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# Immune phenotype predicts new onset diabetes after kidney transplantation

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ABSTRACT

Few data are available concerning immune factors involved in the occurrence of new onset diabetes after transplantation (NODAT). Our objective was to determine an immune profile associated with the subsequent development of NODAT. The secondary objective was to build a predictive model of NODAT. We studied a prospective cohort of incident kidney transplant patients to determine whether pre-transplant immune characteristics could be associated with the occurrence of NODAT. 818 patients were included. We observed a significant inverse correlation between BMI and recent thymic emigrants (RTE) % at transplant time ($p < 0.001$). 177 (17.3%) of 677 non-diabetic patients experienced NODAT in the first year post-transplant. In multivariate analysis, age, body mass index (BMI), use of Tacrolimus, use of anti-thymocyte globulins (ATG), higher B cell count, and lower recent thymic emigrants (RTE) % were associated with NODAT. A differential effect of immune profile was observed in ATG-treated patients and non-ATG-treated patients. B cell count predicts NODAT only in non-ATG-treated patients whereas lower RTE% was associated with NODAT only in ATG-treated patients. Tacrolimus sparing and B cell depletion may efficiently prevent NODAT in selected patients. We identified an immune profile associated with the occurrence of post-transplant diabetes. Further studies should better precise the exact mechanisms involved in this association. Trial registration NCT02843867, registered July 8, 2016 – retrospectively registered https://clinicaltrials.gov/ct2/show/record/NCT02843867.

## 1. Introduction

Post-transplant diabetes is one of the most common complications after kidney transplantation [1]. Its occurrence is associated with increased morbidity and mortality [1–3].

Both innate and adaptive immunity contribute to obesity-induced insulin resistance and type 2 diabetes [4–9]. Post-transplant and type 2 diabetes pathophysiology are much close [1]. However, very few data are available concerning immune factors involved in the occurrence of post-transplant diabetes [1,10]. Furthermore, even when some immunosuppressive treatments have pro-diabetogenic effects, most of them are not related to their immunologic properties [1,11]. Nonetheless, defects or modulation of adaptive immunity could affect insulin sensitivity and modulate the risk of post-transplant diabetes. Thus, whether patients at risk of post-transplant diabetes share a common immune profile is unknown.

We studied a large prospective cohort of incident kidney transplant patients to determine whether pre-transplant immune characteristics are associated with the occurrence of post-transplant diabetes. We further analyzed a predictive model of NODAT and how immune modulation may influence the risk of NODAT.

*Abbreviations:* ATG, anti-thymocytes globulins; BMI, body mass index; CMV, Cytomegalovirus; PRA, panel reactive antibody; NODAT, new onset diabetes after transplantation; RTE, recent thymic emigrants

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## 2. Research design and methods

### 2.1. Patients

Research has been conducted in the 833 first consecutive RTR from the ORLY-EST study. Briefly, ORLY-EST is an observational prospective study [12] including incident RTR in seven French transplant centers (Besançon, Clermont-Ferrand, Dijon, Kremlin-Bicêtre, Nancy, Reims, Strasbourg). The main objective of this study is to describe interactions between immune status and post-transplant atherosclerosis. For each patient, blood samples were collected at time of transplantation and one year after. Sample collection was performed after regulatory approval by the French ministry of health (agreement number # DC-2008-713, June 11th 2009). The ethic committee of Franche-Comté study has approved the study (2008). Patients enrolled in the ORLY-EST study gave their written informed consent. Clinical data were prospectively collected.

Among 833 patients, 156 had pre-transplant diabetes. Calcineurin inhibitors (CNI) and Mycophenolate Mofetil (MMF) were widely used as immunosuppressive regimen.

Cytomegalovirus (CMV) prophylaxis was given according to each center practice. Almost all CMV-exposed patients received valganciclovir for 3 months. All CMV-naïve patients having received a CMV positive kidney received valganciclovir for 3 or 6 months. All patients received Pneumocystis antimicrobial prophylaxis with trimethoprim-sulfamethoxazole for at least 6 months.

### 2.2. Confounding factors

Age, gender, body mass index, diabetes, dyslipidemia, hypertension, smoking habit, and a past history of cardiovascular events (CVE) were analyzed as covariates. Dialysis mode (none, hemodialysis, or peritoneal dialysis), and its duration prior to transplantation were also recorded. HLA mismatches were recorded for HLA-A, -B, and -DR loci. Other relevant immunological parameters such as, pre-transplant panel reactive antibodies (PRA) (0 vs. positive PRA at any level), transplant rank (first vs. second or more), and acute rejection were analyzed as covariates. The use of Tacrolimus (Tac) *versus* Cyclosporine (CsA), and the type of induction were considered as potential covariates. Methods of assessment and definitions of these variables have been previously described in details [12].

