Effector memory CD4^{+} T-cells and dendritic cells are noninvasive biomarkers of late cellular rejection after kidney transplantation

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Introduction. *Diagnosis of the kidney transplant cellular rejection in
the long-term after transplantation remains a challenge. Usual surrogate*

markers are not enough sensitive and specific. Rejection is an immune reaction to donor alloantigens. The kidney transplant biopsy to diagnose a dysfunction is an invasive procedure with the incidence of complications about 12.6% and can lead to transplant loss. In this regard, the search of immunological biomarkers for early noninvasive and accurate diagnosis of kidney transplant rejection is an actual task.

__Material and methods.__ This is a report of the observational retrospective single-center, comparative case-control study in two groups involving 44 patients who underwent kidney transplantation. The first group (REJ) included the patients with the chronic graft dysfunction caused by a biopsy-confirmed late cellular rejection (22 patients). The second group (STA) included the recipients who had no dysfunction in the posttransplant period (22 patients). Flow cytometry of peripheral blood cells was performed to identify immunophenotyping markers of late cellular rejection after kidney transplantation (we determined subpopulations of T, B lymphocytes, and dendritic cells).

__Results.__ As a result of our work, we found significant differences in the absolute count of effector memory T-cells making 0.147 (0.115–0.260) × 10^9 cells/L in REJ group, and 0.106 (0.067–0.136) × 10^9 cells/L in STA group (p = 0.0167). Relative and absolute counts of myeloid dendritic cells were also different between the groups: 0.65 (0.36–0.73) vs. 1.05 (0.67–1.4) % and 0.039 (0.028–0.056) vs. 0.063 (0.049–0.076) × 10^9 cells/L, respectively (p = 0.0009, p = 0.003). The numbers of plasmacytoid dendritic cells were also different between the study groups: 0.0038 (0.0021–0.0054) vs. 0.005 (0.0035–0.007) × 10^9 cells/L for an absolute count (p = 0.0414), and 0.055 (0.04–0.085) vs. 0.09 (0.05–0.12) % for a relative count (p = 0.0197).

Conclusion. *The obtained data showed that the blood level of dendritic cells, which are the main "professional" initiators of immune reaction, and the level of effector helper T memory cells, which constitute the main lymphocyte subpopulation posing a destructive impact on the kidney transplant, can be considered as diagnostic markers of kidney transplant cellular rejection in the long-term after surgery.*

Keywords: kidney transplantation, rejection, immune monitoring

Abbreviations

CFM, cytofluorometry

HLA, human leukocyte antigen

ICC, immunocompetent cells

IT, immunosuppressive therapy

KT, kidney transplantation

mDc, myeloid dendritic cells

pDc, plasmacytoid dendritic cells

PRA, panel reactive antibody

REJ, main study group

STA, comparison group

U-test, Mann-Whitney test

Introduction

Currently, kidney transplantation (KT) is the most optimal method of renal replacement therapy. Kidney transplant both improves the quality of life of patients with chronic kidney disease, and also significantly prolongs

their survival. Kaballo et al. showed that a 5-year mortality risk in patients after kidney transplantation is 47% lower than in patients undergoing renal replacement therapy [1].

One of the most significant factors affecting the results of transplantation is the renal allograft rejection reaction. Despite the immunosuppressive therapy (IT), the incidence of acute rejection in the early post-transplant period is about 10%. A cellular rejection in the renal graft in late postoperative period (over 1 year) occurs even more often, develops in 35% of patients and is classified, according to Nair et al., as a late cellular rejection [2]. The development of the late cellular rejection results in immunological processes in kidney graft becoming chronic, and it also constitutes one of the causes for the graft function loss [3].

Currently, the "gold standard" for diagnosing a graft rejection reaction is a percutaneous needle biopsy. However, this is an invasive procedure and it is associated with the risk of complications which, according to literature reports, occur as frequent as in 5.2% to 12.56%, and can lead to the allograft loss in 0.25% of cases [4]. Neither the laboratory markers traditionally used to diagnose the kidney function, nor the ultrasound examination of the graft have sufficient sensitivity and specificity.

