

**Evaluation of CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> T Cell Populations in Peripheral Blood of Patients Following Kidney Transplantation and During Acute Allograft Rejection**

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## ABSTRACT

*Aim:* Regulatory T cells (Treg) are important in mediating immune tolerance and outcomes of allotransplantation. CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> co-expression identifies memory Treg; CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> memory T effectors. We sought to determine CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> expression from the peripheral blood of patients with end stage renal failure, following transplantation and during episodes of acute cellular rejection.

*Methods:* CD4<sup>+</sup> T cells were isolated from peripheral blood leucocytes and analysed for CD25 and CD39 expression by flow cytometry. Treg suppressive function was measured by suppression of autologous effector T-cell proliferation by Treg in co-culture.

*Results:* CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> T cell subsets were tracked longitudinally in the peripheral blood of seventeen patients following renal transplantation. Patients with acute T cell mediated rejection diagnosed on biopsy had reduced CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg ( $p<0.05$ ) and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff ( $p<0.01$ ) cells compared to non-rejecting patients. CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg ( $p<0.05$ ) and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff ( $p=0.057$ ) were reduced in long term transplant patients (> 1 year) compared with controls. Interestingly, remaining CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg in the stable transplant patients displayed more potent suppressive capacity compared with controls (83.2%±3.1% vs. 45.7%±8.0%, nTeff:Treg ratio 8:1,  $p<0.01$ ).

*Conclusion:* CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff in peripheral blood can be tracked in renal transplant patients. Acute cellular rejection was accompanied by reduced mTreg and mTeff. Determining changes in these T cell subsets may help to identify patients with, or at high risk of, renal allograft rejection.

**KEYWORDS:** Treg, CD39, renal transplantation, acute cellular rejection, kidney

Abbreviations:

ESRF – end stage renal failure

CMR – cell mediated rejection

KTR – kidney transplant recipient

Treg – regulatory T cells

Teff – effector T cell

mTreg – memory Treg

mTeff – memory T effector cells

nTeff – naïve T effector cells

CD39 - ectonucleoside triphosphate diphosphohydrolase 1, E-NTPDase1

CD73 - ecto-5'-nucleotidase, ecto5'NTase

## **INTRODUCTION**

Regulatory T cells (Treg) are central in mediating immune homeostasis. This is particularly relevant in transplantation where the balance between Treg and the pathogenic alloreactive T effector cells (Teff) they suppress is vital for preservation of graft function and the induction and maintenance of tolerance.

Treg are defined by the expression of various markers however no-one marker has total specificity. Treg have traditionally been defined by  $CD4^+CD25^+FoxP3^+$  expression, however recent data in mice and humans shows FoxP3 is also expressed by pathogenic cells with Th17 potential (1, 2).

CD39 (ectonucleoside triphosphate diphosphohydrolase 1, E-NTPDase1) is an integral vascular and immune ectonucleotidase playing critical roles in haemostasis and the immune response. The expression of CD39 by Treg is well established (3-5) and is restricted to a subset of T regulatory memory cells (6) that are capable of suppressing IL-17 production (7). We have previously shown that CD39 in combination with CD4 and CD25 identifies four distinct T cell populations in the peripheral blood of mice and humans (3, 5). Defined as  $CD4^+CD25^+CD39^+$  (activated/memory Treg, mTreg);  $CD4^+CD25^+CD39^-$  (pro-T helper subset, Th);  $CD4^+CD25^-CD39^+$  (memory T effector cell, mTeff) and  $CD4^+CD25^-CD39^-$  T cells (naïve T effector cell, nTeff), these populations are dynamic and can be tracked in states of inflammation such as renal transplant rejection (5). The extensive role of CD39 in conjunction with CD73 (ecto-5'-nucleotidase, ecto5'NTase) in regulation of the purinergic system and its resultant effect on inflammation and immunity is well established (reviewed in

(8)). Conversion of the pro-inflammatory molecule ATP, via the CD39/CD73 pathway, to adenosine promotes an anti-inflammatory environment. Defects in Treg number and/or function are associated with the emergence of autoimmune disease (9), transplant rejection (10) and inflammation (11). Importantly, the level of expression and activity of CD39 on mTreg cells directly influences their immunosuppressive capacity in many disease states (5-7, 12-14) and increased CD39 expression on mTreg has been linked to operational tolerance in renal transplant patients (15).

