These results are in agreement with other reports indicating that IL-5 is a critical cytokine involved in the induction and augmentation of B-cell–dependent autoantibody responses.[36–38] Furthermore, IFN-γ–dependent immune responses to self-antigens play an important role in the pathogenesis of de novo autoimmunity after heart transplantation.[38]

In this study we also analyzed patients for the development of CAV based on the angiographic evidence of patients in the follow-up period. We determined the levels of Abs to cardiac antigens MYO and VIM in post-HTx patients with and without evidence of CAV. In contrast to control subjects and post-HTx patients without CAV, there was a significant increase in the serum levels of Abs to MYO and VIM in patients with CAV (Figure 3A and B). We also determined $CD4^+$ T-cell responses to MYO and VIM by analyzing the frequencies of $CD4^+$ T cells that secrete IFN-γ, IL-5, IL-17 or IL-10 in patients with CAV. An increased frequency of $CD4^+$ T cells that predominantly secrete IL-17 specifically against MYO and VIM were noted in patients with CAV (Figure 4A and B). A significant decline in the frequency of IL-10 secreting $CD4^+$ T cells, indicating the breakdown of peripheral tolerance, was also noted in patients with CAV (Figure 4A and B). The development of predominantly IL-17–dependent $CD4^+$ T-cell responses to cardiac self-antigens in post-HTx patients with CAV is in agreement with studies indicating an important role for IL-17 in the development of cardiac allograft vasculopathy and other autoimmune diseases in humans.[21,39,40] These findings are supported by recent observations that, in the absence of Th1-mediated responses, CD4 Th17 responses mediate accelerated pro-inflammatory responses leading to allograft rejection and CAV.[40]

Investigators have demonstrated that, in the post-transplant period, higher anti-myosin IgG levels were detected in sera collected during acute rejection than in sera collected during the rejection-free period and that, in absence of an alloimmune response, sensitization of recipient mice with MYO is sufficient to induce rejection of cardiac allografts.[30,41,42] The role of antibodies to vimentin leading to accelerated cardiac rejection has also been demonstrated in a recent study using a mouse model.[43] Furthermore, imaging studies have suggested that anti-MYO Abs could be used as a predictor of transplant survival.[44] Although the role of anti-MYO and anti-VIM Abs in the development of rejection (mostly chronic) after HTx have been demonstrated in studies with small patient cohorts, we have demonstrated these findings in a large group of human heart transplant patients who developed acute AMR and CAV, with a concomitant immune response to the self-antigens MYO and VIM in the early and late post-HTx period, respectively.[42,45] Previous evidence in conjunction with our study findings lead us to strongly suggest that autoimmune-mediated responses play a critical role in the development of chronic rejection post-HTx. Recently, IL-5 and IL-17 have been proposed to mediate key pro-inflammatory responses in the pathogenesis of autoimmune responses.[46,47] We have shown an increased frequency of T lymphocytes producing IL-5 and IL-17 in the peripheral blood lymphocytes of post-transplant patients with acute AMR and CAV. IL-17, in particular, induces the production of numerous cytokines (such as IL-6, G-CSF, GM-CSF, IL-1β, TGF-β and TNF-α), chemokines (including IL-8, GRO-α and MCP-1) and prostaglandins (e.g., PGE_2) from many cell types (fibroblasts, endothelial cells, epithelial cells, keratinocytes and macrophages).[48]

In our study, the presence of increased levels of IL-5 and IL-17 demonstrates the role of autoimmunity in the pathogenesis of acute AMR and CAV, respectively. We have provided evidence strongly suggesting that both alloimmune responses as well as autoimmune responses play an important role in the processes of AMR and CAV in post-HTx patients. Our results also suggest cross-talk between DSA and autoimmunity mediated by IL-17 in the pathogenesis of CAV. Therefore, IL-17 pathway–mediated autoimmunity to self-antigens can represent an important therapeutic option in the management of CAV. Future studies examining the mechanistic basis and signaling pathways involved in this pathogenesis are warranted.

Disclosure statement

The first two authors (D.S.N. and H.I.B.) contributed equally to this study. The authors thank Deepti Saini, PhD, for technical support.

This investigation was supported by the National Institutes of Health (NIH Training Grant T32 HL07776 to D.S.N).

None of the authors have any conflicts of interest to disclose.

