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Regulatory And Transitional B Cells: Potential Biomarkers and Therapeutic Targets in Organ Transplantation

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Abstract

Purpose of the review: Regulatory B cells (Bregs) play a prominent role in various disease settings. While progress has been hindered by the lack of a specific Breg marker, new findings highlight their role modulating the alloimmune response and promoting allograft survival.

Recent findings: Herein, we focus on the recent advances in Breg biology and their role in transplantation. We review studies showing that TIM1 is an inclusive and functional Breg marker in mice that may have human relevance. We highlight the utility of the B cell IL-10/TNFα ratio in identifying underlying immunological reactivity and predicting clinical outcomes in kidney transplantation. This may identify patients requiring more immunosuppression and provide insight into potential therapeutic approaches that can modulate the Breg:B effector cell (Beff) balance.

Summary: Emerging data support Bregs as potent modulators of immune responses in humans. Their ability to promote allograft survival must await development of approaches to expand Bregs in vitro/in vivo. The low IL-10/TNFα ratio reflecting decreased Breg/Beff balance, predicts acute rejection and poorer outcomes in renal transplantation. It remains to be determined whether this paradigm can be extended to other allografts and whether therapy aiming to correct the relative deficiency of Bregs will improve outcomes.

Keywords

regulatory B cells; biomarkers; rejection; effector B cells

Introduction:

Transplantation is the treatment of choice for patients with end-stage organ failure of vital organs such as kidney, liver, heart, and lung(1-3). Though improved immunosuppression has raised one-year graft survival above 90%, depending on the organ type, ~40-70% of the allografts are lost by 10 years(1, 4-6). Long-term allograft survival allografts is often perturbed by on-going immunological injury that leads to premature scarring and allograft loss(4, 7-10). Premature graft loss exacerbates organ shortage and is associated

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with increased mortality, poorer quality of life, and a disproportionate increase in health care costs(11-13). Thus, improving long-term allograft survival is a major unmet need in the field. In this regard, there have been major efforts to identify relevant immunomodulatory cells that might be targeted to improve long-term survival. Mounting evidence suggests that regulatory B cells (Bregs) play a key role in allograft survival. Moreover, Bregs appear to provide insight into an individual patient's immunological reactivity, allowing early risk stratification and pre-emptive modification of immunosuppression according to individual risk. In this review, we will provide an overview of Bregs, their role in transplantation, and discuss their utility as risk stratification tools in clinical transplantation.

Murine and human Bregs:

Bregs were first identified as IL-10-expressing B cells that inhibited spontaneous colitis in T cell receptor-α knock-out mice(14). Subsequently, Bregs were shown to inhibit allograft rejection as well as autoimmunity, infectious, and tumor immunity, largely in an IL-10-dependent manner(15-21). Thus, while lacking a specific marker, Bregs have been frequently identified by their IL-10 expression(22). However, very few splenic B cells in naïve or even immunized mice (1-2%) express IL-10 without *in vitro* stimulation(23, 24). Even then, IL-10+ B cells are relatively rare, comprising only ~5% of all B cells in IL-10-reporter mice(24). Their frequency varies amongst canonical B cell subsets. Different subsets appear to be enriched for IL-10 expression and can adoptively transfer tolerance depending on the disease model. Even in these enriched subsets, IL-10^+ B cells comprise a minority (e.g., 10-15%)(23, 25, 26). ~30% of all B cell IL-10 is expressed by marginal zone B cells and their precursors. Plasma cells are highly enriched for lL-10 expression (~60%) but comprise <1% of all splenocytes. Thus, plasma cells account for less than 30% of total B cell IL-10(24). In contrast, <1% of Follicular (FO) B cells express IL-10. Yet, because FoB comprise ~55% of splenic B cells, they account for ~30% of B cell IL-10(24). Given their very low frequency of IL-10 expression, transferred FO cells are unlikely to show regulatory activity. Therefore, defining Bregs based on the frequency of IL-10+ cells in a given B cell subset as opposed to the contribution of the specific subset to the overall frequency of IL-10+ B cells poses a challenge to our understanding of Bregs in various disease models, highlighting the need for a specific Breg marker(24). Finally, a number of reports have identified Bregs that appear to utilize different immunoregulatory molecules including IL-35, TGFβ, TIGIT, PDL-1, FasL and Granzyme B. How these disparate Bregs relate to IL-10-expressing Bregs was unclear.

