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## **Clinical and immunological follow-up of very long-term kidney transplant recipients treated with calcineurin inhibitors indicates dual phenotypes.**

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## ABSTRACT

Operationally tolerant kidney transplant recipients harbor an immunological signature, associated with low rejection risk, and focused on B lymphocytes. Here, we investigated whether patients with long-term transplantation and still on immunosuppressive therapy would present such a signature of low immunological rejection risk, compared to more recently transplanted patients. Of 114 kidney transplant recipients enrolled, 38 with more than 25 years of graft survival and stable graft function under calcineurin inhibitors, were matched with two different groups of transplanted patients (10-15 and 5-7 years after transplantation). Three phenotypes associated with low immunological rejection risk (Tfh, B and regulatory T cells), initially found in operationally tolerant kidney transplant recipients, and the composite score of tolerance (combination of six transcriptomic markers, age at transplantation and age at sampling) were analyzed. We found that very long-term patients were characterized by a significantly lower percentage of total B cells, a significantly higher proportion of CD24<sup>Hi</sup>CD38<sup>Lo</sup> memory B cells, significantly fewer CD24<sup>Lo</sup>CD38<sup>Lo</sup> naive B cells, and a significantly lower proportion of PD1<sup>Hi</sup>CCR7<sup>Lo</sup> Tfh lymphocytes than more recently transplanted patients. This phenotype is associated with a positive composite score of tolerance in patients transplanted for more than 25 years. Thus, our study suggests a dual phenotype in very long-term kidney transplanted patients with an immunological profile associated with low rejection risk.

**KEY WORDS:** Kidney transplantation, phenotype, B cell, tolerance, rejection

## Introduction

Renal transplantation is the treatment for end-stage chronic kidney disease with the best long-term outcomes for morbidity, mortality and quality of life <sup>1,2</sup>. The need for immunosuppressive therapy is one of its limitations, as it is responsible for opportunistic infections, neoplastic complications, and nephrotoxicity. Conversely, underexposure to immunosuppressants increases the risk of transplant rejection and graft loss <sup>3,4</sup>. The 10-year survival of kidney transplants over time has barely improved. Thus, the current challenge in transplantation is no longer to control early complications, but to maintain stable kidney function over the long term <sup>5,6</sup>. Understanding of immunological mechanisms after transplantation, particularly those associated with low rejection risk, is crucial to improve the long-term prognosis of kidney transplantation.

Low immunological risk of rejection is classically associated with the concept of tolerance. In 2013, a prospective multicenter trial evaluated the discontinuation of immunosuppressive therapy in liver transplanted patients and reported a state of tolerance in 42% of weaned patients <sup>7-9</sup>. Some trials have also been successfully conducted in kidney transplantation, particularly in hematological situations (tolerance induction after combined kidney and hematopoietic stem cell transplantation) <sup>10</sup> or on the basis of cell therapy <sup>11,12</sup>. Some cases of spontaneous operational tolerance have also been described in kidney transplantation <sup>13,14</sup>, with functional criteria (stable graft function, i.e. creatinine <150 µmol/L and nonsignificant proteinuria, without immunosuppressive therapy) <sup>15</sup>. The description of these healthy, barely monitored patients is difficult ; systematic graft biopsy as part of a research protocol to identify them is questionable. Moreover, phenotypic and transcriptomic markers identified in liver transplantation are not applicable to kidney transplantation <sup>8,9,16</sup>.

For several years, we have been interested in this phenomenon of operational tolerance in kidney transplantation <sup>15,17,18</sup>, and we demonstrated that this process that we associated with low immunological risk of rejection is characterized by a particular signature combining biomarkers and

clinical parameters<sup>19</sup>. These tolerant patients display a specific phenotypic profile with increased levels of CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>Lo</sup>FoxP3<sup>Hi</sup> Treg cells<sup>20,21</sup>, increased levels of CD19<sup>+</sup> B cells with mainly CD24<sup>Hi</sup>CD38<sup>Lo</sup> memory B cells<sup>22</sup> and GzmB<sup>+</sup> regulatory B cells<sup>23</sup>, and decreased levels of PD1<sup>Hi</sup>CCR7<sup>Lo</sup> Tfh cells<sup>24</sup>.

The identification of a profile associated with low immunological risk of rejection in patients still on immunosuppressive drugs could allow adaptation of the level of immunosuppression (reduction in dosage or even stopping certain compounds) and thus minimize adverse events. To date, some experimental attempts to wean patients off immunosuppression have failed, likely due to insufficiently robust patient selection based solely on clinical parameters and soon after transplantation<sup>25,26</sup>. The objective of our study was therefore to clinically and immunologically analyze very long-term stable kidney transplant patients (more than 25 years after transplantation) compared to more recently transplanted patients to identify possible markers associated with a low immunological risk of rejection.

# Results

## Demographic characteristics

Seventy-three patients more than 25 years after transplant with a functional graft treated with CNI were identified. Nineteen were removed from the inclusion list (exclusion criteria, loss to follow-up or refusal), and nine could not be included (no outpatient visit during the inclusion periods). Forty-five patients were sampled, and four were subsequently excluded (eGFR <30 mL/min and/or significant proteinuria at the time of inclusion). Fifty-three patients were included in LT group and fifty-two in MT group. For the comparative analysis, only patients with efficient pairing and actual inclusion of both controls were retained. Thus, 114 correctly matched patients (38 in each group) were analyzed (Figure 1).

Demographic data and biological parameters are presented in Table 1. We observed a balanced distribution of sexes (47.4% of men). For most patients, it was the first transplant, with 13 transplants from living donors and 7 kidney-pancreas transplants. These patients had good renal function with an average serum creatinine of 131.5  $\mu\text{mol/L}$  (eGFR 47.2 mL/min) in VLT group, and 119.6  $\mu\text{mol/L}$  (eGFR 53.6 mL/min) and 128.8  $\mu\text{mol/L}$  (eGFR 47.3 mL/min) in LT and MT groups, respectively, and not significantly different between groups. We noted an expected accumulation of the number of patients with nonmelanoma skin carcinoma with posttransplant delay.

Table 2 summarizes the immunosuppression parameters. We found a different distribution of the prescribed CNI, with a clear majority of cyclosporin use in VLT group and tacrolimus use in the other groups ( $p < 0.01$ ) and more patients treated with azathioprine ( $p < 0.01$ ). 15.79% of patients received corticosteroids in VLT group, 21.05% in LT group and 15.79% in MT group ( $p = 0.7846$ ), with no patient treated with a dose higher than 10mg/day. Reported adherence to immunosuppressive therapy was not significantly different between the three groups ( $p = 0.1374$ ), but we observed significantly less partial adherence in VLT group (48.5%) compared to the two

comparison groups (66.7% in LT group and 87.9% in MT group) ( $p=0.0028$ ). The proportion of patients with a history of acute rejection was similar in the three groups ( $p=0.06$ ). Blood levels of leukocytes, especially neutrophils and lymphocytes, were not different between groups. Anti-HLA antibodies were present in 39 patients with a quarter of them being DSAs (all de novo), and did not significantly differ between groups ( $p=0.11$ ). These DSAs were permanent for one patient in VLT group and two patients in LT group, and could be considered marginal for all other patients.

### **Outcomes at one year**

At 1 year, the mean renal function of patients remained good and stable compared to inclusion at 135.4  $\mu\text{mol/L}$  for VLT patients ( $N=37$ ), 120.3  $\mu\text{mol/L}$  in LT group ( $N=35$ ) and 130.6  $\mu\text{mol/L}$  in MT group ( $N=38$ ). There were less viral infections in VLT group (7.9 % in VLT group, 10.5 % in LT group, 28.9 % in MT group,  $p=0.0375$ ). One VLT and three LT patients died during the first year of follow-up, and none returned to dialysis. Cancer was reported in six VLT patients (four skin carcinomas, one melanoma, one lymphoma), four LT patients (three skin carcinomas, one Merkel cell carcinoma), and three MT patients (two skin carcinomas, one pancreatic cancer). Some patients ( $n=5$ ) also had their lymphocyte phenotype controlled at 1 year, showing intraindividual stability over the study period (*data not shown*).

### **Treg phenotype does not differ in the VLT**

We selected a  $\text{CD3}^+\text{CD4}^+$  population, and then we successively distinguished regulatory T cells by a  $\text{CD25}^+\text{CD127}^-$  gate in which we analyzed the expression of CD39, CD15S and intracellular FoxP3 markers. The distribution of the different subpopulations of regulatory T cells (naive, memory) was assessed among  $\text{CD4}^+$  T cells using a  $\text{CD45RAFoxP3}$  gate (Figure 2). We found no significant difference in the number of regulatory T cells between the three groups (2.41% in VLT, 1.77% in LT and 2.17% in MT, Figure 3A). We also found no difference in the  $\text{FoxP3}^{\text{Hi}}$  ( $p=0.16$ ),  $\text{FoxP3}^{\text{Lo}}$  ( $p=0.30$ ) and  $\text{FoxP3}^{\text{Lo}}$  naive ( $p=0.70$ ) population frequencies between the three groups. Among the regulatory molecules, there was no difference in the MFI of CD39 or CD15s

(Figure 3B). Only a significantly higher FoxP3 MFI was observed in LT group compared to the others ( $p=0.04$ ), but no difference was found for VLT (Figure 3C). Altogether, no significant difference in the regulatory T cell phenotype was found in VLT compared to the other groups of transplanted patients.

### **VLTs harbor a higher frequency of PD1<sup>Lo</sup>CCR7<sup>Hi</sup>-activated Tfh cells**

We selected a CD4<sup>+</sup>CD45RA<sup>-</sup>CXCR5<sup>+</sup> population in which CD25<sup>-</sup>CD127<sup>+</sup> gating was used to differentiate Tfh lymphocytes from regulatory follicular T lymphocytes. The expression of CCR7 and PD1, ICOS, and the distribution into Tfh1, Tfh2 and Tfh17 subsets was analyzed in a CXCR3-CCR6 gate among total Tfh cells (Figure 4). We found a nonsignificant trend towards a higher frequency of total CD4<sup>+</sup> T cells in VLT group (53.50%, vs 46.05% and 47.21% in LT and MT, respectively,  $p=0.09$ , *data not shown*). VLT presented a significantly lower proportion of Tfh lymphocytes than LT ( $p<0.01$ ) but not MT (Figure 5A), with no difference between the Tfh subpopulations in the three groups ( $p=0.46$  for Tfh1,  $p=0.07$  for Tfh2,  $p=0.39$  for Tfh17) (Figure 5B). There was no difference in the expression of ICOS between groups but a trend towards a lower MFI of PD1 in the VLT group ( $p=0.07$ , *data not shown*). We found a significantly lower frequency of PD1<sup>Hi</sup>CCR7<sup>Lo</sup> in the VLT (corresponding to highly activated Tfh cells) and a significantly higher frequency of PD1<sup>Lo</sup>CCR7<sup>Hi</sup> ( $p<0.01$ ) (Figure 5C). Altogether, VLT patients displayed fewer PD1<sup>Hi</sup>CCR7<sup>Lo</sup>-activated Tfh cells.

