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Original Research

Higher Expression of Activated $CD8^+$ T Lymphocytes ($CD8^+CD25^+$, $CD8^+CD69^+$ and $CD8^+CD95^+$) Mediate Early Post-Transplant Acute Tubular Injury in Kidney Recipients

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Abstract

Background: Acute kidney injury (AKI) is a leading cause of early post-transplant kidney damage. Furthermore, acute tubular necrosis (ATN) is appointed as the most prevalent form of AKI, a frequent multifactorial process associated with high morbidity and mortality, yet giving rise to delayed graft function (DGF) and, ultimately, allograft dysfunction. Common factors such as prolonged cold ischemia time, advanced donor age, cadaveric versus living donor, donor history of hypertension, as well as donation after cardiac death have all been deemed risk factors for ATN. With the increasing number of older cadaveric and cardiac donors in the donation process, ATN could have a detrimental impact on patient welfare. Therefore understanding the underlying process would benefit the transplant outcome. We aimed to prospectively monitor several T cell subsets in a cohort of kidney transplant recipients (KTrs) to investigate whether there is an adaptive immune-mediated involvement in the ATN process. **Methods**: Peripheral blood was collected from 31 KTrs at different time points within the first-year post-transplantation for *in vitro* stimulation with Concanavalin-A (Con-A) in a humidified 5% CO_2 incubator at 37 °C for 72 hours. Upon cell stimulation, flow cytometry was applied to quantify the surface expression through the median fluorescence intensity (MFI) of $CD4^+CD25^+$, $CD8^+CD25^+$, $CD4^+CD38^+$, $CD8^+CD38^+$, $CD4^+CD154^+$, $CD8^+CD154^+$, $CD4^+CD69^+$, $CD8^+CD69^+$, $CD4^+CD95^+$, and $CD8^+CD95^+$ T cells. Statistical analysis was carried out with SPSS Statistics IBM v.25 (IBM Corp, Armonk, NY, USA). MFIs values were compared using a univariate analysis by a nonparametric U-Mann Whitney test. ROC analysis was applied to define cut-off values most capable of stratifying patients at high risk of ATN. Spearman's rank-order coefficient test was applied to correlate biomarkers with allograft function. Multivariate regression independently validated $CD8^+$ T lymphocytes as surrogate biomarkers of ATN. A p-value < 0.05 was considered statistically significant. **Results**: KTrs who developed ATN upon transplantation had significantly higher expression of CD25, CD69, and CD95 on $CD8^+$ and lower expression of CD95 on $CD4^+$ T lymphocytes than patients with stable graft function. ROC curve analysis showed that MFIs $\geq$1015.20 for $CD8^+CD25^+$, $\geq$2489.05 for $CD8^+CD69^+$, $\geq$4257.28 for $CD8^+CD95^+$, and $\leq$1581.98 for $CD4^+CD95^+$ were capable of stratifying KTrs at high risk of ATN. Furthermore, patients with an MFI below any cut-off were significantly less likely to develop ATN than those with other values. The allograft function was correlated with the $CD4^+CD95^+/CD8^+CD95^+$ ratio in KTrs who developed ATN. The multivariate analysis confirmed that, within the first-month post-transplant, MFI values of $CD8^+CD25^+$, $CD4^+CD95^+$, and $CD8^+CD95^+$ T lymphocytes, along with donor age, serum creatinine, and GFR were independent risk factors to ATN. Moreover, we were also able to corroborate previous immune factors of importance in immune-mediated response to the allograft, such as the patient's maximum panel reactive antibody (PRA) or the maintenance immunosuppression therapy. **Conclusions**: Our results demonstrate evidence for the implication of $CD8^+$ T lymphocytes in the development of ATN early in the post-transplant phase. Post-transplant monitoring of activated $CD8^+$ T lymphocytes may help identify which patients require further clinical intervention to prevent graft damage.

Keywords: acute tubular necrosis; immune-mediated damage; $CD8^+$ T lymphocytes; biomarker; kidney transplantation

1. Introduction

Kidney transplantation has become a routine procedure to rescue patients with end-stage renal disease (ESRD), resulting in good overall one-year survival [1–3]. However, despite the continuous improvement in the clinical management of kidney transplant (KT) recipients (KTrs), acute kidney injury (AKI) remains a common cause of post-transplant kidney damage in this population. The most common cause of AKI is acute tubular injury (ATI), a frequent multifactorial process following KT. Biopsies obtained one-week after transplantation often show widespread tissue damage causing detachment of the basement membrane and tubular dysfunction with typical signs of denuded tubules with mitotic figures, that in most cases resolve thanks to the high regenerative ability of the kidney [4,5]. However, little is known about the relationship between histological parameters of acute renal damage and transplant outcome. However, it has been directly linked with delayed graft function (DGF) after renal transplantation [6,7], and to some extent, the morphological characteristics of ATI immediately after KT correlates with graft function following DGF recovery [5]. Nevertheless, this arsenal of traditional diagnostic and clinical tools provides little to no information about the cause. However, the higher incidence of ATN in pre-sensitised re-transplanted patients has led to the hypothesis that, in some cases, DGF may be mediated by an immunological insult [8–10].

Several factors can trigger ATI, some of which are directly associated with both donors as well as organ retrievals, such as a prolonged cold ischemia time, older donor age, cadaveric *vs*. life donor, donor history of hypertension, donor serum creatinine levels (dsCr), or donation after cardiac death [11–13]. The main clinical manifestation in patients suffering post-transplant ATI is oliguria, which in some cases may require dialysis for a certain period (several days to months).

Traditionally studies have determined ATN as a relatively innocent complication of renal transplantation; therefore, recommendations were to avoid discarding kidneys in patients that might suffer from this complication as the medical community did not find a rise in mortality nor allograft loss in patients [14], albeit more recent research in this field has shown otherwise. Indeed, ATN might not, by itself, jeopardise the short- and long-term outcome of transplantation. However, some forms of acute injury of the graft may eventually cause other complications such as graft rejection, fibrosis, and even graft dysfunction [15,16]. In this scenario, it is important to discover what pathogenic mechanisms are involved in acute tubular damage to propose them as surrogate markers of transplant outcome or even use them as potential therapeutical targets to diminish the deleterious effect they may cause.

Few biomarkers can be used to predict early post-transplant DGF, of which dsCr stands as the primary; though useful, it is otherwise unspecific on its own. Recent research has attempted to address the scarcity of its specificity by bringing in other biomarker technologies ranging from molecular biology to soluble plasma proteins covering both donor and recipient characteristics. In this instance, albeit donor-derived cell-free DNA (dd-cfDNA) proved its feasibility unequivocally as a surrogate biomarker for the diagnosis of active as well as subclinical kidney rejection [17–20], it failed to accomplish identifying patients with either DGF or ATI [21,22]. In contrast, a few studies have indicated that donor factors can be used as predictors of DGF. In this case, donors' immune-derived mediators such as interleukin 2 (IL-2) along with terminal serum creatinine and blood glucose levels showed, in a recent study conducted by Zhao S. *et al*. [23], an average positive predictive value to DGF. Furthermore, other molecules indicative of tubular injury were eventually tested as surrogate markers of DGF both at the expression level using the recipient's protocol renal biopsies (mRNA-based studies) and in their soluble/secreted forms in peripheral blood/urine, respectively. Donors' urinary levels of neutrophil gelatinase-associated lipocalin (NGAL), liver-type fatty acid-binding protein (L-FABP), interleukin 18 (IL-18), and kidney injury molecule 1 (KIM-1) were assessed at the time of organ procurement. Although NGAL was the only marker showing higher sensitivity and specificity to diagnose severe ATI, its discriminatory values were modest [24]. Nevertheless, all these donor proteins showed differential expression and, more importantly, their levels do not respond equally to all forms of stimuli, thus triggering different biological pathways [24,25], which may allow a more accurate definition of AKI.

Many studies have been conducted to understand the poor sensitivity and specificity values that sCr levels display assessing early post-transplant kidney damage. The current trend in this topic focuses on the donor, and despite all the efforts, the utmost tested markers have shown minimal capacity in the diagnosis of either DGF or ATI thus far.

Currently, there is an increasing number of cadaveric non-heart-beating donors, which might incorporate known risk factors that could increase the incidence of DGF. As AKI will have a detrimental impact on patients' post-transplant outcomes, it is importance to determine putative biomarkers capable of predicting the occurrence of ATI in recipients rather than donors. The recipient's immune response to the graft is pivotal, delivering long-lasting organ survival and better transplant outcomes. Thus far, acute rejection (AR) reflects the gold alloimmune process conditioning graft survival, and therefore, to date, the vast majority of research focuses on the patient alloresponse against the graft to elucidate this phenomenon. However, current research has not focused on the patient immune responses implicated in the development of post-transplant ATN.