As in mice, human Bregs are typically identified based on their IL-10 expression following *in vitro* stimulation with mitogens for 24-96 hours(23, 27). In addition to canonical subsets such as $CD24^{hi}$ $CD38^{hi}$ transitional B cells (TrBs), $CD24^{hi}$ $CD27^+$ memory B cells, and plasmablasts, other B cell subsets such as $CD39^{hi}$, $CD1d^{hi}CD25^{hi}CD71^{hi}CD73^-$, $TNFR2^+$, and $CD271^+CD431^+CD11b^+$ B cells are enriched for IL-10+ cells, depending on the nature and duration of the *in vitro* stimulus(28-32). However, prolonged *in vitro* stimulation can significantly alter the phenotype of these cells, limiting our understanding of their true physiological significance. Taken together, despite the recognition for the prominent role of Bregs in various disease settings, further progress has been thwarted by the lack of a broad and inclusive marker or a key transcription factor.

TIM-1 (T cell immunoglobulin and mucin family-1) as an inclusive and functional marker for Bregs:

We identified B cells as the target of a tolerogenic anti-TIM-1 monoclonal antibody (RMT1-10) that ameliorates experimental autoimmune encephalomyelitis (EAE) and prolongs allograft survival in mice(33). In mice, 10-15% of B cells express TIM-1 and TIM-1$^+$ B cells are 10-25-fold enriched for IL-10 expression compared to TIM-1− B cells across all canonical B cell subsets. Unlike most other Breg markers, TIM-1 encompasses ~75% all IL-10+ B cells(33). Adoptive transfer of TIM-1+, but not TIM-1−, B cells from alloimmunized hosts promotes islet allograft tolerance in an allospecific manner(33). Moreover, anti-TIM-1 treatment increases TIM-1 and IL-10 expression, resulting in an ~4-fold increase in the frequency and number of IL-10+ B cells, and this is essential for prolongation of islet allograft survival by anti-TIM-1(24). Infusion of apoptotic cells induces IL-10$^+$ Bregs(34). Phosphatidyl serine (PtdS), exposed on the surface of apoptotic cells, is a natural ligand for TIM-1. PtdS, binds to WT TIM-1+ B cells and induces IL-10 expression, but does not bind to, or induce IL-10 in WT TIM-1− B cells or TIM-1+ B cells from mice expressing the TIM-1Δmucin mutant, which exhibit decreased IL-10+ B cells.(35). Taken together, TIM-1 is a major PtdS receptor on B cells and TIM-1 signaling plays a functional role in Breg maintenance and induction. Transcriptional analysis of TIM-1+ and TIM-1− B cells from WT and TIM-1Δmucin mice reveals that TIM-1 not only regulates IL-10, but a range of inhibitory cytokines and co-inhibitory molecules including, IL-10, Ebi3, GITRL, Fgl2, CLTLA-4, Lag3, and TIGIT(19). Furthermore, B cell-specific deletion of TIM-1 results in age-related spontaneous multi-organ lymphocytic infiltration and systemic autoimmunity manifest by EAE-like paralysis, skin lesions, and colitis(19). Deletion of TIGIT in B cells results in a less severe phenotype with lower penetrance of paralytic disease. Taken together, TIM-1 is a specific functional, inclusive marker that may link Bregs that utilize a variety of mechanisms of action.

Blair initially showed that IL-10+ B cells are most enriched within TrBs(29). Moreover, TrBs were more suppressive *in vitro* compared to other canonical B cell subsets, findings corroborated by our group(29, 36). By comparison, TIM-1 expression on human B cells has not been extensively studied. In this regard, Aravena reported that ~5% of human peripheral blood B cells and 15%-35% of the TrBs express TIM-1(37). 90% of TIM-1$^+$ TrBs expressed IL-10, and TIM1$^+$ but not TIM1$^-$ B cells suppressed pro-inflammatory cytokine expression by autologous T cells *in vitro*. Moreover, TIM-1+ B cells were reduced in frequency and lose their *in vitro* suppressive activity in patients with systemic sclerosis(37).