In this study we tracked the CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> T cell subsets in patients with end stage renal failure (ESRF) awaiting transplantation and in the first 3 months post-transplant and correlated them with episodes of acute cell mediated rejection (CMR). Further, analysis of T cell proliferation and Treg suppressive function in stable renal transplant recipients was assessed.

## **METHODS**

### **End stage renal failure and kidney transplant recipients**

Peripheral blood samples were obtained prospectively from kidney transplant recipients (KTR) with ESRF recruited from the Nephrology Unit at St. Vincent's Hospital, Melbourne, Australia. 10ml of blood was drawn for analysis in those that received a renal transplant during the early post-operative period (day 1–6), at the time of indicated biopsy (biopsy for an unexpected rise in serum creatinine) and at their 3 month protocol biopsy. Written

informed consent was obtained from all patients and healthy controls and this study was approved by the St. Vincent's Hospital (Melbourne) Human Ethics Committee.

### **Human peripheral blood mononuclear cell (PBMC) preparation and Treg isolation**

PBMC were obtained by Ficoll gradient and CD4<sup>+</sup> T cells isolated using MACS microbeads (Miltenyi Biotec Australia, Macquarie Park, NSW, Australia). For phenotypic analysis CD4<sup>+</sup> T cells were stained with anti-human CD4-PE-Cy7, CD39-APC and CD25-APC-Cy7 (BD Biosciences Australia, North Ryde, NSW, Australia & eBioscience, San Diego, CA, USA). Samples were analysed on a FACSCanto (BD Biosciences) and expression assessed using FlowJo software (Tree Star, Ashland, OR, USA).

### **mTreg Suppression and nTeff Proliferation Assays**

Peripheral blood samples for the suppression and proliferation analysis were sourced from controls and KTR (stable >1 year) who required venesection but were otherwise well: the KTR for polycythaemia; the controls for iron overload. PBMC were obtained by Ficoll gradient and CD4<sup>+</sup> T cells purified using the CD4<sup>+</sup> T cell Isolation Kit II and AUTOMACS (Miltenyi Biotec Australia, Macquarie Park, NSW, Australia). CD4<sup>+</sup> cells were stained as described above and CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>-</sup> nTeff populations were isolated following flow cytometry cell sorting (FACS Aria, BD Biosciences, San Jose, CA). nTeff cells were stained with CFSE using the CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes™, Thermo Scientific, Melbourne, Australia). nTeff were activated using Treg Suppression Inspector, human (Miltenyi Biotec). A total of 5 x 10<sup>4</sup> nTeff cells/well

cultured in a 1:2 cell:bead ratio in a final volume of 220 $\mu$ L of complete medium ((RPMI-1640 containing Glutamax, 1mM Pen-Strep, 1mM Na-Pyruvate (Gibco™, Thermo Scientific, Melbourne, Victoria, Australia), 10% Human AB serum (Invitogen™, Thermo Scientific) and 50mM 2-mercaptoethanol (Sigma-Aldrich, Castle Hill, NSW, Australia)). To assess suppressive activity, mTreg were co-cultured at nTeff:Treg ratios of 1:1 to 8:1 for 4 days. Following culture, wells were washed and cells were incubated with mouse anti-human CD4-APC Ab (BD Biosciences) at 1:100 dilution for 30 minutes at room temperature. Cells were washed and resuspended in PBS + 2% BSA. 7AAD (BD Biosciences) was added to exclude dead cells prior to FACS analysis. CD4<sup>+</sup> cells were gated and nTeff proliferation assessed using FlowJo Version 7.6 Proliferation Platform (Tree Star), on a FACSCanto (BD Biosciences).

### **Statistical Analysis**

Two-tailed Student's *t*-test and one-way ANOVA were used for comparison of flow cytometry data with  $p < 0.05$  considered significant. Results are represented as mean  $\pm$  standard error of the mean (SEM). Statistical analysis was performed using statistical software GraphPad Prism version 5.01 (GraphPad Software Inc., La Jolla, CA, USA).

## **RESULTS**

### **Patient demographics and clinical characteristics of kidney transplant recipients.**

Seventeen acute transplant recipients were recruited. Transplant specific data is detailed in Table 1. Patient 1-11 did not experience rejection, although three KTR underwent a clinically indicated biopsy due to an unexplained rise in creatinine, whereas patient 12-17 had an episode of acute cellular rejection diagnosed on biopsy (Tables 1&2). All 17 patients received the same immunosuppressive regimen of induction with basiliximab followed by maintenance therapy with a calcineurin inhibitor, mycophenolate mofetil and prednisolone.