### 2.3. Lymphocyte subsets

#### 2.3.1. T and B cell immunophenotypic analysis

Absolute numbers of $CD4^+$ and $CD8^+$ T cells were determined on fresh samples by a single platform flow cytometry approach using TetraCXP® method, Flow-Count® fluorospheres and FC500 cytometer (Beckman Coulter, Villepinte, France) according to manufacturer's recommendations. PBMCs were isolated by density gradient centrifugation (Pancoll, Pan-Biotech GmBH Aidenbach, Germany) and cryopreserved. After thawing, PBMCs were washed twice in RPMI 1640 + GlutaMAX™-I medium (Invitrogen, Cergy-Pontoise, France) containing 10% fetal calf serum (Invitrogen), thereafter referred as complete medium. Cells were stained with the following conjugated antibodies directed against: CD3, CD4, CD8, CD31, CD45RA, CD45RO, CD16, CD19 (Supplementary figure 1), CD56. Cell debris and doublets were excluded on the basis of side *versus* forward scatter. Cells were analysed on a FACS CANTO II cytometer (BD Biosciences) using FACS Diva (BD Biosciences) software.

Recent thymic emigrants (RTE) were defined as $CD45RA^+CD31^+CD4^+$ T cells [12]. Data were analysed by considering the percentage of RTE among $CD4^+$ T cells (RTE frequency or RTE%) and the absolute numbers of circulating RTE/$mm^3$ (Supplementary figure 2).

### 2.4. Outcomes

Post-transplant diabetes was defined according to current recommendations (Symptoms of diabetes plus random plasma glucose ≥ 200 mg/dL (11.1 mmol/L) OR fasting plasma glucose ≥ 126 mg/dL (7.0 mmol/L) OR 2 h-plasma glucose after an oral glucose ≥ 200 mg/dL (11.1 mmol/L) OR HbA1c ≥ 6.5% occurring after transplantation [13]. We restricted analysis to the first-year post-transplant to really consider transplant-related diabetes.

### 2.5. Statistical analysis

Arithmetic mean was calculated and expressed as + SD. For normally distributed variables, *t* test was used for continuous variables and chi-2 test for dichotomous variables. Abnormally distributed variables were either log-transformed or split in tertiles.

Correlations were calculated through Spearman test. Multiple regressions were used to determine factors associated with insulin sensitivity and secretion.

Using log rank tests on Kaplan Meier nonparametric estimates of the survival without NODAT distribution in the first-year post-transplant, we selected variables with a *p* value lower than, or equal to, 0.20. The selected variables were included into a Cox proportional hazards model, and a backward stepwise selection process was performed, this time at a classical $\alpha = 0.05$. Results are expressed as hazard ratio (HR) and 95% confidence interval (CI), with a *p* value testing the null hypothesis: HR = 1. Therefore when *p* value is $< 0.05$, HR is significantly different from 1, either greater than 1 (*i.e.*, risk of acute rejection is increased) or $< 1$ (*i.e.*, risk of acute rejection is decreased). Assumptions of Cox models (log-linearity, proportionality of risk in time) were met in this analysis.

Wald test was used to test potential interactions between variables.

Receiving operator characteristic (ROC) curve analysis was performed to assess the predictive performance of the model [14].

## 3. Results

### 3.1. Study population

Characteristics of the study population were depicted in Table 1.

### 3.2. Immune phenotype and pre-transplant diabetes

We observed a significant inverse correlation between BMI and RTE frequency at transplant time ($R = -0.16$; $p < 0.001$) (Fig. 1).