In contrast to the above observations, Hasan reported that a greater proportion of human peripheral blood memory B cells (CD24^{hi}CD27$^+$CD39^{hi}) express IL-10 and PD-L1 compared to TrBs(38). Also, in contrast with studies above, Hasan found that memory B cells (MemBs) were enriched for TIM-1, TIGIT, and granzyme B compared to TrBs(38). Some of this discrepancy in IL-10 expression may relate to the kinetics of *in vitro* stimulation since a greater proportion of TrBs expressed IL-10 24 hours after stimulation (despite minimal TIGIT and TIM-1 expression) compared to memory B cells, while more memory B cells expressed IL-10 after 48 hours. We cannot directly extrapolate which

in vitro conditions best reflect what occurs *in vivo*. Nonetheless, in this study, MemBs were stronger suppressors of autologous T cells *in vitro* than TrBs. Further, this *in vitro* suppressive activity was IL-10, PD-L1, TIGIT, and Granzyme B dependent(38). In addition to inhibiting T cells, TIGIT+ MemBs downregulated expression of co-stimulatory molecules and pro-inflammatory cytokines by immature monocyte-derived dendritic cells (MDDCs) *in vitro.* Such MDDCs were more efficient in suppressing CD4+ T cells *in vitro*. Finally, $TIGIT^+$ MemBs were reduced in kidney and liver allograft recipients with donor specific antibody (DSA) compared to those without DSA(38).

As in murine models, Bregs might form the basis for cell therapy in clinical transplantation. Addressing this, Shankar showed that CD154-stimulation of human B cells for 2 weeks drives a >900-fold expansion of IL-10+ B cells(39). Almost 75% of such expanded B cells express TIM-1 and were shown to suppress CD4+ T cell proliferation in a TIM-1 dependent fashion. Importantly, these expanded B cells prolonged allograft survival in a humanized mouse skin transplant model(39). Furthermore, the authors observed increased frequency of skin Tregs in mice that received expanded human Bregs, echoing findings in mice, where increased Bregs result in increased Tregs(23, 33).These data highlight the potential for *ex vivo* expanded TIM-1+ human Bregs to be the basis for cell therapy. In summary, these diverse studies underscore the potential importance of TIM-1 and TIGIT as functional human Breg markers that may be relevant in autoimmune disease and clinical transplantation.

IL-10/TNFα ratio as a measure for Breg:Beff balance

Thus far, this review has focused on Bregs. Yet, the net modulatory effect of B cells on immune response likely results from the balance of the opposing effects of both Bregs and Beffs. Beffs express a variety of proinflammatory cytokines and strongly promote auto-, allo- and infectious immunity in mice and humans(40-42). This notion is supported in studies of multiple sclerosis (MS)(43, 44). B cells from patients with MS expressed less IL-10 and more pro-inflammatory cytokines such as TNFα, lymphotoxin, and GM-CSF than B cells from healthy controls(43, 44). Further, such B cells failed to suppress T cell proliferation *in vitro*. Importantly, in MS patients who responded clinically to B cell depletion with Rituximab, the reconstituting B cells have a normalized IL-10/TNFα ratio and were now suppressive *in vitro*(44). In clinical transplantation, indirect evidence for an imbalance in Bregs and Beffs emerged when attempts were made to prevent antibody-mediated rejection by depleting B cells in the peri-transplant period. Surprisingly, this led to a marked increase in early acute T cell–mediated rejection in kidney transplant patients, and significantly increased CAV at one year in cardiac transplant recipients(45, 46). The differential responses to B cell depletion in MS versus transplantation might be explained by the fact that B cells in patients with MS express increased TNFα and decreased IL-10 compared to healthy subjects. By depleting all B cells, the predominance of Beffs is disrupted, and the B cell compartment is reconstituted with B cells expressing normal TNFα and IL-10, reflecting normal Breg:Beff balance. In contrast, transplant patients generally lack active autoimmunity, and we hypothesize their balance of Bregs and Beff cells is less skewed. Early B cell depletion may remove Bregs at a critical time early

post-transplantation, and skew re-emerging B cells towards Beff, promoting the alloimmune response.

