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Chemokine Receptors CCR1, CCR3, CCR7 and Chemokines CX3CL1 and CCL5 are Significantly Up-Regulated and Very Reliable for Acute Rejection Diagnosis of Kidney Transplants

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Abstract

Background: The allo-immune response following organ transplantation constitutes one of the main determinants concerning both short- and long-term outcomes in renal graft recipients. Chemokines and their receptors play a diversified and important role, either homeostatic or inflammatory and direct different immune-competent cell types to the allograft. While deeply studied in the last two decades, controversy persists as a result of chemokines' pleiotropic actions. We report our analysis of CCR1, CCR3, CCR7, CCL5 and CX3CL1 expression or synthesis by graft-infiltrating cells in human kidney transplants (KTx). At the same time, we tested their robustness in diagnosing acute rejection. **Methods:** Fine-needle aspiration biopsies (Fnab) were performed either on days 7 or 14 post-transplantation among stable KTx and on the day of acute rejection (AR) diagnosis. Fnab cytopreparations were studied by the enzymatic avidin-biotin complex staining for CCR1, CCR3, CCR7 and CX3CL1. From another subgroup of cases, Fnab samples were cultured for 48 hours and the supernatants were analysed for CCL5 by ELISA. **Results:** The group of AR cases showed a significantly up-regulated expression of CCR1, CCR3, CCR7 and CX3CL1 and a significantly higher synthesis of CCL5. The positive predictive values were respectively 92%, 97%, 85%, 76% and 78% and negative predictive values were by the same order, 100%, 73%, 100%, 98% and 83%. **Conclusions:** Our study permits us to advance that CCR1 and CCR3 play a significant and non-redundant role in acute rejection, and it is the first report of CCR3 association with rejection, probably related to CCL5. The presence inside the graft of significant up-regulation for CCR7 surmises that part of antigen presentation may be performed there without being re-

stricted to secondary lymphoid sites. Our results with CX3CL1 confirm other reports.

Keywords

Kidney Transplants, Aspiration Biopsies, Acute Rejection, Chemokines, Chemokine Receptors

1. Introduction

An enlarging proportion of the worldwide population may suffer from chronic kidney disease which was ranked among the first fifteen causes of death [1]. With few exceptions of clinical conditions persisting as absolute contraindications kidney transplantation remains the best treatment alternative providing better long-term survival and better quality of life [2] [3].

Once an organ is available for transplantation several challenges must be answered. The most complex and difficult is the recipient's innate and adaptive immune response, both encompassing an afferent and efferent phase. During the early years of organ transplantation, the bulk of our attention was directed to the efferent phase of the adaptive response. However, a consensus is easily achieved about the primordial importance of the afferent phase where chemokines and chemokine receptors mediate both the leukocyte infiltration of the transplanted organ and the cross-talk between antigen-presenting cells and T and B cells in lymphoid tissues [4].

Actually, chemokines constitute the largest family of cytokines while the chemokine receptors are the biggest branch of the γ subfamily of rhodopsin-like 7TM receptors [5]. To complicate the huge diversity of chemokines and chemokine receptors and their ability to bind to two or more receptors [5], they may also be functionally classified into homeostatic, inflammatory, and dual inflammatory/homeostatic subtypes. They are involved in the immune system development and basal leukocyte trafficking as well as in the emergency trafficking of leukocytes to sites of infection or tissue injury or both [6], respectively. The early recognition of the major importance of chemokines and chemokine receptors upon the fate of a transplanted organ [7] was disturbed by an unreachable unified interpretation brought by divergent results on different animal models and transplanted organs [8]. Usually, targeting either a single chemokine or a chemokine receptor turned out to be ineffective in prolonging allograft survival. But not always, as exemplified by RANTES (now CCL5) inhibition that seems to be deprived of a significant therapeutic effect, while one of its receptors, CCR5 appeared to be influential in allograft outcome [9]. Of major interest, one group reported significantly better renal transplant survival among patients who were homozygous carriers of CCR5Δ32, leading to an inactive receptor [10].

Through the reports of several groups over the last two decades, we learned that intra-graft chemokine expression profile is dependent on whether the graft

is syngeneic or allogeneic [11]. Furthermore, in situations of rejection, the set of chemokines identified depends on the time interval since grafting [12], as well as among other important subdivisions, we could classify the chemokines as those that drive leukocytes into or out of the allograft [13].

Notwithstanding, these differentiating findings, several chemokine receptors could be found in quite different immunocompetent cells. As relevant examples, CCR1 may be expressed by activated T helper1 cells, memory T helper1, monocytes, and immature dendritic cells, while CCR7 may be found on naïve T cells, activated T helper1, activated T helper2, and mature dendritic cells [8]. Strictly pertinent to our study, while it is known that immature blood-derived dendritic cells may express any of CCR1 to CCR6, a question can be raised about which chemokine receptors are expressed by tissue-resident dendritic cells [14]. In addition, a few non immunocompetent cells may express chemokine receptors, like mesangial cells (CCR1 and CXCR3) and endothelial cells (CCR2, CXCR1, CXCR2, and ACKR1) [15], both widely represented in a renal allograft.

In human renal transplantation CCR5 and CCL5 have been reported to be associated with acute rejection but not with chronic allograft nephropathy, along with a lower expression of CCR1 and CCL2 [16], though the low CCR1 expression was not confirmed by others [17]. Also, whereas CXCL8 was reported to be higher during acute rejection in serum and urine of renal transplants [18], we did not find that CXCL8 synthesis by graft-infiltrating cells was changed for the same clinical condition [19]. Somewhat differently, another group reported that CCR1 expression was the same in stable and acute rejection cases, CCR3 was absent and mRNA for CCL3 was up-regulated in acute rejection [20]. On the contrary, we have reported that CCL3 synthesis was significantly higher during chronic but not acute rejection [19]. The studies of CXCR3 and CXCL9, CXCL10, and CXCL11 have produced more consistent results, strongly suggesting a causal association of these chemokines and chemokine receptors with acute rejection [21].

Some of these studies were done in urine which is really a two-sided coin method. On the one hand, is a uniquely safe and comfortable examination for the patient, but not infrequently is not available during the early days post-transplantation due to anuria. On the other hand, variable urine amounts may come from badly functioning native kidneys which can potentially confound the interpretation. Most studies were done on core transplant biopsies, performed under direct ultrasound guidance with automated biopsy needles. This method is moderately uncomfortable for the recipient and has a reduced but still considerable risk of bleeding and hematoma after the procedure [22], along with the difficulty of programming it in a very busy department of interventional radiology. For a few decades, we have recurred to fine-needle aspiration biopsy (Fnab) which is a highly safe and almost complication-free method following the technique described by P Haÿry [23]. This way we get access to graft-infiltrating lymphocytes and monocytes-macrophages together with parenchymal cells. More, we have reported that these immunocompetent cells are significantly different when com-

pared to peripheral blood circulating lymphocytes [24] and are significantly associated with acute rejection, although flow cytometry analysis of peripheral blood lymphocytes was not reliable to identify an acute rejection episode [24]. Our past experience has ensured us that Fnab constitutes an easy, cheap and ethically acceptable gateway into the scrutiny of post-transplant immune events.

We selected to study the expression of CCR1, CCR3, CCR7, and CX3CL1 in Fnab sampling graft-infiltrating cells and CCL5 synthesis in cultures of Fnab cells in kidney transplant recipients.

