Anti-HLA Antibodies and Kidney Allograft Outcomes in Recipients with Donor Bone Marrow Cell Infusion

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ABSTRACT

Background: Anti-HLA-antibodies are known to affect the allograft survival in transplant recipient patients. **Objective:** The aim of this study was to evaluate the association between anti-HLA antibodies and kidney allograft outcomes, particularly in recipients with concurrent donor bone marrow cell infusion (DBMI). **Methods:** Between June 2006 and May 2007, forty living unrelated donor kidney transplants consisting of 20 recipients with DBMI and 20 without infusion entered into the study and were monitored prospectively for one year. Pre- and post-transplant (days 14, 30, and 90) sera were screened for the presence of anti-HLA class-I and II antibodies, and subsequently positive sera retested with ELISA specific panel for antibody specification. **Results:** Of 40 patients, 9 (22.5%) experienced acute rejection episodes (ARE) (6/20 cases in non-infused versus 3/20 in DBMI patients). The prevalence of anti-HLA antibodies before and after transplantation were higher in patients with ARE compared to non-rejecting ones (88.8% vs. 38.7%, p=0.01 and 66.6% vs. 25.8%, p=0.04, respectively). A total of 10% (4/40) of patients developed donor specific anti-HLA antibodies (DSA) and in this regard 2 patients from the control group experienced ARE. All 3 rejecting patients in DBMI group were negative for DSA and positive for non-DSA. The lower titer of post-transplant anti-HLA antibodies were shown in DBMI patients compared to pre-transplantation titer. Additionally, the average serum creatinine levels during one year follow up and even in those patients with ARE were lower compared to controls. **Conclusion:** Our findings reveal an association between pre- and post-transplant anti-HLA antibodies, and ARE and also early allograft dysfunction. It suggests that lower incidence of ARE, undetectable DSA, lower titer of antibodies concomitant with a decrease in serum creatinine level, better allograft function and lower percentages of PRA in DBMI patients, could be the probable manifestations of partial hypo-responsiveness against allografts.

Keywords: Allograft, Bone Marrow, HLA, Infusion, Kidney

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INTRODUCTION

A significant number of studies revealed an increased risk for early acute rejection, primary dysfunction, and/or decreased allograft survival in patients who had pre- and post-transplant anti-HLA antibodies. Thus, it seems reasonable to perform anti-HLA antibody screening and identification in order to identify patients at higher risk for acute rejection episodes (ARE), graft loss and chronic rejection episodes. Hence, they could be useful for better prediction and management of allograft outcomes (1,2).

The majority of animal and human studies have provided evidence that chronic allograft dysfunction is the end result of a pathophysiologic process that involves both T and B cell mechanisms along with non immune factors. In addition, alloantigen recognition by T cells via indirect pathway can provide help for antibody responses by allospecific B cells. Thus, de novo production of anti-HLA antibody may be the climax of an early and specific T-B cell cooperation (1,3,4).

On the other hand, one of the most important protocols for induction or maintenance of clinical tolerance in organ transplantation is donor bone marrow cell infusion (DBMI) which has been extensively studied in renal transplant recipients (5-8). It is believed that, infused donor bone marrow cells (DBMCs) have some immunomodulatory effects on anti-donor reactivity and it may promote donor specific hypo-responsiveness (9-12).

It is well established that graft survival and function are associated with post-transplant development of anti-HLA antibodies as compared to recipients without antibodies (13). A cohort evaluation of 235 kidney transplant recipients followed for more than 5 years showed a significant correlation between the development of circulating donor specific anti-HLA antibodies and graft survival. Donor specific HLA antibodies were present in 52% of patients experiencing graft failure versus 1.8% of those with a functioning graft. It was also shown that anti-HLA antibodies can be directed against class-I (50%) and class-II (41%) HLA molecules or both (9%) (13,14). Hollarron et al., reported that 20% of their patients developing donor directed HLA-I antibodies within 3 months after kidney transplantation, experienced rejection episodes (15). Worthington et al., retrospectively investigated 112 kidney transplant recipients who lost their allograft as a result of the presence of anti-HLA antibodies and compared them with 123 patients who had functioning allograft for more than 5 years. They showed that 50.9% of patients with allografts loss produced anti-HLA antibodies, compared to 1.6% in patients with stable graft function (14).

