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Matchmaking the B cell signature of tolerance to regulatory B cells

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Abstract

Confirmation of clinical tolerance requires the cessation of immunosuppressive drugs, which evokes immune reactivation and allograft rejection in all but the rare individuals that successfully transition into a state of operational transplantation tolerance. Therefore the safe conduct of trials in transplantation tolerance requires two conditions: a sensitive and reliable means to identify individuals still being maintained on immunosuppression that are most likely to exhibit tolerance after immunosuppression is withdrawn, and a non-invasive means that assesses the quality or robustness of the tolerant state. Two recent studies attempting to identify a gene signature in peripheral blood of spontaneously tolerant kidney transplant recipients made the unexpected observation that tolerant, but not immune suppressed transplant recipients, exhibited enriched B cells and B cell transcripts in their blood. In concert with the emerging appreciation of a specialized subset of regulatory B cells that possess immunomodulatory function, these observations raise the possibility that regulatory B cells play a critical role in the maintenance of tolerance to renal allografts in transplant patients. This review summarizes these recent findings and speculates on the relationship of regulatory B cells to the maintenance of transplantation tolerance.

Keywords

Transplantation Tolerance; Biomarkers; Regulatory B cells

One of the major challenges to achieving successful transplantation tolerance in the clinic is the lack of a non-invasive way to accurately diagnose the tolerant state and to identify the recipients in which tolerance is most likely to occur (1). The cellular and genetic signatures of tolerance were described in two tour de force studies from the Immune Tolerance Network (ITN) and the Reprogramming the Immune System for the Establishment of Tolerance (RISET) (2, 3). These groups retrospectively identified 25 (ITN) and 11 (RISET) kidney transplant recipients that had stopped their immunosuppressive drugs but had not rejected their grafts. The median time of suspended immunosuppression was 13 and 9 years, and the state of operational tolerance was 1–32 (median=13) and 2–21 (median=3) years for the ITN and RISET study, respectively. The average HLA mismatch was 0.63–0.83 of 6 in the ITN study and 4.0 of 10 in the RISET study, the latter demonstrating that matching HLA loci is not a strict requirement for achieving operational tolerance.

T cells have long been recognized as the critical mediators of acute rejection and regulatory T cells have been extensively demonstrated to play a critical role in tolerance (4, 5). Thus, the expectation going into these investigations was that the signature of tolerance would

point to dampened T cell function and a prominent T cell-mediated regulation. However what emerged was an enriched B cell signature in operationally tolerant recipients relative to stable immunosuppressed (s-IS) recipients. This mini-review summarizes these recent observations, speculates on their significance in the context of regulatory B cells (Bregs), and discusses future directions.

The nature of the B cell signature of tolerance: Cellular Analysis

Flow cytometric assays were conducted on peripheral blood samples to compare the lymphocyte subsets within drug-free tolerant and s-IS (maintained on Calcineurin or mTOR inhibitors, an anti-proliferative agent and steroids) transplant patients, as well as healthy controls. Comparing the tolerant to s-IS groups had the potential to reveal two variables simultaneously – the effect of immune suppression and a tolerance signature. Comparing the tolerant group to non-immunosuppressed healthy controls was essential for differentiating between these two variables, and providing possible mechanistic insights. The results of these studies are summarized in Table 1.

In Sagoo et al. (3), flow cytometric analysis of thawed peripheral blood revealed a statistically significant increase in B cells and to a lesser extent NK cells, and a corresponding decrease in T cells, in the peripheral blood of the operationally tolerant group compared to s-IS group within both the RISET and ITN cohorts. In the comparison between tolerant and healthy control groups, a similar pattern of enriched B cells and reduced T cells was observed within the thawed PBMC of RISET patients, but not in that of the ITN cohort (Table 1). Sagoo et al. (3) speculated that the greater HLA mismatches manifested in the RISET cohort compared to that of the ITN could lead to the induction of a more robust tolerance signature. However, when thawed PBMCs from 23 ITN and 6 RISET samples were separately analyzed by the Newell et al. (2) study, significant increases in the percentages of total B cells, as well as in naïve and transitional B cells, were observed in the tolerant compared to s-IS groups but not between the tolerant and the healthy control groups for both the ITN and RISET cohorts.