Recently, our group reported that liver transplant recipients who developed acute cellular rejection (ACR)

within the first-month post-transplant had a significant increase of percentages from two different subsets of activated T lymphocytes than those with stable graft function. Indeed, as liver recipients who reject showed higher percentages of activated $CD4^+CD154^+$ and $CD8^+CD154^+$ T lymphocytes, we recommended the use of these two subsets as cell-mediated immunity (CMI) biomarkers to monitor the alloresponse against the graft to prevent ACR [26]. These results, amongst others, indicate that immune-mediated reactions to the graft occurring early following transplantation are of pivotal importance to prevent any acute insult, for instance, AR.

To this purpose, our group concentrated on the recipient's immune system throughout the implementation of a functional assay allowing us to characterise several T lymphocyte subsets following *in vitro* stimulation that could meet our primary hypothesis of this research, specifically, recipient's T lymphocytes mediate reactions to the graft leading to post-transplant ATN. This functional assay can assess the activation status of both $CD4^+$ and $CD8^+$ T lymphocyte subsets throughout a flow-cytometric multiparametric panel of immunophenotypic markers such as CD25, CD38, CD69, CD154, and CD95.

This novel approach was applied in this study to prospectively monitor the recipient's $CD4^+$ and $CD8^+$ T lymphocytes at baseline and also at several time points along the first-year post-transplantation (the first and second-week; the first, second, third, and sixth-month; and the first-year) to investigate whether they played a role in AKI, and thus to correlate them with the development of this early post-transplant complication proposing them as surrogate CMI biomarkers for its diagnosis. To this end, consultant immunologists thereafter can advise clinicians on the risk of ATN and/or DGF based on the distribution of T lymphocytes.

2. Materials and Methods

2.1 Study Design

The primary endpoint of this study was to investigate whether, in the course of DGF, due to ATN, there is an immune-mediated insult to the allograft delivered by the recipient's T lymphocytes. To this purpose, we established the biopsy-proven diagnosis of ATN following transplantation in KTrs as the clinical outcome of DGF.

Thirty-one consecutive first-time KTrs were enrolled in this unicentre prospective observational study at the Immunology Department of the Clinical University Hospital "Virgen de la Arrixaca" (HUVA) in Murcia, Spain. Additionally, 17 healthy volunteers were also included as controls. For patients and control subjects, a sample consisting of 10 mL of whole peripheral blood was drawn in a sodium heparin container by venipuncture phlebotomy. This protocol of blood extraction was followed at different time points within the first-year post-transplantation (first and second-week; first, second, third and sixth-month; first-year) as shown in Fig. 1. Socio-demographic data (age, sex) from donors and recipients, alongside clinical, pathological and immunological data were collected in a unified database. Post-transplant complications were also registered (AR, opportunistic infections).

Inclusion criteria for participation in the study were as follows: ABO match, first-time kidney transplant, immunosuppressive therapy based on tacrolimus (TRL) with or without mycophenolate mofetil (MMF), as well as HIV-negativity. Paediatric, re-transplant, or simultaneous kidney-pancreas or liver-kidney transplant patients were excluded.

All patients gave informed consent for their samples to be manipulated and stored for current and future research before recruitment to the study. The study was conducted following the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Clinical University Hospital "Virgen de la Arrixaca" (HUVA) (PI15/01370).

2.2 T Lymphocyte Activation and Flow Cytometry Analysis

Patients enrolled in this study were prospectively monitored for percentages of $CD4^+$ and $CD8^+$ T lymphocytes as well as measuring the upregulation of various important activation and functional markers (CD25, CD38, CD154, and CD95) applying a modified protocol used by Barten MJ *et al*. [27] and as previously described elsewhere [26,28]. Briefly, 10 mL of whole peripheral blood (WPB) was drawn for *in vitro* stimulation purposes according to our own validated standard operating procedure for T lymphocyte stimulation. WPB samples were diluted with RPMI-1640 (1:10) (BioWhittaker®, Lonza, Belgium) and added into flat-bottom 24-well tissue culture microtiter plates. Patient samples were polyclonally activated with lectin mitogenic concanavalin A (Con-A), which is known for its ability to interact with specific components of the T-cell receptor [29] (1 mg/mL; Sigma-Aldrich®, St. Louis, Missouri, USA), reaching a final concentration of 15 µg/mL and a final volume of 2 mL per well. Cell-culture microtiter plates were incubated in a humidified 5% CO_2 incubator at 37 °C for 72 hours (Fig. 1).

Upon *in vitro* stimulation, cell cultures were prepared for multi-colour stain using a combination of mouse anti-human monoclonal antibodies (MoAb) (BD® Biosciences BD, San Jose, CA, USA). Stimulated samples were incubated with a panel of MoAb according to the manufacturer's instructions following erythrocyte lysis by adding 2 mL of 1× lysis buffer (BD FACS™ Lysing Solution). The panel of MoAb included a mouse $IgG1_K$ anti-human CD3-FITC, CD4-APC, CD8-PerCPCy™ 5.5, CD25-PE, CD38-PE, CD69-PE and CD95-PE (BD® Biosciences BD, San Jose, CA, USA) and mouse $IgG1_K$ anti-human CD154-PE (Beckman Coulter®, Marseille, France). In all cases, isotype control antibodies were used to assess the positivity of each fluorochrome; IgG1-FITC and IgG1-PE (Beck-

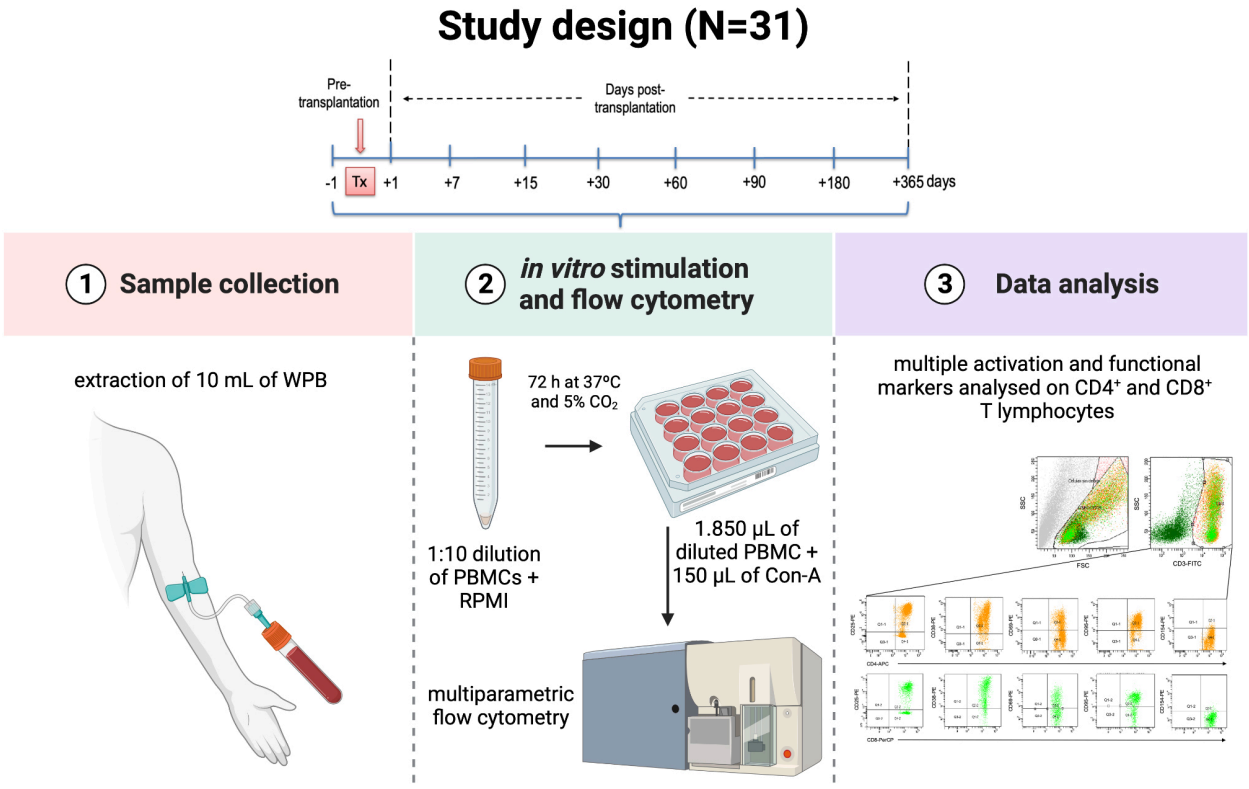

Fig. 1. Study design. Kidney transplant recipients enrolled in this prospective observational study were appointed for sample collection at different time points during the first-year post-transplantation. Following blood extraction, samples were activated *in vitro* using polyclonal stimulus to assess T lymphocytes for several surface markers using multi-parametric flow cytometry.

man Coulter®, Marseille, France), mouse $IgG1_K$-APC and mouse $IgG1_K$-PerCP (BD® Biosciences BD, San Jose, CA, USA). Mean fluorescence intensity (MFI) was used as a relative measurement of molecule expression. Finally, the patient's samples were analysed in a flow cytometer (FACScanto™ II, BD® Biosciences BD, San Jose, CA USA) following the acquisition of 50,000 events from the activated $CD3^+$ T lymphocyte gate. Data analysis was carried out with BD FACSDiva™ Software 6.1.3 (FACScanto™ II, BD® Biosciences BD, San Jose, CA USA) for the percentage and level of expression of CD25, CD38, CD69, CD95, and CD154 on $CD4^+$ and $CD8^+$ T lymphocytes, as shown in Fig. 1.

