Reduced human transitional B cell T1/T2 ratio is associated with subsequent deterioration in renal allograft function

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Human transitional B cells express relatively high IL-10 and low TNF-α levels, which correlate with B regulatory activity *in vitro*. Herein, we aim to further define B regulatory phenotype and determine whether B regulatory activity can serve as a prognostic marker for renal allograft dysfunction (graft loss or 2-fold fall in estimated glomerular filtration rate). Transitional B cells can be divided into T1 and T2 subsets based on surface phenotype. T1 cells express a significantly higher ratio of IL-10 to TNF-α than T2 cells or other B subsets. When analyzed in 45 kidney transplant recipients 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 kidney transplant recipients 2 years after transplant. Again, the T1/T2 ratio was strongly and independently associated with allograft dysfunction over the ensuing 5 years. In these clinically quiescent patients, a low T1/T2 ratio identified a 41-patient subgroup in which 35% developed allograft dysfunction, with 25% losing their allografts. However, none of the 56 patients with a high ratio developed graft dysfunction. In both the initial study and validation groups, the T1/T2 ratio was a much stronger predictor of graft dysfunction than donor-specific antibodies or the estimated glomerular filtration rate. Thus, the T1/T2 ratio, a relative measure of expressing an anti-inflammatory cytokine profile, is a novel prognostic marker that might inform individualized immunosuppression.

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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% to 35% of recipients lose their transplants[2,3] with a resultant 3-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 after 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, noninvasive 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 (TrBs) represent a critical developmental stage between immature B cells in the bone marrow and mature B cells in the periphery.[9] In humans, TrBs 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 in the context of clinical transplantation tolerance.[12–14] We recently showed that various B cell subsets in peripheral blood of healthy human subjects express IL-10. However, TrBs express less pro-inflammatory TNF-α, resulting in a higher ratio of IL-10 to TNF-α than do other B subsets, and this correlated with suppressive function *in vitro*.[10] Moreover, TrBs from renal transplant recipients with rejection specifically exhibited a decreased ratio of IL-10 to TNF-α and loss of *in vitro* Breg activity, suggesting that TrB cells or cytokines might serve as a marker for renal allograft outcomes.

TrBs are comprised of less mature T1 cells and more mature T2 cells.[15,16] In humans, both T1 and T2 TrBs are found in the peripheral blood and spleen.[17] While T1 and T2 TrBs 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 ratio of

IL-10 to TNF-α than the T2 subset. Moreover, a reduced T1/T2 ratio at the time of for-cause biopsy was associated with allograft deterioration over subsequent 5 years. Importantly, the T1/T2 ratio was prospectively validated in a distinct cohort of clinically quiescent patients 2 years after 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

TrBs 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^{-} (Figure 1a).[17] While 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] When applied to healthy subjects, these gates lead to an approximately 25:75 ratio (T1/T2). Such gating was established in healthy subjects, and then the same settings were applied in a nonbiased manner to patients. Compared to T2 cells, T1 cells