CCR1 is broadly expressed on both hematopoietic and nonhematopoietic cells. CCR1 is also expressed on monocytes, memory cells and dendritic cells [5] but it can be deactivated by LPS and other activating agents in the presence of IL-10 in a way that both dendritic cells and monocytes will down-regulate CCR7 and up-regulate CCR1, CCR2, and CCR5 expression [25]. CCR1 antagonist drugs were reported to be effective in a preclinical model of heart transplantation and renal fibrosis [5] but human clinical trials in rheumatoid arthritis have been disappointing [5]. On the other hand, CCR1 is one of the CCL5 receptors which have been associated with acute rejection. Thus, our study was done with conflicting past reports on CCR1.

CCR3 was originally identified as the eosinophil receptor for eotaxin-1, now CCL11, but it is now known to have a wide pattern of binding, including RANTES (now, CCL5) [5]. CCR3 is also highly expressed on different cells beyond eosinophils, namely endothelial cells, epithelial cells, dendritic cells and Th2 lymphocytes [5]. The role CCR3 may play post-transplantation has not been clarified, although eosinophils have variably been implicated in the alloimmune response [27]. However, data on CCR3 remain contradictory, with its expression being reported both in resident cells of allografts without inflammation and in 61% of human cardiac allograft biopsies undergoing a rejection crisis [28]. At the same time, the absence of CCR3 expression in human kidney transplant rejections [20] was reported, although in this paper the number of samples with acute rejection was only eight.

CCR7 seems to play a non-redundant role upon the traffic between central lymphoid organs and peripheral tissues of dendritic cells, B, and T cells [5]. Immature dendritic cells do not express CCR7 but upon antigen exposure, they upregulate CCR7 which will mediate dendritic cells' migration to lymph nodes, followed by CCR7 ligation to CCL21 expressed on lymphatic endothelial cells [6]. In some animal models, CCR7 appears to be absolutely required for T cells to exit from peripheral tissues towards secondary lymphoid structures [29]. Contrary to what was anticipated, CCR7 is downregulated (together with CXCR5) which facilitates T cell exit from lymph nodes back to the inflamed peripheral sites [29]. CCR7-deficient mice exhibit a longer heart and skin allograft survival and a delayed cellular infiltration of allografts [30]. Of potential interest, CCR7 expression is observed in mesangial cells and its receptor, CCL21 can be found among podocytes [31]. The part of CCR7 within the deployment of anti-allograft response is

not settled. While DJ Lo described increased CCR7 transcript in biopsies of acutely rejecting kidney transplants and even higher transcription among subclinical rejection [32], KW Kim reported a different scenario where $CCR7^{+}/CD8^{+}$ T cells were significantly decreased in kidney transplants with acute rejection [33].

CX3CL1 (previously fractalkine) exists in a stalked and a soluble form and is expressed and produced by a variety of resident cell types, including endothelial cells, renal tubular cells, mesangial cells, and podocytes [34] which are of interest concerning kidney transplants. It is known that renal tubular cells attract monocytes, dendritic cells and natural killer cells in a CX3CL1-dependent manner [35]. CX3CL1 expression in tubular epithelium is upregulated by IFN-γ and TNF-α [35]. There is also evidence that CX3CL1 is involved in T helper cells, natural killer cells and monocyte-macrophage migration [5]. The only CX3CL1 receptor is CX3CR1 [5] and the magnitude of CX3CR1 expression in the allograft interstitial macrophages and dendritic cells was reported to correlate with the outcome in acute rejection of human kidney recipients [36]. On the contrary, no significant difference was observed in a group of 163 kidney transplant recipients when comparing different CX3CR1 genotypes [37]. Measuring urinary elimination, Peng observed that CX3CL1 was the best discriminator between acute rejection and acute tubular necrosis compared with other molecules previously strongly associated with acute rejection such as CXCL9, CXCL10, perforin, and granzyme B [38].

CCL5 (formerly RANTES) is expressed as an immediate early gene in epithelial and endothelial cells within minutes of stimulation while it is upregulated only after 3 - 5 days after activation in T lymphocytes [15]. In rat models of organ transplant, CCL5 expression was found to be upregulated during acute rejection [15] and treatment with a CCL5-receptor antagonist significantly reduced the vascular and tubular injury in acute rejection [39]. In humans, CCL5 was signalled to be significantly associated with a plethora of different renal diseases [40]. The association of CCL5 expression with acute rejection in human kidney recipients has been reported, first in a study done by Pattison back in 1994 [26]. CCL5 was incorporated into a set of ten IFN-γ stimulated genes which performed quite well in the diagnosis of acute rejection in human kidney transplantation recurring to renal biopsy samples [41]. On the other hand, plasma CCL5 decreased significantly post-transplant as compared to pre-transplant, probably as an effect of the immunosuppressive drug therapy [42]. In our study, we tested the ability to synthesize CCL5 by graft-infiltrating cells.

2. Patients and Methods

Table 1 summarizes patients' demographics and characteristics.

This study included 136 KTx, 75 males and 61 females, between the ages of 20 and 68 years. Each patient provided adequate Fnab samples according to the criteria defined by P Haÿry [23] and all received an organ from a deceased donor. The study group was not of consecutive KTx, it included the cases where Fnab

Table 1. Demographics and characteristics. Recipients are all adults, aged between 20-68 years old, and transplanted with kidney deceased donors. AR diagnosis was done on the biopsy-gun biopsy, done at the same time as Fnab, and classified according to Banff criteria and secured by a positive response to treatment or by histologic reassessment of graft nephrectomy. DN-diabetes mellitus, IgA-IgA nephropathy, RPGN-rapidly progressive glomerulonephritis, SLE-systemic lupus erythematous, FSGS-focal segmental glomerulosclerosis, TIN-tubulointerstitial nephritis, HTN-hypertension, CGN-chronic glomerulonephritis, PKD-adult polycystic kidney disease, DGF-delayed graft function. Serum creatinine values were significantly higher in the rejection group ($p = 0.01$) either when compared with day 7 or day 14 - 30.

Phenotype Characteristic	Kidney Transplant Recipients (n = 136)	
Gender		
Female	61	
Male	75	
Cause of ESRD		
DN	26	
IgA	13	
RPGN	10	
SLE	6	
FSGS	6	
TIN	37	
HTN	5	
CGN	22	
PKD	11	
KTx		
First	124	
Re-KTx	12	
DGF	28	
Serum Creatinine (mg/dL)		
7 day	3.6 (1.3 - 7.4)	
14 - 30 days	2.6 (1.3 - 10)	
Rejection day	5.1 (2.3 - 10)	
Immunossupression	**Stable Recipients**	**Rejection Recipients**
CsA	52	26
TAC + MMF	42	16
Anti-IL2αR	17	2

was possible for logistic reasons and when the sample proved to be of quality following P Haÿry [23]; the acute rejection frequency of KTx in the transplant unit averages 15% of cases, much lower than the prevalence that might be inferred had this group consisted of consecutive transplants. While for 124 cases this was their first KTx, twelve were second-time recipients. Each patient was

treated from the outset with a calcineurin inhibitor, 78 with cyclosporine A (CsA) and 58 with tacrolimus (TAC), plus mycophenolate mofetil (MMF) and prednisolone, with the exception of the second grafts that received quadruple sequential therapy, including two to five doses of thymoglobulin, according to the number of blood lymphocytes. Furthermore, in seventeen of first KTx an IL-2Rα-chain antibody was added. The therapeutic target whole blood levels for CsA, and TAC during the first three months post-KTx were 150 - 250 ηg/mL, and 6 - 12 ηg/mL, respectively.

Their original diseases were hereditary nephritis/tubulointerstitial nephritis/congenital obstructive urologic disease (n = 37), diabetes mellitus (n = 26), unknown/chronic glomerulonephritis (n = 22), IgA nephropathy (n = 13), rapidly progressive glomerulonephritis (n = 10), focal segmental glomerulosclerosis (n = 6), polycystic kidney disease (n = 11), hypertension (n = 5), and systemic lupus erythematous (n = 6). All had a panel of reactive antibodies of less than 10% with the exception of the second KTx.