Details pertaining to four long-term (>1 year) renal transplant recipients are presented in Table 3. Maintenance therapy for these patients included mycophenolate mofetil and prednisolone. Patient 18 and 20 were on cyclosporine, patient 19, and 21 on tacrolimus. These patients had polycythemia requiring regular venesection. In addition blood was analysed from four non-transplant, non-immunosuppressed individuals undergoing venesection for iron overload to act as a comparator.

#### **CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> T cells can be tracked in kidney transplant recipients.**

The CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> T cell subsets in peripheral blood were isolated as shown in Figure 1. CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff populations were assessed longitudinally in eleven patients (patient 1-11) both prior to and following transplantation (Figure 2). None of these patients experienced allograft rejection although three patients (patient 1, 3 and 5) did undergo a clinically indicated biopsy due to an unexplained rise in creatinine. In these patients no cause for the rise in serum creatinine was evident on biopsy and it was attributed to acute calcineurin toxicity. Some expected inter-patient variation in population numbers was noted over time however, as previously reported (5)



immunosuppression did not have a significant impact on CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> T cell populations over time.

**CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff are lowered in patients with T cell mediated rejection in the peri-transplant period.**

CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> T cell populations were compared in those patients with a documented episode of acute cell mediated rejection (patient 12-17) and with those without rejection (patient 1-11). Patients with acute T cell mediated rejection had fewer CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg (**ESRF:** 1.4±0.6 vs. 3.2±0.9, **Tx<7 days:** 1.1±0.5 vs. 2.4±0.4, **indicated biopsy:** 0.8±0.2 vs. 1.9±0.4; *p*<0.05 and **protocol biopsy:** 0.8±0.3 vs. 1.9±0.3) and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff (**ESRF:** 1.3±0.5 vs. 2.9±0.9, **Tx<7 days:** 1.0±0.2 vs. 3.5±1.0, **indicated biopsy:** 1.0±0.3 vs 3.0±0.1; *p*<0.01 and **protocol biopsy:** 0.9±0.4 vs 1.8±0.5) at all time points compared with patients without rejection, this difference reaching significance at the time of indicated biopsy (Figure 3).

**CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> T cell populations are reduced in immunosuppressed kidney transplant recipients.**

CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> ARIA sorted samples were collected for the purpose of T cell suppression assays. The percent of CD25<sup>+/-</sup>CD39<sup>+/-</sup> T cells within the CD4<sup>+</sup> population of controls and long-term (>1 year) KTR (patient 18-21) were recorded based on gating of individual populations. A reduction of mTreg (1.9±0.4 vs. 6.9±0.7, *p*<0.05) and mTeff

( $1.4 \pm 0.4$  vs.  $4.6 \pm 1.1$ ,  $p=0.057$ ) populations in renal transplant patients compared with controls was observed (Figure 4A and 4B respectively).

**CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg in stable kidney transplant recipients display more potent suppressive capacity.**

nTeff and mTreg from controls and KTR (patient 18-21) were assessed for their proliferative and suppressive capacity respectively. Figure 5A shows the proliferative capacity of CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>-</sup> nTeff. Although some variation within the immunosuppressed KTR group was observed, overall there was no significant difference in proliferative capacity of the nTeff population between KTR as a group and controls. However, CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg in KTR beyond one year post-transplant displayed more potent suppressive capacity compared with controls (nTeff:Treg ratio 8:1,  $83.2\% \pm 3.1\%$  vs.  $45.7\% \pm 8.0\%$ ,  $p < 0.01$ ) (Figure 5B).

**DISCUSSION**

The differential expression of CD25 and CD39 identifies four distinct human CD4<sup>+</sup> T cell populations and importantly, distinguishes between Treg and pathogenic populations that secrete pro-inflammatory cytokines (5). The data presented in this series of experiments supports our previous observation that these populations are dynamic and can be monitored in renal transplant patients (5). Alterations in the mTreg population have also been documented in other inflammatory conditions (16-19) highlighting their dynamic nature and

raising questions of their stability in states of inflammation. In the renal transplant setting the possible prognostic value of assessing Treg number and/or ratio Treg:Teffector in peripheral blood and at the graft site has yielded varying results and interpretations recently reviewed in Braza et al (20). We have previously reported that in healthy controls and stable patients with ESRF or following transplantation, no significant difference in the percentage of these T cell subsets is observed and Treg:Tmeff populations are in a ratio of 1:1. However during episodes of acute antibody mediated rejection in KTR, this ratio was heavily skewed to the CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff population and appeared proportional to the severity of rejection histologically (5).

