

Clinical Significance of the Pre-Transplant CXCR3 and CCR6 Expression on T Cells In Kidney Graft Recipients

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ABSTRACT

Background. T cells play a fundamental role in the processes that mediate graft rejection, tolerance, and defense against infections. The CXCR3 and CCR6 receptors, highly expressed in Th1 (type 1 T helper cells)/Tc1 (T cytotoxic cells, type 1), Th1-Tc1, and Th17-Tc17 lymphocytes, respectively, participate in cell migration toward inflamed tissues. The altered expression level of CXCR3 and CCR6 has been associated with different clinical events after renal transplantation, such as acute rejection (AR) and chronic graft dysfunction, but data are still limited. In this study, we evaluated the expression of the receptor CXCR3 and CCR6 in peripheral blood T lymphocytes from kidney transplant recipients (KTR) and their association with viral infections, AR, and allograft function.

Methods. Through flow cytometry, the peripheral blood expression of CXCR3 and CCR6 in T cells was evaluated in a pretransplant collection of KTR. The levels of these T subpopulations and their association with the incidence of AR, kidney graft function, viral infections, cytomegalovirus, and BK virus were studied. Adverse clinical events and graft function were monitored during the first year post transplant.

Results. KTRs with low pretransplantation levels of Th17 (CD4+CXCR3-CCR6+) (tertile 1, Th17<16.4%) had a higher risk of suffering AR during the first year post transplantation ($P = .033$). KTRs with viral infections or reactivations during the first 3 months post transplantation had significantly lower levels of Tc17 (CD8+CXCR3-CCR6+) and higher levels of Th1 (CD4+CXCR3+CCR6-). In patients with cytomegalovirus reactivations, the viral peak correlates negatively with the pretransplant levels of Th1 ($r = -0.606$, $P = .037$).

Conclusions. Pretransplantation assessment of Th1-Th17 and Tc1-Tc17 levels may help predict post-transplant clinical events such as AR and reactivation of viral infections.

B and T lymphocytes are essential to the biological processes that control renal transplant tolerance and rejection through several effector mechanisms, including antibody formation, antigen presentation to T cells, and the release of cytokines and chemokines [1]. Regulatory B cells can interact with various cell populations directly by preventing the response of Th1 and Th17 cells, indirectly promoting the growth of regulatory T cells (Treg) [2,3]. Different studies have shown that TGF- β secretion inhibits Th1 responses [4]. Mice were deficient

The contribution of I. Legaz and M. Muro is equal and the order is arbitrary

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in TGF- β increase of Th1 and Th17 responses, crucial for Treg induction [5].

Moreover, T lymphocytes play a fundamental role in the transplantation field in the processes that mediate graft rejection and tolerance and in the defense of the receptor against infection [6,7]. The efficacy of effector T cells is counterbalanced by Tregs that infiltrate many tumors and suppress local effector T cell responses [8,9].

The chemokine receptors CXCR3 and CCR6 are highly expressed in Th1-Tc1 lymphocytes, and interleukin (IL)-17 secreting CD4 (Th17) and CD8 (Tc17) T cells, respectively, participate in cell migration toward inflamed tissues in several immunologic processes and pathologies [10,11] as tumor processes, genetic diseases, or hyperplasia induced by HPV, between others [12–14]. CXCR3 is highly expressed by effector T lymphocytes [10] and is in a higher proportion of CD4+CD8+ T lymphocytes than CD4 T cells [15].

Altered lymphocyte level expressing CXCR3 and CCR6 has been associated with different clinical events after solid organ transplantation, such as acute rejection (AR) and chronic graft dysfunction, but data are still limited [10,11,16–20]. These receptors and their associated ligands have been expressed in murine and human lungs and cardiac allograft rejection [10,21].

For this reason, our study aimed to evaluate the expression of the receptors CXCR3 and CCR6 in T lymphocytes of peripheral blood in kidney transplant recipients and their association with reactivation of viral infections, AR development, and allograft function.

MATERIAL AND METHODS

The study's design, clinical parameters, and demographic information

A total of 169 consecutive adult kidney transplant recipients (KTR) were evaluated at the University Clinic Hospital "Virgen de la Arrixaca" (Spain) and retrospectively analyzed. All follow-up samples were used in this analysis, but only the patients whose kidney grafts obtained Donor Specific Antibodies Luminex findings for detecting anti-HLA antibodies for at least 1 month after transplantation were included.