2.3 Acute Tubular Necrosis Diagnosis

The recipients enrolled in the present study were classified into two study groups named as follows, with acute tubular necrosis (ATN) or without ATN (NoATN). Seven patients (22.6%) experienced DGF due to ATN within the first-year post-transplantation and were therefore included in the ATN study group, whereas the remaining patients (n = 24, 77.4%) were included in the NoATN study group. At our institution, KTrs experiencing DGF with oliguria are subjected to a "for-cause biopsy". Socio-demographic, clinical, and immunological characteristics, as well as the outcomes, were compared between the two groups.

In this context, renal allograft biopsies are commonly evaluated by experienced pathologists based on the current Banff classification [30–32]. There is no current definition of ATI endorsed by the Banff classification. Currently, ATI is included as a diagnosis in Banff diagnostic category 6, which is characterised by the lack of other apparent causes but histological evidence of acute tissue injury in the context of a diagnosis of acute or active ABMR with antibody-mediated changes [30]. Therefore, ATI was diagnosed from patient biopsies with DGF clinically manifested as oliguria that did not show antibody-mediated changes but others unrelated to acute or chronic rejection. Variable tissue changes used to diagnose ATI were described as vacuolisation, brush border loss, and pyknotic nuclei of tubular epithelial cells with signs of tubular dilatation with or without tubulitis, or the presence of any of the following, interstitial inflammation, glomerulitis, tubular atrophy, and luminal casts [33,34].

2.4 Induction and Maintenance Immunosuppression Therapy

KTrs received immunosuppression therapy based on our institution's standard practice of care. Accordingly, induction therapy consisted of either rabbit anti-thymocyte globulin or anti-CD25 monoclonal antibody-based therapy. Therefore thymoglobulin was provided to high-

immune-risk patients, whereas basiliximab was given to low-immune-risk patients. From our data, 87.1% (n = 27) received basiliximab and 12.9% (n = 4) received thymoglobulin.

Maintenance immunosuppression therapy was based on the administration of either tacrolimus (TRL, Prograf®, Astellas Pharma, United Kingdom) with a target dose of 5 mg/day or mycophenolate mofetil (MMF, CellCept®, Roche Pharma, Switzerland) with a target dose of 2000 mg/day. All patients in this study were under the same immunosuppressive conditions. Additionally, KTrs also received methylprednisolone as the main corticosteroid-based therapy (Dacortin® 20 mg/day). Steroids were gradually reduced to 5 mg/day from day 1 until day 90 post-transplantation. Due to the number of side effects associated with glucocorticoids, patients with stable graft function were weaned from steroids at the sixth-month post-transplantation. A total of 90.3% (n = 28) of KTrs received double immunosuppression therapy based on TRL and MMF, whereas the remaining patients were on a monotherapy regimen, either with TRL (3.2%, n = 1) or MMF (6.5%, n = 2).

2.5 Statistical Analysis

Demographic, clinical, and immunological data were collected in a unified database (Microsoft Access 11.0; Microsoft Corporation, Seattle, WA, USA), and statistical analysis was performed using the SPSS 20.0 software (SPSS Inc., Chicago IL, USA). The Kolmogorov–Smirnoff test was used to assess whether the demographic and clinical patient data, as well as the percentages and MFI data of the T lymphocyte subsets adjusted to parametric distribution. All variables were distributed nonparametrically. Therefore, quantitative data were presented using the median with an interquartile range of 25 and 75, whereas qualitative data were presented as absolute and relative frequencies.

Pearson's X^2 and two-tailed Fisher's exact tests were carried out to detect differences comparing bivariant categorical variables between groups, whereas the Kruskal–Wallis test was applied for variables with more than two categories. Odds ratios (OR) and 95% confidence intervals (CI) were used to calculate the specificity, sensibility, as well as positive and negative predictive values of our models. Additionally, the U Mann–Whitney test was used to compare unpaired continuous variables, and Spearman's rank-order correlation coefficient test was used to measure the strength and direction of associations existing between nonparametric paired continuous variables.

Receiver operating characteristic (ROC) curves were used to identify the optimal cut-off points for those surrogate biomarkers deemed significant to stratify patients at high risk of ATN. Cut-off points were calculated based on the best Youden-index (sensitivity + specificity-1) [35]. The area under the ROC curve (AUC) was analysed as follows, an area of 0.6–0.7 was considered acceptable; an area of 0.7–0.8, excellent; and an area of >0.8, outstanding [36].

Any demographic, clinical, and immunological variable statistically significant at the univariate pre-transplant cross-sectional analysis as well as any known variable with clinical importance, was finally assessed in a backward stepwise multivariate logistic regression [37]. Multivariable logistic regression analysis was applied to confirm positive associations. Relative hazard ratios (HR) and their 95% confidence intervals (CI) were calculated to estimate the likelihood of the occurrence of ATN. A level of $p \leq 0.05$ was accepted as statistically significant.

3. Results

3.1 Patient's Demographic, Immunological, and Clinical Characteristics

Within the first-year post-transplantation, 22.6% of KTrs (n = 7) were diagnosed with DGF due to biopsy-proven ATN versus 77.4% (n = 24) who did not. Furthermore, from the ATN group, only one patient (14.3%) additionally developed AR, whereas 25% (n = 6) of recipients from the NoATN group did not reject the allograft during the study. Nevertheless, neither the presence nor the absence of ATN was correlated with the occurrence of AR in our cohort of patients (p = 0.551, OR = 1.750, 95% CI = 0.251–12.207 and OR = 0.875, 95% CI = 0.598–1.280, respectively).

Overall, the median [IQR] donor age was 55 [42–61], and the median [IQR] recipient age was 53 [41–59] with no significant impact on ATN (p = 0.679 and p = 0.125, respectively) despite age being previously associated with DGF and ATN. However, some intrinsic and extrinsic immunological and clinical characteristics of the patients differed significantly from the ATN and NoATN study groups. For instance, the presence of preformed anti-HLA antibodies (42.9% *vs*. 16.7%, p = 0.012, OR = 3.750, 95% CI = 1.295-10-862), and the maximum (6.42 ± 1.37 *vs*. 4.46 ± 1.27, p = 0.005), as well as current (4.71 ± 1.56 *vs*. 3.21 ± 0.87, p = 0.032) panel reactive antibody (PRA), showcased significant differences between patients diagnosed with and without ATN. Moreover, the dose of MMF (mg/day) was significantly higher in NoATN than in ATN (1890.83 ± 30.56 *vs*. 1500.25 ± 0.89, p = 0.006). Post-transplant immunosuppressive therapy was also significantly correlated with ATN, suggesting that in patients treated with a double therapeutic regimen (TRL + MMF), 78.57% (n = 22) had stable allograft function within the first-month post-transplant, compared with 21.43% (n = 2) who developed ATN (p = 0.019). Finally, renal function was significantly impaired in patients with ATN compared with patients without ATN, as showed by serum creatinine (13.06 ± 7.07 *vs*. 2.48 ± 0.25, p = 0.000000143) and glomerular filtration rate (32.71 ± 6.18 *vs*. 63.78 ± 3.32, p = 0.00012). All remaining patient characteristics were not significant. These data are depicted in Table 1.

Table 1. Demographic, immunological and clinical data at baseline[1] and within the 1st month posttransplantation[2].

