ORIGINAL ARTICLE

Compartment-specific expression of natural killer cell markers in renal transplantation: immune profile in acute rejection

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SUMMARY

Natural killer (NK) cells have been implicated in graft dysfunction. Here, we formulated hypothesis that distinct patterns of expression NK cells markers correlated with acute rejection in kidney transplantation. Therefore, we studied the pattern of NK cell markers CD56, CD57, and CD16 in different compartments of biopsies obtained from recipients diagnosed with acute graft rejection, with or without donor-specific antibodies (DSA). DSA-negative biopsies-from patients with acute T-cell mediated rejection (aTCMR) had an increased expression of CD56+ and CD57+ cells ($P = 0.004$ and $P = 0.001$) in the interstitial compartment in comparison with DSA-positive biopsies from patients acute antibody-mediated rejection (aABMR) with (aABMR C4d+) and without C4d deposition (aABMR C4d-). CD16+ cells was increased ($P = 0.03$) in the glomerular compartment in DSA-positive biopsies. We assume that CD16+ expression and antibody-dependent cellular cytotoxicity (ADCC) in microvascular injury can be associated with aABMR. IFN-γ release from cytoplasmic granules of NK cell could be associated with aTCMR. Our findings suggest that NK cells need to be carefully evaluated because variations in NK cell marker expression might imply the involvement of different immune system pathways in graft rejection.

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Introduction

Studies of immunohistochemical markers that can assist in identifying biopsies that are indicative of renal allograft rejection and the incorporation of the use of such markers in the routine practice of diagnostic pathologists are important for the management of renal graft dysfunction. Various studies have focused on interpreting natural killer cells (NK cells) in post-transplant biopsies and in identifying their associations with the immune status of patients. The results of those studies support new theories about the role that NK cells in biological functions such as the innate and adaptive immune responses [1].

In humans, NK cells compose up to 20% of the peripheral blood mononuclear cells [2]. NK cells express activating and inhibitory receptors. The recognition of NK cells involves the initial binding to potential target cells; activating and inhibitory receptor interactions with ligands available on the target; and the integration of signals transmitted by these receptors, which determines whether the NK cell detaches and moves on or stays and responds. The NK cells respond by reorganizing and releasing cytotoxic granules, as well as by transcribing and secreting cytokines. Inhibitory receptors are responsible for recognizing class I self-major histocompatibility complex (MHC) antigens [3]. Therefore, NK cells can respond to allografts that lack host-type class I MHC antigen. The first activating NK receptor identified, which is also the one that is the most well characterized, is CD16, a Fc-receptor for immunoglobulin G (IgG) responsible for antibody-dependent cellular cytotoxicity (ADCC) [4].

One recent study found associations between NK cells and donor-specific antibody (DSA) transcripts that are selective for antibody-mediated rejection (ABMR), indicating that NK cells play a role in this type of rejection [5]. Another study revealed that NK cell transcripts are more numerous in T-cell-mediated rejection (TCMR) and in late ABMR [6].

In experimental studies of solid organ transplantation, Hirohashi *et al.* [7] showed that NK cells induce DSA-associated chronic allograft vasculopathy in heart transplantation. The authors proposed a novel pathway of action between NK cells and grafts, in which specific Fc receptors are required in order to interact with non-complement-fixing DSA. This mechanism had previously been described as related to CD16, which has a low affinity for antibodies expressed by NK cells [8].

In renal transplantation, DSA positivity is an important factor that is associated with graft dysfunction. Sensitive methods that reveal these antibodies in sera, such as Luminex and other solid-phase assays, have led to advances in the study of graft pathology, especially ABMR.

A report from the 2013 Banff conference established defining criteria for a diagnosis of C4d-negative ABMR [9]. This new classification not only takes into consideration histological and serological evidence of acute tissue injury but also outlines new histological criteria, defining moderate microvascular injury as a combined glomerulitis-peritubular capillaritis score ≥ 2, which could be evidence of recent antibody interaction with vascular endothelium in cases with deposition of C4d-negative antibodies. For all ABMR diagnoses, it is currently recommended that the lesion be categorized as C4d-positive or without evident C4d deposition. In either case, serological evidence of DSA is an unequivocal criterion [9]. It has long been known that the pathogenesis of ABMR involves endothelial damage that is related to DSA- or complement-mediated injury [10,11]. That pathogenesis is usually supported by the analysis of histological features in biopsies of transplant recipients.

Based on the above considerations, we hypothesized that NK cell immunostaining would show various profiles in acute rejection in kidney transplantation. Therefore, we studied the pattern of NK cell staining in different compartments of biopsies obtained from kidney transplant recipients, all diagnosed with acute graft injury, with or without serological evidence of DSA.