### **VLTs display fewer B cells with an increase in switched IgM-IgD<sup>-</sup> memory B cells**

We selected the different B cell subpopulations as follows: memory (CD19<sup>+</sup>CD24<sup>Hi</sup>CD38<sup>Lo</sup>), naive (CD19<sup>+</sup>CD24<sup>Lo</sup>CD38<sup>Lo</sup>) and transitional B cells (CD19<sup>+</sup>CD24<sup>Hi</sup>CD38<sup>Hi</sup>). IgD and IgM stainings were analyzed on the memory subpopulation confirmed on the CD27<sup>Hi</sup>CD38<sup>Lo</sup> gate. The expression of CD9 and intracellular IL-10 and granzyme B was analyzed on total CD19<sup>+</sup> B cells (Figure 6). We found that VLT harbored a significantly lower frequency of total CD19<sup>+</sup> B cells compared to the other two groups ( $p<0.01$ ) (Figure 7A), with a significantly higher level of



CD24<sup>Hi</sup>CD38<sup>Lo</sup> memory B cells (28.01% vs 19.81% and 20.53% in LT and MT groups, respectively, p=0.02) and a lower proportion of CD24<sup>Lo</sup>CD38<sup>Lo</sup> naive B cells (61.79% vs 71.21% and 71.10% in LT and MT, p=0.01) (Figure 7B). No difference was observed for CD24<sup>Hi</sup>CD38<sup>Hi</sup> transitional B cells. VLT also harbored significantly fewer IgM<sup>+</sup>IgD<sup>+</sup> memory B cells (p=0.02) and more IgM<sup>-</sup>IgD<sup>-</sup> memory B cells (p<0.01). No difference was observed in the expression of surface CD9, intracellular granzyme B or intracellular IL-10 markers, either in terms of MFI or frequency of cells (Figure 8). Subgroup analysis after censoring patients treated with azathioprine, which is known to interfere mainly with B cells, showed similar results with a significantly lower proportion of total B cells (p=0.0114) and a persistent trend to less naive B cells (p=0.0620) and more memory B cells (p=0.1485) in VLT ; surface immunoglobulins differed likewise (supplementary table S1). Altogether, VLT patients presented a lower frequency of total B lymphocytes with a significant increase in switched IgM<sup>-</sup>IgD<sup>-</sup> memory B lymphocytes and a concurrent decrease in naive B cells.

### **VLTs harbor a positive cSoT score**

The cSoT score was calculated for 90 patients (30 from each group) and was significantly different between the 3 groups, with a higher score in VLT patients corresponding to a profile of low immunological risk of rejection (Figures 9 and 10).

## Discussion

In this prospective study, we compared the characteristics of a population of kidney transplant recipients after more than 25 years of transplantation with those of two populations of more recently transplanted patients (10-15 years and 5-7 years), all with a functional graft and treated with CNI. The objective was to look for an aggregation, with time since transplantation, of a phenotype that could be associated with low immunological risk of rejection ; such a phenotype could be used to preemptively detect candidates for alleviation of immunosuppression. Apart from immunosuppressive therapy (the oldest transplanted patients were treated more often with cyclosporine and azathioprine), the three populations did not differ in clinical parameters including renal function, leukocyte count or existence of anti-HLA antibodies (including DSAs). Analysis of the lymphocyte phenotypes evidenced significant differences for the patients transplanted for more than 25 years compared with the other groups of patients with a decreased frequency of activated PD1<sup>+</sup> Tfh lymphocytes <sup>24</sup>, a decrease in the percentage of total B lymphocytes and naive B lymphocytes, an increase in the proportion of switched memory B lymphocytes, also associated with low immunological risk of rejection <sup>23</sup>, and no difference for the regulatory T cell phenotype.

Very few reports rely on very long-term cohorts of kidney transplant recipients and their factors of success. A study published in 2012 characterized patients transplanted for more than 30 years <sup>27</sup>. Nearly half of them had a history of acute rejection, a high incidence of cancer (51%) and elevated mortality associated with these cancers. Their phenotype analysis did not reveal any significant difference compared to recently transplanted patients (less than 3 years). Previous studies from our group showed an increase in the percentage of total B lymphocytes in patients defined as tolerant associated with low immunological risk of rejection compared to stable patients <sup>14,22,23,28</sup>. Their transcriptomics data also support the existence of a B lymphocyte signature <sup>19,29</sup>. Former studies showed a higher proportion of B cells with regulatory properties and an increase in

memory B lymphocytes, with an inhibitory profile <sup>22</sup>. Interestingly, B lymphocytes of the VLT group are fewer and more often IgD<sup>-</sup>, indicating an activated B lymphocyte phenotype that has also been shown to be associated with suppressive activity <sup>30</sup>, and also observed in tolerant patients <sup>19,30</sup>. This profile is concordant with the lower proportion of activated Tfh lymphocytes (PD1<sup>Hi</sup>CCR7<sup>Lo</sup>) observed in the VLT group <sup>24</sup>.

Thus, on one hand VLT patients have protolerogenic profile characterized by higher regulatory B cells. On the other hand, LT and MT patients have pro-immunogenic profile characterized by higher IgD<sup>+</sup>IgM<sup>-</sup> B cells. Retention of stimulating IgD receptors, combined with the downregulation of anergizing IgM receptors are critical for the accumulation of mature anergic B cells to increase the peripheral BCR repertoire in order to encounter foreign antigens. This profile may be due to immune under-responsiveness in VLT, which is not the case since occurrence of viral infections were less frequent in this group, in accordance with previous ones reporting that the majority of operational tolerant patients presented a normal humoral response after influenza vaccination <sup>31</sup>.

Comparative mRNA analysis highlights a positive cSoT in favor of a profile of low risk of rejection in the VLT group <sup>19</sup>. This result is not surprising since the cSoT was purposely designed with the time since transplantation being one of its components, underlining the important influence of this parameter <sup>14,19,32</sup>. However, time since transplantation is not the only parameter, and we found this score to also be positive in some patients in the other two groups. One of the main differences between the three groups of transplanted patients (in addition to posttransplantation delay) is immunosuppressive therapy: VLT patients were mainly treated with cyclosporine and more often with azathioprine, while patients in the two other groups were mainly treated with a combination of tacrolimus and MMF. This is a major limitation of our study, as immunosuppressive regimen is a confounding variable which has to be considered. This difference is explained by a change in recommendations and habits of prescription <sup>33</sup>. One could hypothesize that this influenced

the phenotypes and cSoT calculation since immunosuppressive therapies have a direct effect on lymphocyte count and function <sup>34</sup>. Immunosuppressive therapy can affect numerous gene expression, related to B cell or regulatory T cell metabolisms. Previous studies aimed to assess whether immunosuppressants modify these signatures, by comparing tolerant patients to stable patients, but very few compare patients under different courses of immunosuppressive therapy <sup>13</sup>. However, it has been previously shown that CNI slightly affect gene expression, and that the cSoT was not influenced by some treatments <sup>19</sup>. This is confirmed in our study: after censoring patients treated with azathioprine (assuming a strong influence of this treatment), differences remained significant for the percentage of total B cells with a preserved trend regarding the proportions of memory and naive B cells in accordance with a profile of low risk of rejection in VLT group.

Another bias of our study is the selection of VLT, which excluded de facto patients with worst outcomes and intercurrent events only to keep patients with good transplant function. The study was designed to find any data suggestive of tolerance hidden among stable patients. An important point is the fact that these patients with long-term graft survival, despite their signature of low risk of rejection in accordance with their clinical status, are likely not tolerant regarding their compliance with the treatment. These results are consistent with lack of increased level of B cells expressing granzyme B, which are associated in tolerant patients with an active mechanism of tolerance <sup>23</sup>. VLT patients likely succeeded in maintaining good graft function for such a long time at least partially because of their adherence to immunosuppressive therapy, even if adherence is decreasing over years. Thus, one could hypothesize that among these nonadherent patients exists unrecognized tolerant patients, i.e. stable without immunosuppression. According to the literature, approximately 3-5% of patients monitored after kidney transplantation are tolerant and this percentage increases with time <sup>17,29,35</sup>. Obviously immunosuppressive therapy will not be stopped after more than 25 years after transplantation in stable patients, even less if this patient presents other characteristics of high risk of rejection such as DSAs. A previous study showed how hard and

tricky it can be to wean immunosuppressive therapy, even among selected patients <sup>25</sup>. Yet extrapolation of this phenotype to recently transplanted patients throughout prospective clinical trials in large cohorts of such patients might allow a reasonable reduction in immunosuppressive treatments in patients identified as having a profile of low risk of immunological rejection, to reduce the side effects of immunosuppressants <sup>4,25</sup> and the cost of transplantation.