To further analyse the association between immune phenotype, obesity, and diabetes, we studied obese patients (body mass index

**Table 1**
Clinical characteristics of the study population.

| Characteristics (n = 677) | |
|---|---|
| Age (years) [Range] | 51 + 14 [18–84] |
| Male gender (%) | 64% |
| Body mass index (kg/$m^2$) [Range] | 24.8 + 4.3 [17.3–46.2] |
| Smoking (%) | 22% |
| Hypertension (%) | 84% |
| Dyslipidemia (%) | 34% |
| Pre-transplant CV (%) | **12%** |
| Pre-transplant dialysis (%) | 89% |
| Dialysis duration (months) | 39 + 36 |
| Pre-transplant CMV exposition (%) | 55% |
| PRA (% sensitized patients) | 31% |
| ATG use (%) | 30% |
| Early steroid withdrawal (%) | 12% |
| Tacrolimus use (%) | 63% |
| mTORi use (%) | 3% |

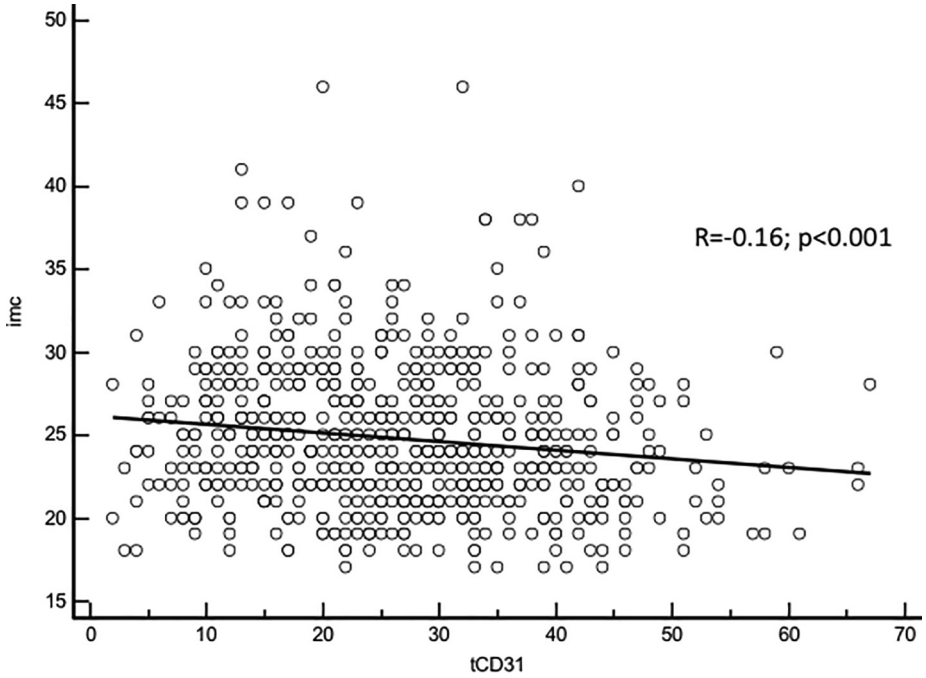


**Fig. 1.** Correlation between body mass index (BMI) and recent thymic emigrants (RTE) frequency at transplant time.

above 30 kg/m$^2$, n = 113) with and without diabetes before transplantation. Thirty obese patients with type 2 diabetes were compared to 83 obese patients without diabetes. Mean BMI were not different between the 2 groups (33.1 ± 3.8 vs 33.6 + 2.9 kg/m$^2$; p = 0.536). In multivariate analysis, diabetes was associated with age (RR 1.15 [1.08–1.23] for each increase of one year, p < 0.001) and lower RTE frequency (RR 3.98 [1.37–11.11], for %RTE under the median value; p = 0.011). Same results were observed when RTE were replaced by CD4/CD8 ratio (RR 1.54 [1.03–2.32] for each decrease of one unit of CD4/CD8, p = 0.039). There were no differences concerning other immune parameters.

### 3.3. New-onset diabetes

Among 677 patients without pre-transplant diabetes, One hundred and seventeen patients (17.3%) experienced new onset diabetes in the first year post-transplant.

In univariate analysis, age (< 0.001), BMI (0.003), use of Tacrolimus (0.003), B cell count (0.084), CD4/CD8 ratio (0.038), recent thymic emigrant (RTE) frequency (0.020) were significantly associated with the occurrence of post-transplant diabetes. Use of ATG (0.192) was marginally associated and maintained in the multivariate analysis.

Other immune cell subsets were not associated with post-transplant diabetes (Table S1).