In this cohort, we sought to assess the CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff populations in individual patients prior to and following transplantation including during episodes of acute cell mediated rejection. We successfully followed seventeen individual patients longitudinally, sampling at ESRF, within 7 days of transplantation, at the time of indicated biopsy and at the 3-month protocol biopsy (Figure 2). When the CD4<sup>+</sup>CD25<sup>+/-</sup>CD39<sup>+</sup> T cell numbers from patients with and without acute cell mediated rejection were compared, a consistent reduction was observed in both CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff populations both prior to transplant, in the peri-transplant period, significantly at the time of indicated biopsy and remaining reduced thereafter (Figure 3). Reduced numbers within these T cell subsets during this time period may indicate a predisposition to rejection due to a less regulated inflammatory environment. Indeed, increased frequency of CD39<sup>+</sup> Treg and the level of CD39 expression on Treg has

been associated with better suppressive capacity in a variety of autoimmune and inflammatory disorders (6, 7, 21, 22).

We also sought to assess  $CD4^+CD25^+CD39^+$  mTreg function in a cohort of stable KTR. To obtain adequate cell numbers for our assay, peripheral blood samples were obtained from controls and stable KTR who required venesection. Interestingly, where we previously reported no difference in  $CD4^+CD25^{+/-}CD39^+$  T cell population numbers between healthy controls and stable KTR (5), in the current study we noted a decrease in both percentage of mTreg and mTeff in KTR compared to controls. The main difference between this and our previous study is that patients recruited for Treg functional assessment were polycythemic necessitating venesection. Indeed a recent series of papers (23-25) has shown that polycythemia in the setting of underlying myeloproliferative neoplasms may alter Treg number. Although variation in T cell number is often attributed to the effects of immunosuppression (reviewed in (26)), in this instance, the impact of polycythemia should be considered.

Although the desired effect of immunosuppression is to target pathogenic T effector cells, we have shown that in the  $CD4^+CD25^-CD39^-$  nTeff population, no difference in the proliferative capability between control and KTR was observed (Figure 5A). Interestingly, despite immunosuppression, the suppressive capacity of the  $CD4^+CD25^+CD39^+$  mTreg in stable immunosuppressed KTR is similar to that of controls at nTeff:Treg ratios of 1:1 and 2:1 (Figure 5B). Of particular note is the ability of mTreg to sustain suppression in the presence

of greater nTeff numbers suggesting that this population is particularly potent on a per cell basis.

Braza *et al* have recently shown that increased proportions of CD4<sup>+</sup>CD45RA<sup>-</sup>FoxP3<sup>hi</sup> mTregs are found in operationally tolerant renal transplant patients (15). Additionally, San Segundo *et al* also described increased numbers of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>Foxp3<sup>+</sup> were associated with better graft outcomes in KTR (27). Based on our previous phenotyping data (5), we postulate that these populations are equivalent to the mTreg population we describe herein. Indeed, Braza showed that the CD4<sup>+</sup>CD45RA<sup>-</sup>FoxP3<sup>hi</sup> T cells also expressed higher levels of CD39. Additionally, their work showed that this T cell subset displayed increased FoxP3 TSDR (Treg specific demethylation region), increased GITR (glucocorticoid-induced TNF-related receptor) and had greater suppressive properties in operationally tolerant recipients compared to those from patients with stable graft function (15). Notably, a recent study by Roederer *et al* (28) confirms that the frequency of CD4<sup>+</sup>CD39<sup>+</sup> Treg cells is highly heritable and specifically due to the genetic control of CD39 cell surface expression. Although we have not directly assessed the expression levels of CD39 on mTreg in this study, the increased levels of the CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg subset in our stable graft patients compared with those who experienced T cell mediated rejection suggests that the stable KTR may be less susceptible to CMR. These studies highlight the importance of assessing both cell frequency and cell surface CD39 expression and we propose the assessment of CD39 as a Treg marker should be considered in future KTR studies.