References

1. Kfoury AG, Hammond ME. Controversies in defining cardiac antibody-mediated rejection: Need for updated criteria. J Heart Lung Transplant 2010;29:389-94.
2. Michaels PJ, Fishbein MC, Colvin RB. Humoral rejection of human organ transplants. Springer Semin Immunopathol 2003;25:119-40.
3. Kfoury AG, Hammond ME, Snow GL, et al. Early screening for antibody-mediated rejection in heart transplant recipients. J Heart Lung Transplant 2007;26:1264-9.
4. Almuti K, Haythe J, Dwyer E, et al. The changing pattern of humoral rejection in cardiac transplant recipients. Transplantation 2007;84:498-503.
5. Fishbein MC, Kobashigawa J. Biopsy-negative cardiac transplant rejection: etiology, diagnosis, and therapy. Curr Opin Cardiol 2004;19:166-9.
6. Wu GW, Kobashigawa JA, Fishbein MC, et al. Asymptomatic antibody-mediated rejection after heart transplantation predicts poor outcomes. J Heart Lung Transplant 2009;28:417-22.
7. Reed EF, Demetris AJ, Hammond E, et al. Acute antibody-mediated rejection of cardiac transplants. J Heart Lung Transplant 2006;25:153-9.
8. Uber WE, Self SE, Van Bakel AB, et al. Acute antibody-mediated rejection following heart transplantation. Am J Transplant 2007;7:2064-74.