## Methods

### Population

All kidney and simultaneous pancreas-kidney transplant recipients more than 25 years after transplant, followed at Nantes University Hospital, were identified using the DIVaT database and entered in this prospective study (referred to as the very long-term follow-up group, “VLT”). Extraction was performed in April 2016 (patients transplanted before December 31, 1991) and completed in February 2018 (patients transplanted in 1992). The inclusion criteria were: patient over 18 years of age with a functional renal transplant (eGFR CKD-EPI >30 mL/min) and current treatment with CNI (tacrolimus, cyclosporin). Pediatric patients at the time of transplantation, second transplants, and simultaneous kidney-pancreas transplants were included. The exclusion criteria were significant proteinuria (> 1g/L or 1g/24h or 1g/g of creatininuria), active cancer (excluding nonmelanoma skin cancer), current treatment with a mTOR inhibitor (e.g., sirolimus or everolimus), and full HLA-matched living donor transplantation. No patient with a history of donor-specific transfusion was included. The inclusion period ran from October 2016 to July 2018, during patients’ usual transplant follow-up consultation mainly in Nantes University Hospital (five inclusions in other hospitals of our network). Two comparative groups were defined, meeting the same inclusion and exclusion criteria but in more recently transplanted patients: one group included patients who had been transplanted for 10 to 15 years (long-term follow-up group, “LT”), and one group included patients who had been transplanted for 5 to 7 years (middle-term follow-up group, “MT”) (these ranges were obtained by dividing by two and by four the range of the group of interest). A 1:2:2 match on age and sex was made: 2 control patients were assigned to a patient in VLT group to compensate for any loss to follow-up, refusals or exclusion criteria discovered after screening but before inclusion. The objective was to obtain a final 1:1:1 match for the analysis. Informed and written consent was obtained from patients. The research protocol has been filed with

the Research Department of the Nantes University Hospital and the database registered with the CNIL. In accordance with local regulations, this routine care study has not been evaluated by an ethics committee.

### **Clinical and biological parameters**

During the routine outpatient visit, patients were asked to complete BAASIS questionnaire (self-administered questionnaire about immunosuppressive treatment compliance scale), which classified patients as adherent (score =0 on all items), nonadherent (score  $\geq 1$  on any item except “intake time”) and partially adherent (score  $\leq 2$  only on “intake time” item). Blood samples were collected on the day of inclusion (EDTA and PAXGene tubes) in addition to the routine analysis prescribed by the physician if indicated. The data collection was based on the DIVaT database and covered the following elements: age, date of transplantation, transplant rank, type of transplantation (kidney alone or simultaneous kidney-pancreas transplantation), donor status (deceased or living), HLA typing of donor and recipient, initial nephropathy, current immunosuppressive treatment with dosage, history of biopsy-proven acute rejection or chronic rejection, existence of anti-HLA antibodies and DSA (without MFI threshold), history of infectious events and skin carcinoma. Biological analysis included creatinine, eGFR, proteinuria and trough level of CNI. Patients were followed longitudinally with a 6-month and 12-month collection of medical events and biological parameters.

### **PBMC collection**

Blood count was performed from the blood samples on a hematological cytometer. PBMCs were extracted and isolated by a Ficoll gradient, followed by erythrocyte lysis and platelet separation. The PBMCs collected were then counted under an optical microscope and frozen in vials of  $10^7$  cells in a solution of 90% of their own plasma and 10% DMSO at  $-80^{\circ}\text{C}$  for 48h and then in liquid nitrogen. For further analysis, PBMCs were thawed in a water bath, cleaned from

DMSO and then incubated for 16h at 37°C in RPMI culture medium enriched with fetal calf serum, streptomycin, penicillin and glutamine. This incubation was carried out to avoid cellular remains, eliminate dead cells and allow certain markers on the extracellular side of the membrane that were weakly expressed and particularly repressed after freezing to be re-expressed (mainly the chemokine receptors studied in the Tfh phenotype) <sup>36,37</sup>.

## **Cytometry and transcriptional analysis**

Cell labeling was performed on thawed PBMCs. All antibodies were from BD Bioscience (San Jose, CA), and details for each phenotype antibodies with their concentration can be found in the supplementary material. Flow cytometry was analyzed with BD-FACSDiva and interpreted with FlowJo V10 softwares. First, for the three phenotypes, gating was performed on the cell size and granularity to delimit lymphocytes, and then a second gating was performed to remove doublets. A last common gating was performed on a viability marker (Figure 11), and then a specific gating was performed for each phenotype (described in supplementary material).

PAXgene tubes were processed on the day of sampling and frosted at -20°C for 48h and then at -80°C. The tubes were thawed (by stasis at room temperature), and RNA was extracted using a PAXgene Blood RNA Kit (Qiagen/PreAnalytiX, Hilden, Germany) according to the manufacturer's instructions <sup>38-40</sup>. The total mass of RNA extracted was measured by spectrophotometry. Finally, the transcriptomic study was carried out using Nanostring nCounter technology, adapted in our unit particularly for the calculation of cSoT (analysis of expression of the following genes: AKR1C3, CD40, CTLA4, ID3, MZB1, and TCL1A ; two clinical parameters: age at transplantation and age at sampling) <sup>19</sup>. All patients were analyzed at the same time and on the same plate to avoid possible interplate variability.



## **Statistics**

Data were processed using LibreOffice Calc 6 and GraphPad Prism 7 softwares. Clinical data were analyzed using two-factor ANOVA with repeated measures (quantitative variables) and Chi-square test (qualitative variables). In cases of cytometry data and missing data (some data could not be analyzed due to technical problems or excessive cell mortality), these analyses were not feasible, and the samples were thus considered unmatched. One-factor ANOVA without repeated measures was performed, with an additional Kruskal-Wallis test if the distribution of variance differed between groups. The alpha level of significance was set at 5%. The analysis of flow cytometry and transcriptomic data was blinded to the group to which each patient belonged.

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## **DISCLOSURE**

The authors declare conflict of interest with Roche Pharma, Novartis and Sanofi and Astellas laboratories for supporting the DIVAT cohort.

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## **AUTHORS' CONTRIBUTIONS**

Amaury Dujardin: Conceptualization, Methodology, Experimental realization, Formal analysis, Statistical analysis, Writing - original draft, review and editing. Linh Bui : Editing and Reviewing of manuscript, Additional statistical analysis. Mélanie Chesneau: Methodology, Experimental realization, Formal analysis. Florian Dubois: Methodology, Experimental realization. Richard Danger: Methodology, Experimental realization (transcriptomic analysis). Clarisse Kerleau: Database management, Data extraction. Pierrick Guérif: Conceptualization, Patients care, Samples management. Jacques Dantal: Conceptualization, Methodology, Writing - review and editing. Sophie Brouard: Conceptualization, Methodology, Writing - review and editing.

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## **ACKNOWLEDGMENTS**

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DIVaT Database : Données Informatisées et Validées en Transplantation, [www.divat.fr](http://www.divat.fr), approved by the CNIL (French National Data Protection Commission), n°914184.

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## Figures

**Figure 1.** Flow chart of the study. \* event occurrence between screening and day of inclusion.

**Figure 2.** Gating strategy for the regulatory T cell phenotype.

**Figure 3.** Regulatory T cells phenotype. (A) Percentage of regulatory T cells among CD4+ lymphocytes (mean with SD) (B) MFI of CD39 and (C) MFI of FoxP3 on regulatory T cells (boxes : 1st – 3rd quartiles, whiskers : minimum – maximum).

**Figure 4.** Gating strategy for the Tfh phenotype. Figures D, E, F are derived from Tfh lymphocytes defined in figure C.

**Figure 5.** Tfh phenotype, \*\* for  $p < 0.01$ . (A) Percentage of Tfh lymphocytes among CD4+ lymphocytes (mean with SD). (B) Tfh subsets distribution depending of post-transplantation delay. (C) PD1 Hi/lo distribution depending of post-transplantation delay.

**Figure 6.** Gating strategy for the B phenotype. Figures B, C, D, E, F are derived from figure A. Figure G is derived from figure B.

**Figure 7.** B cell phenotype, \* for  $p < 0,05$ , \*\* for  $p < 0,01$ . (A) Percentage of B cells (mean with SD). (B) B cells subsets distribution depending on the post-transplantation delay.

**Figure 8.** Expression of regulatory molecules in B cells. MFI of (A) CD9, (B) Granzyme B and (C) IL-10, among all B cells (box : 1st – 3rd quartile, whiskers : minimum – maximum).

**Figure 9.** Values of cSoT.

**Figure 10.** Illustration of cSoT values, among the three groups and depending on post-transplantation delay.

**Figure 11.** Common gating strategy.

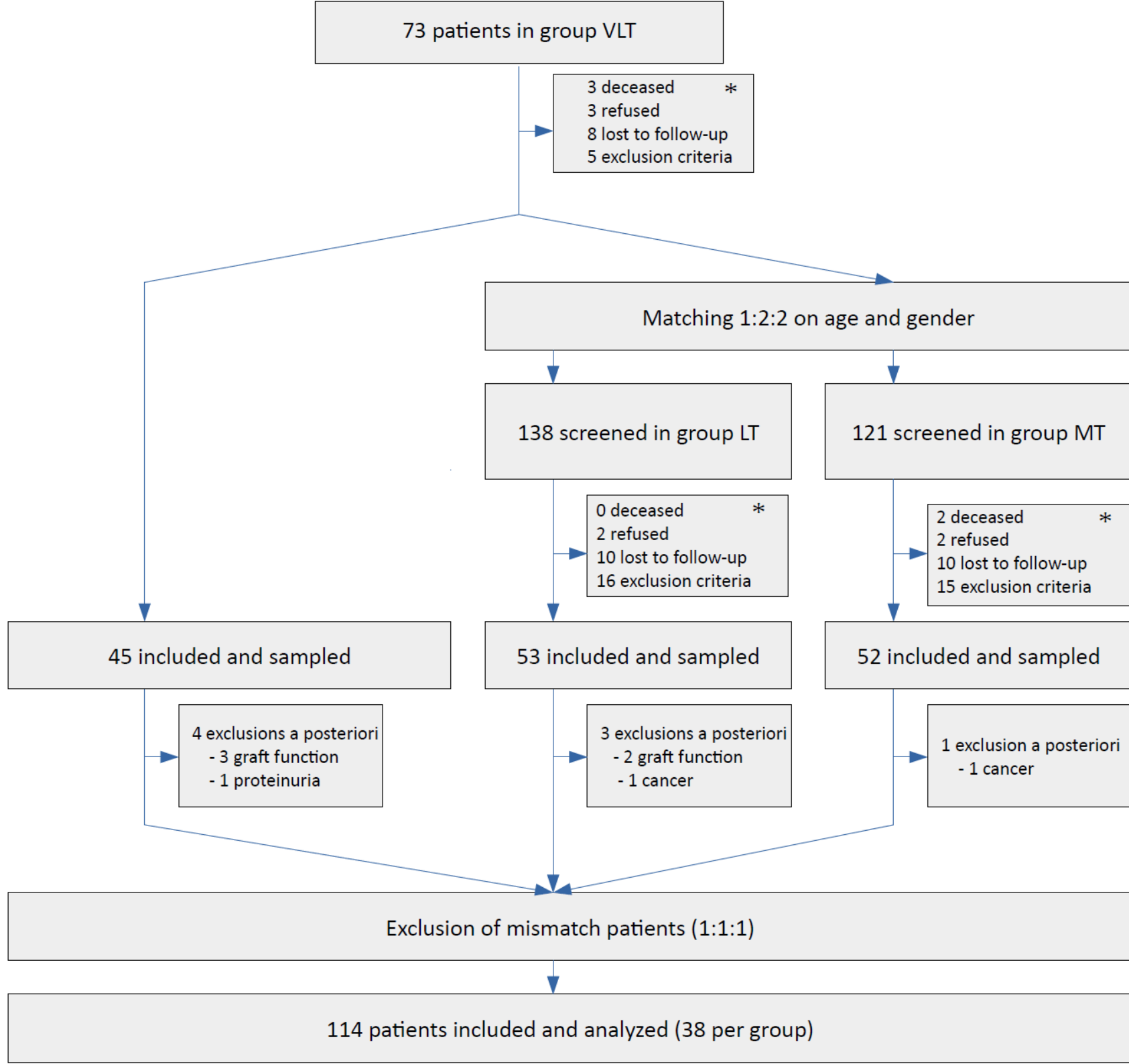
**Table 1.** Demographic characteristics and transplantation data. Percentages are presented with number of patients : % (n), medians with 1st and 3rd quartiles : median (1st – 3rd quartile), means with standard deviation : mean (SD).