Patients and methods

Patients

This study included for-cause biopsies obtained from renal transplant recipients who underwent transplantation between January 2007 and December 2011 at the Renal Transplantation Center of the Department of Urology of the University of São Paulo School of Medicine *Hospital das Clínicas*, in the city of São Paulo, Brazil. The biopsies, obtained between 2009 and 2012, were examined in the Pathology Department of the Hospital. The study was approved by the Research Ethics Committee and Institutional Review Board of the *Hospital das Clínicas* (Certificate no. 03770112.5.3001.0071).

We collected demographic data, as well as data related to human leukocyte antigen (HLA) mismatches; primary diagnosis; initial and induction immunosuppression therapy; pretransplant and post-transplant HLA; and donor characteristics. The biopsies were ret-

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rospectively reviewed by two independent renal pathologists. In cases of diagnostic disagreement, a third pathologist was asked to evaluate the biopsy.

We evaluated HLA matching by determining the number of HLA-matched antigens at loci A, B, C, DP, DQ, and DR. All of the transplant recipients exhibited a negative pretransplant cytotoxic crossmatch. Pretransplant panel-reactive antibodies were determined by Luminex assay (LabScreen®, One Lambda, CA, USA). Pretransplant and post-transplant DSA with MFI > 300 was performed with Luminex single-antigen bead assays for class I and II HLAs (LabScreen® Single Antigen; One Lambda, CA, USA). A *de novo* DSA was defined as a new DSA detected by single-antigen assay that was not present prior to transplant and was identified in the biopsy.

Histopathology

For the histopathological analysis, tissue samples were fixed in Duboscq-Brazil, embedded in paraffin, and sectioned in a cryostat. Each section (3 μm in thickness) was then stained with hematoxylin and eosin, periodic acid-Schiff, methenamine-silver, and Masson's trichrome. We evaluated all biopsies according to the Banff criteria [9,12], establishing individual scores for the glomerular, interstitial, vascular, and peritubular capillary compartments. We divided the biopsies into two groups: acute TCMR (aTCMR), which included all biopsies meeting the criteria for aTCMR or with borderline changes suggestive of such; and acute ABMR (aABMR). The biopsies in the aABMR group were further divided into two subsets: those with no apparent C4d deposition (aABMR-C4d−); and those with evident C4d deposition (aABMR-C4d+).

Immunofluorescence

Biopsy sections were placed on silanized slides and dried at room temperature for 20 min. Endogenous peroxidase activity was blocked with avidin and biotin solutions, 20 min in each solution at room temperature. Frozen sections were then stained for C4d by 30 min of incubation at room temperature with an anti-C4d monoclonal antibody (Quidel, San Diego, CA, USA), diluted 1:100 in bovine serum albumin. After being washed in phosphate buffered saline, the sections were incubated for another 30 min at room temperature with biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA), also diluted 1:100 in bovine serum albumin. The sections were again washed, after which they were incubated for another 30 min at room temperature with fluorescein isothiocyanate-conjugated streptavidin (SA-5001; Vector Laboratories), also diluted 1:100 in bovine serum albumin. The slides were then washed in saline and mounted in the usual manner.

Sections were examined under immunofluorescence microscopy at 400× magnification. The pathologists who scored C4d in the peritubular capillaries were blinded to the clinical and morphological data. Linear staining for C4d2 or C4d3 in the peritubular capillaries was interpreted as positive. When immunofluorescence microscopy data were not available, we studied the immunohistochemistry for C4d.

Immunohistochemistry

Amplification was carried out on anti-rabbit and anti-mouse polymers and with an immunohistology-based amplification system kit (Reveal-Biotin-Free Polyvalent HRP-SPB-999; Spring Bioscience, Pleasanton, CA, USA). The silanized slides were prepared as follows: deparaffinization for 30 min in an oven at 60 °C; immersion in xylene for 10 min at room temperature, followed by a second immersion in xylene; dehydration by immersion in absolute ethanol for 2 min (twice) and in 95% alcohol for 2 min (twice); and rehydration in tap water. Subsequently, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in six cycles of 5 min each, followed by incubation with 0.4% casein in phosphate buffered saline with surfactants and stabilizers. After the slides had been washed in running tap water, immunohistochemistry markers were identified through antigen retrieval by heating the slides in a pressure cooker at 95 °C for 4 min with 10 mM of citric acid buffer at pH 6.0.