Newell et al. (2) confirmed that the increased percentages of B cells in the peripheral blood of tolerant individuals was due to increases in absolute numbers of B cells (Table 1). Increased CD20 mRNA in the urinary sedimentary cells extended these observations and suggested that B cells may also be enriched in the grafts of tolerant recipients. Comparative phenotypic analysis revealed that tolerant compared to s-IS groups (but not when compared to healthy controls) were significantly enriched for naïve and transitional B cells, even though the transitional B cells remained a minor (2–3%) subset. These observations are consistent with immunosuppression preferentially inhibiting the generation memory B cells while allowing the accumulation of naïve and transitional B cells. A significant increase in the numbers of memory or CD86⁺ B cells/ μ l of peripheral blood from tolerant compared to healthy controls was noted. However, when percentages were compared between these two groups, memory (18.8 vs. 11.8%) and CD86⁺ B cell populations (7.7% vs. 2.6%) were only modestly enriched in the tolerant group while the percentages of transitional and naïve B cells were not significantly different. Thus these data do not strongly support a conclusion of preferential accumulation of memory B cells during tolerance.

In a different study of tolerant patients by Pallier et al. (6) also reported an increase in total B cells in the peripheral blood in the tolerant compared to the s-IS groups but not when compared to the healthy control group. They concluded that a major effect of immunosuppression was the reduction of B cell numbers, especially of activated/memory B cells. Comparison of tolerant versus healthy control group revealed a non-statistically

significant trend towards increased total and activated B cells but no preferential enrichment of memory Bm5 or CD27⁺ B cells with tolerance (Table 1).

Overall the cellular analyses from these studies consistently observed an enrichment of naïve and transitional B cells in tolerant compared with s-IS patients, and that in the absence of immunosuppression, the peripheral blood of transplant recipients approached that of healthy controls.

The nature of the B cell signature of tolerance: Transcriptome Analysis

In an effort to discover additional features of the tolerant state both the ITN and Riset groups analyzed the transcriptome of blood circulating leukocytes, the rationale being that, in addition to verifying the cellular analysis, this approach had the potential to identify novel molecules since it interrogated for changes in transcript levels of 47,000 (2) or 4,607 genes (3). Additionally, multiplex real-time PCR was performed. The top 10–20 transcripts that were differentially expressed between the tolerant versus the s-IS groups are listed in Table 2. Many of the transcripts enriched in tolerant recipients corresponded to abundantly expressed immunoglobulin heavy and light chain genes expressed only by B cells. Several genes associated with B cell function were also enriched in the tolerant compared to the s-IS group. Most of these genes were highly ranked in only one study but not in other, with 4 exceptions, namely MS4A1, TCL1a, STAP1 and FCRL.

Both Newell et al. (2) and Sagoo et al. (3) found increased expression of MS4A1 (membrane-spanning 4-domains, subfamily A, member 1), which encodes the B lymphocyte-specific CD20 cell surface molecule involved in B cell activation. CD20 transcripts were also observed to be unregulated in urine sedimentary cells following real-time PCR gene expression analyses (2). Additionally, TCL1a (T cell leukemia/lymphoma 1a) transcripts were enriched in the ITN and the Riset studies (2, 3). TCL1a is expressed mainly by immature T cells as well as pre- and naïve B cells, where its expression decreases at more mature stages of B cell development (7). TCL1 interacts with the Akt kinase to promote Akt signaling, which via the PI3K – Akt signaling axis plays broad roles in cell activation, survival, and proliferation. STAP-1 (signal transducing adaptor family member 1) or BRDG1 (BCR downstream signaling 1) was identified by Newell et al. (2) in a gene array analysis and confirmed by quantitative PCR to be enriched in the PBL of tolerant recipients. STAP-1 has been shown to act downstream of the Tec family of protein tyrosine kinases to enhance BCR-mediated activation of the cellular transcription factor, cAMP-response element binding protein (CREB) (8).

The Fc Receptor Like (FCRL) molecules, namely FCRL1 (2, 3), FCRL2 (3) and FCRLA (2) were increased in the tolerant group compared to the s-IS group. This ancient class of receptors shows homology to Fc Receptors, however, they do not bind circulating antibodies or antibody complexes but serve as inhibitory receptors that modulate B cell activation (9). The cytoplasmic tail of FCRL1 and 2 contain ITIM motifs specialized in recruiting phosphatases that counteract kinases activated by the BCR or other activating receptors. Of note, expression in B cells is increased during chronic HIV and hepatitis B infection, and circulating B cells from these infected individuals were significantly hyporesponsive to mitogenic signaling (10). If FCRL1 and FCRL2 also mediate inhibitory signaling like FCRL4, then their increased expression within B cells from operationally tolerant recipients would be consistent with down-modulated B cell responses. FCRLA is a soluble resident endoplasmic reticulum protein and associates with multiple Ig isotypes within the secretory pathway, suggesting a role in Ig assembly (11). Its expression is restricted to B cells, is most abundant in germinal center and marginal zone B cells and is found at significant levels in resting blood B cells (12).