9. Stewart S, Winters GL, Fishbein MC, et al. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. J Heart Lung Transplant 2005;24:1710-20.
10. Bharat A, Kuo E, Steward N, et al. Immunological link between primary graft dysfunction and chronic lung allograft rejection. Ann Thorac Surg 2008;86:189-95.
11. Bharat A, Narayanan K, Street T, et al. Early posttransplant inflammation promotes the development of alloimmunity and chronic human lung allograft rejection. Transplantation 2007;83:150-8.
12. Kobashigawa JA. Cardiac allograft vasculopathy in heart transplant patients: pathologic and clinical aspects for angioplasty/stenting. J Am Coll Cardiol 2006;48:462-3.
13. Hornick PI, Mason PD, Yacoub MH, et al. Assessment of the contribution that direct allorecognition makes to the progression of chronic cardiac transplant rejection in humans. Circulation 1998;97:1257-63.
14. Baker RJ, Hernandez-Fuentes MP, Brookes PA, et al. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. J Immunol 2001;167:7199-206.
15. Uretsky BF, Murali S, Reddy PS, et al. Development of coronary artery disease in cardiac transplant patients receiving immunosuppressive therapy with cyclosporine and prednisone. Circulation 1987;76: 827-34.
16. Ramzy D, Rao V, Brahm J, et al. Cardiac allograft vasculopathy: a review. Can J Surg 2005;48:319-27.
17. Taylor DO, Edwards LB, Boucek MM, et al. The Registry of the International Society for Heart and Lung Transplantation: twenty-first official adult heart transplant report—2004. J Heart Lung Transplant 2004;23:796-803.
18. Davis SF, Yeung AC, Meredith IT, et al. Early endothelial dysfunction predicts the development of transplant coronary artery disease at 1 year posttransplant. Circulation 1996;93:457-62.
19. Fedoseyeva EV, Tam RC, Popov IA, et al. Induction of T cell responses to a self-antigen following allotransplantation. Transplantation 1996;61:679-83.
20. Haque MA, Mizobuchi T, Yasufuku K, et al. Evidence for immune responses to a self-antigen in lung transplantation: role of type V collagen-specific T cells in the pathogenesis of lung allograft rejection. J Immunol 2002;169:1542-9.
21. Fukami N, Ramachandran S, Saini D, et al. Antibodies to MHC class I induce autoimmunity: role in the pathogenesis of chronic rejection. J Immunol 2009;182:309-18.
22. Goers TA, Ramachandran S, Aloush A, et al. De novo production of K-alpha1 tubulin-specific antibodies: role in chronic lung allograft rejection. J Immunol 2008;180:4487-94.
23. Dunn MJ, Rose ML, Latif N, et al. Demonstration by western blotting of antiheart antibodies before and after cardiac transplantation. Transplantation 1991;51:806-12.
24. Linke AT, Marchant B, Marsh P, et al. Screening of a HUVEC cDNA library with transplant-associated coronary artery disease sera identifies RPL7 as a candidate autoantigen associated with this disease. Clin Exp Immunol 2001;126:173-9.
25. Laguens RP, Argel MI, Chambo JG, et al. Anti-skeletal muscle glycolipid antibodies in human heart transplantation as markers of acute rejection. Correlation with endomyocardial biopsy. Transplantation 1996;62:211-6.
26. Lawson C, Holder AL, Stanford RE, et al. Anti-intercellular adhesion molecule-1 antibodies in sera of heart transplant recipients: a role in endothelial cell activation. Transplantation 2005;80:264-71.
27. Golocheikine A, Nath DS, Basha HI, et al. Increased erythrocyte C4D is associated with known alloantibody and autoantibody markers of antibody-mediated rejection in human lung transplant recipients. J Heart Lung Transplant 2010;29:410-6.
28. Fields RC, Bharat A, Steward N, et al. Elevated soluble CD30 correlates with development of bronchiolitis obliterans syndrome following lung transplantation. Transplantation 2006;82:1596-601.
29. Dhaliwal A, Thohan V. Cardiac allograft vasculopathy: the Achilles' heel of long-term survival after cardiac transplantation. Curr Atheroscler Rep 2006;8:119-30.
30. Fedoseyeva EV, Kishimoto K, Rolls HK, et al. Modulation of tissue-specific immune response to cardiac myosin can prolong survival of allogeneic heart transplants. J Immunol 2002;169:1168-74.
31. Jindra PT, Hsueh A, Hong L, et al. Anti-MHC class I antibody activation of proliferation and survival signaling in murine cardiac allografts. J Immunol 2008;180:2214-24.
32. Horne PH, Zimmerer JM, Fisher MG, et al. Critical role of effector macrophages in mediating CD4-dependent alloimmune injury of transplanted liver parenchymal cells. J Immunol 2008;181:1224-31.
33. Wasowska BA, Lee CY, Halushka MK, et al. New concepts of complement in allorecognition and graft rejection. Cell Immunol 2007; 248:18-30.
34. Braun RK, Molitor-Dart M, Wigfield C, et al. Transfer of tolerance to collagen type V suppresses T-helper-cell-17 lymphocyte-mediated acute lung transplant rejection. Transplantation 2009;88:1341-8.
35. Yamada Y, Sekine Y, Yoshida S, et al. Type V collagen-induced oral tolerance plus low-dose cyclosporine prevents rejection of MHC class I and II incompatible lung allografts. J Immunol 2009;183:237-45.
36. Herron LR, Coffman RL, Bond MW, et al. Increased autoantibody production by NZB/NZW B cells in response to IL-5. J Immunol 1988;141:842-8.
37. Hsu LW, Goto S, Lin YC, et al. Prolongation of heart allograft survival of rats treated by a Th2 inhibitor. Transplant Immunol 2003;11:385-8.
38. Braun MY, Desalle F, Le Moine A, et al. IL-5 and eosinophils mediate the rejection of fully histoincompatible vascularized cardiac allografts: regulatory role of alloreactive CD8(+) T lymphocytes and IFN-gamma. Eur J Immunol 2000;30:1290-6.
39. Burlingham WJ, Love RB, Jankowska-Gan E, et al. IL-17-dependent cellular immunity to collagen type V predisposes to obliterative bronchiolitis in human lung transplants. J Clin Invest 2007;117:3498-506.
40. Yuan X, Paez-Cortez J, Schmitt-Knosalla I, et al. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. J Exp Med 2008;205:3133-44.
41. Fedoseyeva EV, Zhang F, Orr PL, et al. De novo autoimmunity to cardiac myosin after heart transplantation and its contribution to the rejection process. J Immunol 1999;162:6836-42.
42. Morgun A, Shulzhenko N, Unterkircher CS, et al. Pre- and post-transplant anti-myosin and anti-heat shock protein antibodies and cardiac transplant outcome. J Heart Lung Transplant 2004;23:204-9.
43. Mahesh B, Leong HS, McCormack A, et al. Autoantibodies to vimentin cause accelerated rejection of cardiac allografts. Am J Pathol 2007;170:1415-27.
44. Isobe M, Haber E, Khaw BA. Early detection of rejection and assessment of cyclosporine therapy by 111In antimyosin imaging in mouse heart allografts. Circulation 1991;84:1246-55.
45. Jurcevic S, Ainsworth ME, Pomerance A, et al. Antivimentin antibodies are an independent predictor of transplant-associated coronary artery disease after cardiac transplantation. Transplantation 2001;71: 886-92.
46. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol 2007;96:41-101.
47. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity 2004;21:467-76.
48. Aggarwal S, Gurney AL. IL-17: prototype member of an emerging cytokine family. J Leukoc Biol 2002;71:1-8.