At least ninety-four KTx remained rejection-free for the first year post-KTx, 52 were treated with CsA and 42 with TAC, including 17 with IL-2Rα-chain antibody. Forty-two KTx developed an acute rejection episode at a median of 13.1 ± 409 days post-KTx, 27 episodes occurred during the first month, six cases during the second and third month, and nine cases after the third month post-KTx. Twenty-six of these acute rejection cases were treated with CsA and sixteen with TAC, including two KTx receiving IL-2Rα-chain antibody, and three with quadruple therapy. Every acute rejection episode was diagnosed by a biopty-gun biopsy done at the same time as the Fnab procedure and read by an independent pathologist following the standardized Banff criteria [43]. The rejection diagnosis was further secured by either a positive response to treatment or by histologic reassessment of graft nephrectomy. Acute rejection was treated with either 1) 3 pulses of IV 250 - 500 mg methylprednisolone, 2) thymoglobulin, minimum of two doses, or 3) 5 - 12 sessions of plasmapheresis and IgG at 0.4 gr/kg weight, both when the episode was graded IIa or greater and when c4d positivity was observed along with donor-specific antibodies. Only two cases proved to be treatment resistant, and both patients had their graft surgically removed before the first month post-KTx.

All patients received prophylaxis with ganciclovir/valganciclovir when the donor was positive and the recipient was negative for CMV, and whenever thymoglobulin was administered. Furthermore, each KTx received cotrimoxazol as prophylaxis for Pneumocystis jirovecii during the first six to twelve months post-surgery.

Among the rejection-free cases, Fnab was done on day seven (in close to 80% of cases) and on days 14 or 30 post-KTx for the remaining KTx, 90 - 150 min after the morning intake of immunosuppressive drugs, and on the day of the biopty-gun biopsy among rejection patients. The corresponding blood samples were drawn in parallel with the Fnab procedure. As a rule, one patient provided one sample for analysis.

Immunocytochemistry studies

Fnab samples were submitted to 700 rpm cytocentrifugation for 10 min and kept at −70˚C until testing. On the analysis day, cytoslides were brought back to room temperature and submitted to immunocytochemistry procedure by the enzymatic Avidin Biotin Complex (ABC) method using the detection system UltraVision™, HRP/DAB (Horseradish Peroxidase/Three, 3'Diaminobenzidine Tetrahydrochloride) from Thermo Scientific, UK. All the incubations were done at room temperature. Briefly, the cytoslides were hydrated in ethanol 95˚ and incubated with hydrogen peroxide for 15 min to peroxidase blocking and rinsed in distilled water and Tris Buffered Saline (TBS) solution at pH = 7.4. Next, an incubation of 10 min with Ultra V Block from Lab Vision™ for unspecific immunoglobulin blocking was done. After removal of the excess of the unspecific serum, the primary antibody was added at the appropriated dilution, followed by 60 minutes of incubation. In the end, cytoslides were washed in distilled water and dipped for 10 min in TBS just before the secondary antibody addition, at a concentration of 4 μg/mL of goat anti-mouse or rabbit anti-goat and 10 min of incubation. We rinsed the excess of the secondary antibody with TBS, followed by 10 min of incubation with Streptavidin Peroxidase, 10 min rinsing in TBS and 10 min of incubation after DAB Chromogen and DAB Substrate addition. Finally, hematoxylin from Mayer's Hematoxylin, Dako Cytomation was applied, followed by 2 min rinse in running tap water and 1 min dehydration with ethanol 95˚ before finishing the cytoslide preparation with a coverslip with Entellan® mounting medium. The primary antibodies used included a goat polyclonal IgG at 15 μg/mL, for CX3CL1, a mouse IgG_{2B} at 8 μg/mL for CCR1, a rat IgG_{2A} at 15 μg/mL for CCR3, and a mouse IgG_{2A} at 25 μg/mL for CCR7, each acquired from R&D. In all cytoslide preparations, every negative and positive kidney tubular cell (R) and lymphocyte and monocyte-macrophage (L/M) were counted in order to obtain the absolute values of positive cells, as well as the ratio of positive cells for both R and L/M cells in an attempt to correct the variation in cellular numbers present in the Fnab samples.

ELISA studies in Fnab culture supernatants

Fnab samples were cultured as previously described [44]. Briefly, samples were aspirated into 6 mL of RPMI medium with sodium heparinate at 15 - 20 UI/mL and cell suspensions were adjusted to a final concentration of 2.5×10^5 cells/mL. Cultures were done in RPMI medium supplemented with penicillin, gentamicin and l-glutamine, 10% autologous serum from the daily blood sample drawn concurrently with Fnab procedure and 10 U/mL of rIL-2. After 48 hours of incubation at 37˚C and 5% of CO_2 the supernatants were harvested and kept at −70˚C until testing.

For CCL5 study, Fnab culture supernatants were quantified by ELISA according to the manufacturer's instruction, Endogen™.

Statistical analysis

The statistical analysis included the determination of median, SD, and inter-

quartile ranges. Comparisons for serum creatinine and whole blood immunosuppressor drug levels were done by unpaired Student's T test and comparative analysis for CCR1, CCR3, CCR7, CX3CL1 and CCL5 results by Mann-Whitney U test. The correlations between the chemokines and their co-receptors with serum creatinine and blood drug levels were tested using Spearman correlation. When indicated, the EasyROC statistical software, version 1.3.1. was used to evaluate the sensitivity, specificity, negative predictive value (NPV), positive predictive (PPV) and areas under the curve (ROC).

This study was approved by the Faculty of Medicine of University of Porto and Hospital of S. João of Porto joint Committee of Ethics. Informed consent was obtained from each patient and the study was conducted in compliance with the Good Clinical Practice Guidelines declaration of Helsinki and Istanbul.

3. Results

The median and interquartile range for serum creatinine among rejection-free cases on day seven post-KTx was 3.6 mg/dL and 1.3 - 7.4 mg/dl, respectively, 5.1 mg/dL and 2.3 - 10 mg//dL, respectively for acute rejection group (P = 0.01) and 2.6 mg/dL and 1.3 - 10 mg/dL on days 14 and 30 combined post-KTx (P > 0.05 on comparing day seven versus days 14 - 30 combined on stable cases and P = 0.01 for days 14 - 30 combined versus acute rejection day). Seventeen cases of delayed graft function were remarked among rejection-free cases and eleven among acute rejection cases. The whole blood levels for CsA, and TAC were within the limits of the transplant unit protocol in almost every case and no episode of calcineurin toxicity or clinically important CMV disease occurred. Of interest, both CsA and TAC blood levels were significantly lower in acute rejection group as compared with all the rejection-free KTx, P = 0.001 and P = 0.004, respectively. No significant difference was observed when comparing HLA matching for rejection-free versus acute rejection KTx although a significant correlation was observed between the presence of anti-HLA antibodies pre-KTx and acute rejection (P = 0.004).

In Table 2 we present the CCR1 results, and in Figure 1 we show the statistical analysis. The best diagnostic performance was achieved by the absolute value of positive cells for CCR1, the optimal cut-off being 13 translated into a positive predictive value of 0.917 and a negative predictive value of 1.00. No correlation was observed between blood drug levels and CCR1 expression. A positive but non-significant correlation was observed between IRF3 expression and creatinine values among acute rejection cases.