In multivariate analysis, older age (HR, 1.04; 95%CI, 1.02 to 1.06 for each increase of 1 year in age; $p < 0.001$), higher BMI (HR, 1.08; 95%CI, 1.03 to 1.13 for each increase of 1 kg/m$^2$ in BMI; $p = 0.001$), use of Tacrolimus (HR, 2.93; 95%CI, 1.77 to 4.85; $p < 0.001$), use of ATG (HR, 1.58; 95%CI, 1.00 to 2.48; $p = 0.047$), and higher B cell count (HR, 1.12; 95%CI, 1.03 to 1.25 for each increase of 50/mm$^3$ in B cells; $p = 0.007$) were risk factors for new-onset diabetes. Higher RTE frequency was found to be protective against post-transplant diabetes (HR, 0.80; 95%CI, 0.62 to 0.94 for each increase of 10% in %RTE $p = 0.011$) (Table 2). Similar results were obtained with absolute number of RTE. Higher CD4/CD8 ratio was found to be protective for post-transplant occurrence of diabetes (HR, 0.79; 95%CI, 0.66 to 0.94 for each increase of 1 in CD4/CD8; $p = 0.019$) when RTE was removed from the model.

A risk score was derived from logistic regression. ROC curve AUC was 0.71 (95% CI: 0.67–0.74) (Fig. 2A). Immunological parameters (B cell count and %RTE) significantly improved ROC curve AUC (0.71 + 0.03 vs 0.68 + 0.03; $p = 0.041$) (Fig. 2B). Hosmer-Lemeshow test revealed good calibration ($p = 0.259$). 84% of patients were correctly classified.

### 3.4. Effects of B cell according to ATG use

Because ATG affects post-transplant B cell count, we separately analysed the effect of B cell on NODAT in patients having or not received ATG. Patients having received ATG had lower B cell count one-year post-transplant (84 ± 87/mm$^3$ vs 103 ± 106/mm$^3$; p = 0.034).

Whereas B cell remained strongly associated with NODAT occurrence in non-ATG treated patients (HR, 1.05; 95%CI, 1.02 to 1.09 for each increase of 50/mm$^3$ in B cells; $p = 0.002$), the effect was lost in those having received ATG (HR, 0.93; 95%CI, 0.74 to 1.13 for each increase of 50/mm$^3$ in B cells; $p = 0.521$) (p for interaction = 0.042).

### 3.5. Effects of RTE according to ATG use

Because ATG decreases RTE count [15], we separately analysed the effect of RTE on NODAT in patients having or not received ATG. Patients having received ATG had lower RTE frequency one-year post-transplant (18 ± 12% vs 25 ± 12%; p < 0.001).

Whereas RTE was strongly associated with NODAT occurrence in ATG treated patients (HR, 0.74; 95%CI, 0.55 to 0.91 for each increase of 10% RTE; p = 0.002), the effect was lost in those not having received ATG (p for interaction = 0.021).

## 4. Discussion

We reported that higher B cell count and lower thymic function were independently associated with post-transplant diabetes. This specific immune phenotype enhances discrimination and predictability from traditional risk factors. Intriguingly, the impact of immune phenotype seems to be modulated by induction therapy. Altogether, our results suggest that adaptive immunity plays a major role in post-transplant insulin resistance and diabetes.

Naïve CD45RA$^+$CD31$^+$ CD4$^+$ T cells represent an excellent cellular

**Table 2**
Pre-transplant risk factors for post-transplant diabetes (Multivariate Cox model) in the whole cohort, in ATG-treated patients, and in non ATG-treated patients.

| | Whole cohort (n = 677) | | | ATG-treated patients (n = 203) | | | Non-ATG-treated patients (n = 474) | | |
|---|---|---|---|---|---|---|---|---|---|
| | HR | 95%CI | p | HR | 95%CI | p | HR | 95%CI | p |
| Age (for each supplementary year) | 1.04 | 1.02–1.06 | < 0.001 | 1.04 | 1.02–1.09 | < 0.001 | 1.03 | 1.01–1.05 | 0.002 |
| BMI (for each supplementary 1 kg/m$^2$) | 1.08 | 1.03–1.13 | 0.001 | 1.10 | 1.01–1.09 | 0.022 | 1.08 | 1.02–1.15 | 0.014 |
| Tacrolimus | 2.93 | 1.77–4.83 | < 0.001 | 4.82 | 1.87–12.42 | 0.001 | 2.18 | 1.20–3.97 | 0.011 |
| ATG | 1.58 | 1.00–2.48 | 0.047 | | | | | | |
| B cell count (for each increase of 50/mm$^3$) | 1.12 | 1.03–1.25 | 0.007 | 1.09 | 0.83–1.30 | 0.579 | 1.15 | 1.03–1.27 | 0.019 |
| %RTE (for each increase of 10%) | 0.80 | 0.62–0.94 | 0.011 | 0.74 | 0.55–0.91 | 0.006 | 0.95 | 0.77–1.19 | 0.221 |

HR (Hazard ratio), CI (Confidence interval).
ATG Anti-Thymocyte Globulins.
BMI Body Mass Index.
RTE Recent Thymic Emigrants.