While the transcriptome analysis of PBL confirmed the cellular data of an enriched B cells signature of tolerance, it is unclear why only a limited number of B cell-specific transcripts were observed in all three analyses. Potential heterogeneity of B cell subsets within individuals and poor probe performance during hybridization are possible explanations. Interestingly, the largest class of transcripts enriched in tolerant recipients represents cell surface receptors and signaling molecules suggesting that circulating B cells may not be in a state of immunological ignorance, but may have perceived the allograft and increased their expression of negative regulators to dampen their ability to respond to BCR engagement in a cell intrinsic manner. To this end, the observations of Pallier et al. (6) are potentially insightful, where tolerance was reported to be associated with an mRNA profile that was enriched for negative regulators of B cells function, namely inhibitory FcγRIIA over activating FcγRIIB, and the B-cell scaffold protein with ankyrin repeats-1 (BANK-1), which is a negative modulator of CD40-mediated Akt activation.

Does the B cell signature imply a role for regulatory B cells in tolerance to renal allografts?

There is an expectation that the immunologic signature associated with operationally tolerant individuals would provide mechanistic insight into the maintenance of the tolerant state. Regulatory B cells have recently emerged as potential players in dampening immune responses, especially in murine models of autoimmunity. While our understanding of Bregs in humans is still in its infancy, it is nonetheless tempting to speculate that expanded Bregs function to maintain a state of transplantation tolerance.

B cells with the capacity to regulate T cell function were originally described by Shimamura et al. (13–15), and by studies a decade later by Wolf et al. (16) who reported that mice deficient in B cells were unable to recover from experimentally-induced encephalomyelitis (EAE) whereas mice sufficient in B cells recovered. Since then, Bregs have been shown to exert immune-suppressive actions in models of colitis, arthritis and EAE (17, 18). Marginal Zone (MZ) and B1 B cells as well as at least two phenotypically distinct subsets of Bregs have been characterized as having suppressive activities – transitional 2 marginal zone precursor (T2-MZP) and B10 cells (Table 3). These Breg subsets express high levels of CD1d, CD21, CD24 and IgM and moderate levels of CD19, and secrete the immunomodulatory cytokine IL-10. Additionally, T2-MZP Bregs are positive for CD93 and CD23, while B10s uniquely express CD5. The relationship of B10 cells to T2-MZP, classical MZ B cells and B1 cells is currently unresolved.

The production of IL-10 is the sine qua non for Bregs in many studies and IL-10 producing B cells in the peripheral blood of healthy humans have been reported (19). IL-10 production can be observed in approximately 0.6–5% of peripheral blood B cells stimulated by Toll-like receptor ligation (CpG or LPS) in combination with CD40 ligation. Furthermore, B10 and B10 precursors are present at higher frequencies (> 10%) in patients with autoimmune disease (rheumatoid arthritis, systemic lupus erythematosus, Sjogren syndrome, blistering skin disease and multiple sclerosis) (20). The phenotype of human Bregs is consistent with immature/transitional CD19⁺CD24^{hi}CD38^{hi}CD27⁻ B cells or memory CD24^{hi}CD27⁺, the majority of which express CD38⁺ (20–22). In addition, human CD40-activated B cells are potent antigen-presenting cells capable of inducing and expanding FoxP3-expressing regulatory T cells *in vitro* (23, 24). These observations suggest another potential mechanism for immune suppression by human Bregs, similar to their mouse counterparts, in addition to their secretion of IL-10.

Although IL-10 was not identified in the transcriptome analysis, Newel et al. (2) looked for the presence of intracellular IL-10 in sorted transitional B cells stimulated with PMA and

ionomycin. They observed significantly increased frequencies of transitional B cells expressing IL-10, but not TGF β , in the tolerant and healthy controls compared to the s-IS group (2). Sagoo et al. (3) reported no significant differences in IL-10, TGF β and IFN γ in total B cells stimulated with PMA and ionomycin from all study groups, although there was a trend towards B cells from tolerant recipients producing more TGF β relative to IFN- γ . Pallier et al. (6) investigated the production of IL-10, as well as TNF α and IL-6, following the stimulation of total B cells with CD40 $\pm$ CpG. There was no significant differences in the production of all three cytokines by B cells from tolerant compared to s-IS and healthy controls. With the caveat that different stimulatory conditions were used in the study by Pallier et al. (6), the results of these three studies are not necessarily contradictory as the percentage of IL-10 producing B cells in the ITN study was only 0–5% of transitional B cells, and transitional B cells constituted only 2–3% of total B cells. It is likely that such a modest increase in IL-10 production in this minor subset of B cells would be undetectable when total B cells were investigated.