The frequency of donor specific or non specific anti-HLA antibodies detected after kidney transplantation is extremely variable, ranging from 1.6% to 60% due to multiple factors including the type of assays, immunosuppressive regimens, the type of patient population analysis or the variable times of sample collection (1,16). The aim of this study was to evaluate the association between pre- and post-transplant anti-HLA antibodies and kidney allograft outcomes during one year follow up, particularly in recipients with a concurrent DBMI to find out the probability of sensitization or immunomodulation following donor cell infusion. We further investigated the impact of these cells on early post-transplant outcomes such as acute rejection, allograft dysfunction, patient and graft survival and graft loss.

MATERIALS AND METHODS

During the period of June 2006 through May 2007, a total of 40 living unrelated donor renal transplant candidates who had consecutively accepted our study were included in this study and were monitored prospectively for one year. Inclusion criteria consisted of the primary transplant, and the absence of previous transfusion. Informed consent was obtained from all donors and recipients according to protocols approved by TUMS ethical committee. These patients were divided in two groups comprising; 20 kidney recipients who concurrently received donor bone marrow cell infusion (DBMI group), and another 20 patients with kidney allograft alone as the control group. Both groups received the same baseline immunosuppressant that was instituted 24 hours prior to renal transplantation and the protocol consisted of a triple drug regimen of cyclosporine A (5mg/kg/day BD), mycophenolate mofetil (MMF) (2 gram/day BD) and prednisolone (2 mg/kg/day). Because of live donation, we preferentially did not use antibody induction therapy.

Bone Marrow Cell Preparation and Infusion. Donor bone marrow cells were prepared from iliac crest at the time of donor nephrectomy by the aspiration of 150-200 milliliters of bone marrow using Jamshidi syringe. Mononuclear cells (MNC) were isolated using hydroxyethyl starch (HES 6%, plasmastrile, Fresenus, Germany) as described by Adkins et al., (17) with a brief modification. Aliquots (0.5 ml) of MNC suspension in HES were assayed for the absolute count of total nucleated cells, and then for the flowcytometric determination of the percentage and the absolute number of $CD34^+$ $CD45^+$ hematopoietic progenitor cells by RPE conjugated anti-CD34 and FITC labeled anti-CD45 and isotype matched negative control (DACO, Denmark) according to the ISHAGE guidelines (18). The average number of donor cells infused post operatively was $2.19\times10^9 \pm 1.13\times10^9$ mononuclear cells/each patient including $2.66\times10^7 \pm 1.70\times10^7$ $CD34^+$ progenitor cells.

HLA Typing. After DNA extraction using salting-out procedure, HLA-typing for donors and recipients was performed by standard PCR-SSP technique, to determine HLA-A, B, DR alleles using HLA-A B DR low resolution typing kit (Heidelberg, Germany). Pre-transplant panel reactive antibody (PRA) analysis and WBC cross match were done by complement dependent cytotoxicity method.

Anti-HLA Antibody Screening and Identification. A total of 160 serum samples from 40 patients in both groups were collected during follow up and frozen at -70˚C until testing; four samples for each patient including one pre-transplant and three sequential samples at days 14, 30, and 90 post transplant were collected based on the probability of the higher occurrence of acute rejection during the first 3 months of post operation as reported by similar studies (13-16). Sera were screened for the presence of anti-HLA class I and class II IgG antibodies by solid phase enzyme linked immunosorbant assay (ELISA) kit (Ab Screen, HLA class I and II, Biotest, Dreieich, Germany) according to the manufacturer's instructions. Results equal to or greater than twice the mean of the negative controls were defined as positive. Reaction strength was scored as follows: 2 = weak positive (OD $\leq$ 2× cutoff), 4 = positive (OD = 5× cutoff), 6 = positive (OD = 8× cutoff), and 8 = strong positive (OD = 12× cutoff). Subsequently, positive sera were re-tested with an ELISA against HLA class I and class II specific panels using 40 antigens for class I and 30 for class II (Ab Identification, HLA class I and II, Biotest, Dreieich, Germany) to confirm the screening result and identify HLA antibody specificity or post-transplant PRA levels. Specificity analysis was performed using the Ab identification software provided by the kit manufacturer.