Variables	Total KTr (N = 31)[1]	ATN (n = 7)[2]	NoATN (n = 24)[2]	*p* value[#]
Donor age, median [IQR]*	55 [42–61]	54 [47–69]	56 [41–61]	0.679
Recipient age, median [IQR]*	53 [41–59]	57 [49–61]	53 [40–59]	0.125
Recipient gender (male/female), n (%)	24 (77.4)/7 (22.6)	5 (71.4)/2 (28.6)	19 (79.2)/5 (20.8)	0.667
Pre-transplant sensitisation⌉ (No/Yes)	24 (77.4)/7 (22.6)	4 (57.1)/3 (42.9)	20 (83.3)/4 (16.7)	0.012[b]
Maximum PRA, median [IQR]**	0 [0–7]	7 [0–14]	0 [0–0]	0.005[a]
Current PRA, median [IQR]**	0 [0–4]	0 [0–9]	0 [0–0]	0.032[a]
HLA-A mismatch (0/1/2)	2 (6.5)/17 (54.8)/12 (38.7)	1 (14.3)/4 (57.1)/2 (28.6)	1 (4.2)/13 (54.2)/10 (41.7)	0.574
HLA-B mismatch (0/1/2)	4 (12.9)/15 (48.4)/12 (38.7)	2 (28.6)/2 (28.6)/3 (42.9)	2 (8.3) /13 (54.2)/9 (37.5)	0.287
HLA-DRB1 mismatch (0/1/2)	6 (19.4)/18 (58.1)/7 (22.6)	2 (28.6)/5 (71.4)/0 (0)	4 (16.7)/13 (54.2)/7 (29.2)	0.257
Total lymphocyte (%), median [IQR]	10.6 [4.6–21.7]	10.6 [4–13]	10.5 [4.7–23.9]	0.028[a]
Total lymphocyte (cells/mm^3), median [IQR]	870 [500–1540]	1000 [500–1600]	850 [523–1480]	0.129
Total leukocyte ($\times 10^9$/L), median [IQR]	8.4 [5.9–12.8]	10.9 [7.3–13.3]	7.9 [5.7–12.7]	0.847
Induction therapy (Thymoglobulin/Basiliximab), n (%)	4 (12.9)/27 (87.1)	0 (0)/7 (100)	4 (16.7)/20 (83.3)	0.550
Post-transplant treatment (TRL+MMF/MMF or TRL), n (%)	28 (90.3)/3 (9.7)	6 (85.7)/1 (14.3)	22 (91.7)/2 (8.3)	0.019[c]
Dose of TRL (mg/day), median [IQR]	13 [10–16]	11 [10–14]	13.5 [9.8–16.3]	0.262
Dose of MMF (mg/day), median [IQR]	2000 [800–2000]	2000 [500–2000]	2000 [1800–2000]	0.006[a]
Serum creatinine (mg/dL), median [IQR]	5.9 [4.5–7.3]	7.3 [5.5–8.9]	5.4 [4.2–6.8]	0.000000143[a]
Glomerular filtration rate (mL/min/1.73 m^2), median [IQR]	61 [54–76]	40 [32–47]	68 [60–78]	0.00012[a]
Dialysis time (years), median [IQR]	3 [2–5]	4 [2–6]	3 [2–5]	0.132
Acute Rejection (No/Yes), n (%)	24 (77.4)/7 (22.6)	6 (85.7)/1 (14.3)	18 (75)/6 (25)	0.551

N, total number of individuals; n, number of patients in each group; ATN, acute tubular necrosis study group; NoATN, non-acute tubular necrosis study group; KTr, kidney transplant recipients; IQR, interquartile range; PRA, panel reactive antibody; HLA, human leukocyte antigen; TRL, tacrolimus; MMF, mycophenolate mofetil; DSA, donor-specific antibodies.

[1]Values correspond to patient's characteristics at baseline.

[2]All comparisons were made between ATN and NoATN study groups within the 1st month posttransplantation.

*Age is expressed in years.

**PRA is expressed in %.

⌉Includes preformed DSA and NoDSA anti-HLA antibodies.

[#]*p* values marked in bold are statistically significant ($p \leq 0.05$).

[a]*p* value obtained comparing continuous variables between ATN versus NoATN patients in univariate analysis using two-tailed Mann-Whitney U test.

[b]*p* value obtained comparing categorical variables between ATN versus NoATN patients in univariate analysis using two-tailed Pearson's Chi-square test.

[c]*p* value obtained comparing categorical variables between ATN versus NoATN patients in univariate analysis using two-tailed Kruskal-Wallis test.

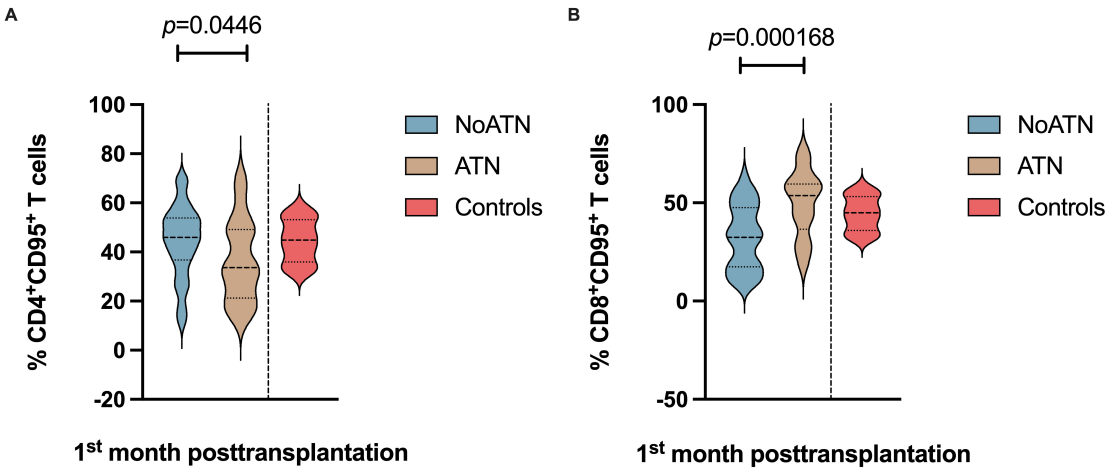

Fig. 2. Stratification analysis in KTrs within the first-month post-transplantation. (A) Differences in the percentage of $CD4^+CD95^+$ T lymphocytes* between ATN, NoATN and controls. (B) Differences in the percentage of $CD8^+CD95^+$ T lymphocytes* between ATN, NoATN and controls. ATN, KTrs with acute tubular necrosis; NoATN, KTrs without acute tubular necrosis; KTrs, kidney transplant recipients. *The mean percentages of $CD4^+CD95^+$ and $CD8^+CD95^+$ T lymphocytes at 7, 15 and 30 days post-transplantation were used in this analysis.

3.2 Differences in the Percentages and Expression Level of CD25, CD38, CD69, CD154 and CD95 on $CD4^+$ and $CD8^+$ T Lymphocytes

As ATN is most frequently diagnosed within the first days following surgery, we focused our stratification analysis on different $CD4^+$ and $CD8^+$ T lymphocyte subsets from day one to one-month post-transplant. We calculated the mean of the distribution for NoATN vs ATN of $CD4^+CD95^+$ (Fig. 2A, 44.31 $\pm$ 1.74 *vs.* 36.59 $\pm$ 3.68, p = 0.0446) and $CD8^+CD95^+$ (Fig. 2B, 24.31 $\pm$ 3.71 *vs.* 53.82 $\pm$ 1.26, p = 0.000168) T lymphocytes from 7, 15 and 30 days post-transplantation, where both showed statistically significant differences. No differences were observed between either of the T lymphocyte subsets and the control group. Furthermore, the percentages of $CD4^+CD25^+$, $CD8^+CD25^+$, $CD4^+CD38^+$, $CD8^+CD38^+$, $CD4^+CD154^+$, $CD8^+CD154^+$, $CD4^+CD69^+$ and $CD8^+CD69^+$ T lymphocytes from both study groups showed no differences at any other time point during the study (**Supplementary Table 1**).

The expression, measured as MFI, of the different surface markers, did not differ much from what we observed regarding the distribution of T cell subsets. Mean MFI values were calculated for the different T lymphocyte subsets and used in the analysis. Albeit not seen as statistically significant following the univariate stratification analysis, the expression of CD25 on $CD8^+$ T lymphocytes showed a trend towards a higher MFI in ATN than in NoATN patients (Fig. 3A, 2704.44 $\pm$ 825.37 vs 1632.12 $\pm$ 476.17, p = 0.058). $CD8^+CD69^+$ T lymphocytes showed differences between both study groups, where patients with ATN had statistically significantly higher MFI values than patients without ATN (Fig. 3B, 4495.10 $\pm$ 604.85 *vs.* 3985.43 $\pm$ 549.94, p = 0.035). Furthermore, the level of CD95 again indicated an inverse expression depending on the T lymphocyte subset in such a way that patients with ATN had a lower expression of CD95 on $CD4^+$ (Fig. 3C, 4495.10 $\pm$ 604.85 *vs.* 3985.43 $\pm$ 549.94, p = 0.004) compared with $CD8^+$ (Fig. 3D, 4567.11 $\pm$ 642.20 vs 2861.08 $\pm$ 575.36, p = 0.012) T lymphocytes. None of the T lymphocyte subsets differed significantly from controls, as shown in Fig. 3.