The ability of Treg to regulate the inflammatory response has identified them as a clinical therapeutic candidate in the transplantation setting. Indeed, Treg cellular therapy for KTR is currently being assessed in clinical trials (<https://clinicaltrials.gov/ct2/show/NCT02088931>; <https://clinicaltrials.gov/ct2show/NCT021298810>). However, contradictory to this study and that published by Braza et al (15) and San Sagundo et al (27), a recent multicentre study showed that a high proportion of pre-transplantation CD4<sup>+</sup>CD25<sup>hi</sup>CD62L<sup>+</sup>CD45RO<sup>+</sup> activated Treg predicted acute rejection in renal transplantation (29). These data highlight the inherent difficulties defining Treg and the consequent challenges associated with using Treg therapeutically. The heterogeneity of this population makes development and targeting Treg to specific clinical settings increasingly complex: not only do Treg employ a range of suppressive molecular mechanisms, it is now apparent that these mechanisms vary depending on location and the condition of the microenvironment. Such changes in the local milieu have the potential to positively or negatively impact Treg function.

In this study we have demonstrated that:

- CD4, CD25 and CD39 identify mTreg, mTeff cell populations in the peripheral blood which can be tracked in renal transplant patients.
- mTreg and mTeff cell populations in peripheral blood are lower in renal transplant patients who experience an episode of CMR at all time points reaching statistical significance at the time of rejection.

- mTreg in transplant patients with stable graft function display more potent suppressive capacity compared with controls.

We propose that determining changes within these T cell subsets, in conjunction with serum creatinine and calcineurin inhibitor levels, may help identify patients with, or at risk of, renal allograft rejection. Furthermore, the suppressive potency of the CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg population suggests these markers could be considered in Treg isolation for clinical purposes.

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#### FIGURE LEGENDS

**Figure 1:** Representative patient analysis and gating of peripheral blood mononuclear cells (PBMC). A maximum of 100,000 events within the lymphocyte gate were collected. **(A)** Forward Scatter (FSC-A) versus Side Scatter (SSC-A) dot plot of CD4<sup>+</sup> T cells isolated from PBMC of control or renal patient with gated peripheral blood lymphocytes (PBL) identified based on size and granularity. **(B)** and **(C)** indicate doublet removal from lymphocyte population. **(D)** PBMC gated based on CD4 expression. **(E)** CD4<sup>+</sup> population examined for the expression of CD25 and CD39 and proportion of CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg and CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff were determined. **(F)** Population hierarchy of PBMC analysis.

**Figure 2:** CD4<sup>+</sup> T cell subpopulations sampled in eleven patients (Pt) with end stage renal failure (ESRF), within 7 days of transplantation (Tx<7 days), at the time of clinically indicated biopsy and 3-month protocol biopsy. **(A)** CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg cells; **(B)** CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff cells.

**Figure 3:** Patients with cell mediated rejection (CMR – black bar) and those that did not reject (grey bar) prior to transplantation (ESRF), within the first seven days of transplantation (Tx<7days), at the time of a clinically indicated biopsy for a rise in creatinine and at the time of protocol biopsy **(A)** CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg cells and **(B)** CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff cells.

**Figure 4:** % CD4<sup>+</sup> T cell subsets in controls and KTR (>1year) following ARIA sort. **(A)** CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg cells, **(B)** CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>+</sup> mTeff cells.

**Figure 5:** CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>-</sup> nTeff proliferative and CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg suppressive capacity in KTR (>1year) kidney transplant recipients (KTR) and controls. **(A)** % proliferation of CD4<sup>+</sup>CD25<sup>-</sup>CD39<sup>-</sup> nTeff cells and **(B)** CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> mTreg suppressive activity in stable (>1year) KTR (circle) and controls (square).

## TABLES:

**Table 1:** Demographics and clinical characteristics in KTR.

Patient	Age	Gender	Tx type	Primary disease	DGF	3-month Se Cr	3-month eGFR	HLA mismatch
Patient 1 <sup>#</sup>	47	M	DD	Solitary kidney; HT	No	237	26	3
Patient 2	56	F	DD	T2D	No	74	52	3
Patient 3 <sup>#</sup>	66	F	DD	Reflux	No	118	40	4
Patient 4	33	M	LRT	GN	No	107	60	2
Patient 5 <sup>#</sup>	34	F	DD	Alports	Yes	200	25	2
Patient 6	49	M	DD	APCKD	No	140	50	1
Patient 7	53	M	LRT	IgA	No	101	67	0
Patient 8	54	M	LURT	APCKD	No	108	60	3
Patient 9	41	M	DD	IgA	No	140	49	1
Patient 10	48	M	DD	nephrocalcinosis	No	139	48	5
Patient 11	52	M	DD	T1D	No	74	70	4
Patient 12 <sup>†</sup>	37	M	DD	Reflux	No	178	38	3
Patient 13 <sup>†</sup>	38	M	DD	IgA	No	104	70	3
Patient 14 <sup>†</sup>	58	M	DD	HT	Yes	131	49	3
Patient 15 <sup>†</sup>	61	M	DD	HT	Yes	145	43	3
Patient 16 <sup>†</sup>	34	M	LRT	FSGS	No	130	55	2
Patient 17 <sup>†</sup>	34	M	DD	FSGS	No	142	50	2