Delayed graft function (DGF) was defined as a requirement for dialysis within the first week after surgery. The acute rejection was determined according to a biopsy proven pathological analysis or a clinical manifestation which required treatment by pulse steroid /ATG therapy.

Returns of the patients to dialysis, transplant nephrectomy, or death were indices of graft loss. The median time of follow up was one year for patients with functioning allograft and one month for those with graft loss.

Statistical Analysis. All statistical analyses were performed using SPSS, version 11.5 for Windows. Significant differences between frequencies were determined by Chi-square analysis and Fisher's exact test. Averages were compared using paired and unpaired Student's *t*-test. Two-tailed p values less than 0.05 was reported as statistically significant. In addition, multiple logistic regression analysis was applied to determine the association of acute rejection with prognostic factors including pre- and post-transplant anti-HLA antibodies, serum creatinine level, delayed graft function and graft loss.

RESULTS

All patients in this study received allograft from living unrelated donors. Infusion of donor cells was perfectly tolerated and no GVHD was shown following this protocol. The number of HLA mismatches (A- B- DR) was nearly the same between both groups (median of HLA mismatches were 4 in DBMI and 5 in control groups, Table 1). Demographic and clinical characteristics and critical risk factors are summarized in Table 1 with no significant differences between patient groups.

Acute Rejection Episode (ARE), Graft Loss and Patients' Survival in DBMI and Control Groups. Of the 40 patients, 9 cases (22.5%) showed acute rejection episodes (ARE) during the follow up period. The number of patients undergoing acute rejection episodes appeared more frequent but not statistically significant in the control group [3 cases (15%) in DBMI group versus 6 (30%) in controls (p=0.45)]. All 3 patients with ARE in DBMI group showed controlled rejection episodes by pulse steroid and/or ATG therapy. While, among the 6 patients with ARE in the control group, two cases were diagnosed as biopsy proven acute humoral rejection, and one of the two lost his graft subsequently. Another case showed acute humoral and cellular rejection at day 25 and returned to dialysis (graft loss). The remaining 3 cases with ARE in the control group were reversed by drug treatment (methyl-prednisolone and/or ATG), therefore, histological analysis was not performed on them.

One year patient and graft survival was 100% and 95% in DBMI group and 100% and 90% in the controls, respectively. During the first year of follow up, chronic rejection was not diagnosed in either group.

Association of Acute Rejection with Pre and Post Transplantation Anti-HLA Antibodies. Screening of anti-HLA antibodies by ELISA demonstrated that 50% of all patients (20 cases including 11 cases in the control and 9 in the DBMI groups) were sensitized to HLA antigens (class I or II or both) prior to transplantation. Whereas, post-transplant antibodies were seen in 14 (35%) patients (7 cases for each group). Among 7 antibody positive patients after transplantation in DBMI group, 3 cases were those with the pre-transplant antibody and with ARE, but the titer of antibodies was decreased to half and one/third of the titer compared to pre-transplantation level (Table 2).

Table 1. Demographic and clinical data of kidney transplant patients (N=40).