The slides were then washed again, in distilled water, with three exchanges of Tris buffer at pH 7.4, after which they were incubated with the following primary antibodies: anti-CD56 (1:600, clone 123C3.D5; Novocastra, Nussloch, Germany); anti-CD57 (1:600, clone NK-1; DBS, Pleasanton, CA, USA); anti-CD16 (1:10 000, clone 2H7; Monosan, Uden, the Netherlands); anti-CD68 (1:40 000, clone KP1; Dako, Carpenteria, CA, USA); anti-CD3 (1:2000, clone F7.238; Dako); anti-CD4 (1:800, clone 4B12; Dako); and anti-CD8 (1:800, clone C8/144B; Dako).

For all primary antibodies, the slides were incubated for 18 h at 4 °C, after which they were again washed in distilled water, with three exchanges of Tris buffer, for 2 min. The anti-rabbit and anti-mouse polymers were then applied. Subsequently, primary antibodies recognizing polymers bound to the tissue and, after another

washing with Tris, that binding was revealed using 3,3′-diaminobenzidine, at 1.74% in stabilizer solution, as chromogen. Counterstaining was performed with Mayer's hematoxylin (Merck, Darmstadt, Germany). After dehydration in a graded alcohol series and clearance with xylene, sections were mounted in Entellan mounting medium (Merck). Positive and negative controls were included for all reactions.

Immunostaining analysis

In all of the biopsies, we used immunoperoxidase staining to assess the numbers of CD56+ cells, CD57+ cells, CD16+ cells, as well as CD68+ cells, CD3+, CD4+ cells, and CD8+ cells in various tissue compartments: interstitial (including the peritubular capillaries); glomerular; and vascular (the intima and muscle layers of arteries). In the interstitial compartment, the numbers of positive cells were counted in consecutive fields at a magnification for 400× and averaged. In the glomerular and vascular compartments, the mean number of positive cells was calculated for each glomerulus or artery respectively. Figure 1 shows the distribution of CD56+, CD57+, and CD16+ cells in the interstitial compartment and the lack of expression for CD56 and CD57 in glomerulus that showed positivity for CD16 staining.

Statistical analysis

Results are expressed as medians and ranges for continuous variables (except for donor and recipient ages, which are expressed as means and ranges). When the data distribution was non-Gaussian, we calculated medians rather than means. For categorical data, comparisons were based on the chi-square (χ^2) test and Fisher's exact test. For nonparametric data, we used the Kruskal–Wallis test, followed by Dunn's multiple comparisons test, and we used the Mann–Whitney test for comparisons between two groups. Correlations between expressions were estimated by Spearman's correlation coefficient. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed with the Statistical Package for the Social Sciences, version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

We evaluated a total of 74 consecutive biopsies, obtained from 59 patients. The biopsy samples were collected between 2009 and 2012. All the 59 patients were found to have a median time of 42 days after transplantation (Table 1). Of those 74 biopsies, 38 tested positive for DSA and were therefore classified as aABMR, the remaining 36 being classified as aTCMR. None of these biopsies indicated both TCMR and ABMR. Table 1 also shows the demographic and clinical characteristics of the patients.

Among the 38 aABMR biopsies, class I DSAs were detected in 21 (55.2%); class II DSAs were detected in 12 (31.6%); and 13.2% ($n = 5$) class I and II DSAs were detected in 5 (13.2%); and C4d positivity was detected

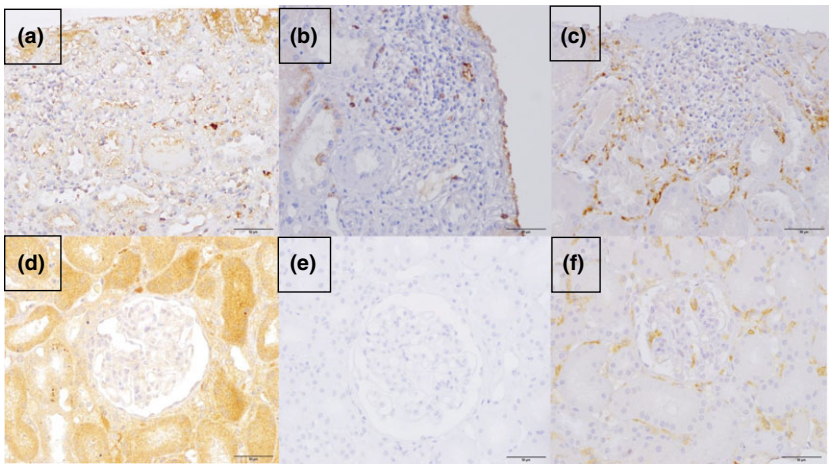

Figure 1 Pictures of original staining results for natural killer cell markers: CD56, CD57, and CD16. A, B, and C: CD56+, CD57+, and CD16+ cells in interstitium respectively. D and E: lack of expression for CD56 and CD57 cells in glomerulus respectively. F: CD16+ cells in glomerulus. Pictures D and E were chosen because they represent the same area where CD56 and CD57 staining results were negative and where the CD16 staining was positive in the same glomerulus. All figures related to interstitial and glomerular compartments correspond to the same field in the same biopsy, respectively, showing that immunohistochemical markers do not coincide each other (400×).

