Title: Reduced Human Transitional B cell T1/T2 Ratio is Associated with Subsequent Deterioration in Renal Allograft Function

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Abstract

Human Transitional B cells (TrB) express relatively high IL-10 and low TNF-α levels, which correlate with B regulatory (Breg) activity *in vitro*. Herein, we aimed to further define Breg phenotype and determine whether Bregs can serve as a prognostic marker for renal allograft dysfunction (graft loss or two-fold fall in eGFR). TrBs can be divided into T1 and T2 subsets based on surface phenotype. T1 cells express a significantly higher IL-10:TNF-α ratio than T2 cells or other B subsets. When analyzed in 45 kidney transplant recipients (KTRs) at the time of late for-cause biopsy, the T1/T2 ratio was independently associated with allograft dysfunction over the next 5 years. Next, the T1/T2 ratio was examined in an independent set of 97 clinically stable KTRs 2 years post-transplant. Again, the T1/T2 ratio was strongly and independently associated with allograft dysfunction over the ensuing five years. In these clinically quiescent patients, a low T1/T2 ratio identified a sub-group (n=41) in which 35% developed allograft dysfunction (with 25% losing their allografts), whereas no patients with a high ratio developed graft dysfunction (n=56). In both the initial study and validation groups, T1/T2 ratio was a much stronger predictor of graft dysfunction than DSA or eGFR. Thus, the T1/T2 ratio, a relative measure of Bregs expressing an anti-inflammatory cytokine profile, is a novel prognostic marker that might inform individualized immunosuppression.

Introduction

Kidney transplantation is the treatment of choice for patients with end-stage kidney disease, conferring improved quality of life and survival while reducing costs (1). Despite remarkable short-term outcomes, there is continued graft loss such that by 10 years, 25-35% of recipients lose their transplants (2, 3). Importantly, return to dialysis following graft loss is associated with a three-fold increased risk of death and significantly increased costs (4, 5). Although the etiology for late allograft loss may be multifactorial, prior immune-mediated damage is common (6, 7). The inability to accurately assess adequacy of immunosuppression or predict adverse long-term outcomes, precludes individualization of therapy based on actual risk. While surveillance biopsies within the first year post–transplant might help predict subsequent clinical course, these are performed in <20% of US transplant centers (8). Late surveillance biopsies in clinically stable patients are not standard of care. Thus, non-invasive biomarkers that can predict long-term outcomes in clinically quiescent patients are sorely needed to identify patients who might benefit from less versus more immunosuppression or invasive monitoring.

Transitional B cells (TrB) represent a critical developmental stage between immature B cells in the bone-marrow and mature B cells in the periphery (9). In humans, TrB have gained recent attention because of their purported regulatory B cell (Breg) activity, based upon their relatively high-level expression of the anti-inflammatory cytokine IL-10 (10, 11). Bregs play an important role in murine models of autoimmunity and transplant tolerance, and their frequency and/or function is decreased in SLE and MS (12, 13). In the context of clinical transplantation, TrB are increased in tolerant renal transplant recipients compared to stable recipients on immunosuppression (14). We recently showed that various B cell subsets in peripheral blood of healthy human subjects express significant IL-10. However, TrB express less pro-inflammatory TNF-α, resulting in a higher IL-10/TNF-α ratio than other B subsets, and this correlated with suppressive function *in vitro* (10). Moreover, TrB from renal transplant recipients with rejection specifically exhibit a decreased IL-10/TNF-α ratio and loss of *in*

vitro Breg activity suggesting that TrB cells or cytokines might serve as a marker for renal allograft outcomes.

TrB are comprised of less mature T1 cells and more mature T2 cells (15, 16). In humans, both T1 and T2 TrB are found in the peripheral blood and spleen (17). While T1 and T2 TrB utilize distinct maturation and survival signals (18-21) little is known about their cytokine expression or significance (9, 16, 22). Here, we show that the T1 subset expresses a markedly higher IL-10/TNF-α ratio than the T2 subset. Moreover, a reduced T1/T2 ratio at the time of for-cause biopsy was associated with allograft deterioration over subsequent five years. Importantly, the T1/T2 ratio was prospectively validated in a distinct cohort of clinically quiescent patients two years post-transplantation, as an independent marker for allograft deterioration. Such risk-stratification may improve outcomes by allowing individualization of therapy.

Results:

$CD24^{hi}CD38^{hi}$ Transitional B cells are phenotypically and functionally heterogeneous

TrB are identified in humans by their high-level expression of CD24 and CD38 ($CD24^{hi}CD38^{hi}$) (17, 23, 24). We further characterized the phenotype of TrB in peripheral blood from 5 healthy volunteers. As previously defined, T1 cells were $CD19^{+}CD27^{-}CD24^{+++}CD38^{+++}$ whereas T2 cells were $CD19^{+}CD27^{-}CD24^{++}CD38^{++}$and naïve B cells were $CD19^{+}CD24^{+}CD38^{+}CD27^{-}$ (17) (Fig 1A). While the distinction between T1 and T2 cells is not absolute, gates were established and applied consistently, as previously described (17, 25). Compared to T2 cells, T1 cells expressed higher levels of CD5, CD20, and especially, IgM, and CD10 (Fig 1B, 1C). IgD expression was somewhat reduced, while CD1d (a general TrB marker) and CD86 did not differ.