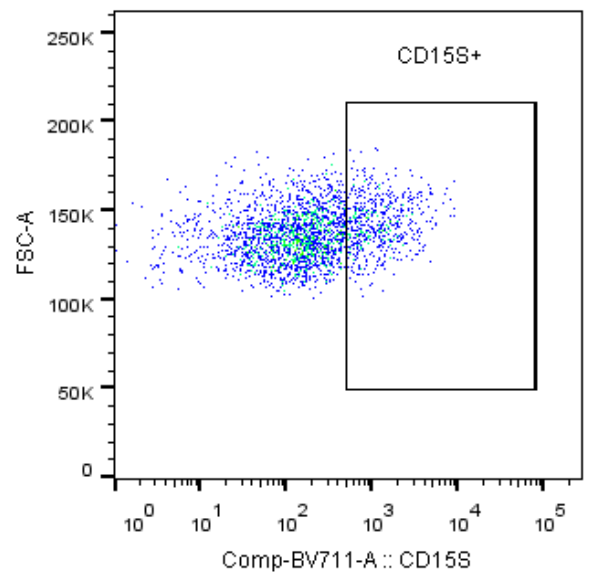
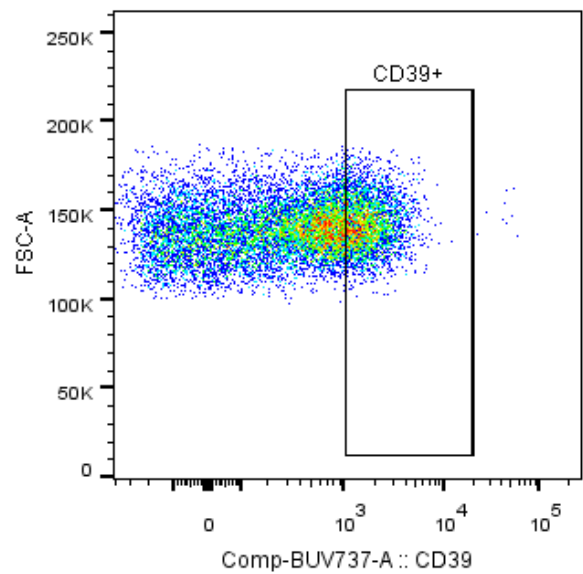
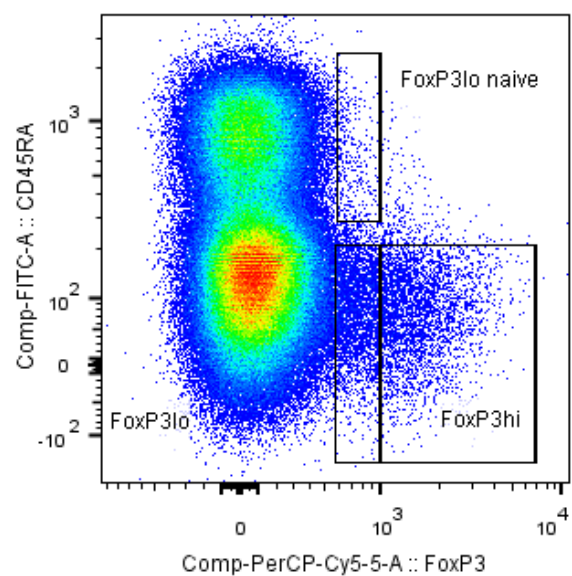
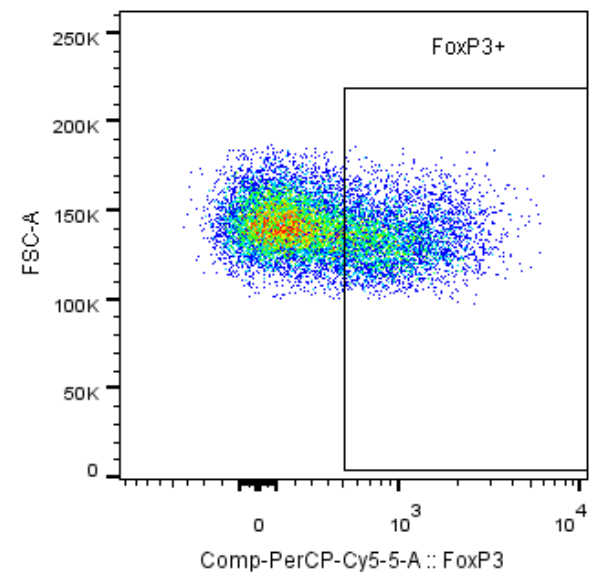
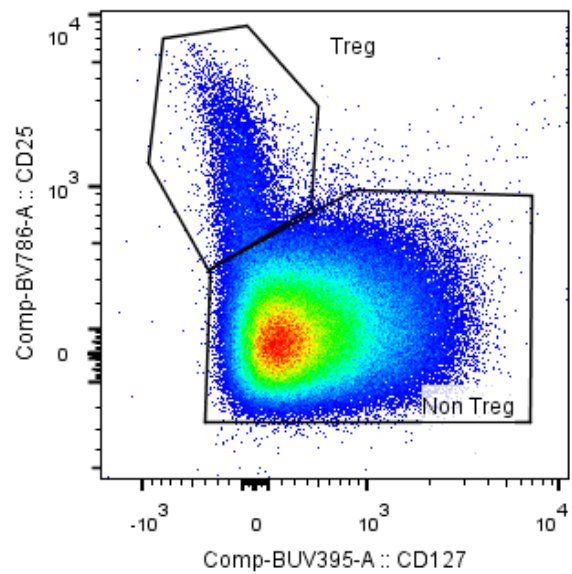
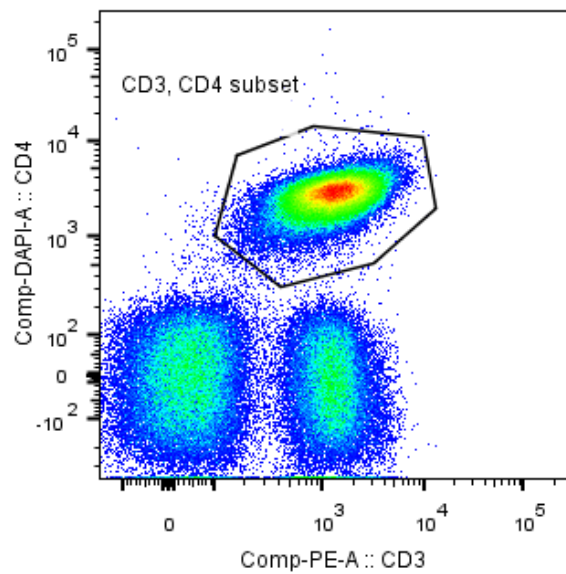
<b>Demographic characteristics</b>	<b>All patients (n=114)</b>	<b>VLT (n=38)</b>	<b>LT (n=38)</b>	<b>MT (n=38)</b>	<b>P-value</b>
Male, %	47.37 (54)	47.37 (18)	47.37 (18)	47.37 (18)	-
Age at inclusion (years), median	60.04 (52 - 70)	60.18 (52.5 – 71.25)	60.49 (55 - 70)	59.36 (52 - 70)	0.3342
Post-transplantation time (years), median	-	28.48 (25.54 – 30.92)	13.33 (12.04 – 14.42)	6.73 (6.02 – 7.31)	< 0.0001
Kidney alone / combined pancreas – kidney graft, %	93.86 / 6.14 (107/7)	97.37 / 2.63 (37/1)	86.84 / 13.16 (33 / 5)	97.37 / 2.63 (37 / 1)	0.0876
Deceased / living donor, %	88.60 / 11.40 (101/13)	94.74 / 5.26 (36/2)	86.84 / 13.16 (33 / 5)	84.21 / 15.79 (32 / 6)	0.3234
N° of graft, mean	1.16 (0.39)	1.11 (0.31)	1.21 (0.47)	1.16 (0.37)	0.4551
BMI, mean	25.09 (4.65)	24.17 (4.04)	24.45 (4.89)	26.39 (4.73)	0.1029
Kidney function					
Serum creatinin, µmol/L	126.64 (32.89)	131.51 (36.89)	119.59 (30.80)	128.83 (30.01)	0.2112
GFR (CKD-EPI), mL/min/1.73m <sup>2</sup>	49.37 (15.26)	47.23 (13.53)	53.62 (17.85)	47.25 (13.46)	0.1264
Proteinuria, g/L, mean	0.184 (0.32)	0.183 (0.29)	0.163 (0.21)	0.206 (0.43)	0.8360
Skin cancer, %	25.44 (29)	36.84 (14)	23.69 (9)	15.79 (6)	0.1037