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Table 1. Demographic and clinical characteristics of patients diagnosed with acute renal allograft rejection, collectively and by type of rejection.

Characteristic		All patients (n = 59)	aTCMR (n = 33)	aABMR (n = 26)	P value
Recipient age, in years, mean (range)		43.5 (16–63)	41.2 (16–63)	46.3 (23–62)	ns
Female recipients, n (%)		32 (54.2)	13 (39.4)	19 (73)	0.017
First transplantation, n (%)		52 (88.2)	32 (97)	20 (76.9)	0.036
HLA mismatches, n (%)					
A1		42 (71.2)	23 (69.6)	19 (73.0)	ns
A2		51 (86.4)	30 (90.9)	21 (80.7)	ns
B1		48 (81.3)	25 (75.7)	23 (88.4)	ns
B2		47 (79.6)	27 (81.8)	20 (76.9)	ns
DR1		42 (71.2)	23 (69.6)	18 (69.2)	ns
DR2		43 (72.8)	24 (72.7	19 (73.0)	ns
Primary disease, n (%)					
Hypertension		6 (10.1)	5 (15.1)	1 (3.8)	ns
Diabetes mellitus		3 (5.0)	3 (9.0)	0 (0)	ns
Glomerulonephritis		2 (3.3)	2 (6.0)	0 (0)	ns
Pyelonephritis/reflux		6 (10.1)	1 (3.0)	5 (19.2)	ns
Polycystic disease		3 (5.0)	2 (6.0)	1 (3.8)	ns
Systemic lupus erythematosus		3 (5.0)	1 (3.0)	2 (7.7)	ns
Unknown		36 (61)	19 (57.5)	17 (65.3)	ns
Initial immunosuppression therapy, n (%)					
MPA/Tac/corticosteroid		54 (93.1)	29(50)	25(43.1)	ns
MPA/Tac		2 (3.4)	2(3.4)	0(0.0)	ns
MPA/corticosteroid		2 (3.4)	2(3.4)	0(0.0)	ns
Donor age, in years, mean (range)		41.3 (21–70)	40 (21–65)	47(25–70)	0.035
Post-transplant days at biopsy, median (minimum-maximum)		42 (2–970)	120 (4–820)	20 (2–970)	0.084
Scr at last visit (mg/dl, mean ± SD)		2.52 ± 2.31	2.16 ± 1.80	2.97 ± 2.80	ns
Graft loss		22.0%	18.1%	26.9%	ns
Deceased donors, n (%)		35 (59.3)	14 (42.4)	21 (80.7)	0.0036
Induction with thymoglobulin, n (%)		34 (59.6)	11 (35.4)	23 (88.4)	<0.0001
Pre-existing	n (%)	8 (53.3)	2 (13.3)	6 (40)	
DSA class I	Mean MFI ± SD	2614.56 ± 18.1	1014.4 ± 0.0	2904.36 ± 22.1	0.021
Pre-existing	n (%)	4 (26.6)	0 (0)	4 (26.6)	
DSA class II	Mean MFI ± SD	2521.24 ± 38.1	0.0 ± 0.0	2521.24 ± 38.1	<0.0001
Pre-existing	n (%)	3 (20)	0 (0)	3 (20)	
DSA class I & II	Mean MFI ± SD	2228.1 ± 27.3	0.0 ± 0.0	2228.1 ± 27.3	<0.0001
De novo	n (%)	16 (61.5)	0 (0)	16 (61.5)	
DSA class I	Mean MFI ± SD	3119.25 ± 21.4	0.0 ± 0.0	3119.25 ± 21.4	<0.0001
De novo	n (%)	7 (26.9)	0 (0)	7 (26.9)	
DSA class II	Mean MFI ± SD	4112.12 ± 19.2	0.0 ± 0.0	4112.12 ± 19.2	<0.0001
De novo	n (%)	3 (11.5)	0 (0)	3 (11.5)	
DSA class I & II	Mean MFI ± SD	4252.11 ± 14.2	0.0 ± 0.0	4252.11 ± 14.2	<0.0001

aTCMR: acute T-cell-mediated rejection; aABMR: acute antibody-mediated rejection; HLA: human leukocyte antigen; MPA: mycophenolic acid and mycophenolatemofetil; Tac: tacrolimus.

in 19 (50.0%). The Banff scores for the aTCMR biopsies revealed the following: borderline-suspicious grafts in 7 (19.4%); grade 1A rejection in 5 (13.9%); grade 1B rejection in 11 (30.5%); grade 2A rejection in 9 (25.0%); grade 2B rejection in 1 (2.7%); and grade 3 rejection in 3 (8.4%). In the sample as a whole, the mean serum creatinine and estimated glomerular filtration rate at biopsy were 5.5 ± 3.61 and 20.2 ± 3.61 respectively.