The estimated allograft loss was a return to dialysis. Finally, 34 KTRs were chosen for the specialized investigation of T cell monitoring, whose unique clinical and demographic characteristics are reported in Table 1.

The KTRs were separated into 2 groups based on whether AR occurred within the first year after transplantation (AR Group) or not (NAR Group). A total of 34 KTRs were evaluated, and 5 (14.7%) experienced AR in the first year after transplantation, compared with 29 (85.2%) who sustained stable renal function free of rejection. Acute cellular rejection (ACR) was identified in 4 KTRs with AR and acute humoral rejection in one. Before transplantation, 5 patients in the NAR group showed non-Donor Specific Antibodies anti-HLA antibodies, unlike none in the AR group.

Immunosuppressive Treatment

Prednisolone, oral tacrolimus (Prograf, Astellas, Ireland), and mycophenolate mofetil (CellCept, Roche, Switzerland) comprised the same triple immunosuppressive therapy given to all participants (Dacortin, Merck, Spain). To maintain a trough level of tacrolimus (FK) in whole blood between 8 and 12 ng/mL during the first month postoperatively, between 7 and 10 ng/mL during the first 2 months following transplant, and between 5 and 8 ng/mL after that, a tacrolimus (FK)-based protocol was started at the dose of (0.10-0.15 mg/kg/day). Mycophenolate mofetil was administered daily at a dose of 2000 mg, and within the first month after surgery, depending on the patient's white blood cell level, the dosage was reduced to 1000 to 1500 mg [22,23].

Methylprednisolone was administered intravenously at doses of 500, 250, and 125 mg/day on the day of transplantation, days 1 to 2, and days 3 to 4 after surgery, respectively. Oral prednisolone was started on day 5 after surgery at the dose of 20 mg and was then tapered to 5 to 10 mg/day within 2 to 3 months after transplant. Some patients were treated with induction therapy based on thymoglobulin or basiliximab, as previously reported, depending on the immunologic risk before transplantation [22].

Kidney rejection diagnosis, graft function, and viral infections

A rise in serum creatinine of at least 20% over the pretransplant level and biopsy-confirmed rejection were considered signs of allograft ACR. As amended in 2017, Specimens were examined with light microscopy and immunofluorescence labeling using the Banff classification and a marker for classical complement activation (C4d) [24]. Acute humoral rejection must be diagnosed for distinct histologic

Table 1. Kidney Recipients' Demographic Data and Clinical Characteristics Included in CXCR3 and CCR6 Expression Studies

	NAR (n = 29)	AR (n = 5)	P*
Age (y)	56.2 \pm 1.59	60.1 \pm 6.71	.358
Sex (male/female) n/(%)	16 (54.3)/13 (45.7)	3 (60)/2 (40)	1.000
HLA mismatches [†]	4.1 \pm 0.17	4.5 \pm 0.64	.524
Live donor (%)	2 (5.7)	1 (20)	.338
Prefomed anti-HLA antibodies (%)	5 (17.1)	1 (20)	1.000
Induction therapy (Tim/Bas)	9 (31.4)/4 (14.3)	3 (60)/0 (0)	.386
Delayed graft function (%)	7 (23.5)	2 (40)	.279
Rejection type (cellular/humoral)	-	4 (60)/1 (40)	-

Quantitative data were expressed as the mean value \pm SD.

AR, acute rejection; Bas, basiliximab; NAR, no acute rejection; SD, standard deviation; Tim, thymoglobulin.

* For qualitative and quantitative comparisons, the nonparametric Mann-Whitney U test and Fisher's exact test were used. P values less than .05 were regarded as significant.

[†] Total genetic differences between the donor and recipient for the HLA-A, HLA-B, and HLA-DRB1 genes.

features, positive C4d staining in peritubular capillaries, and concurrent Donor Specific Antibodies [23,25].

Increased maintenance immunosuppression and pulse steroids (500-mg methylprednisolone boluses) were used to treat mild acute cellular rejection (Banff grade I). Antithymocyte globulin was used to treat all other ACR.

Additionally, steroid-sensitive rejections (ACR Banff grade I) and steroid-insensitive rejections (ACR Banff grade II and III, as well as antibody-mediated rejection) were identified as types of AR episodes. Pulse steroids and intravenous immunoglobulin (0.25 g/kg during the final session and 1 g/kg [maximum 140 g] divided into 2 doses combined with plasmapheresis) were also used to treat AR (3 sessions a day, every 5 days). Later, as previously reported, we gave patients 500 mg of anti-CD20 (Rituximab, Roche pharmaceuticals) intravenously [22].