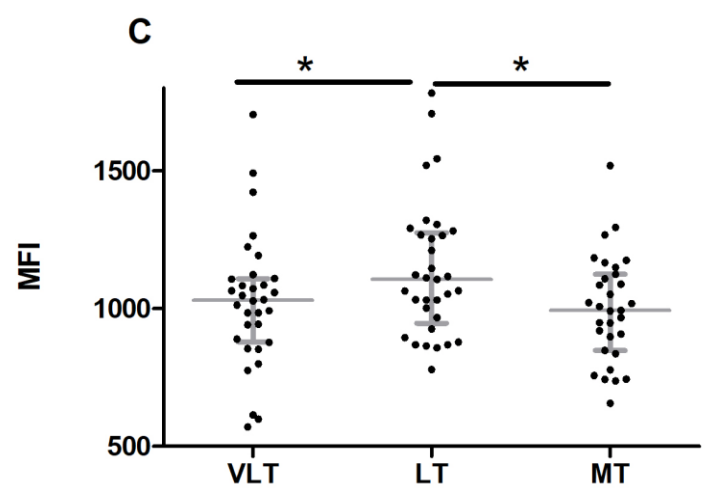
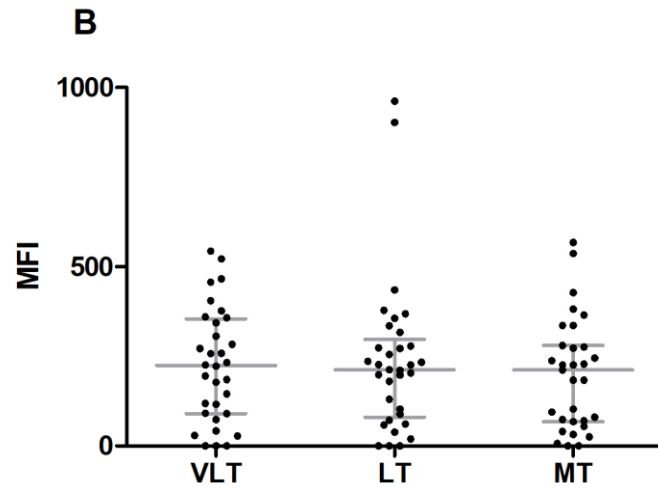
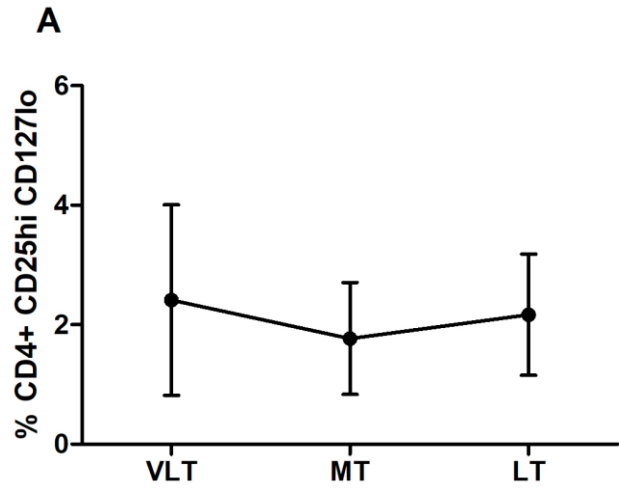


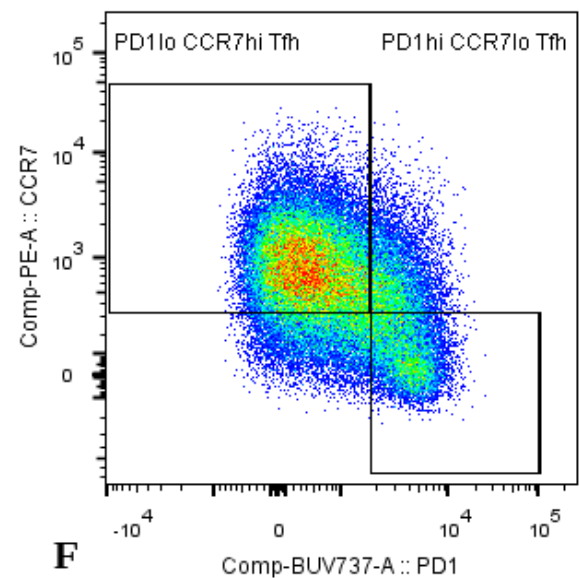
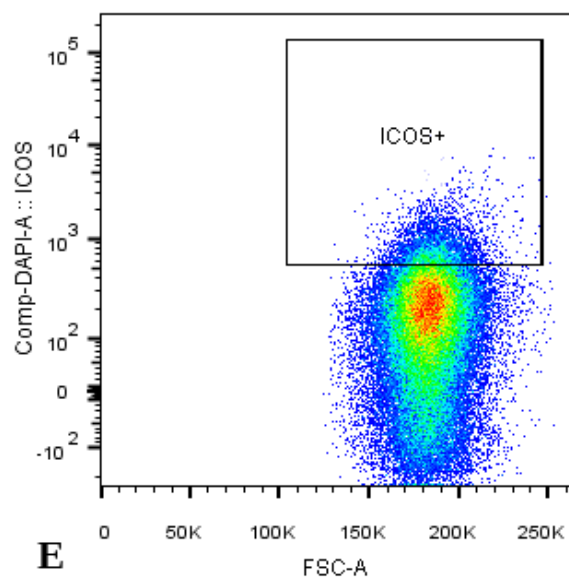
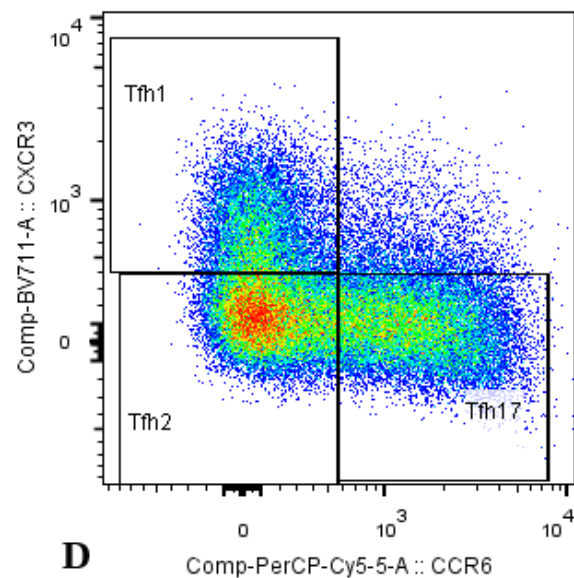
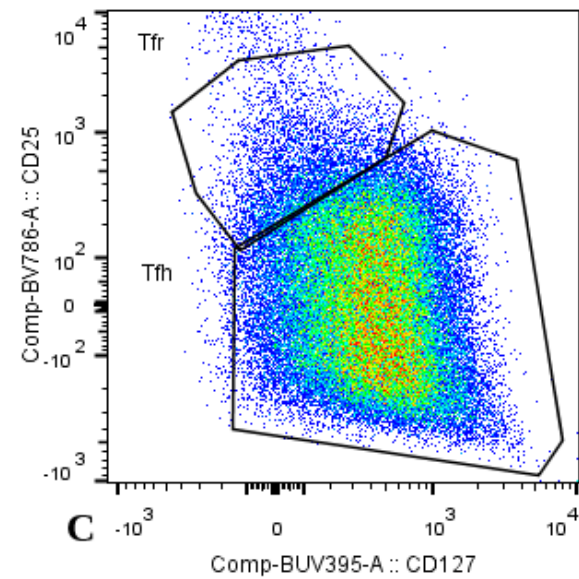
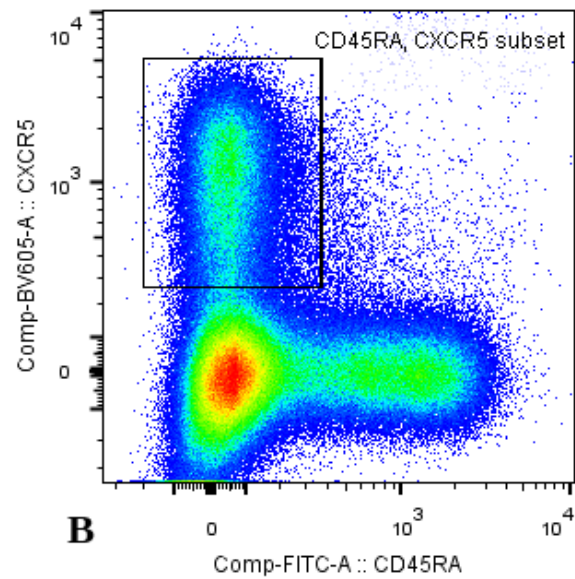
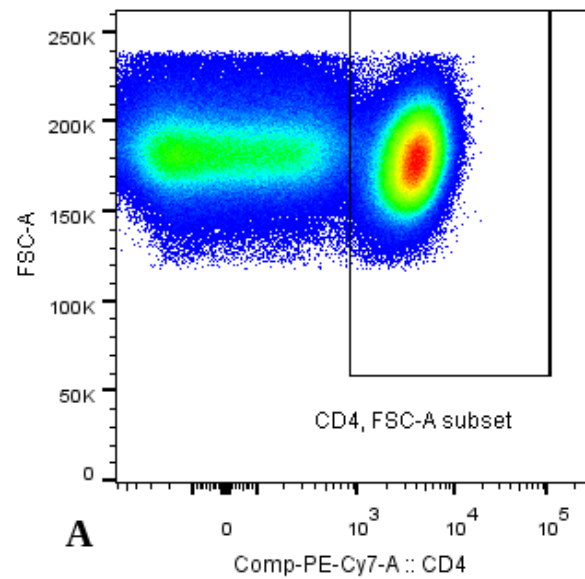
**Table 2.** Immunosuppressants and immunological data. Percentages are presented with number of patients : % (n), means with standard deviation : mean (SD). T0, through level, MMF, mycophenolic acid.