The functional repertoire of T1 and T2 cells in terms of cytokine expression is unknown. To address this, ($CD19^{+}$) B lymphocytes purified from peripheral blood of 15 healthy volunteers, were stimulated with CPG and CD40L for 48hrs, as described (26). Analysis of intracellular cytokines revealed that a similar percentage of T1 and T2 cells expressed IL-10 (Figs 1D, 1E). However, a significantly lower proportion of T1 cells expressed TNF-α, resulting in a higher IL-10/TNF-α ratio compared to T2 cells

(Figs 1F, 1G). This includes a lower proportion of cells that simultaneously express both cytokines (Figs 1D, 1I). Nonetheless, the T2 subset still expressed significantly more IL-10 and less TNF-α than naive B cells. The IL-10/TNF-a ratio within the T1 subset is significantly higher when compared to regardless of whether cytokines were measured after 12, 24, or 48hrs after *in vitro* stimulation (Fig 1H). Moreover, the same trend with the IL-10/TNF-a ratio was noted when the subsets were analyzed even in renal transplant patients with immunosuppression (Fig-S1). Thus, the IL-10/TNF-α ratio differs in these B cell subsets, and the T1 subset had the most anti-inflammatory cytokine profile.

T1/T2 ratio risk stratifies renal transplant recipients at the time of late for-cause allograft biopsy

We previously showed that the TrB IL-10/TNF-α ratio was reduced in patients with allograft rejection. Since the measurement of cell surface phenotype is less technically demanding than measurement of cytokines, we extended our studies to determine whether TrB subsets could be used to risk-stratify outcomes in 45 cross-match negative renal allograft recipients undergoing a for-cause allograft biopsy for low-grade proteinuria or worsening eGFR ~3-12 years after transplantation (99 ±65 months). Patients were predominantly first deceased donor allograft recipients (81%) induced with basiliximab and maintained on calcineurin inhibitors (CNI) and mycophenolate mofetil (MMF). (For demographics, see Table-I). Of these 45 patients, 25 had histological evidence of rejection with 70% showing mixed T cell and antibody-mediated rejection and 30% showing antibody-mediated rejection alone. B cell subset analysis was performed at the time of biopsy before any therapeutic intervention, and patients were followed for a further 5 years.

Based on renal function during the 5-year follow-up, patients were divided into those with stable function (Group-S; n=17) versus those with further functional deterioration (Group-D; n=28). Functional deterioration was defined as halving of eGFR or graft loss, from the time of B cell subset analysis. As shown in Fig 2A-F, despite comparable B cell numbers between groups, group-D patients had a significantly lower frequency and absolute number of TrB, along with lower absolute numbers of both T1 and T2 cells. (Representative FACs plots in patients and healthy controls are shown in Fig S2). However, compared to Group-S, Group-D patients exhibited a greater reduction in the number of

T1 than T2 cells (Fig 2D, 2E), resulting in a marked decrease in their T1/T2 ratio (Group-S, 0.27±0.02 vs. Group-D, 0.11±0.02; $p<0.0001$; Fig 2F). Of note, TrB IL-10/TNF-α ratio, which we showed reflects the regulatory potential of TrB (10), was also reduced in Group-D, and paralleled the T1/T2 ratio (Fig S3).

We next assessed the ability of the T1/T2 ratio (at time of biopsy) to predict risk for subsequent allograft deterioration. As shown in Figs 3A and 3B, the T1/T2 ratio was a strong predictor of allograft deterioration with an AUC of 0.88 on ROC (Receiver Operating Characteristic curve) analysis ($p<0.0001$). At a T1/T2 cut-off value of ≤ 0.17, this marker had a sensitivity of 88.2% and a specificity of 78.6%. Based on this optimal cut-off, all 45 patients were then divided into those with a T1/T2 ratio ≤ 0.17 (low ratio, n=24) and those with a ratio >0.17 (high ratio, n=21). As shown (Fig 3C), over the next 5 years, a striking 95% of those with a low ratio had allograft deterioration (2-fold fall in eGFR or graft loss) compared to only 30% of the patients with a high T1/T2 ratio ($p<0.0001$). Concordantly, those with a low T1/T2 ratio had a significantly greater decline in eGFR than those with a high ratio ($p<0.0001$; Fig 3D). More importantly, the incidence of death-censored graft loss was markedly higher in patients with a low T1/T2 ratio compared to those with a high ratio (60% vs. 15%, respectively; $p<0.0001$; Fig 3E).

As noted above, these patients were heterogeneous, and the 25 out of 45 patients with biopsy-proven rejection could have contributed to the adverse outcomes. To address this, we separately analyzed patients with biopsy-proven rejection. Notably, amongst the 25 patients with allograft rejection, 100% of patients with a low T1/T2 ratio had allograft deterioration, compared to 40% within the high ratio group ($p=0.014$; Fig 3F). A similar non-significant trend was noted when the groups were compared for the change in eGFR over five-years (Fig 3G). In these 25 patients, the association between the severity of the Banff histological scores and the T1/T2 ratio was analyzed in detail. As shown in Fig 4A, a low T1/T2 ratio tended to identify patients with more severe inflammation (higher “t”, “i”, and “ptc” scores) and chronic damage (higher ‘cg’, ‘ct’ ($p=0.03$), and ‘ci’ scores) than those with a high ratio. However, analysis of AUC-ROC for these histological parameters revealed that they lack predictive capacity (Fig 4B). In contrast, the T1/T2 ratio strongly classified graft dysfunction vs.

stable function amongst these rejecting patients (AUC 0.92, p=0.0085). Thus, the T1/T2 ratio not only identified patients with worse acute and chronic histological changes, but is also a significantly more sensitive and specific marker for subsequent deterioration.

T1/T2 ratio predicts the course of renal allograft function in an independent validation set

We next sought to validate the ability of the T1/T2 ratio to identify patients at risk for renal allograft deterioration in an independent cohort of clinically stable patients analyzed approximately two years post-transplant. This validation set utilized 97 of 116 patients enrolled in a randomized controlled trial comparing alemtuzumab induction and tacrolimus monotherapy (n=51) to basiliximab induction with tacrolimus and MMF therapy (n = 46) who had PBMC available, and agreed to participate (Demographics summarized in Table 2). 17.5% of these patients had a rejection episode within the first year that was treated with intravenous methylprednisolone and addition of maintenance prednisone except for one patient who received plasmapheresis and IVIg along with corticosteroids for antibody-mediated rejection (see Table S1 for further details).