In mice, we identified TIM-4+ as a marker for B cells expressing IFNγ, which promote infectious, autoimmune, alloimmune, and anti-tumor immunity(47-50). However, in humans, there are currently no markers for Beffs(23, 25). In fact, the three main canonical B cell subsets (transitional, naïve and memory) isolated from human peripheral blood all express pro-inflammatory cytokines like TNFα in addition to IL-10(36). Transitional B cells (TrB), have higher IL-10 and lower TNF expression (higher IL-10/TNFα ratio) than the other B cell subsets, and importantly, this ratio correlates directly with their *in vitro* Breg activity(27, 36). Thus, both regulatory and effector B cells are present in varying proportions in each of the major canonical B cell subsets and their relative number dictates Breg:Beff balance. Co-expression of proinflammatory cytokines by B cell subsets shown to be enriched for IL-10+ Bregs was corroborated by Glass et al(27). Further, they observed that IL-10/TNFα ratio within total B cells was significantly higher in operationally tolerant liver transplant recipients compared to those on maintenance immunosuppression. Thus, IL-10/TNFα expression provides a measure of the Breg:Beff balance amongst total B cells and individual B cell subsets.

The importance of a predictive biomarker in renal transplantation

As noted above, the rate of chronic allograft loss has changed little with time. Late biopsies reveal scarring and frequently, a degree of acute inflammation(51, 52). This suggests that earlier or ongoing inflammation, often clinically silent, contributes to long-term and irreversible damage. In this regard, longitudinal analysis of renal transplant patients with serial biopsies in the first-year post-transplantation, reveals that early rejection (0-4mos) that resolves with treatment does not significantly affect graft survival. However late rejection, whether recurrent or de novo, clinical or subclinical, is associated with worse 5-year outcomes(23). ~60% of all rejection in the first year in this study was subclinical. In many centers where surveillance biopsies are not routinely performed, subclinical rejection might smolder on undetected (53-56). Moreover, late rejection is already associated with increased premature scarring. This highlights the need for early predictive biomarkers for subsequent rejection and poor outcomes.

T1B IL-10/TNFα ratio as a predictive biomarker for renal allograft rejection and outcomes

Since Breg:Beff balance might reflect immune status, we examined the TrB IL-10/TNFα ratio in peripheral blood of renal transplant patients undergoing late for-cause biopsies for graft dysfunction(36). While IL-10 expression alone did not differ, patients with acute rejection (AR) had a significantly lower T1B IL-10/TNFα ratio than those with graft dysfunction without AR, healthy controls, or patients with stable allograft function. Furthermore, in patients with AR, a low TrB IL-10/TNFα ratio at the time of their allograft biopsy was associated with inferior subsequent 3-yr graft survival.

Upon further assessment, we found that the most immature TrB cells (T1 subset) have an even higher IL-10/TNFα ratio than the relatively more mature T2 TrB cells(57). Moreover, the ratio of T1/T2 TrBs generally parallels the TrB IL-10/TNFα ratio. In stable renal allograft patients 2 years post-transplant, a T1/T2 ratio below the median was associated with a fall in GFR and 25% graft loss over the ensuing 5 years, whereas patients with a T1/T2 ratio above the mean had stable GFR and no graft loss(57). In summary, these data suggest that TrB IL-10/TNFα ratio (or T1/T2 ratio as a surrogate) might identify a patient's underlying immune reactivity and be predictive of future clinical course. Contemporaneously, several other studies showed that a low TrB number is associated with increased incidence of AR and reduced eGFR(58-61). However, neither Bregs nor Beff based on cytokine expression were examined.

We next asked whether B subsets or their cytokines could serve as an early biomarker for transplant outcomes. Serial blood draws were performed in adult renal allograft patients who underwent surveillance biopsies (3mos and 12mos) in addition to for-cause biopsies. In the training set (n=162), of all parameters examined, the T1B IL-10/TNFα ratio measured 3 months post-transplant was found to best differentiate between patients with and without AR in the first year, and was further examined as a biomarker(62). The T1B cytokine ratio 3 months post-transplantation strongly predicted subclinical or clinical AR at any time in the first year (ROC AUC 0.89, with a sensitivity and specificity of 86% at an optimal cut-off for the ratio at 1.3). Moreover, in patients who had no early rejection (0-4mos), the T1B cytokine ratio could predict *de novo* late AR (5-12mos) with an average lead time of 8 months (ROC AUC 0.9, sensitivity 87%, specificity 91%). The T1B cytokine ratio was validated in both an internal validation cohort (82 patients) and an independent external validation cohort of 102 patients. Overall, 44% of the patients were categorized as high-risk, and these patients had an ~12-fold increase in incidence of subsequent late AR compared to the low-risk individuals (58% vs. 5%). The high-risk group also had significantly more early rejection (60%), almost half of which recurred by 12 months despite treatment. The T1B IL-10/TNFα ratio was unaffected by key demographic or clinical features (e.g. medication adherence, opportunistic viral infections, HLA mismatch).