Overall, the expanded B cell population portending a role for Bregs in the maintenance of tolerance remains an intriguing possibility. Resolution of this issue will require a better phenotypic definition of Bregs in humans, and a mechanistic understanding of how these cells suppress alloreactive immune responses *in vivo*.

Speculation on Future Directions

We recognize that the non-invasive diagnosis of tolerance should optimally be based on procured peripheral blood and urine sedimentary cells, the latter proximally sampling the kidney graft. In support of this approach are reports of specific NK and $\gamma\delta$ TCR $^{+}$ T cell-enriched signatures in patients tolerant to liver allografts (25–27) and gene signatures predictive of chronic allograft nephropathy (25–27). Indeed, the absence of an enriched B cells marker in patients tolerant to liver allografts compared to those maintained on monotherapy of calcineurin inhibitor or mycophenolate mophetil has been used to argue for the B cell signature being specifically indicative of tolerance to renal allografts. Nonetheless, a cautionary note was raised by the recent report by Cobbold et al. (28), where biomarkers of transplantation tolerance were sought in three different locations, in the graft, draining lymph node and spleen, in three different mouse models of skin allograft tolerance. They observed that the pattern of gene expression within long-term surviving tolerant grafts was similar to syngeneic grafts, but distinct from rejection, and that these differences were only observed within the graft organ but not in the draining lymph node or spleen. These observations raise two important points: that an immunological marker of tolerance, beyond a lack of inflammation, may not be discernable in stable tolerance, and that if it existed, it may be most prominently expressed in the grafted organ.

The current observations raise two important questions; will the B cell signature be replicated in a larger cohort of tolerant patients and, more importantly, is it indicative of tolerance or simply a manifestation of an absence of immunosuppression. The latter question can be addressed by clinical studies that prospectively test whether this tolerance signature is observed in only a small subset of stable immunosuppressed transplant recipients, and whether weaning those individuals off immune suppression would be more successful compared to historical controls. Another approach is to test in pre-clinical models whether a similarly enriched B cell signature in the blood and other tissue compartments is observed in tolerance to kidney but not to liver allografts. Analysis of the fate of alloreactive B cells, and of when and how B cells may promote tolerance in these pre-clinical models would provide insights into whether this matchmaking of the B cell signature to Bregs and the acquired stated of transplantation tolerance proves to be a match made in heaven or a hasty shotgun marriage.

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Abbreviations

BANK-1	B-cell scaffold protein with ankyrin repeats-1
BRDG1	BCR downstream signaling 1
CpG	Unmethylated single-stranded synthetic DNA containing cytosine phosphodiester guanine
CREB	cAMP-response element binding protein
EAE	Experimentally-induced encephalomyelitis
FCRL	Fc Receptor Like
IQR	interquartile range
ITN	Immune Tolerance Network
MS4A1	Membrane-spanning 4-domains, subfamily A, member 1
mTOR	Mammalian target of rapamycin
PI3K	Phosphatidylinositol 3-kinases
RISET	Reprogramming the Immune System for the Establishment of Tolerance
s-IS	Stable immunosuppressed
STAP-1	Signal transducing adaptor family member 1
T2-MZ	Transitional-Marginal Zone B cells
TCL1a	T cell leukemia/lymphoma 1a
TOL	Tolerant

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Table 1

Summary of Cellular Comparisons in tolerant (TOL) versus stable-immunosuppressed (s-IS) transplant recipients and healthy controls (Healthy)