PBMC samples were obtained ~2 years post-transplantation, a time when patients were clinically quiescent with a mean baseline eGFR of 50.2 ±9 ml/min. No patients were actively being treated for acute rejection but 13 patients had *de novo* DSA. Over the 5-years from the time of PBMC analysis, 85 patients had stable function (group-S; n=85) whilst 12 had graft deterioration (group-D; n=12). 8 of the 12 patients with deterioration had for-cause biopsies, and all showed marked tubulointerstitial scarring with glomerulosclerosis. In addition, two had chronic antibody-mediated rejection and one exhibited cellular rejection.

Even in this validation cohort, patients with graft deterioration (Group-D) had a significantly lower frequency and absolute number of TrBs, and absolute number of T1 cells, and a trend towards less T2 cells compared to patients with stable function (Fig 5A-F). Again, the T1/T2 ratio differed by a reasonable magnitude (Group-D, 0.1±0.01 vs. Group-S, 0.2±0.01; p=0.0001). ROC analysis confirmed that the T1/T2 ratio was a strong predictor of allograft deterioration, with an AUC of 0.824; a sensitivity of 100% and a specificity of 66% (reflecting less patients who subsequently

develop allograft deterioration in these stable patients) using the same optimal cut-off of 0.17 used in the training set (Fig 6A-B). Nonetheless, when patients were divided into those with a low (≤0.17, n=41) or high (>0.17, n=56) T1/T2 ratio, 35% of those with a low T1/T2 ratio developed subsequent allograft deterioration over five years and 25% suffered graft loss (Fig 6C, 6D). In stark contrast, none of the patients in the high T1/T2 ratio group developed allograft deterioration ($p<0.0001$ Fig 6C; $p=0.0002$; Fig 6D). In fact, patients with a high ratio exhibited stable eGFR over five years (Fig 6E). It is noteworthy that a low T1/T2 ratio was associated with graft deterioration regardless of immunosuppressive regimen, as is seen by separate analysis of patients who received Alemtuzumab (n=51; Fig S4A, B) versus Basiliximab based therapy (n=46; Fig S4C, D).

TrB T1/T2 ratio predicts allograft dysfunction independently of and stronger than clinical parameters

We next asked if the TrB T1/T2 ratio is associated with graft dysfunction independently of currently accepted clinical standards (i.e., eGFR, serum DSA) and histological evidence of rejection. Multivariate Cox analysis (Table 3), showed that the TrB T1/T2 ratio was independently associated with a decline in allograft function in both the initial study (adjusted HR 0.6; 95% CI 0.4-0.9; $p=0.02$) and the validation cohorts (adjusted HR 0.35; 95% CI 0.14-0.9; $p=0.02$). We also assessed whether or not the TrB parameters were influenced by the renal function. Whilst a weak negative correlation was noted between T1/T2 ratio and index eGFR at blood sampling in the validation cohort (Fig S5), the multivariate analysis included eGFR as a covariate and T1/T2 ratio was associated with graft deterioration independently of baseline renal function. Tacrolimus trough levels were not significantly different between the high and low T1/T2 groups for 8 months (test set) and 18 months (validation set) before the ratio was determined (Fig S6). Although, in the validation set, more patients with low T1/T2 ratio were on maintenance corticosteroids, a low T1/T2 ratio was associated with graft deterioration irrespective of steroid usage (Fig S7) Indeed, comparing ROC curves for the prediction of graft deterioration in both study and validation groups reveals that while T1/T2 ratio strongly classifies patients with stable vs. graft dysfunction and achieves statistical significance (AUC

0.88 p<0.0001 training set; AUC 0.82, p=0.0003, validation set); DSA and eGFR have low AUCs (0.56-0.66) and do not reach significance. Thus, the T1/T2 ratio is both independent of, and superior to, clinical parameters for predicting late allograft decline.

Discussion:

In addition to humoral immunity, B cells shape effector T cell responses through antigen presentation, costimulation, and production of regulatory and pro-inflammatory cytokines (12, 22, 27-39). This is buttressed by studies in genetically modified mice and humans demonstrating that both pro-inflammatory and anti-inflammatory cytokines specifically expressed by B cells profoundly influence the immune response to infections, autoimmunity and transplantation (22, 29, 32, 40, 41). Unfortunately, no specific markers exist for either inflammatory or regulatory B cells. While several human B cell subsets are enriched for IL-10, we showed that these same subsets co-express TNFα and that human Breg activity correlates best with the ratio of IL-10/TNF-α expression (10, 11, 13, 26). Thus, TrBs exhibit the highest IL-10/TNF-α ratio and demonstrate potent IL-10-dependent regulatory activity *in vitro* and suggested that Bregs may correlate with immunological activity in transplantation (10). We now show that T1 cells have a significantly higher IL-10/TNF-α ratio than T2 cells and while both subsets are reduced in patients with subsequent dysfunction, the reduction in T1 cells is much more pronounced, resulting in a marked fall in T1/T2 ratio compared to patients with stable function. Thus, the T1/T2 ratio largely parallels the fall in IL-10/TNF-α ratio we previously identified. Now using T1/T2 ratio as a marker for Breg activity, we show that Bregs may serve as a prognostic marker for long-term allograft outcome.