Parameters	DBMI group (N=20)	Control group (N=20)	P value
Recipients' age (years) (Mean ± SD)	43 ± 14	45 ± 18	NS
Recipients' gender (M/F)	(13 / 7)	(14 / 6)	NS
Donors' age (years) (Mean ± SD)	36 ± 13	32 ± 8	NS
Pre-transplant PRA (CDC)			
0 – 5 %	18	19	NS
5- 10 %	2	1	NS
Number of HLA mismatches (A - B - DR)			
Median	4	5	NS
Mean ± SD	4.25 ± 1.18	4.75 ± 0.94	NS
Cold ischemia time in minutes (Mean ± SD)	52 ± 9.6	51 ± 14.9	NS
Warm ischemia time in minutes (Mean ± SD)	4.2 ± 0.8	4.1 ± 0.85	NS
CMV infection Positive (Post Tx)	7	7	NS
Serum creatinine (mg/dl) (Mean± SD)			
1 weeks after Tx	2.94 ± 1.3	2.85 ± 1.27	NS
2 weeks after Tx	2.23 ± 1.1	2.70 ± 2.65	NS
Delayed Graft Function (DGF)	3	4	NS
Hospitalization after discharge due to CMV infection/increased Cr	9 (4/5)	8 (4/4)	NS

DBMI: donor bone marrow infusion; PRA: Panel Reactive Antibody; CDC: Complement Dependent Cytotoxicity; Tx: Transplantation; CMV: Cytomegalovirus; DGF: Delayed graft function; Cr: Creatinine; NS: Not significant.
Note: Data were not statistically significant between both groups of the study.

In addition, the remaining 4 antibody positive patients without ARE had a lower titer of antibody and even in one case, the titer was 4 times lower (at day 90) than its pre-transplantation level. In controls, the post-transplant number of antibody positive patients, with and without ARE, were 3 and 4 cases, respectively. The titer of those antibodies was similar to that of pre-transplantation value and interestingly one patient with ARE who lost his graft had a titer 2 times higher than its pre-transplantation level (Case 5, Table 2).

Susceptibility to post-transplant morbidity (requiring hospitalization) including viral, fungal and bacterial infection and raised creatinine levels appeared to be not statistically significant (9/20 in DBMI and 8/20 in controls). However, hospitalization due to CMV infection was the same for both groups (4 cases, Table 1).

Post Transplantation Donor Specific Antibodies and PRA Screening in Patients with ARE. In DBMI group, among 7 antibody positive patients after transplantation, 3 patients with AR were positive for non-donor specific antibody (non-DSA) against HLA class I and II antigens (Table 2), while three cases without AR did not show antibodies against HLA antigens, possibly due to the low titer of the antibodies. Only one patient had both DSA and non-DSA with an antibody titer of one fourth compared to its pre-transplantation level (data not shown).

Table 2. Anti-HLA antibody screening and identification in patients with ARE from both groups.

AR patients	Donor HLA-mismatches (A, B, DR)	Anti-HLA Abs(Class I/II) Pre-Tx	Post -Tx (days) 14	30	90	Ab Identification DSA / Non-DSA	Time of AR (in days)	Serum Cr levels (mg/dl) at the time of AR	Allograft Biopsy
DBMI									
1	A2,74; B14; DR1	HLA-I: 4+ HLA-II: N	N N	2+ N	N N	Neg / (A, B)	27	3.4	NB
2	A24,26; B49,51; DR9,11,52, 53	HLA-I: 6+ HLA-II: 4+	4+ 2+	4+ N	2+ N	Neg / (A, B)	35	3.3	NB
3	A3; B18,44; DR11	HLA-I: 6+ HLA-II: 2+	4+ 2+	** **		Neg / (A,B,DR)	12	5.9 **Mean: 4.2 ±1.47^{x}**	NB
Controls									
1	A2,11; B52; DR1	HLA-I: N HLA-II: 2+	N N	N N	N N	Neg / Neg	140	4.1	AHR
2	A2; B27,52; DR10	HLA-I: 2+ HLA-II: 6+	N 4+	N 2+	N 4+	Neg / (DR)	18	2.9	NB
3	A3, 24; B57; DR4,11	HLA-I: N HLA-II: 2+	2+ 2+	2+ 2+	ns ns	A24, B57/ (DR)	28	4.5	NB
4	A3; DR11	HLA-I: N HLA-II: N	N N	N N	N N	Neg/ Neg	16	3.3	NB
5[§]	A26; B27,38; DR13,12	HLA-I: N HLA-II: 4+	N 6+	N 8+	§ 	DR12,13/ (DR)	25	8.2	AHR & ACR
6[§]	A2,30; B38,51; DR14	HLA-I: N HLA-II: 2+	N N	§ 		Neg/ Neg	13	12 **Mean: 5.8 ±3.6^{y}**	AHR