We also study the expression levels of these T subpopulations and their association with the incidence of AR and kidney graft function (the equation initially recommended for estimating glomerular filtration rate was that of the formula modification of diet in renal disease), and viral infections cytomegalovirus (CMV) and BK.

By identifying and assessing 2 kinds of CMV antibodies, the CMV detection was classified as either a present or previous infection: 1. within a week or 2 of the initial exposure, IgM antibodies can be found in the blood as a result of the body's early response to a CMV infection. After a brief increase, IgM levels (titers) reduce and drop below detectable levels after a few months. When latent CMV was reactivated, IgM antibody levels increased once more. 2. Several weeks after the first CMV infection, IgG antibodies are subsequently generated. IgG levels increase while the infection is active and stabilize once the infection with CMV is over and the virus is no longer active. GeneProof CMV PCR Kit was also used to detect CMV by real-time PCR technique (Cat. N° CMV/ISEX/025) (GeneProof, a.s., Brno, Czech Republic).

BK infection presence was detected by real-time PCR technique by using the GeneProof BK/JC Virus (BK/JC) PCR Kit (cat. N°BKJC/ISEX/025) (GeneProof a.s. Brno, Czech Republic).

Monitoring of T cells expressing CXCR3 and CCR6 by flow cytometry

Flow cytometry evaluated the expression of CXCR3 and CCR6 biomarkers in T lymphocytes (CD4 and CD8) in peripheral blood in a pretransplant (waiting list) collection of KTR. Later we study the levels of these T subpopulations and their association with AR incidence, kidney graft function (estimated using the formula modification of diet in renal disease), and viral infections (CMV and BK). Adverse clinical events and graft function were monitored during the first year post transplant.

Monoclonal antibodies were used to label peripheral blood samples by accepted flow cytometry labeling procedures. In a nutshell, 50 L of peripheral blood sample were treated for 10 minutes in the dark with 5 L of each monoclonal antibody. After the lysing step, this sample was incubated for 7 minutes at room temperature with 3 mL of BD FACS Lysing Solution (Becton Dickinson BD, Bioscience, San Jose, Calif, United States). After lysing, the material was centrifuged at 1800 rpm for 5 minutes before being rinsed with PBS, as described in a prior study [22,25].

All samples were collected using a FACS Canto II flow cytometer after labeling (Becton Dickinson BD, San Jose, Calif, United States). The FACS Diva program was used to evaluate the results (Becton Dickinson BD, San Jose, Calif, United States). By combining the absolute lymphocyte count from a Medonic cell counter M16 (Boule Medical, Sweden) with the relative frequencies determined by flow cytometry,

the absolute number of the various cell subpopulations was determined, as previously reported [22].

Statistical analysis

The results have been expressed as the mean \pm standard error of the mean for quantitative data or as percentages for categorical data. The χ^2 or Fisher's exact test was used to compare categorical variables. The verification of the normality of the data was carried out using the Kolmogorov-Smirnov test. The Mann-Whitney U test was used to compare 2 groups with variables that do not adjust to normality. For the comparison of 3 or more groups, the Kruskal Wallis test and Dunn's post hoc test with Bonferroni correction for multiple comparisons were used, and the correlation analyses were carried out using the Spearman index, as previously published [25].

The Wilcoxon nonparametric test for related samples was used to compare 2 related groups over time. The Friedman test was employed with Wilcoxon post hoc analysis to compare 3 or more comparable groups.

For all statistical tests, P values of .05, or p -corrected .05 in the event of multiple comparisons, were considered significant. The graphics and statistical analysis were produced using the SPSS software (version 22, Chicago, IL, United States), GraphPad Prism (version 6, San Diego, Calif, United States), and the R programming language for this Latest R Studio Integrated Development Environment version 3.4.

RESULTS

Subtypes of T cells in kidney transplant recipients to CXCR3 and CCR6 expression

After monitoring cytometrically pretransplantation and evaluating the expression of biomarkers, the following results were found.