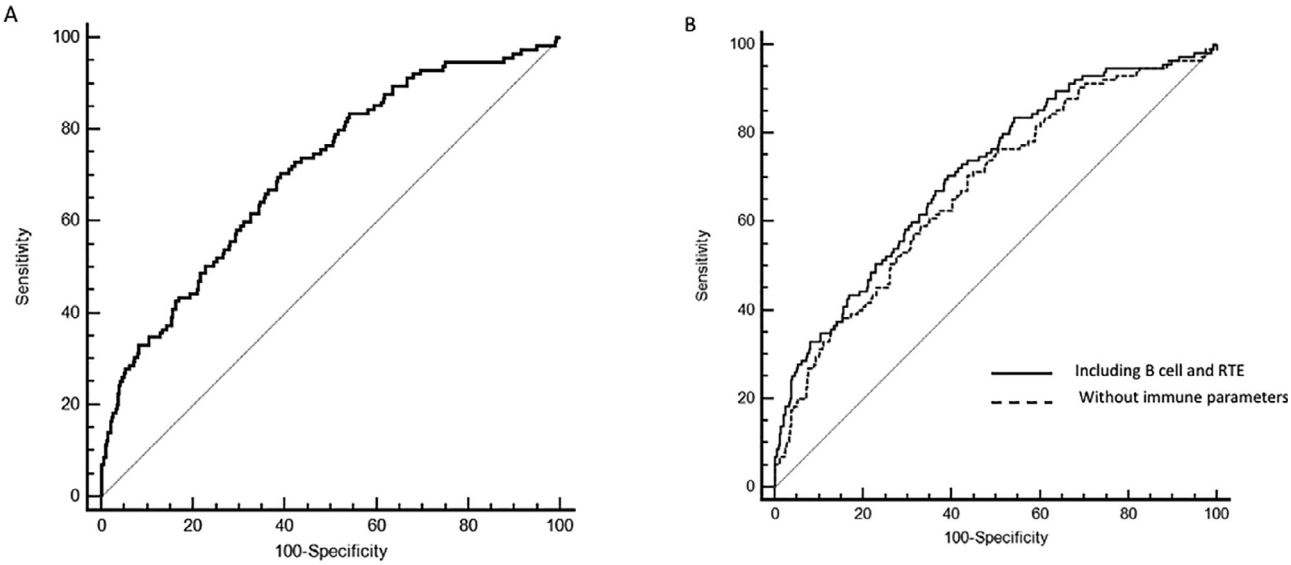


**Fig. 2.** ROC curve of model prediction of diabetes (A: in the whole cohort; B: comparison of models with and without immunological parameters).

phenotype to appreciate thymic activity [15]. CD45RA$^+$ CD31$^+$ CD4$^+$ T cells in peripheral blood are not fully identical with RTEs but provide a RTE rich subset. More accurate tools to peripherally analyze thymic function using blood samples, such as the sj/beta-TREC ratio, are very sophisticated and expensive and therefore clinically not useful. Compelling data suggest that the determination of CD31$^+$CD45RA$^+$CD4$^+$ T cells is as efficient as T-cell Receptor Excision Circle (TREC) to measure thymic activity [15]. Strong correlations between TREC content in CD4$^+$ T cells and frequencies of CD31$^+$CD4$^+$ T cells have been described in numerous populations [15], as well as in end-stage renal disease patients [12]. Higher TREC contents are identified in Human CD31$^+$ CD4$^+$ T cell subsets [16]. However, compared to TREC measurement, cytometric analysis of CD31$^+$CD4$^+$ T cells is highly standardized and results are not affected by homeostatic proliferation. Finally, measurement is easy and quick, warranting possible clinical application. Consequently, we believed that our results suggest that pre-transplant thymic function, as assessed by CD31$^+$CD45RA$^+$CD4$^+$ T cells, predicts NODAT.