AR: Acute rejection; Pre-Tx: Pre transplant; Ab: Antibodies; DSA: Donor specific antibodies, N: negative, ns: no sample, NB: No Biopsy, DBMI: Donor bone marrow infused group; AHR: acute humoral rejection; ACR: acute cellular rejection; **x** versus **y**, p=0.36; **§**: patients with graft loss.
** This case was excluded from further monitoring because of uncontrolled bleeding at day 16 due to surgical problems.

Among 9 antibody positive patients in the control group, two cases with ARE showed both DSA (against HLA class I and II antigens) and non-DSA (against HLA-II antigens) and one case showed only non-DSA against class II antigens (Table 2). Of four cases without ARE, 2 patients were negative for antibody identification probably due to a lower titer of antibodies. One of the other two cases were positive for both DSA and non-DSA against HLA-I antigens and the other was positive for non-DSA against class I antigens (data not shown).

Of 14 antibody positive patients after transplantation, 7 cases showed an increase in PRA level (4th week post operatively) while, the other 7 cases were negative for post-transplant PRA assay probably due to the low titer of those antibodies against respective antigens in the panel. A seven times increase in PRA against a panel of HLA class II antigens (30 antigens) was shown for one patient in the control group compared to its pre-transplant level (10 to 70% with a concurrent rejection and graft loss, Table 3).

Table 3. PRA percentages in antibody positive patients with or without acute rejection (ARE).

DBMI patients	PRA % (Pre-Tx)*	PRA% (Post-Tx)**
S. M. (NR)	10 %	26.6% (class-II)
A. Sh. (AR)	5.0%	7.0% (class-I)
J. J. (AR)	0.0%	2.5% (class-I)
E..P. (AR)	10%	30 % (class-II)
Control patients	PRA % (Pre-Tx)	PRA% (Post-Tx)
F. H. (AR) $^{\Phi}$	10 %	70 % (class-II)
SM. M. (NR)	0.0%	5.0 % (class-I)
A.H. (NR)	5.0%	32.5% (class-I)

Note: 40 antigens for HLA class I and 30 antigens for class II antibody identification were used.
AR: Acute Rejection; NR: Non- Acute Rejection; * PRA by CDC method; ** PRA by ELISA method.
$^{\Phi}$ the graft was lost within the 4th week of post transplantation.

A similar increase in PRA level against HLA class I antigens (40 antigens) was shown for two other cases but without AR (0% into 5% and 5% into 32.5%, separately). In DBMI group, 3 patients undergoing AR and one without AR showed a 2-3 times increase in PRA level against HLA class I/II antigens, which is lower compared to the controls (16.5% versus 35.8%, Table 3).

Table 4. Average serum creatinine levels at days 14, 30 and months 3, 6 and 12 after transplantation in both groups.

Serum Cr (Mean ± 1 SD)	Control (N=20)	DBMI (N=20)	P Value
Day 14	2.70 ± 2.62	2.23 ± 1.10	0.47
Day 30	2.33 ± 2.15	1.60 ± 0.42	0.19
Month 3	1.55 ± 0.46	1.51 ± 0.43	0.80
Month 6	1.60 ± 0.72	1.42 ± 0.35	0.33
Month 12	1.37 ± 0.29	1.28 ± 0.36	0.43

Measuring the serum creatinine levels as a surrogate marker of graft dysfunction at days 14, 30 and months 3, 6 and 12 after transplantation indicated a lower creatinine in DBMI patients particularly in those cases with acute rejection compared to the controls (Table 4 and Figure 1).