3.3 The Expression of CD25, CD69, and CD95 on T Lymphocytes Identifies Patients at High Risk of ATN

Following the univariate stratification analysis, we carried out a study to validate, from the pool of significant T lymphocyte subsets, those of importance as surrogate biomarkers for ATN in KTrs within the first-month post-transplantation.

The AUC analysis showed that out of all $CD4^+$ and $CD8^+$T lymphocyte subsets, only a few were capable of stratifying statistically significant recipients at high risk of ATN with reasonable sensitivity and specificity (Fig. 4). Therefore, we tested the capability of our four different predictive models, where we found that CD25 and CD69 expression on $CD8^+$ T lymphocytes correlated directly with the risk of ATN. Accordingly, 84.8% of patients with MFI values of $CD8^+CD25^+$ $<$1015.20 did not develop ATN compared with 15.2% that, regardless of being below the cut-off, developed ATN (OR = 4.738, 95% CI = 1.662–13.508, p = 0.002). Similarly, 76.2% of KTrs with an MFI of $CD8^+CD69^+$ $\geq$2489.05 developed ATN, whereas 23.8% of patients developed ATN despite being stratified as low risk (OR = 3.491, 95% CI = 1.151–10.590, p = 0.022).

In addition, the expression of CD95 on $CD4^+$ or $CD8^+$ T lymphocytes differed in stratifying KTrs. A total of 88.6% of patients with MFI values of $CD4^+CD95^+$

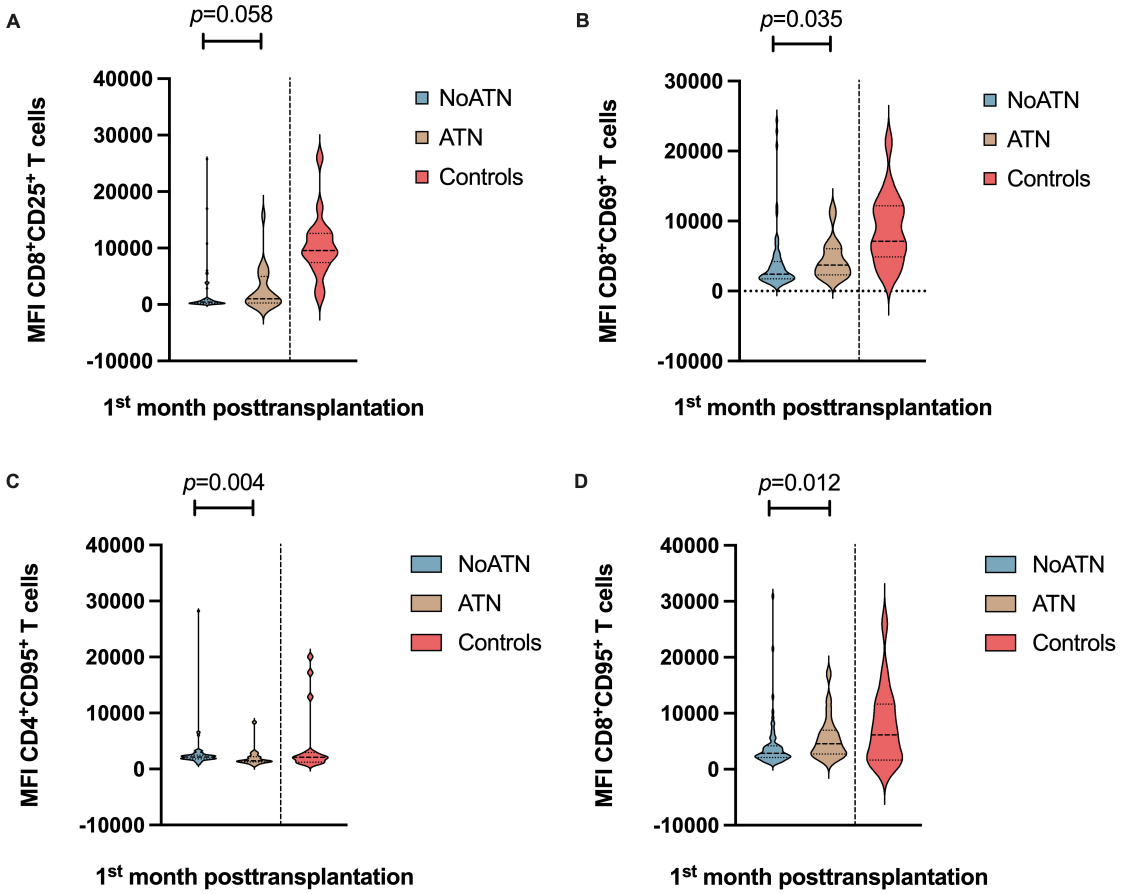

Fig. 3. Stratification analysis in KTrs within the first-month post-transplantation. (A) Differences in the MFI of $CD8^+CD25^+$ T lymphocytes* between ATN, NoATN and controls. (B) Differences in the MFI of $CD8^+CD69^+$ T lymphocytes* between ATN, NoATN and controls. (C) Differences in the MFI of $CD4^+CD95^+$ T lymphocytes* between ATN, NoATN and controls. (D) Differences in the MFI of $CD8^+CD95^+$ T lymphocytes* between ATN, NoATN and controls. ATN, KTr with acute tubular necrosis; NoATN, KTr without acute tubular necrosis; KTr, kidney transplant recipients; MFI, median fluorescent intensity. *Mean MFI of $CD8^+CD25^+$, $CD8^+CD69^+$, $CD4^+CD95^+$ and $CD8^+CD95^+$ T lymphocytes at 7, 15 and 30 days post-transplantation were used in this analysis.

>1581.98 did not develop ATN compared with 11.4% who experienced otherwise (OR = 14.393, 95% CI = 4.434–46.721, p = 0.0000587). Contrarily, MFI values of $CD8^+CD69^+$ <4257.28 were capable of stratifying patients at low risk of ATN, showing that 84.1% of KTrs with levels below the cut-off were free of ATN, whereas 15.9% were diagnosed with ATN (OR = 3.644, 95% CI = 1.310–10.132, p = 0.011).

The specificities, sensitivities, and positive and negative predictive values of the four different predictive models for ATN are depicted in Table 2.

3.4 The $CD4^+CD95^+/CD8^+CD95^+$ Ratio Correlates with the Allograft Renal Function and Could be Used as a Surrogate Biomarker of ATN

Recipients who experienced ATN had significantly lower glomerular filtration rates (GFR) compared with those who displayed stable allograft function within the first-month post-transplantation (Fig. 5A, 32.71 ± 6.18 vs 63.78 ± 3.32, p = 0.000120). Furthermore, the impairment in renal function was accompanied by a significant increase in serum creatinine levels, as shown in Fig. 5B (13.06 ± 7.07 vs 2.48 ± 0.25, p = 0.000000143).

Interestingly, we calculated the ratio of $CD4^+CD95^+/CD8^+CD95^+$ and compared it against both study groups showing that ATN patients had an inversion of this ratio compared with NoATN patients (Fig. 5C, 0.56 ± 0.09 vs 1.09 ± 0.31, p = 0.023).

Furthermore, we assessed the $CD4^+CD95^+/CD8^+CD95^+$ ratio using a ROC curve analysis to ascertain the cut-off point to stratify KTrs at high risk of allograft failure in the absence of rejection. Recipients were tested before transplantation and within the first-month post-transplantation. Albeit the ROC curve analysis at baseline did not reach statistical significance (AUC = 0.470, 95% CI = 0.211–0.729, p = 0.813), it was different when used in the first-month post-transplantation. A $CD4^+CD95^+/CD8^+CD95^+$ ratio ≤0.32 accurately stratified KTrs at high risk of DGF due to ATN (Fig. 5D, AUC = 0.664, 95% CI = 0.522–0.806, p = 0.023) with