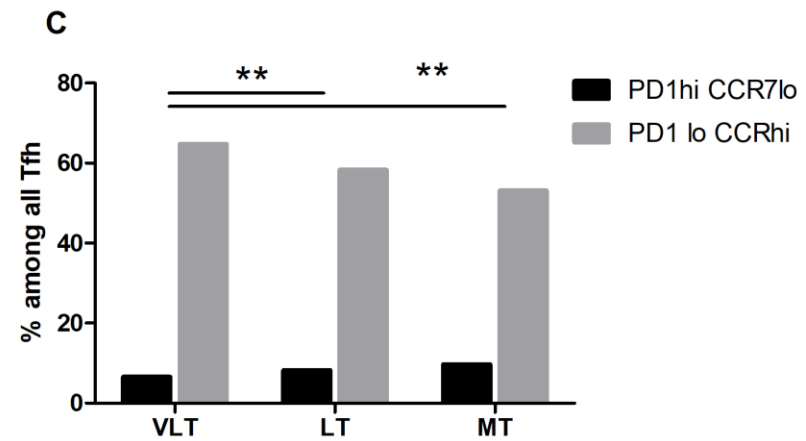
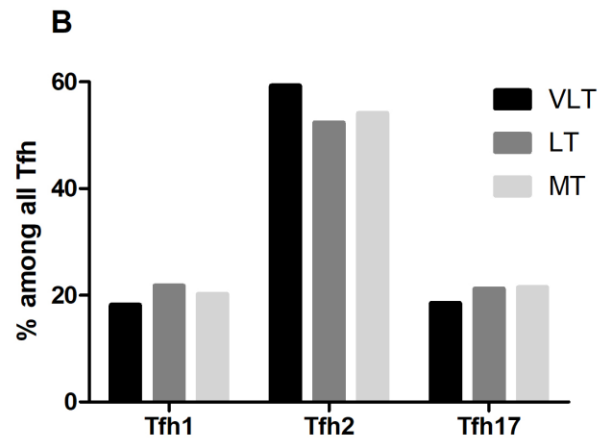
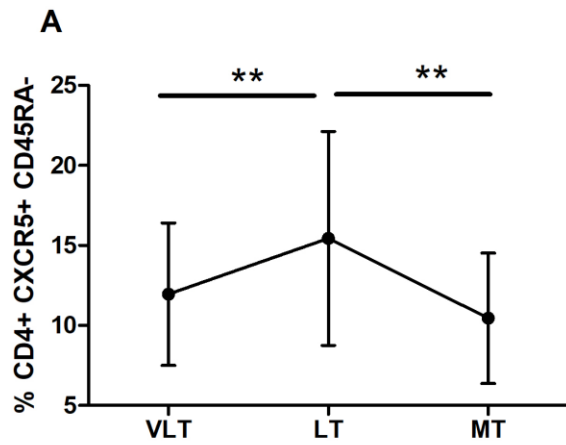
	<b>All patients (n=114)</b>	<b>VLT (n=38)</b>	<b>LT (n=38)</b>	<b>MT (n=38)</b>	<b>P-value</b>
Tacrolimus, %	61.40 (70)	7.89 (3)	86.84 (33)	89.47 (34)	< 0.0001
<i>Extended release, %</i>	58.57 (41)	0	54.54 (18)	69.69 (23)	0.3216 †
<i>Mean dose</i>	4.53 (3.29)	4.33 (NA)	4.19 (3.53)	4.87 (3.15)	0.3519 †
<i>Mean T0, ng/mL</i>	6.09 (1.44)	4.76 (NA)	6.01 (1.53)	6.29 (1.24)	0.42 †
Ciclosporin, %	38.60 (44)	92.11 (35)	13.16 (5)	10.53 (4)	< 0.0001
<i>Mean dose</i>	147.95 (48.23)	144.14 (49.49)	140 (22.36)	191.25 (46.26)	0.1685
<i>Mean T0, µg/L</i>	83.72 (30.15)	82.2 (28.61)	76.8 (16.48)	113 (56.47)	0.2070
MMF, %	60.52 (69)	29.73 (11)	76.31 (29)	76.31 (29)	< 0.0001
Myfortic / Cellcept, %	34.78 / 65.22 (24 / 45)	45.45 / 54.55 (5 / 6)	37.93 / 62.07 (11 / 18)	27.59 / 72.41 (8 / 21)	-
<i>Mean dose</i>	885.65 (314.4)	592.73 (236.9)	944.83 (231.68)	937.59 (355.36)	0.0024
Corticosteroids	17.54 (20)	15.79 (6)	21.05 (8)	15.79 (6)	0.7846
<i>Mean dose</i>	5.98 (2.26)	5.33 (2.09)	6.88 (2.59)	5.42 (1.88)	0.2281
Azathioprine	16.67 (19)	31.59 (12)	2.63 (1)	15.79 (6)	0.0032
<i>Mean dose</i>	50.00 (15.08)	61.84 (25.51)	100 (NA)	79.17 (29.23)	0.0119 ‡
<b>Blood count</b>					
Leukocytes	6704 (2176)	6946 (2411)	6919 (2224)	6248 (1838)	0.2729
Neutrophiles	4381 (1896)	4345 (2210)	4677 (1899)	4119 (1536)	0.4384
Lymphocytes	1480 (664)	1604 (607)	1479 (748)	1360 (621)	0.2841
Monocytes	647 (248)	693 (230)	634 (230)	615 (281)	0.3692
Hemoglobin	13.17 (1.84)	12.76 (1.66)	13.31 (2.16)	13.42 (1.64)	0.2881
Platelets	214395 (64708)	228184 (51196)	213184 (74897)	201816 (64790)	-
History of acute rejection, %	13.16 (15)	23.69 (9)	7.89 (3)	7.89 (3)	0.0631
<b>Antibodies</b>					
A/B/DR mismatches	3.13 (1.42)	3.19 (1.24)	2.92 (1.74)	3.26 (1.22)	0.5467
Anti-HLA, %	34.21 (39)	21.05 (8)	39.47 (15)	42.11 (16)	0.1084
DSA, %	8.77 (10)	5.26 (2)	13.16 (5)	7.89 (3)	0.4643

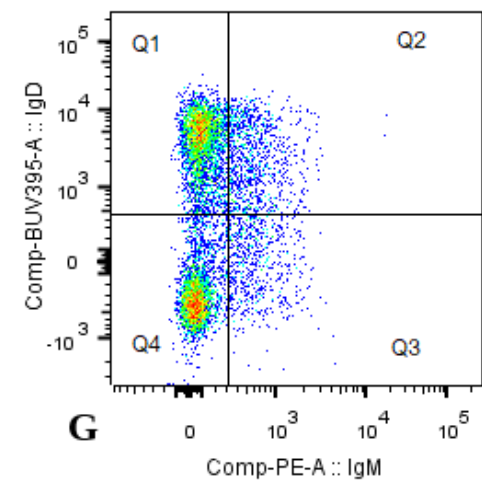
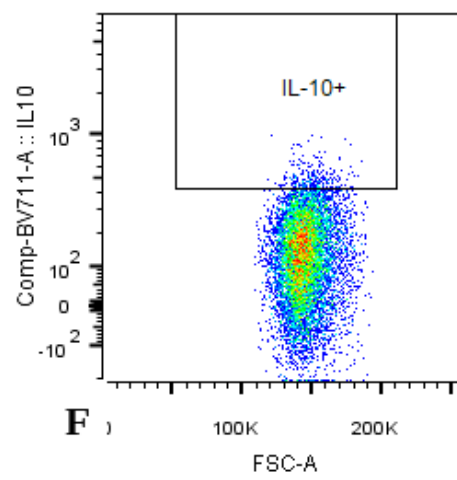
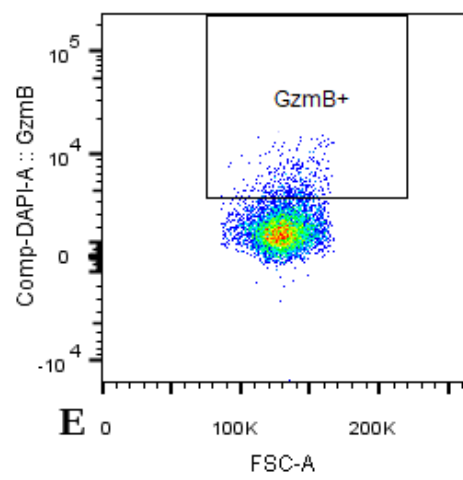
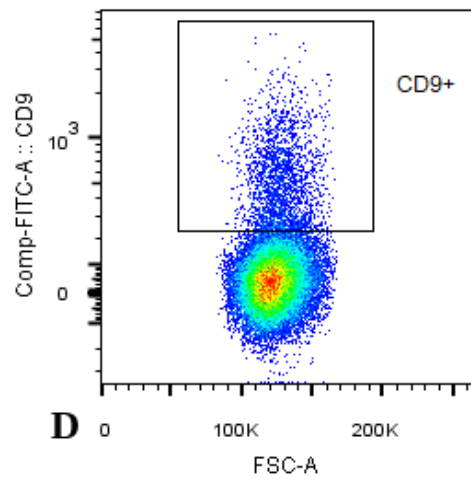
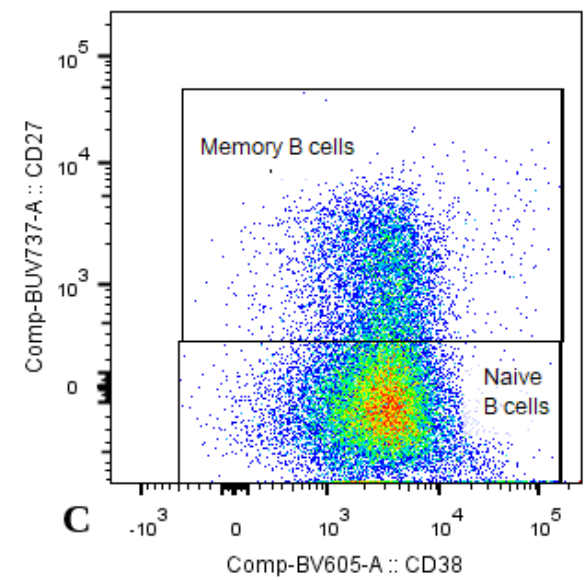
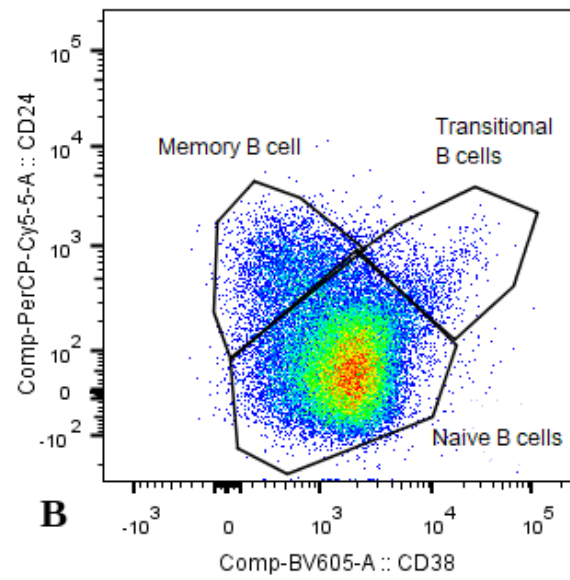
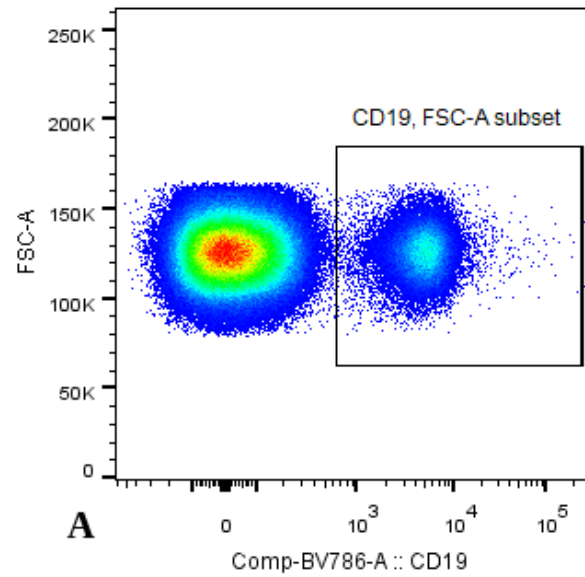