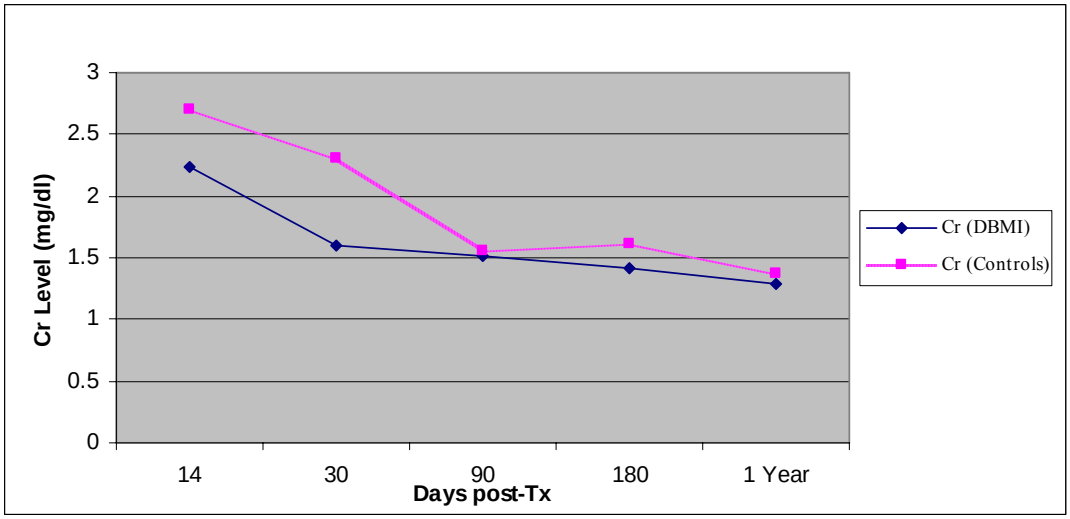

Figure 1: comparison of serum creatinine levels between both groups within the first year of transplantation.
Cr, Creatinine; DBMI, donor bone marrow infusion; Tx, transplantation

Multivariate analysis showed that the incidence of acute rejection was significantly associated with a high serum creatinine (p=0.006), delayed graft function (p=0.035) and graft loss (p=0.005) in the control patients. Similar analysis in DBMI patients showed that acute rejection was associated with post-transplant anti-HLA antibodies (p=0.01) and the graft loss (p=0.015, Table 5).

Table 5: Association of ARE with prognostic factors in a multivariate analysis for both groups.

Prognostic factors	DBMI P value*	Control P value
Pre-Tx anti-HLA antibodies	0.06	0.06
Post-Tx anti HLA antibodies	0.01	0.24
High serum creatinine	0.09	0.006
Delayed Graft Function	0.33	0.035
Graft loss	0.01	0.005

Lower levels of serum creatinine are apparent in all time intervals for infused patients compared with controls.
DBMI: Donor bone marrow infusion group; Cr: Creatinine.
*P-values are shown for the association of ARE with different variables for both groups separately.

DISCUSSION

Despite the lower incidence of acute rejection using newer generations of immunosuppressants, this type of rejection still remains a problem in kidney transplantation. This problem could also be a risk factor for the development of chronic allograft dysfunction or chronic rejection which is the major cause of graft loss (19). According to the ex vivo and in vivo findings, infusion of DBMC in kidney allograft patients could have a positive role in helping to induce or maintain partial donor specific hypo-responsiveness. That could be due to the down regulatory effect of DBMC on recipient anti-donor immunity (20). Nonetheless, the role of DBMI in facilitating tolerance/hypo-responsiveness needs to be studied more extensively (21).

There is ample evidence showing that de novo production of anti-HLA antibodies is associated with rejection, graft loss and poor prognosis. Thus, HLA antibodies could be used as an indicator of patient alloreactivity, so that it can distinguish immunologic graft failures from nonimmunologic ones (16,22).