As shown in Table 2, CD56+ (P = 0.007) and CD57+ (P = 0.003) expression in the interstitial compartment was higher for the aTCMR group than for the aABMR-C4d+ and aABMR-C4d− subsets. In the vascular compartment, CD57+ expression was also higher for

Table 2. Comparison among the acute T-cell mediated rejection group and the two subsets of the acute antibody-mediated rejection group, in terms of the immunostaining for natural killer cell markers in the different compartments of biopsies obtained from patients diagnosed with acute renal allograft rejection.

				aABMR							
	aTCMR (n = 36)			C4d− (n = 19)			C4d+ (n = 19)				
Variable	Med	Min	Max	Med	Min	Max	Med	Min	Max	P*	Relationships†
CD56 interstitial	0.4	0.0	17.0	0.08	0.0	1.17	0.25	0.0	1.35	0.007	aTCMR > aABMR-C4d−
CD56 glomerular	0.0	0.0	1.1	0.0	0.0	0.91	0.07	0.0	0.66	ns	
CD56 vascular	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	ns	
CD57 interstitial	2.6	0.0	44.0	0.53	0.05	7.82	0.8	0.17	8.7	0.003	aTCMR > aABMR (both)
CD57 glomerular	0.9	0.0	30.8	0.28	0.0	3.8	0.28	0.0	6.57	ns	
CD57 vascular	0.0	0.0	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.005	aTCMR > aABMR (both)
CD16 interstitial	5.3	0.1	70.0	2.4	0.23	23.4	4.72	0.09	64	ns	
CD16 glomerular	1.3	0.0	11.0	2.0	0.5	13.09	2.68	0.4	6.8	ns	
CD16 vascular	0.0	0.0	4.0	0.0	0.0	1.0	0.0	0.0	4.25	ns	
CD68 interstitial	5.0	0.1	64.5	2.62	0.0	20.4	5.16	0.12	76.6	ns	
CD68 glomerular	0.6	0.0	16.5	0.78	0.0	6.47	1.53	0.0	11.75	0.004	aTCMR < aABMR-C4d+
CD68 vascular	0.0	0.0	8.0	0.0	0.0	3.0	0.0	0.0	4.6	ns	
CD3 interstitial	40.4	3.2	87.6	4.54	0.0	44.9	10.6	0.0	42.1	0.000	aTCMR > aABMR (both)
CD3 glomerular	0.8	0.0	8.6	0.0	0.0	3.08	0.42	0.0	3.6	0.027	aTCMR > aABMR-C4d−
CD3 vascular	0.0	0.0	6.3	0.0	0.0	0.0	0.0	0.0	4.0	0.024	aTCMR > aABMR-C4d−
CD8 interstitial	28.0	1.9	77.1	4.7	0.1	42.8	10.0	0.18	81.1	0.000	aTCMR > aABMR (both)
CD8 glomerular	0.6	0.0	7.6	0.09	0.0	3.3	0.8	0.0	5.0	ns	
CD8 vascular	0.0	0.0	4.5	0.0	0.0	0.0	0.0	0.0	2.6	0.042	aTCMR > aABMR-C4d−
CD4 interstitial	1.8	0.0	74.5	0.19	0.0	16.2	0.65	0.0	22.2	0.001	aTCMR > aABMR-C4d−
CD4 glomerular	0.0	0.0	0.4	0.0	0.0	0.8	0.0	0.0	2.3	ns	
CD4 vascular	0.0	0.0	6.0	0.0	0.0	0.0	0.0	0.0	4.5	ns	

aTCMR: acute T-cell mediated rejection; aABMR: acute antibody-mediated rejection; C4d−: without C4d deposition, C4d+: with C4d deposition; ptc: peritubular capillary (compartment); i: interstitial (compartment); g: glomerular (compartment); v vascular (compartment).

*Kruskal–Wallis test.

†Dunn's multiple comparisons test.

aTCMR group (P = 0.005). aTCMR group also showed higher expression for CD3+ (in the interstitial, glomerular, and vascular compartments, P = 0.000, P = 0.027, and P = 0.024, respectively) and for CD8+ (in the interstitial and vascular compartments, P = 0.000, and P = 0.042 respectively). The expression of CD4+ in the interstitial compartment was also higher in aTCMR group (P < 0.001).