A promising trend in a non-invasive diagnosis of the rejection reaction in KT is a cytofluorometry (CFM). The use of this technique makes it possible to quantify simultaneously the numbers of practically all subpopulations of peripheral blood leukocytes. However, at present phenotypic biomarkers that would reliably reflect immunological processes in the graft have not been identified and no protocols have been developed on the clinical use of CFM as a method for diagnosing the rejection reaction in a renal allograft.

In this regard, the **goal of our work** was to study the subpopulations of peripheral blood lymphocytes in patients after KT and to develop the CFM criteria for diagnosing a late cellular rejection.

Material and methods

An observational, retrospective, single-center, analytical, comparative study in two groups was conducted in the "case-control" design, and included 44 kidney transplant recipients who were investigated in the *Republican Scientific-Practical Center of Organ and Tissue Transplantation at City Clinical Hospital No. 9,* Minsk, Republic of Belarus. The patients underwent KT from a brain-death donor in the period between 2004 and 2013.

The inclusion criteria in the study were the following: the recipients after transplantation of a renal allograft only; transplantation from a donor with brain death; the follow-up period of no less than 4 years; the recipients aged from 18 to 70 years old; the presence of the kidney graft in the study period; receiving immunosuppression; the possibility to obtain a voluntary informed to participate in the study. The exclusion criteria were as follows: a high risk of immune complications at the time of transplantation (no human leukocyte antigen [HLA] matches in the donor-recipient pair, the recipient's serum panel reactive antibodies [PRA] > 15%); infectious complications and oncological diseases.

The allocation into study groups was based on the characteristics of the post-transplantation course and the results of the kidney graft histology examination.

The main study group (REJ) consisted of 22 recipients with a chronic post-transplant dysfunction due to late cellular rejection. The late cellular

rejection of the kidney graft developed both after an acute rejection crisis of the allograft in 9 (40.9%) of 22 patients, and without previous acute graft dysfunction with a slowly progressing chronic injury in 13 (59.1%) patients. Later, the renal graft cellular rejection was confirmed by histological examination and by the assessment according the International Standardized Banff Classification Criteria. No other causes of chronic graft dysfunction were revealed by histological examination in patients of this group.

The comparison group (STA) included 22 recipients who had no renal graft dysfunction in the post-transplant period. The group was formed using a random number method.

Among 44 study participants, there were 21 men (47.7%), and 23 women (52.3%) (Table 1). The median age of study participants was 49 (39.5-55.5) years. The prevalent pathology leading to the end-stage chronic kidney disease was chronic glomerulonephritis in 34/44 (77.3%) patients. Hemodialysis as a renal replacement therapy before transplantation was given to 38 (86.4%) recipients. The duration of being on the dialysis therapy averaged 71.8 (39-105) months. The time of graft preservation did not exceed 24 hours and averaged 8.5 (6.75-10) hours. The donor-recipient pairs most often matched in two MHC HLAs class I in 21 (47.7%) of 44 patients. The PRA level of pre-existing antibodies at the time of enrollment in the study did not exceed 15%. Pre-existing antibodies were detected at the time of transplantation in 7 (15.9%) study participants.

IT was standard, performed according to the Clinical Management Protocol for Patients after KT, and did not differ between the study participants of the two groups (see Table 1).