In Table 3 we present the findings concerning CCR3 and in Figure 2 we show the statistical analysis. The best diagnostic performance was equally achieved by the ratios of positive cells over renal parenchymal cells or lymphocytes-monocytes, both with positive predictive values over 0.95 and cut-offs defined as 0.10 and 0.21, respectively. Again, no significant correlation was observed with drug blood levels and the correlation between CCR3 and serum creatinine was positive

Table 2. CCR1 expression in Fnab cells. Results are expressed as the absolute number of positive cells, the ratio of positives cells over renal parenchymal cells (R), and the ratio of positives cells over a total of lymphocyte plus monocyte/macrophage (LM); values are given as median ± SD and lower and upper quartiles between brackets.

CCR1	Stable ($n = 31$)	Acute rejection ($n = 15$)	Mann-Whitney P
Absolute number	5.6 ± 11.9 [0 - 6]	25.5 ± 26.5 [13 - 29]	<0.000
Positive cells/Rcells	0.02 ± 0.04 [0 - 0.02]	0.087 ± 0.11 [0.1 - 0.12]	0.002
Positive cells/LM cells	0.03± 0.05 [0 - 0.03]	0.16 ± 0.27 [0.05 - 0.09]	0.0002

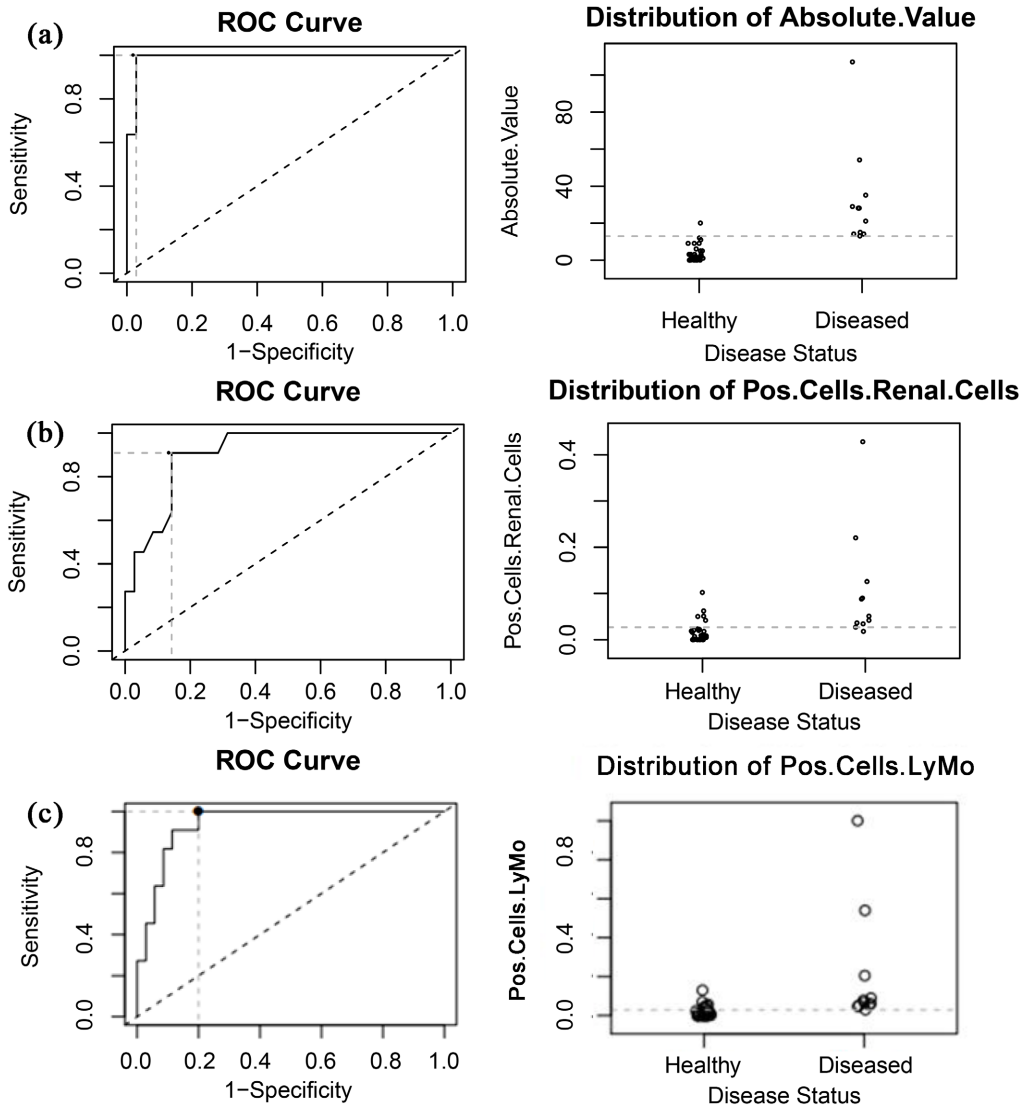

Figure 1. CCR1 chemokine receptor could segregate rejection free (RF) patients from those with acute rejection (AR). The score model was practiced on 31 RF and 15 AR samples to generate a scale Q score ranging from 0 to 100 for Absolute Value (VA) and from 0 to 0.5 for PosCells/RenalCells (Pos/RCel) and for PosCells/LyMo (Pos/LM) ratios respectively. The AUC of the ROC curves and the distribution of RF and AR are shown in the figure. (a) For VA the Youden optimal cut-off method set a threshold at 13 with a corresponding sensitivity of 100% and specificity of 97.1%. The AUC of the ROC curve was 0.989 ($p < 0.000$). (b) For the ratio Pos/RCel, the Youden optimal cut-off method set a threshold at 0.027 with a corresponding sensitivity of 90.9% and specificity of 85.7%. The AUC of the ROC curve was 0.910 ($p < 0.000$). (c) For the ratio PosLM the Youden optimal cut-off method set a threshold at 0.03 with a corresponding sensitivity of 100% and specificity of 80% The AUC of the ROC curve was 0.940 ($p < 0.000$).

Table 3. CCR3 expression in Fnab cells. Results are expressed as the absolute number of positive cells, the ratio of positives over renal parenchymal cells (R), and the ratio of positives cells over a total of lymphocyte plus monocyte/macrophage (LM); values are given as median ± SD and lower and upper quartiles between brackets.

CCR3	Stable ($n = 21$)	Acute rejection ($n = 21$)	Mann-Whitney P
Absolute number	64.4 ± 107 [9 - 78]	123 ± 97 [60 - 193]	0.001
Positive cells/Rcells	0.16 ± 0.32 [0.02 - 0.17]	0.53 ± 0.57 [0.15 - 0.5]	0.0002
Positive cells/LM cells	0.15 ± 0.2 [0.03 - 0.2]	0.5 ± 0.34 [0.24 - 0.8]	0.0001