Figure 2 shows the expression of CD56+ and CD57+ in the interstitial compartment, as well as that of CD16+ and CD68+ in the glomerular compartment, between the aTCMR and aABMR groups. The expression of CD56+ and CD57+ in the interstitial compartment was significantly higher in the aTCMR group (P = 0.004 and P = 0.001 respectively). As shown in Table 3, staining for CD57+ cells in the glomerular compartment was significantly more intense in the aTCMR group than in the aABMR group (P = 0.046). In that same compartment, the median expression of CD16+ cells was 1.3 for the aTCMR group and 2.3 for the aABMR group (P = 0.030), whereas that of CD68+ was 0.6 for the aTCMR group and 1.5 for the aABMR group (P = 0.01).

In the interstitial compartment of the aTCMR group biopsies, we found significant correlations between the expressions of the following markers: CD56 and CD57 (r = 0.429; P = 0.009); CD56 and CD16 (r = 0.489; P = 0.003); and CD57 and CD16 (r = 0.483; P = 0.003). Similar correlations were observed for the aABMR group (Table 4). In that same compartment, we observed no correlation between CD68+ expression and that of CD56+, in either group. In the glomerular compartment, the significant correlations were between the expressions of CD56 and CD57 in the aABMR

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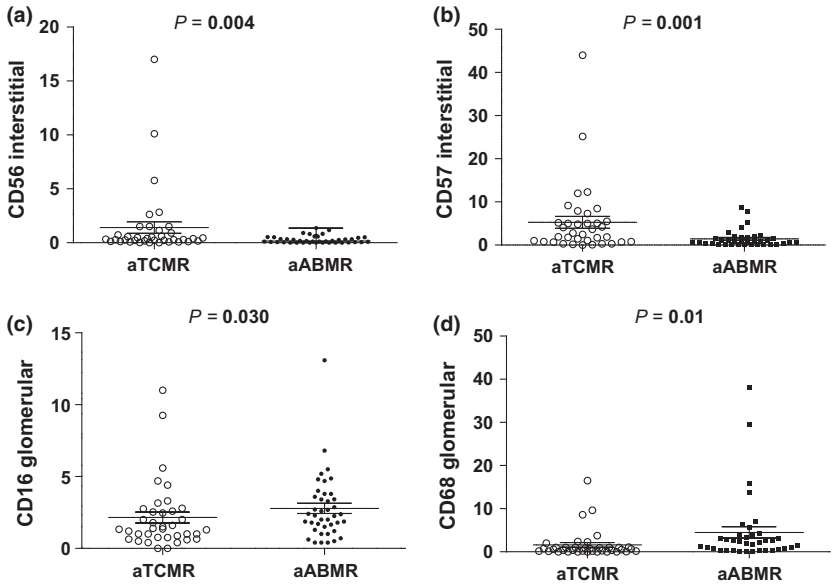

Figure 2 Comparisons between the acute T-cell-mediated rejection (aTCMR) and acute antibody-mediated rejection (aABMR) groups, in relation to CD56+ and CD57+ cells in the interstitial compartment, as well as CD16+ and CD68+ cells in the glomerular compartment.

group ($r = 0.355$; $P = 0.029$); between those of CD56 and CD16 in the aABMR group ($r = 0.407$; $P = 0.011$); and between those of CD16 and CD68, in the aTCMR group ($r = 0.36$; $P = 0.029$) and aABMR group ($r = 0.562$; $P = 0.000$).

Discussion

Natural killer cells have been the subject of research in recent publications involving pathology of renal transplantation. In 2008, Zhang *et al.* demonstrated that in situations of ischemia-reperfusion injury, renal tubular epithelial cells signal to NK cells. Hence, NK cells are able to enhance the inflammatory response by recruiting neutrophils and releasing perforin [13]. Subsequent studies showed that activated NK cells can mediate the apoptosis of tubular epithelial cells [14] and that can also contribute to the cell-mediated alloresponse and acute rejection [15,16]. Using an experimental model of MHC-compatible kidney transplantation, another study demonstrated that the activity of NK cells could persist over time despite this MHC compatibility and could mediate long-term transplant kidney injury independent of T and B cells [17]. Studies have also shown that, depending upon the types of NK cell receptors engaged and the nature of cytokine released, early NK cell activation can triggering acute rejection or tolerance [18–20].

In our study, we used immunohistochemical markers related to NK cells (CD56 and CD57), including low-affinity IgG Fc receptor (CD16) and extending these analyses to encompass macrophage/monocyte markers (CD68), in biopsies obtained from renal transplant recipients. Our objective was to evaluate the role that NK cells can play in the context of acute renal allograft rejection with or without the presence of DSA.