Table 1. Comparison of demographic and clinical characteristics between the study groups

Characteristic	REJ	STA	Significance level
Gender			
male	12 (54.5%)	9/22 (40.9%)	p = 0.365
female	10 (45.5%)	13/22 (59.1%)	
Age, years	47.5 (35-56)	49 (43-55)	p = 0.622
Pathology			
chronic glomerulonephritis	18 (81.8%)	17 (77.3%)	p = 0.73
congenital anomaly of the urinary tract	1 (4.55%)	1 (4.55%)	
polycystic disease	1 (4.55%)	1 (4.55%)	
diabetes	1 (4.55%)	3 (13.6%)	
genetic pathology	1 (4.55%)	0 (0%)	
HLA matches			
$\leq$ 3 mismatches	18 (81.8%)	19 (86.4%)	p = 0.68
> 3 mismatches	4 (18.2%)	3 (13.6%)	
PRA at time of transplantation	4 (18.2%)	3 (13.6%)	p = 0.679
Period on dialysis therapy, months	70 (46-107.5)	60 (17-86)	p = 0.336
Cold ischemia time, hours	8.75 (7-10)	7.5 (6.5-20)	p = 0.411
Induction IT			
antithymocyte globulin	9 (40.9%)	14 (63.6%)	p = 0.13
basiliximab	13 (59.1%)	8 (36.4%)	
Calcineurin inhibitor			
cyclosporin A, mg/day	150 (100-175)	150 (125-150)	p = 0.472
cyclosporine concentration, ng/mL	76 (63.1-83)	70.1 (63.7-87)	p = 0.87
tacrolimus, mg/day	4 (2-5)	3 (2.5-3)	p = 0.344
tacrolimus concentration, ng/mL	5.2 (4.95-5.9)	5.8 (5.18-5.95)	p = 0.7
Antimetabolite			
mycophenolic acid, mg/day	1000 (1000-2000)	1000 (1000-1000)	p = 0.66
azathioprine, mg/day	100 (75-100)	100 (100-100)	p = 0.264
Glucocorticosteroids			
methylprednisolone, mg/day	4 (2-4)	2 (2-4)	p = 0.378
Graft function			
primary graft dysfunction	12 (54.5%)	2 (9%)	**p = 0.0014**
serum creatinine, μmol/L	163 (101-218)	76.4 (65-93)	**p <0.01**
one time proteinuria, g/L	0.315 (0.046-0.789)	0.046 (0-0.186)	**p <0.01**
Glomerular filtration rate, ml/min	46.9 (27-75.4)	82 (70-98)	**p <0.01**

Note: the results are presented as a median (1st quartile; 3d quartile)

The study protocol was approved by the Ethics Committee of *City Clinical Hospital No. 9,* Minsk. A voluntary informed consent to participate in the study was obtained from every patient. All patients underwent CFM assay with the calculation of the absolute and relative counts of peripheral blood leukocyte subpopulations.

Immunophenotype of peripheral blood cells was determined by means of the eight-color CFM technique using a FACSCanto II on a flow cytofluorimeter (Becton Dickinson, USA) equipped with three lasers (488 nm, 633 nm, 405 nm). The data were analyzed using the FACSDiva computer software, version 6.

To determine the counts of leukocyte subpopulations, 100 μL of peripheral blood was incubated with appropriate monoclonal antibodies in the amount according to the manufacturer's prescription for 15 minutes at 4° C. The erythrocytes were then lysed with a solution of ammonium chloride for 10 minutes at 4° C. The cells were then pelleted by centrifugation at 1500 rpm for 5 minutes. The supernatant was removed; the cells were suspended in 200 mcl of phosphate-buffered saline (PSB). For further analysis, the cells were loaded in the volume of at least 10 000 events in a T-lymphocyte region; at least 500 events in the region of dendritic cells and at least 3000 events in the B-lymphocyte region.