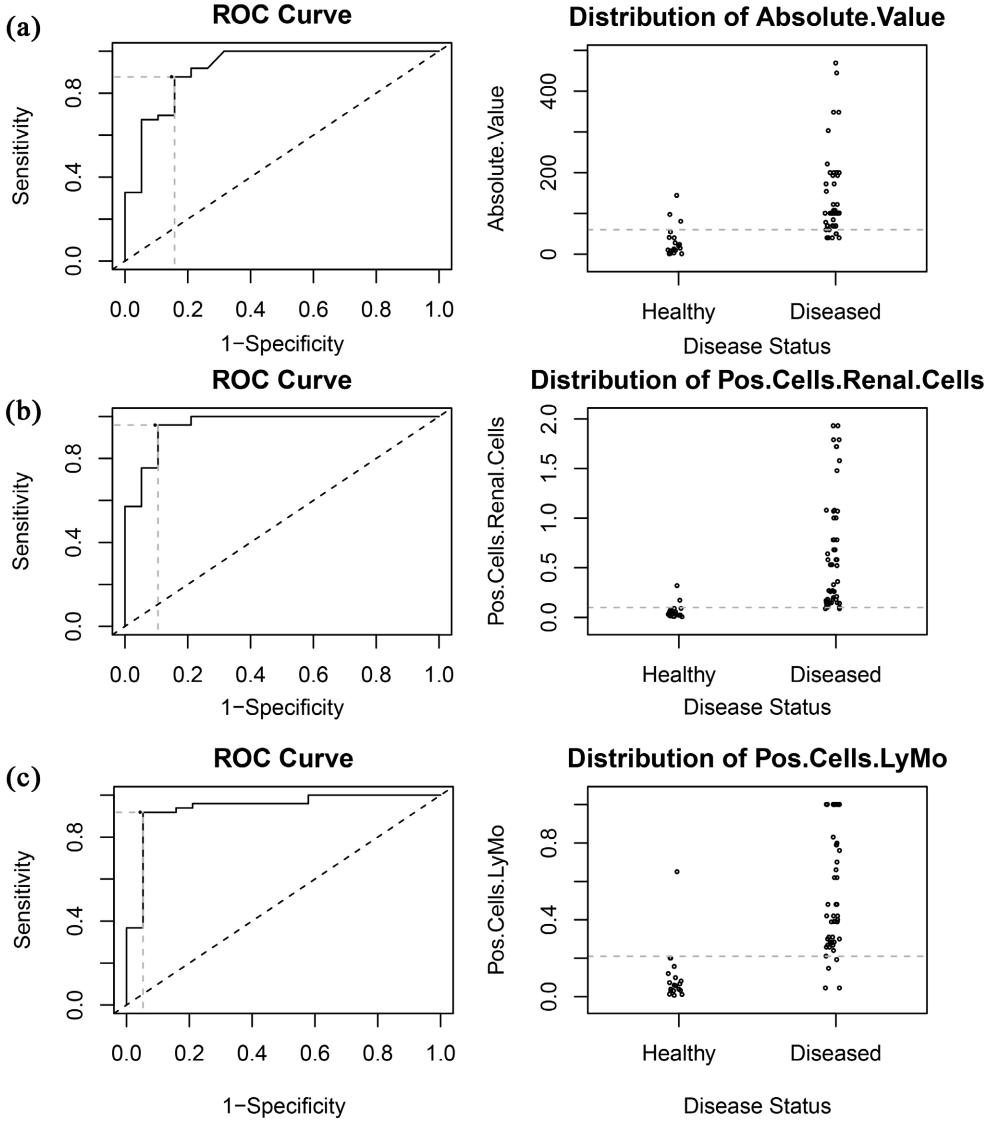

Figure 2. CCR3 chemokine receptor could segregate rejection free (RF) patients from those with acute rejection (AR). The score model was practiced on 21 RF and 21 AR samples to generate a scale Q score ranging from 0 to 500 for Absolute Value (VA), from 0 to 2.0 for PosCells/RenalCells (Pos/RCel) and from 0 to 1 for PosCells/LyMo (Pos/LM) ratios respectively. The AUC of the ROC curves and the distribution of RF and AR are shown in the figure. (a) For VA the Youden optimal cut-off method set a threshold at 60 with a corresponding sensitivity of 87.8% and specificity of 84.2%. The AUC of the ROC curve was 0.918 ($p < 0.000$). (b) For the ratio Pos/RCel, the Youden optimal cut-off method set a threshold at 0.10 with a corresponding sensitivity of 95.9% and specificity of 89.5%. The AUC of the ROC curve was 0.960 ($p < 0.000$). (c) For the ratio Pos/LM the Youden optimal cut-off method set a threshold at 0.21 with a corresponding sensitivity of 91.8% and specificity of 94.7% The AUC of the ROC curve was 0.940 ($p < 0.000$).

but not significant.

In Table 4 the results for CCR7 are displayed, while Figure 3 shows the statistical analysis. The most discriminating measurement was the absolute number of positive cells for CCR7 as can be seen in Figure 3, with a cut-off value of nine positive cells. Once again we did not find any significant correlation with immunosuppressive drug levels or serum creatinine.

In Table 5 we show the findings for CX3CL1 and its statistical analysis is portrayed in Figure 4. The best differentiation parameter was reached by the ratio of positive cells for CX3CL1 among lymphocytes and monocytes-macrophages using as cut-off 0.56. Of note, the PPV was lower for CX3CL1 as compared to the best PPV reached by the CCR1, CCR3, and CCR7. Again, no significant correlation was observed with drug blood levels, while the correlation with creatinine serum was positive but non-significantly.

In Table 6 we show the values measured for CCL5, which were significantly higher in the rejection group (P = 0.004). Statistically for a cut-off of 1250 pg/mL, the sensitivity was 80.2%, specificity 76.9%, PPV 78.6% and NPV 83.3%. Following the pattern described previously, no correlation was observed between CCL5 synthesis and drug blood levels, while being positive the correlation between CCL5 and serum creatinine, it was not significant.

4. Discussion

Our study was done in a group of human renal transplant cases that clearly highlights significant differences between acute KTx rejection and the stable ones. The expression of CCR1, CCR3, CCR7 and CX3CL1 in graft-infiltrating cells from Fnab samples subjected to incubation, synthesized significantly higher amounts of CCL5. Although not entirely so, our findings seem to be in line with the trend previously observed in others' reports. Besides, we are the first to analyse these chemokines and chemokine receptors' positive predictive values for acute rejection in human KTx. We believe that the positive predictive values of 91.7%, 97.0% and 84.6% achieved for CCR1, CCR3, and CCR7, respectively, are of major significance, suggesting a non-redundant part played by these factors in the early alloimmune response in KTx. Yet, CX3CL1 and CCL5 predictive positive values of 76.0% and 78.6%, respectively, albeit satisfactory do not allow a similar interpretation.

Chemokines and their receptors have been extensively studied along the last

Table 4. CCR7 expression in Fnab cells. Results are expressed as the absolute number of positive cells, the ratio of positives cells over renal parenchymal cells (R), and the ratio of positives cells over a total of lymphocytes plus monocyte/macrophage (LM); values are given as median ± SD and lower and upper quartiles between brackets.

CCR7	Stable ($n = 37$)	Acute rejection ($n = 13$)	Mann-Whitney P
Absolute number	2.4 ± 4.4 [0 - 3]	33 ± 33 [12 - 38]	<0.000
Positive cells/Rcells	0.007 ± 0.01 [0 - 0.007]	0.13 ± 0.17 [0.01 - 0.24]	<0.000
Positive cells/LM cells	0.007± 0.013 [0 - 0.006]	0.21 ± 0.24 [0.04 - 0.37]	<0.000