In an attempt to explore the relationship between DBMI, post transplant antibody production and allograft outcome, we analyzed the effect of DBMI on sensitization and renal allograft function. The main findings of the present study were the lower incidence of ARE, lower titer of post transplant antibodies concomitant with a decrease in serum creatinine level, undetectable DSA and lower percentage of PRA in DBMI patients compared to the controls during early follow up.

One year follow up of patients demonstrated that, despite the same immunosuppressive therapeutic regimen in both groups and no antibody induction therapy (e.g. using ATG), DBMI did not enhance sensitization after transplantation.

In contrast to the similar incidence of DGF, ARE and PRA in both infused and non- infused patients in the study of Ciancio et al., (23), we observed more DGF (4 vs. 3 cases), two times more incidence of ARE (30% vs. 15%), and higher percentage of PRA in controls compared to the DBMI patients.

In a study by Ciancio et al., (8) who followed up 63 infused patients and 213 controls for six years, the incidence of ARE was not statistically different between both groups

and the test group demonstrated more prevalence of CMV infection. However, the incidence of ARE among infused patients from our study was half of the controls (15% vs. 30%) and, the rate of CMV infection was the same between both groups. Mean levels of the serum creatinine during follow up period were lower in DBMI patients for both studies, indicative of more stable graft function in the infused patients compared to the controls.

According to a study by Mathew et al., (10), lower percentages of PRA against HLA antigens and lower incidence of ARE, have been shown for infused patients compared to the controls. Conversely, their results indicated higher CMV infection one year post operatively. Similar incidence of CMV infection (20%) was seen in both groups in our study (Table 1).

As indicated in a previous study by Israeli et al., (24), we also showed twice or even more than twice an increase in PRA percentages in patients with or without rejection versus their pre-transplantation PRA. Remarkably, the mean percentage of post transplant PRA in the controls was 2 times higher than those of the DBMI patients (35.8% vs. 16.5%, Table 3).

Analysis of the relationship between DSA and the incidence of AR for both groups showed that 2 cases with ARE in the controls developed post transplant DSA against HLA class I and class II antigens, separately (Table 2). Whereas, no antibodies directed against mismatched HLA antigens were detected in DBMI patients with AR. More importantly, non specific antibodies in those patients showed an obvious decrease in the titers, post operatively (Table 2).

Testing of serial serum samples from antibody positive patients in DBMI (with or without ARE) displayed a great decrease in the antibody titers so that, two patients with ARE were found to have a half to one third of antibody titer compared to the pre-transplantation condition (Table 2). Interestingly, a similar decrease was shown for the other 4 cases without ARE and one showed a 4 times decreased titer at day 90 versus day 14 and 30 after transplantation (data not shown).

In the controls, rising of the titer of both DSA and non DSA was found in two patients with ARE (one graft loss, Table 2). The remaining antibody positive cases in this group did not show any decrease in antibody titer after transplantation (data not shown).

Since the same immunosuppressive regimens are used in both groups, observation of the lower production of antibodies in DBMI group may be somehow due to the immunomodulatory effects of donor bone marrow cells through different mechanisms such as augmentation of microchimerism, induction of suppressor T cells with regulatory influence on B cell function, and other unknown factors (9,20).

It has been demonstrated that the presence of anti-HLA antibodies before transplantation increases the incidence of delayed graft function and ARE (25). Likewise, we found the pre-transplant sensitization in 3 patients from the controls and one in the DBMI group who underwent DGF. Totally, there were 7 cases with DGF (4 in the rejecter versus 3 in the non rejecter patients, p=0.03, OR=7.47, Table 6).

Table 6. Association between ARE and different risk factors in all patients.