Tx – transplant; DD – Deceased Donor; LRT – Living related transplant; LURT – Living unrelated transplant; HT – Hypertension; T1D – type 1 diabetes; T2D – type 2 diabetes; GN –

Glomerulonephritis; APCKD – adult polycystic kidney disease; IgA – IgA nephropathy; FSGS – Focal and segmental glomerulosclerosis; DGF – delayed graft function; Se Cr – serum creatinine; eGFR – estimated glomerular filtration rate; HLA mismatch – human leukocyte antigen mismatch.

# Patient with no rejection at time of indicated biopsy. † Patient with rejection at time of indicated biopsy.

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**Table 2:** Banff staging score for KTR with rejection at time of indicated biopsy.

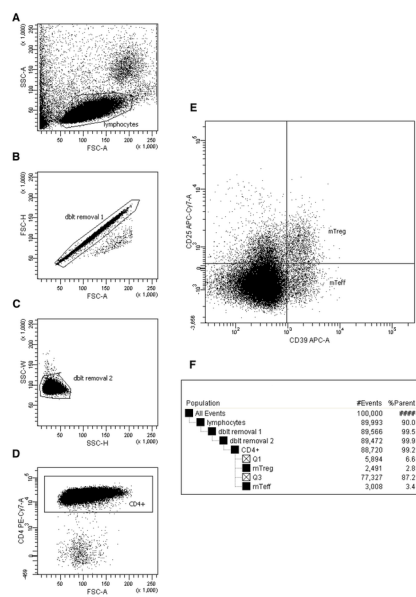
Patient	Diagnosis	Banff acute rejection score	Banff chronic rejection score
Patient 12	Borderline (15-20%) acute T cell mediated rejection. C4d negative.	i1, t2, g0, v0, ptc0	ci0, ct0, cg0, cv0, ah0, mm0
Patient 13	Acute T cell mediated rejection. Grade IB. C4d negative.	i2, t3, g0, v0, ptc0	ci1-2, ct1-2, cg0, cv0, ah0, mm0
Patient 14	Borderline (10%) acute T cell mediated rejection. C4d negative.	i1, t1-2, g0, v0, ptc0	ci0, ct0, cg0, cv0, ah0, mm0
Patient 15	Borderline (15-20%) acute T cell mediated rejection. C4d negative.	i1, t1, g0, v0, ptc0	ci<1, ct<1, cg0, cv0, ah1, mm0
Patient 16	Suspicious for suspicious/borderline acute interstitial rejection (10%). C4d negative.	i<1, t<1, g0, v0, ptc0	ci<1, ct<1, cg0, mm0, cv0, ah0
Patient 17	T-cell mediated interstitial. C4d negative.	i2, t1-2, g0, v0, ptc0	ci1-2, ct1-2, cg0, mm0, cv1, ah1

KTR – kidney transplant recipient

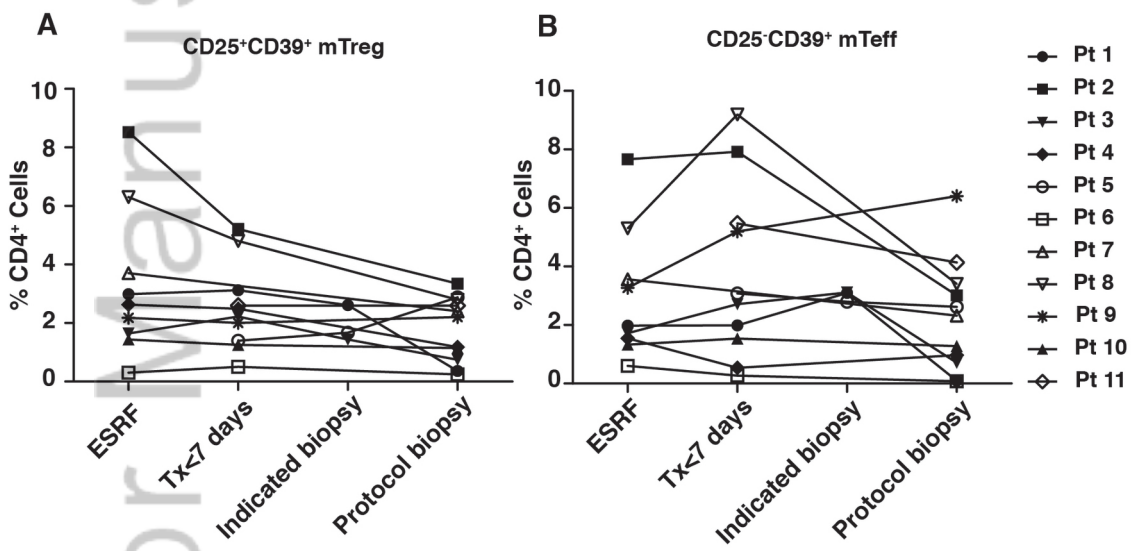
**Table 3:** Demographics and clinical characteristics in KTR (>1 year).