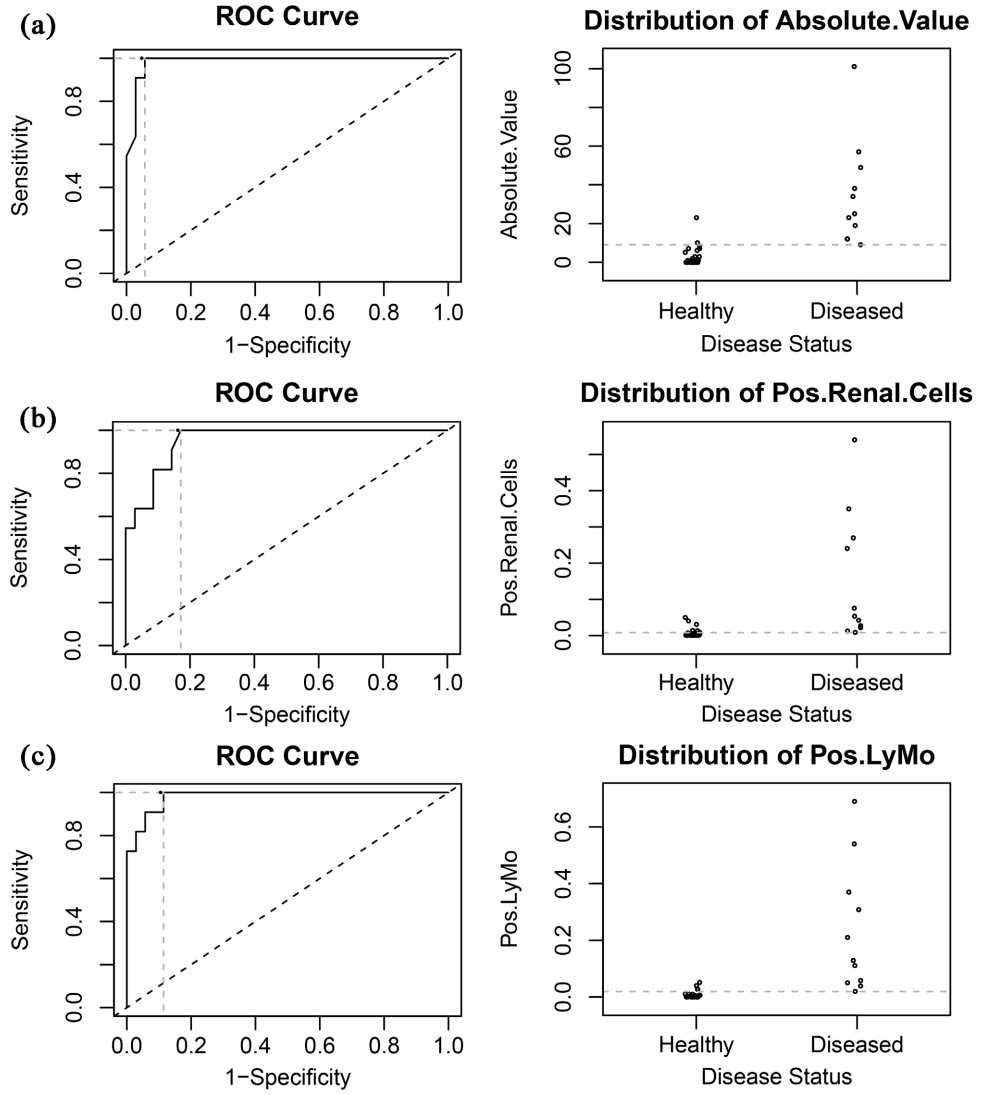

Figure 3. CCR7 chemokine receptor could segregate rejection free (RF) patients from those with acute rejection (AR). The score model was practiced on 37 RF and 13 AR samples to generate a scale Q score ranging from 0 to 100 for Absolute Value (VA), from 0 to 0.5 for PosCells/RenalCells (Pos/RCel) and from 0 to 0.8 for PosCells/LyMo (Pos/LM) ratios respectively. The AUC of the ROC curves and the distribution of RF and AR are shown in the figure. (a) For VA the Youden optimal cut-off method set a threshold at 9 with a corresponding sensitivity of 100% and specificity of 94.3%. The AUC of the ROC curve was 0.986 ($p < 0.000$). (b) For the ratio Pos/RCel, the Youden optimal cut-off method set a threshold at 0.008 with a corresponding sensitivity of 100% and specificity of 82.9%. The AUC of the ROC curve was 0.954 ($p < 0.000$). (c) For the ratio Pos/LM the Youden optimal cut-off method set a threshold at 0.019 with a corresponding sensitivity of 100% and specificity of 88.6% The AUC of the ROC curve was 0.982 ($p < 0.000$).

Table 5. CX3CL1 expression in Fnab cells. Group A, all stable KTx; group B, acute rejection group. Results expressed as the absolute number of positive cells, the ratio of positives over renal parenchymal cells - R, and the ratio of positives over a total of lymphocyte-monocyte/macrophage - LM cells; values given as median ± Sd and lower and upper quartiles between brackets.

CX3CL1	Stable ($n = 42$)	Acute rejection ($n = 8$)	Mann-Whitney P
Absolute number	10.1 ± 14.4 [1 - 17]	80.9 ± 76.6 [28.5 - 102]	0.0001
Positive cells/Rcells	0.028 ± 0.05 [0.00 - 0.034]	0.22 ± 0.23 [0.06 - 0.33]	0.0001
Positive cells/LM cells	0.04 ± 0.087 [0.00 - 0.04]	0.387 ± 0.23 [0.19 - 0.58]	0.0001