Within the TrBs, T1 cells represent the most immature bone marrow emigrants (15, 16, 20, 42, 43). It is known that T1 and T2 cells are tightly regulated by BCR and CD40-CD40L-mediated survival signals (18, 19). Altered negative selection of T1 cells is felt to promote self-reactivity and antibody-mediated autoimmune disease (15, 18, 24, 43). However, our data now demonstrate that T1 cells exhibit a highly anti-inflammatory cytokine profile, suggesting that they function as Bregs and appear to be highly sensitive markers for graft decline. Future studies will be required to determine whether

the changes in T1 cell number reflect altered survival versus release from the marrow, and whether altered cytokine expression reflects differences in activation cues as opposed to differentiation/survival of distinct subpopulations. T1 and T2 subsets were identified by their relative expression of the surface markers CD24 and CD38, as is standard in the field (17, 24).

Chronic allograft loss resulting from ongoing subclinical immunological damage remains a major problem affecting transplant outcomes (7). In patients at risk for immunological reactivity, aggressive immunosuppression may improve allograft survival. At the same time, there has been a concerted effort in the field to minimize immunosuppression to reduce side effects. Unfortunately, current clinical parameters cannot sufficiently risk stratify patients to achieve this. Using protocol biopsies, studies reveal that subclinical rejection occurs in up to 30% of patients in the first year, and treatment improves outcomes (44, 45). Despite this, protocol biopsies are not commonly employed , due to the added risks, costs, and unresolved issues such as the frequency and timing of repeat biopsies (8). It is therefore essential to develop noninvasive markers that will allow risk-stratification. This is especially true in the large majority of patients with stable function late after transplantation, when protocol biopsies are not performed.

Several biomarkers have been shown to predict clinical acute rejection, including CD3 and IP-10 mRNA in urine, expression of a 17-gene set in peripheral blood (46, 47) and measurement of urinary chemokines such as CCL2 and CXCL9 (48-51). Clinical parameters such as elevated serum creatinine at 1 year or early low-grade proteinuria, have limited sensitivity and specificity for predicting late allograft loss (52-57). Although the above markers show promise (48-51), they need to be prospectively validated and the influence of confounders (e.g. baseline GFR, DSA and immunosuppressive drug levels) addressed before their clinical applicability can be assessed.

We now showed that the T1/T2 ratio at the time of for-cause biopsies is an independent predictor of allograft deterioration over five years. Allograft deterioration defined as halving of eGFR or dialysis dependency is an established surrogate for the progression of kidney disease (58-60). Moreover, we have analyzed death censored graft loss and ΔeGFR (change in eGFR) separately for all the groups of patients, showing that the findings having a low T1/T2 ratio is associated with worse renal function

regardless of which cut-off or parameter is used. . Especially, in the subset with rejection, 100% with a low ratio exhibited allograft deterioration compared to 40% with a high ratio. While patients with a low T1/T2 ratio had worse histological scores, it is noteworthy histology alone was not predictive of allograft deterioration. Our findings are in general agreement with recent observations that a low transitional B cell number is associated with renal transplant rejection (61, 62). But our data adds to the current body of literature as these previous observations were on the incidence of early rejection episodes and long-term outcomes were not assessed.

Use of the T1/T2 ratio was validated prospectively in an independent set of clinically quiescent patients. The T1/T2 ratio outperformed current clinical standards, including DSA and eGFR in predicting allograft dysfunction, and it is independent of these key confounders. The results from the validation set are striking in that T1/T2 ratio identifies a group, comprising nearly half of the patients, who have a very low risk for graft deterioration and might safely tolerate reduced immunosuppression. In contrast, the other half of the patients has a low T1/T2 ratio and despite apparent quiescence are at substantial risk for developing allograft dysfunction and 25% will lose their kidneys in 5 years. This group may benefit from intensification of therapy and invasive monitoring. We have shown that the T1/T2 ratio serves as a predictive biomarker over the ensuing 5 years both in stable patients two years post-transplant, and also in patients undergoing for-cause renal biopsy from 3-12 years post-transplant. This implies that the exact timing of the T1/T2 ratio is unlikely to be important. Of course serial sampling is required to truly determine how the ratio varies in individual patients over time. Notwithstanding, our results show that when measured in the two contexts noted above, the T1/T2 ratio serves as a valid biomarker for subsequent renal function. This is further supported by the findings of Shabir et al. who showed that in the early peri-transplant period, a increase in the number of TrB cells were associate with decreased incidence of rejection (61). Whether those patients exhibit worse function on longer follow-up is unknown, however, we are in the process of examining the role of T1/T2 ratio and other parameters as markers for outcome, early in the post-transplant course. Clearly, the T1/T2 ratio as a predictive marker for renal allograft

function needs to be confirmed in additional and larger patient cohorts and further trials will be required to test the clinical implications.

It is possible that some of the patients in our validation set with apparently 'stable' grafts at two years may already have subclinical rejection and/or chronic damage. However, routine protocol biopsies in stable patients, late after transplantation is not standard of care. By risk stratifying patients, the T1/T2 ratio may identify patients who need such invasive monitoring at a time when therapy might have an impact. It is noteworthy that in patients with late rejection, the T1/T2 ratio outperforms renal allograft biopsies in predicting subsequent allograft damage. Allograft histology detects current inflammation and response to previous injury at a snapshot in time. Our data suggest that T1/T2 ratio is a much more sensitive marker for the risk of subsequent deterioration in patients with late rejection. Although tacrolimus levels were comparable between patients with high and low ratio, T1/T2 ratio seems to be influenced by the usage of maintenance corticosteroids. This was in agreement with our previous observation that the B cell cytokine expression is influenced by the use of corticosteroids. (10). However, we have shown that T1/T2 ratio was associated with graft deterioration whether or not patients were on maintenance corticosteroids (Fig-S). Furthermore, alemtuzumab induction leads to a B cell phenotype characterized by more transitional and naïve B cells. In line with this observation, patients with alemtuzumab induction had a higher T1/T2 ratio than those with basiliximab. However, irrespective of the induction therapy, more patients with a low T1/T2 ratio had graft deterioration suggesting that this marker could be used in patients treated by both these induction regimens.