Importantly, high-risk patients defined by the biomarker had a 3-fold increased incidence of interstitial fibrosis with inflammation (IF+i), and 1.5-fold increase in interstitial fibrosis and tubular atrophy (IFTA) at 1 year, decreased 5-year GFR, and a ~5-fold increase in premature graft loss by 5 years. Thus, the biomarker predicts early and persistent inflammation that translates into premature fibrosis and increased risk for allograft loss. Early prediction of subsequent AR provides lead time to increase immunosuppression in patients who exhibit heightened alloreactivity before further damage and maturation of the immune response occur.

The true clinical applicability of this biomarker needs to be confirmed in future studies. However, the T1B cytokine ratio is distinct from other transplant biomarkers in that it identifies an immunological imbalance (Breg:Beff) that might be amenable to therapeutic manipulation. Supporting this, incubation of B cells with anti-TNF *in vitro*, augments the T1B IL-10/TNFα ratio and these B cells then exhibit increased *in vitro* Breg activity. Moreover, treatment of rheumatoid arthritis patients with anti-TNFα increases IL-10+ B

cells *in vivo*, supporting the notion that TNF-blockade may modulate Breg:Beff balance(63). These data provide a rationale for use of agents, targeting inflammatory cytokines produced by B cells in patients with a skewed Breg:Beff balance.

Conclusion

Bregs play an important role modulating immune responses. TIM-1 is a broad functional marker for Bregs in mice that can utilize different suppressive mechanisms including co-inhibitory molecules like TIGIT in addition to cytokines like IL-10. In humans, TIM-1^+ B cells appear to be enriched in either the immature transitional or the memory B cell subsets. B cells expressing TIM1 and TIGIT inhibit T cells and DC activation *in vitro*. While it remains unclear whether TIM-1 is an inclusive or functional marker for human Bregs, it is a tantalizing target for their identification and expansion. However, important questions remain: (a). Do TIM-1+ or TIGIT+ human B cells truly express anti-inflammatory and not pro-inflammatory cytokines? (b). Can these be used for Breg-based cell therapy, and will they enhance allograft survival? (c). Does their functional repertoire remain stable, even in an inflammatory milieu? Further studies are needed to address these questions.

The identification of the T1B IL-10/TNFα ratio as a biomarker, highlights the importance of Breg:Beff balance in identifying a patient's immunological "set-point". Identification of inclusive markers for both Bregs and Beffs in humans could be an important advance for immune monitoring. In the meantime, the IL-10/TNFα ratio within the T1B subset strongly predicts renal transplant rejection, scarring and graft loss. Why the cytokine ratio within the T1B subset per se, turns out to be most predictive is not clear. While TrBs are short-lived, it is possible that they maintain their original cytokine profile when they mature or are activated in immune or parenchymal tissues(64). Whether this biomarker is predictive in other organ transplants, or changes with therapy and can be used as a therapeutic guide, remains to be determined. Nonetheless, the ability to identify high-risk patients based on an individual patient's Breg:Beff balance could advance therapeutic strategies aimed at shifting this balance towards Bregs. The involvement of Bregs and Beffs in allograft survival makes further understanding of their biology imperative, even if to avoid unintended results when B-directed therapies are used aiming to limit antibody-mediated rejection.

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to etsbalish T1B IL-10/TNFα ratio, a measure of Breg:Beff balance in human peripheral blood as an early predictive biomarker for renal transplant rejectio and subsequent clinical course].

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Key Points:

- TIM-1 is an inclusive marker for Bregs that utilize disparate suppressive mechanisms.
- TIM-1 and TIGIT+ human B cells modulate immune responses and serve as markers for human Bregs.
- Ex vivo expanded IL-10 expressing human B cells express TIM-1 and have a potential for Breg based cell therapy.
- The ratio of IL-10/TNFα expression mirrors Breg:Beff balance in individual B cell subsets.
- A low ratio of IL-10/ TNFα expressing T1B cells is an early predictive biomarker for acute rejection and subsequent clinical course in renal transplantation.