Considering the NK cells and macrophages/monocytes as constituents of the innate immune response [21], we also studied markers of the adaptive immune response, such as CD3, CD4, and CD8. We complemented this panoramic immunohistochemical assessment of immune cell markers in acute rejection by counting cells in the different compartments. Various studies have reported that the manifestations of renal allograft rejection differ among compartments, for example, vasculopathy having received considerable attention at the Banff conference [22] discussions about microcirculation injury especially in terms of to the importance of the glomeruli, peritubular capillaries [23], and interstitium [24], as well as in specific situations within each type of rejection.

Our results show that there was a predominance of interstitial CD56+, CD57+, CD3+, and CD8+ cells in the aTCMR biopsies. CD56 is a traditional marker of the immunophenotype of NK cells, which constitute a distinct lymphocyte subtype. CD56 is a 140-kDa isoform known as a neural cell adhesion molecule; it can be found on NK cells and on a minority of T lymphocytes [25]. CD57+ cells can include NK cells and TCD8+ cells [16].

Table 3. Comparison between the acute T-cell mediated rejection group and the acute antibody-mediated rejection group, in terms of the immunostaining for natural killer cell markers in the different compartments of biopsies obtained from patients diagnosed with acute renal allograft rejection.

	aTCMR (n = 36)			aABMR (n = 38)			
Variable	Med	Min	Max	Med	Min	Max	P*
CD56 interstitial	0.4	0.0	17.0	0.1	0.0	1.4	0.004
CD56 glomerular	0.0	0.0	1.1	0.0	0.0	0.9	ns
CD56 vascular	0.0	0.0	0.0	0.0	0.0	0.0	ns
CD57 interstitial	2.6	0.0	44.0	0.8	0.1	8.7	0.001
CD57 glomerular	0.9	0.0	30.8	0.3	0.0	6.6	0.046
CD57 vascular	0.0	0.0	2.3	0.0	0.0	0.0	0.001
CD16 interstitial	5.3	0.1	70.0	3.1	0.1	64.0	ns
CD16 glomerular	1.3	0.0	11.0	2.3	0.4	13.1	0.030
CD16 vascular	0.0	0.0	4.0	0.0	0.0	4.3	ns
CD68 interstitial	5.0	0.1	64.5	3.5	0.0	76.6	ns
CD68 glomerular	0.6	0.0	16.5	1.5	0.0	11.8	0.004
CD68 vascular	0.0	0.0	8.0	0.0	0.0	4.6	ns
CD3 interstitial	40.4	3.2	87.6	6.4	0.0	44.9	0.000
CD3 glomerular	0.8	0.0	8.6	0.1	0.0	3.6	0.017
CD3 vascular	0.0	0.0	6.3	0.0	0.0	4.0	0.010
CD8 interstitial	28.0	1.9	77.1	6.6	0.1	81.1	0.000
CD8 glomerular	0.6	0.0	7.6	0.4	0.0	5.0	ns
CD8 vascular	0.0	0.0	4.5	0.0	0.0	2.6	0.019
CD4 interstitial	1.8	0.0	74.5	0.5	0.0	22.2	0.002
CD4 glomerular	0.0	0.0	0.4	0.0	0.0	2.3	ns
CD4 vascular	0.0	0.0	6.0	0.0	0.0	4.5	ns

aTCMR: acute T-cell mediated rejection; aABMR: acute antibody-mediated rejection.

*Mann–Whitney test.

The pathogenesis of aTCMR typically involves antigen-presenting cells known as dendritic cells. The mechanism of recognition between T lymphocytes and MHC promoted by antigen-presenting cells of the donor (direct pathway) or recipient (indirect pathway) is the basis of the acute alloimmune response, and the presence of MHC molecules determines the differentiation into CD8+ and CD4+T cells [26].

Natural killer cells recognize their targets via a large complex of receptors. NK cell activity is mediated by a balance between the activating receptors responsible for the killing activity of the cells and the inhibitory receptors that modulate their cytotoxicity [27]. NK cells are also known to be major producers of interferon gamma (IFN-γ) in pathological conditions. The production of IFN-γ might modulate the response of T cells in peripheral lymphoid organs. This modulation occurs after the NK cells migrate to these secondary organs. After that migration, there is IFN-γ-mediated interaction between naïve T cells and NK cells [28].

Our interpretation is that NK cells, together with other components of the immune system, such as CD8+ T cells, might have participated in or even enhanced the aTCMR processes observed in our study, given that the NK cell production of IFN-γ increases the recruitment of alloantigen-specific T cells [29]. However, we found a predominance of CD16+ and CD68+ cells in the glomerular compartment in the cases of aABMR.