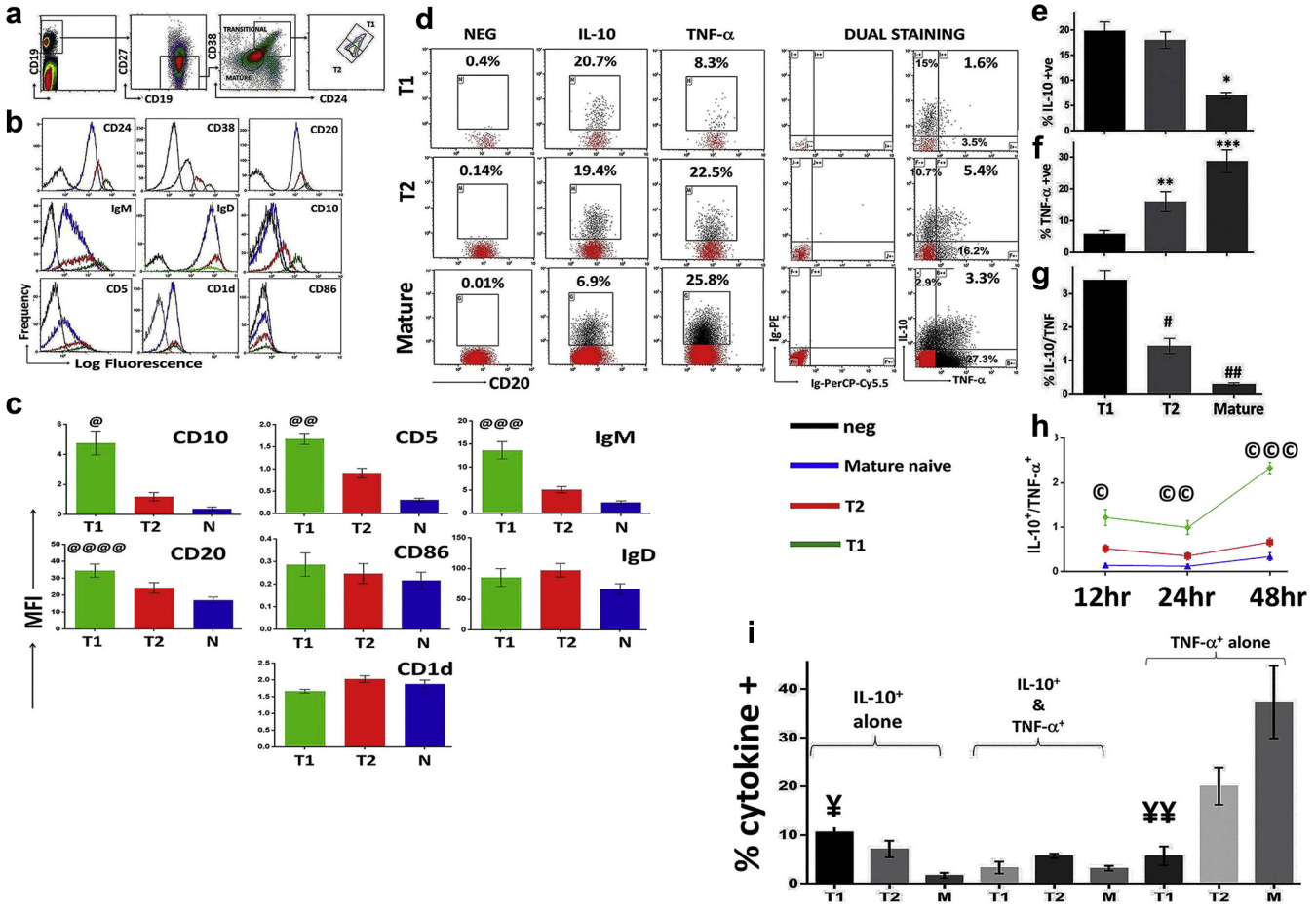

Figure 1 | Phenotypic characterization of Transitional B subsets. (**a**) Human B cells are classified into transitional T1, T2, and mature naïve based on the surface expression of CD19, CD24, CD38, and CD27. (**b**) When the 3 subsets of cells are analyzed for the expression of other markers, T1 cells 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 48 h with the addition of PIB in the last 5 h of culture. (**c**) Bar graphs compare the mean fluorescence intensity (MFI) values for CD10, CD5, IgM, CD20, CD86, IgD, and CD1d across T1, T2, and naïve B subsets. (**d**) Scatter plots 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+ 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; and $P = 0.02$, T2 vs. mature). (**g**) Cumulative results of the ratio of IL-10 to TNF-α within each subset (n = 15, $P < 0.0001$, # T1 vs. T2; $P < 0.0001$, ## T1 vs. mature; and $P = 0.0001$, T2 vs. mature). (**h**) Cumulative results from 5 healthy volunteers comparing the ratio of IL-10 to TNF-α between T1, T2, and naïve B subsets when analyzed at 12, 24, and 48 h. (**i**) Both IL-10 and TNF-α are analyzed by dual cytokine staining (cumulative results from 5 individual experiments) ($P < 0.0001$, ¥T1 vs. mature naïve; $P < 0.0001$, ¥¥T1 vs. mature naïve; and $P = 0.04$, T1 vs. T2). The bars in each graphic represent mean, while the error bars represent SEM. Statistical analysis was by one way ANOVA with Dunnet's *post hoc* correction for multiple comparisons.