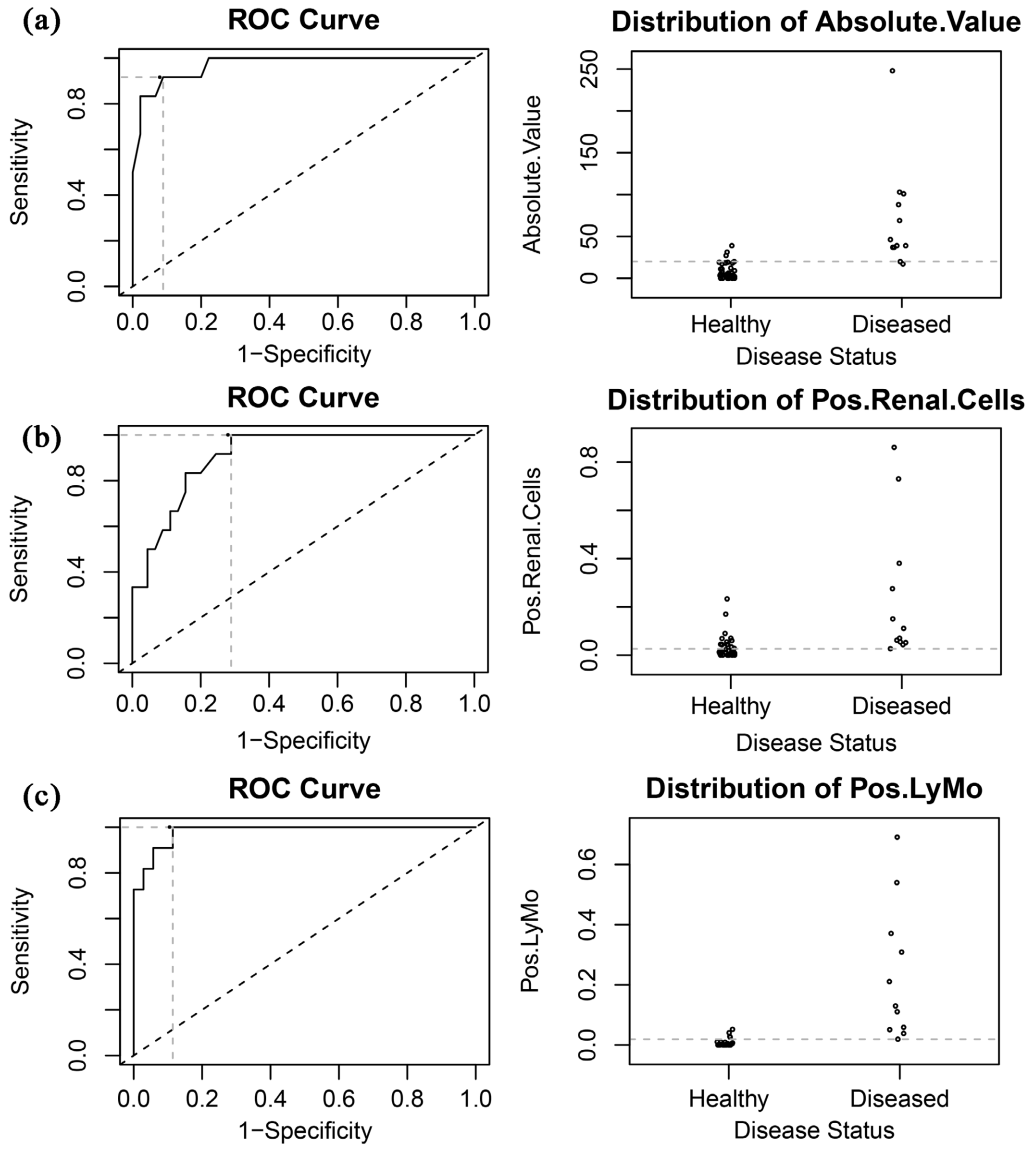

Figure 4. CX3CL1 chemokine could segregate rejection free (RF) patients from those with acute rejection (AR). The score model was practiced on 42 RF and 8 AR samples to generate a scale Q score ranging from 0 to 250 for Absolute Value (VA), from 0 to 1.0 for PosCells/RenalCells (Pos/RCel) and from 0 to 0.8 for PosCells/LyMo (Pos/LM) ratios respectively. The AUC of the ROC curves and the distribution of RF and AR are shown in the figure. (a) For VA the Youden optimal cut-off method set a threshold at 20 with a corresponding sensitivity of 91.7% and specificity of 91.1%. The AUC of the ROC curve was 0.970($p < 0.000$). (b) For the ratio Pos/RCel, the Youden optimal cut-off method set a threshold at 0.026 with a corresponding sensitivity of 100% and specificity of 0.711%. The AUC of the ROC curve was 0.909 ($p < 0.000$). (c) For the ratio Pos/LM the Youden optimal cut-off method set a threshold at 0.056 with a corresponding sensitivity of 83.3% and specificity of 93.3% The AUC of the ROC curve was 0.942 ($p < 0.000$).

Table 6. CCL5 in Fnab sample culture supernatants. Results are expressed as pg/mL; values given as median ±SD and lower and upper quartiles between brackets.

	Stable *n* = 13	Acute rejection *n* = 13	Mann-Whitney P
CCL5 pg/mL	924 ± 538 [548 - 1239]	1470 ± 250 [1294 - 1700]	0.004

two decades in multiple diseases and in several animal models. In fact, they seemed to play very important roles in three temporal phases following organ transplantation: 1) early immune driven alloantigen-independent injury (the ischemia-reperfusion injury); 2) acquired immune driven alloantigen-dependent injury; 3) chronic injury. CCL5 along with several other chemokines and chemokine-receptors, including CCR1 [45], CCL2 and CCL3 [15] [46], was one of the first chemokines to reveal itself important in the alloimmune response. In all chemokine studied it was possible to observe higher synthesis in Fnab culture samples during acute rejection, although non-significantly, except for CCL3 during chronic rejection [19]. Of interest, our negative results for CCL2 were also confirmed by others [20].

Immature dendritic cells express inflammatory chemokine receptors CCR1, CCR2, CCR5, and CXCR1 but during maturation, these receptors decrease in expression to give place to CCR4, CXCR4, and CCR7 that are up-regulated. It is suggested that CCR7 plays a pivotal role in migration of dendritic cells to secondary lymphoid tissue where they encounter CCR7 positive naïve T cells, which upon activation down-regulate CCR7 expression [8] [47]. Not unexpectedly, this complexity was associated with the inability in prolonging allograft survival by targeting a single chemokine or a chemokine receptor [4]. Nevertheless, it would be reasonable to speculate that a comprehensive study regarding the role of chemokines and chemokine receptors may play and enable their use as a molecular or immunohistological biomarker in transplant biopsies. This could bring about changes in immunosuppressive protocols with improved organ outcomes for recipients, as has been sought for a variety of other human diseases [5] [48].

The case for the pivotal role of CCR1 in the anti-allograft response seems very steady. In a mice model of heart transplantation a targeted deletion of CCR1 gene together with cyclosporine A treatment has been associated with acute rejection absence and no sign of chronic rejection 50-200 days after transplantation [49]. Also, in a mouse model of renal ischemia-reperfusion injury, CCR1 contributed to macrophages and neutrophils accumulation in the kidney and supported the production of CCL5 by the kidney [50]. Furthermore, in a Fischer to Lewis renal transplantation model, a CCR1 antagonist decreased the infiltration of proliferating mononuclear cells, down-regulated the expression of acute phase reactive and proinflammatory genes and significantly improved allograft function and the histology at day 21 post-grafting [51].

In a small group of human KTx, CCR1 mRNA was significantly increased both in acute rejection biopsies and chronic nephropathy cases, and was correlated with CCL5 and serum creatinine [17], which we did not observe in our patients. In another group studied by Mayer *et al.* [17], CCR1 protein expression was restricted to monocytes, CD20-positive B cells and DC-SIGN-positive dendritic cells which are in accordance with our observation of CCR1 absence in kidney parenchymal cells and endothelial cells present in Fnab samples. We did not find any correlation between CCR1 and calcineurin inhibitors blood levels

or serum creatinine, but its discriminating value for acute rejection was very convincingly, especially the positive and negative predictive values. Also, whereas in stable cases only about 3% of immunocompetent mononuclear cells expressed CCR1, in rejection patients this value was significantly up-regulated to around 16% (**Table 1**). As a consequence we believe that our findings strongly suggest a non-redundant role for CCR1 in KTx, probably partly linked to CCL5 binding as discussed below. As far as we know there is no drug development concerning CCR1 in organ transplantation but it is noteworthy that statins can silence CCR1 [5].

CCR3, whose role in organ transplantation is far from established, was originally identified as the eosinophil receptor for CCL11 (eotaxin-1), but now it is known as highly promiscuous [5]. CCR3 is said to be associated with Th2 lymphocytes, not commonly linked with acute rejection. However and despite its high expression on the rejection patients, CCR3 may be not a good surrogate marker for Th2 cytokines like IL-4 and IL-13 [52]. Moreover, CCR3 can be expressed in Th1 cells, which could display an increased migratory potential when under the influence of IL-12 [53]. Furthermore, the removal of Th2 cells from the acute rejection picture may be unwarranted [27]. More, in human heart transplantation, an increase in eosinophils usually precedes allograft rejection [54] while plasma CCL11 has been closely associated with cyclosporine A dose and plasma prednisolone concentration which risk correlates with acute rejection [54]. It should be remembered that CCL11 activity is exclusively directed through CCR3 [54]. CCL11 may also attract T cells and macrophages in addition to eosinophils and enable the recruitment of more inflammatory cells into the graft. Altogether, a triple connexion would be easily postulated between CCL11, CCR3 and eosinophils with allograft rejection [55] [56]. However, in human heart transplants, CCR3 expression was studied in endomyocardial biopsies and it was found to be most intense at sites of focal T cell infiltrates and was associated non-significantly with acute rejection [28], while CCR3 mRNA was described as absent in acute rejection biopsies in human renal transplants [20].

On the contrary, we observed a significantly higher CCR3 expression among acute rejection samples and again with a very strong positive predictive value. Of major importance, in Fnab samples from stable KTx, CCR3 expression was around 15% in immunocompetent mononuclear cells but this value reached a little bit more than 50% in acute rejection cases (**Table 2**). It must be emphasized that CCR3 is closely related to CCR1, it binds to a plethora of inflammatory chemokines, like CCL5 and CCL11 [5] and it is up-regulated by TNF-α [5] a potential link capable of explaining the high CCR3 expression in our KTx rejection group.