Sagoo et al (3)			
RISSET Patients	TOL; n=11	s-IS; n=30	Healthy; n=19
% B cells	8–48 (28)	0–24 (12); p<0.001	7–15 (11); p<0.05
% NK cells	3–30 (19)	0–23 (12); p<0.01	3–18 (11)
% T cells	35–80 (58)	72–96 (84); p<0.001	74–91 (83); p<0.001
%CD4 ⁺ CD25 ^{int}	7–44 (26)	17–72 (45)	29–47 (38); p<0.01
%CD4 ⁺ CD25 ^{hi}	0.5–15 (8)	1.5–9 (5)	3.5–6.2 (5)
ITN Patients	TOL; n=24	s-I; n=34	Healthy; n=31
% B cells	4–22 (13)	0–20 (10); p<0.001	7–20 (14)
% NK cells	4–23 (14)	2–38 (20); p<0.01	4–16 (10)
% T cells	48–80 (64)	52–92 (72); p<0.001	64–85 (75)
%CD4 ⁺ CD25 ^{int}	25–61 (43)	28–68 (48); p<0.05	16–53 (35)
%CD4 ⁺ CD25 ^{hi}	2–9 (6)	1–13 (7)	1–8 (5)
Newell et al. (2)			
ITN Patients	TOL; n=19	s-IS; n=30	Healthy; n=42
% Total B cells (Cells/ μ l)	1–17 (9)	0–8 (4); p<0.01	2–13 (8)
Total B cells/ μ l	176	120	287
% Transitional of CD19 ⁺	0–4.5 (2)	0–0.2 (0.1); p<0.01	0.5–4 (2)
% Naïve of CD19 ⁺	50–95 (70)	10–85 (48); p<0.01	50–90 (73)
Memory of CD19 ⁺ Cells/ μ l	54.2	n/a	20.8; p<0.03
CD86 ⁺ of CD19 ⁺ Cells/ μ l	22.0	n/a	4.5; p<0.01
RISSET Patients	TOL; n=6	s-IS; n=14	Healthy; n=18
% Total B cells	4–13 (8)	3–8 (6); p<0.05	4–13 (8)
% Transitional	0.3–0.5 (0.4)	0–1.5 (0.75); p=0.07	0.4–6 (3)
% Naïve	41–80 (61)	21–78 (50); p<0.05	41–81 (61)
Pallier et al. (6)	Cells/μl (% B cells)		
	TOL; n=12	s-IS; n=34	Healthy; n=29
B Cells	325	60; p<0.01	220
Activated Bm2	155 (48%)	10; p<0.001 (17%)	90 (41%)
Memory Bm5	76 (23%)	7; p<0.001 (12%)	76 (35%)
CD27 ⁺ Memory	74 (23%)	12; p<0.001 (20%)	60 (27%)

Data are extracted from Sagoo et al. (Figures 1–2; (3)) and indicate the 5th and 95th; Newell et al. (Figures 6–7; (2)) and indicate 1.5 interquartile range (IQR) and Pallier et al. (Figure 1; (6)) Data in bold are mean or median values.

The p values indicate statistically significant differences between TOL versus s-IS or healthy controls and are highlighted as grey cells.

IS: Immunosuppression with CN/mTOR inhibitors, anti-proliferative agents and steroids.

% CD3⁺ T, CD19⁺ B and CD56⁺CD3[−] NK cells are of total lymphocytes; % B and T cell subsets are of total B and T cells, respectively.

Naïve/mature B: $CD19^{+}CD27^{-}CD24^{int};CD38^{int}$ T1/T2 transitional B: $CD19^{+}CD27^{-}CD24^{+}CD38^{hi}$; memory B: $CD19^{+}CD27^{+}IgD^{-}CD24^{+}CD38^{-}/int$; Activated Bm2: $IgD^{+}CD38^{+}$; memory Bm5: $IgD^{-}CD38^{+/-}$

Transcriptome Analysis revealing the top-ranking genes that differ from tolerant versus stable-immunosuppressed transplant recipients

Table 2

PBL MicroArray (2)		PBL RT-PCR (2)		PBL MicroArray(3)	
* Gene Rank	Official symbol	Gene Rank	Official symbol	Gene Rank	Official symbol
1	STAP1	8	STAP1	1	CD79B
2	Cd72	13	MS4A1	2	TCL1A
3	BCNPI	14	FCRLA	3	HS3ST1
4	TCL1A	15	FAM129C	4	SH2D1B
7	FCRL1	16	PPAPDC1B	5	MS4A1
8	FCRLA	20	IRF4	6	TLR5
10	PPAPDC1B			7	FCRL1
				8	PNOC
				9	SLCB1
				10	FCRL2

* Skipped Gene Ranks were Ig heavy or light chain transcripts.
Genes upregulated in at least 2 studies (bold) or 3 studies (grey cells).

Table 3

Markers on B cell and Regulatory B cell subsets (20, 29, 30)

Phenotype of Human CD19 ⁺ Subsets in Blood						
Virgin Naive	Activated Naive	Bm2: Pre-GC	Bm3: GC centroblast	Bm4: GC centrocytes	Bm5: Memory	B-regs Transitional
IgD ⁺ CD38 ⁻ CD27 ⁻	IgD ⁺ CD38 ⁺ CD27 ⁻	IgD ⁺ CD38 ⁺⁺	IgD ⁻ CD38 ⁺⁺	IgD ⁻ CD38 ^{+/-} CD27 ⁺	IgM ^{hi} IgD ^{hi} CD38 ^{hi} CD24 ^{hi} CD5 ⁺ CD1d ^{hi}	IgD ⁺ CD38 ⁺ CD27 ⁺ CD5 ^{+/-} CD1d ^{low}