In NK cells, there can also be expression of CD16, a cell surface receptor capable of allowing NK cells to detect antibodies (via FcγRIIIA) and to exert ADCC. CD16 is a low-affinity receptor that binds opsonized antibodies and promotes intracellular signaling through the subunit containing an immunoreceptor tyrosine-based activation motif to exert ADCC [8]. The ADCC control is mediated by CD16 and regulated by immunoreceptor tyrosine-based inhibition motifs expressed by antigen-presenting cells [30]. Although CD16 is expressed on NK cells, neutrophils, and basophils, it is not expressed on B lymphocytes or on T lymphocytes belonging to certain subfamilies [31].

The predominance of CD16+ cells in the aABMR (and therefore DSA-positive) biopsies in our study could indicate the involvement of NK cells via the ADCC mechanism. In ADCC, target cells opsonized by IgG antibodies bind to FcγRIIIA (CD16) on NK cells and induce the release of cytotoxic granules, triggering apoptosis of the target cells [32].

We found it interesting that there was a predominance of CD16+ cells in the glomerular compartment, an area involved in injury to the microcirculation, which is being extensively studied as being likely associated with ABMR.

A recent study analyzing the expression of selective transcripts found evidence that NK cells and the CD16a Fc receptor are involved in ABMR. The data derived from that study suggest a model of ABMR that includes microvascular injury and repair induced by cognate recognition involving antibody and CD16a, thus triggering IFN-γ release and NK cell-mediated ADCC [33]. CD16a can be expressed by NK cells and by monocytes. There is also a CD16b variant, which has an extracellular domain similar to that of CD16a but is expressed in neutrophils. It is known, however, that CD16b does not mediate the same functions as CD16a [34].

The elevated number of CD68+ cells in the glomerular compartment of aABMR biopsies might simply reflect a connection between those cells and the HLA

Table 4. Correlations among natural killer cell stainings (CD56+, CD57+, and CD16+ cells) and CD68+ cells evaluated in biopsies obtained from patients diagnosed with acute renal allograft rejection, by compartment and type of rejection.

Compartment		aTCMR			aABMR		
Interstitial	Statistic	CD57	CD16	CD68	CD57	CD16	CD68
CD56	*r**	0.429	0.489	0.313	0.336	0.356	0.178
	P	0.009	0.003	ns	0.039	0.028	ns
CD57	*r**		0.483	0.435		0.595	0.514
	P		0.003	0.008		0.000	0.001
CD16	*r**			0.609			0.535
	P			0.000			0.001
		aTCMR			aABMR		
Glomerular	Statistic	CD57	CD16	CD68	CD57	CD16	CD68
CD56	*r**	0.100	0.149	0.149	0.355	0.407	0.182
	P	ns	ns	ns	0.029	0.011	ns
CD57	*r**		0.273	0.191		0.251	0.067
	P		ns	ns		ns	ns
CD16	*r**			0.364			0.562
	P			0.029			0.000

aTCMR: acute T-cell mediated rejection; aABMR: acute antibody-mediated rejection.

*r**: Spearman's correlation coefficient.

antibodies adhered to endothelial cells. That would corroborate the findings of other studies that found an association between the presence of macrophages/monocytes and ABMR in renal transplantation [35,36].

It seems that the glomerular and tubulointerstitial compartments differ in terms of the recruitment and activation of immune cells. This discussion could extend to the study of biopsies obtained from kidney transplant recipients. In relation to the distinctive profiles of immunohistochemical markers by renal parenchyma compartments, there is at least one plausible hypothesis. A study of compartment-specific expression of dendritic cell markers suggested that the glomerular compartment represents a special immunological microenvironment and that the cells infiltrating in the glomeruli and tubulointerstitium were of varying types, as evidenced by the differences among their surface markers [37].

Taking into account the specific limitations of using immunohistochemical markers for the identification of NK cells, we can suggest that these cells are involved in the pathogenesis of acute rejection in kidney transplantation. On the basis of our findings, we can also suggest that these markers can express themselves in different ways and in different compartments depending on the type of rejection. Our data could lay the groundwork for further studies seeking evidence to support the participation of NK cells in acute rejection in general and in ABMR in particular.

Authorship

DCdosS: PERFORMED CONCEPTION AND DESIGN OF STUDY, collected clinical and histological data, performed histological evaluation of the biopsy specimens, developed and performed the immunohistochemical analysis, analyzed data and wrote the paper. EFC: assisted in study design. NOSC: assisted in study design and supervised the analysis of clinical data. DSRD: perfomed histological evaluation of the biopsy specimens. DMACM: assisted in study design and supervised the analysis of HISTOLOGICAL AND IMMUNOHISTOCHEMICAL DATA OF BIOPSY SPECIMEN.

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