In conclusion, we have shown that TrB are comprised of T1 and T2 cells that are phenotypically and functionally distinct. The T1 cells, in particular, exhibit the most regulatory cytokine profile of all B cells. The relative loss of T1 cells in the setting of late allograft dysfunction leads to a fall in T1/T2 ratio. Moreover, the T1/T2 ratio has been tested and validated as a marker for late graft deterioration in renal transplant recipients in this small phase-2 biomarker study (63).

Methods:

Study participants:

PBMCs were collected from healthy volunteers and renal transplant recipients after obtaining informed consent according to the Declaration of Helsinki. The study was approved by the Regional Ethics Committee (Ref No: 08/H1311/41; 06/Q1206/64).

Renal transplant recipients followed in our out-patient clinic who developed proteinuria of 0.5g/d assessed by urine protein/creatinine ratio on three consecutive random urine samples and/or deteriorating function (change in 1/creatinine over one year) were advised to undergo for-cause allograft biopsy (53, 54, 64, 65). 45 consecutive patients agreeing to undergo biopsy and participate were studied. Biopsies were scored according to the BANFF-2007 criteria (66).

Validation Group: Adult renal transplant patients enrolled in a randomized controlled trial *(European clinical trials database number: 2006-000830-11)* comparing alemtuzumab induction and maintenance tacrolimus monotherapy (n=51) with basiliximab induction and maintenance tacrolimus and MMF therapy (n=46) with low-medium immunological risk (absence of preformed DSA, previous early (<3 months) immunological graft loss, and $\leq$2 HLA-DR mismatch) agreed to participate. PBMC were collected for B cell analysis 24.9 (95% CI 23–27) months following transplantation and patients were followed for 5 additional years.

Outcome measures: All patients were followed-up for 5 years from the time of B subset analysis. The primary outcome analyzed was allograft deterioration as defined by halving of eGFR or death censored graft loss (58-60). Change in renal function was measured by ΔeGFR over the five years of follow-up. For the sake of eGFR calculations, patients with graft loss were ascribed to an eGFR value of '0'. Death censored graft loss was also examined separately.

Immunosuppression:

Patients either received Alemtuzumab (Day-0: Alemtuzumab-30mg, Methylprednisolone-1g with Tacrolimus monotherapy (trough levels: 9-14 ng/mL for the first 3 months; 4-9 ng/mL thereafter)) or

Basiliximab (day-0 and day-4-basiliximab-20 mg; methylprednisolone-1g on d0 with tacrolimus (target levels as above) and MMF (1.5 g/day) therapy). No routine maintenance steroids were given.

Antibodies and flow cytometry:

All experiments were performed on fresh cells on the same day of blood sampling. Anti-human mAbs included PerCP-CD19(BD), PerCP-Cy5.5-CD20(Ebioscience), APC-CD20(BD), PE-CD24(Ebioscience), FITC-CD24(BD), APC-CD38(Ebioscience), FITC-CD27(Ebioscience), PE-CD27(BD), APC-CD27(BD), FITC-CD5(BD), PE-CD1d(BD Bioscience), APC-CD10(Ebioscience), PE-IgD(BD), FITC-IgM(BD), PE-CD86(BD), , FITC-CD40(BD), PE-IL10(BD), AF488-TNF-α(BD). B cells in whole blood were stained after RBC-lysis using optimized antibody concentrations, as per manufacturer's instructions and analyzed on BD-FACS Calibur or BD-LSR-Fortessa Flow cytometers. TrB subsets were defined by the relative surface expression of CD24 and CD38 within the $CD19+CD27^-$ subset (9). Whilst the distinction between T1 (CD24+++CD38+++) and T2 cells (CD24++CD38++) is somewhat arbitrary using known markers, earlier studies defined cut-offs based on initial B cell repopulation by T1 cells followed by T2 cells in patients treated with Rituximab (17, 25) or in healthy volunteers (24). Such a gating strategy, though arbitrary, when applied to healthy subjects, lead to an approximately 30:70 ratio (T1:T2) and showed that the gated T1 cells have significantly stronger expression of CD10 and IgM when compared to either the T2 or naïve B cell subsets, consistent with their being the earliest bone marrow emigrants (24, 67). Of all the markers assessed, CD24 and CD38 provided the best differentiation of these populations. Also this gating strategy demonstrated marked differences in the cytokine polarization of T1, T2 and naïve B subsets. These gates were established in healthy volunteers and the same gates were objectively applied to the patients. Furthermore it should be noted that in most patients when there is a fall in the T1/T2 ratio, the T1 population is virtually not detected in the peripheral blood (see Fig S2). Such gating was established in healthy subjects and then the same settings were applied in a non-biased manner to patients.

DSA detection

Serum collected at time of biopsy (training set) or B cell analysis (validation set) was analyzed for the presence of HLA-specific antibodies by Luminex technology. A normalized median fluorescent intensity (MFI) of >1000 was considered positive on the basis of local validation.

Cell culture-cytokine detection

Human PBMCs were prepared by ficoll-paque density gradient centrifugation. B lymphocytes were isolated from PBMCs by by positive selection with anti-CD19 magnetic beads (Miltenyi Biotec). Enriched B cells were suspended ($1X10^6$ cells/ml) in RPMI 1640 supplemented with 10% FCS and 1% Penicillin-Streptomycin-L-glutamine and stimulated with CpG-ODN2006 (10µg/ml) and CD40L (1µg/ml), as described (10). PMA (50ng/ml), Ionomycin (1µg/ml), and Brefeldin A (1µl/ml) were added for the last 5h of culture. Cultured cells were stained with antibodies to CD20, CD24, CD38 and CD27, fixed, permeabilized (Cytofix/Cytoperm kit; BD Biosciences) and then stained for intracellular cytokines.