Fig 1 shows that KTRs with low levels of pretransplant Th17 (CD4+CXCR3-CCR6+) lymphocytes (tertile 1, Th17<16.4%) have a higher risk of suffering AR during the first year after transplantation ($P = .033$). In this sense, increased infiltration of

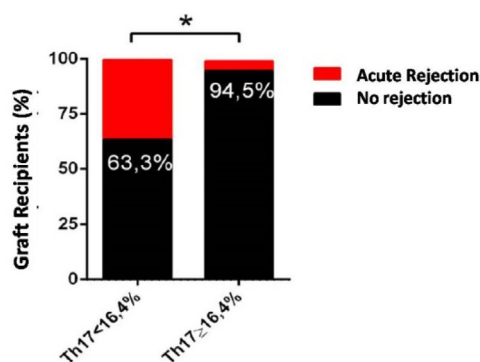


Fig 1. Monitoring of subtypes of T cells in kidney transplant recipients concerning CXCR3 and CCR6 expression showing Th17 subclass. The Wilcoxon test for paired samples was used to illustrate the relative frequencies (black) and absolute values (red) of the T lymphocyte subpopulations at pretransplantation and post-transplantation for pretransplantation (pre). Values of $P < .05$ were considered statistically significant. * $P < .05$.

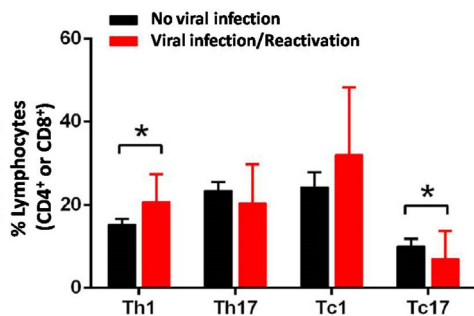


Fig 2. Monitoring of subtypes of T cells in kidney transplant recipients regarding CXCR3 and CCR6 expression shows different T cells subclass to viral infections. The Wilcoxon test for paired samples was used to illustrate the relative frequencies (black) and absolute values (red) of the T lymphocyte subpopulations at pre-transplant and post-transplantation concerning pretransplantation (pre). Values of $P < .05$ were considered statistically significant. * $P < .05$.

Th17 lymphocytes into inflamed tissues of kidney allografts could explain the low levels at the peripheral level in patients at risk of AR episodes.

Subtypes of T cells in kidney transplant recipients for CXCR3 and CCR6 expression

On the other hand, KTRs with viral infections or viral reactivations during the first 3 months post transplantation have significantly lower levels of Tc17 (CD8+CXCR3-CCR6+) cells and higher levels of Th1 (CD4+CXCR3+CCR6-) lymphocytes (Fig 2).

Besides patients with CMV reactivations, the viral peak also correlates negatively with pretransplant levels of Th1 cells ($r = -0.606$, $P = .037$). Th1-Tc1 lymphocytes are specialized in eliminating intracellular pathogens; therefore, the increase in lymphocytes with this particular phenotype may reflect an early activation by viral antigens.

DISCUSSION

In the present study, the expression of the receptors CXCR3 and CCR6 in T lymphocytes of peripheral blood from KTRs collected pre transplantation were evaluated, and their association with reactivation of viral infections, AR development, and allograft function post transplant were analyzed.

Flow cytometry was used to monitor the expression of CXCR3, and CCR6 receptors on T cells in peripheral blood of the KTRs collected pre transplantation.

Chemokines and their receptors are critical in all inflammatory, autoimmune, and immune response processes and even in infectious processes, as has been extensively published in the literature [12-14,26-30]. For example, CXCR3 expression is higher in allograft tissue than in isograft control. It could suggest that CXCR3 is a promising imaging target for immune cell infiltration in early-stage acute rejection [31] or that a prevalent CXCR3+ phenotype of circulating follicular Th cells could

indicate humoral dysregulation in children with Down syndrome [13]. Additionally, it has been shown that these circulating follicular Th cells participate in B-cell-mediated alloreactive reactions in kidney and liver transplantation. So, the results of Zhang et al [11] found that when comparing patients with AR to those with NAR, the proportion of CXCR3-CCR6-CXCR5+CD4+ T cells was significantly higher, and the proportion of CXCR3-CCR6+CXCR5+CD4+ T cells was significantly lower.

Additionally, in individuals with AR, there was a favorable correlation between the percentage of CXCR3-CCR6-CXCR5+CD4+ T cells and the percentage of B cells. In follicular-like formations in liver transplant tissues from AR patients, B cells and Tfh cells have been found. These findings suggested a potential role for CXCR3-CCR6-CXCR5+CD4+ T cells in AR after liver transplantation. Other authors indicate kidney failure is associated with T cell exhaustion and imbalanced Tfh cells [17]. Finally, Shino et al [20] also found a correlation between bronchoalveolar lavage CXCR3 chemokines and lung allograft histopathology.