To detect the immunophenotype of dendritic cells, natural killers, T- and B-lymphocytes, the following monoclonal antibodies were used: CD45-PerCP (ExBio, Czech Republic), CD45RA-FITC (Beckman Coulter, USA), CD62L-PE (Beckman Coulter, USA), CD127 -PC7 (Beckman Coulter, USA), CD25-APC (Beckman Coulter, USA), CD4-APC-Cy7 (ExBio, Czech Republic), CD3-Pacific Blue (Beckman Coulter, USA), CD8-Krome Orange (Beckman Coulter, USA), CD11-PE (ExBio, Czech Republic), CD123-PC7

(Beckman Coulter, USA), CD56-APC (ExBio, Czech Republic), CD19-APC-Alexa Fluor 750 (Beckman Coulter, USA), HLA-DR-Krome Orange (Beckman Coulter, USA), CD38-FITC (Beckman Coulter, USA), IgD-PE (Becton Dickinson, USA), CD27-PC7 (Beckman Coulter, USA), CD5-APC (Beckman Coulter, USA), IgM-Pacific Blue (Beckman Coulter, USA).

The main subpopulations of immunocompetent cells (ICCs) were gated according to the developed and standardized protocol.

The statistical analysis of the quantitative data in groups was made using the Mann-Whitney's U test. The results of the quantitative data comparison are presented as REJ Median (25% quartile to 75% quartile) vs. STA Median (25% quartile to 75% quartile), the significance level of differences. To compare the qualitative data in the study groups, the χ^2 test was used. The results of the qualitative data comparison are presented as REJ Absolute rate (Percentage) vs. STA Absolute rate (Percentage), the significance level of differences. To identify the correlation between the data types, the Spearman criterion was used for quantitative data, and the association criterion for the qualitative data. The correlation between the qualitative and quantitative data was estimated using a rank-biserial test. The statistical significant difference between the obtained results was set at $p \leq 0.05$.

Results

Characteristics of study participants

Among the study participants, an acute graft dysfunction in the long-term occurred in 9 (40.9%) of 22 recipients.

Meanwhile, the development of acute rejection crisis was associated with the occurrence of chronic graft dysfunction (the association coefficient

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0.65). The results of the needle allograft biopsy (NAB) analyzed at patient's inclusion in the study showed that Banff Grade 1A rejection was detected in 13 (59.1%) of 22 recipients, Grade 1B rejection was identified in 2 (9%), and Grade 2A rejection was found in 3 (13.6%) recipients. Initial manifestations of chronic graft rejection were noted in 4 (18.3%) participants of the REJ group.

The statistical analysis of results demonstrated no impact of the clinical factors on the incidence of the kidney graft immunological dysfunction developed. A strong correlation was found between the rejection reaction and the primary allograft dysfunction. No relationship between the grade of histologically confirmed renal allograft rejection and the number of ICC subpopulations was found.

Cytofluorometry results

The CFM results (Table 2) showed significant differences between the groups in the absolute count of CD4+ effector memory T-cells: 0.147 (0.115-0.260) vs. 0.106 (0.067-0.136) $\times 10^9$ cells/L; p = 0.0167 (Fig. 1A).

Significant differences between the REJ and STA groups were also found in the counts of myeloid and plasmacytoid dendritic cells (mDc and pDc). Thus, the relative count of mDc was lower in the group with histologically confirmed immune conflict and made 0.65 (0.36-0.73) vs. 1.05 (0.67-1.4); p=0.0009. Accordingly, the absolute numbers of mDc in peripheral blood in the groups were 0.039 (0.028-0.056) vs. 0.063 (0.049-0.076) $\times 10^9$ cells/L; p =0.003 (Fig. 1B, C).

A)

B)

C)

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Difference in the relative counts of plasmacytoid dendritic cells