Statistical analysis:

Statistical analysis was performed by SPSS. Categorical variables were compared using Chi-square test and continuous variables using Mann-Whitney U test for data with skewed distribution and independent sample student t-test for normally distributed data. Multiple groups were analyzed by one-way ANOVA with post-hoc Dunnet test used as adjustment for multiple comparisons. ROC curve analysis and comparison of the AUC between the ROC curves was performed as per Delong et al. (68). The cut-off value for T1/T2 ratio determined by optimal sensitivity and specificity on ROC curve analysis and used to categorize patients as having a high or low ratio. Graft survival/stability was assessed by Kaplan-Meier method and curves for high and low T/T2 ratio groups compared by Log rank test. A multivariate Cox proportional hazards model was used to assess the independence of T1/T2 ratio from other parameters. A p-value <0.05 was considered statistically significant.

Conflict of Interest: None of the authors declare any conflict of interest in relation to this submission

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Table-1: Demographic characteristics-patients with late for-cause biopsy

Variable	Total study population	T1/T2 ratio<0.17	T1/T2 ratio>0.17	P-value
Number	45	24	21	
Recipient age	51 (14.3)	48.6 (14.0)	54.3 (14.0)	0.3
Recipient gender-male%	71%	65.4%	80%	0.2
Cause of ESRD				0.3
Diabetes & hypertension	6.6%	4.1%	9.5%	
Glomerular	28.9%	25%	33%	
Inherited	13.3%	12.5%	14.3%	
Other	51%	58.3%	42.8%	
Time since transplant	99 (64)	110 (75)	82 (39)	0.3
Donor age	42.5 (15.5)	41 (14.6)	43.2 (16.2)	0.6
Donor type				0.9
Live	14%	12%	19%	
DBD	67%	69%	62%	
DCD	19%	19%	19%	
Donor cause of death				0.2
Trauma	24%	26.5%	21%	
Vascular	55%	47%	64%	
Others	21%	26.5%	15%	
HLA mismatches	**2.25 (1.4)**	**2.8 (1.3)**	**1.7 (1.2)**	**0.01**
Induction				0.9
Alemtuzumab	4.4%	4%	5%	
Basiliximab	95.6%	9%	95%	
Maintenance Immunosuppression				
Cyclosporine/Tacrolimus	86%	81%	89.5%	
Mycophenolate/Azathioprine	77%	81%	74%	
Prednisolone	**35.5%**	**52%**	**11%**	**0.005**
Baseline eGFR	35.3 (15)	33.3 (17)	32.9 (15)	0.7
Allograft rejection at baseline	**55.5%**	**87%**	**24%**	**<0.0001**

For all continuous variables means are presented with standard deviation in the parenthesis. For all categorical variables, data is presented as percentage of total

*Groups compared by either independent samples t test or Mann Whitney U test as appropriate

**ESRD End Stage Renal Disease

***Inherited: includes polycystic kidney disease and congenital or inherited renal or systemic diseases like Fabry's disease

****Others: includes reflux disease, pyelonephritis, stones and unknown causes

$ DBD Donation after brain Death

$$ DCD Donation after Cardiac Death

Vascular: includes sub-arachnoid hemorrhage, stroke or any cause of cerebral ischemia

Others: includes live grafts along with miscellaneous causes of donor death including septicemia etc.

Table-2: Demographic characteristics-validation set

Variable	Total study population	T1/T2 ratio<0.17	T1/T2 ratio>0.17	P-value*
Number	97	41	56	
Recipient age	46.9 (14.6)	50.2 (12.2)	45.5 (14.7)	0.1
Recipient gender-male%	78.4%	70.7%	83.9%	0.1
Cause of ESRD**				0.9
Diabetes & hypertension	21.6%	19.5%	23.2%	
Glomerular	23.7%	26.8%	21.4%	
Inherited***	18.6%	17.1%	19.6%	
Other****	36.1%	36.6%	35.8%	
Time since transplant	24.9 (11)	20.9 (10.4)	27.6 (10.7)	**0.003**
Donor age	42.6 (16.1)	42.6 (16.7)	42.7 (15.7)	0.99
Donor type				0.6
Live	18.6%	14.7%	21.4%	
DBD[$]	52.6%	58.5%	48.2%	
DCD[$$]	28.8%	26.8%	30.4%	
Donor cause of death				0.9
Trauma	9.3%	9.8%	8.9%	
Vascular[#]	56.7%	58.5%	55.4%	
Others[##]	34%	32%	35.7%	
HLA mismatches	2.54 (1.4)	2.34 (1.3)	2.67 (1.5)	0.3
Induction				0.06
Alemtuzumab	52.6%	41.5%	60.7%	
Basiliximab	47.4%	58.5%	39.3%	
Maintenance Immunosuppression				0.4
Cyclosporine/Tacrolimus	100%	100%	100%	
Mycophenolate/Azathioprine	52.6%	65.8%	47.1%	
Prednisolone	17.5%	19.5%	16.1%	0.7
Baseline eGFR	50.2 (18.2)	45.9 (19.9)	53.1 (16.3)	0.06
Allograft rejection at baseline	17.5%	19.5%	16.1%	0.7

For all continuous variables means are presented with standard deviation in the parenthesis. For all categorical variables, data is presented as percentage of total

*Groups compared by either independent samples t test or Mann Whitney U test as appropriate

**ESRD End Stage Renal Disease

***Inherited: includes polycystic kidney disease and congenital or inherited renal or systemic diseases like Fabry's disease

****Others: includes reflux disease, pyelonephritis, stones and unknown causes

$ DBD Donation after brain Death

$$ DCD Donation after Cardiac Death

Vascular: includes sub-arachnoid hemorrhage, stroke or any cause of cerebral ischemia

Others: includes live grafts along with miscellaneous causes of donor death including septicemia etc.