We observed lower thymic output in obese patients. Yang et al [17] previously reported that obesity induces acceleration of thymic aging related with defects at multiple levels which include increased apoptosis of developing thymocytes, decrease in T-cell precursor pool, and reduction in RTEs. The same group also suggested that caloric restriction prevents age-related loss of thymic epithelial cells and increases fibroblasts with reduction in epithelial-mesenchymal transition regulators in the thymus [18,19]. Interestingly, it has been reported that metformin partially restores thymic function in type 2 diabetes patients [20]. As previously reported, we observed that obese patients were more prone to infectious complications including post-transplant [21,22]. Therefore, restriction of TCR repertoire diversity as a result of obesity-induced thymic involution may explain the greater risk of infections in obese patients. In obese patients, marked insulin resistance or proven diabetes is associated with a more reduced thymic output [17]. We showed that pre-transplant obese patients with type 2 diabetes have reduced thymic function as compared with obese patients without diabetes. We also observed lower thymic output to be associated with the occurrence of post-transplant diabetes. Concordant with our results, Li et al [23] showed that intra-bone marrow-bone marrow transplantation + concurrent thymic transplantation normalized insulin sensitivity and increased the number of insulin-producing cells in the db/db mouse. Interestingly, this association was restricted to ATG-treated patients. We previously demonstrated that ATG reduces thymic output suggesting a dose-effect of thymic loss on insulin resistance [12]. Collectively, our data suggest that, not only obesity affects thymic function, but also, that compromised T cell immunity may contribute to insulin resistance. These results highlighted the central role of CD4 + T cells in insulin resistance [24].

We reported B cell count to be associated with the occurrence of post-transplant diabetes. Recent studies have shown that obesity induces chronic inflammation within adipose tissue, which in turn leads to systemic metabolic abnormalities and inflammation. Macrophages and T cells are known to play crucial roles in adipose inflammation [25]. More recently, concordant studies reported that total B cells have a pathogenic role in obesity-associated insulin resistance [26]. Thus,

diet-induced obese B cell knockout mice have improved glucose tolerance and insulin sensitivity [27]. Obesity affects B cells by increasing their infiltration to the visceral adipose tissue, including the class switched IgG + B cells [26]. It has been reported that C57Bl/6 mice, fed with a high fat diet (HFD), have marked increases in pre-B cells, immature and mature B cells [28]. Increased B cell lymphopoiesis is at least in part due to increases in leptin, which correlated with increase in bone marrow adipocytes [28]. Peripheral blood B cells from diabetic patients and splenic B cells from obese mice exhibit a pro-inflammatory cytokine profile (increased IL-6, TNF-??, or IL-8 and reduced IL-10 secretion) [27,29]. More recently, Nishimura et al [30] reported that adipose tissue Breg cells maintain homeostasis within adipose tissue and that Breg cell dysfunction contributes to the progression of adipose tissue inflammation in obesity. Finally, Winer et al reported that B cell–depleting anti-CD20 monoclonal antibody improves DIO, whereas transfer of IgG from diet-induced obesity mice induces insulin resistance and glucose intolerance [26]. B cell count was not predictive of post-transplant diabetes in ATG-treated patients. This may be related to ATG-induced B cell depletion. Alternatively, ATG may induce a transient increase in naïve virgin B cells (Bm-1) which may have tolerogenic effects [31].

We used a uniform and sensitive definition of diabetes. Moreover, due to the prospective design of the study, the rate of missing values was very low (< 5%). Finally, the incidence of post-transplant diabetes is that expected in this population [1]. Consequently, any bias of measure is unlikely in this study. We measured blood T and B lymphocytes. Direct analyses of visceral fat adipose tissue immune cells infiltration would be more informative. Further studies should determine whether ATG may deplete or modulate these specific cells. Finally, more precise analyses of immune cell subsets are probably required as well as their dynamics after transplantation.

We identified an immune profile associated with the occurrence of post-transplant diabetes. Both thymic function and B cells interact with insulin resistance and diabetes occurrence. ATG increases the risk of NODAT due in part to its effect on thymic output but could mitigate the role of B cells. Our results also suggest that pre-transplant immune modulation may reduce the risk of this frequent and deleterious complication.

## 5. Contributors

JB, CC, TC, DD, JMR, and PS designed the study concept and drafted the manuscript. DD, TC, CC, EG, CV, and JB participated to acquisition of data and patient follow up. TC, CC, CR, CL, JB and PS participated in T cell subset analysis in patients. DD did statistical analysis. All authors saw and approved the final version of the manuscript. All the authors approved the final manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.08.006.

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