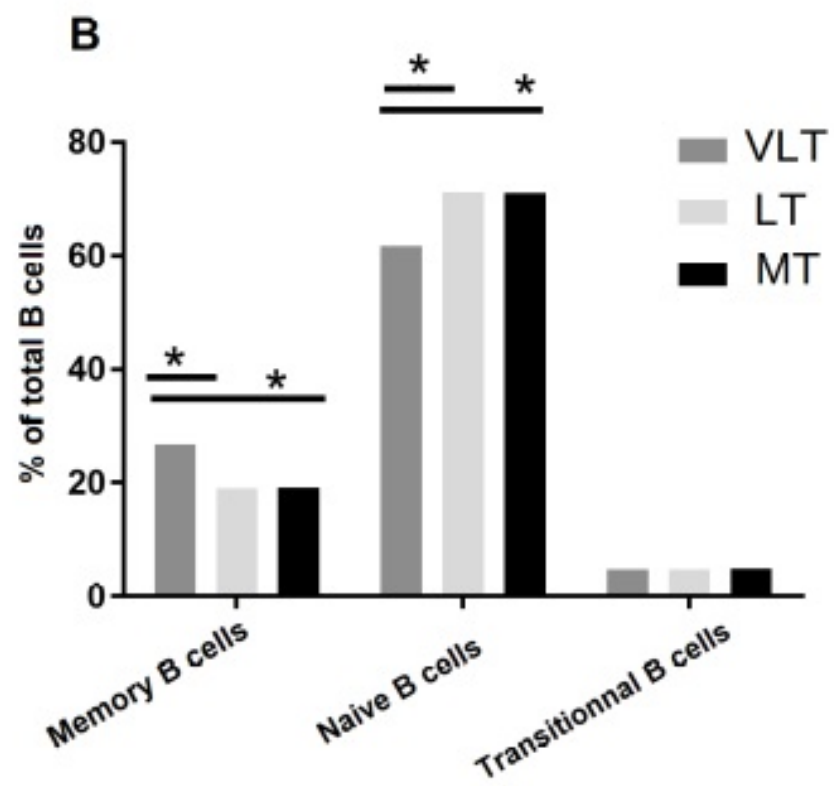
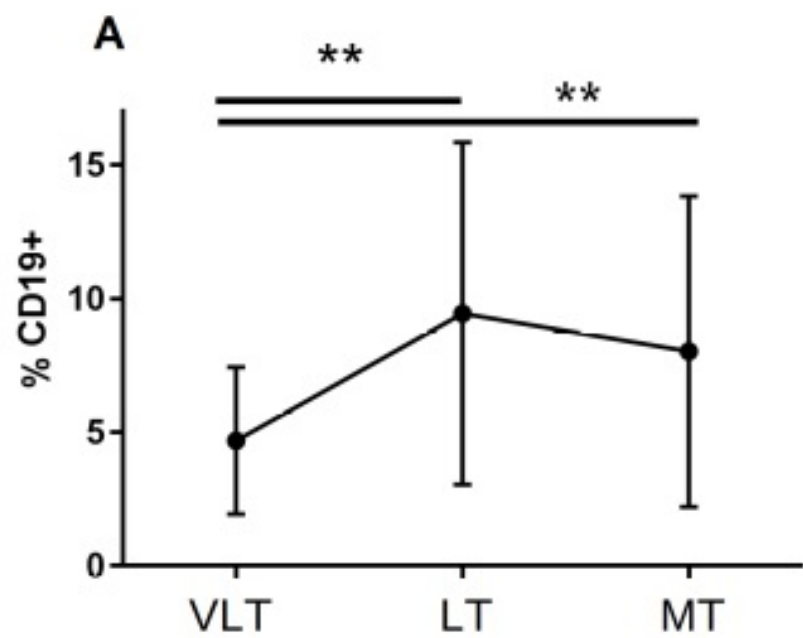




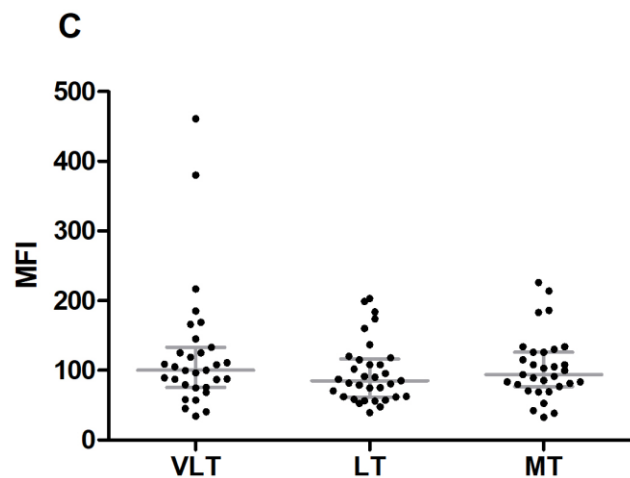
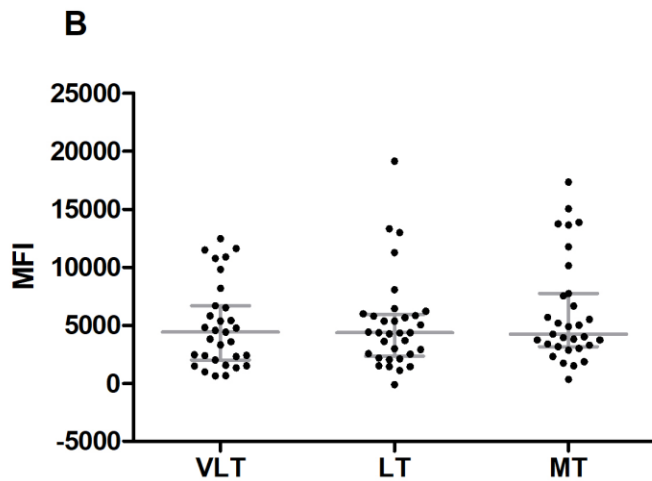
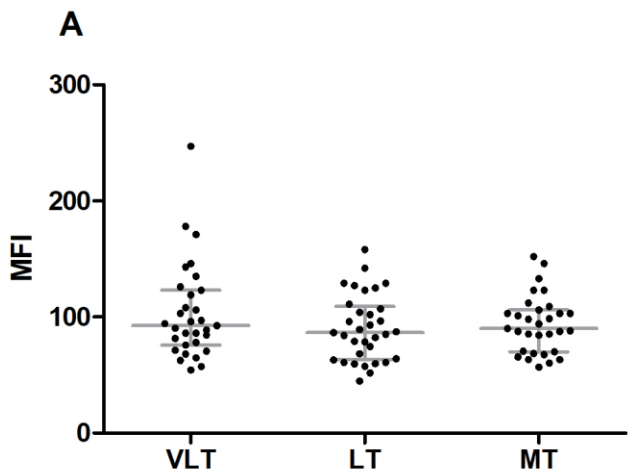