p = 0.0197

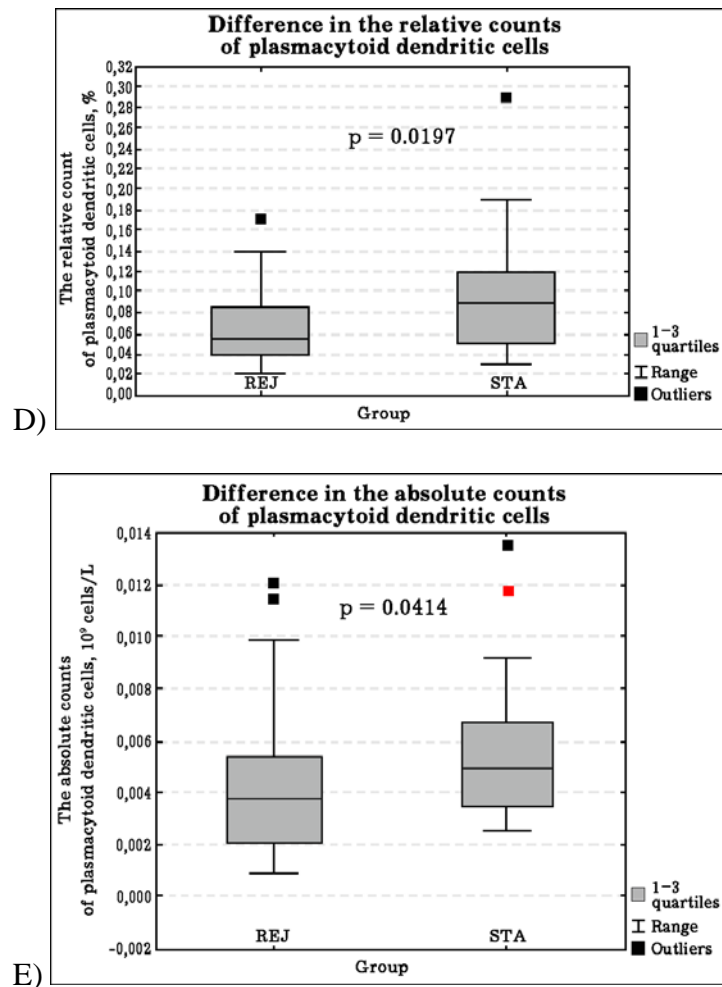**Difference in the absolute counts of plasmacytoid dendritic cells**