Patient	Age	Gender	Tx type	Date of Tx	Se Cr	eGFR	uACR	HLA Mismatch	Drug
Patient 18	48	M	LRT	1998	155	42	6.6	2	CyA 353 µg/L
Patient 19	68	F	DD	2010	99	49	7.4	4	Tac 3.5 µg/L
Patient 20	40	M	LURT	2012	168	43	12.2	4	CyA 726 µg/L
Patient 21	49	M	DD	2012	151	46	3.6	2	Tac 5.4 µg/L

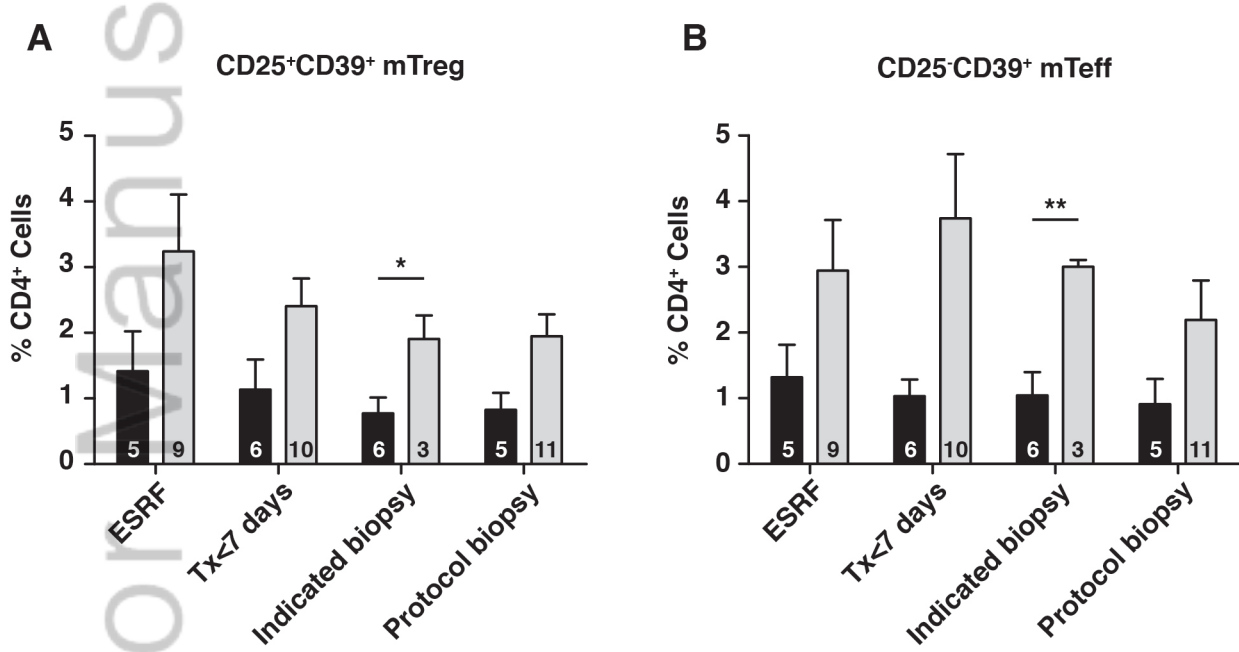
KTR – kidney transplant recipient; Tx – transplant, Se Cr – serum creatinine; eGFR – estimated glomerular filtration rate; uACR – urinary albumin creatinine ratio; HLA – human leukocyte antigen; CyA – 2 hour post dose Cyclosporine level; Tac – pre-dose Tacrolimus level