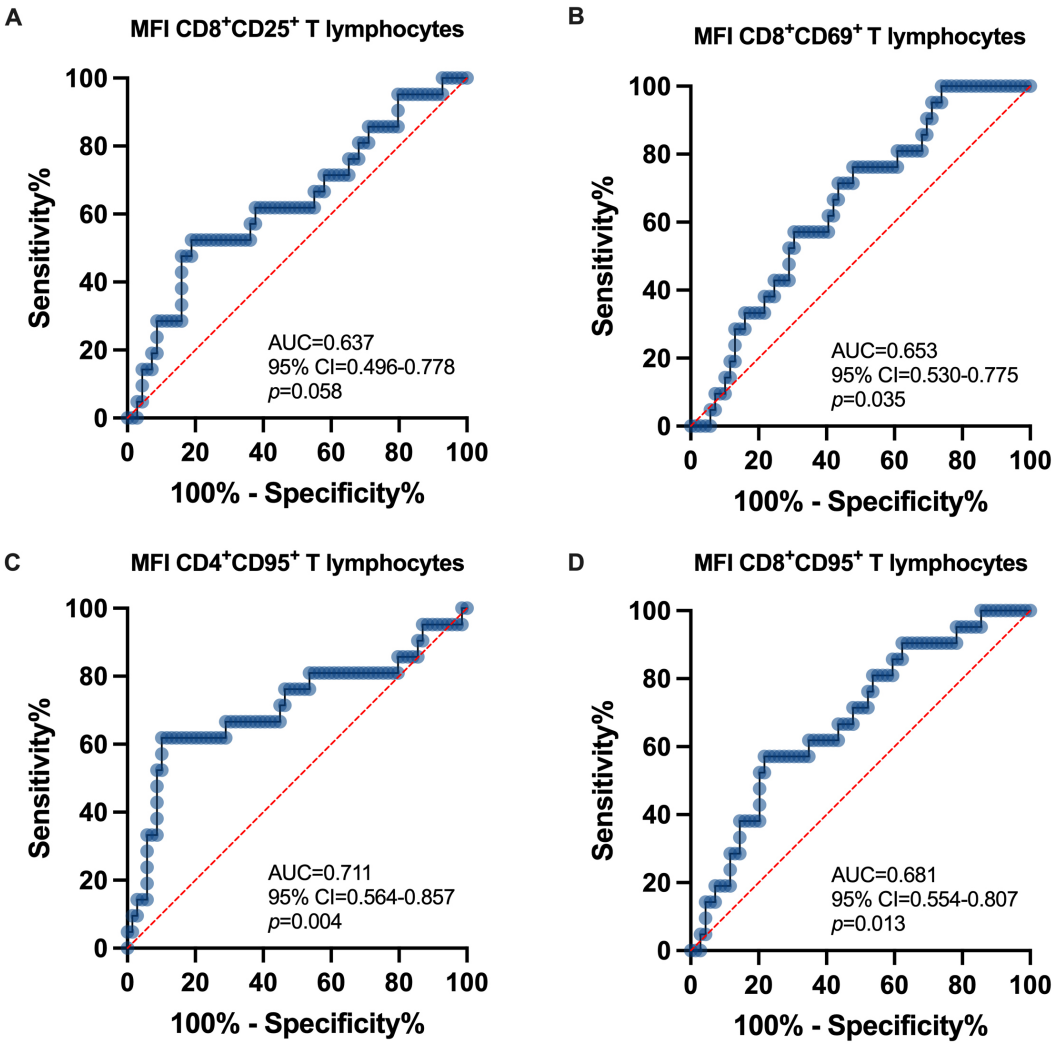

Fig. 4. ROC curve analysis within the first-month post-transplant. (A) AUC⌋, sensitivity and specificity of $CD8^+CD25^+$ T lymphocytes that accurately determined the cut-off‡ for the MFI value to be used as a surrogate biomarker to stratify KTrs at high risk of ATN. (B) AUC⌋, sensitivity and specificity of $CD8^+CD69^+$ T lymphocytes that accurately determined the cut-off‡ for the MFI value to be used as a surrogate biomarker to stratify KTrs at high risk of ATN. (C) AUC⌋, sensitivity and specificity of $CD4^+CD95^+$ T lymphocytes that accurately determined the cut-off‡ for the MFI value to be used as a surrogate biomarker to stratify KTrs at high risk of ATN. (D) AUC⌋, sensitivity and specificity of $CD8^+CD95^+$ T lymphocytes that accurately determined the cut-off‡ for the MFI value to be used as a surrogate biomarker to stratify KTrs at high risk of ATN. ⌋AUC = values considered in this study: 0.6–0.7 as acceptable; 0.7–0.8 as excellent, and >0.8 as outstanding. ‡Youden index (sensitivity + specificity – 1) was carried out to obtain the most accurate cut-off values for the MFI of $CD8^+CD25^+$, $CD8^+CD69^+$, $CD4^+CD95^+$, and $CD8^+CD95^+$ T lymphocytes capable of stratifying patients at high risk of ATN. AUC, area under the curve; 95% CI, 95% confidence interval; ROC, receiver operating characteristic curve; KTrs, kidney transplant recipients; ATN, acute tubular necrosis.

reasonable specificity and sensitivity, as shown in Table 2. In fact, after stratifying KTrs according to the calculated cut-off point, 88.9% of recipients who fell into the high-risk group for DGF due to a $CD4^+CD95^+/CD8^+CD95^+$ ratio $\leq$0.32 were diagnosed with ATN in the first-month post-transplantation, as opposed to 11.1% who did not develop ATN irrespective of a cut-off $\leq$0.32. In our cohort, recipients with a $CD4^+CD95^+/CD8^+CD95^+$ ratio $\leq$0.32 had a 5-fold higher risk of ATN over other recipients (OR = 5.00, 95% CI = 1.64–15.21, $p = 0.005$).

Correlation analysis showed that the $CD4^+CD95^+/CD8^+CD95^+$ ratio was inversely proportional to serum creatinine [Fig. 5E, r_s (8) = 0.226, p = 0.033] whereas for GFR, the $CD4^+CD95^+/CD8^+CD95^+$ ratio showed a positive correlation [Fig. 5F, r_s (8) = 0.378, $p = 0.000239$]. In both cases, an increase in $CD8^+CD95^+$ T lymphocytes over $CD4^+CD95^+$ T lymphocytes was associated with poor allograft function.