p = 0.0414
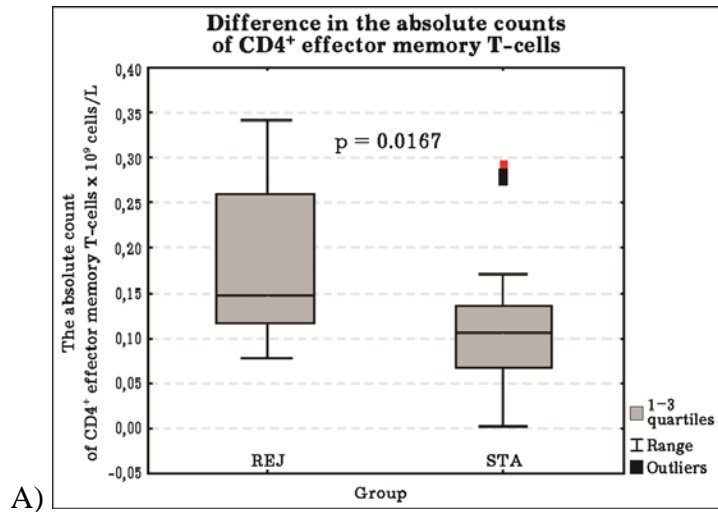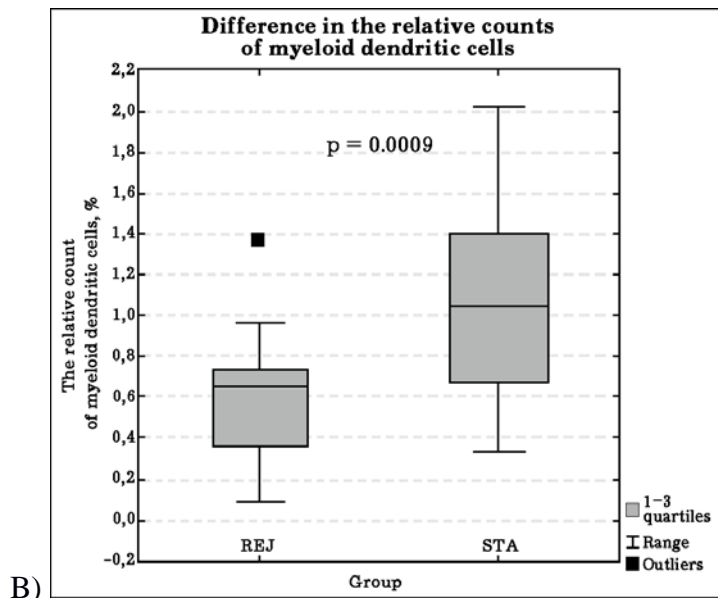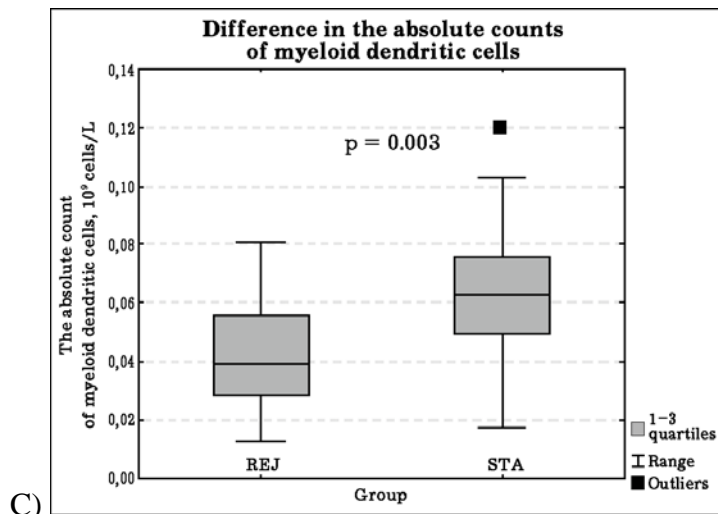
Fig. 1. Differences in subpopulations of immunocompetent cells between the study groups: A, a higher absolute count of effector memory T helper cells in the group of patients with the kidney transplant rejection; B and C, lower relative and absolute counts of myeloid dendritic cells in the group with an immune conflict; D and E, decreased relative and absolute counts of plasmacytoid dendritic cells in the main study group

The reduction in the count of pDc was also detected in the group of recipients with a complicated post-transplantation course. The pDc

percentage in the REJ and STA groups was 0.055 (0.04-0.085) vs. 0.09 (0.05-0.12) %; p=0.0197. The absolute number of pDc was 0.0038 (0.0021 - 0.0054) vs. 0.005 (0.0035-0.0068) $\times 10^9$ cells/L; p=0.0414 (Fig. 1 D, E).

The analysis revealed no trends in the difference in the count of the remaining T-lymphocyte subpopulations, as well as in the counts of B-lymphocyte and natural killer subpopulations (Table 2).

Table 2. The results of immunophenotyping of peripheral blood leukocytes in the study groups