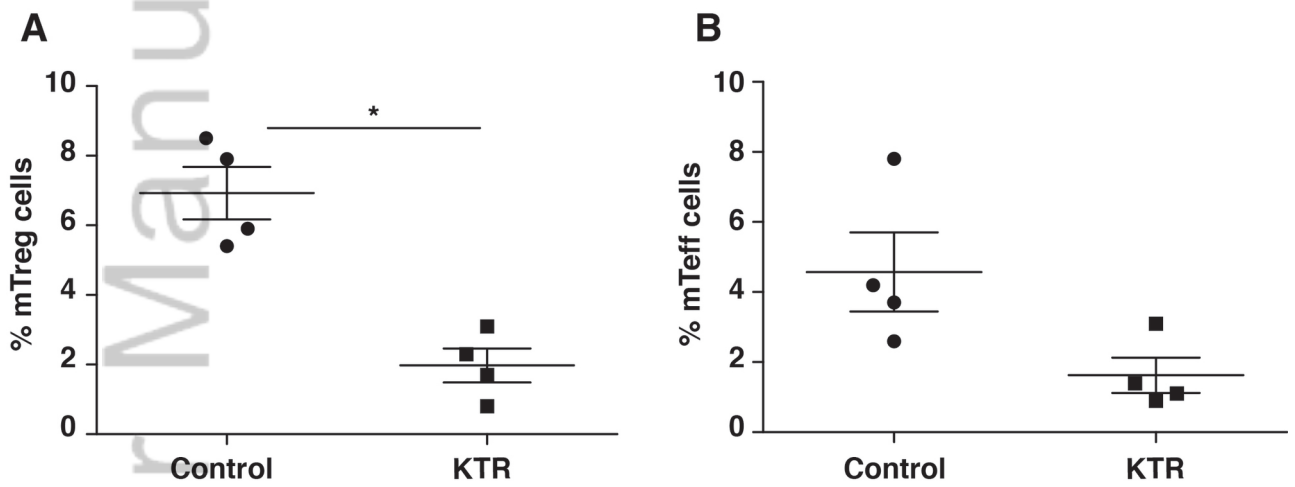
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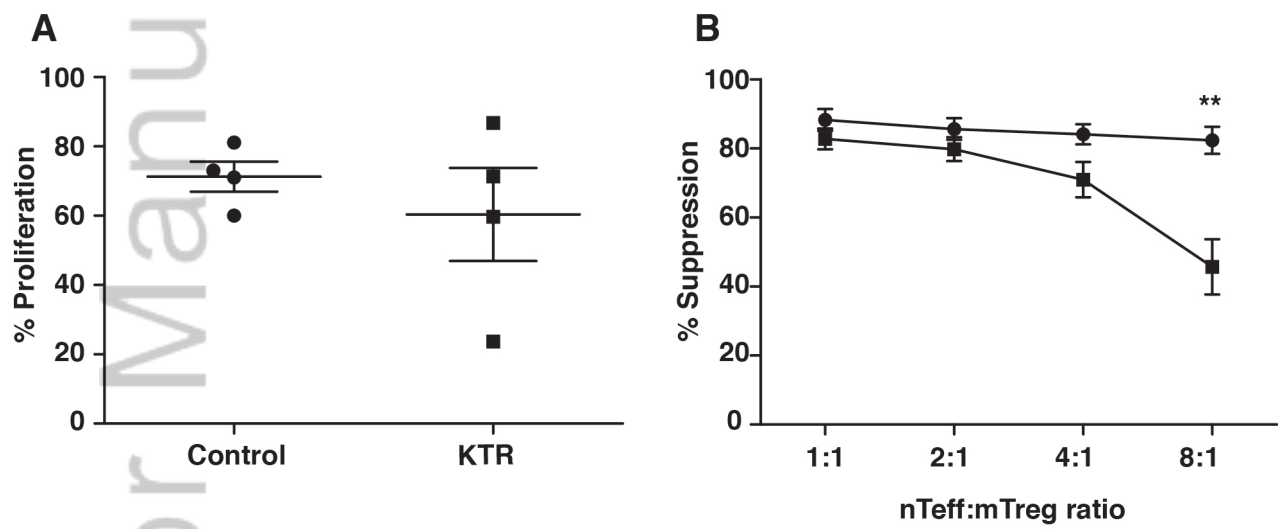
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