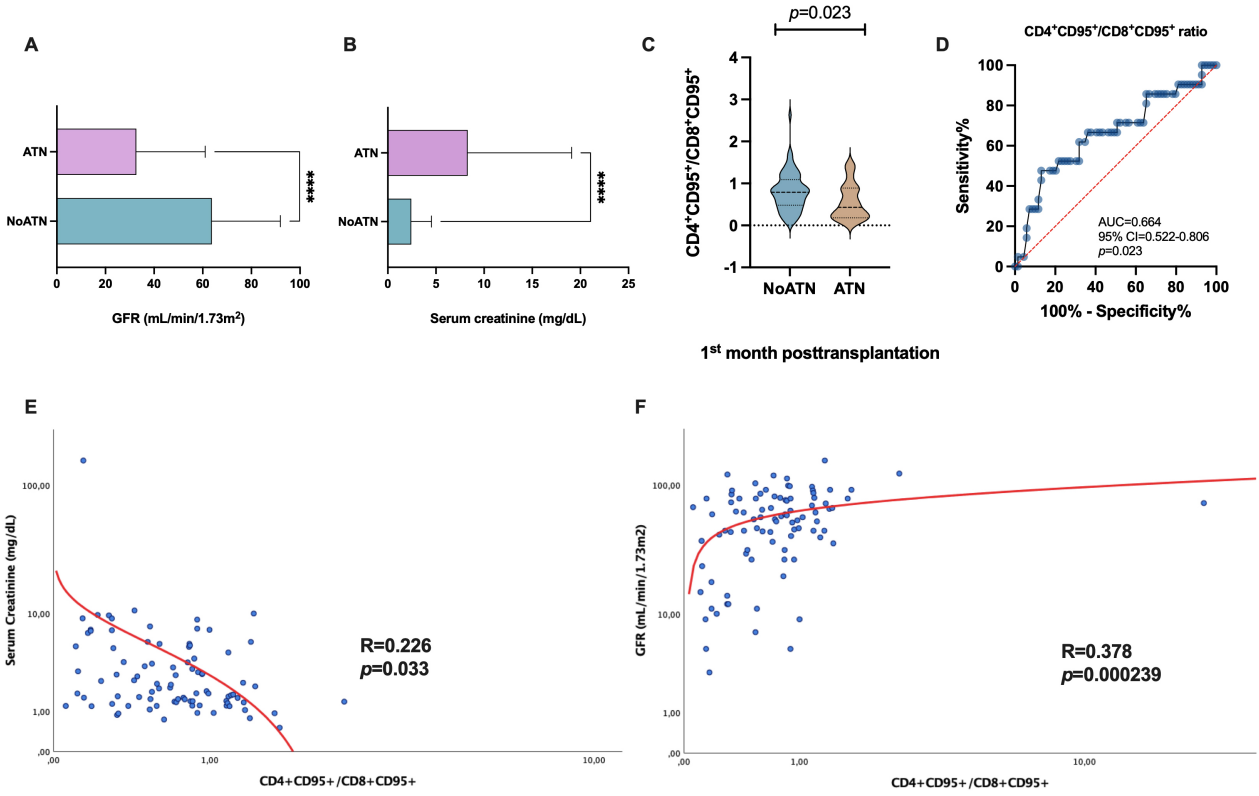

Fig. 5. Analysis of the allograft function and its association with T lymphocytes. (A) Differences in the GFR (mL/min/1.73 m^2) between KTrs with and without ATN. (B) Differences in the concentration of serum creatinine (mg/dL) between KTrs with and without ATN. (C) Differences in the $CD4^+CD95^+/CD8^+CD95^+$ ratio between KTrs with and without ATN. (D) AUC⌋, sensitivity and specificity of $CD4^+CD95^+/CD8^+CD95^+$ ratio that accurately determined the cut-off‡ value to be used as a surrogate biomarker to stratify KTrs at high risk of ATN. (E) Logarithmic-scale correlation (log-log) plot analysis between the concentration of serum creatinine and the $CD4^+CD95^+/CD8^+CD95^+$ ratio with the first-month post-transplantation. (F) Logarithmic-scale correlation (log-log) plot analysis between GFR (mL/min/1.73 m^2) and the $CD4^+CD95^+/CD8^+CD95^+$ ratio with the first-month post-transplantation. ⌋AUC = values considered in this study: 0.6–0.7 as acceptable; 0.7–0.8 as excellent, and >0.8 as outstanding. ‡Youden index (sensitivity + specificity – 1) was carried out to obtain the most accurate cut-off values for the $CD4^+CD95^+/CD8^+CD95^+$ ratio capable of stratifying patients at high risk of ATN. GFR, glomerular filtration rate; KTr, kidney transplant recipient; ATN, acute tubular necrosis; AUC, area under the curve; 95% CI, 95% confidence interval; ROC, receiver operating characteristic curve. **** means a p value ≤ 0.0001.

3.5 CD8 T Cell Subsets Could Stratify KTrs at High Risk of ATN Following Transplantation

Following the stratification analysis, we then analysed whether the differences showed by CD8 T cell subsets between patients with ATN and stable graft function could be used to stratify them into two different groups based on the MFI expression of CD25, CD69 and CD95 in CD8 T cells to ascertain the risk of ATN. ROC curve analysis was applied to find the best cut-off values for $CD8^+CD25^+$, $CD8^+CD69^+$, and $CD8^+CD95^+$ (Fig. 4).

An MFI ≥923 for $CD8^+CD25^+$ (AUC = 0.616, 95% CI = 0.484–0.748, $p = 0.048$), 2747 for $CD8^+CD69^+$ (AUC = 0.652, 95% CI = 0.537–0.767, $p = 0.021$) and 2638 for $CD8^+CD95^+$ (AUC = 0.657, 95% CI = 0.540–0.774, $p = 0.017$) were capable of stratifying KTrs at high risk of ATN within the first-month post-transplantation.

3.6 Activated $CD8^+$ T Cells, Serum Creatinine, GFR, Donor Age, and Presence of Anti-HLA Antibodies Perform as Detrimental Factors in KT

Following univariate analysis, those patients and donor characteristics of clinical importance that showed, by any means, an association with poor allograft function within the first-month post-transplantation were input into a multivariate logistic regression model to ascertain their independency as risk factors for ATN. Certainly, various variables kept their statistical significance toward a poorer outcome by influencing the odds of developing ATN.

From the multivariate analysis, 45.3% of the variation in the development of ATN in KTrs cannot be explained by our predictors (covariables). Our regression model accurately predicted ATN development with high sensitivity and specificity, showing that 95.65% of patients from the low-risk pool did not develop ATN, whereas 80.95% of KTrs stratified as high risk indeed developed ATN within the

Table 2. Sensitivity, specificity, PPV and NPV values, with their 95% confidence intervals, from the predictive models for ATN in KTr within the 1st month posttransplantation based on the MFI values following *in vitro* stimulation of surface markers on $CD4^+$ and $CD8^+$ T lymphocytes, as well as the $CD4^+CD95^+/CD8^+CD95^+$ ratio.

Kidney transplant recipients (N = 31)	Cut-off (MFI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	*p* value[†]
Surrogate biomarker for ATN						
Surface markers on $CD8^+$ T lymphocytes						
$CD8^+CD25^+$ T lymphocytes	1015.20	45.83 (25.55–67.18)	84.85 (73.90–92.49)	52.38 (34.92–69.28)	81.16 (74.62–83.06)	0.002
$CD8^+CD69^+$ T lymphocytes	2489.05	32.65 (19.95–47.54)	87.80 (73.80–95.92)	76.19 (56.18–88.87)	52.17 (46.53–57.76)	0.022
$CD8^+CD95^+$ T lymphocytes	4257.28	40.74 (22.39–61.20)	84.13 (72.74–92.12)	52.38 (34.69–69.50)	76.81 (70.41–82.18)	0.011
Surface markers on $CD4^+$ T lymphocytes						
$CD4^+CD95^+$ T lymphocytes	1581.98	65.00 (40.78–84.61)	88.57 (78.72–94.93)	61.90 (43.99–77.08)	89.86 (82.89–94.18)	0.0000587
Lymphocyte ratio						
$CD4^+CD95^+/CD8^+CD95^+$ ratio	0.32	57.14 (37.18–75.54)	96.77 (88.83–99.61)	88.89 (66.35–97.01)	83.33 (76.48–88.49)	0.005

PPV, positive predictive value; NPV, negative predictive value; ATN, acute tubular necrosis; KTr, kidney transplant recipients; MFI, mean fluorescence intensity; 95% CI, 95% confidence interval.

[†]*p* values obtained using two-tailed Pearson's Chi-square test or two-tailed Fisher exact test accordingly following the application of the best-cutoff value for the different T lymphocyte subsets capable to stratify KTr in high or low risk of ATN.

Table 3. Multivariate logistic model to evaluate the risk of ATN in KTr within the 1st month posttransplantation.

Variables	B coefficient	HR	95% CI	*p* value[⊣]
Donor age (years)	–0.167	0.847	0.728–0.984	0.030
Serum creatinine (mg/dL)	1.073	2.923	1.543–5.536	0.001
Glomerular filtration rate (mL/min/1.73 m^2)	–0.064	0.938	0.882–0.997	0.039
Pretransplant sensitisation (Yes)	1.745	5.726	1.313–24.977	0.020
$CD8^+CD25^+$ (MFI $\geq$1015.20)	1.651	5.212	1.295–20.975	0.020
$CD4^+CD95^+$ (MFI $\leq$1581.98)	2.516	12.376	3.104–49.339	0.000363
$CD8^+CD95^+$ (MFI $\geq$4257.28)	1.306	3.693	1.928–14.689	0.034

ATN, acute tubular necrosis; KTr, kidney transplant recipients; HR, hazard ratio; 95% CI, 95% confidence interval; MFI, Median Fluorescent Intensity.

[⊣]*p* values were calculated using the backwards Wald test.

first-month post-transplantation. Donor age had a protective effect on the risk of ATN, showing that for each year increase in donor age, the recipient was 0.847 times less likely to develop ATN compared with patients transplanted with younger donors (HR: 0.847, 95% CI = 0.728–0.984, $p = 0.003$). Similarly, regarding renal function measured as GFR, a protective effect was also observed. Particularly, patients who maintained stable allograft function in the first-month after transplantation had a 6.2% reduction in the relative risk of ATN (HR: 0.938, 95% CI = 0.882–0.997, $p = 0.039$). Furthermore, serum creatinine showed a significant detrimental effect on the primary outcome, as recipients with higher levels were 1.08% more likely to develop ATN following transplantation (HR: 0.938, 95% CI = 0.882–0.997, $p = 0.039$).

Regarding the immunologic characteristics of the patients, several variables contributed independently to the odds of developing ATN within our predictive model. There were independent pre- and post-transplant variables that affected the risk associated with ATN. Recipients with preformed anti-HLA antibodies were 5.726 times more likely to develop ATN than those without antibodies (HR: 5.726, 95% CI = 1.313–24.977, $p = 0.020$).