Risk Factors	Patients with AR (N= 9; 3 in DBMI & 6 in controls)	Patients without AR (N= 31)	P value	Odds ratio
Cr Levels (mg/dl) at day14 (Mean ± SD)	5.96 ± 3.18	1.87 ± 0.24	0.02	
Pre-Tx Abs positive	8[a] / 9 (88.8%)	12 / 31 (38.7%)	0.01	12.67
Post-Tx Abs positive	6[b] / 9 (66.6%)	8 / 31 (25.8%)	0.04	5.75
DGF	4[c] / 9 (44.4%)	3 / 31 (9.6%)	0.03	7.47
DSA (class I/II)	2[d] / 9 (22.2%)	2* / 31 (6.4%)	0.21	4.14
Non-DSA (class I/II)	6[e]/ 9 (66.6%)	3 / 31 (9.6%)	0.001	18.67
HLA- mismatches	4.66 ± 0.86	4.83 ± 1.17	0.55	

a: 3 cases in DBMI vs. 5 in controls, **b:** 3 cases in DBMI vs. 3 in controls, **c:** 1 patient in DBMI vs. 3 in controls, **d:** both cases belonged to controls, **e:** all 3 patients from DBMI and 3 cases from control groups. ***one patient in DBMI and the other in controls.
Note: because of low sample number in each group, the differences for the risk factors between control and infused groups were not statistically significant, although these differences are obvious.
ARE: acute rejection episodes; DBMI: donor bone marrow cell infusion; Tx: transplantation; DGF: delayed graft function; DSA: donor specific antibodies

The results of a large multi center study showed that patients who developed de novo antibodies after transplantation had a significantly greater chance of graft failure (22,26,27). Based on the humoral theory of transplantation which was proposed by Terasaki et al., (25) and in agreement with other studies (13-15,28), our results suggest that pre-sensitization to HLA antigens as well as post transplant development of antibodies are predictive of subsequent graft failure and are risk factors for the occurrence of ARE in both DBMI and control groups.

Moreover, we demonstrated that 25.8% (8/31) of patients with functioning grafts were antibody positive post operatively compared to 66.6% (6/9) of patients with ARE (p=0.04, OR=5.75, Table 6) in accord with the humoral theory of transplantation (19) and also supported by the study of Mizutani et al., (29). Meanwhile, a strong association was found between production of non DSA and acute rejection episodes (p=0.001, OR=18.67, Table 6). Also, the predictive value of anti-HLA antibodies could be enhanced among patients with higher serum creatinine levels.

We found that average serum creatinine levels at days 14, 30 and months 3, 6 and 12 after transplantation in DBMI patients were lower than the controls (Figure 1 and Table 4). Additionally, multivariate logistic regression analysis showed a significant correlation between ARE and higher serum creatinine (p=0.006), more delayed graft function (p=0.035) and graft loss (p=0.005) in the controls (Table 5). While, a similar analysis in DBMI patients showed a correlation between ARE and post-transplant antibodies (p=0.01) and graft loss (p=0.01, Table 5). It should be considered that the prevalence of pre- and post-transplant antibody in those patients were the same and more importantly, the titer of antibodies was lower after transplantation. In the previous report by our group, the lower levels of proinflammtory makers (IFN-γ and sCD30) as representatives for the initial evaluation of cellular responses, was shown in infused patients (30).

In conclusion, our data confirm that anti-HLA antibodies are associated with allograft outcomes (especially ARE) and they have a predicting value for the poorer outcome of transplantation. More importantly, lower incidence of AR, lower levels of post-transplant antibodies concomitant with a decrease in serum creatinine level, undetectable DSA, lower percentage of post transplant PRA, and better allograft function in DBMI patients compared to the controls are possible primary manifestations of functional immune modulation achieved by the DBMC infusion protocol. In other words, donor cell infusion not only does weaken alloreactivity, but also it may promote the

supportive mechanisms for the induction and/or the maintenance of tolerance through augmentation of microchimerisms and other unknown mechanisms. Taken together, the obvious differences between infused and control patients were seen during early follow up and because of small number of patients in the present study, those differences were not statistically significant. Hence, longer follow up of these patients and evaluation of more clinical and para-clinical findings especially regulatory cell subpopulation analysis and quantification of microchimerism are mandatory to find out the exact role of donor cell infusion in long-term survival and specific hypo-responsiveness in kidney allograft patients.

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