Table-3: Multivariate analysis

	Multivariate Analysis	
	HR (95% CI)	P value
Training set		
DSA	0.9 (0.4-2.3)	0.8
Baseline GFR (per 1ml/min change)	0.97 (0.94-1.01)	0.09
Rejection on biopsy	3.01 (0.9-10.4)	0.08
TrB T1/T2 ratio (per each 0.1 increase in the ratio)	**0.6(0.4-0.9)**	**0.02**
Validation set		
DSA	**3.6 (1.1-12.1)**	**0.04**
Baseline eGFR (per 1ml/min change)	0.97 (0.93-1.01)	0.1
Prior rejection	1.2 (0.3-4.7)	0.8
TrB T1/T2 ratio (per each 0.1 increase in the ratio)	**0.35 (0.14-0.9)**	**0.02**

HR hazard ratio; 95% CI 95% confidence interval; DSA donor specific antibody

Figure Legends:

Fig 1: Phenotypic characterization of Transitional B subsets

(A). Human B cells are classified into transitional T1, T2 and mature naïve based on the surface expression of CD19, CD24, CD38 and CD27. **(B).** When the three subsets of cells are analyzed for the expression of other markers, T1 cells are characterized as $CD20^{hi}$ IgM^{hi} IgD^{lo} $CD10^{hi}$ $CD5^{hi}$ $CD1d^{hi}$ $CD86^{hi}$; T2 cells as $CD20^{int}$ IgM^{lo} IgD^{hi} $CD10^{lo}$ $CD5^{+}$ $CD1d^{hi}$ $CD86^{lo}$ and mature naïve B cells as $CD20^{lo}$ IgM^{lo} IgD^{hi} $CD10^{-}$ $CD5^{lo}$ $CD1d^{hi}$ $CD86^{hi}$. Magnetic bead enriched CD19+ B cells are stimulated with CpG and CD40L for 48hrs with the addition of PIB in the last 5 hrs of culture. **(C).** Bar graphs compare the MFI values for CD10, CD5, IgM, CD20, CD86, IgD and CD1d across T1, T2 and naïve B subsets. **(D).** Scatterplots representing the IL-10 and TNF-α expression after intracellular staining within T1, T2 and mature subsets in a representative healthy volunteer. **(E).** Cumulative results of the percentage of IL10 positive cells within each subset (n=15, *p<0.0001 mature subset

compared to either T1 or T2). **(F).** Cumulative results of the percentage of TNF-α positive cells within each subset (n=15, **p=0.09, T1 vs. T2; ***P<0.0001, T1 vs. Mature & p=0.02, T2 vs. mature). **(G).** Cumulative results of the IL-10/ TNF-α ratio within each subset (n=15, # T1 vs. T2, p<0.0001; ## T1 vs. mature, p<0.0001; T2 vs. mature, p=0.0001). **(H).** Cumulative results from 5 healthy volunteers comparing IL-10/TNF-α ratio between T1, T2 and naïve B subsets when analyzed at 12, 24 and 48hrs. **(I).** Both IL-10 and TNF-α are analyzed by dual cytokine staining (cumulative results from 5 individual experiments) (*T1 vs. mature naïve, p<0.0001; **T1 vs. mature naïve, P<0.0001, T1 vs. T2, p=0.04). The bars in each graphic represent mean whilst the error bars represent SEM. Statistical analysis was by one way ANOVA with Dunnet's post hoc correction for multiple comparisons.

Fig 2: Comparison of B cells, TrB subsets, T1/T2 ratio at for-cause biopsy in patients who remain stable vs. those with dysfunction on follow-up

Patients were divided into those with long-term stable function [S] and those with progressive functional decline [D] over a 5yr follow-up from the time of the for-cause biopsy. Groups S and D were compared (S, n=17; D, n=28) for the absolute number of B cells, TrBs and TrB subsets. Comparison of the absolute B cell number (**A**), percentage of TrBs **(B)**, the absolute numbers of TrBs **(C)**, the absolute numbers of T1 cells **(D)**, the absolute numbers of T2 cells **(E)** and T1/T2 ratio **(F)** between the two groups of patients are shown. Individual values for each patient were shown along with mean and error bars that represent SEM. The groups were compared by Mann-Whitney U test.

Fig 3: T1/T2 ratio can help risk stratify patients at for-cause biopsy and may be a useful biomarker

(A) Comparison of T1/T2 ratio in patients with stable vs. graft dysfunction that shows a very small overlap and the resultant sensitivity, specificity, positive and negative predictive value at the optimal cut-off of 0.17 defined by the AU-ROC. **(B)** ROC curve analysis for T1/T2 ratio as a marker for allograft functional decline defined as either halving of eGFR or graft loss. **(C)** Kaplan Meier survival

curves for allograft functional decline over 5 years from for-cause biopsy, comparing patients with T/T2 ratio>0.17 (high, n=21) or ≤0.17 (low, n=24). **(D)** Comparison of change in eGFR (Δ eGFR) from the time of B cell analysis to 5 year follow-up in patients with high (n=21) or low ratio (n=24). **(E)** Kaplan Meier survival analysis was repeated by analyzing death-censored graft survival and **(F)** comparing only those patients with histological evidence of rejection on the biopsy (high ratio, n=5; low ratio n=20). **(G)** Comparison of change in eGFR from the time of B cell analysis to 5 year follow-up in patients with rejection (high vs. low ratio). Survival curves were censored for death and compared by Log rank test. Each bar in the bar graphs represents mean ± SEM and groups were compared by Mann Whitney U test.