The most important variables we aimed to analyse were the different T lymphocyte subsets to determine any possible underlying immune-mediated pathogenic mechanism causing acute tubular allograft injury. In this respect, we were able to evaluate their potential use as surrogate biomarkers of ATN and, indeed, our predictive model significantly correlated three out of the four T lymphocyte subsets as independent factors contributing to increased risk of ATN. Thus, KTrs with $CD8^+CD25^+$ MFI $\geq$1015.20 within the first-month post-transplantation had a 5.212-fold increased risk of ATN compared with those with levels below the cut-off point (HR: 5.212, 95% CI = 1.295–20.975, $p = 0.020$). Likewise, KTrs with $CD8^+CD95^+$ MFI $\geq$4257.28 were 3.693 times more likely than recipients with levels $<$4257.28 to develop ATN (HR: 3.693, 95% CI = 1.928–14.689, $p = 0.034$). The main predictor and contributor to the increased risk of ATN was the $CD4^+CD95^+$ T lymphocyte population, demonstrating that the lower the decrease below an MFI of 1581.98, the higher the probability of relative risk of ATN, increasing by a factor of 12.376-fold (HR: 12.376, 95% CI = 3.104–49.339, $p = 0.000363$). All these data are shown in Table 3.

4. Discussion

The present study sought to determine whether ATN had an immune-mediated outbreak in which the recipient T lymphocytes played a central role in its presentation. Thirty-one first-time KTrs were enrolled in this single-centre longitudinal observational study for T lymphocyte surveillance. Our research group previously validated an *in vitro* assay that allowed us to assess the degree of expression of several surface markers (CD25, CD38, CD69, CD154, and CD95) on $CD4^+$ and $CD8^+$ T lymphocytes as surrogate biomarkers of AR and opportunistic infection in both liver and kidney recipients [26,28,38]. We aimed to apply the same approach to ATN in KTrs, as some other different outcomes in liver or kidney transplants showed differences in the T lymphocyte subset profile.

Of the 31 KTrs enrolled in this study, 22.6% (n = 7) were diagnosed with DGF due to ATN over the first-month post-transplantation. The diagnosis of ATN following KT relies on changes in specific biochemistry parameters associated with renal function, such as serum creatinine concentration and GFR, that could be accompanied by acute reductions in urine output along with the histological assessment of the allograft [7,39]. Our results showed that, in the ATN group, serum creatinine concentration was significantly higher than that observed in the NoATN group. Similarly, GFR significantly differed from ATN vs NoATN study groups. Despite the controversy among authors to use both as biomarkers of acute injury, mainly derived from their lack of specificity, the classical assessment of creatinine and GFR of allograft function provided promising results in our predictive multivariate logistic regression model, supporting their continued use. Both parameters correlated significantly with our proposed $CD4^+CD95^+/CD8^+CD95^+$ ratio when comparing ATN vs NoATN recipients (Fig. 5E,F). The combination of both biochemical and cellular parameters early after transplantation could raise the specificity of ATN diagnosis.

Using univariate analysis, the presence of preformed anti-HLA antibodies in wait-listed ESRD patients correlated significantly between the ATN versus NoATN groups and was confirmed as an independent factor contributing to a 5.726-fold increased risk to our primary outcome. Furthermore, albeit not significant in the multivariate logistic regression predictive model, both maximum PRA and current PRA at the time of transplantation were significantly higher in ATN than NoATN in the univariate analysis. These data are consistent with current trends that anti-HLA antibodies appear as an independent risk factor for DGF and ATN earlier after transplantation in KTrs [10]. Both PRA and pre-sensitisation status of ESRD patients are well-established factors used by nephrologists to stratify as high or low immunological risk hence a more aggressive induction therapy is provided before transplantation, reducing the possibility of earlier immune-mediated complications [40,41].

Post-transplant immunosuppression was shown to influence the development of ATN. There was a significantly increased percentage of KTrs from the NoATN group receiving double maintenance therapy compared with the ATN group (78.6% vs 21.4%) during the first-year post-transplant. Additionally, MMF dose (mg/day) was another clinical parameter that significantly affected the development of ATN, whereby the NoATN group had received an overall higher dose than KTrs with acute allograft injury.

These results will require further investigation, as the initial post-transplant immunosuppression regime and induction therapy might benefit KTrs by reducing AR and ATN. Current practice aims to avoid earlier complications arising from alloimmune recognition of the donor's graft, and the vast majority of patients are treated with the calcineurin inhibitor TRL as the primary agent in combination with MMF. As of yet, these drug combinations appear to work well as rejection prophylaxis, but their effect in preventing ATN requires further investigation in future larger and multicentric prospective studies.

To our knowledge, this is the first time an investigation outlines the involvement of the patient's adaptive immune system in earlier post-transplant ATN, whereby T lymphocytes were assessed in whole peripheral blood samples throughout the first-year post-transplantation at different time points. During the first-month post-transplant, patients who developed ATN showed a significantly higher percentage of $CD8^+CD95^+$ and lower $CD4^+CD95^+$ T lymphocytes than NoATN patients. Additionally, this difference was further observed after calculating the ratio between both T lymphocyte subsets, such that patients in the ATN group inverted the ratio towards a higher proportion of $CD8^+CD95^+$ over $CD4^+CD95^+$ T lymphocytes. As already stated, renal allograft function could be assessed by incorporating the ratio together with sCr and GFR, which appeared to be a novel strategy to assess a patient's allograft function, increasing the specificity of both classical markers.

Our results also showed that patients who experienced ATN had increased expression of specific surface activation markers. We observed that CD25 and CD69 were notably overexpressed on $CD8^+$ T lymphocytes. A process deeply implicated with pathogenic renal cell death in AKI and associated with its classical ATN lesion is apoptosis triggered by various insults, such as nephrotoxicity, inflammatory response, acute tubular hypoxia/ischemia, intratubular obstruction or changes in local microvascular blood flow [42,43]. Disbalance in normal cell turnover has been implicated in several pathological states in which excessive apoptosis contributes to atrophy yet promotes fibrosis and organ dysfunction [44].

Cell apoptosis can be triggered by the TNF receptor superfamily member CD95 (Fas, APO-1, TNFRSF6) as a prototypical death receptor expressed by a wide variety of cell types when bound to its natural ligand, CD95L (CD178/TNFSF6) [45]. It is of particular importance that the partnership made by CD95/CD95L not only induces apoptosis but it has now also been established that CD95 has multiple non-apoptotic activities [46,47]. An important anti-apoptotic function in which CD95 participates is the induction of relative cellular resistance to CD95-mediated apoptosis by the upregulation of its surface expression. Other pathways of induced cell apoptosis implicate activated $CD8^+$ cytotoxic effector T lymphocytes through the perforin/granzyme pathway [48,49] and some indirect mechanisms through which tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ) have also been implicated [50].

Consistent with current literature, we were able to characterise that KTrs who developed ATN during the first-month post-transplant had a higher proportion of activated cytotoxic $CD8^+$ T lymphocytes expressing CD25 and CD69 over recipients without ATN. Furthermore, $CD8^+$ T lymphocytes from recipients with ATN overexpressed the surface protein CD95 on the $CD4^+$ compartment compared with the NoATN group. Following these observations, we implemented a predictive model with high specificity and negative predictive value (NPV) but with modest sensitivity based on the best cut-off values assessed by ROC curve analysis, whereby T lymphocyte subsets could be used as surrogate biomarkers for the diagnosis of ATN. We attribute the low sensitivity resulting from our analysis to the relatively low number of patients included in this study. Therefore, to raise the ability of our model to better detect ATN, a more extensive study deems mandatory. Nevertheless, the otherwise high specificity and NPV of this novel non-invasive assay ensure that clinicians could benefit from its use to exclude a $CD8^+$ T lymphocyte immune-mediated acute insult to the allograft earlier after transplantation.

Beyond the aforementioned limitations of our study, we performed an appropriate study design by including comprehensive prospective immune surveillance at various time points using an already validated functional *in vitro* immunophenotypic assay that reduces potential co-founders. However, the small number of patients included due to the relatively short follow-up period will require a larger cohort and longer surveillance to validate our preliminary results.

5. Conclusions

To our knowledge, this is the first study addressing the role of $CD8^+$ T lymphocytes in KTrs developing ATN following KT. In conclusion, we have added to the current knowledge in transplant immunology a potential mechanism by which the recipient's adaptive immune system, through activated T lymphocytes, contributes to acute tubular damage allowing the possibility for use as surrogate markers of ATN and also opening possibilities for new therapeutical targets to control deleterious effects that might affect long-term transplant outcome.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Author Contributions

FB and MM, conceptualization and designed the research study. RA and VJC, performed the research and implemented the methodology. FB, RA and MRMQ, biomarker determination and data acquisition. JDLPM and SL, provided help and advice on patient clinical data and management. FB and IL, analyzed the data. FB and AM, statistical analysis. MRMQ and AM, clinical data. FB, VJC, and RA wrote-preparation of the draft manuscript. FB and MM revised and edited the final version of the manuscript. FB and MM, supervision of the project. MM, visualization, project administration, and funding acquisition. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All patients gave their informed consent for their samples to be manipulated and stored for current and future research before they were recruited to the study. The study was conducted following the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the University Hospital "Virgen de la Arrixaca" (HUVA) (PI15/01370).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2806119.

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