Altered lymphocyte level expressing CXCR3 and CCR6 has been associated with different clinical events after solid organ transplantation, such as acute rejection and chronic graft dysfunction, but data are still limited [11,17-19,27-29]. For this reason, our study evaluated the expression of the receptor CXCR3 and CCR6 in peripheral blood T lymphocytes from KTRs and their association with viral infections, AR, and allograft function.

Later we study the levels of these T subpopulations and their association with AR, kidney graft function (estimated using the formula modification of diet in renal disease), and viral infections (CMV and BK). Adverse clinical events and graft function were monitored during the first year post transplant.

In this sense, we found that KTRs with low levels of pretransplantation Th17 (CD4+CXCR3-CCR6+) lymphocytes (Tertile 1, Th17<16.4%) had a higher risk of suffering AR during the first year after transplantation ($P = .033$). In this sense, increased infiltration of Th17 lymphocytes into inflamed tissues of allograft could explain the low levels at the peripheral level in patients at risk of suffering from AR episodes.

Our results showed that KTRs with viral infections or viral reactivations during the first 3 months post transplantation had significantly lower levels of Tc17 (CD8+CXCR3-CCR6+) cells and higher levels of Th1 (CD4+CXCR3+CCR6-) lymphocytes. On the other hand, in patients with CMV reactivations, the viral peak also correlated negatively with pretransplant levels of Th1 cells ($r = -0.606$, $P = .037$). Th1-Tc1 lymphocytes are specialized in eliminating intracellular pathogens; therefore, the increase in lymphocytes presenting this particular phenotype may reflect an early activation by viral antigens and could be an early infection biomarker.

Classically, CMV infection has been associated with kidney graft loss, and in experimental models, this injury is accelerated by CMV-induced Th17 lymphocyte infiltrates [19]. A study by Dhital et al [19] demonstrated that murine CMV-induced intra-graft Th17 cells had a Th1/17 phenotype co-expressing IFN- γ and/or TNF- α and that murine CMV promoted intragraft

expression of CCL20 and CXCL10, which was associated with recruitment of CCR6⁺ CXCR3⁺ Th17 cells.

On the other hand, different regulatory cells are involved in attenuating or potentiating the immune response, such as type 1 Treg cells that promote the generation of tissue-resident memory CD8 T cells [32]. Besides, CXCR3 identified human naive CD8⁺ T cells with enhanced effector differentiation potential [33], and different clusters of B cells and regulatory B cells could be implicated in allograft rejection or tolerance, which could also establish a kind of personalized medicine according to the results of different biomarkers analyzed in the evolution of the transplant [22,34,35].

In this sense, De Simone et al [33] have identified 2 discrete subsets of human CD8⁺ T naïve (T_N) cells, defined by the absence or presence of CXCR3 receptor. The more abundant CXCR3⁺ T_N cell subset displayed an effector-like transcriptional profile and expressed TCRs with physicochemical characteristics indicative of enhanced interactions with peptide-HLA class I Ags. These cells frequently produced IL-2 and TNF in response to nonspecific activation directly ex vivo. They differentiated readily into Ag-specific effector cells in vitro. These findings support the notion that effector differentiation is shaped by heterogeneity in the preimmune repertoire of human CD8⁺ T cells.

Our study has several limitations, as the size of the sample used for the types of rejection groups is small. Therefore, the conclusions of our work should be validated in larger cohorts. In addition, the design of a single-center study may limit extending our general conclusions. However, as a point in favor, we have the advantage that our patients have been optimally characterized for years, as can be seen in our scientific production, with stable and standardized immunosuppression protocols, with constant and periodic clinical and immunologic function monitoring, allowing us a large amount of tracking data. Lastly, it is also a strength that all the biopsies evaluated have been done by the same pathologist, which limits the eventual variability in the histopathological evaluation of the transplant samples. New advances and new bioinformatic resources and tools, as recently published [36], could help confirm our preliminary results and establish more consistent models.

In conclusion, our data imply that pretransplantation evaluation of Th1-Th17 and Tc1-Tc17 levels may be beneficial for predicting post-transplant clinical events like reactivation of viral infections and AR. This analysis is a noninvasive tool for enhancing the immunologic follow-up of patients undergoing kidney transplantation, but additional research will be required to support the findings, establish cut-off values, and develop standardized methods and protocols.

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