ICC Subpopulation	REJ	STA	p
T-lymphocytes, $\times 10^9$ cells/L CD3$^+$	1.25 (0.816-1.9)	1.35 (0.915-1.672)	0.805
T-helpers, $\times 10^9$ cells/L CD3$^+$ CD4$^+$	0.297 (0.21-0.415)	0.254 (0.158-0.453)	0.581
T-killers, $\times 10^9$ cells/L CD3$^+$ CD8$^+$	0.423 (0.31-0.73)	0.4755 (0.276-0.683)	0.699
T-regulatory cells, $\times 10^9$ cells/L CD3$^+$ CD4$^+$ CD25^{++} CD127$^-$	0.0124 (0.0084-0.023)	0.014 (0.0057-0.03)	0.860
"Naive" T-helpers, $\times 10^9$ cells/L CD3$^+$ CD4$^+$ CD45RA$^+$ CD62L$^+$	0.205 (0.143-0.412)	0.2695 (0.18-0.421)	0.318
CD4$^+$ central memory T-cells, $\times 10^9$ cells/L CD3$^+$ CD4$^+$ CD45RA- CD62L+	0.253 (0.184-0.345)	0.248 (0.158-0.453)	0.879
CD4$^+$ effector memory T-cells, % CD3$^+$ CD4$^+$ CD45RA$^-$ CD62L$^-$	22.45 (18.0-28.3)	16.55 (9.5-27.3)	0.069
CD4$^+$ effector memory T-cells, $\times 10^9$ cells/L CD3$^+$ CD4$^+$ CD45RA$^-$ CD62L$^-$	0.147 (0.115-0.260)	0.106 (0.067-0.136)	0.0167
CD4$^+$ effector T-lymphocytes, $\times 10^9$ cells/L CD3$^+$ CD4$^+$ CD45RA$^+$ CD62L$^-$	0.009 (0.006-0.028)	0.0135 (0.006-0.027)	0,824
"Naive" T-killers, x 10^9 cells/L CD3$^+$ CD8$^+$ CD45RA$^+$ CD62L$^+$	0.139 (0.066-0.195)	0.162 (0.087-0.25)	0.318
CD8$^+$ central memory T-cells, $\times 10^9$ cells/L CD3$^+$ CD8$^+$ CD45RA$^-$CD62L $^+$	0.029 (0.012-0.039)	0.024 (0.015-0.05)	0.991
CD8 $^+$ effector memory T-cells, $\times 10^9$	0.06 (0.043-0.089)	0.041 (0.015-0.086)	0.163

cells/L $CD3^+ CD8^+ CD45RA^- CD62L^-$			
$CD8^+$ effector T-lymphocytes, $\times 10^9$ cells/L $CD3^+ CD8^+ CD45RA^+ CD62L^-$	0.197 (0.118-0.367)	0.22 (0.078-0.31)	0.392
B-lymphocytes, $\times 10^9$ cells/L $CD19^+$	0.068 (0.047-0.086)	0.063 (0.026-0.096)	0.392
"Naive" B-lymphocytes, $\times 10^9$ cells/L $CD19^+ 27^- IgD^+ IgM^+$	0.038 (0.02-0.056)	0.028 (0.011-0.064)	0.366
"Non-switched" memory B-cells, $\times 10^9$ cells/L $CD19^+ 27^+ IgD^+ IgM^+$	0.008 (0.005-0.014)	0.0075 (0.0036-0.01)	0.392
"Switched" memory B-cells, $\times 10^9$ cells/L $CD19^+ 27^+ IgD^- IgM^-$	0.012 (0.009-0.017)	0.017 (0.006-0.025)	0.565
Regulatory B-cells, $\times 10^9$ cells/L $CD19^+ CD38^{++} CD24^{++} IgM^+ IgD^+$	0.0002 (0.00007-0.0006)	0.0002 (0.00002-0.00065)	0.842
Plasmacytes, $\times 10^9$ cells/L $CD19^+ 27^+ CD38^{++} IgD^- IgM^-$	0.00065 (0.0004-0.0014)	0.001 (0.0005-0.0025)	0,255
B-1a-lymphocytes, $\times 10^9$ cells/L $CD19^+ CD5^+ CD20^+$	0.00076 (0.0004-0.00099)	0.001 (0.0003-0.00215)	0.286
Bm-1-lymphocytes, $\times 10^9$ cells/L $CD19^+ IgD^+ CD38^-$	0.021 (0.014-0.032)	0.019 (0.01-0.024)	0.489
Bm-2-lymphocytes, $\times 10^9$ cells/L $CD19^+ IgD^+ CD38^+$	0.023 (0.0115-0.042)	0.017 (0.0075-0.035)	0.286
Bm-2'-lymphocytes, $\times 10^9$ cells/L $CD19^+ IgD^+ CD38^{++}$	0.00005 (0.0-0.00019)	0.00016 (0.0-0.0004)	0,275064
eBm-5-lymphocytes, $\times 10^9$ cells/L $CD19^+ IgD^- CD38^+$	0.011 (0.0074-0.014)	0.01 (0.0044-0.024)	0,787210
Bm-5-lymphocytes, $\times 10^9$ cells/L $CD19^+ IgD^- CD38^-$	0.0058 (0.0047-0.007)	0.006 (0.003-0.011)	0.823543
Myeloid dendritic cells,% $Lin- CD14^- HLA-DR^+ CD11c^+ CD123^-$	0.65 (0.36-0.73)	1.05 (0.67-1.4)	0.0009
Myeloid dendritic cells, $\times 10^9$ cells/L $Lin^- CD14^- HLA-DR^+ CD11c^+ CD123^-$	0.039 (0.028-0.056)	0.063 (0.049-0.076)	0.003
Plasmacytoid dendritic cells,% $Lin^- CD14^- HLA-DR^+ CD11c^- CD123^+$	0.055 (0.04-0.085)	0.09 (0.05-0.12)	0.0197
Plasmacytoid dendritic cells, $\times 10^9$ cells/L $Lin^- CD14^- HLA-DR^+ CD11c^- CD123^+$	0.0038 (0.0021-0.0054)	0.005 (0.0035-0.007)	0.0414
Natural killers, $\times 10^9$ cells/L $CD16^+ CD14^- CD56^+$	0.118 (0.036-0.2)	0.059 (0.0256-0.14)	0.342