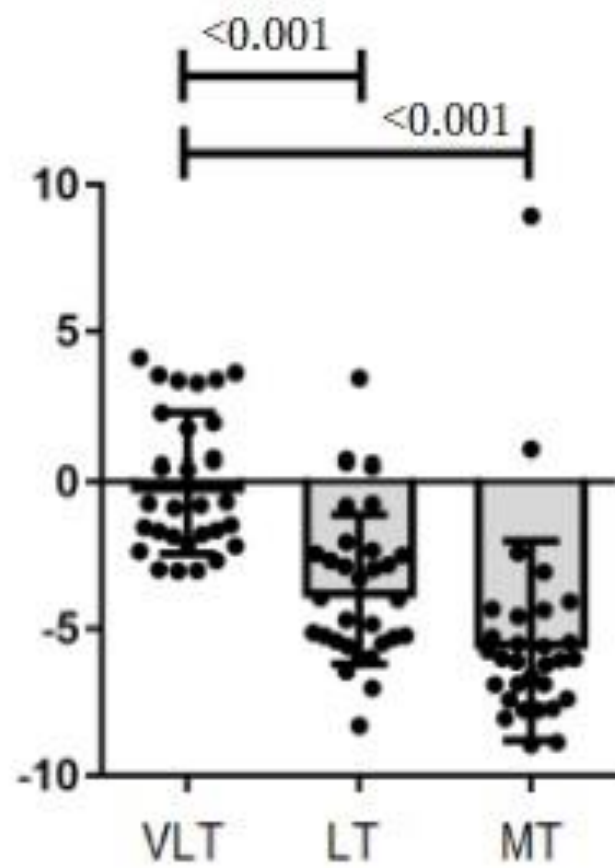


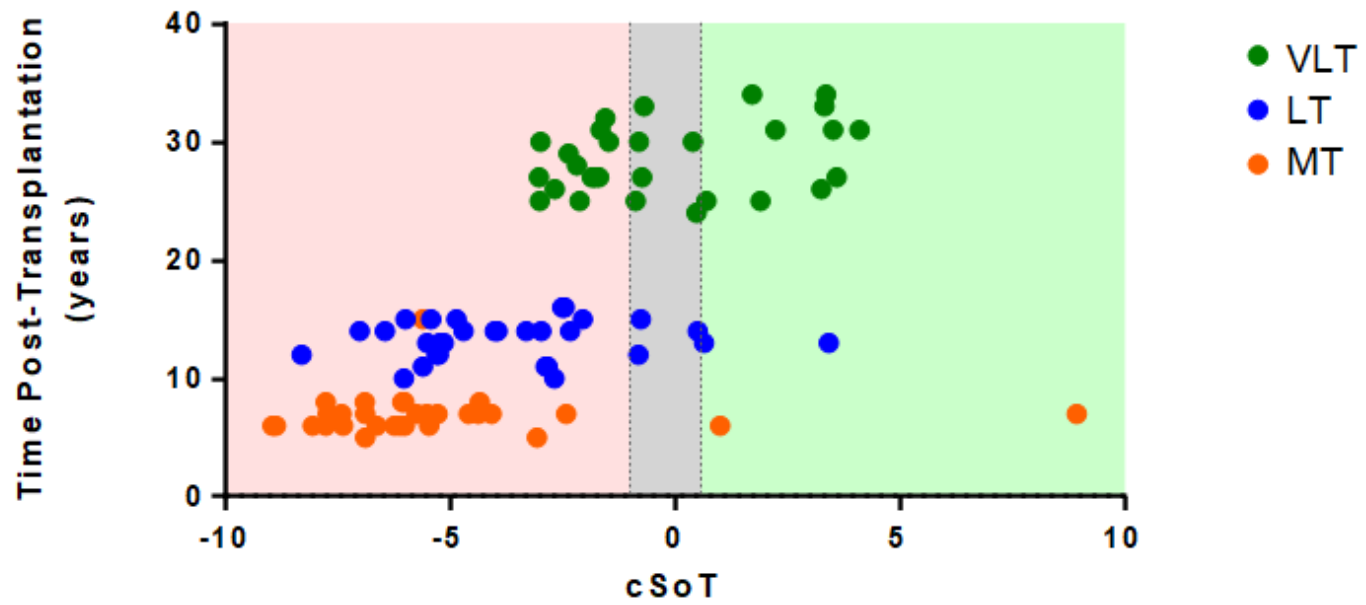


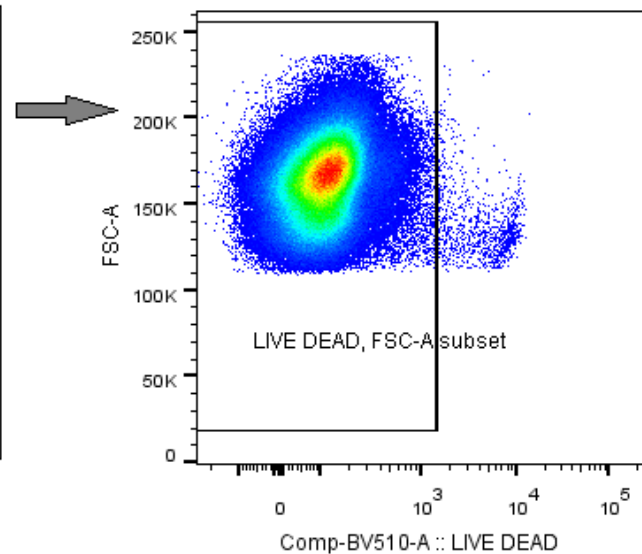
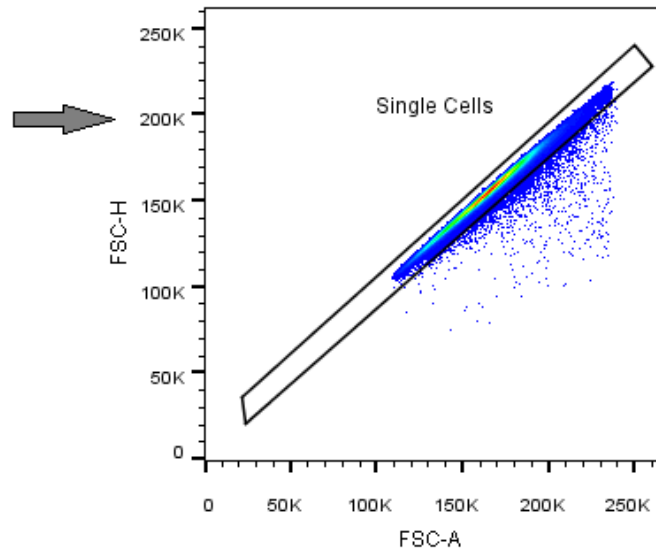
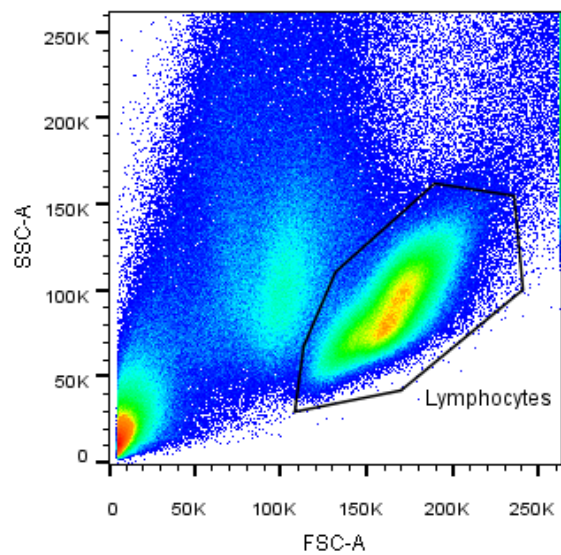




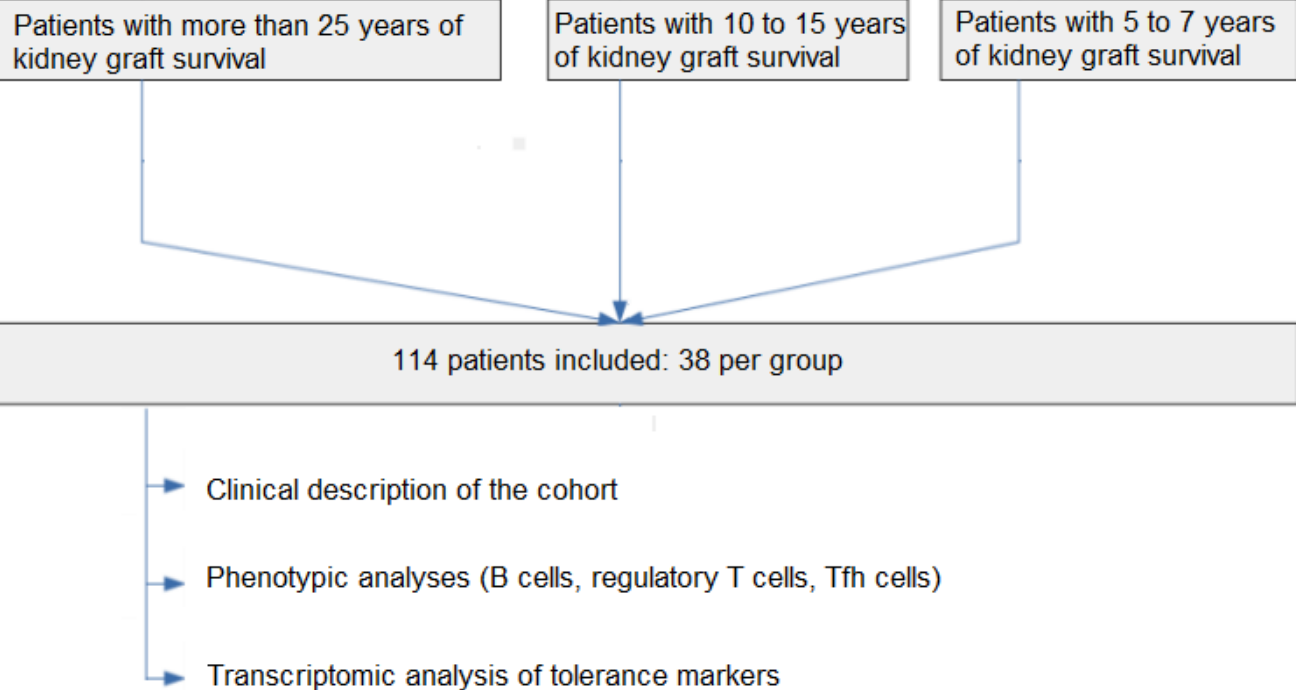




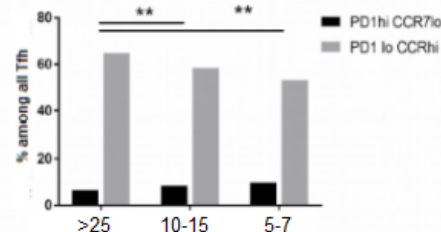




# Clinical and immunological follow-up of very long-term kidney transplant recipients treated with calcineurin inhibitors indicates dual phenotypes.

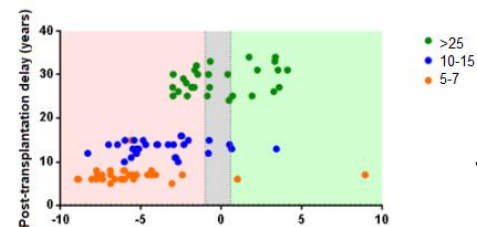
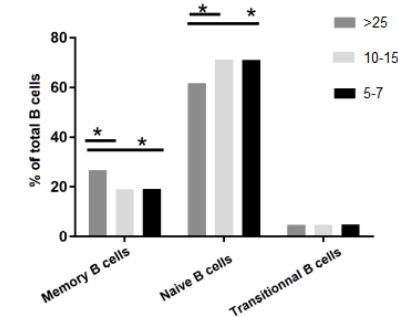


Patients with more than 25 years of kidney graft survival had:



- Less activated Tfh cells ( $p=0.0017$ )

- More memory B cells ( $p=0.0247$ ), and less naive B cells ( $p=0.0130$ )



- A higher score of tolerance ( $p<0.001$ )

## CONCLUSION:

This study suggests a dual phenotype in very long-term kidney transplanted patients with an immunological profile associated with low rejection risk.