CCR7 has only two ligands and regulates trafficking of dendritic cells and lymphocytes [5]. Following antigen capture by dendritic cells, CCR7 up-regulation along with the inflammatory chemokine receptor down-regulation is responsible for mediate the migration of mature dendritic cells and T cells towards lymph nodes—a pathway absent at least during the first trimester post-surgery—and

mostly important, early on post-grafting CCR7 mediates recirculation of T cells from blood into lymph nodes [5]. Then, upon activation, both Th1 and Th2 cells down-regulate CCR7 expression and up-regulate inflammatory chemokine receptors, which facilitate the exit of cells from lymph node towards inflamed sites [57]. Therein, CCR7 may play two opposing roles on the immune response, both a priming of the adaptive response and the necessary role for central tolerance establishment [58]. In fact, the proportion of $CCR7^+/CD8^+$ T cells were significantly decreased in a group of human KTx with acute cellular rejection [33], being that $CCR7^+CD8^+$ T cells effectively suppressed T cell proliferation. Moreover, the proportion of $CCR7^+/CD8^+$T cells in peripheral blood negatively correlated with effector T cells [33].

CCR7 expression was also described to be up-regulated in both subclinical rejection and acute cellular rejection in renal allograft biopsies [32] and the pattern that up-regulates this chemokine profile suggests that alloimmune response in human renal transplantation is associated with CD8Th1 effector phenotype, paralleling our previous reports [59] [60]. Our group of KTx showed a significantly higher CCR7 expression in graft-infiltrating cells with a consistent positive predictive value. While $CCR7^+$ cells were quite rare among Fnab samples from stable patients, they were pretty abundant during acute rejection reaching 20% of positive cells in the immunocompetent mononuclear cell compartment (**Table 3**).

The membrane-anchored form of CX3CL1 which was studied here, promotes strong selectin- and integrin-dependent adhesion of mononuclear cells expressing CX3CR1 [5]. CX3CL1 may have an antiapoptotic function and monocyte survival depends on membrane CX3CL1 which may be relevant to the pathology of atherosclerosis and other vascular diseases [5] [61]. In a mouse model of heart transplant treatment with a neutralizing anti-CX3CR1 antibody and no additional immunosuppression prolonged graft survival significantly [62].

CX3CL1 may be expressed by the renal tubular epithelium and $CX3CR1^+$ monocytes and T cells are widely expressed in inflammatory renal tissues [63]. The CX3CL1/CX3CR1 axis is activated by IFN-γ, IL-1, TNF-α, IL-6, IL-10, and CD40 ligand. While Th1 cells respond to CX3CL1, Th2 cells do not [64]. CX3CL1 mRNA was reported to increase in tubular epithelial cells from acutely rejecting KTx, but a bit surprisingly, it was also described to be absent in infiltrating leukocyte subsets, although there was a co-localization between CX3CL1, T cells and macrophages [65]. One group reported that both CX3CR1 and CX3CL1 in serum were good predictors of acute rejection in human KTx [63] and that serum CX3CL1 level was more sensitive than the increases of serum creatinine for rejection diagnosis [66]. Urinary CX3CL1 was reported to differentiate KTx suffering acute tubular necrosis from those enduring acute rejection [38] and seemed to identify irreversible acute rejections [38]. This very promising report suffers from the limitation associated with the significant number of rejecting KTx that do not produce urine.

We observed a significantly higher expression of CX3CL1 among acutely rejecting KTx, and although the PPV was the lowest in this group of molecules, the AUC was 0.97 when analysing the absolute numbers of $CX3CL1^+$ cells. More, we did not observe any correlation with serum creatinine or calcineurin inhibitors blood levels, as we did for the other factors analysed.

We preferred to study CCL5 synthesis in Fnab sample cultures, since we have previously reported to produce different sets of cytokines depending on the clinical KTx status [19] [44] [60]. Intrarenal CCL5 production was observed following activation of dendritic cells with Flt3 in mice [67]. CCL5 is expressed 3-5 days after naïve T cell activation and is chemoattractive of T lymphocytes, monocytes, and natural killer cells among other cells [40]. CCL5 induces metalloproteinase expression which is required to facilitate cells movement through extracellular matrix under chemokine gradients [40]. CCL5 has been reported to associate with a wide variety of kidney diseases, encompassing acute glomerulonephritis, acute renal failure, renal carcinoma and transplant rejection [40]. A comprehensive analysis of plasma CCL5 showed a significant decline post-KTx as compared to pre-KTx. We could speculate an effect of immunosuppressive treatment [42], although CCL5 was already lower among pre-KTx as compared to healthy controls [42].

The first description of CCL5 in KTx was made J Pattison *et al.* back in the nineties [26] and it has been confirmed more recent by others' reports [15] [42]. In biopsy samples, in which the CCL5 gene was also incorporated into a set causally related to the differentiation and activation of macrophages and B cells, a very good performance was observed, either in the diagnosis of acute rejection or in the degree of anti-graft reaction [41]. We also observed a significantly higher CCL5 production by graft-infiltrating cells of acutely rejecting patients with an acceptable PPV of 78.5%. We cannot know if our results would be better if the culture incubation time was longer than the 48 hours select, but contrary to previous reports, ours do not point for a non-redundant role of CCL5 in KTx acute rejection.

Our study is endowed to strongness and weakness. Among the former, we used a friendly method to obtain graft-infiltrating cells, which according to our previous reports has better capacity to tell what is going on as compared to cells collected from the peripheral blood [59]. Our data also confirmed some other results and advanced an original finding concerning CCR3, while at the same time proposes another set of markers that could help in refining the analysis of transplant biopsies. To the best of our knowledge, this is the first time that positive predictive values results for those set of chemokine receptors and CX3CL1 are presented.

Notwithstanding, our study presents a few clear-cut weaknesses. The low number of cases did not allow a subdivision of different kinds of rejection, specially the cellular and humoral ones, or irreversible rejection episodes. Yet the safety and very little patient discomfort, we were reluctant to propose a sequen-

tial Fnab sampling protocol, though we do not doubt that this would carry additional and very useful information.

This was a unique and basic study from a single center, demanding a replication from others, particularly including more cases treated with polyclonal or monoclonal antibodies. Furthermore, it is a retrospective study and as a result of the limited amount of each sample we were unable to analyse simultaneously several factors in each patient.

One could also rightly argue that our Fnab samples are contaminated by variable amount of peripheral blood. However, we have repeatedly shown that cells in Fnab samples are different from those in peripheral blood, suggesting that what we are seeing reflects, albeit imperfectly, the ongoing events within the renal graft that do not show up in peripheral blood flow [24] [59].

5. Conclusion

Despite the limited number of cases, we believe that our data are quite consistent and add further information about the anti-allograft response, particularly during the important early post-transplantation period. Furthermore, while our data partially replicate reports by other authors, we added information about the positive predictive value of CCR1, CCR3, CCR7 and CCL5. These, despite being indirect indicators, are robust in their non-redundant role. Moreover, we bring CCR3 to the foreground, which has not been reported in kidney transplants. We surmise that future advances in immunosuppressive drugs should take into account these results, as well as other previous studies on chemokines and their receptors.

Compliance with Ethics Guidelines

This study was approved by the Committee of Ethics of the Center of Saint John Hospital and of the Faculty of Medicine of the University of Porto, application nº 22930505. All procedures were followed in accordance with the Helsinki Declaration of 1975 and subsequent revisions. Informed consent was received from all the patients involved in this study.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

Fine-needle aspiration biopsies: Fnab

Kidney transplants: KTx

Acute rejection: AR