Note: the results are presented as Me (1st quartile; 3d quartile).

Thus, Table 2 shows that among the studied lymphocyte subpopulations, the counts of CD4$^+$ effector memory T-cells, as well as myeloid and plasmacytoid dendritic cells, were statistically significantly different between the study groups.

Discussion

The study results have demonstrated that increased counts of CD4$^+$ effector memory T-cells and a decreased number/count of mDc in the blood of kidney transplant recipients are reliable noninvasive markers of late cellular rejection.

We believe that the data we have obtained are not casual and can be explained by the role of T lymphocytes and mDc in the immune response to alloantigens.

So, CD4$^+$ effector memory T-cells serve as some of the main components of an active immunological memory that support the activation and proliferation of donor-specific CD8$^+$ T-lymphocytes and B cells that, in turn, represent the main substrate of the graft rejection [5]. The increase in the count of this T-lymphocyte subpopulation in blood is most likely associated with the increased immune response to the graft alloantigens resulting in the development of the graft rejection and dysfunction.

Dendritic cells are also one of the main initiators of the immune response, ensuring the capture, processing, and presentation of the antigen to T-lymphocytes. In the system of transplantation immunity, the interaction of CD4$^+$ T-lymphocytes with mDc is the main triggering mechanism of the T-cell response. The decrease in the number of mDc in peripheral blood is associated with their migration to the inflammatory focus, i.e. to the graft

[6]. It was demonstrated that the detection of mDc in the renal graft tissues directly correlates with the allograft rejection Grade according to Banff classification. At the same time, the mDc density in renal graft tissues in the period of acute rejection is an independent predictor of the graft functional loss within 1 year [6]. Dendritic cells are a resident subpopulation, and apparently because of that, their count lacks increasing in blood of the recipients with an active immune response to alloantigens [8].

From the foregoing it follows that the differences we have found postoperatively in the immunophenotype of peripheral blood lymphocytes between the patients with a normal graft function and those with the developed late cellular rejection are objective and pathogenetically well-grounded.

Conclusion

The relevance of results we have obtained lies in the possible effective, rapid, and non-invasive diagnosis of the cellular rejection reaction in the renal graft in patients after kidney transplantation in the long-term.

Cytofluorimetry with the assessment of T-lymphocyte and dendritic cell subpopulations can be used for the differentiated diagnosis of renal allograft dysfunction without the risk of causing complications for both the graft and the patient.

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