Fig 4: Analysis of the Banff histological lesions in patients with rejection in relation to the T1/T2 ratio (A). Comparison of the Banff scores in the rejection group (n=25) with a low (≤0.17) or high (>0.17) T1/T2 ratio. The top panel shows stacked bar charts that compare the proportion of patients with a Banff score of '0', '1' or '>1' for each of the lesions. The middle panel shows the mean Banff scores with error bars representing the SEM. (B). ROC-AUCs are shown for the T1/T2 ratio in the group of patients with rejection for the prediction of allograft dysfunction along with derived histological indices of inflammation (t+I score, g+ptc score) and chronic damage (ct+ci score). The proportions were compared by the Chi square test while all the means were compared by the Mann Whitney U test.

Fig 5: Comparison of B cells, TrB subsets, T1/T2 ratio in patients who remain stable vs. those with dysfunction on follow-up: validation set

Patients were divided into those with long-term stable function [S] and those with progressive functional decline [D] over a 5yr follow-up from the time of the B cell subset analysis. Groups S and D were compared (S, n=85; D, n=12) for the absolute number of B cells, TrBs and TrB subsets. Comparison of the absolute B cell number (**A**), percentage of TrBs **(B)**, the absolute numbers of TrBs **(C)**, the absolute numbers of T1 cells **(D)**, the absolute numbers of T2 cells **(E)** and T1/T2 ratio **(F)** between the two groups of patients are shown. Individual values for each patient were shown along with mean and error bars that represent SEM. The groups were compared by Mann-Whitney U test.

Fig 6: T1/T2 ratio can help risk stratify clinically quiescent patients: results from the validation set

(A) ROC curve analysis for T1/T2 ratio as a marker for allograft functional decline defined as either halving of eGFR or graft loss. **(B)** Comparison of T1/T2 ratio in patients with stable vs. graft dysfunction that shows the degree of overlap and the resultant sensitivity, specificity, positive and negative predictive value at the optimal cut-off of 0.17 defined by the AU-ROC. Patients were divided into those with high (n=56) or low (n=41) T1/T2 ratio based on the cut-off value of 0.17 **(C)** Kaplan Meier survival curves for allograft functional decline comparing patients with high or low ratio. **(D)** Kaplan Meier survival curves for death censored graft loss comparing patients with high or low ratio. **(E)** Comparison of change in eGFR (Δ eGFR) from the time of B cell analysis to 5 year follow-up in patients with high or low ratio. Survival curves were compared by Log rank test. Bar charts represent mean ± SEM and groups compared by Mann Whitney U test.

Fig 7: T1/T2 ratio as a prognostic biomarker-comparison with DSA and eGFR: ROC curve analysis

ROC curves with optimal sensitivity and specificity for eGFR at for-cause biopsy, DSA positivity and T1/T2 ratio in patients with late for-cause biopsy are shown in Fig A. ROC curve analysis with optimal sensitivity and specificity for baseline eGFR, DSA positivity and T1/T2 ratio in clinically quiescent patients with late for-cause biopsy is shown in Fig B.

Supplementary materials:

Fig-S1: Comparison of IL-10, TNF-a and IL-10/TNF-a ratio between T1 and T2 subsets in renal transplant patients

45 patients who underwent a for-cause biopsy had their TrB cells analyzed for the expression of IL-10, TNF-a and IL-10/TNF-a ratio at the time of the for-cause biopsy before any therapeutic

intervention. The respective cytokines were compared between the T1 and T2 populations. The groups were compared by Mann Whitney-U test.

Fig S2: Gating strategy for T1 and T2 cells and representative patients with high and low T1/T2 ratio

Cells were analysed from B cell gate (CD19+)

Fig S3: Comparison of TrB IL-10: TNF-α ratio in Stable patients (S) vs allograft deterioration (D) after a for-cause biopsy

$p<0.0001$, Mann Whitney U test

Fig S4: Comparison of Allograft dysfunction in patients with either low or high T1/T2 ratio stratified by the immunosuppression regimen: validation set

Patients were divided into those with high or low T1/T2 ratio at a cut off of 0.17. Here rate of allograft dysfunction was analysed by Kaplan Meier method in patients who received alemtuzumab induction with tacrolimus monotherapy (A) and change in eGFR compared (B). The analysis was repeated in patients who received basiliximab induction with tacrolimus and MMF therapy (C and D). Death censored graft survival with stable function was analyzed from the date of B cell subset analysis. Curves were compared by Log rank test. The mean change in eGFR was calculated from the date of B cell analysis for 5 years. The groups were compared by Mann Whitney U test.

Fig S5: Correlation between eGFR and the TrB subsets

Scatter plots demonstrating the correlation between T1/T2 ratio, absolute number of TrB, T1 and T2 subsets in the original patient cohort and the validation cohorts. Correlations analyzed by the Spearman's correlation coefficient

Fig S6: Tacrolimus levels: training and validation sets

In the test set, tacrolimus levels are shown at 8 months and 4 months prior to the analysis of T1/T2 ratio and at the time of the analysis. In the validation set, tacrolimus levels are shown from the time of transplantation till 36 months post transplantation. T1/T2 analysis was performed between 12 to 24 months post-transplantation.

Fig S7: Kaplan Meier Analysis: Allograft deterioration and corticosteroid usage

In the test set, more patients in the low T1/T2 ratio group were on maintenance corticosteroids. To examine the influence of corticosteroids on the ability of T1/T2 ratio to predict graft deterioration, Kaplan Meier analysis was performed and the survival curves were compared between the low and high ratio groups in those with and without maintenance steroids. The curves were compared by Log Rank test.

Table S1: Details of rejection episodes-validation set

Allograft biopsies were scored as per the Banff 2007 criteria